Serendipita SPECIES FROM AUSTRALIAN NATIVE ORCHIDS AND THEIR IMPACTS ON TOMATO GROWTH AND PROTECTION AGAINST POWDERY MILDEW

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ABSTRACT

Most plants form symbiotic associations with mycorrhizal fungi, which provide water and inorganic nutrients to the host and in exchange obtain plant-derived carbohydrates for their own growth. Fungi in the Serendipitaceae form mycorrhizal associations with a wide variety of plant species including grasses, orchids, ericoids and bryophytes. *Serendipita indica* is the most extensively studied species in the Serendipitaceae, due to its ease of culture on artificial media and its ability to colonise the roots of various plant species. Previous studies have shown that colonisation with *S. indica* enhances the growth of the shoots and roots of many plants and protects plants against biotic and abiotic stresses. The first aim of this project was to isolate and identify *Serendipita* spp. from native Australian orchids including *Caladenia* spp. and *Bulbophyllum* spp. in south east Queensland. Five known *Serendipita* spp. were isolated from seven *Caladenia* species and identified using morphological and molecular methods. Two likely new species of *Serendipita* were also isolated from *Bulbophyllum schillerianum* and *B. bracteatum*. The fungal isolates from *B. minutissimum* and *B. shepherdii* also included endophytic *Preussia* and *Tulasnella* respectively. Inoculating plants with a recently described *Serendipita* species, *S. whamiae,* increased the fresh weight of tomato and improved immunity against the powdery mildew pathogen, *Golovinomyces lycopersici*. Semi-quantitative RT-PCR analysis showed no difference in the expression of nutrient transporters between *Serendipita*-colonised tomato and non-colonised plants. In *Serendipita*-colonised plants challenged with powdery mildew, there was inconsistent expression pattern of a β-1,3-glucanase gene and no expression in chitinase, callose 11 synthase, phenylalanine ammonia lyase and JA-Lipoxygenase genes. *Serendipita* inoculants may represent an alternative eco-friendly and non-chemical approach for enhancing plant growth and pathogen control in agriculture and horticulture.

CERTIFICATION OF THESIS

I certify that the experimental work, results, analyses and conclusions reported in this thesis are entirely my own effort, except where otherwise acknowledged. I also certify that the work is original and not been previously submitted for any other award, except where otherwise acknowledged.

Principal Supervisor: Associate Professor **John Dearnaley**

Associate Supervisor: Dr. **Bree Wilson**

Associate Supervisor: Professor **Levente Kiss**

Student and Supervisors signatures of endorsement are held at the university.

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AMF: arbuscular mycorrhizal fungi

CFU: colony forming units

Ca: calcium

cDNA: complementary deoxyribonucleic acid

Cq: quantitation cycle

ISR: induced systemic resistance

JA: jasmonic acid

MAMP: microbe-associated molecular pattern

µl: microliter

min: minute

OB: oxidative burst

PAL: phenylalanine ammonia lyase

PAMPs: pathogen associated molecular patterns

PCR: polymerase chain reaction

+PM: powdery mildew infection

PNM: plant nutrient media

PR: pathogenesis-related proteins

qRT-PCR: quantitative real time –PCR

RNA: Ribonucleic acid

Ser+: *Serendipita* presence

Ser-: *Serendipita* absence

SAR: systemic acquired resistance

CHAPTER 1: LITERATURE REVIEW

1.1 Interactions between mycorrhizal fungi and plants

Most plants form symbiotic associations with mycorrhizal fungi, which provide water and inorganic nutrients to the host and in exchange obtain plant-derived carbohydrates for their own growth (Nehls et al., 2010; Habib et al., 2013). There are various types of mycorrhizas, such as arbuscular mycorrhizas, ectomycorrhizas, orchid mycorrhizas, ericoid mycorrhizas and ectendomycorrhizas (Selosse and Le Tacon, 1998) (Figure 1.1).

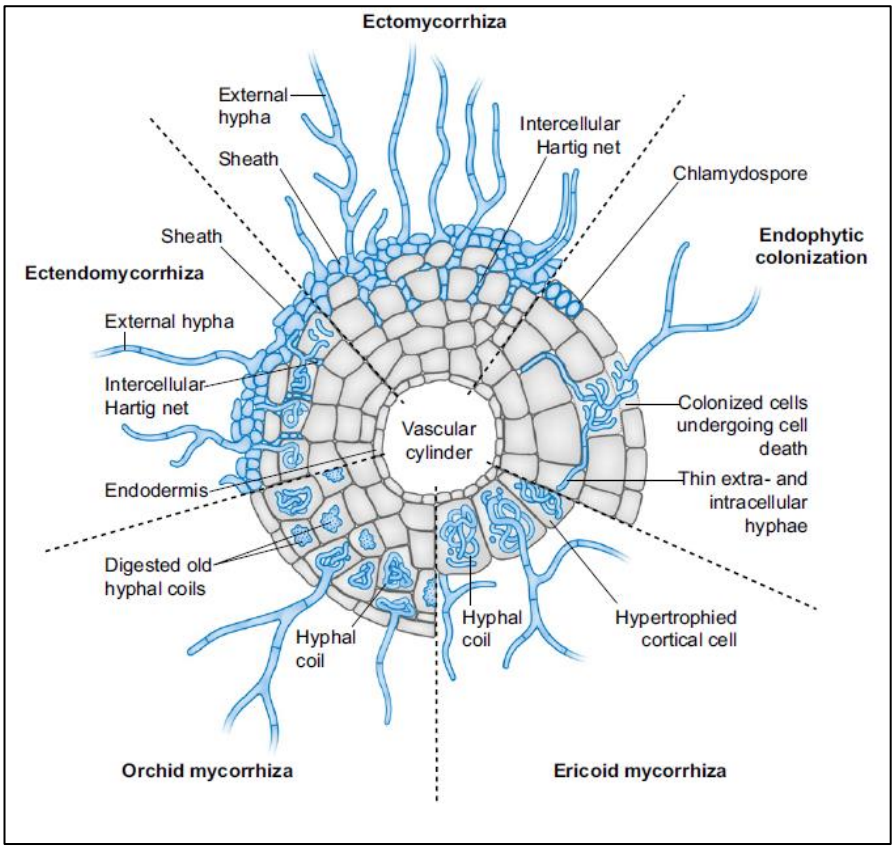


Figure 1. 1: Mycorrhizal fungal interactions with plant roots (modified from Selosse and Le Tacon, 1998).

Briefly, ectomycorrhizas involve the formation of a mantle and Hartig net of intercellular hyphae on root cells. Arbuscular mycorrhizas, ericoid mycorrhizas, orchid mycorrhizas and ectendomycorrhizas are classified as endomycorrhizas. Each

type of endomycorrhiza involves colonisation of root cells intracellularly by fungal hyphae, and each has a distinctive morphology (Peterson et al., 2004).

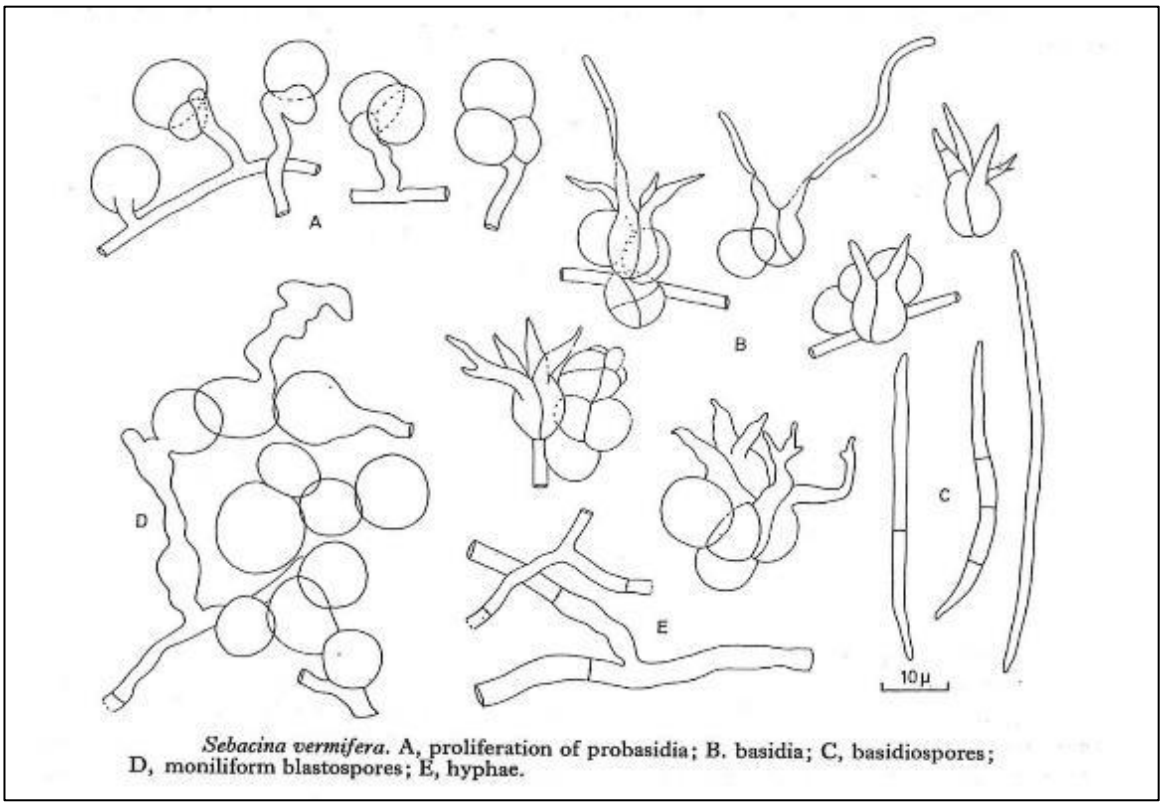
1.1.1 Orchid mycorrhizas and *Serendipita* fungi

Previously, the interaction between orchids and mycorrhizal fungi was considered an anomalous association because it was believed that fungus received no benefits from the host orchid (Alexander & Hadley, 1985). However, recent studies of orchid mycorrhizas have suggested that the flow of nutrients is bidirectional between the plant and its fungal partner, where the plant depends on coiled hyphae (pelotons) of the fungus to receive essential elements such as phosphorus and nitrogen (Cameron et al., 2006) and the fungus receives nitrogen in the form of ammonium (NH^{4+}) (Fochi et al. 2017) and carbon in the photosynthate from the orchid (Cameron et al., 2006; Latalova & Balaž, 2010). Green photosynthetic orchids are mixotrophic most of their life cycle and also receive carbon at adult and embryonic stages (Selosse & Roy 2009; Selosse & Martos 2014).

The Basidiomycete genera *Tulasnella*, *Serendipita* and *Ceratobasidium* are typical partners of green orchids (Garcia et al., 2006; Whitehead et al., 2017). *Serendipita* belong to the Sebacinales order which was developed by the French mycological brothers Charles and Louis-Rene Tulasne. They erected the *Sebacina* genus in 1871, based on the unusual longitudinally septate basidia present in these fungi (Tulasne & Tulasne, 1871). The family Sebacinaceae was established by Wells and Oberwinkler based on morphological characteristics, such as longitudinal septate basidia, absence of clamp connections and relatively thick-walled hyphae (Wells & Oberwinkler, 1982). The Sebacinales currently includes two families; the Sebacinaceae and the Serendipitaceae (as amended by Oberwinkler et al., 2014).

Oberwinkler described the first Serendipitaceae species in 1964. He classified the isolate in the *Sebacina* genus due to the presence of longitudinally septate basidia (Warcup & Talbot 1967; Figure 3) and named it *vermifera* based on worm-like basidiospores (Oberwinkler, 1964). Oberwinkler described the teleomorph stage of the fungus, where the probasidia were sub-globose to ovate and 8-10 µm in diameter (Figure 4A), metabasidia with cruciate septa and basidia with forked, branched and septate sterigmata (Figure 4.B). The basidiospores were usually filiform, cylindrical,

aseptate or formed 1-3-septa and were straight or bent (Figure 4C). In addition, Oberwinkler described anamorphic monilioid blastospores, which were present in clusters, spherical in shape, thin-walled, hyaline (clear) in appearance and 9-14 µm diameter (Figure 4D). Hyphae were characterised by Oberwinkler, as being thin-walled, hyaline, without clamp connections and with small dolipores in the septa (Figure 4E).



Sebacina vermifera. A, proliferation of probasidia; B. basidia; C, basidiospores; D, moniliform blastospores; E, hyphae.

Figure 1. 2: *Serendipita vermifera* (formerly *Sebacina vermifera*); anamorphic and teleomorphic stages (Warcup & Talbot 1967).

In 1967, Warcup & Talbot described fungal strains isolated from Australian orchids as having similar features to Oberwinkler's *Sebacina vermifera* (Warcup & Talbot 1967). *Serendipita vermifera* was later identified on rotting timber in the United Kingdom (Roberts, 1993). Molecular analyses have shown that Warcup's strains consist of combination of diverse species of *Serendipita* (Weiss et al., 2004, 2011; Deshmukh et al., 2006). Recently, some of these species have been named as *S. australiana*, *S. secunda*, *S. warcupii*, *S. talbotii*, *S. occidentalis*, *S. rarihospitum* and *S. communis* (Oktalira et al, 2021).

The most extensively studied Serendipitaceae species is *Serendipita indica*, due to its ease of culture on artificial media and its ability to colonise the roots of various plant species (Waller et al., 2005; Sherameti et al., 2008; Fakhro et al., 2010; Jogawat et al., 2013). *Serendipita indica* was named due to its pear-shaped asexual spores and was originally isolated and described from the arbuscular mycorrhizal fungus (AMF) *Funneliformis mosseae* from the Thar desert in India (Verma et al., 1998). Other Serendipitaceae species include *S. williamsii,* which was identifed from the roots of *Trifolium repens* (Milligan & Williams 1987), *S. herbamans* from the herbaceous plant *Bistorta vivipara* (Riess et al., 2014), *S. restingae* from the orchid *Epidendrum fulgens* (Fritsche et al., 2021) and *S. whamiae,* which was isolated from the collar region (immediately above the tuber) in the stem of the orchid *Eriochilus cucullatus* (Crous et al., 2020).

1.2 Mycorrhizal/*Serendipita* fungi as plant growth promoters

Several previous studies have shown the positive impacts of mycorrhizal fungi on tomato under abiotic and biotic stress (Nzanza et al., 2012; Aseel et al., 2019; Leventis et al., 2021; Wang et al., 2021; Detrey et al., 2022; Hallasgo et al., 2022). For example *Rhizophagus irregularis* was shown to increase the plant length and the fresh weight of root in colonised tomato by 41% and 48% respectively under salinity stress compared to control plants. Another study demonestrated that mycorrhizal fungi could support tomato growth under drought stress, where *Septoglomus constrictum*-colonised tomato plants have showed higher root and shoot dry weight (14% and 18% respectively) than non-mycorrhizal plants under drought stress (Duc et al., 2018). When tomato plants were infected with the bacterial pathogen *Ralstonia solanacearum, Glomus versiforme* reduced around 80% of the *R. solanacearum* population on the root surface by inducing soluble phenols contents in root tissue (Zhu & Yao, 2004).

One year after discovering *S. indica,* Varma and colleagues (1999) investigated the function of *S. indica* as a plant growth promoter. They noted the positive impact of *S. indica* on the growth of the shoots and roots of maize (*Zea mays),* poplar (*Populus tremula)* and parsley (*Petroselinum crispum)* (Varma et al.,1999). An improvement of

plant biomass was also observed in *S. indica* colonised *Arabidopsis* which involved an increase in the number and size of rosette leaves (Peskan-Berghofer et al., 2004). *S. indica* enhanced the shoot biomass 1.65 times compared with the controls in black gram (*Vigna mungo)* (Kumar et al., 2012). *S. indica* also enhanced both shoot and root biomass of wheat (*Triticum aestivum)* (Serfling et al., 2007), *Chlorophytum* sp. (Gosal et al., 2010), Chinese cabbage (Sun et al., 2010) and fennel seedlings (Dolatabadi et al., 2011). Research also showed an increases in nutrient absorption and biomass in Chinese fir (Wu et al., 2019). In the medicinal plant *Adhatoda vasica*, *S. indica* increased the growth rate of colonised plants compared to control plants (Rai & Varma, 2005). In addition, *S. indica* induced early flowering and increased the chlorophyll and phosphorus content of the medicinal plant *Coleus forskohlii* (Das et al., 2012). Moreover, *S. indica* increased plant biomass, the number of leaves and roots and the length of shoots and roots of *Centella asiatica* (Satheesan et al., 2012). Additional studies where *Serendipita* spp. improved plant growth are summarised below (Table 1.1).

Table 1. 1: Impacts of *Serendipita* on plant growth parameters. *Serendipita* increased shoot and root length, grain yield and fruit and overall fresh weight in multiple studies.

Plant	*Serendipita* sp.	Plant part	Increase rate	Reference
Switchgrass (*Panicum virgatum* L.)	*S. vermifera*	Overall plant biomass	Increased from 77% to 113% increase compared to the control	Ghimire et al., 2009
Spilanthes calva	*S. indica*	Shoot length	Increased by 87%	Rai et al., 2001
Withania somnifera	*S. indica*	Shoot length	Increased by 63%	Rai et al., 2001
Barley	*S. indica*	Grain yield	Increased by 11%	Waller et al., 2005
Tomato	*S. indica*	Fruit fresh weight	Increased between 50% and 100%	Fakhro et al., 2010
Anthurium andraeanum	*S. indica*	Root length	Increased by 58%	Lin et al., 2019
Arabidopsis	*S. indica*	Fresh weight	Increased by 30%	Sun et al., 2014
Arabidopsis	*S. indica*	Shoot fresh weight	Increased by 6.6 fold compared to the control	Venneman et al., 2020

The effects of inoculating both *S. indica* and the nitrogen-fixing bacterium, *Azotobacter chroococcum* on *Artemisia annua* L. (sweet wormwood plant) have been studied. The impacts of this dual interaction improved plant growth, increased phosphorus and nitrogen levels and enhanced photosynthetic pigment concentration (Arora et al., 2016). Furthermore, another *Serendipita* species, *S. vermifera* has shown a positive impact on growth parameters of many plants (Weiss et al., 2016), where *S. vermifera* enhanced the stalk length of *Nicotiana attenuata* (Barazani et al., 2005), shoot growth and fresh weight in barley (Deshmukh et al., 2006) and improved root and shoot length and dry mass in switchgrass (Ghimire et al., 2009).

1.3 *Serendipita* induced protection against plant stress

Colonisation with *Serendipita* enhances protection against both biotic and abiotic plant stresses (reviewed in Weiss et al., 2016). Barley plants colonised with *S. indica* showed higher resistance to *Fusarium culmorum* than non-colonised plants (Waller et al., 2005; Harrach et al., 2013). The fresh weight of roots and shoots decreased two fold in *Serendipita*-colonised barley under *Fusarium* stress, while the losses were 12 fold in the absence of *Serendipita* (Waller et al., 2005). In wheat, Serfling et al, (2007) showed that *S. indica* protects plants from the stem base pathogen *Pseudocercosporella herpotrichoides* (= *Tapesia yallundae*), *F. culmorum* and *Blumeria graminis* f.sp. *tritici* (Serfling et al, 2007), and *Serendipita* increased germination rate under pathogen stress to 90.7% compared with control plants which was 71.4% (Serfling et al., 2007). In another study, *S. indica* protected wheat from crown rot disease caused by *Fusarium culmorum* and *F. graminearum* (Rabiey et al., 2015). *Serendipita indica* enhanced the growth of infected plants and increased seed emergence and root biomass more than infected wheat without *S. indica* (Rabiey et al., 2015). *S. indica* showed an ability to attenuate the development of the vascular wilt fungus *Verticillium dahliae* in tomato (Fakhro et al., 2010) and *Arabidopsis* (Sun et al., 2014). *S. indica* conferred protection of *Arabidopsis* against *Alternaria brassicae,* the pathogen responsible for leaf blight disease (Johnson et al., 2013). In maize, *S. indica* protected plants from the root pathogen *Fusarium verticillioides* and improved the biomass and root length and number as compared with non-colonised plants (Kumar et al., 2009). Moreover, *S. indica* controlled black root rot disease caused by

Thielaviopsis basicola and *Fusarium* wilt disease in tomato and controlled *Rhizoctonia* root rot disease in barley (Baltruschat & Kogel, unpublished data).

S. indica co-inoculated with other beneficial microorganisms can also confer biological control against pathogens. For example, *S. indica* and fluorescent *Pseudomonas* strains showed an ability to control *Fusarium oxysporum* f. sp *lycopersici* in tomato (Sarma et al., 2011). In addition, the interaction of *S. indica* with other beneficial fungi such as *Sebacina vermifera*, *Trichoderma viride* and *Trichoderma harzianum*, reduced the severity of *Fusarium oxysporum* f. sp. *lentis*, the causal agent of lentil wilt (Dolatabadi et al., 2012).

Besides its potential to control plant pathogens, *S. indica* has been shown to induce plant resistance to herbivorous insects. A study demonstrated the role of the fungus in improving tolerance of rice plants to rice water weevil *Lissorhoptrus oryzophilus* infestation (Cosme et al., 2016). The plants colonised with *S. indica* produced more shoot and root biomass, tillers, and total root length was higher compared with plants infested with larvae without *S. indica* (Cosme et al., 2016). This result contrasted with an older study, where the researchers found that *Serendipita vermifera* had negative impacts on *Nicotiana attenuata* plants, where the expression of trypsin proteinase inhibitors which reduce the feeding of the herbivore *Manduca sexta*, was reduced in the presence of the symbiont (Barazani et al., 2005).

1.4 Cellular and molecular aspects of *Serendipita*-plant interactions

1.4.1.1 *Serendipita*-plant colonisation

Previous studies showed that *S. indica* colonises roots of barley (Deshmukh et al., 2006; Schafer et al., 2009) and *Arabidopsis* (Jacobs et al., 2011) mainly in the maturation zone and infrequently in the meristematic zone. After inoculation, hyphal growth occurred on the root surface and intercellularly, which led to cell wall digestion of the host plant (Jacobs et al., 2011). Hyphae become intracellular after three to seven days post inoculation. This is followed by death of colonised host cells, although cell death did not cause root necrosis and did not negatively impact root growth (Deshmukh et al., 2006). Chlamydospores form in the rhizodermis within 10-14 days post inoculation in barley plants (Deshmukh et al., 2006).

Plant genes play a role in root colonisation by *Serendipita* species. In barley, the HvBI-1 gene encodes the BAX inhibitor-1 protein that inhibits cell death, whilst *S. indica* repressed the BAX inhibitor-1 protein to activate cell death and successfully colonise barley roots (Deshmukh et al., 2006). However, overexpression of the HvBI-1 gene suppressed *S. indica* colonisation in barley roots (Deshmukh et al., 2006). Another study demonstrated that the level of gibberellic acid can control the degree of barley root colonisation by *S. indica*, where gibberellic acid mutant barley lines showed a reduction of *S. indica* colonisation compared with control plants (Schafer et al., 2009).

1.4.1.2 Gene expression changes in *Serendipita*-plant interactions

Serendipita fungi affect the gene expression of host plants during different stages of colonisation (Schafer et al., 2009). In *Serendipita*-colonised barley plants seven days after inoculation, expression analysis showed up-regulation of genes for gibberellin, auxin and abscisic acid synthesis. In contrast, the levels of genes for salicylic acid, jasmonic acid, and ethylene production were reduced (Schafer et al., 2009). In the *Dendrobium officinale* orchid interaction with a Serendipitaceae strain, high levels of signal transduction pathway associated genes such as calcium-dependent protein kinases were recorded (Zhao et al., 2013). Also, *S. indica* reprogrammed the physiological pathways of the dancing-lady orchid *Oncidium* by inducing genes involved in phytohormone signalling, cell wall metabolism and transcription factor regulation (Ye et al., 2014).

At the beginning of the interaction, plants identify *Serendipita* fungi as pathogens (Schafer et al., 2009). Therefore, the microbe-associated molecular pattern (MAMP) mechanism is triggered, and genes of pathogenesis-related proteins are up-regulated such as PR1 in barley (Schafer et al., 2009) and *Arabidopsis* (Pedrotti et al., 2013). The *Serendipita* fungus inhibits further up-regulation of resistance genes in plants by decreasing the production of hydrogen peroxide (Camehl et al., 2011). *Serendipita* fungi have been shown to overcome defence barriers via regulation of the jasmonic acid pathway in *Arabidopsis* roots. This regulation repressed early root MAMP-triggered immunity (MTI) such as the oxidative burst and late MTI such as salicylic acid and glucosinolate production (Jacobs et al., 2011). Furthermore, gene expression

analysis of *Serendipita*-colonised barley roots showed induction of genes for proteins that may have immune suppressive function (Zuccaro et al., 2011).

Serendipita indica-colonised plants activate systemic resistance against leaf and root pathogens (reviewed in Weiss et al., 2016). The presence of *S. indica* in powdery mildew-inoculated barley plants induced genes encoding pathogenesis-related proteins and heat shock proteins (Waller et al., 2008; Molitor et al., 2011). Moreover, *S. indica* induced systemic resistance to barley roots by increasing the levels of ascorbate and glutathione and minimising the activity of lipid peroxide and other antioxidant enzymes (Harrach et al., 2013). During *Arabidopsis* infection with the powdery mildew pathogen *Golovinomyces orontii*, the symbiotic fungus *S. indica* induces systemic resistance through the activation of jasmonic acid signalling (Stein et al., 2008). Furthermore, *S. indica* increased the content of hydrogen peroxide against *Verticillium longisporum* and *Rhizoctonia solani* in *Arabidopsis*, and cellular analysis showed noticable limiting of pathogen mycelium growth in *Serendipita*-colonised plants (Knecht et al., 2010). *S. indica* inhibited the spread of *Verticillium dahliae* fungus to the aerial parts of *Arabidopsis* and blocked the formation of microsclerotia (Sun et al., 2014).

This research is focused on addressing a number of key knowledge gaps regarding Australian *Serendipita*. As most previous Australian studies have focused on identification of new *Serendipita* spp. from Victoria, NSW and Western Australia (eg. Davis et al. 2015) this study focusses on a little studied region in this context, south east Queensland. Also, most previous studies of Australian *Serendipita* have targetted terrestrial orchids (eg. Huynh et al. 2009) this project will seek to isolate and identify *Serendipita* from native epiphytic orchids. Seed and seedling colonisation experiments are common in studies of Australia orchids (eg. Warcup 1971) but the the impacts of Australian *Serendipita* spp on non orchid plants have not been been tested extensively and there is no information on the changes in gene expression in these interactions.

2. Study objectives

This project aims to isolate and identify the *Serendipita* spp. from native Australian orchids including *Caladenia* and *Bulbophyllum* spp. in south east Queensland,

Australia. This project will also monitor the growth impacts of tomato under *Serendipita* colonisation and investigate the effects of *Serendipita* on tomato defense against the powdery mildew pathogen *Golovinomyces lycopersici*.

The specific objectives of this study are to:

1) Isolate and identify via morphology and DNA sequencing, species of *Serendipita* from south east Queensland *Caladenia* orchids, which have been little studied previously.
2) Isolate and identify via morphology and DNA sequencing species of *Serendipita* from Australian *Bulbophyllum* orchids, which have been little studied both nationally and internationally
3) Test the *Serendipita* fungi for their capacity to improve the growth of tomato plants and examine the changes of gene expression in this process.
4) Test the *Serendipita* fungi for their capacity to protect tomato against powdery mildew and examine the changes of defence gene expression in the interaction.

As mentioned above, Australian orchids are a common source of *Serendipita* spp., and therefore this project highlights the importance of the conservation of orchids located in Queensland. The deforestation rates in Queensland have been higher than other Australian territories from 1995 to 2005 (Bradshaw 2012). Furthermore, the woody vegetation in Queensland between 2015 and 2016 has been cleared at a rate of nearly 400,000 ha/year, 33% faster than in 2014-15 (Dept. of Science, Information Technology & Innovation 2017). It is likely during this land-clearing many orchid plants have been lost along with their associated mycorrhizal community. This may mean that options to use some *Serendipita* spp. as ecofriendly agents to enhance immunity and growth of valuable plant species, has been compromised.

CHAPTER 2: ISOLATION AND IDENTIFICATION OF *Caladenia* ASSOCIATED *Serendipita* spp.

2.1 Introduction

Australia has a rich diversity of orchids. For example, southern Australia has over 1000 orchid species (Jones, 2006) and around 197 orchid species have been recorded in south east Queensland (Stanley & Ross, 1989). Orchids form symbiotic interactions with mycorrhizal fungi and absorb all essential nutrients at the early stage of their life cycle (Graham & Dearnaley, 2012). Generally, orchid mycorrhizal fungi are basidiomycetes (Rasmussen, 2002; Bougoure et al., 2005; Dearnaley, 2007; Waterman & Bidartondo, 2008). Bernard (1899) recorded Sebacinaceae fungi as the first orchid symbionts in the common European orchid *Neottia nidus-avis*. Bernard named these orchid-associated fungi 'rhizoctonias' (Selosse et al., 2011), which represent asexual stages of the Cantharellales (Tulasnellaceae and Ceratobasidiaceae) and Sebacinales (Dearnaley et al., 2013). In 1967, the first interaction between Serendipitaceae and terrestrial orchids of Australia was recorded (Warcup & Talbot, 1967). After that, roots of a diversity of Australian orchids such as *Glossodia*, *Elythranthera* and *Eriochilus* spp. were recorded as habitation for Serendipitaceae spp. (Warcup, 1971). Serendipitaceae fungi form fungal pelotons (hyphal coils) in orchid roots and stem collars and are generally easy to isolate and propagate in pure culture (Bougoure et al., 2005).

In addition to association with orchids, there are various types of *Serendipita* interactions with other plants. Intracellular hyphal coils of *Serendipita* were noted in the rhizoids or ventral cells of liverworts (Kottke et al., 2003; Bidartondo & Duckett, 2010; Newsham & Bridge, 2010). *Serendipita* spp. were found associated with liverworts individually (Bidartondo & Duckett, 2010; Newsham & Bridge, 2010) or joined with Tulasnellales in some thalloid liverworts (Kottke et al., 2003). Also, *Serendipita* associate as root endophytes in *Arabidopsis* (Peskan-Berghofer et al., 2004; Sherameti et al., 2005), barley (Deshmukh et al., 2006; Achatz et al., 2010), tomato (Fakhro et al., 2010; Sarma et al., 2011) and wheat (Rabiey et al., 2015). Furthermore, Serendipitaceae associate with members of the plant family Ericaceae as ericoid mycorrhizal symbionts, where the fungi make a sheath surrounding roots

(Setaro et al., 2006a, b), and they can use their cell wall degrading enzymes to act as saprotrophic fungi (Basiewicz et al., 2012).

Caladenia is a large terrestrial orchid genus with over 370 identified species and subspecies (Phillips et al., 2009) in six subgenera (Hopper & Brown, 2001). *Caladenia* are widespread in south western and south eastern Australia, a few species have been recognized in New Zealand and one species has been identified in New Caledonia and Indonesia (Phillips et al., 2009). *Caladenia* species can be found in temperate woodlands, forests, subalpine woodlands and the margins of the arid zone (Jones, 2006; Hoffman & Brown, 2011) and have been shown to associate almost exclusively with *Serendipita* spp. (Warcup 1971; Huynh et al. 2009; Swarts et al., 2010; Wright et al., 2010; Oberwinkler et al., 2014). *Serendipita* occur in *Caladenia* stems and mainly colonise the collar region subtending the leaf (Ramsay et al., 1986). Although south east Queensland has a large number of terrestrial orchid species recorded (Stanley & Ross, 1989), only a few studies have isolated *Serendipita* from *Caladenia* species, including *C. carnea* from Helidon Hills (Bougoure et al., 2005) and *C. atroclavia* from the Stanthorpe region (Dearnaley et al., 2009).

In fungal taxonomy, PCR amplification and sequencing of the ribosomal DNA is commonly used as a molecular tool to identify fungal isolates and build phylogenetic trees to compare various species (Bougoure et al., 2005). The internal transcribed spacer (ITS) is a variable region located between the genes for the small subunit (SSU) and the large subunit (LSU) ribosomal RNA, and is of particular taxonomic usefulness (White et al., 1990; Bruns et al., 1991; Gardes & Bruns, 1993; Boysen et al., 1996; Kuninaga et al., 1997; Takamatsu, 1998). A number of recent studies have used ITS sequences to distinguish different *Serendipita* spp. in Australia (Davis et al. 2015; Phillips et al. 2016; Oktalira et al., 2021).

In recent years there has been an increase in the number of formally named *Serendipita* spp. *Serendipita restingae* sp. nov. was recently characterised from the terrestrial orchid *Epidendrum fulgens* in Brazil (Fritsche et al., 2021). *Serendipita whamiae* was described from parson's bands orchid, *Eriochilus cucullatus* in the Stanthorpe region of Queensland and named in honour of the well-known naturalist of the Stanthorpe region, Dell Wham (Crous et al., 2020). Seven new *Serendipita* species associated with

Australian terrestrial orchids (largely *Caladenia* spp.) have also been formally described e.g. *S. australiana, S. communis, S. occidentalis, S. rarihospitum, S. secunda, S. talbotii* and *S. warcupii* (Oktalira et al., 2021).

In this part of the project, the fungal associates of *Caladenia* orchids from a previously little explored Australian bioregion are investigated for the first time. Stem collars were obtained from *Caladenia* orchids growing at five sites in south east Queensland. Cultures of isolated fungi were obtained and these were examined macroscopically and microscopically to detect their morphological closeness to *Serendipita*. The ITS-DNA sequences of the isolated fungi were BLAST searched against the GenBank database and phylogenetically analysed to determine their relationship to existing *Serendipita* taxa.

The specific objectives of this component of the study are:

a) To isolate *Serendipita* fungi from multiple *Caladenia* spp. in south east Queensland
b) To identify *Serendipita* fungi using morphological and molecular taxonomic procedures
c) To further characterise isolated *Serendipita* fungi using phylogenetic approaches.

2.2 Methods

2.2.1 Sample collection and media preparation

Whole plants of seven *Caladenia* species were obtained from five locations in south east Queensland (Figures 2.1 and 2.2). Samples were kept on ice while in transit to the laboratory, then around 0.5 cm portions of colonised stems were removed from plants at the USQ laboratory. To kill any root surface-dwelling microorganisms, these stem portions were surface-sterilised by 30 seconds immersion in commercial bleach (0.05% NaOCl) and rinsed 3 times in sterilised distilled water. Aseptically, each stem portion was finely sliced and squashed with a sterilised scalpel blade to release pelotons. Sterile distilled water was mixed with the crushed stem material and divided between 3 x 90 mm petri dishes (3 replicates per stem), and cooled, molten potato dextrose agar (PDA), (Bacto Labs, Liverpool NSW) was poured over the crushed

material. Plates were sealed with parafilm and incubated at 22°C in the dark (Memmert incubator, VIC, Australia). Every 14 hours the plates were checked for fungal growth by light microscopy (E600 photomicroscope; Nikon, Tokyo, Japan). Colonies were assessed for similarity to *Serendipita* fungi in terms of slowness of radial growth (≤2 mm/day), right-angled hyphal branching and the absence of sexual spores as per González-García et al. (2006). *Serendipita*-like colonies were cut from the PDA aseptically and sub-cultured onto a new plate of PDA and this step was repeated until a number of pure isolates were obtained.

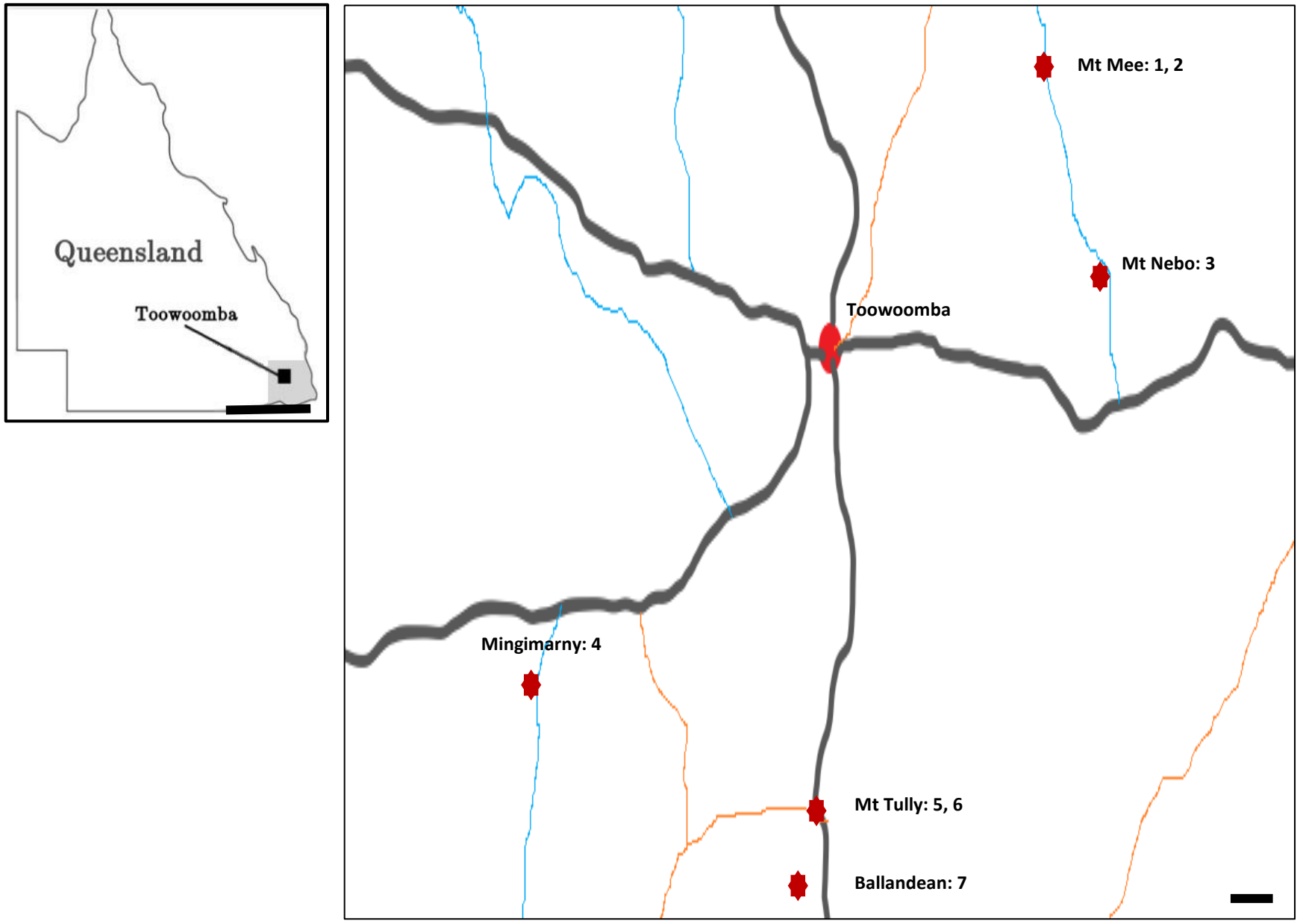


Figure 2.1: (A) Location of the study region in Queensland. (B) *Caladenia* collection locations: 1; *C. catenata*, 2; *C. gracillimum*, 3; *C. picta*, 4; *C. caerulea*, 5; *Caladenia.* sp., 6; *C. fuscata*, 7; *C. filamentosa.* Scale bars are is 200 km and 10 km.

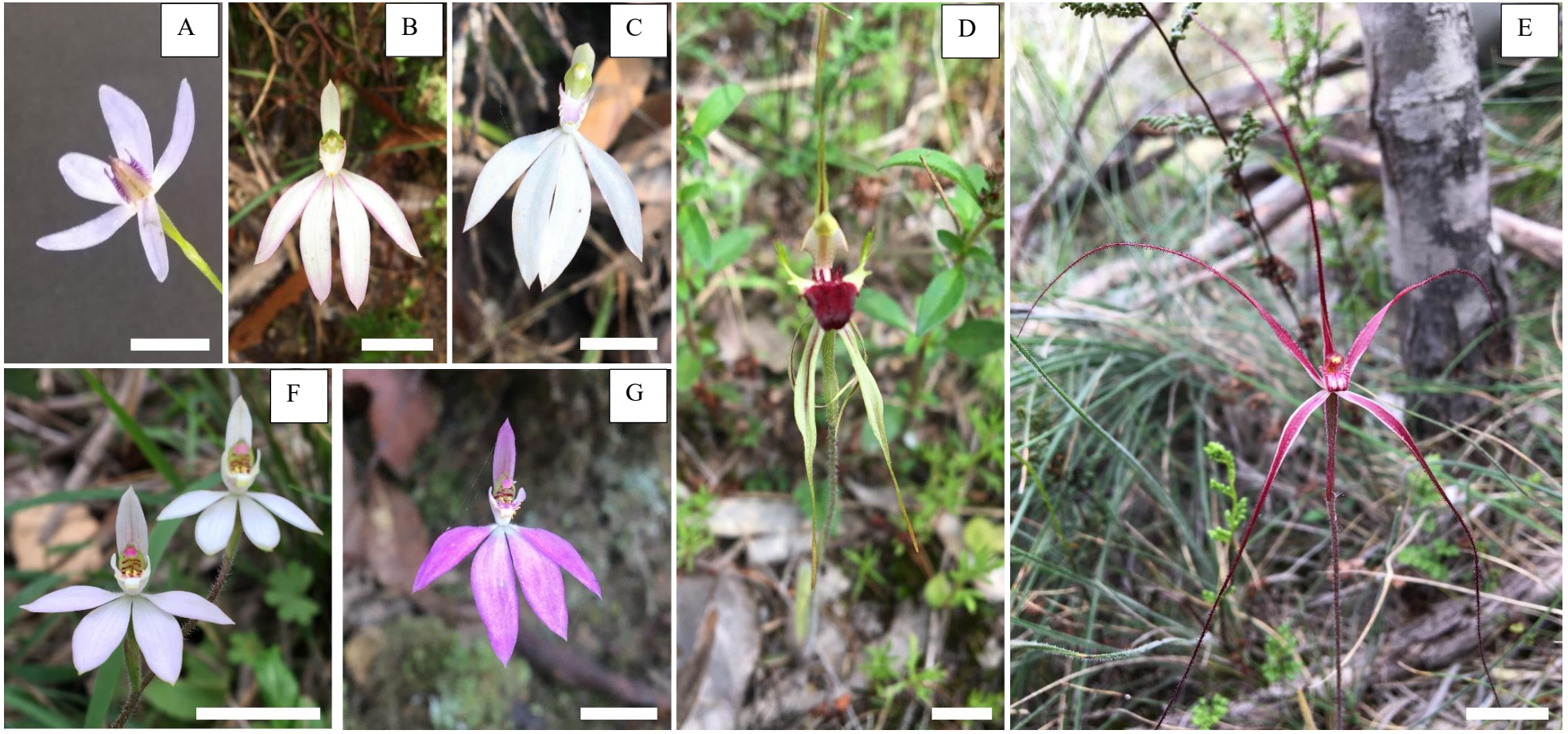


Figure 2. 2: (A-G) Flowers of the *Caladenia* species sampled in this study. A: *Caladenia caerulea,* B: *C. picta*, C: *C. catenata,* D: *Caladenia.* sp, E: *C. filamentosa*, F: *C. fuscata,* G: *C. gracillimum*. Scale bars in A-F are 1 cm. Scale bar in G is 0.5 cm.

2.2.2 Fungal DNA extraction

After macroscopic and microscopic observation of all cultures, one plate from each distinct fungal isolate was selected for DNA extraction, PCR and sequencing from each orchid species. Aseptically, DNA was extracted from pure fungal cultures using an Extract-N-Amp Plant PCR Kit (Sigma-Aldrich, Australia) following the manufacturers' instructions. Approximately 100 mg of the mycelial sample was ground with a sterile micropipette tip in a microcentrifuge tube and then, 100 µl extraction buffer was added. The ground tissue was incubated for 10 min at 95°C in a heating block before being mixed using a vortex. After that, 100 µl of dilution solution was added to the same tube to stop the extraction process.

2.2.3 DNA amplification by PCR

PCR amplification involved adding 1 µl of the extracted DNA to 7 µl sterile milli-Q water, 10 µl of PCR ready mix (Sigma-Aldrich) and 1 µl each of the fungal specific primer ITS1F (Gardes & Bruns 1993) or ITS1 primer and ITS4 primer (White et al. 1990). All PCR reactions were performed in duplicate and a control tube containing 1 µl of sterile H_2O instead of DNA was included. Samples were amplified with 35 cycles of 95°C for one min, 50°C for one min and 72°C for one min, with a final elongation step of 10 min at 72°C using PCR express machine (Thermo Hybaid, UK).

2.2.4 Electrophoresis

An electrophoresis apparatus (Bio-Rad, Gladesville, NSW, Australia) was used to separate the PCR products. Two microlitres of the PCR product was run on a 1% agarose gel containing GelRed nucleic acid stain (Fisher Biotec, Wembley, WA, Australia) and viewed under UV light using a Quantum ST4 gel documentation system (Vilber Lourmat, Fisher Biotech, Wembley, WA, Australia). The gel was photographed using a Quantum capture ST4® image acquisition and analysis software package.

2.2.5 DNA purification, sequencing and phylogenetic analysis

PCR products were purified in preparation for sequencing using a DNA purification kit (Qiagen, Doncaster, VIC, Australia) as per the manufacturers' instructions. Two

microliters of the resulting purified DNA were run on a 1% w/v agarose gel to check the concentration of the purified sample. Samples containing between 17 ng and 30 ng of DNA were sent to the Australian Genome Research Facility (AGRF) in Brisbane for Sanger sequencing. Upon receipt of the returned chromatograms, these were viewed using the Chromas® 2.0 program to check for contamination and quality. A BLAST search against the National Centre for Biotechnology Information (NCBI) database<http://www.ncbi.nlm.nih.gov/> was used to determine the closest species matches. MEGAX software analysis (Kumar et al., 2018) was used to explore the phylogenetic context of the sequenced *Serendipita* fungi by comparing them with other species that have been characterised previously. For aligning sequences, a Clustal-W approach was used with default settings of 15 and 6.66 for both pairwise and multiple parameters. The statistical method Maximum Likelihood was used for constructing phylogenetic trees. A bootstrapping value of 1000 was used under "test of phylogeny". A Kimura 2-parameter model was used under Model/Method; Gamma distributed was selected under "rates among sites" and Partial deletion was chosen under "Gaps/Missing Data treatment".

2.3 Results

2.3.1 Morphological identification of fungal isolates

Subculturing onto a new plate of PDA was repeated until several pure isolates were obtained. Contaminated plates (i.e. fast growing ascomycetes, yeasts and bacteria) were excluded from the sequence analysis. Table 2.1 shows the number of all isolated microbial plates for each *Caladenia* spp.

Table 2. 1: Number of all isolated microbial plates from each *Caladenia* spp. Possible *Serendipita* spp., Ascomycetes, yeast and bacteria were isolated from all *Caladenia* orchids studied.

Source	Possible *Serendipita* spp.	Other microbes			Total
		Ascomycetes	Yeasts	Bacteria	Total
C. gracillimum	10	26	9	20	65
C. catenata	10	16	1	10	37
C. filamentosa	10	3	1	8	22
C. fuscata	6	8	0	22	36
Caladenia sp.	7	10	1	3	21
C. caerulea	16	3	9	0	28
C. picta	15	20	16	0	51

Prior to peloton isolation, cross sections were made of *C. gracillimum, C. catenata, C. fuscata, Caladenia* sp and *C. filamentosa* stems to confirm fungal colonisation. Not all species showed pelotons inside their cortical tissues (Figure 2.3).

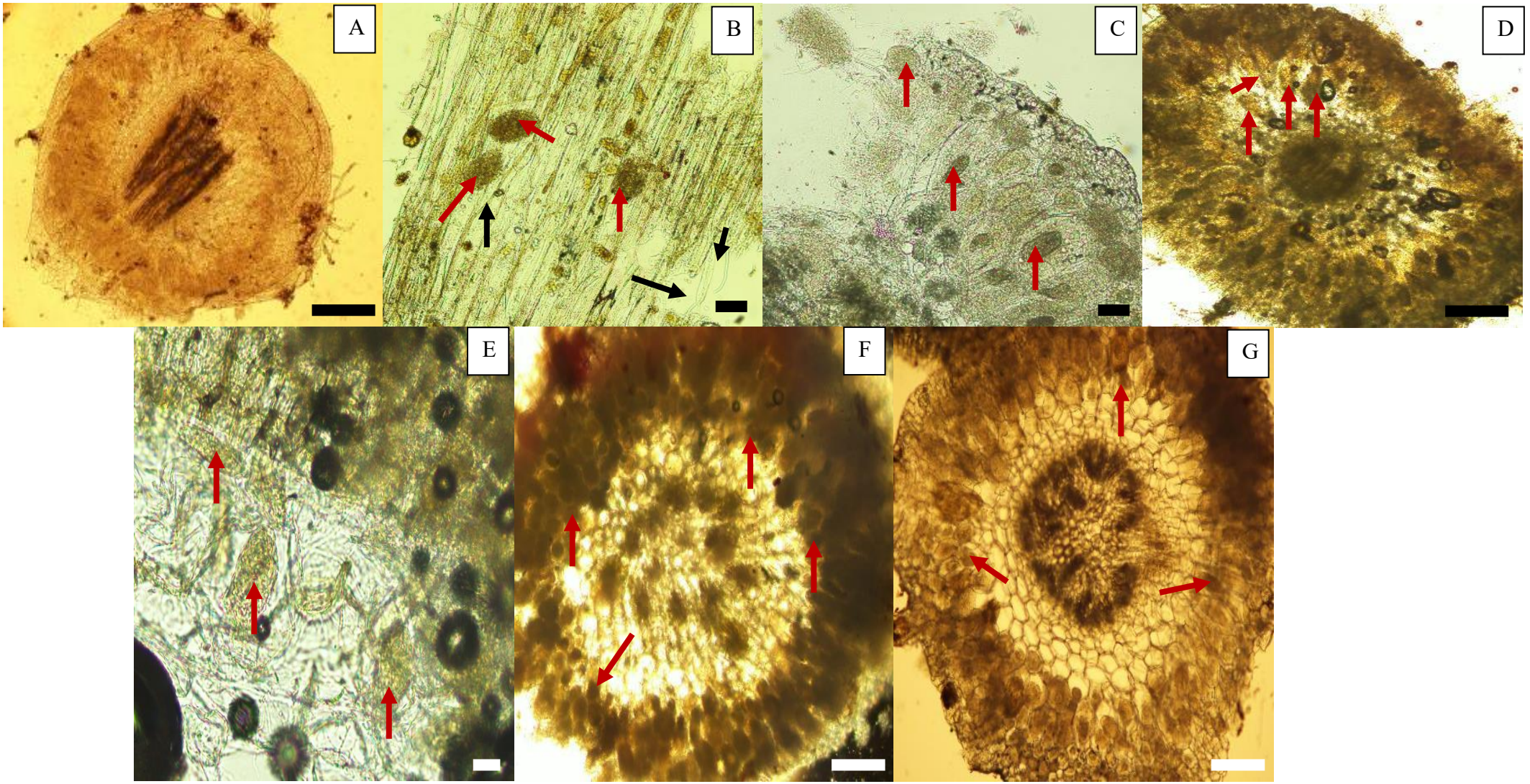


Figure 2. 3: No obvious pelotons inside *C. gracillimum* stem (A). Pelotons (red arrows) and ascomycete hyphae (black arrows) inside a *C. catenata* stem (B). Pelotons (red arrows) inside stems from *Caladenia* sp (C), *C. fuscata* (D), *C. filamentosa* (E) and *C. caerulea* (F). Scale bars of A, D, F and G are 200 µm, scale bars of B and C are 100 µm, scale bar of E is 50 µm.

Hyphal growth from pelotons was observed on many plates and fine hyphae with right-angled branching was noted in pure fungal cultures from a number of *Caladenia* species (Figure 2.4). Fungal culture colour was white or beige for most *Serendipita*-like isolates. Most cultures grew around 5 cm over two weeks on PDA at 22 °C (Figure 2.5). No teleomorphic spore formation was noted in any of the cultures.

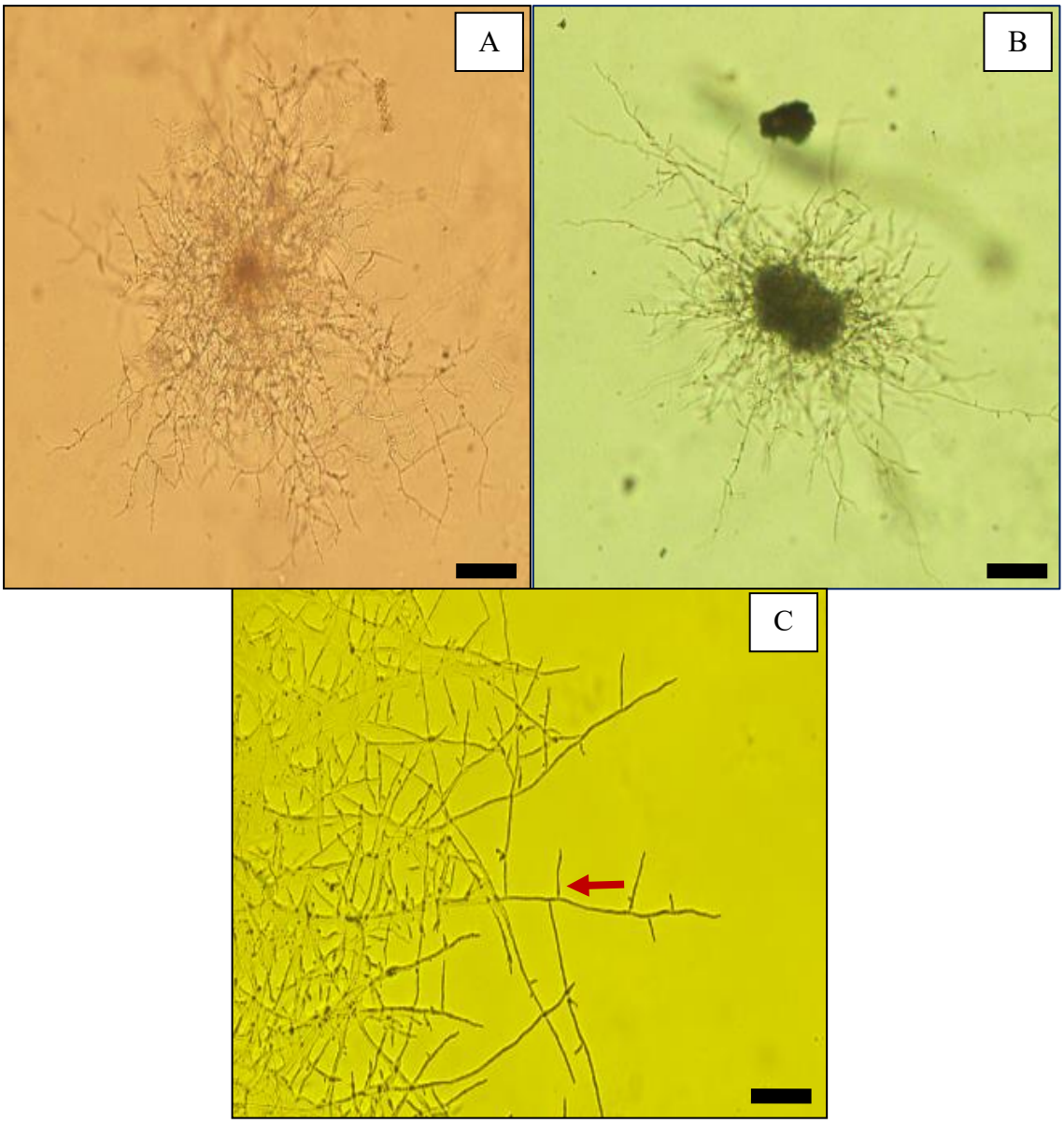


Figure 2. 4: Hyphal growth from pelotons isolated from *C. caerulea* (A) and *C. picta* (B).; C: Right-angled hyphal branch in fungal isolate from *C. caerulea*. Bars are 100µm.

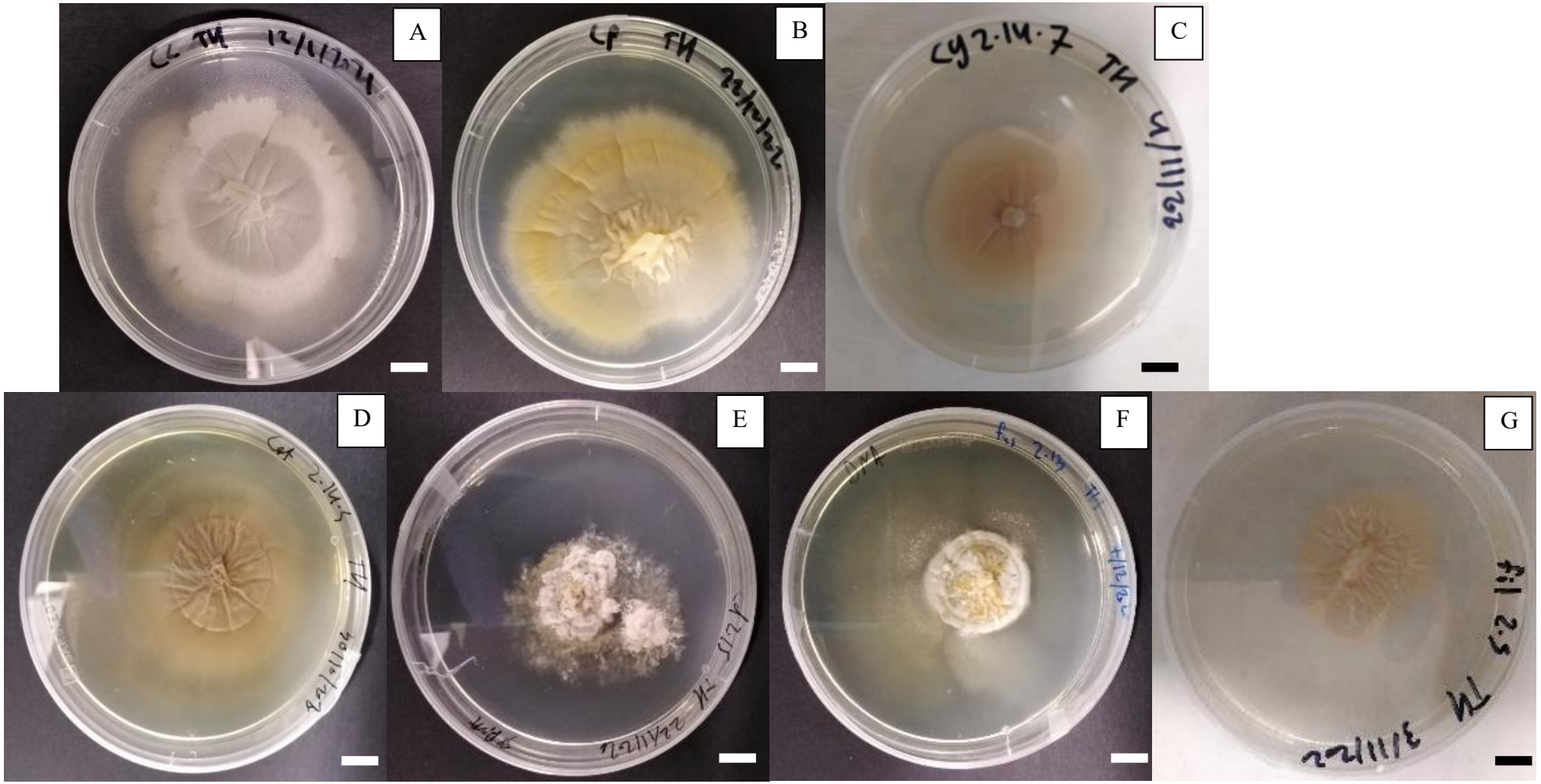


Figure 2. 5: *Serendipita*-like isolated from *Caladenia* spp., A: *C. caerulea,* B: *C. picta*, C: *C. gracillimum,* D: *C. catenata,* E: *Caladenia.* sp, F: *C. fuscata,* G: *C. filamentosa*. Scale bars are 1 cm.

2.3.2 Sequence analysis results

After macroscopic and microscopic inspection, plates of possible *Serendipita* isolates from each orchid species were targeted for ITS-DNA sequencing. The sequencing results are outlined in Table 2.2. BLAST searches of the National Centre for Biotechnology Information (NCBI) database <http://www.ncbi.nlm.nih.gov/> were used to determine closest species matches. All sequences were analysed by both highlighting or removing the "Sequences from type material" option in BLAST (Tables 2.2 and 2.3). The closest type specimen match for the fungal isolates from *C. caerulea, C. picta* and *C. filamentosa* was *Serendipita herbamans* (NR_144842.1), although the highest percentage match was only 83% (over 490 bp) for the fungus from *C. picta*. The ITS-DNA sequence from the *C. fuscata* and *Caladenia* sp. symbionts had distant matches to *Ditangium altaicum* (93% over 236 bp) and *Chaetospermum chaetosporum* (88% over 290 bp) respectively, both taxa from the Sebacinales. The *C. gracillimum* and *C. catenata* symbionts both had distant matches to the type specimen of *Chlamydocillium cyanophilum*, which belongs to the Hypocreales order (Ascomycota). The isolated fungus from *C. caerulea* matched a *Serendipita* sp. isolate (CC1B2_G06) which had been previously isolated from *C. caerulea* (100% similarity over 588 bp, Dearnaley unpublished). Sequences from *C. picta* and *C. fuscata* symbionts had a best match to a *Serendipita* sp. isolate from *Glossodia major* (99% over 590 and 485 bp, respectively). The fungal sequence from *Caladenia* sp was most similar to a *Serendipita* sp. that was isolated from *C. attingens* (98% over 553bp) and the ITS sequence of the *C. filamentosa* isolate most closely matched a *Serendipita* from *Caladenia cairnsiana* (99% over 603bp). The BLAST results of the fungal sequences from *C. gracillimum* and *C. catenata* had a best match to an uncultured and unidentified fungus clone (94% over 550 and 545 bp respectively). BLAST results indicated that the two fungal isolates from *C. gracillimum* and *C. catenata* represent Hypocreales fungi (Ascomycota), Genbank no. (KT269003.1). All of the sequencing results are available in Appendix I.

Table 2. 2: Best alignment hits of BlastN using "Sequences from type material" option. DNA was extracted from plates of possible *Serendipita* spp. from each *Caladenia* spp.

Source	Highest match based on NCBI database (Sequences from type material)	Sequence similarity	Sequence overlap (bp)	Genbank accession no.
Caladenia caerulea	"*Serendipita herbamans* DSM 27534 ITS region; from TYPE material"	82%	387/473	NR_144842.1
Caladenia picta	"*Serendipita herbamans* DSM 27534 ITS region; from TYPE material"	83%	409/490	NR_144842.1
Caladenia fuscata	"*Ditangium altaicum* LE 231836 ITS region; from TYPE material"	93%	219/236	NR_163760.1
Caladenia sp.	"*Chaetospermum chaetosporum* CBS 154.59 ITS region; from TYPE material*"*	88%	255/290	NR_126146.1
Caladenia filamentosa	"*Serendipita herbamans* DSM 27534 ITS region; from TYPE material"	78%	502/645	NR_144842.1
Caladenia gracillimum	"*Chlamydocillium cyanophilum* CBS 246.74a ITS region; from TYPE material"	88%	444/504	NR_153914.1
Caladenia catenata	"*Chlamydocillium cyanophilum* CBS 246.74a ITS region; from TYPE material"	88%	444/504	NR_153914.1

Table 2. 3: Results of BlastN sequences analysis without using "Sequences from type material" setting. Sequences from each possible *Serendipita* spp. plates were used for BlastN analysis.

Source	Highest match based on NCBI database	Sequence similarity	Sequence overlap (bp)	Genbank accession no.
Caladenia caerulea	"*Serendipita* sp. isolate CC1B2_G06"	100%	588/588	MG520176.1
Caladenia picta	"*Serendipita* sp. isolate *G. major* _ TPS _ CLM1864 small subunit ribosomal RNA gene"	99%	583/590	MN872351.1
Caladenia fuscata	"*Serendipita* sp. isolate *G. major* _ EGH _ CLM1862 small subunit ribosomal RNA gene"	99%	481/485	MN872354.1
Caladenia sp.	"*Serendipita* sp. isolate *C. attingens* _ Capel _1224 small subunit ribosomal RNA gene"	98%	544/553	MN872343.1
Caladenia filamentosa	"*Serendipita* sp. isolate CLM2315 small subunit ribosomal RNA gene"	99%	594/603	MT127243.1
Caladenia gracillimum	"Uncultured fungus clone CMH093 18S ribosomal RNA gene"	94%	518/550	KF800184.1
Caladenia catenata	"Uncultured fungus clone CMH093 18S ribosomal RNA gene"	94%	514/545	KF800184.1

2.3.3 Phylogenetic analyses

The ITS sequences of the fungal isolates that were identified as *Serendipita* spp. from *C. caerulea*, *C. picta*, *Caladenia* sp, *C. filamentosa* and *C. fuscata* were further analysed by MEGAX software program and compared with other *Serendipita* taxa including the newly described Australian *Serendipita* species by Oktalira et al. (2021) and Crous et al. 2020 (Figure 2.6).

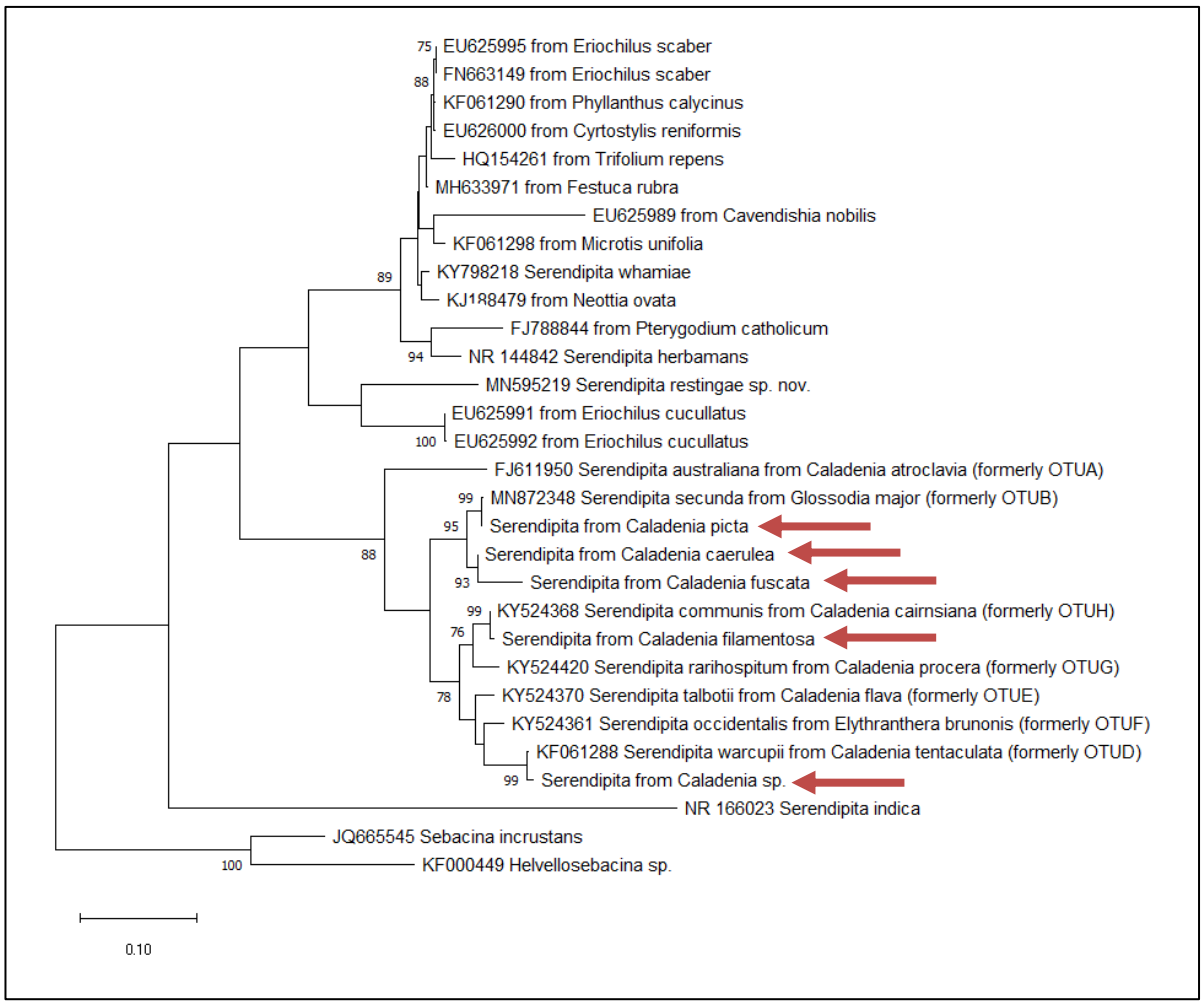


Figure 2.6: Phylogenetic analysis of *Serendipita* spp. isolated from *Caladenia* spp. (red arrows) compared with taxa from other plant species. *Sebacina incrustans* and *Helvellosebacina* sp. are used as outgroups.

The *Serendipita* spp. derived from *C. picta*, *C. caerulea* and *C. fuscata* all appear to be isolates of *Serendipita secunda* as they form a well-supported group (95% bootstrap support) with an isolate of this species from *Glossodia major*. The *Serendipita* isolate from *C. filamentosa* grouped with *Serendipita communis* from *C. cairnsiana* (99% bootstrap support). The *Serendipita* isolate from *Caladenia* sp. grouped with *Serendipita warcupii* from *C. tentaculata* (99% bootstrap support). Thus five of the

seven orchid-fungal partners could be identified as species of known Australian *Serendipita*.

2.4 Discussion

A diversity of ascomycetes, bacteria and yeasts were detected during attempts to isolate *Serendipita* from the seven *Caladenia* spp. Microbial contamination is a common problem in the investigation of orchid mycorrhizas (Warcup, 1985; Clements 1988; Dearnaley et al., 2009) and especially increases when using moist orchid tissues (Warcup, 1981). A variety of different fungal taxa are associated with orchids, but basidiomycetes are the usual mycorrhizal partners (Bougoure et al. 2005; Bougoure & Dearnaley 2005; Dearnaley & Le Brocque 2006; Dearnaley 2006; Bonnardeaux et al. 2007; Irwin et al. 2007). Ascomycetes have been observed as forming mycorrhizas in some orchids (Selosse et al. 2004) but they are rare symbionts. In addition, endophytic bacteria belonging to the genera *Kurthia*, *Pseudomonas*, *Bacillus*, *Xanthomonas*, and *Arthrobacter* have been recorded in Australian terrestrial orchids and may play ecological roles yet to be determined (Wilkinson et al., 1989, 1994a, b). Several studies have suggested that endophytic bacteria have a potential plant growth-promoting role in orchids, where *Sphingomonas* and *Mycobacterium* bacteria promoted 92 % and 88% of seeds of *Dendrobium* orchid to germinate, whereas control plant showed 76% germination rate (Tsavkelova et al., 2016). A *Cymbidium* sp. orchid had increases in root number and root length of approximately 62% and 46% respectively in the presence of *Paenibacillus macerans* bacteria (Faria et al., 2013). *Cymbidium* orchids also had increases of nitrogen and phosphorus content by 68 % and 28 % when colonised with the bacterium *Herbaspirillum frisingense* (Gontijo et al., 2018). The high proportion of bacteria obtained in this study could potentially have been avoided by using antibiotics in the isolation media as is common with studies of this type (eg. Oktalira et al., 2021). However, *Serendipita* have sometimes been shown to be sensitive to antibiotics (Dearnaley, unpublished).

The isolated *Serendipita*-like fungi from *C. picta*, *C. fuscata*, *C. filamentosa*, *C. caerulea* and *Caladenia* sp showed slow growth at approximately 4 mm/day, white/creamy colony colour, right-angled hyphal branching and the absence of a

teleomorph stage. These characteristics were compatible with previously isolated *Serendipita* spp. (Warcup & Talbot, 1967; González-García et al., 2006; Oberwinkler et al., 2013). The fungal isolates from *C. gracillimum* and *C. catenata* also displayed a white/creamy colour but had faster colony growth and under the microscope showed diagonal hyphal branching which give an indication that these isolates did not belong to the genus *Serendipita*. This was reinforced by the molecular analyses that suggested they are Hypocreales.

Many older studies used morphological protocols solely to identify the fungal isolates from Australian native orchids (e.g. Warcup 1971, 1973, 1981, 1991, Ramsay et al., 1986; Clements, 1988; Perkins & McGee, 1995). Molecular approaches using ITS sequence analysis have also been used to identify the fungal partners of Australian terrestrial orchids (Pope & Carter, 2001; Bougoure et al., 2005; Davis et al. 2015; Phillips et al. 2016) and have proven to be superior to morphological protocols. Indeed, much research has shown that analysis of the ITS regions of the rDNA is the most useful approach for identifying fungal species and understanding their biodiversity in the environment (Viaud et al., 2000; Lord et al., 2002; Begerow et al., 2010; Schoch et al., 2012; Hongsanan et al., 2018; Yang et al., 2018; Zhao et al., 2018) despite well-known limitations of fungal identifications based solely on ITS sequences (Kiss, 2012).

Previous studies have identified *Serendipita* spp, associating with Australian orchids. For example, *Serendipita* isolates from various *Caladenia* spp., *Pheladenia deformis* and *Elythranthera brunonis* in Western Australia and Victoria were classified into six operational taxonomic units (OTUs) (Davis et al., 2015). Furthermore, 78 *Serendipita* isolated from 47 *Caladenia* spp. were divided into eight OTUs (Phillips et al., 2016). Here, seven Australian native *Caladenia* species in south east Queensland were sampled to examine the potential occurrence of new *Serendipita* species among them. Five of those seven *Caladenia* spp. have never been included in orchid mycorrhizal studies. *Caladenia caerulea* had been formerly studied (Dearnaley, 2017 unpublished). Previously, *C. catenata* has been collected from the Australian Capital Territory and *Serendipita* spp. have been isolated (Oktalira et al, 2021). In contrast, *Caladenia* sp., *C. picta, C. fuscata, C. filamentosa* and *C. gracillimum* have never been examined for the presence of *Serendipita* inside their stems.

Recently, Australian *Serendipita* spp, previously only with OTUs designations, have been formally described based on morphology and analysis of nuclear ITS and large subunit DNA (Oktalira et al, 2021). The results of sequence comparisons and phylogenetic analysis have confirmed that all isolates of *C. caerulea, C. picta* and *C. fuscata* from Mingimarny, Mount Nebo and Mount Tully respectively, belong to *S. secunda*. The name *S. secunda* indicates that this species was the second *Serendipita* described from a *Caladenia* host (Oktalira et al, 2021). Phylogenetic analysis also showed that the *Serendipita* from *C. filamentosa* in Ballandean is *S. communis* (Oktalira et al, 2021). *Serendipita communis* was originally isolated from *C. cairnsiana* in Western Australia and was given the name due to its common occurrence in Australia (Oktalira et al, 2021). This study considerably extends the range of *Serendipita communis* in Australia. Phylogenetic analysis also demonstrated that the *Serendipita* from *Caladenia* sp. in Mount Tully is *S. warcupii*. This fungus was named after J. H Warcup, whose isolates from three Australian orchids belong to this species (Oktalira et al, 2021). *Serendipita warcupii* was also isolated from the widespread *C. flava* in Western Australia (Sommer et al., 2012; Phillips et al., 2016; Oktalira et al, 2021). The occurrence of *Serendipita* ssp. among multiple host species and over wide geographic ranges has been previously discussed (Davis et al., 2015). *Serendipita* spp. appear to have an ability to inhabit a diversity of habitat types (Tesitelova et al 2015). Their saprotrophic ability (Lahrmann et al., 2015) and ability to live independently of plant hosts, might explain their wide distribution across the Australian continent.

The sequences from the *C. gracillimum* and *C. catenata* fungal isolates were not used in the phylogenetic analysis because they belonged to the Hypocreales order, (Ascomycota). The absence of *Serendipita* mycobionts suggests that *C. gracillimum* may be exceptional among the genus. Dearnaley et al. (2009) noted that not all plants of *C. atroclavia* contained fungal pelotons and it is possible that the orchid cells had completely digested the mycobiont and absorbed the nutrients from the fungal hyphae (Rasmussen & Whigham, 2002, Athipunyakom et al., 2004). Other studies suggest that high levels of soil nutrients such as nitrogen and phosphorus can inhibit plants from taking on mycorrhizal partners (Treseder & Allen, 2002; Mujica et al., 2016), which could be the case here.

Both pelotons and ascomycete hyphae were detected in *C. catenata* stems, and it is likely that the mycorrhizal fungus was overgrown by these latter microbes thus explaining the molecular identification. Some ascomycetes have been recorded as mycorrhizal or endophytic fungi associated with terrestrial orchids. For example, a *Tuber* sp. has been found as a mycorrhizal fungus in *Epipactis* (Bidartondo et al. 2004; Selosse et al. 2004). In addition, species of *Cypripedium* orchids provide a habitat for endophytic *Phialophora* spp. (Shefferson et al. 2005). Five Pezizalean genera *Peziza*, *Terfezia*, *Morchella*, *Geopyxis* and *Wilcoxina* have been associated as mycorrhizal fungi with *Gymnadenia conopsea* (Stark et al., 2009). Members of the Hypocreales order such as *Fusarium* spp. have been reported as endophytic fungi associated with *Grammatophyllum speciosum* seeds (Salifah et al., 2011) and *Himantoglossum adriaticum* (Pecoraro et al., 2013). Another Hypocreales member, *Trichoderma* has been identified as an orchid endophyte in *Wullschlaegelia calcarata*, *Lepanthes caritensis* and *L. rupestris* (Bayman & Otero 2006). The natural role of ascomycete fungi inside orchid roots is still not fully understood (Oliveira et al., 2014), and may range from symbiosis to pathogenesis (Arnold, 2007). Other studies suggested that ascomycetes decompose soil substrate during their colonisation of the outer layer of the roots and this improves nutrient acquisition (Herrera et al., 2010). Further investigation of the ascomycete isolates from *C. catenata* and *C. gracillimum* may be warranted to understand the role of ascomycetes inside orchid roots.

CHAPTER 3: ISOLATION AND IDENTIFICATION OF *Bulbophyllum* ASSOCIATED *Serendipita* spp.

3.1 Introduction

Around 70% of orchid species in the world and 18% in Australia are epiphytic or lithophytic plants (tree or rock-dwelling) (Jones, 2006). Symbioses in epiphytic orchids have only been little studied but some researchers have identified the mycorrhizal partners of plants via DNA sequencing and seed germination confirmation (Nontachaiyapoom et al., 2011; Sathiyadash et al., 2014; Khamchatra et al. 2016). Mycorrhizal interactions are critical to epiphytic orchids as the environment in which they inhabit is more desiccating than soil (Yoder et al., 2000). Epiphytic orchids usually share a substrate with other plants such as mosses and liverworts. In the Costa Rican orchid *Ionopsis utricularioides*, epiphytic plants grew on moss-covered guava trees and had higher mycorrhizal colonisation rates than orchids living on non-mossy trees (Osorio-Gil et al. 2008). The water retention properties of mosses appear significant for epiphytic orchids and their associated mycorrhizal fungi (Osorio-Gil et al. 2008).

In Australia, only a few studies have investigated the mycorrhizal fungi associated with epiphytic orchids. One study identified *Ceratobasidium* spp. colonising the roots of three epiphytic orchids, *Sarcochilus hillii, S. parviflorus* and *Plectorrhiza tridentata* in south eastern Australia (Gowland et al. 2007). Another study showed that a single species of *Ceratobasidium* was associated with the rare epiphytic orchid *Sarcochilus weinthalii*, native to north-east New South Wales and south east Queensland (Graham & Dearnaley 2012).

Bulbophyllum is the largest genus in the Orchidaceae and includes more than 2000 species. The genus is also considered the second largest in the angiosperms after the pea genus *Astragalus* (Frodin, 2004). There have only been a few studies of the mycorrhizal associated fungi of *Bulbophyllum* species. Calvert (2017; unpublished BSc Honours thesis) discovered two species of *Serendipita* in *B. exiguum and B elisae* in south east Queensland, Australia in 2017. Martos and Selosse (2008 unpublished data) identified *Serendipita* isolated from roots of *B. macrocarpum, B. nutans and B.*

longiflorum from Reunion Island in the Indian Ocean, east of Madagascar. Later, it was noted that six species of *Bulbophyllum* located in Reunion Island: *B. pusilum, B. longiflorum, B. densum, B. variegatum, B. nutans* and *B. macrocarpum* had symbionts belonging to both the Sebacinales and Tulasnellaceae without presenting detailed evidence to reinforce their findings (Martos et al., 2012). An Indonesian study identified mycorrhizal fungi belonging to *Tulasnella* sp. associated with *B. beccarii* roots from West Kalimantan (Suryantini et al., 2015).

This thesis chapter reports an investigation of the mycorrhizal fungi from species of native *Bulbophyllum* from multiple locations in south east Queensland. Fungal spp. were isolated from the roots of five different *Bulbophyllum* spp. and were examined macroscopically and microscopically to detect their similarity to *Serendipita* and to characterise them further. Sequencing of ITS and LSU DNA demonstrated that *Bulbophyllum* orchids contain a number of previously unidentified *Serendipita* species.

The specific objectives of this component of the study are:

d) To isolate *Serendipita* fungi from multiple *Bulbophyllum* spp. in south east Queensland
e) To identify *Serendipita* fungi using morphological and molecular taxonomic approaches
f) To further characterise novel *Serendipita* fungi using light and fluoresence microscopy and phylogenetic approaches.

3.2 Methods

3.2.1 Sample collection and media preparation

Root samples were obtained from native Australian species of epiphytic and lithophytic *Bulbophyllum* at four locations in south east Queensland (Figures 3.1 and 3.2). The site at Mount Nebo consisted of subtropical rainforest with the sampled *B. exiguum* colony growing on the trunk of a 15m tree identified as a *Rhodamnia* sp while the *B. schillerianum* colony was growing on the side of a basalt cliff face ovrehanging Stony Creek. The *B. exiguum* colony at Queen Mary's Falls was growing on the side of a basalt boulder in a moist, shaded gully. At Mount Tully *B. exiguum* colonies were

growing inside the crack of a large, split granite boulder close to the summit. *Bulbophyllum bracteatum* and *B. shepherdii* plants sampled at Queen Mary's Falls were growing on basalt boulders on a cliff face. The *B. minutissimum*, plants sampled at a private property in Yalangur QLD, were growing in a dense mat over basalt slabs at the top of a steep, forested hill. Samples were kept on ice while in transit to the laboratory. Small portions of colonised roots (around 0.5 cm) were removed from plants at the USQ laboratory. To kill any root surface-dwelling microorganisms, the root portions were surface-sterilised by 30 seconds immersion in commercial bleach (0.05% NaOCl) and rinsed 3 times in sterilised distilled water. Aseptically, each root portion was finely sliced and squashed with a sterilised scalpel blade to release pelotons. Sterile distilled water was mixed with the crushed root material and divided between 3 x 90 mm petri dishes (3 replicates per root), and cooled, molten PDA poured over the root mixture. Plates was sealed with parafilm and incubated at 22°C in the dark. Every 14 hours, plates were checked for fungal growth by light microscopy. Colonies were assessed for similarity to *Serendipita* fungi in terms of slowness of radial growth (≤2 mm/day), right-angled hyphal branching and the absence of sexual spores as per González-García et al. (2006). *Serendipita*-like colonies were cut from the agar and sub-cultured onto a new plate of PDA. This step was repeated until a number of pure isolates were obtained.

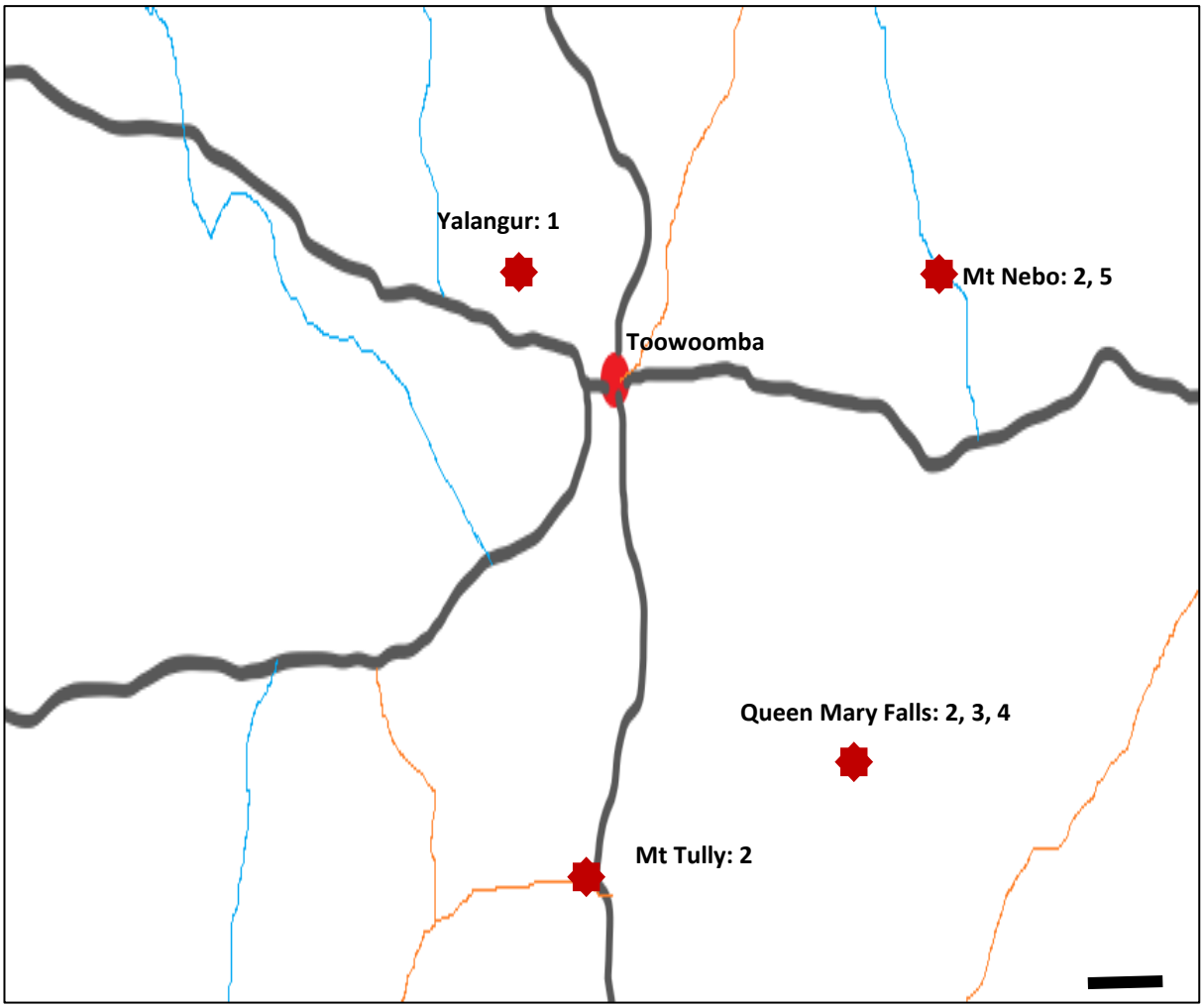


Figure 3. 1: *Bulbophyllum* collection locations: 1; *B. minutissimum*, 2; *B. exiguum*, 3; *B. bracteatum*, 4; *B. shepherdii*, 5; *B. schillerianum*. Scale bar is 20 km.

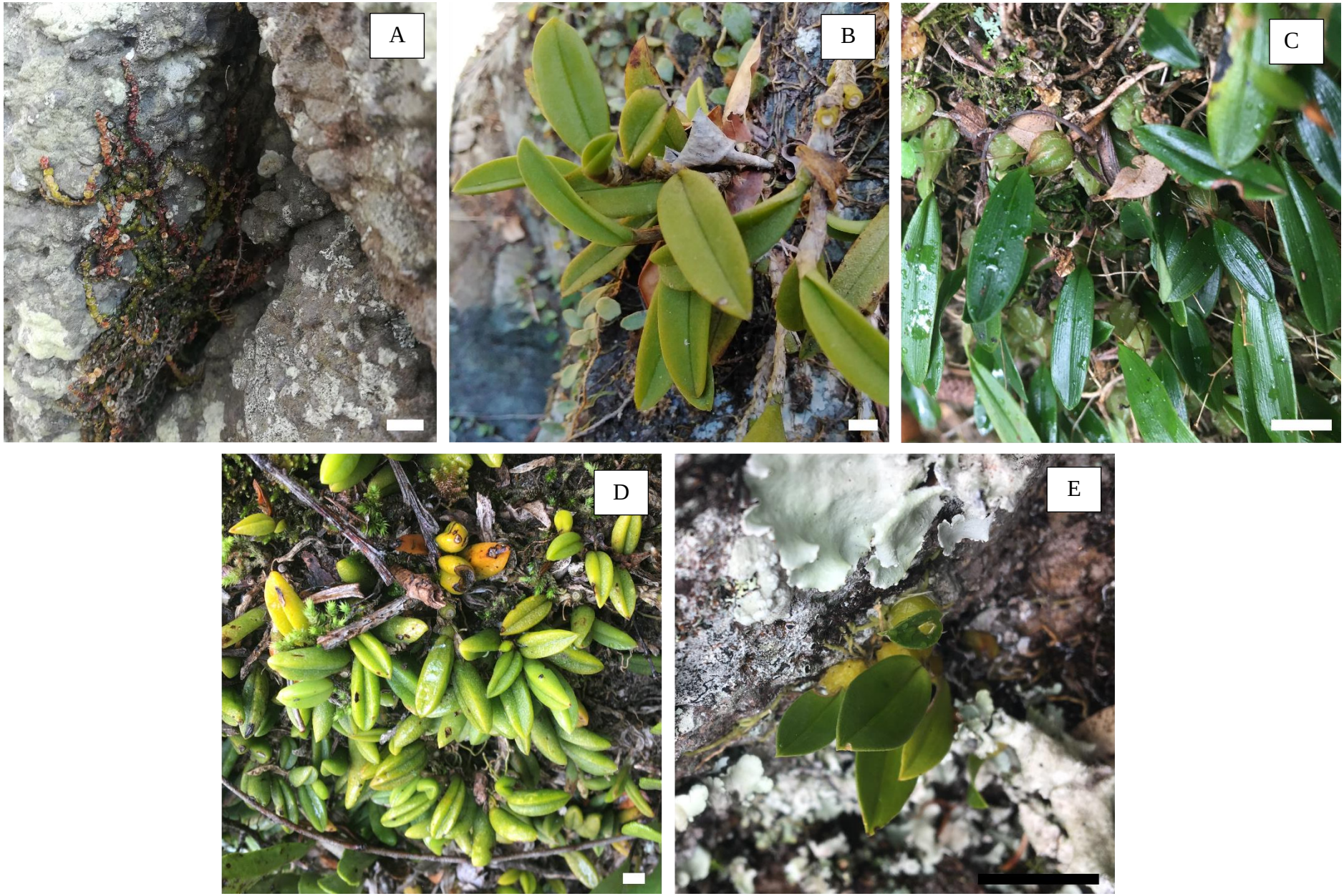


Figure 3. 2: ***Bulbophyllum*** **species in their native habitat. (A)** ***B. minutissimum*****, (B)** ***B. schillerianum*****, (C)** ***B. exiguum*****, (D)** ***B. shepherdii*** **and (E)** ***B. bracteatum*****. The scale bars are 1 cm.**

3.2.2 Fungal DNA extraction

An Extract-N-Amp Plant PCR Kit (Sigma-Aldrich) was used for fungal DNA extraction following the manufacturers' instructions. Approximately 100 mg of the fungal culture was ground with a plastic pestle in a microcentrifuge tube. Then, 100 µl extraction buffer was added, and the mixture was incubated in a heating block for 10 min at 95°C. During the incubation, the mixture was homogenised by vortexing 2-3 times. After that, 100 µl of dilution solution was added to stop the extraction process.

3.2.3 DNA amplification by PCR

PCR was performed by adding 4 µL of the extracted DNA to 4 µL sterile milli-Q water, 10 µL of PCR ready mix (Sigma-Aldrich) and 1 µL of 10 µmoles each of the fungal specific primer ITS1F (Gardes & Bruns 1993) and the ITS4 primer (White et al. 1990). Samples were amplified with 35 cycles of 95°C for one min, 50°C for one min and 72°C for one min, with a final elongation step of ten min at 72°C. In addition, large subunit primers LR7 and LROR (Hopple & Vilgalys 1999) were used for the further characterisation of new *Serendipita* species with the same PCR thermocycling conditions.

3.2.4 Electrophoresis and visualisation by a gel documentation system

A BioRad electrophoresis apparatus was used to separate the PCR products. A 1% agarose gel was prepared by adding 1 g agarose powder to 100 ml TAE buffer. After cooling, 5 µl of GelRed was added for gel staining. Two µl of the PCR product was added to 0.5 µl of running dye and then run on the agarose gel. The finished gel was viewed under UV light using a Quantum ST4 gel documentation system and photographed using a Quantum capture ST4® image acquisition and analysis software package.

3.2.5 DNA purification, sequencing and phylogenetic analysis

Samples containing between 17 ng and 30 ng of DNA were sent to Macrogen (Seoul, South Korea) for purification and sequencing. The returned sequences were viewed using the Chromas® 2.0 program to check for contamination and quality. A BLAST search against the NCBI database was used to determine closest species matches.

MEGAX software analysis was used to explore the phylogenetic context of the sequenced fungi by comparing them with other species that have been sequenced previously. For aligning sequences, a Clustal-W approach was used with default settings of 15 and 6.66 for both pairwise and multiple parameters. The statistical method Maximum Likelihood was used for constructing the phylogenetic tree. A bootstrapping value of 1000 was used under "test of phylogeny". A Tamura-Nei model was used under Model/Method; Gamma distribution with invariant sites was selected under "rates among sites" and Partial deletion was chosen under "Gaps/Missing Data treatment".

3.2.6 Teleomorph production in *Serendipita*

To induce the teleomorphic states of isolated *Serendipita* taxa, fungi were grown on complete yeast medium B (CYMB) (Noel et al., 1995). This was prepared with the following components: 10 g glucose, 5 g bactopeptone, 2.5 g yeast extract, 0.46 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 1 g K_2HPO_4 and 20 g agar (Sigma Aldrich). All components were added to one litre of distilled water and the pH was adjusted to 6.8, before adding the agar. A *Serendipita* mycelial plug (1 cm^3) was incubated for 4 days on CYMB media 90 mm plates in the dark at 27 ºC (Thermoline Scientific, NSW, Australia). Next a mycelial plug from the edge of the growing colony was removed from the CYMB plate and placed on water agar (20 g agar added to 1 litre of distilled water, pH 6.8) for 8 days in the dark at 27 ºC. After that, the water agar plates were placed at 4 ºC under dark conditions for 2 days to induce sporulation by cold shock. Finally, the water agar plates were moved to an incubator at 27 ºC for 11 days (12 hours light/ 12 hours dark). Plates were grown without parafilm sealing the plate due to the importance of aeration in basidium production. Plates were examined daily for probasidium formation.

3.2.7 Colony growth and monilioid cell characterisation

The colony diameter was measured after plates were incubated at 22 ºC in the dark for two weeks (Memmert, Australia). The colony diameter of three petri dishes was measured across two perpendicular axes of the colony. The morphology of monilioid cells including the shape, size and branch characters were also examined by microscopic examination of mycelium growing on PDA with the 10 times objective.

Characteristics of monilioid cells was recorded for 20 separate hyphal branches for each fungal isolate from three plates.

3.2.8 Fluorescence staining of fungal nuclei and septa

Fluorescence staining of nuclei and septa was performed on the potential new *Serendipita* species following Hua'an et al. (1991). Mycelial growth chambers were prepared by placing glass microscope slides on metal rods on filter paper (Whatman, UK) inside 15 cm glass Petri dishes and autoclaving them (Figure 3.3). After these were autoclaved, around 200 µl of molten PDA media was placed on the glass slides and allowed to cool for 10-15 min in a biological safety cabinet (BH2000 model Clyde-Apac, NSW, Australia). An agar block (approximately 1 cm^3) of the fungus was transferred to the middle of the slide. The filter paper underneath the slides was moistened with 1 ml of sterile Milli-Q H_2O, the lid replaced and sealed with parafilm and the chambers were incubated in the dark at 22°C for one to two weeks. After incubation, the slides with a growing fungal colony were removed from the growth chamber and dried in the biological safety cabinet for 10 min. One drop of Hoechst dye (Sigma Aldrich) was then pipetted onto the mycelium and covered with a coverslip. Slides were examined using a UV filter on a E600 fluorescence microscope (Nikon) with a forty times objective.

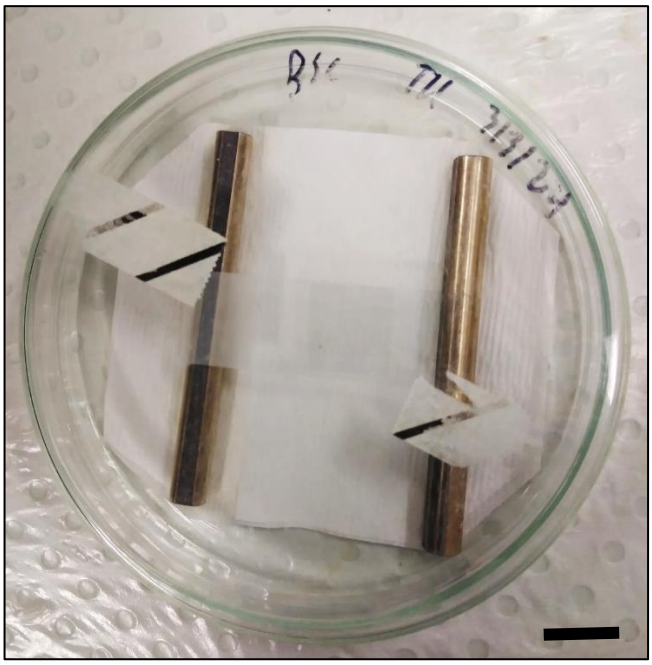


Figure 3. 3: Chamber used for *Serendipita* mycelial growth in preparation for nuclei and septa staining. Scale bar is 1 cm.

3.3 Results

3.3.1 Morphological identification of fungal isolates

Subculturing onto a new plate of PDA was repeated until several pure isolates were obtained. Contaminated plates (ie. fast growing ascomycetes, yeasts and bacteria) were excluded from sequencing analysis. Table 3.1 shows the number of isolated microbial cultures from this part of the study.

Table 3. 1: Total number of isolated microbes from each *Bulbophyllum* spp. The isolated microbe plates included possible *Serendipita* spp., Ascomycetes, yeast and bacteria.

Source	Possible *Serendipita* spp.	Other microbes			Total
		Ascomycetes	Yeast	Bacteria	
B. minutissimum	3	12	1	0	16
B. schillerianum	8	45	0	0	53
B. exiguum	21	130	0	0	151
B. shepherdii	3	3	0	0	6
B. bracteatum	11	40	2	0	53

Sections were made of the sampled orchid roots to confirm mycorrhizal fungal colonisation. A longitudinal hand section was made of *B. schillerianum* roots (Figure 3.4 A). Transverse hand sections were made of *B. bracteatum*, *B. exiguum* and *B. shepherdii* roots (Figure 3.4: B-D). It was difficult to obtain a root cross section for *B. minutissimum* due to its small size.

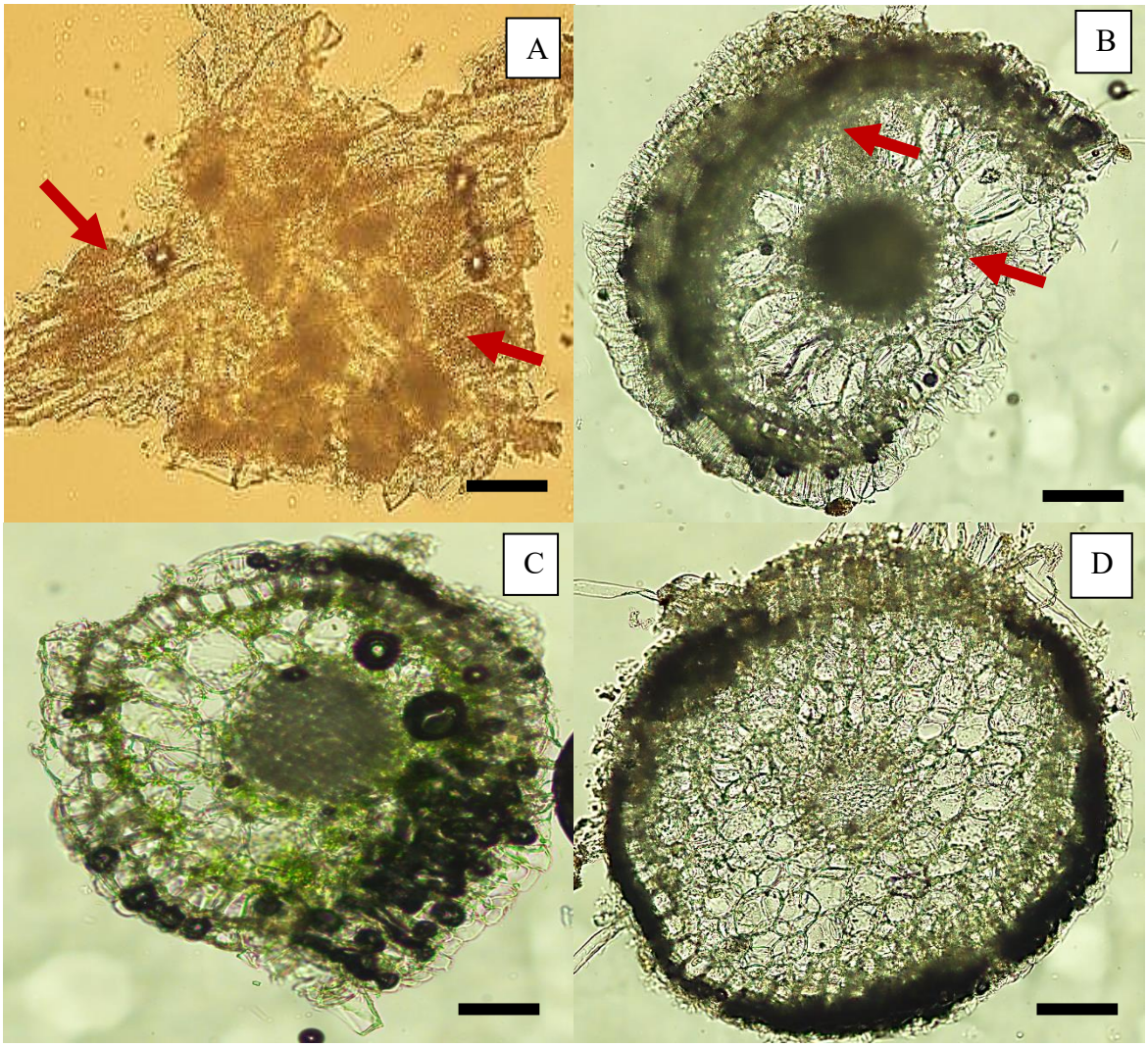


Figure 3. 4: Pelotons (red arrows) inside of *B. schillerianum* (A) and *B. bracteatum* roots (B). No obvious pelotons inside of *B. exiguum* (C) and *B. shepherdii* roots (D). Scale bars of A, B, C & D are 100 µm.

The *Serendipita*-like fungal isolates from *B. bracteatum* had a growth rate of 0.64 mm/day while the *Serendipita*-like fungus derived from *B. schillerianum* had a growth rate of 1.7 mm/day. The colour of the *B. bracteatum* and *B. schillerianum* isolates was creamy white. Fine hyphae with right-angled branching were noted under the

microscope for both of these putative *Serendipita* spp. (Figure 3.5 B & D). Colonies of the putative *Serendipita* fungus from *B. minutissimum* had a growth rate of 3.5 mm/day on PDA. The texture of the *B. minutissimum* isolate was floccose showing frequent white to light cream sectors (Figure 3.5 A). The putative *Serendipita* fungal isolate from *B. shepherdii* also showed a floccose texture on PDA. It had a white to cream colour attaining an approximately 3 mm/day growth rate on PDA (Figure 3.5 C). Finally, the putative *Serendipita* fungus from *B*. *exiguum* had a cream margin with a dark brown colour centre with a 3.5 mm/day growth rate (Figure 3.5 E).

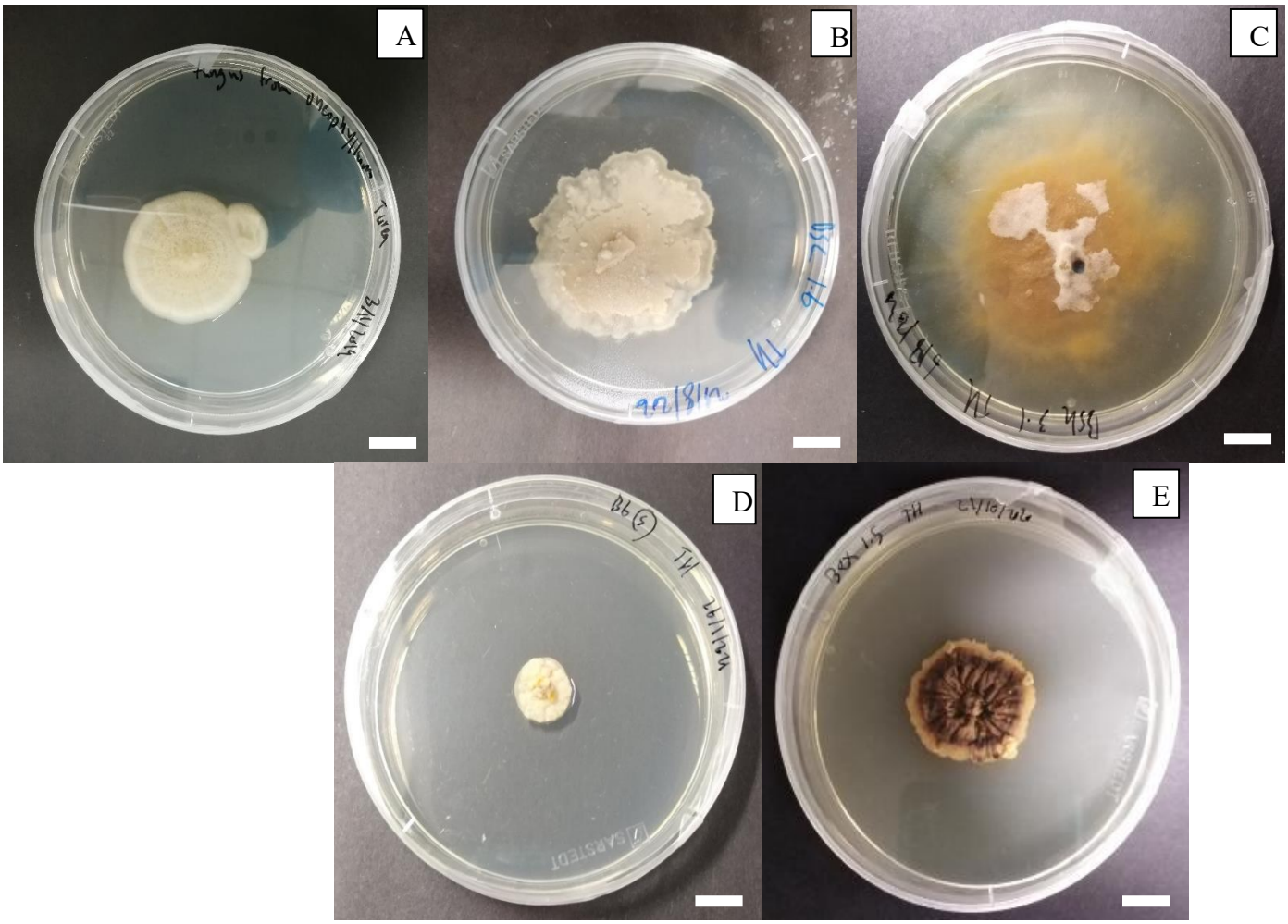


Figure 3. 5: Putative *Serendipita* fungal cultures isolated from *Bulbophyllum* spp., A: *B. minutissimum*, B: *B. schillerianum* C: *B. shepherdii*, D: *B. bracteatum*, E: *B. exiguum*. Bars are 1 cm.

3.3.2 Sequence analysis results

Two positively identified *Serendipita* isolates were obtained from the five *Bulbophyllum* species. These two isolates had similarities lower than 95% to known

fungi when compared against GenBank using the BLAST search with and without "sequences from type material" option (Tables 3.2 and 3.3). The option of "sequences from type material" provided more information of the identity of the isolates. BLAST analysis using the "Sequences from type material" setting indicated that the fungal isolates from *B. bracteatum* and *B. schillerianum* had a closest match to *Serendipita herbamans* (NR_144842.1), ie. *B. bracteatum* 81% over 458 bp and *B. schillerianum* 86% over 353 bp. BLAST analysis without using the "Sequences from type material" setting indicated that the fungal isolates from *B. bracteatum* and *B. schillerianum* had a closest match to different clones of uncultured Sebacinales. For the *B. bracteatum* isolate this was 87% over 907 bp to accession sequence KJ188464.1, and for the *B. schillerianum* isolate this was 92% over 1035 bp to accession sequence KJ188488.1. Analysis of the ITS sequences from the *B. shepherdii* isolate with and without "Sequences from type material" option showed that the fungal isolate belongs to the Tulasnellaceae family and had a closest match to *Tulasnella hadrolaeliae* (87% over 388bp). BLAST analysis of the sequences from the *B. minutissimum* isolate without the "Sequences from type material" option showed that the fungal isolate matched most closely to an unidentified fungal sp. labelled as "isolate B1" and matched most closely to *Preussia isomera* by using the "Sequences from type material" option. Analysis using the type material setting for the fungus from *B. exiguum* showed that it had a closest match to *Nodulisporium indicum*, an Ascomycete in the Xylariales order. This result was reinforced when carrying out a BLAST search without the "Sequences from type material" option. The full sequences from these investigations are available in appendix II. The fungal LSU primers were used to further characterise the sequences from the *B. bracteatum* and *B. schillerianum* symbionts as they clearly were members of the Serendipitaceae but likely novel species of *Serendipita*. Characterisation of the LSU DNA of the other isolated fungi was not conducted as these fungi do not belong to the Serendipitaceae.

Table 3. 2: Best alignment hits of BLASTN using sequences from type material. ITS analysis was used for possible *Serendipita* spp. plates from each *Bulbophyllum* spp.

Source	Highest match based on NCBI database (Sequences from type material)	Sequence similarity (%)	Sequence overlap (bp)	Genbank accession no.
B. bracteatum	"*Serendipita herbamans* DSM 27534 ITS region; from TYPE material"	81%	372/458	NR_144842.1
B. schillerianum	"*Serendipita herbamans* DSM 27534 ITS region; from TYPE material"	86%	302/353	NR_144842.1

B. shepherdii	"*Tulasnella hadrolaeliae* isolate COAD2889 internal transcribed spacer"	87%	339/388	MN385726.1
B. minutissimum	"*Preussia isomera* CBS 318.65 ITS region; from TYPE material"	93%	489/527	NR_103588.1
B. exiguum	"*Nodulisporium indicum* CBS 124.83 ITS region; from TYPE material"	81%	513/631	NR_166005.1

Table 3. 3: Sequences analysis without using "sequences from type material" option. ITS analysis was used for possible *Serendipita* spp. from each *Bulbophyllum* spp. using BLASTN.

Source	Highest match based on NCBI database (without sequences from type material)	Sequence similarity (%)	Sequence overlap (bp)	Genbank accession no.
B. bracteatum	"Uncultured Sebacinales clone LP1-1S 18S ribosomal RNA gene"	87%	792/907	KJ188464.1
B. schillerianum	"Uncultured Sebacinales clone LP27-15S 18S ribosomal RNA gene"	92%	956/1035	KJ188488.1
B. shepherdii	"Uncultured Tulasnellaceae clone 16-14 small subunit ribosomal RNA gene"	95%	569/596	KX587473.1
B. minutissimum	"Fungal sp. isolate B1 II (2-3 a) internal transcribed spacer 1"	97%	506/521	MW603374.1
B. exiguum	"Xylariales BPV101a voucher CEQCA-M1193 internal transcribed spacer 1"	89%	523/586	KC771473.1

Following the results of the ITS analysis, further sequencing of the fungal isolates from *B. bracteatum* and *B. schillerianum* was conducted using PCR amplification LSU primers (Table 3.4 & 3.5). BLAST results of the LSU sequences using the type material setting indicated that the fungal isolates from *B. bracteatum* and *B. schillerianum* had a closest match to *Serendipita herbamans* (KF061285.1), 93% similarity over 1177bp for the *B. bracteatum* isolate and 93% similarity over 1169bp for the *B. schillerianum* isolate. BLAST results of the LSU sequences without using the type material setting indicated that the fungal isolate from *B. bracteatum* had a closest match to Sebacinaceae sp. (JF799765.1), 95% similarity over 1167bp. The fungal isolate from *B. schillerianum* had a closest match to a Sebacinales sp. (JF906111.1), 93% similarity over 1228bp.

Table 3. 4: The results of BLASTN with "sequences from type material". LSU analysis was used for further characterisation of *Serendipita* spp. from *B. bracteatum* and *B. schillerianum*.

Source	Highest match based on NCBI database (Sequences from type material)	Sequence similarity	Sequence overlap (bp)	Genbank accession no.

B. bracteatum	"*Serendipita herbamans* strain S1 18S ribosomal RNA gene"	93%	1094/1177	KF061285.1
B. schillerianum	"*Serendipita herbamans* strain S1 18S ribosomal RNA gene"	93%	1088/1169	KF061285.1

Table 3. 5: Best alignment hits of BLASTN without "sequences from type material". LSU analysis for *Serendipita* spp. from *B. bracteatum* and *B. schillerianum*.

Source	Highest match based on NCBI database (Without sequences from type material)	Sequence similarity	Sequence overlap (bp)	Genbank accession no.
B. bracteatum	"Sebacinaceae sp. 5173 28S ribosomal RNA gene, partial sequence"	95%	1106/1167	JF799765.1
B. schillerianum	"Sebacinales sp. 4035 28S ribosomal RNA gene, partial sequence"	93%	1145/1228	JF906111.1

3.3.3 Phylogenetic analyses

The sequences that matched *Serendipita* spp. from *B. bracteatum* and *B. schillerianum* were phylogenetically analysed using the MEGAX software program and compared with other 37 described sequences of *Serendipita* taxa to determine relatedness to other members of the family Serendipitaceae. In this analysis sequences of *Helvellosebacina* and *Sebacina* were used as outgroups (Figure 3.6). The sequence that matched a *Preussia* from *B. minutissimum* and the sequence of the *Tulasnella* fungus from *B. shepherdii* were also phylogenetically analysed (Figure 3.8 and Figure 3.9).

The *Serendipita* sp. isolated from *B. bracteatum* grouped closely with the mycobiont from *B. elisae* (97% bootstrap support) and included in the same clade was the *Serendipita* sp. isolated from *B. schillerianum* (Figure 3.6). A *Serendipita* sp. previously isolated from *B. schillerianum* (Dearnaley unpublished) did not group with these isolates and was conspecific with an isolate from *B. lamingtonense* (Dearnaley unpublished). Further analysis was performed for the sequences from the *B. bracteatum* and *B. schillerianum* symbionts to check their relatedness using BLASTN align two or more sequences function (Table 3.6). As can be seen in Table 3.6 the two fungi represent different species and had 86% identity over 918 bp. These fungi are related as the phylogenetic analysis of LSU sequences of *B. bracteatum* and *B. schillerianum* grouped them together (96% bootstrap support) in the same clade (Figure 3.7).

Table 3. 6: Comparing the sequences from *B. bracteatum* and *B. schillerianum*. Best alignment hits of BLASTN was used with align two or more sequences function using ITS sequence.

Source	Sequence similarity	Sequence overlap (bp)	Genbank accession no.
B. bracteatum	86%	786/918	Query_15421
B. schillerianum			

The phylogenetic analysis of the *Preussia* isolate from *B. minutissimum* showed that it was related to *Preussia* sp. endophytic isolate BSH2.9 which had been previously identified as *P. africana* (Mapperson et al., 2014) (Figure 3.8). Further analysis was performed for sequences from *B. minutissimum* and a *Preussia* sp. endophytic isolate BSH2.9 (Mapperson et al. 2014) to check if they were the same using BLASTN align two or more sequences function (Table 3.7). Sequence analysis showed that the *Preussia* isolate from *B. minutissimum* and *Preussia* sp. isolate BSH2.9 had 93% identity over 493 bp and thus these are different species of *Preussia*.

Table 3. 7: The result of BLASTN with align two or more sequences function. ITS analysis was used for *Preussia* isolate from *B. minutissimum* and *Preussia* sp. endophytic isolate BSH2.9.

Source	Sequence similarity	Sequence overlap (bp)	Genbank accession no.
B. minutissimum	93%	459/493	Query_64665
Preussia sp. endophytic isolate BSH2.9			

The results of the phylogenetic analysis grouped the *Tulasnella* from *B. shepherdii* with *T. hadrolaeliae* and *T. asymmetrica* (Figure 3.8). Further analysis was performed for sequences from *B. shepherdii* with *T. hadrolaeliae and T. asymmetrica* to check if they were similar using the BLASTN align two or more sequences function. Sequence analysis between the *Tulasnella* from *B. shepherdii* and *T. hadrolaeliae* showed 87% identity over 388 bp. While sequence analysis with *T. asymmetrica* showed no significant similarity. This fungal taxon is quite different from the *Tulasnella* from terrestrial Australian orchids as indicated by substantial separation in the phylogenetic tree (Figure 3.9).

Table 3. 8: Best alignment hits of sequences from *B. shepherdii* with *T. hadrolaeliae*. ITS analysis was used in BLASTN with align two or more sequences function.

Source	Sequence similarity	Sequence overlap (bp)	Genbank accession no.
B. shepherdii	87%	339/388	Query_212725
T. hadrolaeliae			

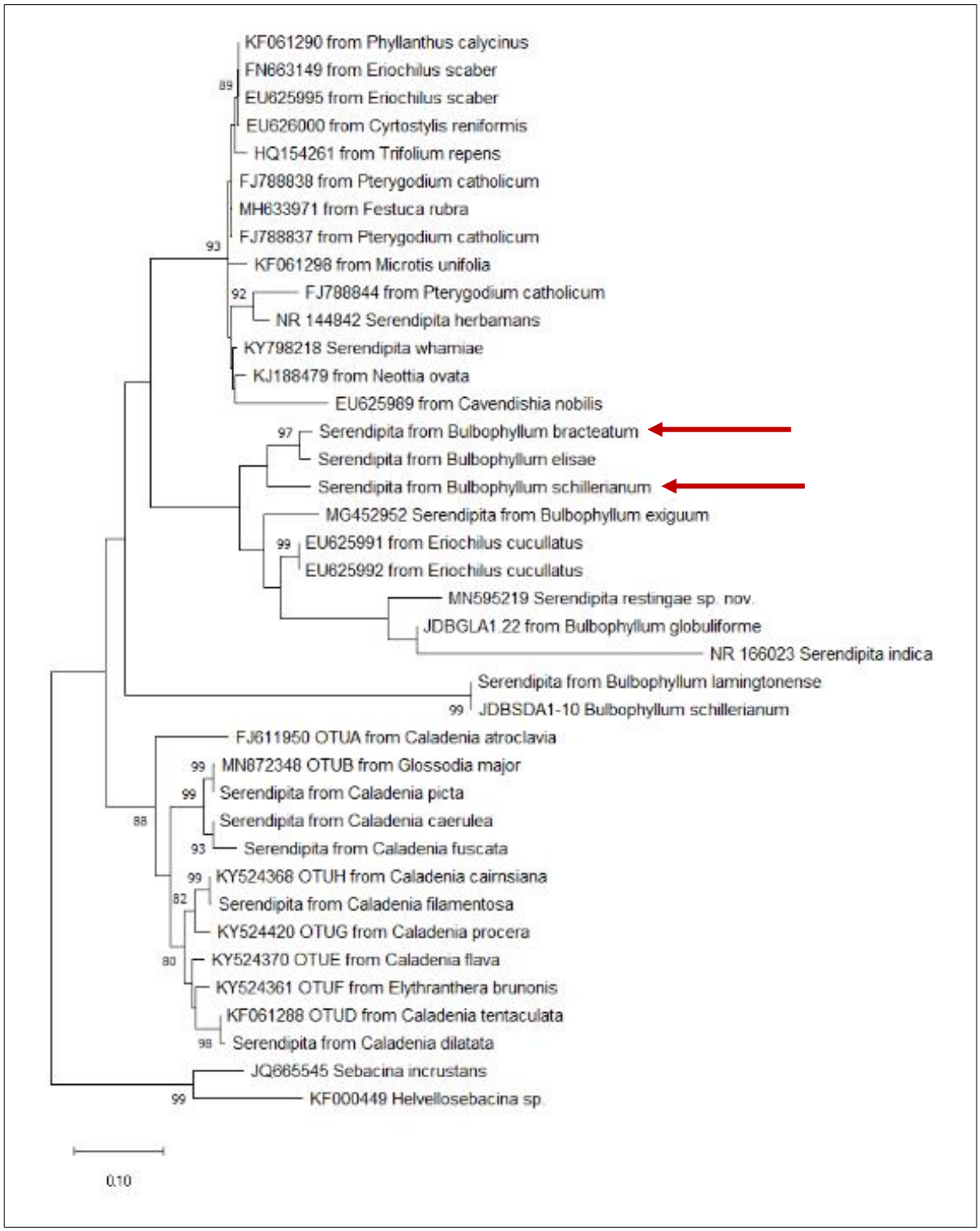


Figure 3. 6: Phylogenetic analysis of ITS-DNA from the *Serendipita* mycobionts isolated from *B. bracteatum* and *B. schillerianum* (red arrows) compared with other *Serendipita* spp from a variety of plant hosts. *Sebacina incrustans* and *Helvellosebacina* sp. were used as outgroups to root the tree.

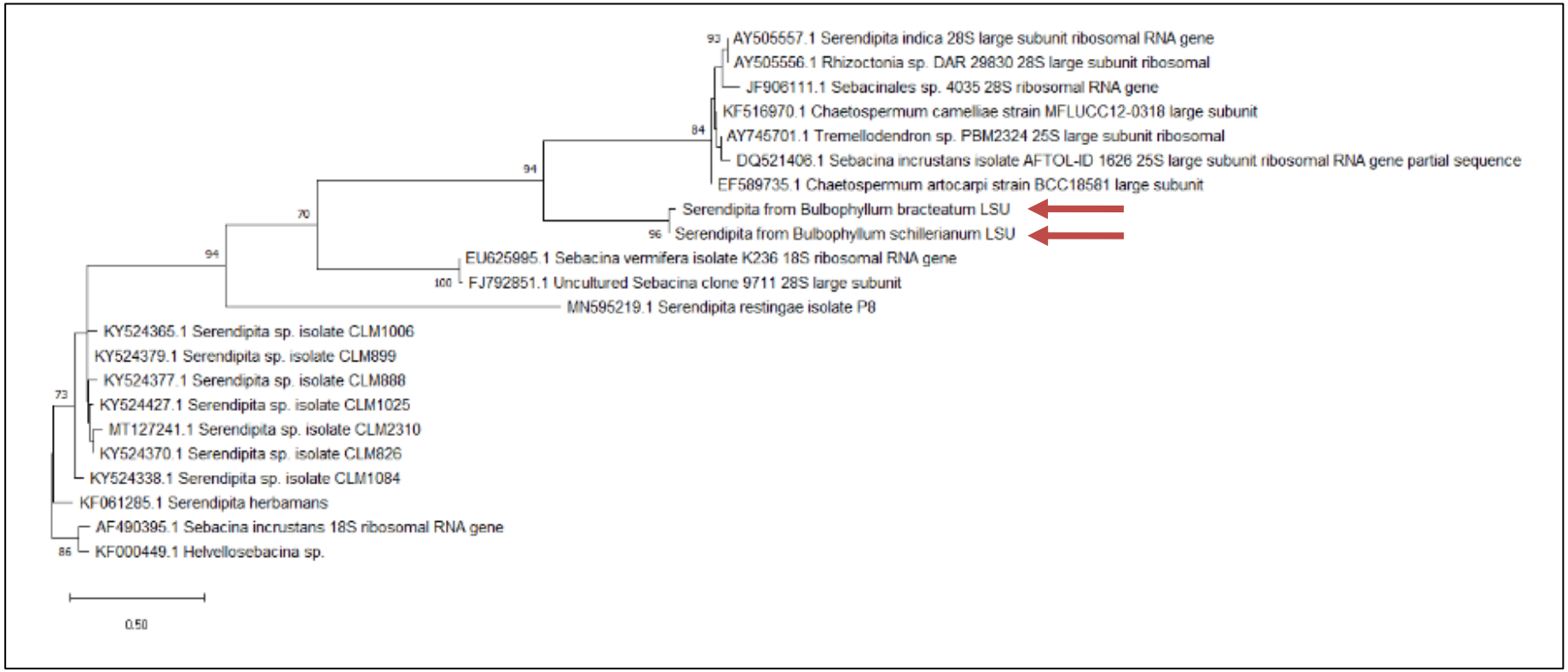


Figure 3. 7: Phylogenetic analysis of LSU-DNA from the *Serendipita spp.* isolated from *B. bracteatum* and *B. schillerianum* (red arrows) compared with other *Serendipita* spp from a variety of plant hosts. *Sebacina incrustans* and *Helvellosebacina* sp. were used as outgroups to root the tree.

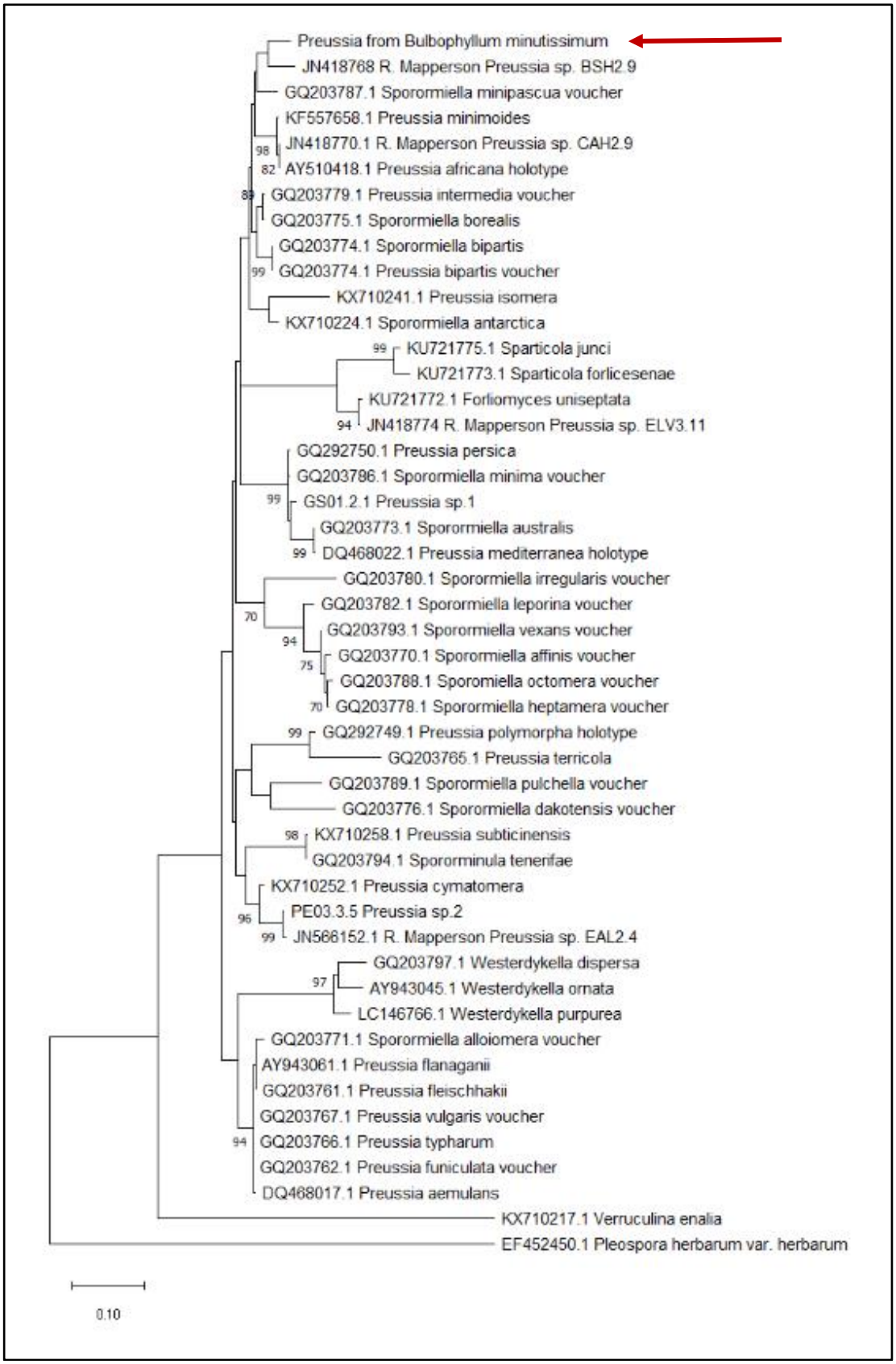


Figure 3. 8: Phylogenetic analysis of ITS-DNA from the *Preussia* fungus isolated from *B. minutissimum* (red arrow) compared with other *Preussia* spp. *Verruculina enalia and Pleospora herbarum* were used as outgroups to root the tree.

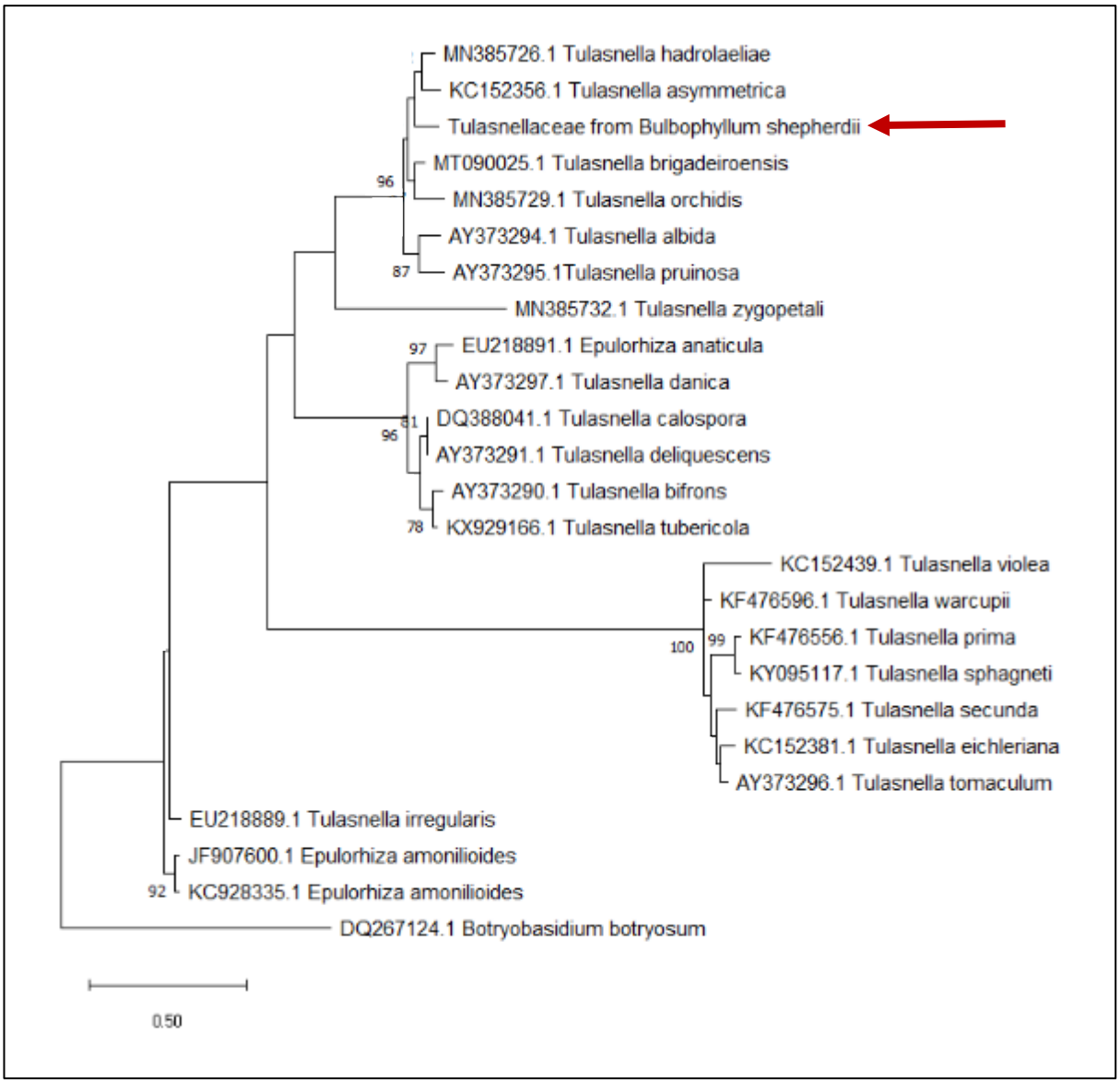


Figure 3. 9: Phylogenetic analysis of ITS-DNA from the fungus isolated from *B. shepherdii* compared with other *Tulasnella* spp. The clade containing *Tulasnella violea* represents *Tulasnella* from Australian terrestrial orchids. *Botryobasidium botryosum* was used as outgroup to root the tree.

3.3.4 Teleomorph production

No obvious teleomorph stages were detected in the fungal isolates from *B. schillerianum* and *B. bracteatum*. Stalked spherical cells, indicative of the early stages of hyphal coils were detected in both cultures (Figures 3.10 & 3.11). The size of monilioid cells was measured in seven week old cultures. The average size of twenty monilioid cells of *Serendipita* from *B. schillerianum* was 3µm and for the *Serendipita* from *B. bracteatum*, 2.9µm. Monilioid cells in both fungal isolates were globose and occurred singly on the ends of hyphae.

Figure 3. 10: Hyphal coil primordia (red arrows) in the *Serendipita* isolated from *B. schillerianum*. Scale bar is 100 µm.

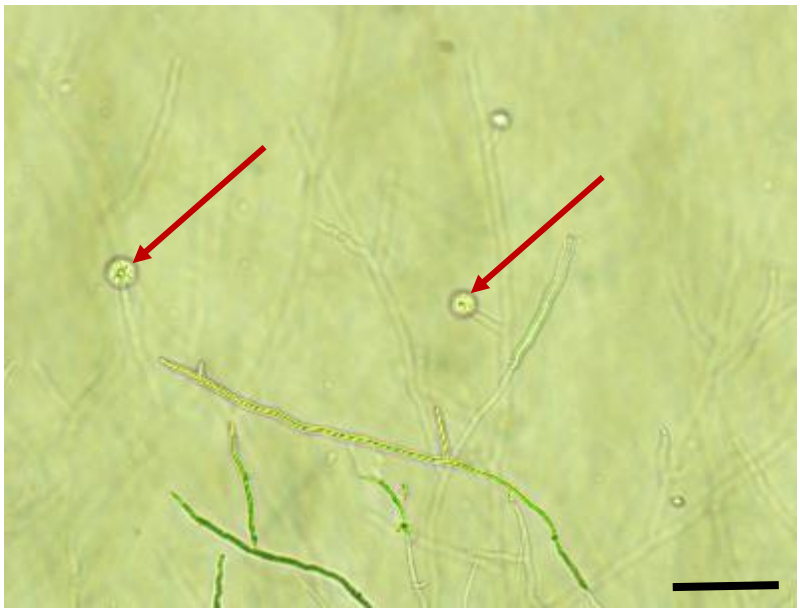


Figure 3. 11: Hyphal coil primordia (red arrows) in the *Serendipita* sp. isolated from *B. bracteatum*. Scale bar is 100 µm.

3.3.5 Growth rate measurement results

The average colony diameter of fungal isolates was determined for the plates after two weeks growth. The mean growth rate for the fungus from *B. schillerianum* was 1.7 mm/day and for the fungus *B. bracteatum* 0.64 mm/day (Table 3.9).

Table 3. 9: Growth rate of fungal isolates from *B. schillerianum* and *B. bracteatum*. The average colony diameter of fungal isolates was determined for the plates after two weeks growth.

B. schillerianum					*B. bracteatum*				
Plate number	1	2	3	Mean	Plate number	1	2	3	Mean
Colony diameter after two weeks growth	22 mm, 24 mm	24 mm, 27 mm	24 mm, 22 mm	24 mm	Colony diameter after two weeks growth	10 mm, 9 mm	9 mm, 8 mm	10 mm, 8 mm	9 mm
Growth rate after two weeks (mm/day)	1.6 mm, 1.7 mm	1.7 mm, 1.9 mm	1.7 mm, 1.6 mm	1.7 mm	Growth rate after two weeks (mm/day)	0.71 mm, 0.64 mm	0.64 mm, 0.57 mm	0.71 mm, 0.57 mm	0.64 mm

3.3.6 Fluorescence staining results

Hyphae were binucleate in the fungus isolated from *B. bracteatum* (Figure 3.12) and were uninucleate in the fungal isolate from *B. schillerianum* (Figure 3.13).

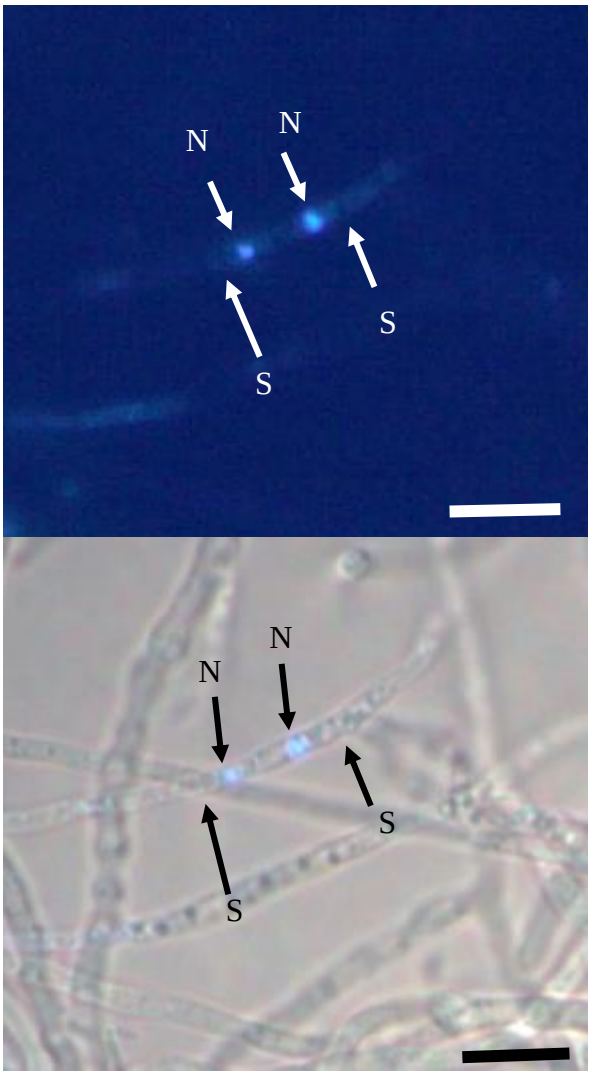


Figure 3. 12: Hyphae stained with Hoechst dye showing binucleate arrangement in the *B. bracteatum Serendipita* isolate. Scale bar is 10 µm.

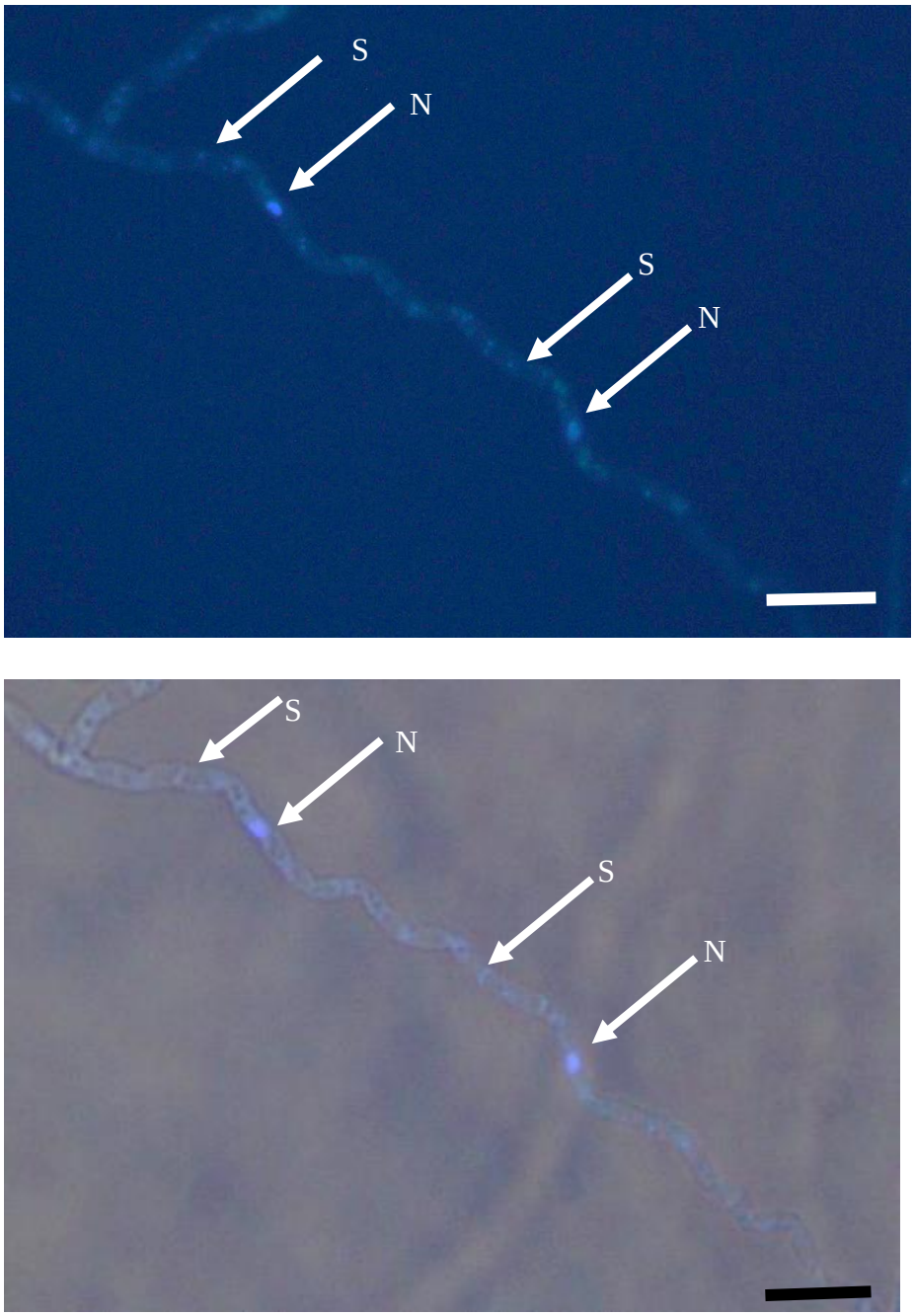


Figure 3. 13: Hyphae stained with Hoechst dye showing uninucleate arrangement in the *B. schillerianum Serendipita* isolate. Scale bar is 10 µm.

3.4 Discussion

This study showed that *Bulbophyllum* spp. provide habitat for many endophytic fungal taxa. Amongst other fungi, *B. bracteatum* and *B. schillerianum* were shown to contain *Serendipita* spp., *B. minutissimum* was colonised by a *Preussia* sp., *B. shepherdii* harboured a *Tulasnella* sp. and *B. exiguum* housed a Xylariales endophyte. The later result contrasts with research conducted on *B. exiguum* by Calvert (2017) who found a novel *Serendipita* species in the orchid of south east Queensland. The absence of a conventional orchid mycobiont in some of these plants might be explained by high external nutrient levels which may have prevented mycorrhizal colonisation (Baum & Makeschin 2000; Treseder & Vitousek 2001; Mujica et al., 2020). The absence of pelotons in the roots of some of the species observed here reinforces this proposition.

The results of BLAST searches showed that fungal sequences from *B. bracteatum* and *B. schillerianum* had closest match to an uncultured Sebacinales GenBank entry (KJ188464.1) with 87% and 92% similarity, respectively. Using the "Sequence from type material" option showed that the sequences had closest match to *Serendipita herbamans* with 81.22% and 85.55% identity respectively. These results suggest that these taxa represent new *Serendipita* species therefore further characterisation was carried out. Sequencing of LSU DNA confirmed their taxonomic novelty. BLAST 2 sequence comparisons, estimations of mycelial growth rates and nuclear staining suggested that they are different species. Teleomorph production would have been helpful to support the molecular and cellular analysis and to enable full characterisation of these species, but unfortunately these were not induced in this study. Teleomorphic features such as probasidia, metabasidia and sterigmata have been recently used to formally describe *S. whamiae* (Crous et al. 2020). It was not surprising to discover *Serendipita* species in these lithophytic plants as this is a common occurrence in orchids in this habitat type (Yokoya et al. 2021). It is possible that lithophytic orchids will harbour different symbionts than epiphytic and terrestrial plants due to the exposed nature of the lithophytic location and the ability of *Serendipita* fungi to promote resistance to drought stress (Ghimire & Craven 2011).
The growth rate of the *Serendipita* isolated from *B. schillerianum* was 2.4 mm/day which is approximately similar to *Serendipita* spp that have been isolated from *Caladenia* spp. (Oktalira et al., 2021). The *Serendipita* isolated from *B. bracteatum*

had a slow growth rate of 0.64 mm/day. *Serendipita whamiae*, recently described from the orchid *Eriochilis cucullatus* (Crous et al. 2020), has a growth rate of 6 mm/day showing that growth rate is a useful taxonomic character in *Serendipita*.

Nuclear staining showed the *Serendipita* spp isolated from *B. schillerianum* and *B. bracteatum* have uninucleate and binucleate septal compartments, repectively. *S. australiana*, *S. talbotii* and *S. secunda* have been shown as having binucleate cells (Oktalira et al., 2021). It is common, however, to observe one fungal isolate containing both uni- and binucleate cells in Basidiomycota such as *Ceratobasidium* (Otero et al. 2002; Hietala et al. 1994) and *Rhizoctonia solani* (Sanford & Skoropad 1955) as well as some Ascomycota (Clutterbuck & Roper 1966; Kaufmann & Philippsen 2009). Thus, cell nuclei number may be limited in taxonomic usefulness for some fungal groups.

Figure 3.6 suggested that *B. bracteatum* and *B. schillerianum* have different *Serendipita* species from those which were isolated from *Caladenia* orchids. Interestingly, *B. schillerianum* and *C. picta* were collected from the same location (Mount Nebo) but had different *Serendipita* spp. further highlighting host specificity and the distinction between the different orchid types (Martos et al., 2012; Jacquemyn et al., 2015; Xing et al., 2019). These phenomena need further exploration, however as a recent study has shown that a new species *S. petricolae* from the terrestrial orchid *Eriochilus cucullatus* terrestrial orchid is the same species as that from the epiphytic orchid *B. globuliforme* (Crous et al., 2022, in press).

An isolated fungus from *B. exiguum* had a closest match to a member of the Xylariales order. This finding concurs with another study of the orchid symbionts in Australian *Bulbophyllum* orchids where ascomycetes from the fungal orders Helotiales, Xylariales and Chaetothyriales were identified (Calvert 2017). Other non-Australian *Bulbophyllum* species from forest ecosyetems have also recently been shown to be common habitat for ascomycete fungi (Cregger et a., 2018; Wang et al., 2019; Trivedi et al., 2020). Many diversity of ascomycete fungi have been associated with orchids including members of the Xylariales, Boliniales, Chaetosphaeriales, Hypocreales, Capnodiales, Pleosporales, Botryosphaeriales, Chaetothyriales, Eurotiales, Thelebolales, Helotiales, Rhytismatales, Phyllachorales and Diaporthales (Richardson

& Currah 1995; Bayman et al. 1997; Tremblay et al. 1998; Yuan et al. 2009; Herrera et al., 2010). Orchid associated ascomycetes have a wide range of nutritional acquisition modes. For example most members of the Xylariales are considered to be saprotrophic, but some orchid associated ascomycetes are endophytic or pathogenic (Petrini & Petrini 1985; Edwards et al., 2003). Members of the Helotiales include an ecologically diverse group of wood, debris and soil saprobes, plant endophytes, plant pathogens and mutualistic ectomycorrhizal (ECM) fungi (Vrålstad et al. 2002; Haug et al. 2004).

The isolated fungal sample from *B. minutissimum* had a closest match to a *Preussia* sp. in GenBank. Various species of endophytic *Preussia* have been recorded in Australia such as *P. africana, P. australis, P. minima, P. cylindrica* and *P. funiculata* (Bell, 2005; Peterson et al., 2009). *Preussia* has been found to be associated with a diversity of Australian dry rainforest plants such as *Alectryon diversifolium*, *Bursaria spinosa*, *Cassine australis*, *Erythroxylum australe*, *Eustrephus latifolius*, *Geijera salicifolia*, *Notelaea venosa*, *Pittosporum angustifolium* and *Pandorea pandorana* (Mapperson et al., 2014). The role of *Preussia* inside the host plant is not well understood (Arenal et al., 2005; Gonzalez-Menendez et al., 2017). *Preussia* probably occupy internal plant tissues, beneath the epidermal cell layers, without causing obvious pathogenic symptoms (Gonzalez-Menendez et al., 2017). Many *Preussia* species have been shown to have antimicrobial activity by producing bioactive secondary metabolites such as preussomerins (Weber & Gloer, 1991; Vilella et al., 2000; Chen & Chen, 2008; Chen et al., 2009) and unamed polyketides (Mapperson et al., 2014). This antimicrobial impact could be a factor that encourages *B. minutissimum* to harbour *Preussia* spp. ie. to confer resistance against possible biotic stress. It is unusual to see *Preussia* associated with orchids, therefore, it would be worthwhile conducting further studies to understand the nature of the interaction between these endophytes and their plant hosts.

The isolated fungus from *B. shepherdii* had closest matches to *Tulasnella* spp. within GenBank. *Tulasnella* species associate with plants in different ways including as mycobionts in orchids (Warcup 1981; Rasmussen & Rasmussen 2009) and liverworts (Kottke et al. 2008), as ectomycorrhizal associates of the nonphotosynthetic liverwort *Cryptothallus mirabilis* (Bidartondo et al. 2003) and as saprotrophs in decayed wood

(Roberts 1994; Cruz et al. 2011). In Australia new *Tulasnella* species have been identified associating with terrestrial orchids such as *T. prima* and *T. sphagneti* in *Chiloglottis*, *T. secunda* in *Drakaea* and *Caleana* and *T. warcupii* in *Arthrochilus oreophilus* (Linde et al., 2017). A recent study showed that *T. australiensis*, *T. occidentalis*, *T. punctata*, *T. densa*, and *T. concentrica* associated with *Cryptostylis* whereas *T. rosea* associated with *Spiculaea ciliata* (Arifin et al., 2021). Few studies have recorded *Tulasnella* associating with epiphytic orchids in Australia. A previous unpublished study has identified a *Tulasnella* sp from three *Bulbophyllum* spp. (*B.exiguum*, *B. bracteatum* and *B. elisae*) across different locations of south east Queensland (Calvert 2017). Recently, four new *Tulasnella* species have been identified from epiphytic orchids from Brazilian Atlantic forest where *T. brigadeiroensis* and *T. hadrolaeliae* were isolated from *Hadrolaelia jongheana* and *T. orchidis* and *T. zygopetali* were isolated from *Zygopetalum maxillare* (Freitas et al., 2020).

Some studies suggested that epiphytic orchids form specific associations with single mycorrhizal fungal species. For example, *B.exiguum* and *B. elisae* only associated with a single species of *Tulasnella* sp (Calvert 2017). In the research presented here, *B. shepherdii* only associates with a single species of *Tulasnella* sp. but additional samples of this orchid from multiple locations would be required to confirm this. Fungal specificity in epiphytes have been explained using the optimal physiology concept (Bonnardeaux et al. 2007; Otero et al., 2007). Under the harsh abiotic conditions typical of the epiphytic state, such as low levels of moisture and nutrients, certain mycorrhizal fungi may optimally increase surface area and improve water and mineral absorbance for plants (Martos et al., 2012). Additionally, high irradiation of epiphytes may allow them to provide fungal partners with more photosynthetically-fixed carbon, leading to greater fungal dependence on epiphytic partners (Martos et al., 2012). It is predicted that epiphytic orchids show heavy mycorrhizal colonisation but this is not the case. For example, it was not possible to isolate mycorrhizal fungi from *B. minutissimum* and little colonisation was seen, therefore further studies are recommended to understand the nature of the interaction between mycorrhizal fungal taxa and epiphytic orchids.

CHAPTER 4: IMPACTS OF *Serendipita* ON THE GROWTH OF TOMATO

4.1 Introduction

The effects of *Serendipita* in enhancing plant growth have been well investigated. *Serendipita* has been shown to enhance the vegetative growth of *Zea mays, Petroselinum crispum, Artemisia annua, Bacopa monnieri Populus tremula* (Varma et al., 1999), *Arabidopsis thaliana* (Peskan-Berghofer et al., 2004), *Hordeum vulgare* (Waller et al., 2005), *Adhatoda vasica* (Rai & Varma, 2005), *Chlorophytum* sp (Gosal et al., 2010) and *Piper nigrum* (Anith et al., 2011). In addition, *Serendipita* enhanced vegetative growth and flowering in *Withania somnifera* and *Spilanthes calva* (Rai et al., 2001). Additionally, *Serendipita* showed positive effects on *Nicotiana tabacum* such as increasing adult plant vegetative growth (Varma et al., 1999; Barazani et al., 2005), seed production (Barazani et al., 2005) and promoting seedling growth (Sherameti et al., 2005).

The levels of nitrogen (N), phosphorus (P) and potassium (K) have been improved in *Serendipita*-colonised chickpea and black lentil plants (Nautiyal et al., 2010; Kumar et al., 2012). Furthermore, *Serendipita* enhanced nitrate and starch metabolism enzymes in *Arabidopsis* roots as an indicator of enhanced N nutrition (Sherameti et al., 2005). Another study, also in *Arabidopsis*, showed increased radio-labelled P absorption in *Serendipita*-colonised plants (Shahollari et al., 2005). Conversely, *Serendipita* showed an inability to support plant nutrition under nutrient-limiting conditions, where an interaction with *Serendipita* did not enhance the content of N and P in tobacco (Barazani et al. 2005), barley (Achatz et al. 2010) and green gram (Ray & Valsalakumar 2010). Moreover, the presence of *Serendipita* lowered the expression of a phosphate transporter leading to the decline of plant growth parameters in potato (Karandashov et al., 2004).

In tomato, *Serendipita* colonisation improved vegetative growth, shortened flowering time and increased the number of fruits and fruit biomass after four weeks. After ten weeks, the colonized plants did not show any difference to the control plants (Fakhro

et al., 2010; Andrade-Linares et al., 2013). Tomato inoculated with a mixture of *Serendipita* and the rhizobacterial strain *Bacillus pumilus* showed improved plant growth after three weeks, as indicated by increased height and fresh and dry weight of shoots and roots (Anith et al., 2015). Long-term plant-*Serendipita* colonisation experiments led to slowing of growth rate and the promotion of programmed cell death (Deshmukh et al., 2006). Another negative impact of long term *Serendipita*-tomato interaction was the induction of blossom-end rot fruit disorder due to calcium (Ca) deficiency (Andrade-Linares et al., 2013). *Serendipita*-colonised tomato showed up-regulation of gibberellin biosynthesis genes which minimize Ca intake and increase Ca transfer into storage organelles, leading to low levels of free Ca in the apoplast. As a result, a limited amount of Ca moves into the fruit (De Freitas et al., 2012).

The interaction between *Serendipita* and plants causes changes in the host transcriptome and proteome. These changes can be studied at both the molecular and cellular levels using *in vitro* co-cultivation systems. These systems allow a balanced symbiosis and plant growth is normal even if there is a deficiency of essential elements such as N, P, K and Fe in the co-cultivation medium. However, at the late stages of the interaction, plants start to show growth deterioration. This leads to a change in the behaviour of the fungus from a mutualist to a parasite (Kaldorf et al. 2005; Johnson & Oelmuller 2009; Oelmuller et al. 2009; Johnson et al., 2013). Maintaining a pH of between 6.5 and 7 in the culture medium is critical to ensuring an efficient interaction in the co-cultivation system (Johnson et al., 2013).

Multiple methods exist to ensure plant roots become colonised with *Serendipita* fungi. Some approaches use liquid media firstly for *Serendipita* propagation such as *Aspergillus* minimal medium (Waller et al., 2005; Kumar et al., 2009), potato dextrose broth (Fakhro et al., 2010) and Kaefer medium and malt yeast peptone (Sefloo et al., 2019) medium. Root colonisation was completed by dipping roots either in the liquid medium (Sefloo et al., 2019) or sowing plants in a mixture of the *Serendipita* culture and sterilised soil (Kumar et al., 2009). *In vitro* co-cultivation systems provide the opportunity to closely study the interaction between *Serendipita* and plant hosts. *In vitro* systems are convenient for gene expression analysis because they keep plants and fungi under sterilised conditions and provide a stable environment for their growth to acquire reproducible and quantitative data. Numerous solid growth media have been

used in *in vitro* co-cultivation system such as plant nutrient medium (PNM) (Sherameti et al., 2008; Johnson et al., 2011; Sun et al., 2014), modified Marx-Melin-Norkrans (MMN) culture medium (Peskan-Berghofer et al., 2004; Sherameti et al., 2005; Daneshkhah et al., 2018) and Murashige-Skoog (MS) medium (Sun et al., 2010; Lee et al., 2011; Lin et al., 2019). *In vitro* co-cultivation systems using PNM have been used to detect the impacts of two dark septate endophytes on tomato (Yakti et al., 2018) and liquid PNM medium has been used to form a symbiosis between *Serendipita* and tomato (Fakhro et al., 2010; Sefloo et al., 2019). In addition, solid PNM was used extensively as a co-cultivation medium between *Serendipita* and *Arabidopsis* (Johnson et al., 2011; Sun et al., 2014; Wawra et al., 2016; Vahabi et al., 2016; Scholz et al., 2018) and barley (Sherameti et al., 2008; Lahrmann et al., 2013; Wawra et al., 2016; Sarkar et al., 2019; Hilbert et al., 2020).

Although the effects of *Serendipita* sp. on improving plant growth have been well studied, to the best of our knowledge, none of the previous studies have examined the role of *Serendipita* sp isolated from native Australian orchids in improving plant growth. In the research presented here, growth improvement of tomato inoculated with Australian orchid derived *Serendipita* sp. will be examined. This will be performed by monitoring the colonisation plants with a range of newly isolated *Serendipita* fungi inside tomato roots growing *in vitro* on PNM medium. Colonised plants will be grown for four weeks *in vitro* and growth parameters such as fresh weight, total plant height and shoot and root length will be measured. To gain insight into the molecular changes occurring in colonised plants, qRT-PCR and semi-quantitative PCR will be used to investigate the expression levels of genes involved with hormone production and nutrient transporter expression.

The specific objectives of this component of the study are:

a) To develop an *in vitro* colonisation system for tomato plants and *Serendipita* fungi
b) To ascertain if *Serendipita* colonisation of tomato plants impacts on growth parameters.
c) To determine if there are changes in growth and nutrient transporter gene expression in *Serendipita*-colonised tomato plants.

4.2 Methods

4.2.1 Co-cultivation system

Tomato seeds (cv. Moneymaker, Mr. Fothergill's Seeds, South Windsor, NSW, Australia) were surface sterilised by soaking for 1 min in 70% ethanol and for 4 min in 2.5% sodium hypochlorite (NaClO). Seeds were then rinsed five times with sterilised water. Seeds were initially grown on MS (Murashige and Skoog 1962) at 4 ℃ for 48 hours in the dark to induce seed germination. Following this, they were transferred to a growth chamber and incubated for ten days at 16/8 hours day/ night cycle at 23/21 ℃ (Yakti et al 2018). At ten days post germination, 40 equally-sized seedlings were transferred to separate 500 ml glass jars with aerated lids, containing approximately 50 ml solid PNM at the base (Johnson et al., 2011). PNM growth media was prepared using the following chemical components (5 mM KNO_3, 2 mM $MgSO_4.7H_2O$, 2 mM $Ca(NO_3)_2$, 0.01 mM $FeSO_4$, 70 mM H_3BO_3, 14 mM $MnCl_2.4H_2O$, 0.5 mM $CuSO_4.5H_2O$, 1mM $ZnSO_4.7H_2O$, 0.2 mM $Na_2MoO_4.2H_2O$, 0.01 mM $CoCl_2.6H_2O$, 10 mM NaCl, and 5 g of agar per 500 ml, pH 5.6) (Johnson et al., 2011). Growth jars were prepared as in figure 4.1. In all experiments, eight weeks old *Serendipita* plates were used for adding hyphal suspension and fungal plugs to the growth jars. The experiments contained two sample groups; each consisting of 20 growth jars. The first group of jars had added *Serendipita* inoculant while the second group had no inoculant added. In the first experiments, 3 ml of hyphal suspension and four *Serendipita* 1 cm^3 PDA plugs were added to each jar. In the second experiments, 1.5 ml of hyphal suspension and two *Serendipita* 1 cm^3 PDA plugs were added to each jar. The hyphal suspension was prepared in a biosafety cabinet by transferring mycelium from a petri dish culture with a sterilised scalpel to 100 ml of sterilised water. The 3 ml inoculation experiments were conducted twice due to necrosis being observed in plants, while the 1.5 ml inoculation experiments were conducted three times. The quantity of fungal propagules in both hyphal suspensions was quantified by hemocytometer (Absher, 1973). After 17 days, plant were harvested and measured and around 50 mg of roots were collected for qRT-PCR purposes. The remaining roots were used for checking colonisation via root clearing (Johnson et al., 2011). For clearing, roots were transferred to a microcentrifuge tube containing 1 M KOH and incubated for 20-30 min at 95 ℃. Roots were then rinsed in tap water, 0.1 M HCl and

then again in tap water. For staining, roots were then boiled in 0.05% Trypan blue in glycerol and lactic acid for 5 min. The stain was removed by washing in 80% glycerol. The roots were mounted under coverslips with 80% glycerol.

The growth parameters, including total plant length, shoot length, root length and fresh weight were measured and compared between two the treatments.

In parallel with the experiments on tomato, experiments using barley plants were performed in case *Serendipita* did not colonise tomato roots. Six barley seeds were surface sterilized with 70% ethanol for 1 min, followed by washing with sterile distilled water. Seeds were subsequently incubated in 12% sodium hypochlorite solution for 1.5 hours before being washed for 6 hours with sterile distilled water. Sterile seeds were placed onto wet filter paper and kept in the dark at room temperature for germination (Sarkar et al., 2019). Four-day-old seedlings were transferred to PNM and were grown in the growth chamber with the above-mentioned conditions.

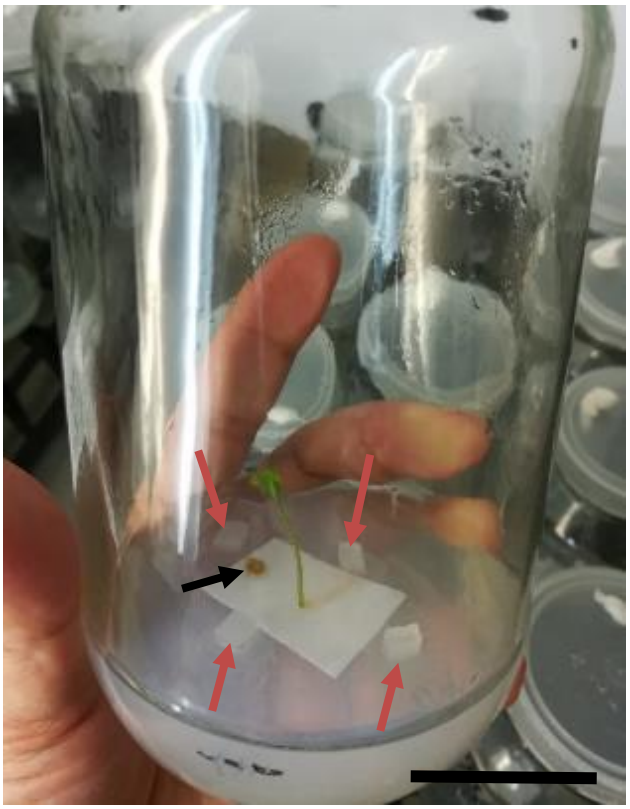


Figure 4. 1: Growth container with 27 day old tomato seedling and *Serendipita* agar plugs (red arrows) on PNM. Black arrow: seed testa. Scale bar is 5 cm.

4.2.2 Statistical analysis

Using the statistical software jamovi 2.2.5 (The jamovi project, 2021), independent samples t-tests were used to analyse data and probability values of less than 5% were used as a measure of significance.

4.2.3 Testing the colonising efficiency of *Serendipita* spp.

Serendipita secunda fungi isolated from *C. caerulea* and *C. picta* were used to colonise tomato and barley under the above-mentioned conditions. The colonisation efficiency of these two fungal taxa was compared with a fast-growing species, *S. whamiae*, which was originally isolated by Crous et al. (2021).

4.2.4 RNA extraction, cDNA synthesis, quantification and testing qPCR primers

Total RNA was extracted from the roots of *in vitro* grown *Serendipita* and non-*Serendipita* colonised tomato using the RNeasy Power Plant Kit (Qiagen, Doncaster, VIC, Australia) following the manufacturers' instructions. Approximately, 50 mg of fresh or frozen plant tissues were added to a bead tube with β-mercaptoethanol and lysis buffer provided by the manufacturer (Qiagen). The tissue were then ground thoroughly using a FastPrep homogenizer (MP Biomedicals, QLD, Australia) at 7 m/s speed for 45 s. After tissue lysis, the released RNA was treated with inhibitor removal technology (IRT) for removing the contaminants that cause PCR inhibition, such as polysaccharides and polyphenolics. Then, the inhibitor-free RNA was captured on a silica-membrane spin filter. The RNA bound to the filter was washed with 100% ethanol and washing buffer to completely flush the membrane of any remaining salts. Finally, the RNA was recovered in RNase-free water. A Denovix spectrophotometer (Wilmington, Delaware, USA) was used to check RNA quality by measuring 280/260 and 260/230 ratios for three roots of *Ser*+ plants and three roots of *Ser*- plants. One µl from each sample suspended in RNase-free water was used for blank measurement. Then, one µl from the sample was loaded on the lower sample surface of the device.
After RNA extraction, cDNA was synthesized from templates using reverse transcriptase and Oligo dT primer from a QuantiNova Reverse Transcription cDNA synthesis kit, (Qiagen) following the manufacturers' instructions. A gDNA elimination

reaction was performed by mixing RNA, gDNA removal mix and RNase-free water. The mixture was then incubated at 45°C for 2 min, then placed on ice and the enzyme with reverse transcription mix added. The reverse transcription reaction was performed with the following protocol: annealing step at 25 ℃ for 3 min, reverse transcription step at 45 ℃ for 10 min and inactivation of the enzyme at 85 ℃ for 5 min.

After cDNA synthesis, all samples from the *Ser*+ and *Ser*- treatments were quantified using Qubit 3.0 flurormeter and its reagents (Invitrogen, Thermo Fisher, USA). Two µl of the cDNA samples were mixed with 198 µl of the working solution then this was vortexed for 2-3 seconds. For the blank assay, 10 µl of the standard solution was mixed and vortexed for 2-3 seconds with 190 µl of working solution. Equal concentrations of cDNA samples were prepared via dilution with sterile RNAse-free distilled water (Qiagen), for further analysis.

Before qRT-PCR analysis, the growth and nutrient transporter primers (Table 4.1) were tested via conventional PCR on tomato root cDNA (colonised with *Serendipita*). The growth and nutrient transporter genes were selected in this study because expression changes have been observed previously in mycorrhizal tomato roots but little is known about the molecular control of the association between plants and *Serendipita* fungi (Balestrini et al., 2007 & 2019; Fiorilli et al., 2009; Zouari et al., 2014). PCR was performed by adding 10 µl of Extract-N-Amp PCR reaction mix, 7 µl sterile distilled water, 1 µl cDNA, 1 µl of each of forward and reverse primers (both at 10 10 µM) to each tube. PCR was performed with the following protocol: initial denaturation at 95 ℃ for 5 min, amplification with 40 cycles of 95 ℃ for 1 min, 50 ℃ for 1 min and 72 ℃ for 1 min, with a final extension at 72 ℃ for 10 min. A BioRad electrophoresis apparatus was used to run the PCR products. Ten µl of the PCR product was loaded on a 1% electrophoresis gel and viewed under UV light using a Quantum ST4 gel documentation system and photographed using a Quantum capture ST4® image acquisition and analysis software package.

Table 4. 1: Primers used for qRT-PCR analysis of the *Serendipita*-tomato growth response. The primers were tested via conventional PCR on tomato root cDNA from *Ser*+ treatment.

Primer name	Accession number	Primer sequence	Melting temp °C	Amplicon size	Reference
Phosphate transporter 4-F	AY804012.2	GAAGGGGAGCCATTTAATGTGG	61	182bp	Rivero et al., 2015
Phosphate transporter 4-R	As above	ATCGCGGCTTGTTTAGCATTTC (GAAATGCTAAACAAGCCGCGAT)	60	As above	
Nitrate transporter 2.3-F	NM_001247198.1	TGTACACTTCCAGTAATGTTAGTT	56	249bp	Fu et al., 2015
Nitrate transporter 2.3-R	As above	GGTACCCAGACGCGATTTGGTGTTA (TAACACCAAATCGCGTCTGGGTACC)	65	As above	
Zinc transporter-F	NM_001322833.1	TCATGTTGGCTTCTGCAGGT	58	279bp	Aoki et al., 2010
Zinc transporter-R	As above	GGTTTCTCCATGCCTCTCCC (GGGAGAGGCATGGAGAAACC)	60	As above	
Potassium transporter 10-F	*Solyc03g097860	CGCAAAGGATCGAATTTATTGAAG	57	84bp	Liu et al., 2019
Potassium transporter 10-R	As above	TCCAATTGGAGTCTCTCTGCAA (TTGCAGAGAGACTCCAATTGGA)	60	As above	
Auxin response factor 3-F	DQ340254.1	TGTTCCTGTGACGCTGATG	57	196bp	Zhang et al., 2015
Auxin response factor3-R	As above	TGTGTTCCTGAGACGAGAGC (GCTCTCGTCTCAGGAACACA)	59	As above	
ACC oxidase 1-F	NM_001247095	TGAGGCTGTTCAAGCTGAGG	59	724bp	Tanigaki et al., 2015
ACC oxidase 1-R	As above	AGCACTTGCAATTGGATCACT (AGTGATCCAATTGCAAGTGCT)	59	As above	
Ubiquitin 1-F	**TC193502	GGACGGACGTACTCTAGCTGAT	62	134bp	Aime et al., 2013
Ubiquitin 1-R	As above	AGCTTTCGACCTCAAGGGTA (TACCCTTGAGGTCGAAAGCT)	58	As above	
Actin-7-like-F	BT013524	CGGTGACCACTTTCCGATCT	60	62bp	Lacerda et al., 2015
Actin7-like-R	As above	TCCTCACCGTCAGCCATTTT (AAAATGGCTGACGGTGAGGA)	59	As above	

Note: All accessions can be found in GenBank, accept (*) in the aramemnon data libraries and (**) in The Institute for Genomic Research Gene Indices.

4.2.5 Quantitative real-time polymerase chain reaction (qRT-PCR)

cDNA was amplified via qRT-PCR using primers for tomato nutrient transporter and growth response genes using a QuantiNova SYBR green quantitative real time PCR kit (Qiagen). qPCR was performed twice with the following protocol: PCR initial activation at 95 ℃ for 2 min, amplification with 40 cycles of denaturation at 95 ℃ for 5 sec and combined annealing/ extension at 50 ℃ for 10 sec. qPCR products were analysed using software of CFX384 real-time detection system (Bio-Rad) via monitoring of a SYBR green fluorescence chart. The housekeeping genes actin and ubiquitin were used for normalisation. QPCR analysis of genomic DNA from *Serendipita* with ITS primers was conducted to check for consistency of pipette usage and optimum machine operation. QPCR analysis using primers to the references genes actin and ubiquitin were used to determine the appropriate cDNA template concentration for analyses.

4.2.6 Semi quantitative RT-PCR Analysis

Expression of nutrient transporter and growth response genes was also studied using the semi-quantitative RT-PCR method. Roots samples from all the treatments were taken, snap frozen in liquid nitrogen, stored at -80 ºC and further used for RNA isolation and cDNA synthesis as described above. The RT-PCR cycling conditions for amplification of each cDNA sample included an initial denaturation step of 95 ºC for 5 min followed by 35 cycles with denaturation at 95 ºC for 1 min, annealing at 50 ºC for 1 min, elongation on 72 ºC for 1 min. A final extension was carried out at 72 ºC for 10 min. After completion of all the steps, the expression levels were observed on 1% agarose gel in 1x TAE buffer with actin and ubiquitin genes as housekeeping controls. A 100 bp DNA ladder molecular weight marker (Axygen, USA) was run on all gels to confirm expected molecular weights and concentrations of the electrophoresed product. The ImageJ software (University of Wisconsin, USA) was used to determine the band intensity by measuring the pixel numbers of each band.

4.3 Results

4.3.1 Colonisation efficiency of *Serendipita* spp.

Although the *Serendipita secunda* isolates from *C. caerulea and C. picta* colonised the tomato seedlings, *S. whamiae* showed heavier cellular colonisation in tomato as evidenced by more extensive hyphal coils in cells (Figure 4.2, A-C). In barley, only *Serendipita secunda* from *C. picta* showed light colonisation in barley roots (Figure 4.2 D). Due to its faster growth rate and more vigorous colonising ability, *S. whamiae* was subsequently used in the growth and bioprotection experiments.

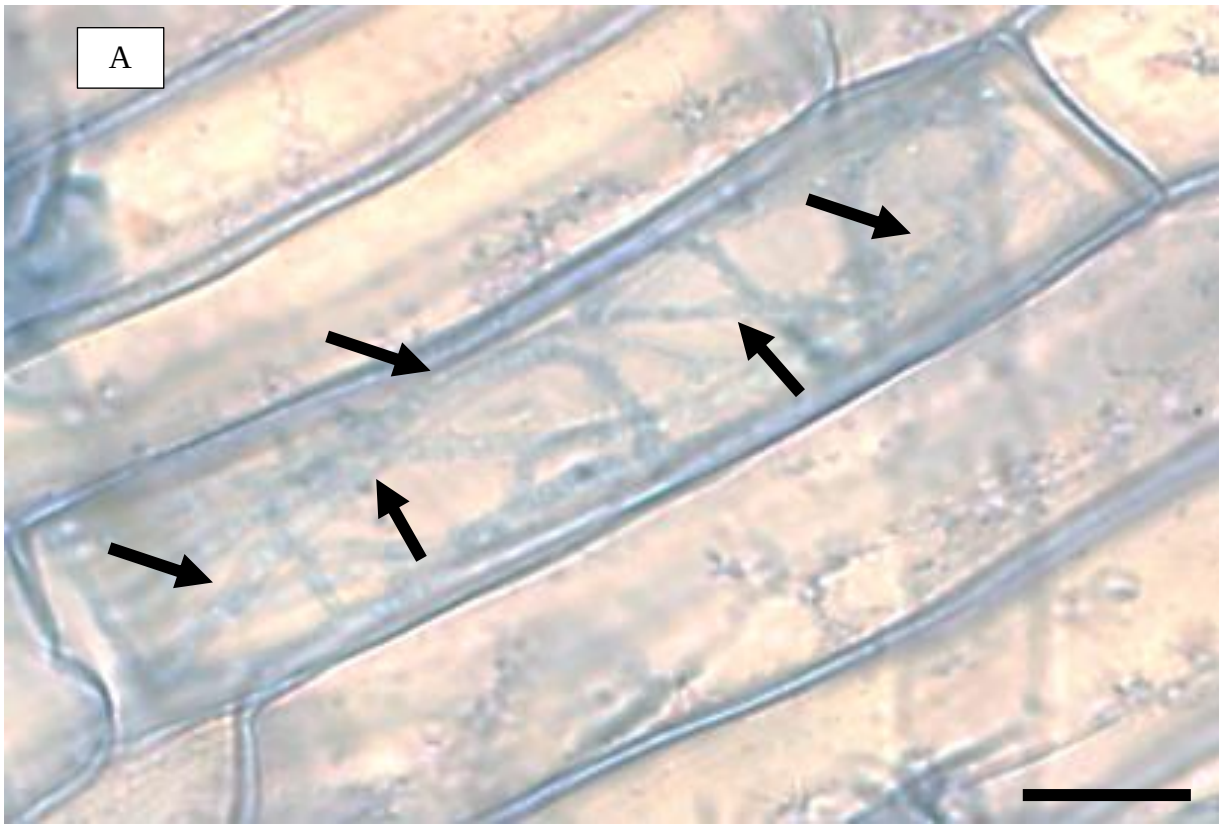


Figure 4. 2A: Tomato roots colonised with hyphae of *S. whamiae* (black arrows).

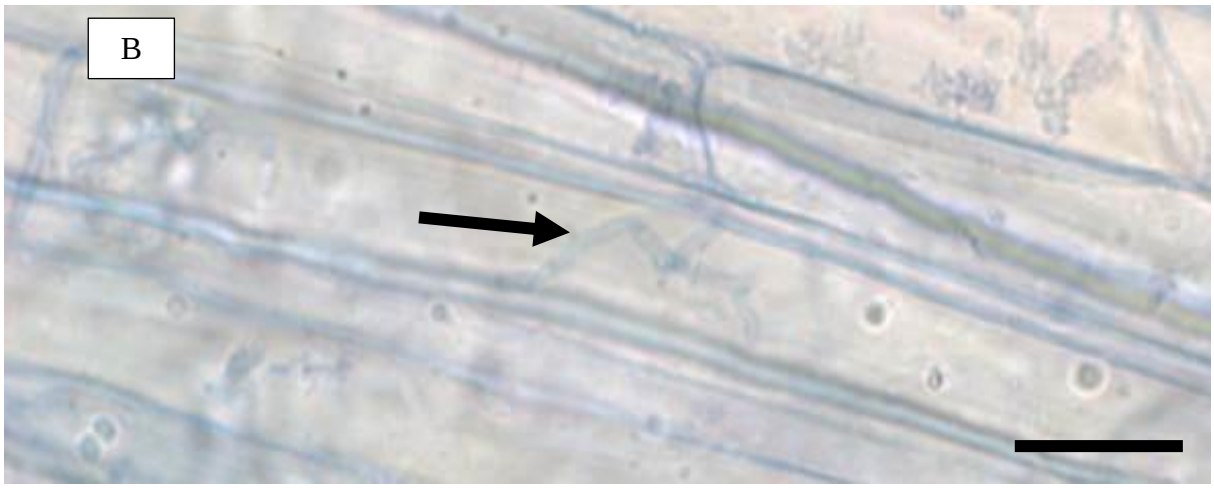


Figure 4. 2B: Tomato roots colonised with hyphae of *Serendipita secunda* from *C. caerulea* (black arrow).

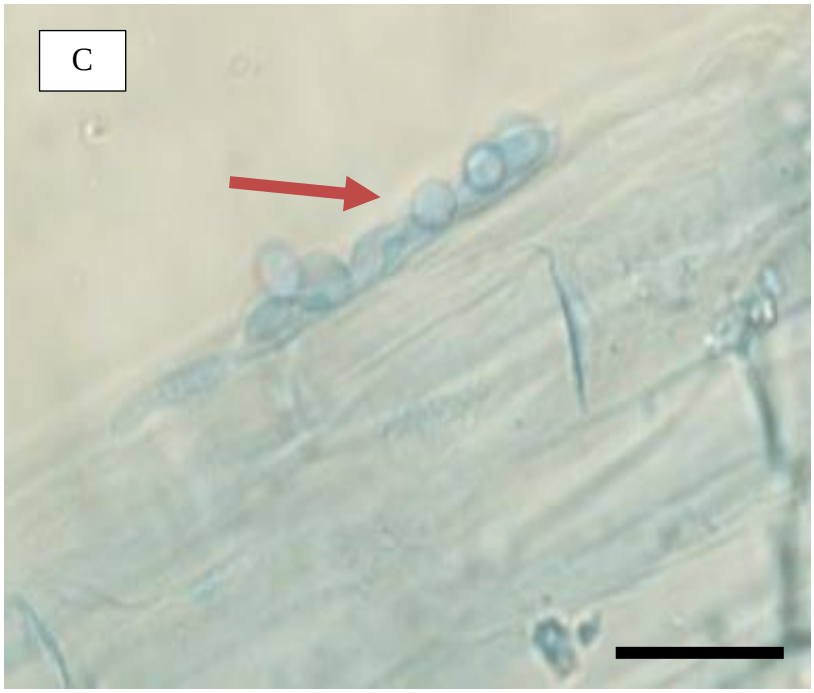


Figure 4. 2C: Tomato roots colonised with chlamydospore of *Serendipita secunda* from *C. picta* (red arrow).

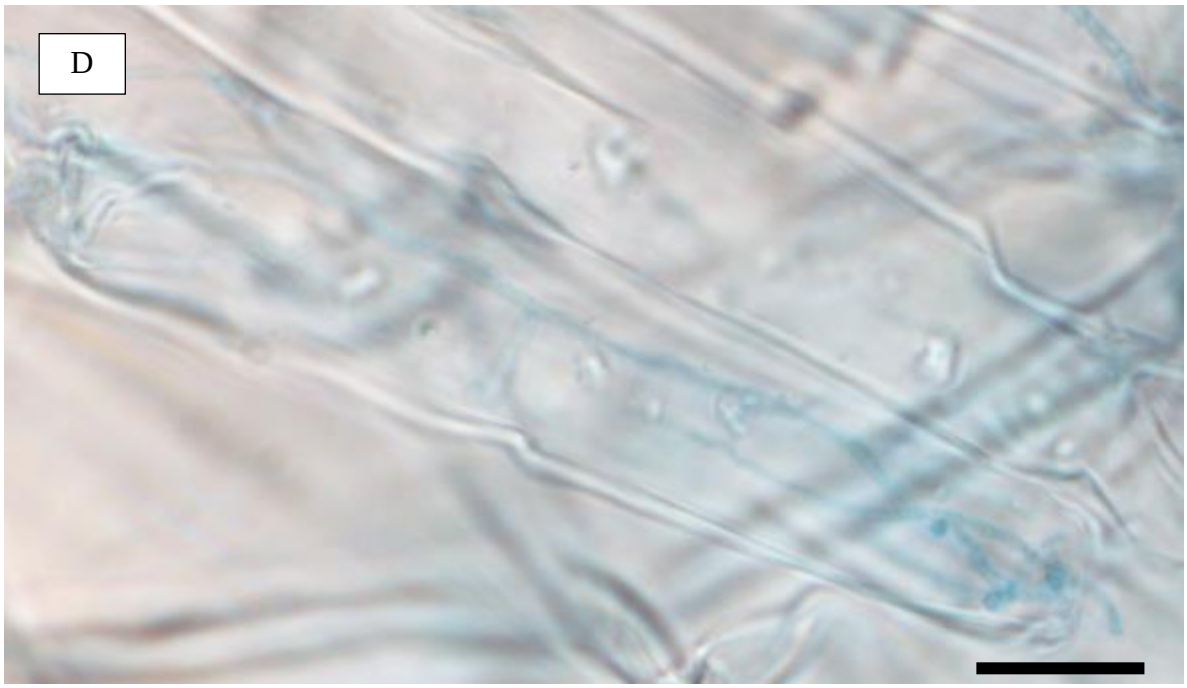


Figure 4. 2D: Barley roots colonised with *Serendipita secunda* from *C. picta* (D). Scale bars are 20 µm.

4.3.2 Measurement of growth parameters in the tomato-*Serendipita* system

The concentration of monilioidal cells and mycelium was determined by hemocytometer as $3x10^5$/ml. According to this, 1.5 ml contains $4.5x10^5$ propagules and 3 ml contains $9x10^5$ propagules. After 17 days, plants from the first experiment (3 ml hyphal suspension and 4 plugs) were harvested and growth parameters were measured (Figure 4.3). The total plant length was greater in control (*Serendipita* -) plants compared to inoculated (*Serendipita* +) plants (experiment one $P<0.05$, repeat of the experiment $P<0.01$) (Figure 4.4). Shoot length was significantly increased in control plants in experiment one ($P<0.01$), but in the repeat experiment there was no significant difference between the two treatments (Figure 4.5). Experiment one showed no significant difference in root length between the two treatments but in the repeat experiment there was greater root length in the controls ($P<0.05$) (Figure 4.6). There was no significant difference in fresh weight between colonised and un-colonised plants in either experiments (Figure 4.7). All values showed a normal distribution in both experiments except for fresh weight, which suggests a violation of the assumption of normality based on Shapiro-Wilk normality test (Appendix V-A).

Figure 4. 3: Tomato with (*Ser*+) and without (*Ser*-) after 17 days at the end of the growth experiment using 3 ml liquid inoculum. Note: the jars in the picture were selected randomly from each treatment.

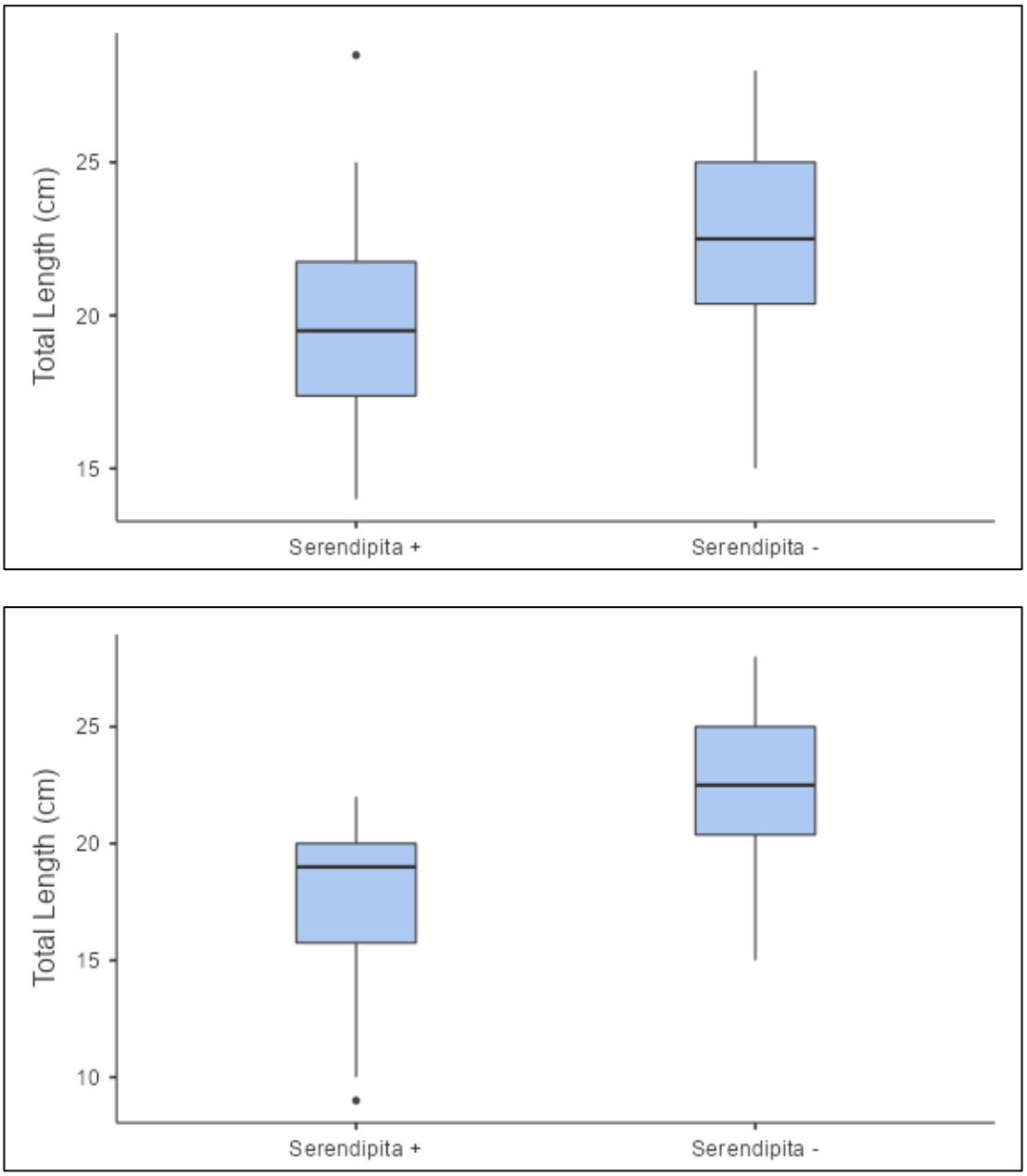


Figure 4. 4: Total length of tomato after 17 days for the *Ser*+/*Ser*- (control) treatments (3 ml inoculum) in experiment one (top) and the repeat experiment (bottom), n=20. Dots are the outlier values of the experiment.

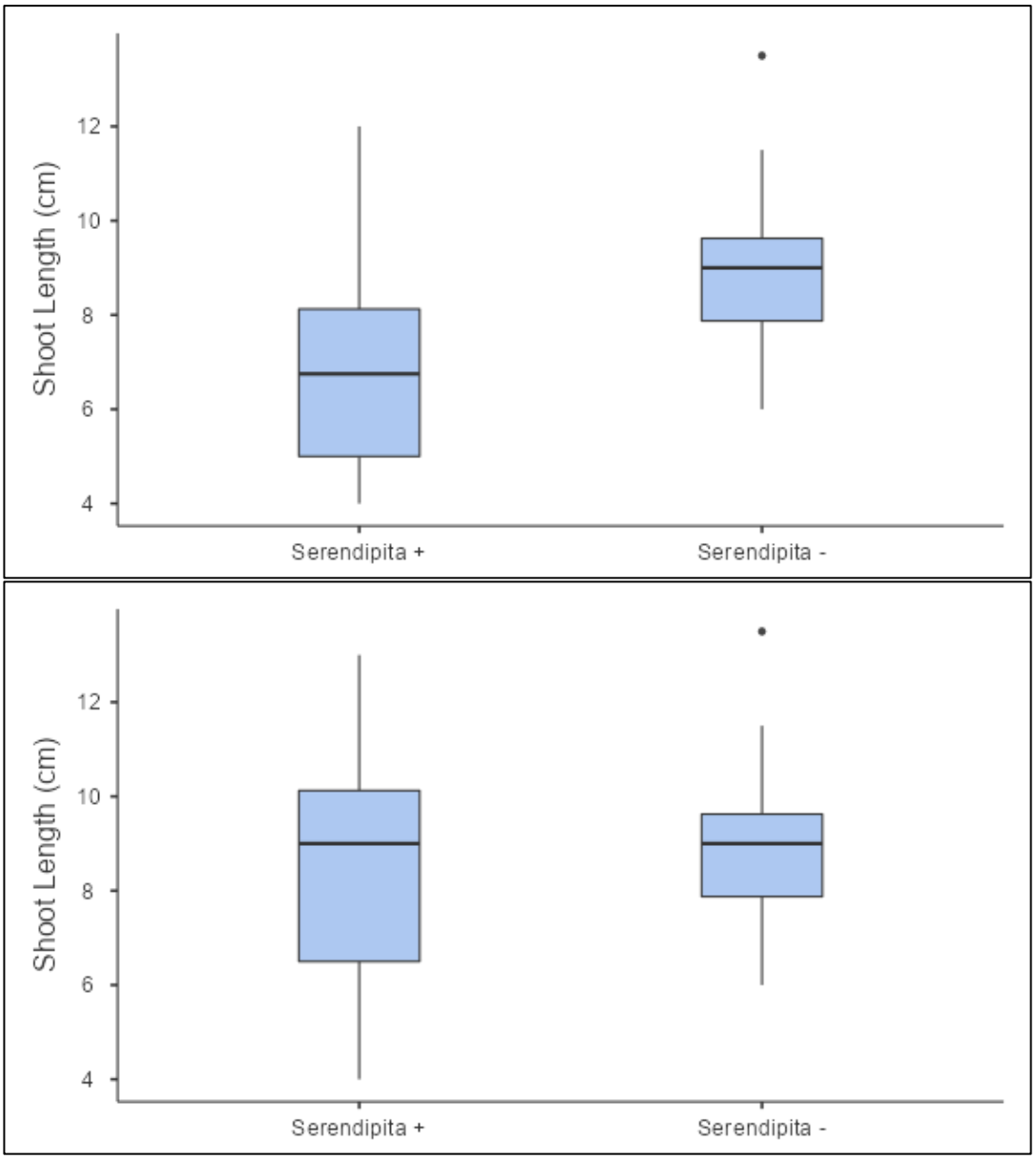


Figure 4.5: Shoot length of tomato after 17 days for the *Ser*+/*Ser*- (control) treatments (3 ml inoculum) in experiment one (top) and the repeat experiment (bottom), n=20. Dots are the outlier values of the experiment.

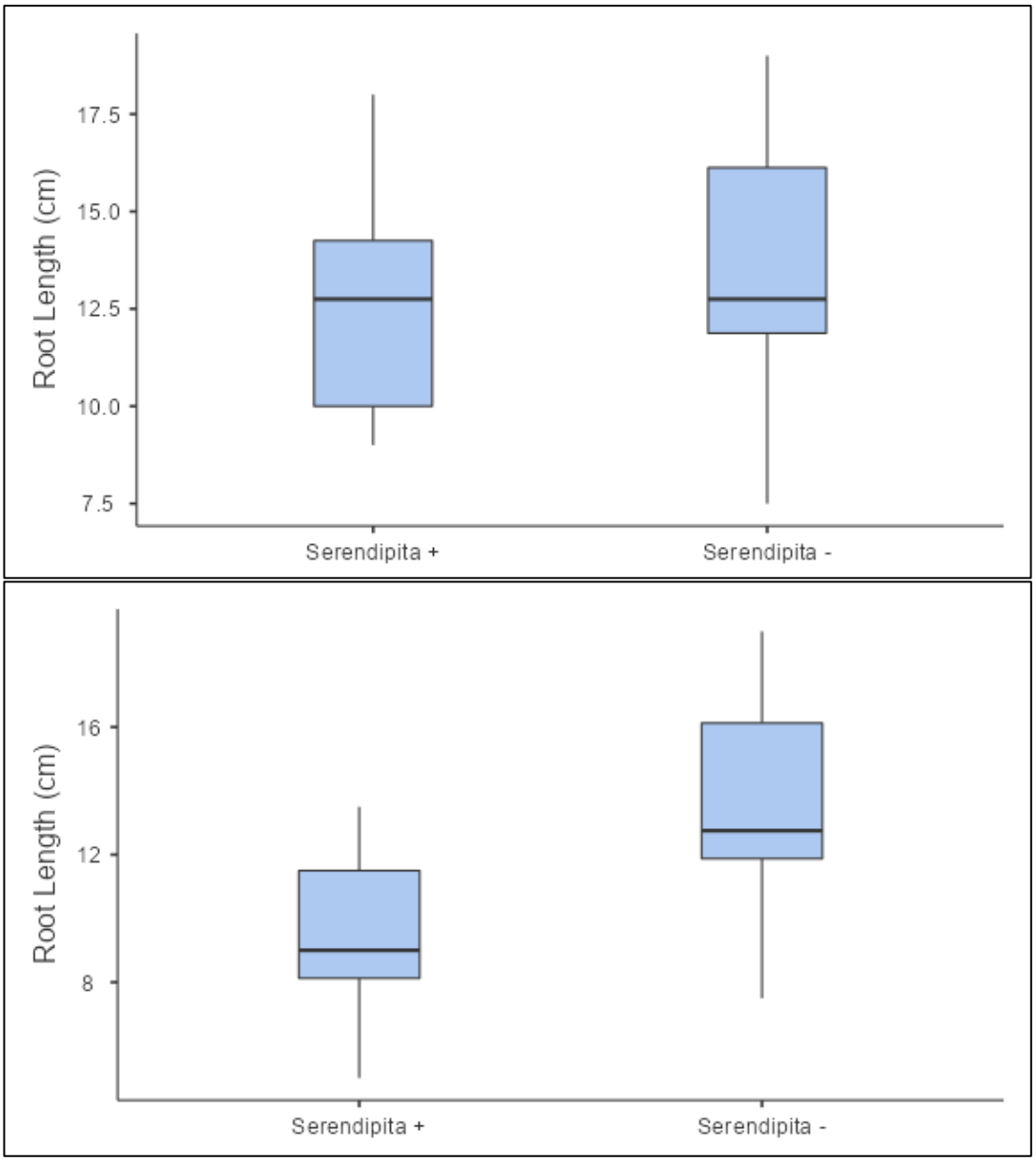


Figure 4.6: Root length of tomato after 17 days for the *Ser*+/*Ser*- (control) treatments (3 ml inoculum) in experiment one (top) and the repeat experiment (bottom), n=20. Dots are the outlier values of the experiment.

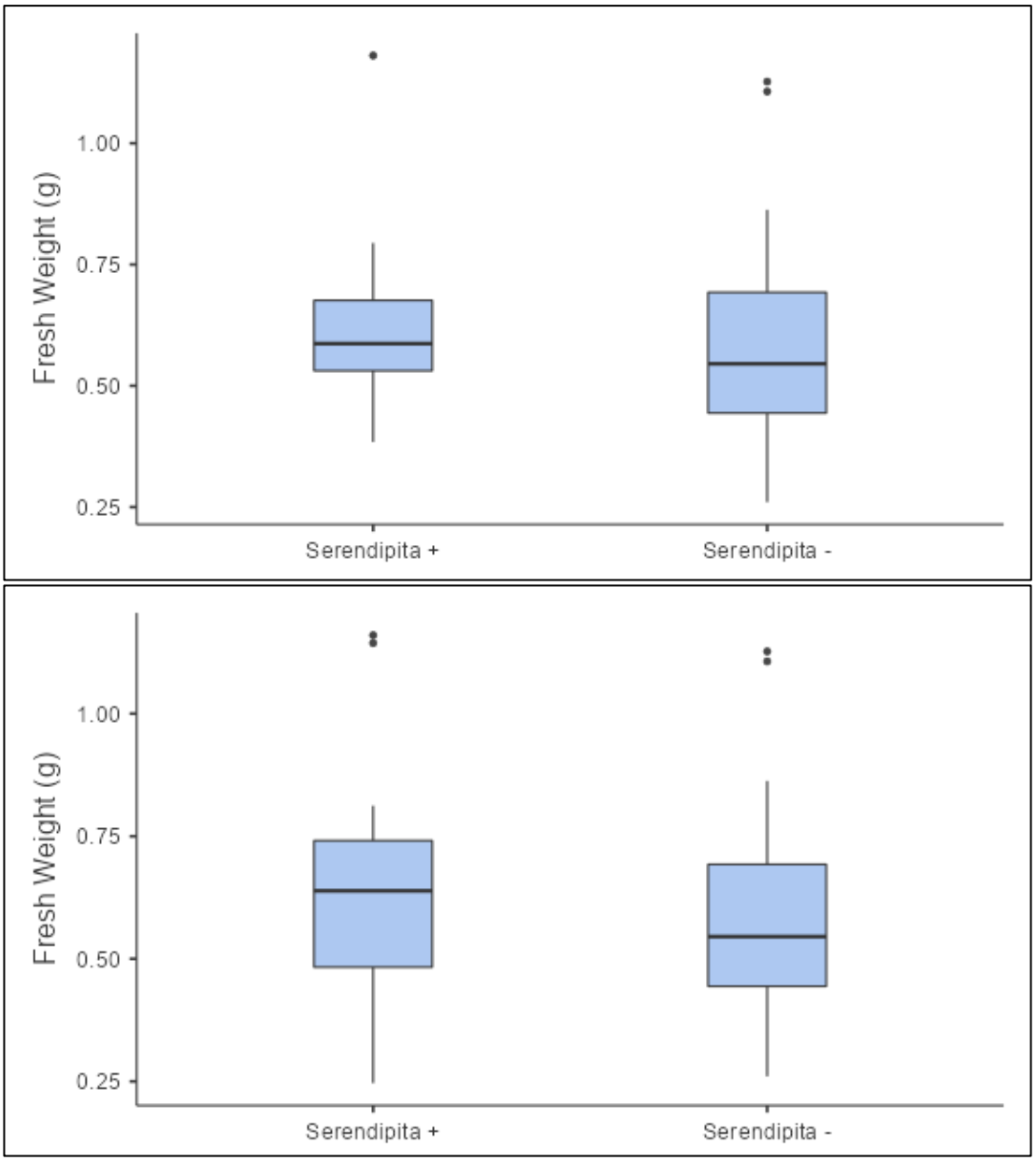


Figure 4. 7: Fresh weight of tomato after 17 days for the *Ser*+/*Ser*- (control) treatments (3 ml inoculum) in experiment one (top) and the repeat experiment (bottom), n=20. Dots are the outlier values of the experiment.

When using 1.5 ml of *Serendipita* hyphal suspension as inoculant there was no significant difference in total plant length in the first and second experiments, but *Serendipita* significantly decreased the total plant length in the third experiment ($P<0.05$) (Figure 4.8). The shoot length increased significantly in colonised plants in the first and third experiments respectively but the treatments displayed no significant difference in the second experiment (Figure 4.9). Control plants showed significantly higher root length in the first and third experiments ($P<0.01$) but there was no

significant difference between the treatments in the second experiment (Figure 4.10). *Serendipita*-colonised plants showed fresh weight enhancement in the first (P=0.079) and the third experiments (P<0.01) respectively but showed no significant difference from controls in the second experiment (Figure 4.11). All values had a normal distribution in the three experiments except fresh weight values which showed a low P-value in the first experiment, suggesting a violation of the assumption of normality based on Shapiro-Wilk normality test (Appendix V-B).

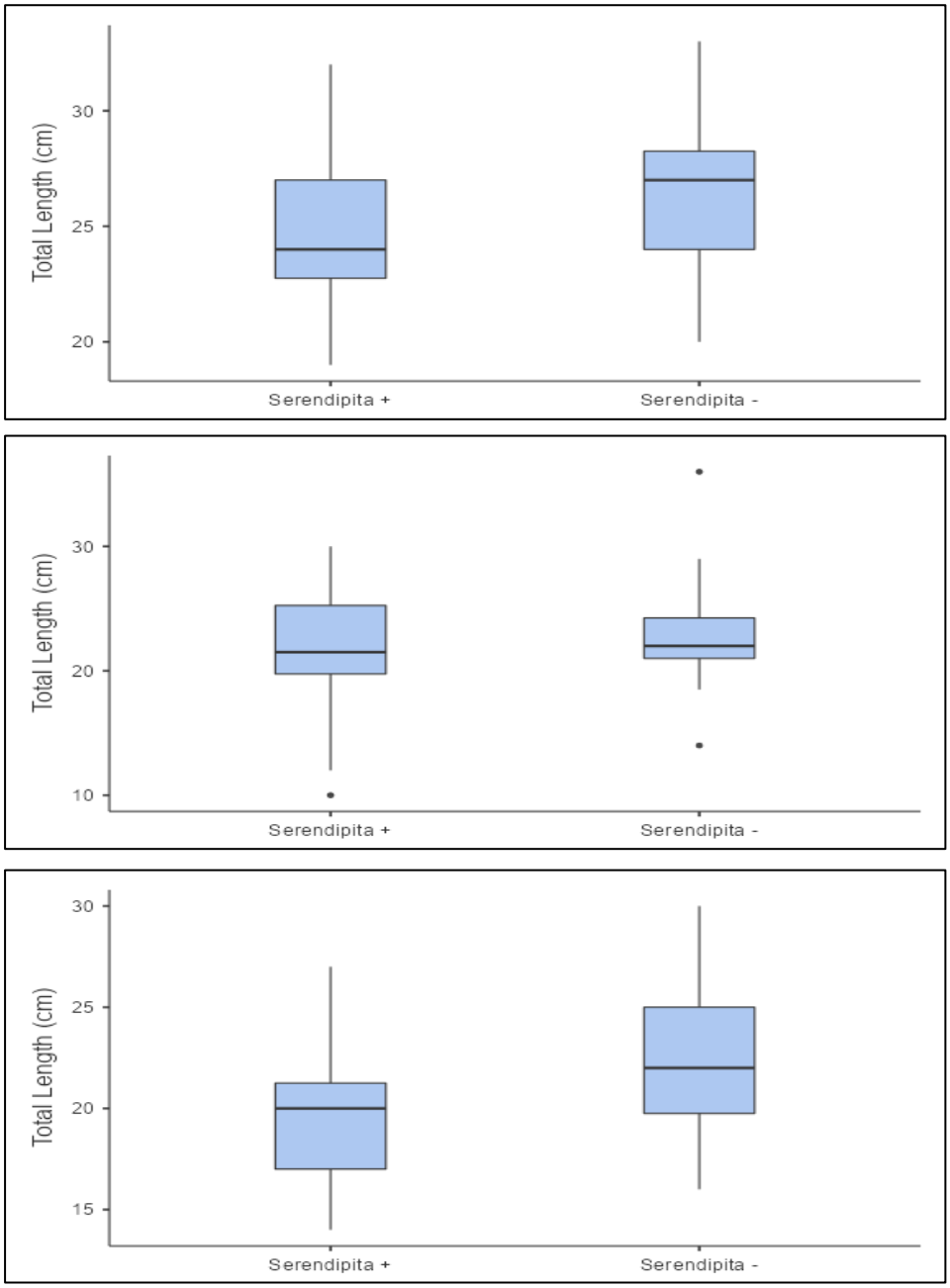


Figure 4.8: Total length of tomato after 17 days for the *Ser*+/*Ser*- (control) treatments (1.5 ml inoculum) in experiment one (top) and the repeat experiments (middle and bottom), n=20. Dots are the outlier values of the experiment.

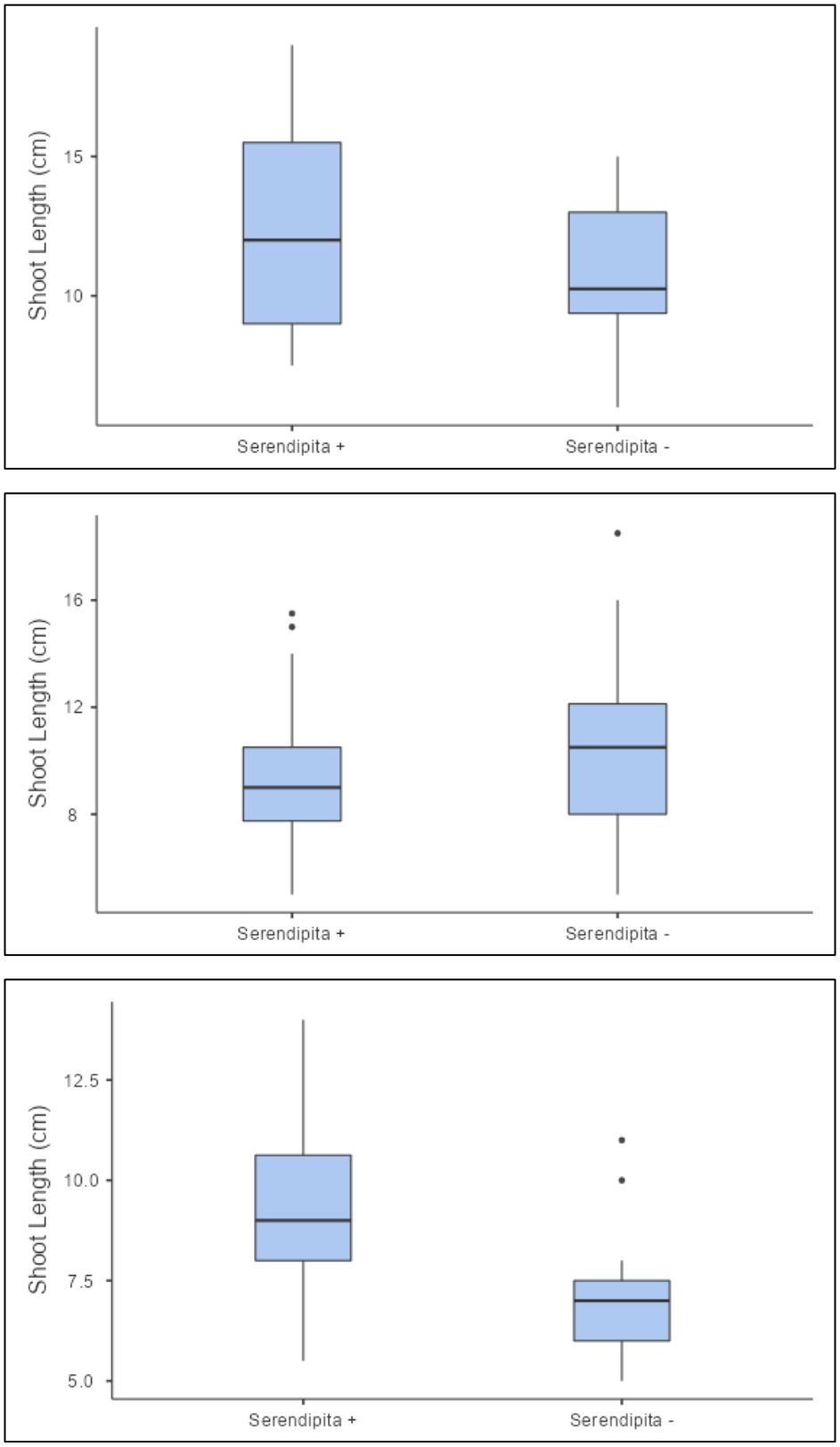


Figure 4.9: Shoot length of tomato after 17 days for the *Ser*+/*Ser*- (control) treatments (1.5 ml inoculum) in experiment one (top) and the repeat experiments (middle and bottom), n=20. Dots are the outlier values of the experiment.

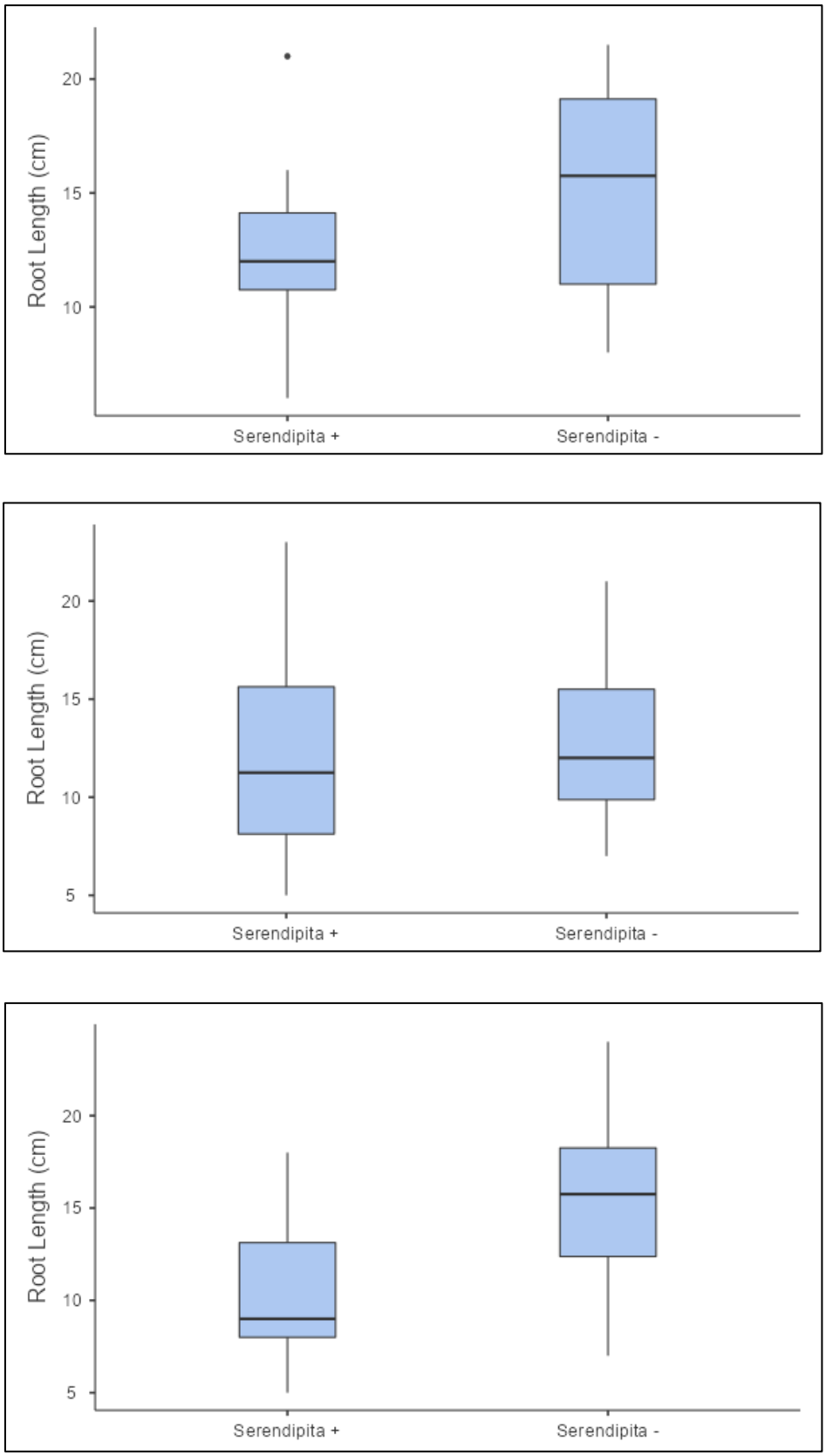


Figure 4.10: Root length of tomato after 17 days for the *Ser*+/*Ser*- (control) treatments (1.5 ml inoculum) in experiment one (top) and the repeat experiments (middle and bottom), n=20. Dots are the outlier values of the experiment.

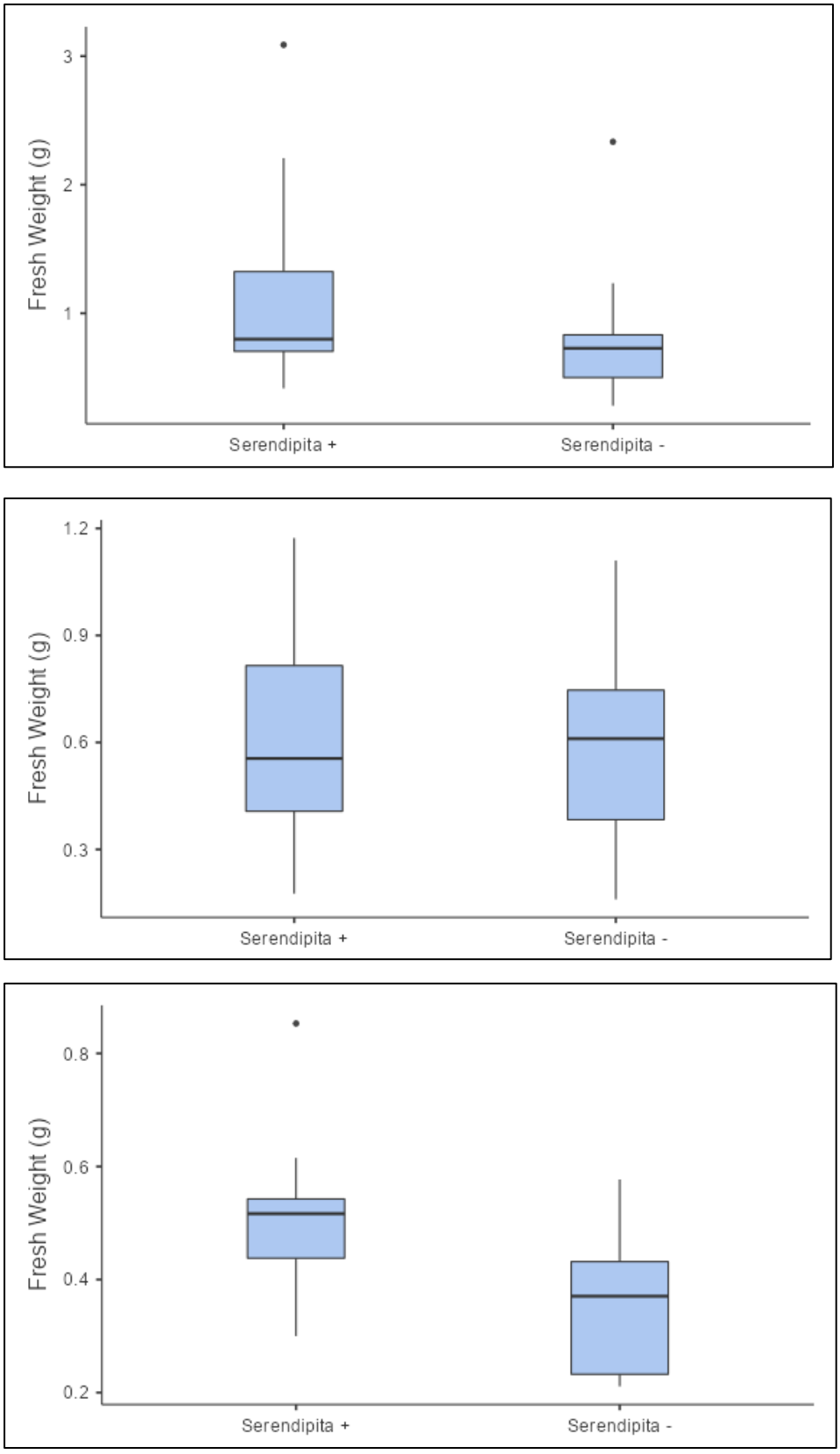


Figure 4.11: Fresh weight of tomato after 17 days for the *Ser*+/*Ser*- (control) treatments (1.5 ml inoculum) in experiment one (top) and the repeat experiments (middle and bottom), n=20. Dots are the outlier values of the experiment.

4.3.3 RNA extraction quality

RNA quantity and quality was checked by a Denovix spectrophotometer for three root samples from each of the *Ser*- and *Ser*+ treatments (Table 4.2). The RNA concentration varied from 47 ng/µl to 57 ng/µl. All *Ser*+ and *Ser*- RNA samples were of acceptable quality with absorbance ratios being around 2.0 at the 260/280 wavelength comparison and in the range of 2.0-2.2 at the 260/230 wavelength comparison.

Table 4. 2: Spectrophotometric analysis of RNA quantity and quality of the tomato roots in *Ser*+ and *Ser*- treatments. High quality RNA was indicated by an absorbance ratio of 2.0 at the 260/280 wavelength and in the range of 2.0-2.2 at the 260/230 wavelength.

Treatment	Concentration (ng/µl)	260/280	260/230
Ser+	47.177	1.8	2.1
Ser+	55.342	1.87	2.16
Ser+	47.389	1.83	2.17
Ser-	57.412	1.95	2.26
Ser-	48.879	1.9	2.18
Ser-	55.66	1.89	2.28

4.3.4 Results of cDNA synthesis and testing qPCR primers

The concentration of cDNA for all samples is outlined in table 4.3.

Table 4. 3: cDNA concentration of tomato roots harvested at 17 days in the *Ser*+ and *Ser*- treatments. Concentrations ranged from 10-30 in *Ser*+ and in the range of 12-21 in *Ser*-.

Treatment	Concentration (ng/µl)
Ser+	26.9
Ser+	30.2
Ser+	10.4
Ser-	18.2
Ser-	11.8

Ser-	20.6

When tested for their efficacy in amplifying tomato cDNA via conventional PCR, four primer sets showed expected band sizes in gels after PCR amplification including those for ubiquitin, actin, potassium transporter and phosphate transporter. The primers for the auxin response gene, ethylene synthesis gene and nitrate transporter were not subsequently used in qRT-PCR due to the absence of, or weak amplification or incorrect band sizes via conventional PCR. Experimentation was continued with the primers for the zinc transporter as the amplicon was slightly less than the expected size and likely due to secondary structure (Figure 4.12).

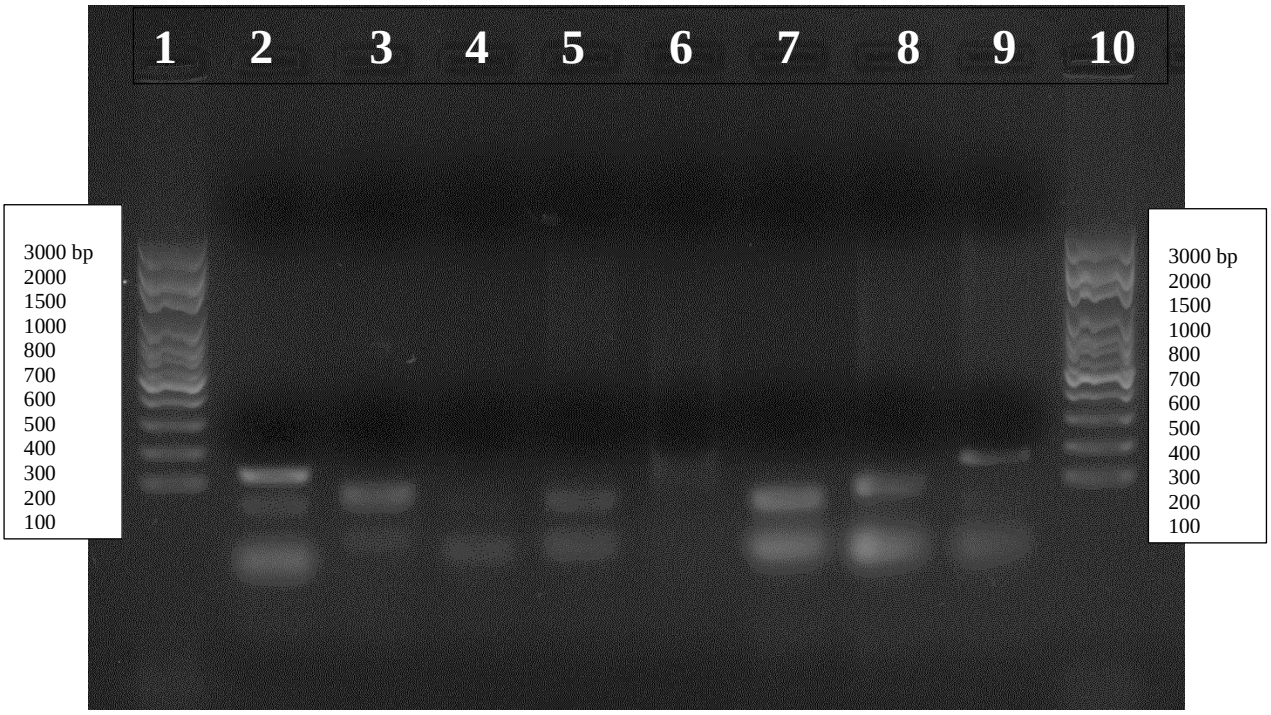


Figure 4. 12: Testing of growth and nutrient transporter primers; 1 & 10: DNA ladder with molecular weight in bp, 2: Ubiquitin, 3: Actin, 4: Auxin response gene, 5: Zn transporter, 6: Nitrate transporter, 7: Ethylene synthesis gene, 8: Potassium transporter, 9: Phosphate transporter.

4.3.5 Quantitative real-time polymerase chain reaction (qRT-PCR)

The cDNA of test genes of three roots from *Serendipita* inoculated plants and three roots of non-inoculated plants were compared via qRT-PCR. To check for errors in pipetting technique and correct operation of the qPCR machine, qPCR reactions were

run on ten identical gDNA samples from *Serendipita* with ITS primers. This analysis showed a consistency of Cq values for the samples (Table 4.4).

Optimization of template cDNA concentrations was carried out with the primers for the reference genes actin and ubiquitin. Undiluted cDNA gave high Cq values for both primers sets (table 4.5) and thus undiluted cDNA (following standardisation) was used in the qRT-PCR experiments.

The qRT-PCR experiment was performed twice. Actin and ubiquitin were used as reference genes, but they did not show consistent quantification within and between experiments and had an absence of amplification in some samples (Appendix VII-A). The Cq values of the nutrient transporter genes were similarly inconsistent (Appendix VII-A).

Table 4. 4: Testing of pipetting technique using gDNA samples. The gDNA was obtained from *Serendipita* and was tested with ITS primers.

Sample number	1	2	3	4	5	6	7	8	9	10
Cq value	31.41	31.43	31.44	31.47	31.80	31.89	32.02	32.03	32.64	33.09

Table 4. 5: Cq values of reference genes actin and ubiquitin at different cDNA concentration. The cDNA samples were obtained from tomato roots of *Ser*+ and *Ser*- treatments.

Reference gene	Sample	Cq
Actin	*Ser*+ undiluted	30.05
Ubi	*Ser*+ undiluted	28.71
Actin	*Ser*- undiluted	30.84
Ubi	*Ser*- undiluted	30.23
Actin	*Ser*+ 1:5	31.69
Ubi	*Ser*+ 1:5	30.14
Actin	*Ser*- 1:5	33.57
Ubi	*Ser*- 1:5	34.23
Actin	*Ser*+ 1:10	33.19
Ubi	*Ser*+ 1:10	30.08
Actin	*Ser*- 1:10	35.16
Ubi	*Ser*- 1:10	33.25

4.3.6 Semi quantitative RT-PCR analysis and bands intensity results

In the semi quantitative RT-PCR analysis, the reference gene ubiquitin largely showed consistency of expression except plant number 2 (*Ser*- treatment) where expression was weak. The reference actin gene showed even expression in both plant treatments. Potassium and zinc transporters showed inconsistent expression between and within treatments. Non-specific amplification using the potassium transporter primers was evident in one plant and therefore this sample was excluded from pixel intensity analysis. There was little expression of the phosphate transporter in both treatments although a band of incorrect size was visible in one plant sample (Figures 4.13- 4.17).

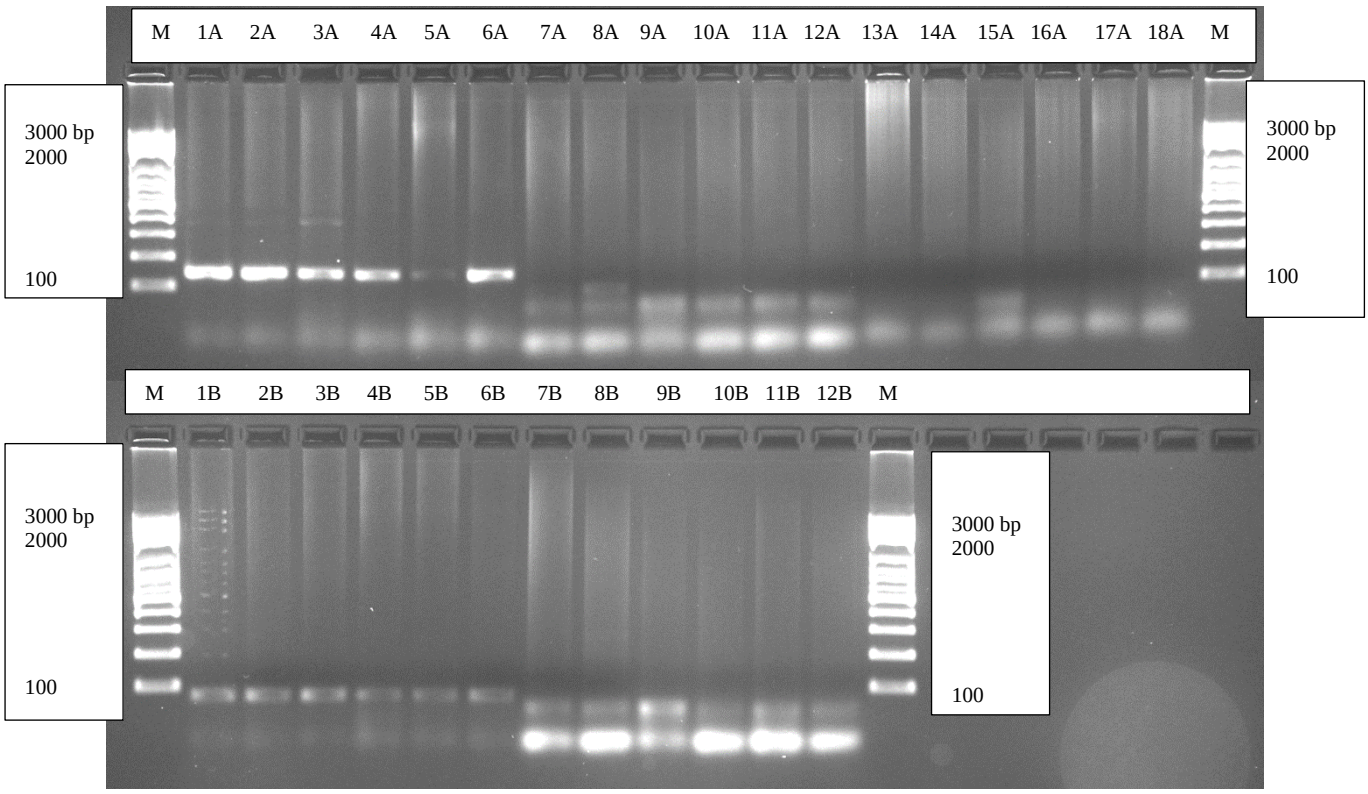


Figure 4. 13: M: marker, 1A-3A: Ubiquitin *Ser*+, 4A-6A: Ubiquitin *Ser*-, 7A-9A: Potassium transporter *Ser*+, 10A-12A: Potassium transporter *Ser*-, 13A-15A: phosphate transporter *Ser*+, 16A-18A: phosphate transporter *Ser*-, 1B-3B: Actin *Ser*+, 4B-6B: Actin *Ser*-, 7B-9B: Zn transporter *Ser*+, 10B-12B: Zn transporter *Ser*-.

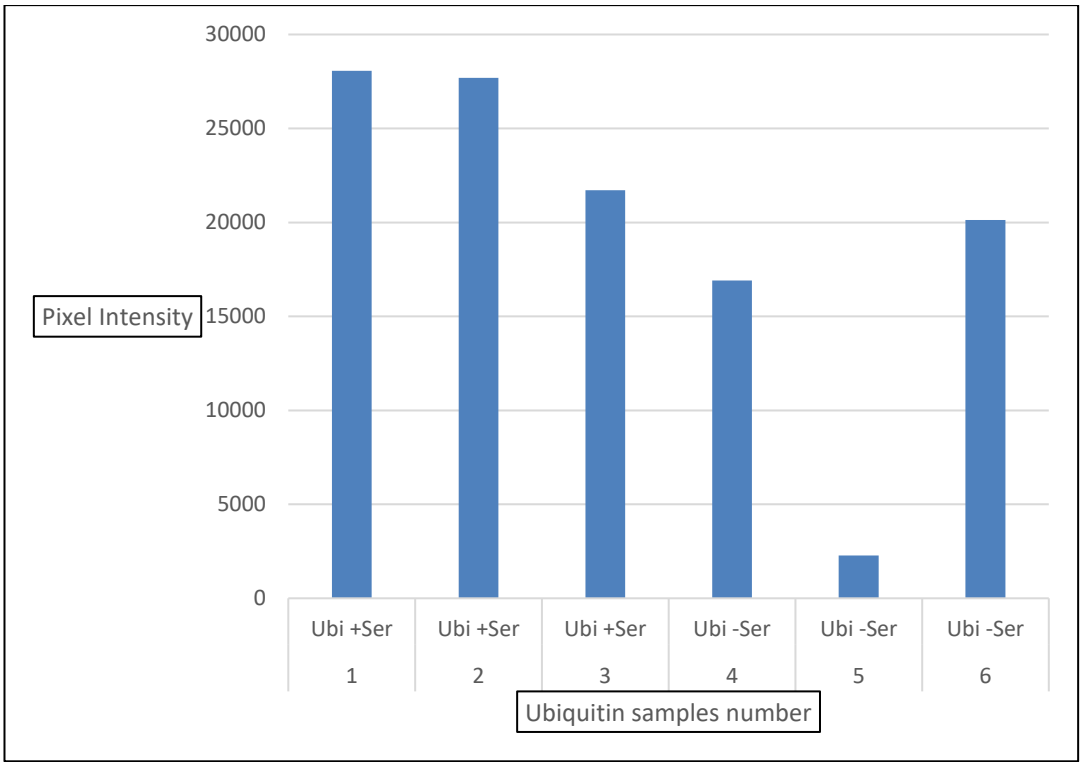


Figure 4. 14: Pixel intensity of ubquitin electrophoretic in *Ser*+ and *Ser*- treatments.

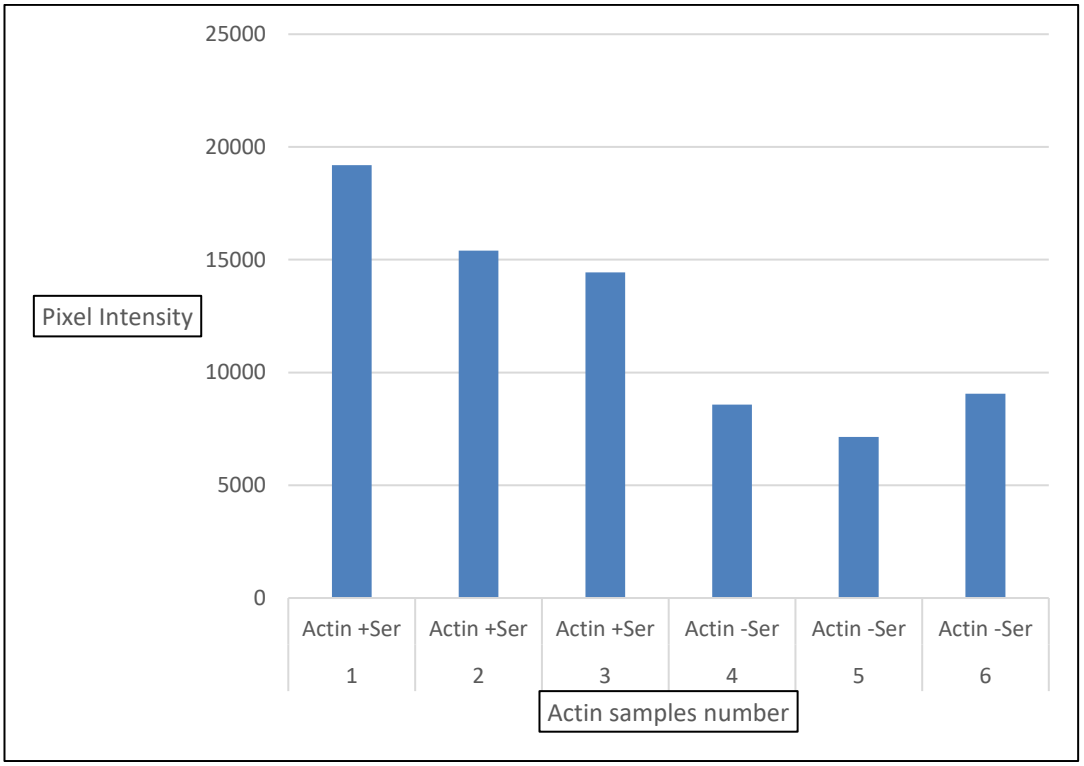


Figure 4. 15: Pixel intensity of Actin electrophoretic in *Ser*+ and *Ser*- treatments.

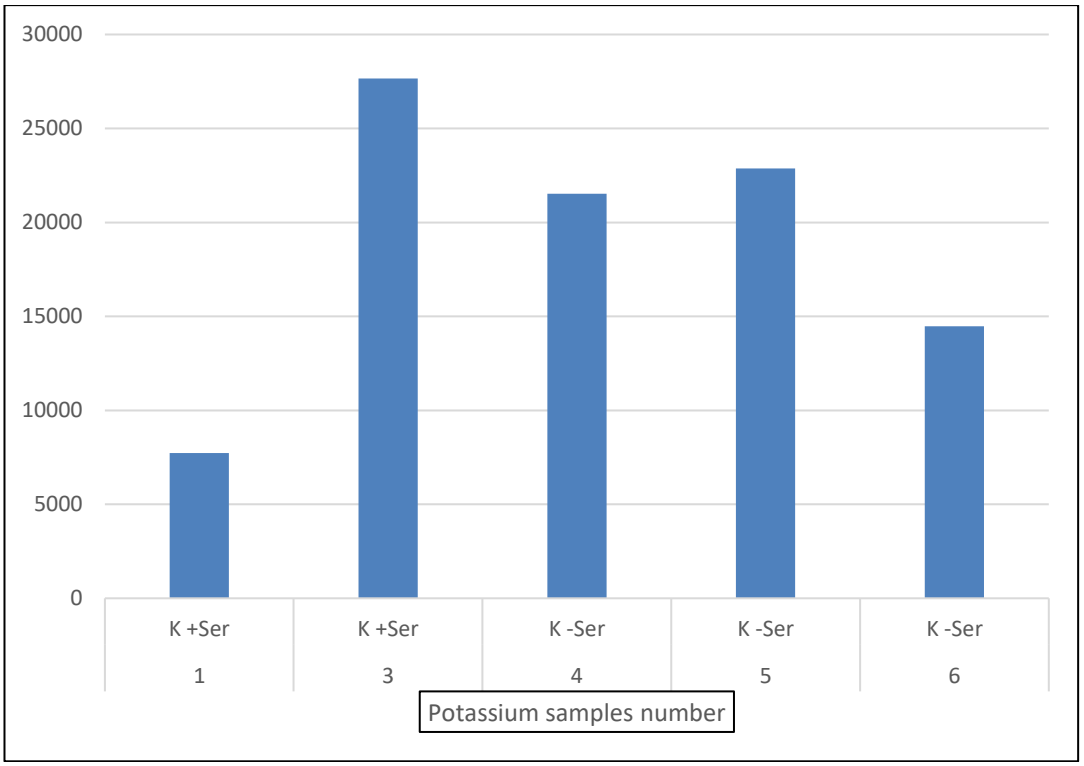


Figure 4. 16: Pixel intensity of K-transporter electrophoretic bands in *Ser*+ and *Ser*- treatments.

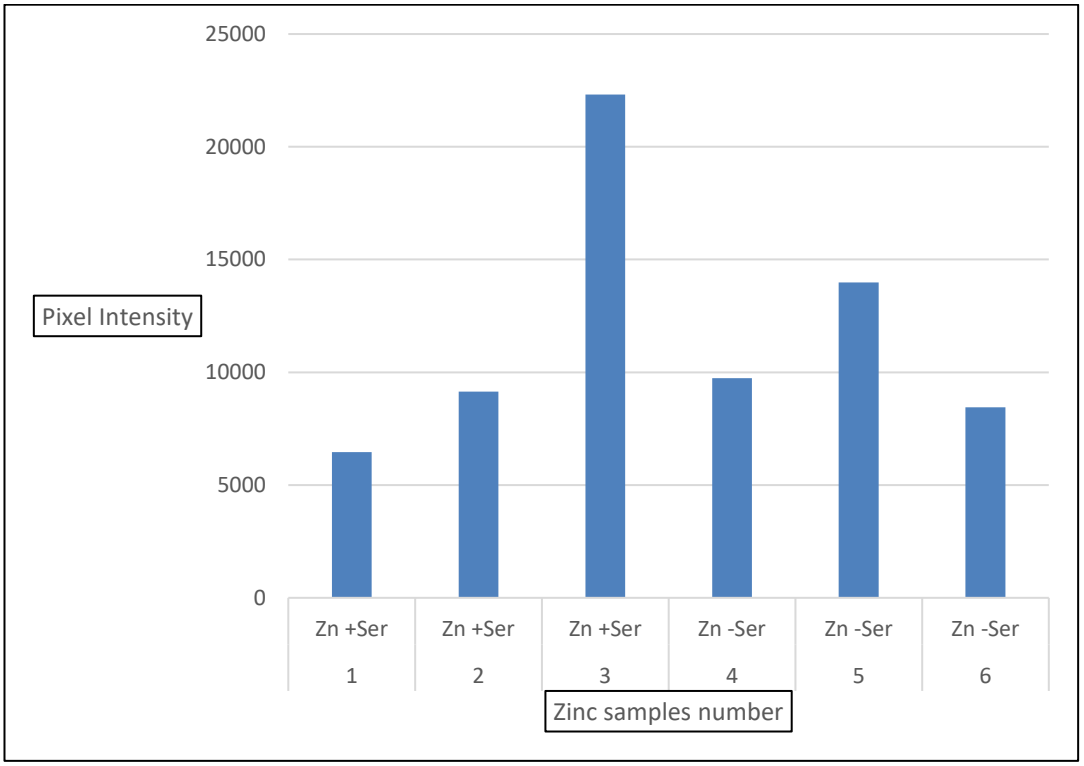


Figure 4. 17: Pixel intensity of Zn-transporter electrophoretic bands in *Ser*+ and *Ser*- treatments.

4.4 Discussion

Many studies have reported the positive impacts of *Serendipita* on growth parameters in different host plant species (Varma et al., 1999; Rai et al., 2001; Druge et al., 2007; Serfling et al., 2007; Meena et al., 2010). *Serendipita* also improves host plant nutrition as indicated by previous studies (Shahollari et al., 2005; Sherameti et al., 2005; Yadav et al., 2010 Nautiyal et al., 2010; Kumar et al., 2012). In this study, although *Serendipita* colonisation largely decreased total plant and root and shoot length, there was an overall increased plant fresh weight compared to controls. This latter result could be due to the symbiont increasing water uptake from the medium as is common in many mycorrhizal associations (Smith et al., 2004; Govindarajulu et al., 2005). Increased expression of aquaporin proteins may be a key component of the increased plant fresh weight observed here. Aquaporins are membrane intrinsic proteins that are responsible for water movement through cell membranes (Maurel et al., 2008; 2015). Aquaporins help plants to maintain water balance during drought stress by modifying the permeability of water membrane (Maurel et al., 2008; Moshelion et al., 2009; Zarrouk et al., 2016). Also, aquaporins have water acquisition roles in mycorrhizal fungi, where they control water uptake and release by the hyphae, and this impacts on hyphal cell expansion, division, and hyphal fusion (Xu & Zwiazek, 2020). Upregulation of aquaporin genes has been observed in mycorrhizal plants such as maize (Chaumont et al., 2001; Barzana et al., 2014), *Picea glauca* (Xu et al., 2015) and *Pinus silvestris* (Peter et al., 2016). Monitoring of aquaporin gene expression in this system is a logical next step here and may provide insight into the molecular control of the association.

Using three ml of *Serendipita* hyphal suspension as inoculum did not improve tomato growth parameters in the two replicates of the growth experiment and necrosis symptoms were been observed on plants. Fakhro et al (2010) and Andrade-Linares et al (2013) mentioned that using high levels of *Serendipita* inoculum (9×10^5 cfu/ml) in nutrient solution, sand or substrate, had negative growth impacts on tomato, while positive results were accomplished using lower concentration (3×10^5 cfu/ml). Compared with using 3 ml of hyphal suspension, 1.5 ml improved shoot length and fresh weight in *Serendipita*-colonised plants in the first and third replicates, and no necrosis was observed in plants. These results highlight the critical importance of

optimising inoculum levels if this technology is to be adopted for use in agriculture or horticulture.

The negative impacts of *Serendipita* on plant growth have been observed when the fungus was cultivated on ammonium instead of nitrate as the sole N source (Kaldorf et al. 2005). This does not match the observations here because the co-cultivation medium PNM contains N in a nitrate form (KNO_3). The other factor that causes negative consequences of *Serendipita* colonisation for plants is low external phosphate content. This phenomenon of negative growth effects under phosphate limited conditions has been observed extensively in mycorrhizal plants (Smith et al., 2003; Smith et al., 2004; Li et al., 2006; Li et al., 2008; Smith et al., 2009). Wheat has shown growth depressions with ten different mycorrhizal fungi species in glasshouse experiments using low phosphate field soils (Graham & Abbott, 2000). Growth depressions with mycorrhizal fungal colonisation have also been observed in barley (Grace et al., 2009), and tobacco (Modjo & Hendrix, 1986; Modjo et el, 1987; Guo et al., 1994). Some studies have suggested that limited phosphate level leads to aggressive mycorrhizal feeding behaviour (Graham & Abbott, 2000), and competition with the host for photosynthesis (Modjo & Hendrix, 1986). Surprisingly, PNM does not contain phosphate although it has been widely used in co-cultivation systems to study the interaction between plants and *Serendipita* fungi. The lack of phosphate in the PNM may explains the weak amplification of the phosphate transporter gene in both treatments. It would be interesting to examine the molecular control of the *Serendipita*-tomato growth response in media that did actually contain phosphate eg. MS media. Furthermore, plants contain a variety of phosphate transporter genes and additional gene expression studies may determine the key molecular controls of the association.

qRT-PCR analysis was used to analyse gene expression changes in tomato plants colonised by *Serendipita* fungi. Although optimum pipetting technique and machine operation was checked, unfortunately, the Cq values of most of the genes investigated, including the reference genes, actin and ubiquitin, were not consistent between replicates of the same treatment and between experiments. This could be caused by qPCR efficiency, inhibitory compounds (in addition to polysaccharides and phenolic compounds which were removed from the mRNA), PCR artefacts, and sampling

variation (Ruiz-Villalba et al., 2021). Also, the low concentration of cDNA may have impacted on the consistency of the Cq values.

In the semi quantitative RT-PCR analysis, no major differences in expression were found in the zinc and potassium transporters between colonised and un-colonised plants. The absence of detection of the phosphate transporter in this analysis after initial verification of primer design suggests that there are continued quality issues (i.e presence of inhibitors or degradation of template over time) with the cDNA that made the semi-quantitative (and qRT-PCR) process unreliable. The lack of any impact of *Serendipita* species on plant nutrient content has been shown previously. For example *S. indica* has no impact on phosphate content in tobacco (Barazani et al. 2007), barley (Achatz et al. 2010) and green gram (Ray & Valsalakumar 2010). Recently, *S. bescii* also did not increase the phosphate content in winter wheat (Ray et al., 2021). In contrast, other studies showed improved phosphate content in *Serendipita*-colonised chickpea (Nautiyal et al. 2010) and black lentil (Kumar et al. 2012). This variation between studies may be explained by differences in experimental conditions including the host plant species and *Serendipita* taxon used (Fakhro et al. 2010).

Arbsucular mycorrhizal colonisation typically improves the phosphate content of plants (Yang et al., 2012; Graham & Abbott, 2000). During the symbiosis there is an upregulation of mycorrhizal specific nutrient transporters such as the StPT3 phosphate transporter in potato (Rausch et al., 2001), and the MtPT4 phosphate transporter in *Medicago truncatula* (Harrison et al., 2002). Arbsucular mycorrhizal colonisation also leads to down regulation of genes for root hair nutrient transporters such as the MtPT1 and MtPT2 phosphate transporters in *Medicago truncatula*, (Liu et al., 1998). Other studies have reported that root-hair potassium transporters are down regulated in *Medicago truncatula* roots colonised with the mycorrhizal fungus *Rhizophagus irregularis* (Gomez et al., 2009; Gaude et al., 2012). There is downregulation of a root-hair Zn transporter MtZIP2 in mycorrhizal *M. truncatula* (Burleigh et al., 2003; Nguyen et al., 2019) while a symbiosis specific gene MtZIP14 is upregulated in mycorrhizal plants (Cardini et al., 2021).

This research shows that the tomato-*Serendipita* interaction has similarities and differences with other mycorrhizal associations. Colonisation leads to an improvement

of plant fresh weight likely due to enhanced water flow from the media via a hyphal conduit. *Serendipita* may cause plant growth depression particularly with high levels of inoculum application and this is likely due to the microbe acquiring excessive amounts of host photosynthate – a situation often observed in arbuscular mycorrhizas. Under the conditions used in the study, *Serendipita* colonisation did not appear to affect the expression of potassium, zinc and phosphate nutrient transporters suggesting that *Serendipita* may not be a major transporter of inorganic substances to the plant.

CHAPTER 5: *Serendipita* AFFECTS TOMATO IMMUNITY AGAINST THE POWDERY MILDEW PATHOGEN *Golovinomyces lycopersici*

5.1 Introduction

5.1.1 Powdery mildew disease in tomato

Several factors limit the production of tomatoes and these include damage caused by insects and microbial pathogens. Of the microbial pathogens, powdery mildew is one of the diseases that threatens tomato crop production in Australia and causes considerable losses up to 44% for Australian farmers as reported in Richards (1993). Powdery mildew is a very common and easily identified disease caused by around 900 species of the Erysiphaceae and affects approximately 10,000 plant species (Takamatsu, 2004). The first record of tomato powdery mildew was in the late 19th century in Australia (Cooke & Massee 1888). Around 100 years later, another species has started to spread in tomato production from the Netherlands, causing serious tomato powdery mildew epidemics across the entire European continent (Jones et al., 2000, 2001; Kiss et al., 2001). Three powdery mildew species infect tomato: *Leveillula taurica, Pseudoidium neolycopersici* and *Golovinomyces lycopersici*. *Leveillula taurica* prefers subtropical conditions (Lindhout et al., 1994). *Golovinomyces lycopersici* (previously known as *Euoidium lycopersici*) has only been reported from Australia (Kiss et al., 2001; Braun et al., 2019; Kiss et al., 2020). *Pseudoidium neolycopersici* is widespread in many parts of the world but has never been found in Australia (Kiss et al., 2001, 2005; Braun & Cook 2012). Powdery mildew symptoms appear on the plant as white to greyish spots or patches (Agrios, 2005). The mycelium grows on the lower leaf side in *Leveillula* infection and on the upperside of the leaf in the case of the other two species that infect tomato (Lindhout et al., 1994). Powdery mildew fungi are obligate biotrophic plant pathogens and acquire nutrients from the infected plant cells through feeding organs called haustoria. During the infection process, spores of the powdery mildew pathogen land and germinate on the plant leaf. After germination, spores form a primary germ tube. Then, an appressorium is formed which produces a penetration peg and invades the leaf tissues. Haustoria develop inside the mesophyll cells in the case of *Leveillula* (Zheng et al., 2013) and inside the epidermal cells in *P. neolycopersici* and *G. lycopersici* (Jacott et al., 2017; Figure 1).

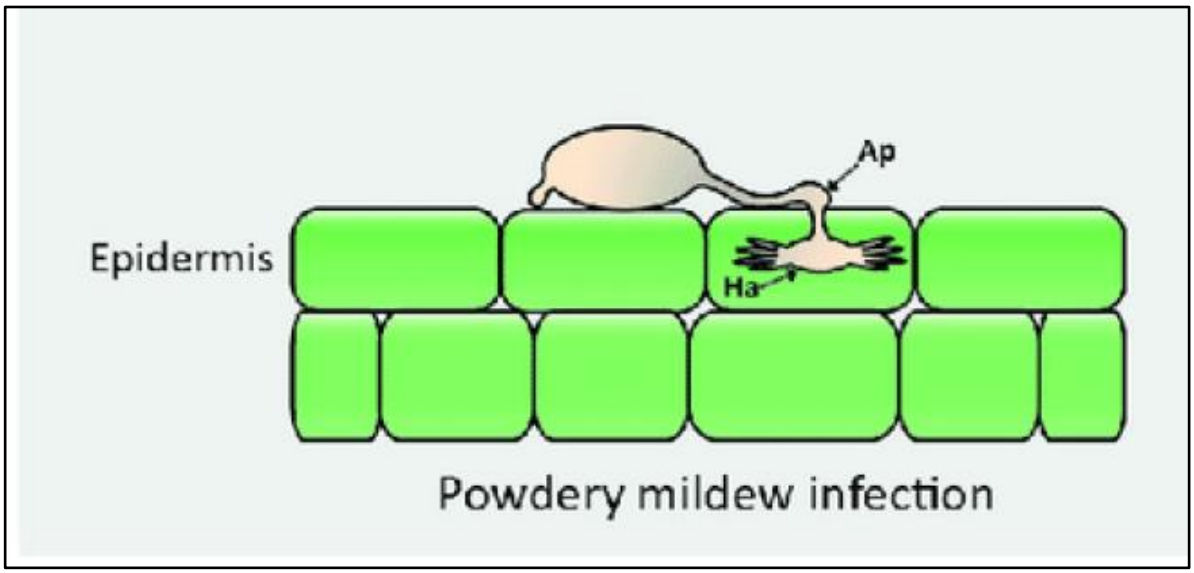


Figure 5. 1: The early stage of the powdery mildew infection process in the cereal powdery mildew, *Blumeria graminis* which is similar to tomato powdery mildew; Ap: appressorium, Ha: haustorium (Jacott et al., 2017).

Fungicides and resistant cultivars are the conventional methods to control powdery mildew in tomato (QDAF, 2018; GRDC, 2019). However, these approaches are not effective in the long-term due to the ability of the fungal populations to evolve resistance (Kiss, 2003), for example resistance to flutriafol and tebuconazole is now commonly observed (GRDC, 2019). Indeed, most available commercial tomato cultivars are susceptible to powdery mildew disease (Kiss et al., 2001). Knocking out of susceptibility genes such as the Mildew Locus O (*MLO*) gene family in tomato confers resistance to powdery mildew (Pessina et al., 2016). However, loss of function of the *MLO* gene may produce undesirable traits such as early senescence, lower plant size, reduced seed production, lower germination rate and lesion-mimic symptoms (Jiang et al., 2016; Kusch & Panstruga, 2017; Polanco et al., 2018).

5.1.2 The tomato-powdery mildew interaction

During the incompatible tomato-powdery mildew interaction, the HR is activated which prevents the formation of fungal haustoria (Huang et al., 1998; Bai et al., 2005). The HR is a resistance strategy that induces cell death of plant leaf tissue to limit the extent of fungal infection (Nimchuk et al. 2003). The oxidative burst (OB) begins HR and includes high accumulation of reactive oxygen species (ROS), such as H_2O_2 which

enhance cell death (Lamb & Dixon 1997; Torres et al., 2006). Callose formation around the area of HR leads to further halting of fungal development, because it is produced before haustoria formation (Seifi et al., 2014). Another chemical that may be induced during powdery mildew infection is suberin (Agrios, 2005). Suberin is present in tissues in underground plant organs such as roots and tubers. Specific organs and cells form suberin as a defence barrier against pathogens (Agrios, 2005). In addition to callose and suberin, lignin deposition limits the spread of pathogens in leaf tissue. Lignin is a component of the secondary cell wall and is induced by wounding and prevents pathogen spread (Kimmins & Wuddah, 1977).

5.1.3 *Serendipita* enhanced resistance to pathogens

Numerous cellular changes occur in tomato during powdery mildew infection. Resistant tomato cultivars respond to the powdery mildew pathogen via membrane enzymes, NADPH oxidases, peroxidases, amine oxidases, and oxalate oxidases (Hückelhoven, 2007). These enzymes activate the oxidative burst (OB) which includes reactive oxygen species and callose accumulation (Seifi et al., 2014). In contrast, susceptible cultivars such as Moneymaker show a weak induction of OB and low accumulation of reactive oxygen species and callose (Li et al. 2007, 2012; Seifi et al., 2014). It is hypothesised that colonising Moneymaker plants with *Serendipita* may lead to enhanced resistance to biotic stress. The activation of resistance mechanisms in *Serendipita*-colonised plants has been recorded in previous studies. The concentration of hydrogen peroxide has been shown to increase in the leaves of *Serendipita*-colonised susceptible wheat after powdery mildew infection (Serfling et al., 2007). Furthermore, *Serendipita*-colonised barley restricted haustoria formation by a powdery mildew pathogen with a hypersensitive reaction, which included a host-cell-death response and cell-wall associated defence (Waller et al., 2005). Increasing levels of antioxidant enzymes such as those associated with the activation of the glutathione–ascorbate cycle have been observed in barley roots treated with *Serendipita* against a *Fusarium* root rot pathogen (Waller et al., 2005; Harrach et al., 2013). Moreover, superoxide dismutase, catalase and peroxidase antioxidants increased in *Serendipita*-colonised plants under biotic stress (Kumar et al., 2009; Harrach et al., 2013; Lin et al., 2019).

During pathogen infection stress, the SA-pathway is commonly activated in plant tissues leading to systemic acquired resistance (Vernooij et al., 1994; Ryals et al., 1996; Vlot et al., 2009; Spoel et al., 2012). Contrastingly, during plant interactions with beneficial microbes the JA/ET signalling pathway is activated leading to induced systemic resistance (ISR) (Hoffland et al., 1995; Pieterse et al., 2000; Van Loon & Bakker, 2005; Ahn et al., 2007; Stein et al., 2008). Although these are separate plant responses, some studies have shown that non-pathogenic microbes can trigger both pathways (Audenaert et al., 2002; Mathys et al., 2012; Sun et al., 2014; Lin et al., 2019).

Serendipita fungi have been reported to reduce the expression of defence genes so as to overcome the immune system of the host plant (Schafer et al., 2009; Camehl et al., 2011; Pedrotti et al., 2013). Genes encoding the antioxidant enzymes monodehydroascorbate reductase and dehydroascorbate reductase are down-regulated by *Serendipita* to allow successful colonisation (Vadassery et al., 2009; White & Torres, 2010; Foyer & Shigeoka, 2011; Hamilton et al., 2012). *Serendipita* also suppressed salicylic and jasmonic dependent genes to allow host colonisation (Waller et al., 2005; Akum et al., 2015).

This part of the project will investigate for the first time, the combination of tomato, *Serendipita* as a mycorrhizal fungus and tomato powdery mildew pathogen *Golovinomyces lycopersici*. Previous studies have shown the interaction between two of the mentioned components, such as *Serendipita* against powdery mildew in barley (Waller et al., 2005), wheat (Serfling et al, 2007) and *Arabidopsis* (Stein et al., 2008). In addition, one study has shown *Serendipita* increases the resistance of tomato to the *Verticillium dahliae* pathogen (Fakhro et al., 2010). Plants will be colonised with *Serendipita* fungi and then challenged with *G. lycopersici.* The number of infected plants will be compared between the two treatments. Gene expression comparisons will also be utilised to understand the molecular basis of the interactions. The possible cellular and molecular changes occurring in pathogen-challenged *Serendipita* colonised-tomato is clarified in Figure 5.2.

The specific objectives of this component of the study are:

a) To develop a powdery mildew inoculation procedure for *Serendipita*-colonised tomato plants

b) To ascertain if *Serendipita* colonisation of tomato plants increases resistance to the powdery mildew pathogen.

c) To determine if there are changes in defence gene expression in bioprotected tomato plants.

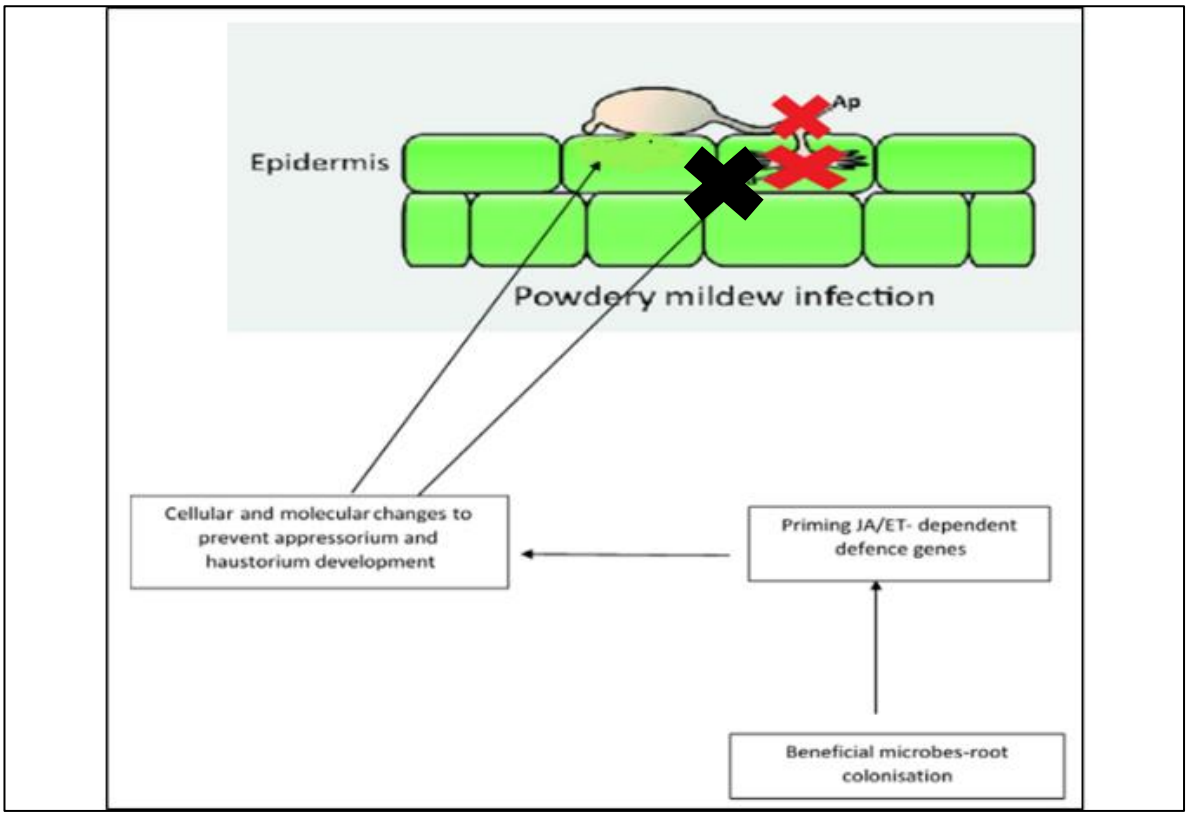


Figure 5. 2: Possible pre-penetrative cellular (black cross) and molecular changes occurring in a *Serendipita* mediated bio-protective response to powdery mildew (modified from Jacott et al., 2017). Red crosses indicate sites of possible powdery mildew infection inhibition during plant invasion.

5.2 Methods

5.2.1 Maintenance of powdery mildew cultures

The powdery mildew species *G. lycopersici* is an obligate biotrophic pathogen; therefore, it does not grow on artificial media. Thus, continuously infected tomato plants were used as source of inoculum. Tomato seeds (cv. Moneymaker) were planted in plastic pots containing fertilised soil. At 10-14 days after germination, the leaves of these tomato seedlings were inoculated with spores of the powdery mildew pathogen

by gently rubbing them with existing infected leaves. The process of reinfection was repeated for around three years to maintain the powdery mildew as a source of infection for lab experiments.

5.2.2 Development of infection protocols

Two methods were tested for the transfer of powdery mildew inoculum to plants. The first method involved using part of an infected leaf and placing it in a microcentrifuge tube with sterilised distilled water and then vortexing it briefly. Conidia viability was determined by adding 100µl of the suspension onto a glass microscope slide and after 0, 5 min, 10 min and 20 min, viewing spore morphology. The second method used a dry cotton bud to remove conidia from the leaves of tomato plants infected with *G. lycopersici* and gently brushing them on a dry microscope slide to check their viability over time.

5.2.3 Challenging of *Serendipita* colonised tomato plants with powdery mildew

Tomato seeds (cv. Moneymaker) were surface sterilised by soaking for 1 min in 70% ethanol and for 4 min in 2.5% NaClO. Tomato seeds were grown on MS at 4 ℃ for 48 hours in the dark to induce seed germination. This was followed by incubation in a growth chamber for ten days with a 16/8 hours day/ night cycle at 23/21 ℃ (Yakti et al 2018). At 10 days post germination, the seedlings were transferred to 40 aerated glass jars containing PNM (Johnson et al., 2011) for seven days. The jars were divided into two treatments; the first treatment consisted of 20 plants each colonised by *Serendipita* by adding two *Serendipita* mycelial plugs and 1.5 ml of hyphal suspension together in one jar. The control treatment did not have *Serendipita* plugs or hyphal suspension added. The inoculation procedure involved removing conidia from leaves of tomato plants infected with *G. lycopersici* with a dry cotton bud and gently brushing these onto the terminal leaves of the *in vitro* grown plants. After 10 days, the number of infected plants was recorded as indicated by obvious areas of powdery mildew mycelium using the dissecting microscope. The growth parameters such as total plant length, shoot length, root length and fresh weight were also recorded. *Serendipita* colonisation was also checked via root clearing (KOH boiling, trypan blue staining; Johnson et al., 2011).

5.2.4 Statistical analysis

All of the experiments were conducted in triplicate and results were tabulated as mean ± standard deviation (SD). The statistical software jamovi 2.2.5 (The jamovi project, 2021) was used to perform independent sample t-tests and probability values of $P< 0.05$ were considered to be significant. To measure the differences in the number of infected plants in *Ser*+/PM+ and *Ser*-/PM+ treatments, (0) and (1) scores were given to non-infected and infected plants respectively. The same software jamovi 2.2.5 was used to perform a Chi-squared tests and the probability values of $P< 0.05$ were considered to be significant.

5.2.5 cDNA synthesis, quantification and testing qPCR primers

Total RNA was extracted using the RNeasy Power Plant Kit (Qiagen) following the manufacturers' instructions from the leaves of *in vitro* grown *Serendipita* and non-*Serendipita* colonised plants. Fresh or frozen plant tissues were added to bead tubes with β-mercaptoethanol and lysis buffer provided by the manufacturer (Qiagen). Then, the tissue were homogenized using a FastPrep homogenizer at 7 m/s speed for 45 s. After cell lysis, the released RNA was treated with inhibitor removal technology (IRT). The inhibitor-free RNA was captured on a silica-membrane spin filter. The RNA bound to the filter was washed to remove contaminants. Finally, the RNA was recovered in certified RNase-Free Water. A Denovix spectrophotometer was used to check RNA quality by measuring 280/260 and 260/230 absorbance ratios for three leaves of *Ser*+/PM+ plants and three leaves of *Ser*-/PM+ plants. One µl of the suspension buffer was used for blank measurement. One µl from the sample was loaded on the lower sample surface of the device.

After mRNA extraction, cDNA was synthesized from template RNA using reverse transcriptase and Oligo dT primer using a QuantiNova Reverse Transcription cDNA synthesis kit, (Qiagen) following the manufacturers' instructions. Quantification of cDNA concentration was carried out as per section 4.2.4.

To further study the impact of *Serendipita* in promoting immunity against the powdery mildew pathogen, genes involved with defence via ROS (e.g., the NADPH oxidase gene), wall modification (e.g. callose synthase) and direct pathogen destruction (e.g.

chitinases) were monitored via qRT PCR. The primer details are below and indicated are the forward and reverse sequences, the melting temperature, amplicon size and the source. Chitinase primers were designed using the Genbank website (Table 5.1)

Table 5. 1: Details of defence-related primers including the forward and reverse sequences, the melting temperature, amplicon size and source. The primers were tested via conventional PCR on tomato leaf cDNA from the *Ser*+/PM+ treatment.

Primer name	Accession number	Primer sequence	Melting temp	Amplicon size	Reference
NADPH oxidaseA-F	*Solyc01g099620	GAGAGTAGGATTCAGCGGT	56	173bp	Li et al., 2015
NADPH oxidaseA-R	As above	GCCTCTTTTCGAGCTTGCT (AGCAAGCTCGAAAAGAGGC)	57	As above	
PAL-F	M83314	ACGGGTTGCCATCTAATCTG	57	197bp	Aime et al., 2013
PAL-R	As above	AGCTCTTTTCCTGGCTGAAA (TTTCAGCCAGGAAAAGAGCT)	56	As above	
Callose synthase11-F	**KR70638	GAAGGACGAGAGAGAGATATGG	59	149bp	Adkar-Purushothama et al., 2015
Callose synthase11-R	As above	CTGAAGCAGAATCAAGGAACG (CGTTCCTTGATTCTGCTTCAG)	59	As above	
Callose synthase12-F	**KR706382	TGAGGAGGCACTGAAAATGAGGAAC	64	195bp	Adkar-Purushothama et al., 2015
Callose synthase12-R	As above	CGGATTTTCAGGGGGTTGGCT (AGCCAACCCCCTGAAAATCCG)	66	As above	
Chitinase 17-F	Z15139.1	TAGCTGGGCAAGCAATTGGA	58	202bp	This study
Chitinase 17-R	As above	ATGACACCGTAGCCTGGTTG	59	As above	
JA-Lipoxygenase D-F	U37840.2	GGCTTGCTTTACTCCTGGTC	58	72bp	Sun et al., 2017
JA-Lipoxygenase D-R	As above	AAATCAAAGCGCCAGTTCTT (AAGAACTGGCGCTTTGATTT)	55	As above	
β-1,3-glucanase -F		GCGGTGTTCAGCCTGGATG	59.1	94bp	Chandrasekaran & Chun 2016
β-1,3-glucanase-R		AGCATGAGCAAGAAGTATGTTGTG	55.7	As above	

Note: All accession numbers can be found in GenBank, accept (*) in the aramemnon data libraries and (**) can be found in European Molecular Biology Lab (EMBL).

Before qRT-PCR analysis, the defence gene primers were tested for their efficacy via conventional PCR. PCR was performed by adding 10 µl of Extract-N-Amp PCR reaction mix, 7 µl sterile distilled water, 1 µl cDNA (from a *Serendipita* and PM treated plant), 1 µl of each of forward and reverse primers in each microtube. PCR cycling included the following protocol: initial denaturation at 95 ℃ for 5 min, amplification with 40 cycles of 95 ℃ for 1 min, 50 ℃ for 1 min and 72 ℃ for 1 min, and final extension at 72 ℃ for 10 min. Ten µl of the PCR products were then run on a 1% electrophoresis gel and viewed under UV light using a Quantum ST4 gel

documentation system (VilberLourmat, Fisher Biotech, Wembley, WA, Australia) and photographed using a Quantum capture ST4® image acquisition and analysis software package.

5.2.6 Quantitative real-time polymerase chain reaction (qRT-PCR)

cDNA was amplified via qRT-PCR using primers for defence-related genes and QuantiNova SYBR green quantitative real time PCR kit (Qiagen). qPCR was performed twice with the following protocol: PCR initial activation at 95 ℃ for 2 min, amplification with 40 cycles of denaturation at 95 ℃ for 5 sec and combined annealing/ extension at 50 ℃ for 10 sec. PCR products were analyzed using appropriate software via monitoring of a SYBR green fluorescence chart and melting curve of PCR products to check reaction stability and presence of primer dimers. Housekeeping genes for actin and ubiquitin were used for normalisation. . As mentioned in chapter 4, qPCR amplification of ITS regions of genomic DNA from *Serendipita* was used to check the efficacy of the pipetting technique and machine operation. Also, qPCR amplification using primers to the reference genes actin and ubiquitin was used to optimise template concentrations.

5.2.7 Semi quantitative RT-PCR Analysis

Expression of defence response genes was also studied using the semi-quantitative RT-PCR method. Leaf samples from all the treatments were taken, snap frozen in liquid nitrogen, stored at -80 ºC and further used for RNA isolation and cDNA synthesis as described above. The RT-PCR cycling conditions for amplification of each cDNA sample included an initial denaturation step of 95 ºC for 5 min followed by 35 cycles with denaturation at 95 ºC for 1 min, annealing at 50 ºC for 1 min, elongation on 72 ºC for 1 min. A final extension was carried out at 72 ºC for 10 min. After completion of all the PCR steps, the gene expression levels were observed on 1% agarose gel in 1x TAE buffer with actin and ubiquitin genes as housekeeping controls. A 100 bp DNA ladder molecular weight marker (Axygen, USA) was run on all gels to confirm expected molecular weights and concentrations of the electrophoresed product. ImageJ

software (University of Wisconsin, USA) was used to determine the band intensity by measuring the pixel numbers of each band.

5.3 Results

5.3.1 Assessment of infection protocols

After rinsing portions of infected leaves in distilled water and placing spores on slides, the powdery mildew spores showed cytoplasm shrinkage away from the cell wall, which indicated a lack of viability (Figure 5.3 A-D). In contrast, dry cotton bud application to slides showed continuous viable spores; therefore, this procedure was used subsequently to transfer the inoculum to the plants in the jars (Figure 5.4).

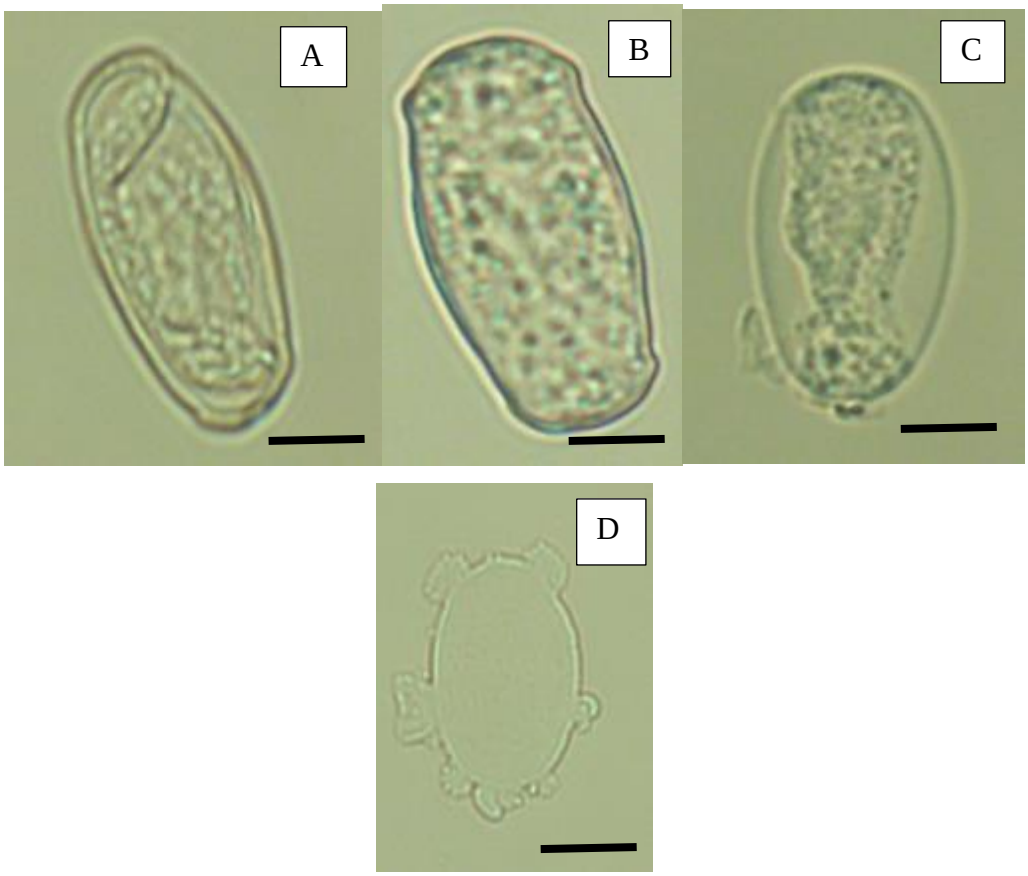


Figure 5. 3: Spores in distilled water (A) zero time, (B) 5 min (C) 10 min, 20 min (D). Note the burst spore at 20 min. Scale bar is 50 µm.

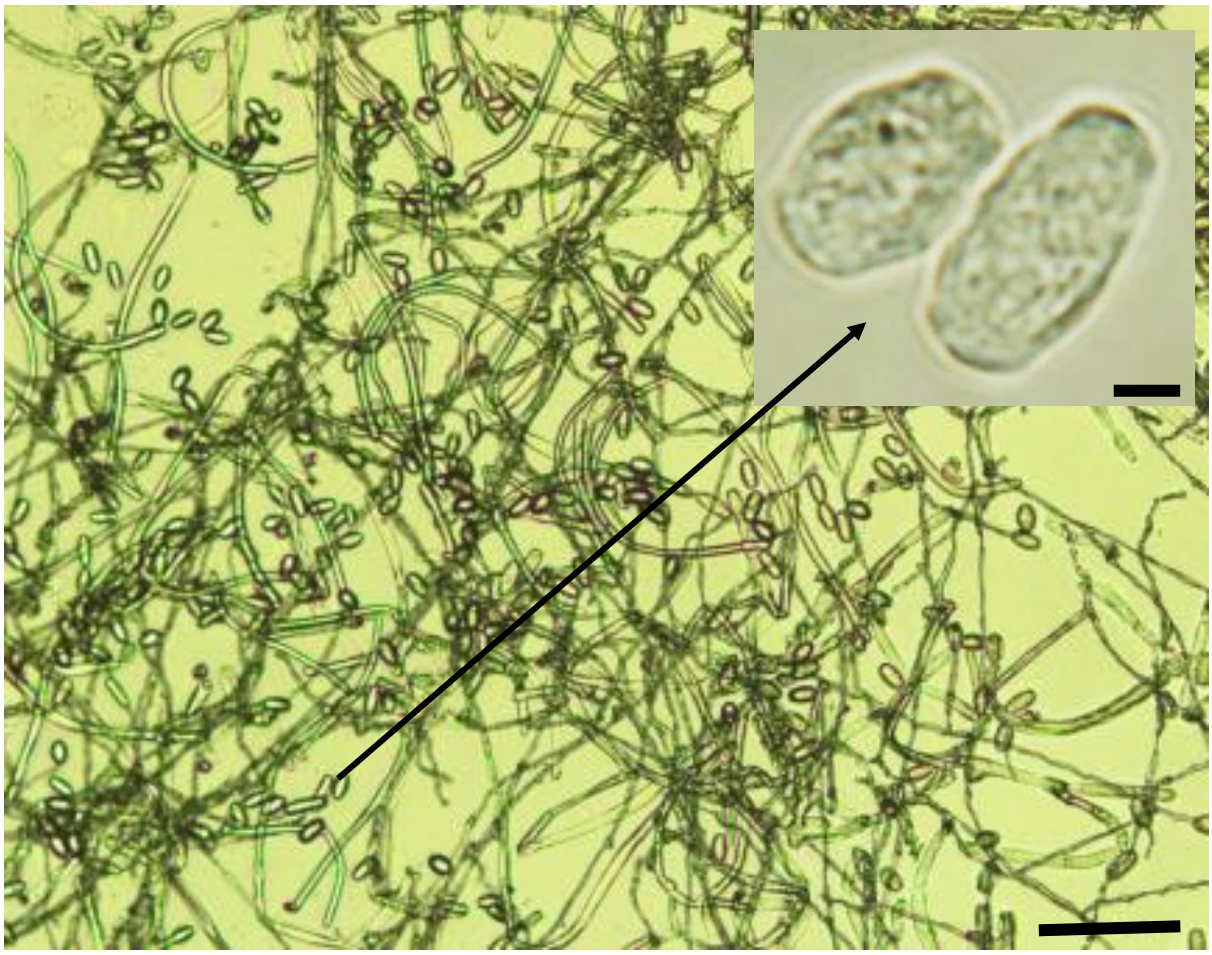
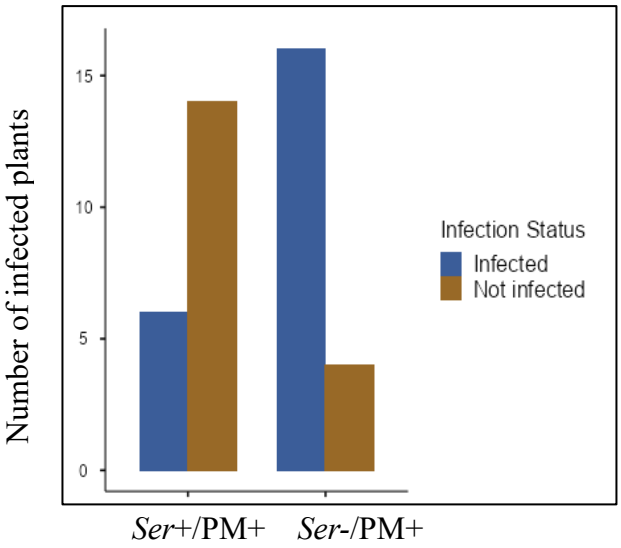


Figure 5. 4: PM inoculation procedure with cotton wool bud. Spores maintained viability continuously with this procedure (inset) Scale bar is 100 µm. Scale bar of inset is 20 µm.

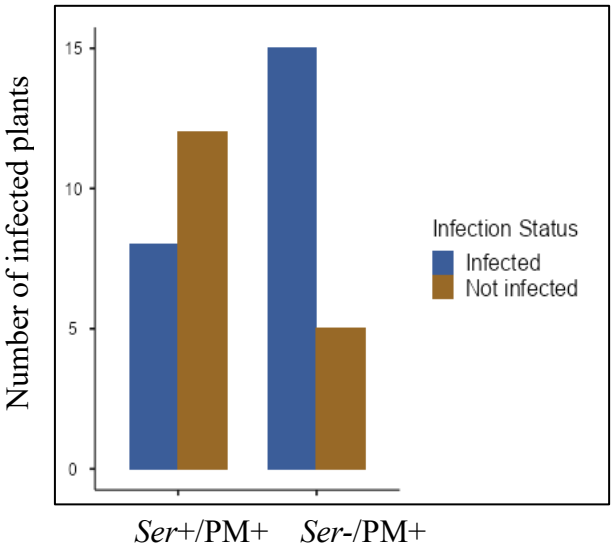
5.3.2 Challenging of *Serendipita* colonised tomato plants with powdery mildew

The symptoms of powdery mildew disease were monitored 10 days post inoculation (Figure 5.6 A-B). In all three experiments, the number of infected plants was significantly lower in *Serendipita* colonised plants (Figure 5.5). Difference in two proportions at 95% confidence intervals was -0.5 in the first experiment and -0.35 in the second and third experiments (Appendix VII).

Exp 1, P<0.01
Average of infected plants in *Ser*+/PM+ = 0.30, SD = 0.47
Average of infection plants in *Ser*-/PM+ = 0.80, SD = 0.41



Exp 2, P<0.01
Average of infected plants in *Ser*+/PM+ = 0.40, SD = 0.50
Average of infected plants in *Ser*-/PM+ = 0.75, SD = 0.44



Exp 3, P<0.01
Average of infected plants in *Ser*+/PM+ = 0.45, SD = 0.51
Average of infected plants in *Ser*-/PM+ = 0.80, SD = 0.41

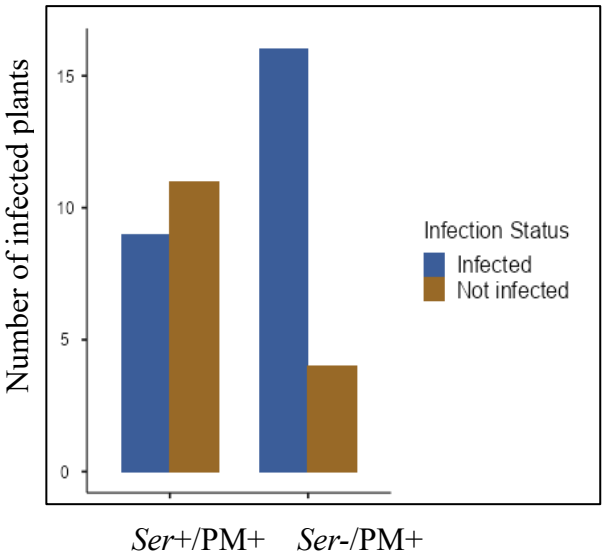


Figure 5. 5: Number of infected tomato plants in *Ser*+/PM+ and *Ser*-/PM+ treatments from the three experiments, n=20. The LHS boxes contain average and standard deviation (SD) of infected plants after 10 days from PM infection.

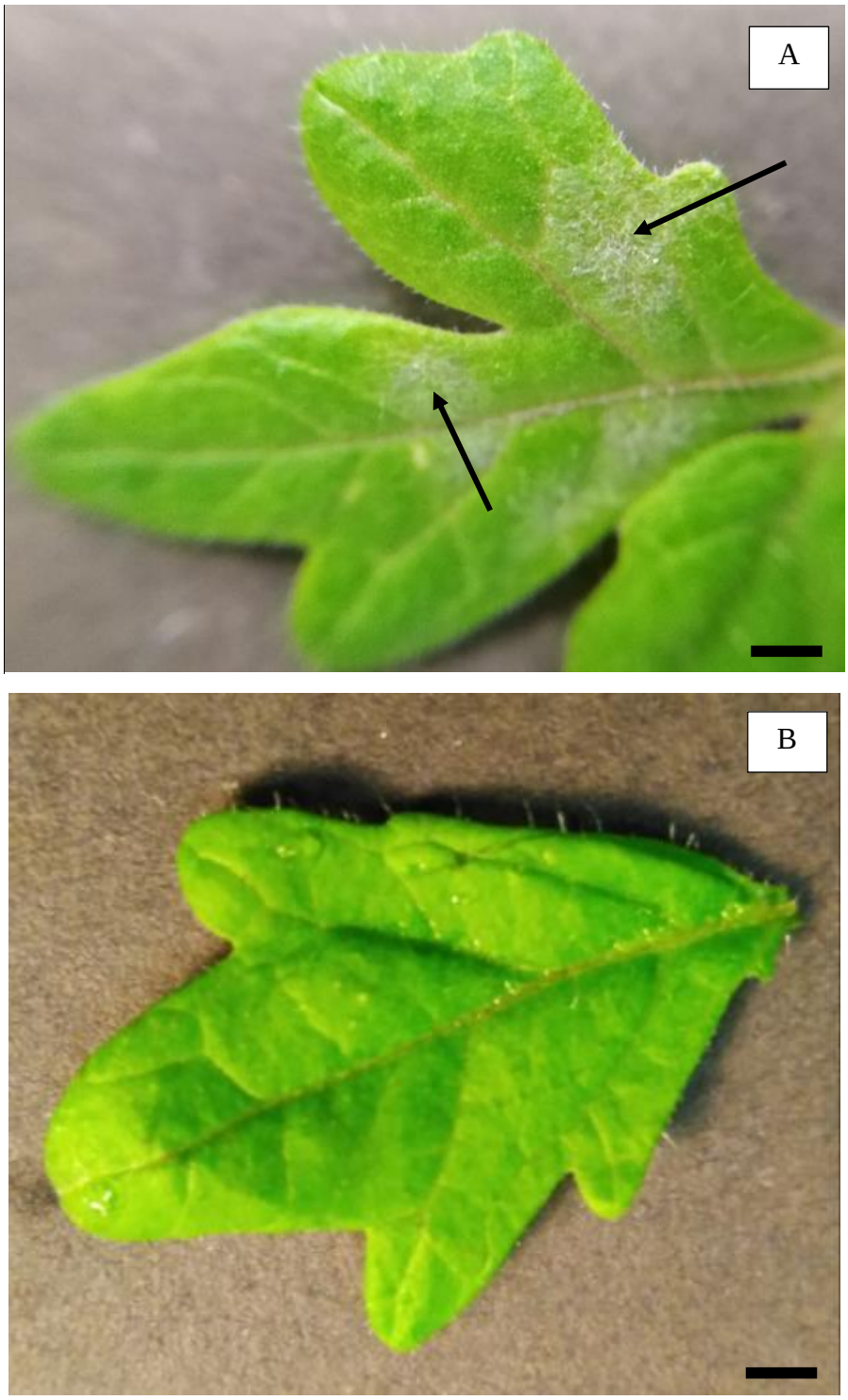


Figure 5. 6: Leaves of *Ser*- (A) and *Ser*+ (B) plants and powdery mildew infection (Black arrows). Scale bar is 1 cm.

5.3.3 Measurement of growth parameters in the bio-protection experiment and statistical analysis

Growth parameters were measured in plants ten days post inoculation with powdery mildew spores. The total plant length was not significantly different between the two treatments in all three experiments (Figure 5.7). Shoot length was not significantly different between the two treatments in the first and third experiments but in experiment two there was significantly higher shoot length in *Serendipita* colonised plants ($P<0.01$) (Figure 5.8). Both experiments one and three showed no significant difference in root length between the two treatments but in experiment two there was significantly higher root length in the non-*Serendipita* colonised plants ($P<0.05$) (Figure 5.9). Fresh weight was improved in *Serendipita*-colonised plants in two experiments ($P<0.01$ in experiment one, $P<0.05$ in experiment two) but was not significantly different between the two treatments in the third experiment (Figure 5.10). Most values showed a normal distribution in the three experiments except the values of shoot length in the second and third experiments and fresh weight values in the third experiment showed a low p-value in the first experiment, which suggests a violation of the assumption of normality based on Shapiro-Wilk normality test (Appendix VI).

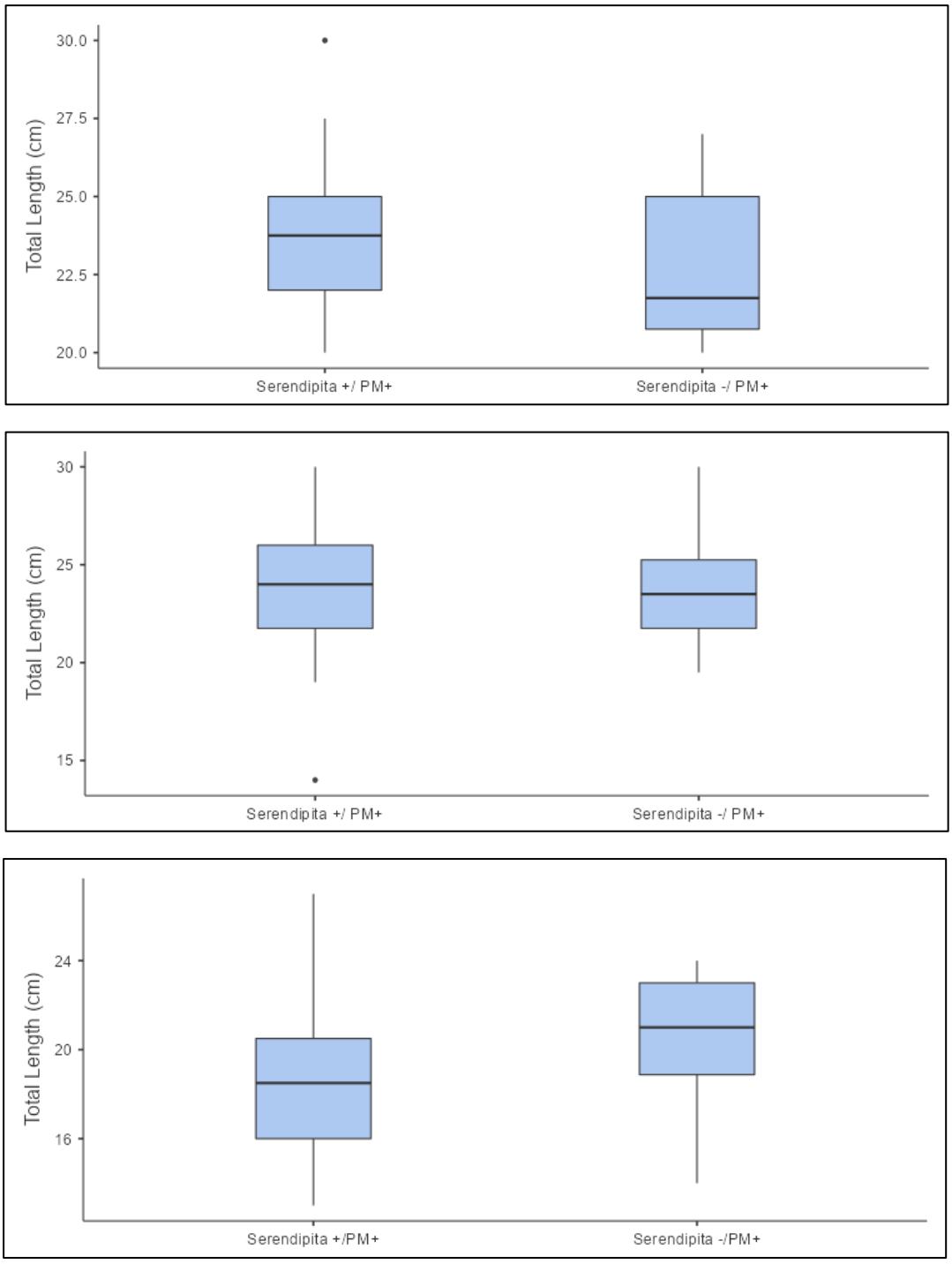
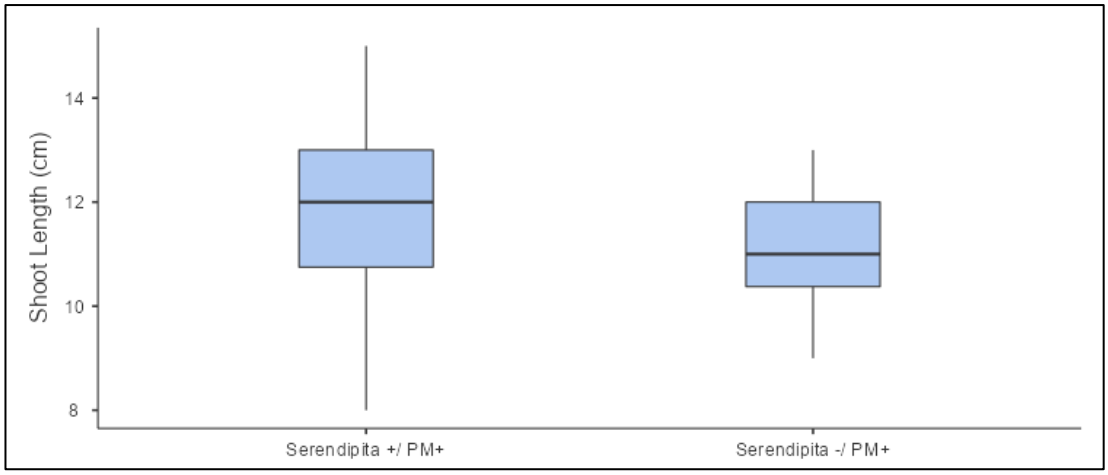
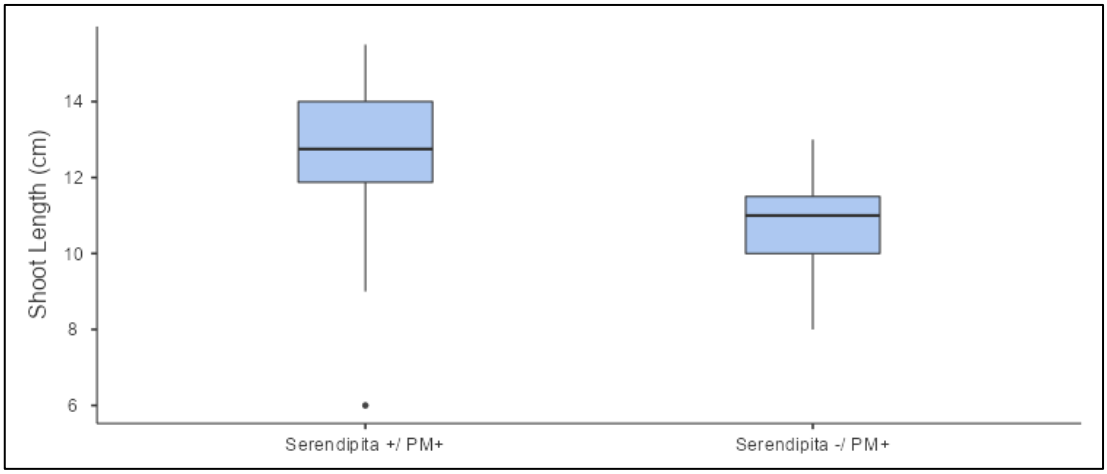


Figure 5. 7: Total length of tomato after 20 days for the *Ser*+/PM+ and *Ser*-/PM+ (control) in experiment one and the repeat experiments (middle and bottom), n=20. Dots are the outlier values of the experiment.





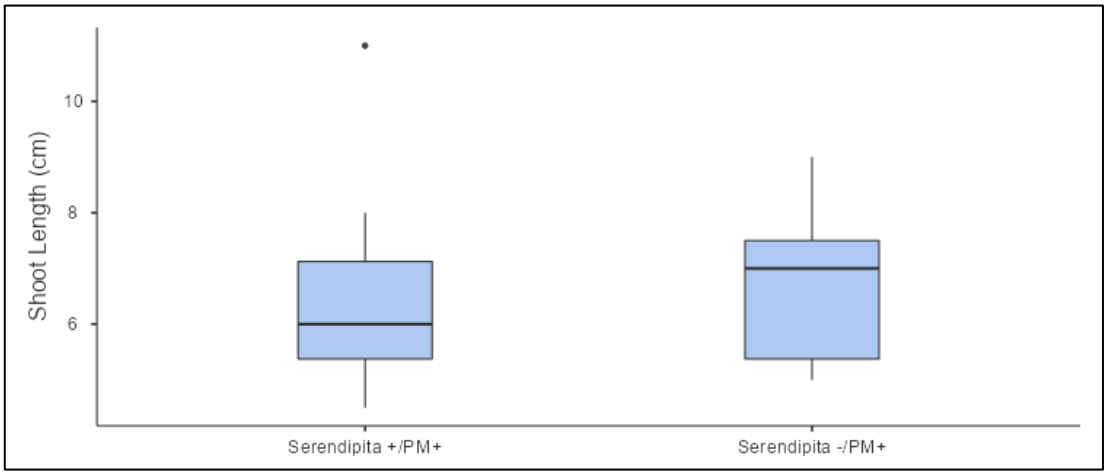


Figure 5. 8: Shoot length of tomato after 20 days for the *Ser*+/PM+ and *Ser*-/PM+ (control) in experiment one and the repeat experiments (middle and bottom), n=20. Dots are the outlier values of the experiment.

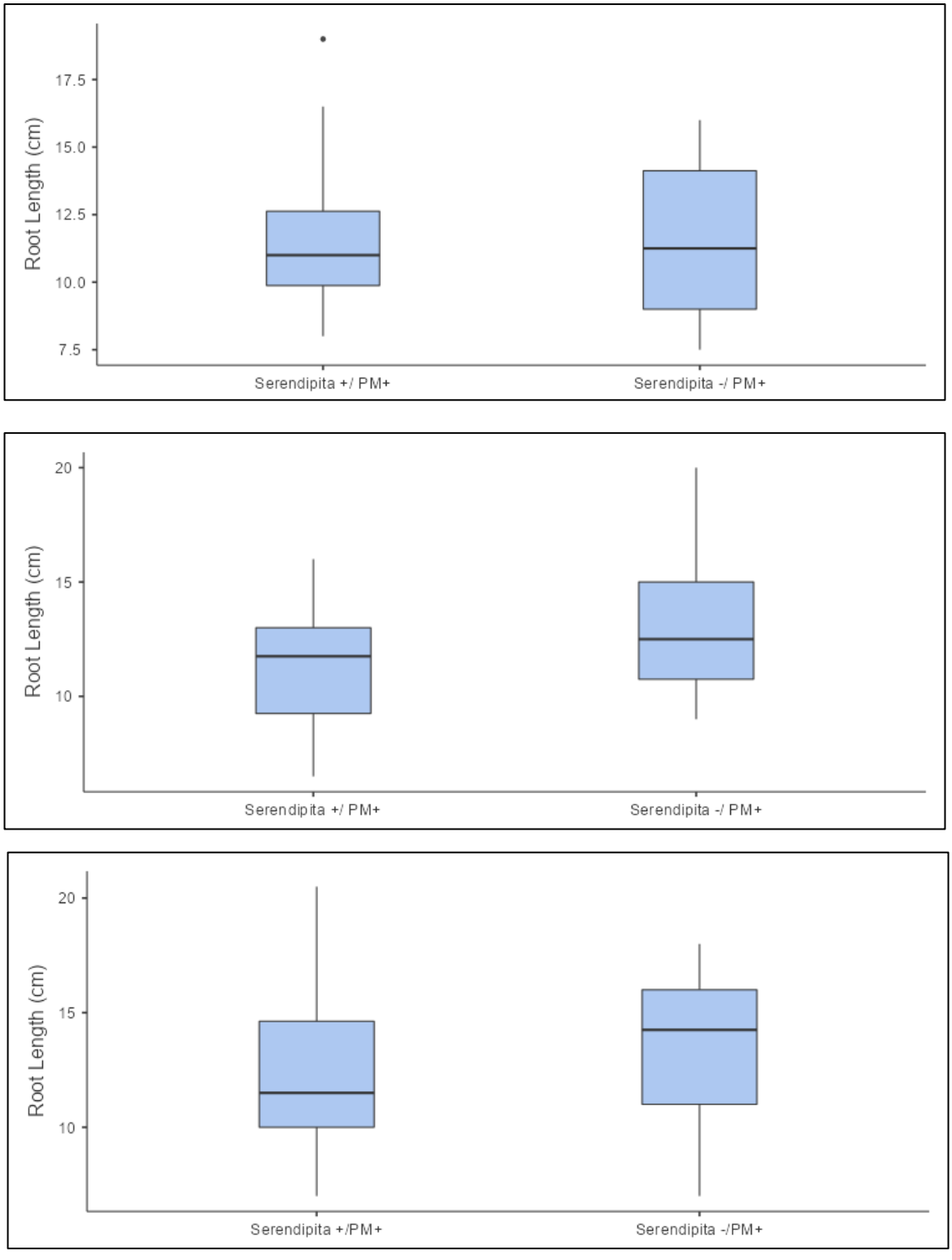


Figure 5. 9: Root length of tomato after 20 days for the *Ser*+/PM+ and *Ser*-/PM+ (control) in experiment one and the repeat experiments (middle and bottom), n=20. Dots are the outlier values of the experiment.

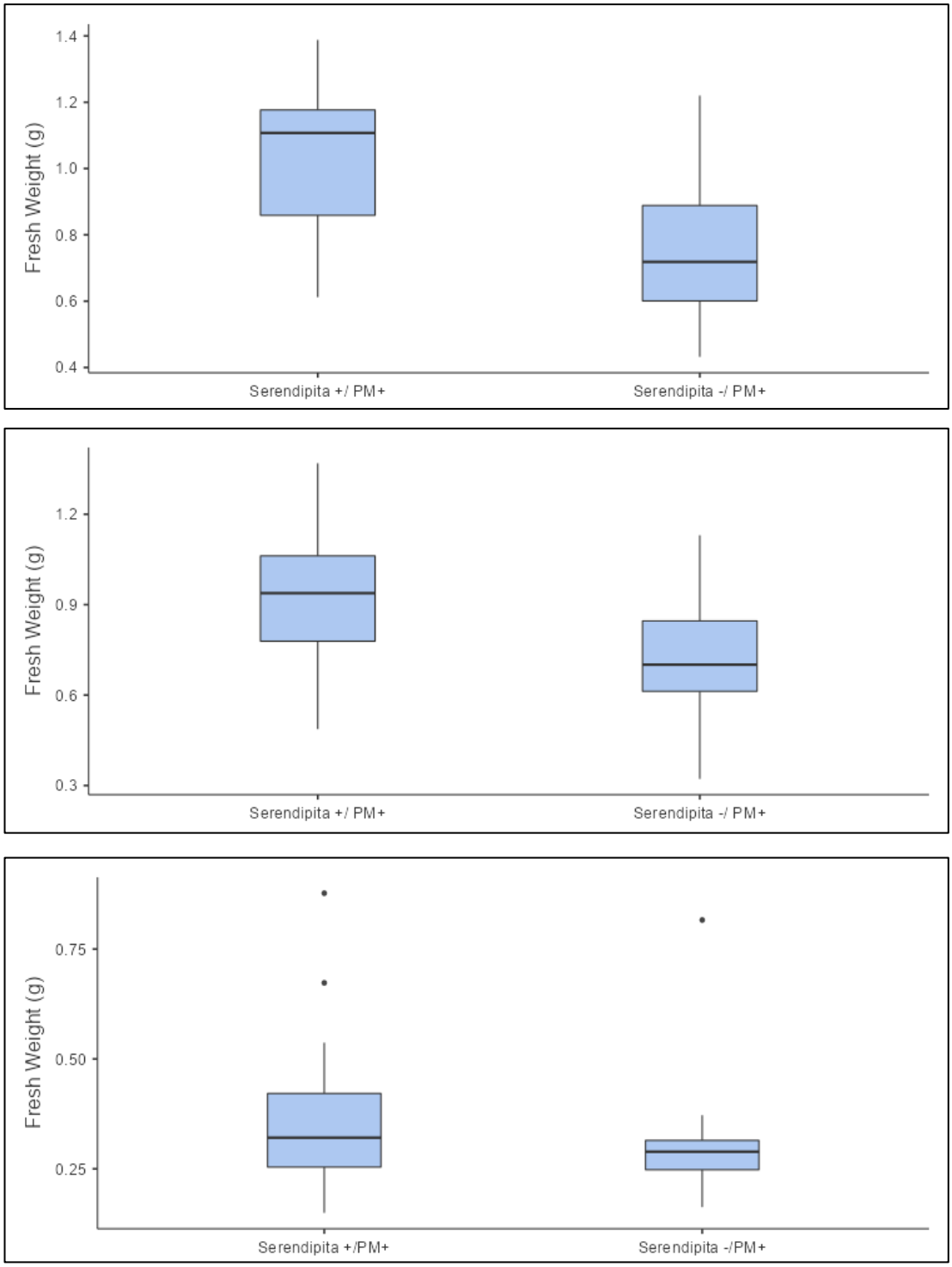


Figure 5. 10: Fresh weight of tomato after 20 days for the *Ser*+/PM+ and *Ser*-/PM+ (control) in experiment one and the repeat experiments (middle and bottom), n=20. Dots are the outlier values of the experiment.

5.3.4 RNA extraction quality

RNA quantity and quality was checked by a Denovix spectrophotometer (Wilmington, Delaware, USA) for three leaf samples from each of the *Ser*+/PM+ and *Ser*-/PM+ treatments (Table 5.2). The RNA concentration varied from 44ng/µl to 79ng/µl. All *Ser*+ and *Ser*- RNA samples were of acceptable quality with absorbance ratios being around 2.0 at the 260/280 wavelength comparison and in the range of 2.0-2.2 at the 260/230 wavelength comparison.

Table 5. 2: RNA quality of tomato leaves in *Ser*+/PM+ and *Ser*-/PM+ treatments. RNA samples had a ratio of 2.0 at the 260/280 wavelength and were in the range of 2.0-2.2 at the 260/230 wavelength.

Treatment	Concentration (ng/µl)	260/280 nm	260/230 nm
Ser+/PM+	50.52	2.08	2.1
Ser+/PM+	50.822	2.05	2.07
Ser+/PM+	69.377	1.95	2.28
Ser-/PM+	45.496	2.13	2.19
Ser-/PM+	44.334	2.06	2.19
Ser-/PM+	79.405	2.08	2.25

5.3.5 cDNA synthesis, quantification and testing qPCR primers

After cDNA synthesis, all samples from the *Ser*+/PM+ and *Ser*-/PM+ treatments were also quantified. The concentration of cDNA for all samples are indicated in table 5.3.

Table 5. 3: cDNA concentration of tomato leaves at 20 days in *Ser*+/PM+ and *Ser*-/PM+ treatments. cDNA concentration were in the range 10-13ng/µl in *Ser*+/PM+ and in the range of 15-22ng/µl in *Ser*-/PM+.

Treatment	Concentration (ng/µl)
Ser+/PM+	10.3
Ser+/PM+	13.0
Ser+/PM+	9.89
Ser-/PM+	15.2

Ser-/PM+	21.5
Ser-/PM+	19.5

The seven primer sets amplified expected band sizes in gels after PCR amplification of the extracted cDNA (from *Ser*+/PM+ leaves). These were primers to the NADPH oxidase gene, PAL gene, two callose synthase genes, chitinase gene, JA-Lipoxygenase gene and β-1,3-glucanase gene (Figure 5.11).

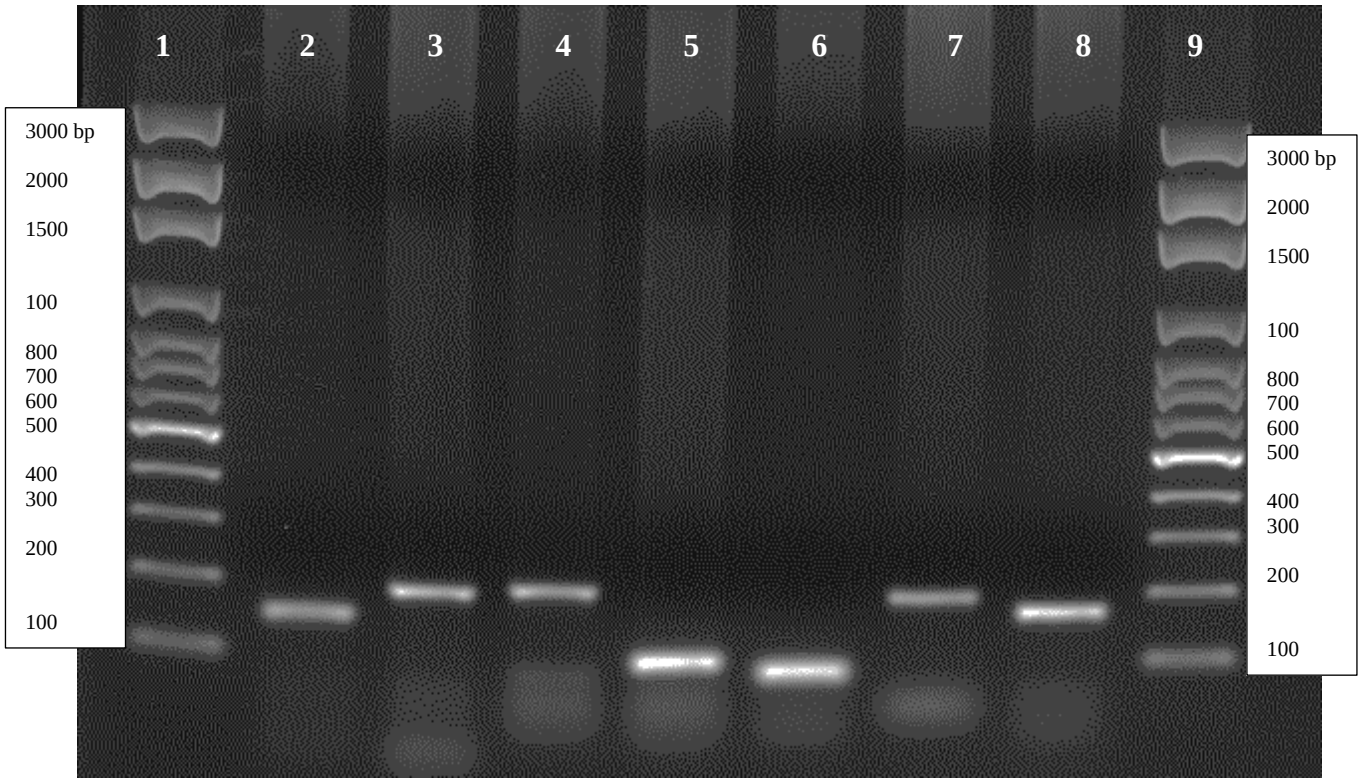


Figure 5. 11: Testing of defence primers. Lane 1 & 9: DNA ladder with molecular weight in bp, 2: Callose 11 synthase, 3: Callose 12 synthase, 4: Chitinase, 5: β-1,3-glucanase , 6: JA-Lipoxygenase, 7: PAL, 8: NADPH oxidase.

5.3.6 Quantitative real-time polymerase chain reaction (qRT-PCR)

The cDNA of three leaves from *Ser*+/PM+ plants were used for qRT-PCR and compared with the cDNA of three leaves of *Ser*-/PM+ plants. As mentioned in chapter 4, to check for errors in pipetting technique and correct operation of the qPCR machine, qPCR reactions were run on ten identical gDNA samples from *Serendipita* with ITS primers. This analysis showed a consistency of Cq values for the samples

(Table 5.4). As mentioned in section 4.3.5, optimization of template concentrations was also carried out using primers to the reference genes actin and ubiquitin. High Cq values were obtained with undiluted template and thus this was the concentration used for the qRT-PCR experiments (Table 4.5).

The qRT-PCR for this bio-protection experiment was performed twice Actin and ubiquitin were used as reference genes, but they did not show consistent quantification cycle (Cq) values in both experiments (Appendix VII-B). The Cq values of the defence genes were similarly inconsistent (Appendix VII-B).

Table 5. 4: Testing of pipetting technique using gDNA. The gDNA samples from *Serendipita* were tested with ITS primers.

Sample number	1	2	3	4	5	6	7	8	9	10
Cq value	31.41	31.43	31.44	31.47	31.80	31.89	32.02	32.03	32.64	33.09

5.3.7 Semi quantitative RT-PCR analysis and bands intensity results

Semi-quantitative RT-PCR analysis also showed an inconsistency in expression of the two reference genes. The β-1,3-glucanase gene was also inconsistently expressed in the *Ser*+/PM+ and *Ser*-/PM+ treatments. The chitinase, PAL, callose and jasmonic acid genes showed no expression in the *Ser*+/PM+ and *Ser*-/PM+ plants. Although the NADPH oxidase gene were amplified in both treatments the amplicons were of the wrong size (ie. <100bp) (Figures 5.12- 5.16).

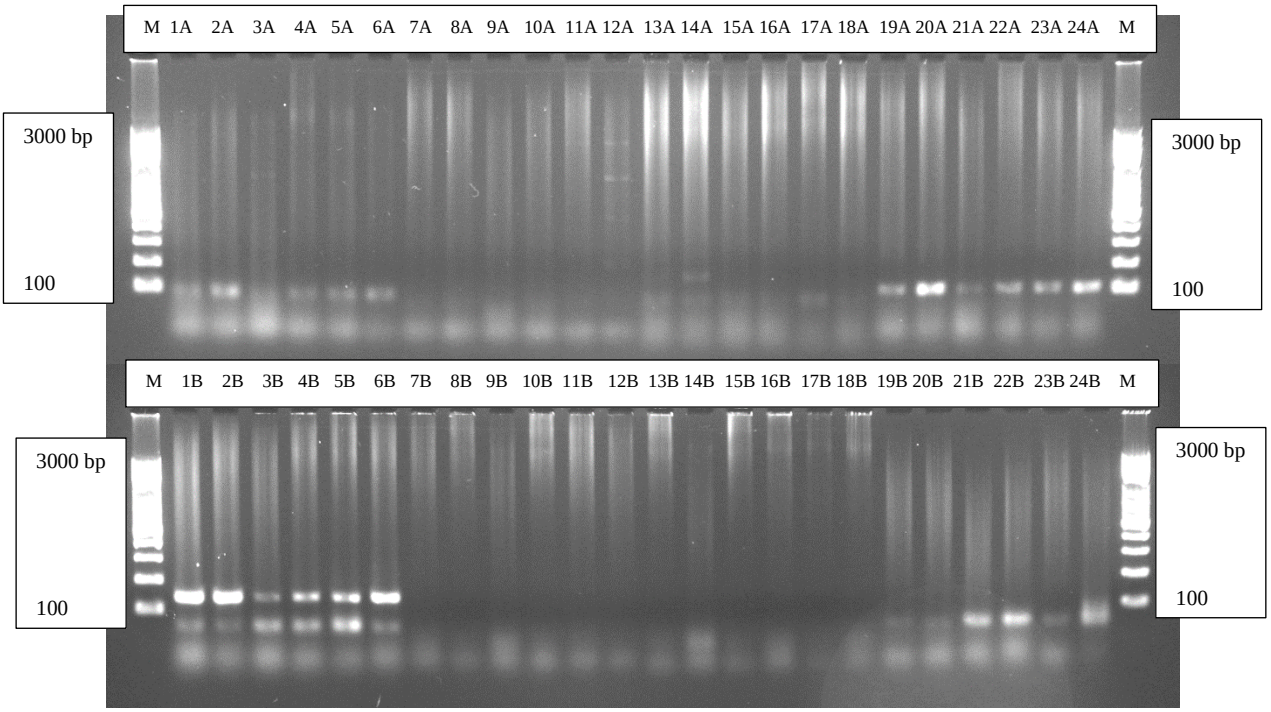


Figure 5. 12: Semi-quantitative RT-PCR analysis of defence genes. M: marker, 1A-3A: Actin *Ser*+, 4A-6A: Actin *Ser*-, 7A-9A: chitinase *Ser*+, 10A-12A: chitinase *Ser*-, 13A-15A: callose 11 synthase *Ser*+, 16A-18A: callose 11 synthase *Ser*-, 19A-21A: β-1,3-glucanase *Ser*+, 22A-24A: β-1,3-glucanase *Ser*-, 1B-3B: ubiquitin *Ser*+, 4B-6B: ubiquitin *Ser*-, 7B-9B: PAL *Ser*+, 10B-12B: PAL *Ser*-, 13B-15B: JA-Lipoxygenase *Ser*+, 16B-18B: JA-Lipoxygenase *Ser*-, 19B-21B: NADPH oxidase *Ser*+, 22B-24B: NADPH oxidase *Ser*-.

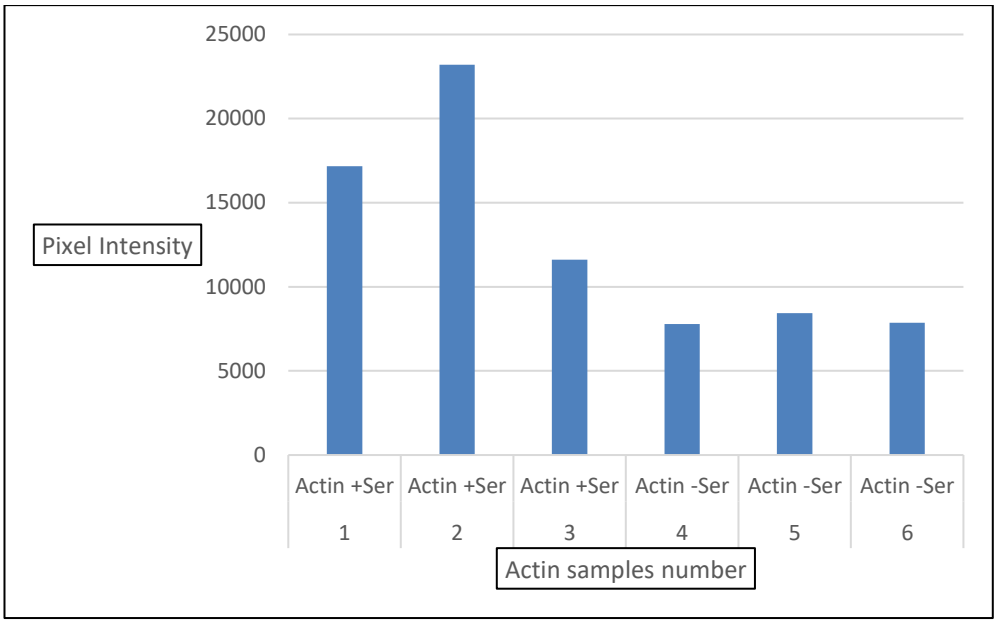


Figure 5. 13: Pixel intensity of Actin bands in *Ser*+/PM+ and *Ser*-/PM+ treatments.

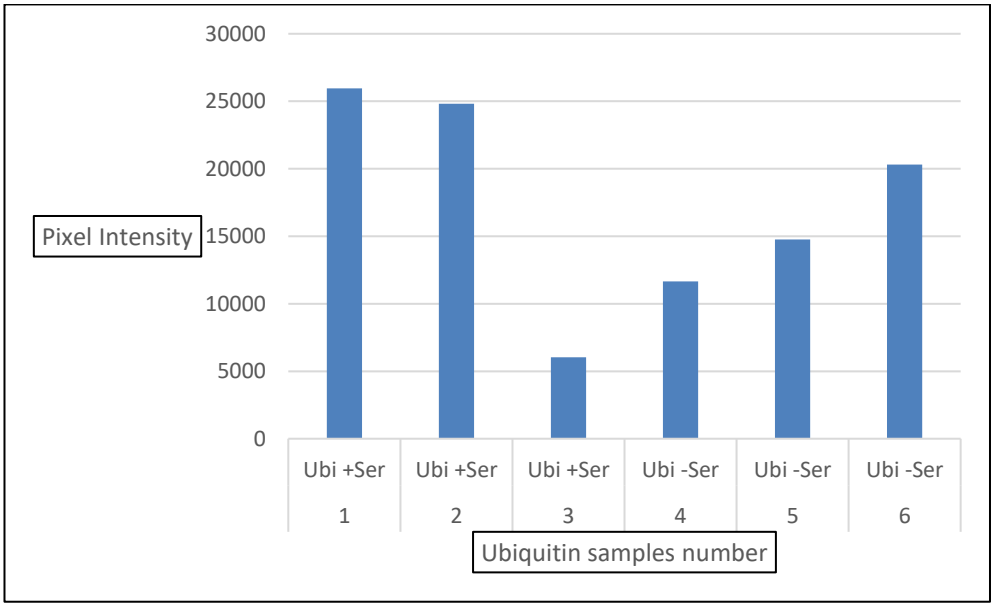


Figure 5. 14: Pixel intensity of Ubiquitin bands in *Ser*+/PM+ and *Ser*-/PM+ treatments.

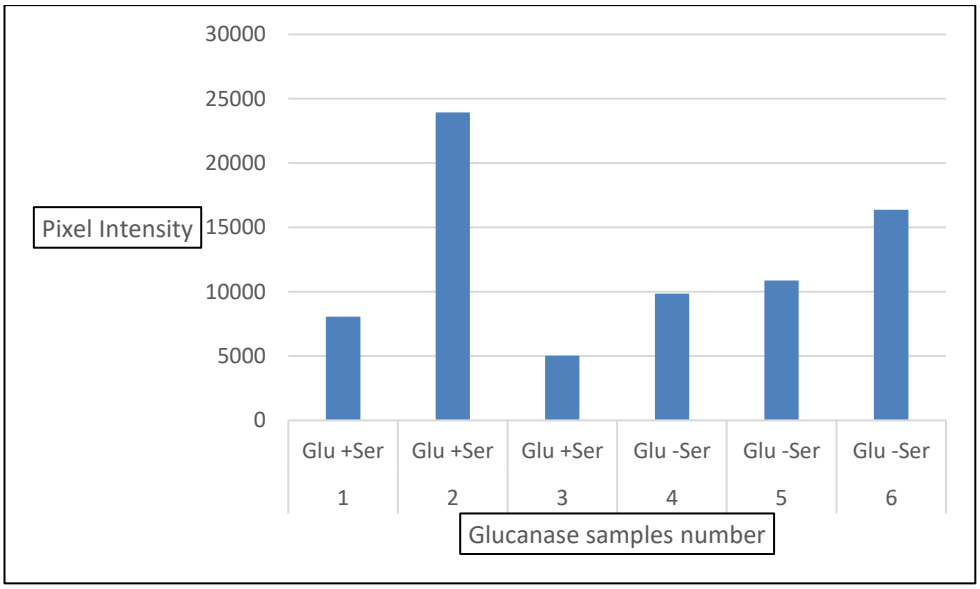


Figure 5. 15: Pixel intensity of Glucanase bands in *Ser*+/PM+ and *Ser*-/PM+ treatments.

5.4 Discussion

In this study, the fresh weight of tomato was enhanced in the presence of *Serendipita*, similar to the results reported in Chapter 4, despite the plant being challenged by a powdery mildew pathogen. This is consistent with previous studies which have shown that *Serendipita* improves plant growth parameters when under biotic challenge. For example, *Serendipita* improved root and shoot fresh weight of barley compared with control plants infected with *F. culmorum* alone (Waller et al., 2005; Harrach et al., 2013). Also, *S. indica* enhanced biomass and root length of maize under *F. verticillioides* infection as compared with control plants (Kumar et al., 2009). It is unclear why water transport continues to be enhanced in the mycorrhizal plants during pathogen attack, but it potentially could relate to the increased levels of hydrolytic enzymes that are being synthesised in the response (Nafady et al., 2022; Harrach et al., 2013; Waller et al., 2005).

Plant defence enhancement via mycorrhizal colonisation has been previously investigated in tomato (Song et al., 2015). For example, *Rhizophagus irregularis* (formerly, *Glomus intraradices*) has been reported to improve the immunity of tomato plants under interaction with the false root-knot nematode *Nacobbus aberrans* (Lax et al., 2010). The total plant length, dry weight of roots and shoots and the number of

galls were measured in the presence and absence of the symbiont. Mycorrhizal plants showed a lower number of galls and increased plant length and biomass (Lax et al., 2010). *Funneliformis mosseae* enhanced defence gene expression in tomato plants under root-knot nematode (*Meloidogyne incognita)* infection. The expression of defence genes was examined by suppressive subtractive hybridisation and up-regulation of the phenylpropanoid pathway and reactive oxygen species (ROS) metabolism were identified as the main reason that the infection rate in mycorrhizal tomato plants was lower compared to non-mycorrhizal plants (Vos et al., 2013).

Unlike the data reported in chapter 4, the two reference genes (actin and ubiquitin) showed inconsistent expression patterns within and between treatments and thus they were not able to be used to gauge changes in defence gene expression. Other researchers have found inconsistencies in reference genes in plants growing under stress conditions (Lanubile et al., 2010; Le et al., 2012; Muller et al., 2015). For example, the common housekeeping gene GAPDH was down regulated in maize plants infected with *Fusarium verticillioides* infection (Lanubile et al., 2010). Other housekeeping genes will thus need to be used when repeating these experiments. Semi-quantitative RT-PCR analysis also showed inconsistent expression pattern of the β-1,3-glucanase gene in and between the *Ser*+/PM+ and *Ser*-/PM+ plants. This variability may be due to genetic variation of the individual plants within the same treatment, (Dolatabadian & Fernando, 2022; Kover & Schaal, 2002) but as mentioned it is hard to quantify the expression of genes when the housekeeping genes do not provide a reliable benchmark. The absence of detection of the other defence genes in this analysis after initial verification of primer design suggests that there are continued quality issues (i.e presence of inhibitors) with the cDNA that make the semi-quantitative (and qRT-PCR) process unreliable.

β-1,3-glucanase inhibits the growth of pathogens by hydrolysing the major structural component of the fungal cell wall, beta-1,3-glucans (Shi et al., 2006; Singh et al., 2014; Su et al., 2016). The induction of β-1,3-glucanase genes has been observed in many mycorrhizal plants under biotic stress, where the AMF *Funneliformis mosseae* (formerly, *Glomus mosseae*) has been found to reduce the impact of *Phytophthora parasitica* pathogen on tomato by inducing chitosanase, β-1,3-glucanase and superoxide dismutase (Pozo et al., 2002). Also, in *Funneliformis mosseae*-colonised

tomato plants, a high level of chitinase and β-1,3-glucanase conferred resistance to early blight disease (Song et al., 2015). Despite the inconsistent expression patterns, β-1,3-glucanase genes were clearly activated in both the *Ser*+/PM+ and *Ser*-/PM+ treatments. β-1,3-glucanase belong to the PR-2 protein family which are usually produced in SAR defence mechanisms against pathogens. This research shows that, β-1,3-glucanase can also be induced by ISR responses which are activated in plants under colonisation by beneficial microorganisms. The mechanism of (ISR) is somewhat similar to systemic acquired resistance (SAR), but there are key differences between them. First, ISR is induced by beneficial microorganisms and SAR is induced by pathogens. Second, ISR activates the jasmonic acid and ethylene pathways during microbial colonisation and SAR activates salicylic acid production following pathogen infection (Schumann and D'Arcy, 2010; Pieterse et al., 2014; Figure 5.15). Finally, ISR is not thought to produce PR proteins (Molitor et al., 2011; Pieterse et al., 2014).

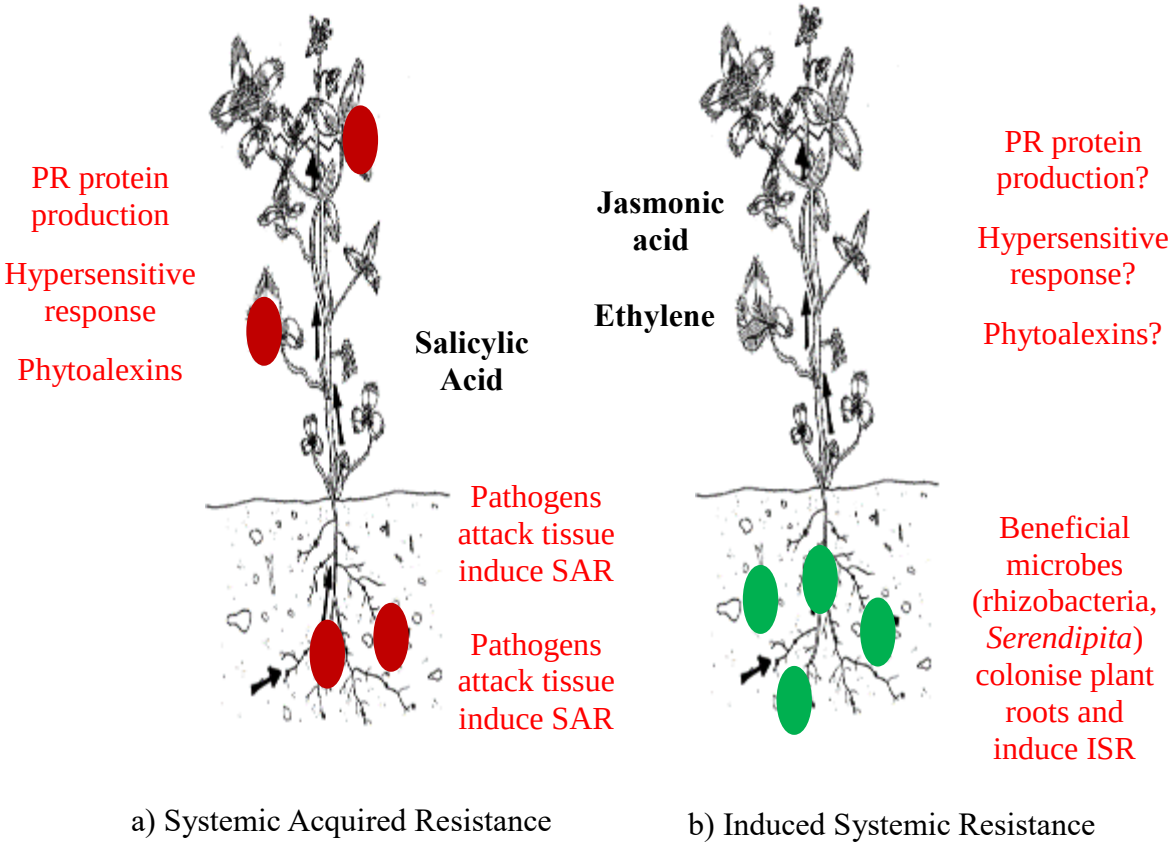


Figure 5. 16: Signalling differences between SAR and ISR (modified from Vallad and Goodman, 2004).

The above differences between ISR and SAR are still controversial especially the second and third aspects, where the results of some studies showed that beneficial *Pseudomonas aeruginosa* activated the salicylic acid pathway instead of the jasmonic acid and ethylene pathways in the defence mechanism against *Botrytis cinerea* in bean (De Meyer et al., 1999) and tomato (Audenaert et al., 2002). PR proteins eg. chitinase, chitosanase and β-1,3-glucanase are also synthesised when mycorrhizal tomato plants are attacked by *Phytophthora parasitica* (Pozo et al., 2002). Also, ISR caused the production of PR proteins in the leaves of tomato infected with *Alternaria solani* (Song et al., 2015). A number of studies have investigated the capability and mechanism of beneficial microorganisms in promoting ISR (Shoresh et al., 2005; Liu et al., 2007; Segarra et al., 2009; Mathys et al., 2012). Additional studies are summarised in the table (5.5) below.

The *Ser*+/PM+ treatment in this study showed a lower number of infected plants than *Ser*-/PM+ treatment, which may be explained by a rapid defence response of *Serendipita* colonised plants. ISR prepares plant defences to be immediately ready upon pathogen attack, which is called priming. ISR priming increases the sensitivity of cells to hormones rather than increasing their synthesis locally and induces rapid cellular defence responses, such as the oxidative burst (Ahn et al., 2007), cell-wall strengthening (Heil & Bostock, 2002), accumulation of defence-related enzymes (Rahman et al., 2014) and the production of secondary metabolites (Yedidia et al., 2003).

Table 5. 5: ISR induction and the potential mechanism of action that have been mentioned in the previous studies. The main defence molecules are jasmonate, ethylene, salicylic acid and PR proteins.

Plant	Pathogen	Mycobiont	Mechanism of action	Reference
Banana	*Fusarium oxysporum* f.sp. *cubense*	Rhizobacteria *Pseudomonas fluorescens*	Upregulating defence genes: chitinase, β-1,3-glucanase and phenolic compounds	Thangavelu et al., 2003
Rice	*Xanthomonas oryzae* pv. *oryzae*	*Bacillus*	Activation of defence enzymes such as phenylalanine ammonia lyase, peroxidase, and the synthesis of phenolics, phytoalexins and lignin	Chithrashree et al., 2011
Tomato	*Botrytis cinerea*	Gram-positive bacterium *Micromonospora*	Induction of jasmonate defences	Martínez-Hidalgo et al., 2015
Arabidopsis	*Pseudomonas syringae* pv. *tomato*	The beneficial bacteria Paraburkholderia phytofirmans (PsJN strain)	Induction of jasmonate, ethylene, salicylic acid and reactive oxygen species pathways	Timmermann et al., 2019
Arabidopsis	*Pseudomonas syringae* pv. *tomato*	*Penicillium* sp. GP16-2 strain	Activation of the transcriptional factor NPR1 and up regulation of jasmonate and ethylene gene expression	Hossain et al., 2008
Maize	*Curvularia lunata*	*Trichoderma harzianum*	Increasing jasmonate and ethylene metabolic induction	Saravanakumar et al., 2016
Anthurium andraeanum	*R. solanacearum*	*S. indica*	Higher levels of the antioxidative enzymes superoxide dismutase, catalase and peroxidase, increasing the expression of jasmonate related genes and PR proteins	Lin et al., 2019

Priming is not only related with enhanced cellular defence, but also with molecular defence such as alterations in the expression of transcription factors. For example in rhizospheric bacteria-mediated ISR in *Arabidopsis* the MYC2 transcription factor, has been shown to up-regulate jasmonic acid levels (Pozo et al, 2008). In addition, members of the transcription factor family APETALA2/Ethylene response factor (AP2/ERF) have been involved in the activation of jasmonic acid and ethylene genes in ISR-induced plants (Memelink, 2009). Transcription factors are assumed to remain

latent under stress-free conditions, but they grant a quick response to the plant under any potential pathogen stress (Pieterse et al., 2014). The priming mechanism does not include only increased sensitivity of defence gene expression, but also includes acceleration in cellular responses. For example, in *Pseudomonas fluorescens*-colonised *Arabidopsis* plants, high callose accumulation occurred rapidly during infection with the downy mildew pathogen *Hyaloperonospora arabidopsidis*. Rapid stomata closure was induced when *Arabidopsis* plants previously colonised with a beneficial *Bacillus subtilis* FB17 strain, were infected with a pathogenic *Pseudomonas syringae* (Rudrappa et al., 2008).

All of the defence genes studied here were initially detected in *Serendipita* and PM colonised tomato leaves. When the PCR was repeated there was little expression of the chitinase, callose 11 synthase, PAL and JA-Lipoxygenase genes in any plants. This was likely due to the presence of inhibitors or template degradation. Interestingly *Serendipita* colonization has been previously shown to repress PR proteins in barley (Schafer et al., 2009; Sarkar et al., 2019) and *Arabidopsis* (Pedrotti et al., 2013), suppress jasmonate related genes in rice (Cosme et al., 2016) and reduce the oxidative burst in *Arabidopsis* (Jacobs et al., 2011). It has been suggested that *Serendipita* fungi downregulate the expression of certain defence genes to avoid the immune system of host plants (Schafer et al., 2009; Camehl et al., 2011; Pedrotti et al., 2013).

This part of the study shows that *Serendipita*-colonisation improves the immunity of tomato plants to the powdery mildew pathogen, at least at a phenotypic scale. *Serendipita*-colonised tomato plants showed little expression of the majority of defense genes analysed although β-1,3-glucanase was expressed inconsistently within and between treatments. Other key defence genes might be worthy of investigation in this system such as the PR protein thaumatin to endeavour to understand the molecular basis of the bio-protective response. Inoculating crop species with the symbiont may provide a sustainable and chemical free method for controlling plant disease in horticultural and agricultural situations. This technology will need to be trialled under glasshouse and field situations to confirm its usefulness. Such trials could investigate different *Serendipita* species, crop plant varieties and pathogens to ascertain the most appropriate combinations to use to maintain yields over growing periods.

CHAPTER 6: RECOMMENDATIONS AND CONCLUSIONS

Serendipita fungi are common symbionts of Australian orchids including those in Western Australia (Sommer et al., 2012; Davis et al., 2015; Phillips et al., 2016; Vohnik et al., 2016; Reiter et al., 2020), Victoria (Huynh et al. 2009; Wright et al., 2010; Setaro et al., 2012; Reiter at al., 2020) and South Australia (Warcup 1967 & 1971). This project investigated the *Serendipita* partners of orchids in south east Queensland because only a few studies have previously focussed on this region (Bougoure et al., 2005; Dearnaley et al., 2009). Although the *Caladenia* species investigated in this study did not contain new *Serendipita* species, this work has considerably extended the range of some of those species. For example, *S. communis* and *S. warcupii*, have previously only been known from Western Australia. Queensland has a number of still un-investigated *Caladenia* species eg. *C. chaematophylla* in the tropical northern region (Jones 2006) and further investigations of this type may reveal additional new *Serendipita* species or extend the range of well-known fungal taxa.

The fungal symbionts within *B. bracteatum* and *B. schillerianum* represent new *Serendipita* species. This was confirmed via sequencing of ITS and LSU DNA, mycelial growth rates and nuclear staining. Teleomorphic states of these two fungi will need to be induced to complete life cycle descriptions before formal taxonomic description. Although *in vitro* methods were unsuccessfully trialled here, soil over agar procedures will likely produce better results in this regard (Crous et al. 2020). These *Bulbophyllum*-associated fungal taxa are quite different from those colonising *Caladenia* spp. Future molecular taxonomic analyses with additional DNA barcodes, e.g., RPB1 and TEF1a, may conceivably elevate these fungi to a second genus within the Serendipitaceae. Queensland has many unstudied epiphytic orchid species (Jones, 2006) and there is clearly much potential for discovery of novel mycorrhizal fungal taxa.

Serendipita colonisation improved the fresh weight of tomato which is compatible with previous reports that have shown the ability of the fungus to improve plant biomass (Varma et al., 1999; Rai et al., 2001; Druge et al., 2007; Serfling et al., 2007). This physiological response may be associated with increased aquaporin expression

which enhances water movement through cell membranes. Aquaporins are key to the function of other mycorrhizal-plant interactions (Maurel et al., 2008 & 2015; Chaumont et al., 2001; Barzana et al., 2014), and it would be illuminating to study expression of such genes under *Serendipita* colonisation using qRT-PCR or RNA-Seq protocols.

This study provided an opportunity to investigate the molecular and cellular features of ISR during the interaction of *Serendipita*-colonised tomato plant with the powdery mildew pathogen *Golovinomyces lycopersici*. *Serendipita indica* appears to play a similar role as rhizospheric bacteria; because the fungus activates ISR by inducing jasmonic acid and ethylene signalling and the transcriptional regulator non-expressor of PR genes1 (NPR1), during colonisation of *Arabidopsis* roots (Stein et al., 2008; Jacobs et al., 2011; Franken, 2012; Pedrotti et al., 2013). The transcriptional regulator NPR1 is important for activation of jasmonic/ethylene-dependent ISR and salicylic acid-dependent SAR. The function of NPR1 in ISR seems to be different to that in SAR. In SAR, NPR1 activates PR genes, while in ISR; NPR1 is thought to operate without activation of PR genes (Pieterse et al., 2014). Furthermore, in salicylic acid signalling, NPR1 operates in the nucleus (Dong, 2004), while in jasmonic/ethylene signalling, NPR1 functions in the cytosol (Spoel et al., 2003; Ramirez et al., 2010; Pieterse et al., 2012). Further molecular studies are necessary to clarify the function of NPR1 in the regulation of ISR elicited by beneficial microbes. The *Serendipita*-tomato-powdery mildew system provides an opportunity to study the role of NPR1 in ISR further, particularly given the possible role of the ß-1,3 glucanase protein in the bio-protective response observed here.

Priming induced by mycorrhizal fungi is an effective strategy to inhibit the stress caused by plant pathogens and represents a potential approach to enhance plant protection in agricultural systems instead of chemical methods (Walters et al., 2013). This technology still needs to be tested for its utility under glasshouse and field conditions. Studying *Serendipita* induced priming during the interaction between the plants and the pathogens in the field will be challenging, where many biotic and abiotic stresses such as pathogens, herbivores, drought and salinity stresses happen at the same time (Mauch-Mani et al., 2017). Little is known about how plants set their defensive

priorities, which makes it difficult to predict their cellular and molecular reaction and their resistance response under field conditions (Mauch-Mani et al., 2017).

This project focused on the role of *Serendipita* in tomato performance under biotic stress. The tomato-*Serendipita* model could also be tested for its usefulness in combating abiotic stress such as drought, salinity and heavy metal toxicity in agricultural and horticultural systems. A potential way forward here would be to set up *in vitro* study systems which would intially confirm the protectant capabilities of the fungi (eg. Ghimire and Craven 2011). Experiments could then be conducted in glasshouses conditsions and ultimately in field conditions.

Quantitative RT-PCR is a powerful, modern technique that analyses gene expression in different cell populations. It has many advantages such as firstly, simplicity, where the protocol requires few reagents such as a polymerase, dNTPs, fluorescent dye, and a pair of primers. Secondly, qRT-PCR is a sensitive technique that quantifies samples with very few copies of messenger RNA. Thirdly, a well-designed assay is specific for a single molecular target. Fourthly, a well-designed trial will give results in a wide range of reaction conditions and finally, the cost of reagents is affordable (Bustin & Kessler, 2010). In this study, the low concentration of RNA/cDNA, presence of inhibitors, or template degradation present in the growth and bio-protection experiments may have impacted on the inconsistency of Cq values obtained. Future studies of this type should use greater concentrations of high quality starting material to more accurately monitor gene expression via qRTPCR.

An alternative to qRT-PCR in future studies of this type may be RNA-Seq. RNA-Seq is becoming the method of choice for gene expression analyses in research. It provides an overview of complete cellular mRNA populations and is highly sensitive in detecting changes in single nucleotides in transcripts, microsatellites, and allelic variants. It has high levels of reproducibility and accuracy. Moreover, RNA-Seq does not need a reference genome, and it detects the expression of genes in low levels of starting materials that are not detected by other gene expression techniques (Segundo-Val & Sanz-Lozano, 2016).

Serendipita fungi enhance plant resistance against pathogens, increase nutrient uptake, and improve survival under drought, salinity, and heavy metal stress (reviewed in

Weiss et al. 2016). *Serendipita* could be developed as agricultural inoculants for Australian farmers and other world regions. In India, 10^9 CFU of *Serendipita* was formulated with magnesium silicate, which acts as a carrier. This commercial product is called Rootonic bio-fertilizer and is still under trial but may become widely used in India (Singhal et al., 2017). Much developmental work needs to be done including testing Australian *Serendipita* fungi under glasshouse and field conditions. Different fungal-plant combinations should be studied and inoculum production conditions, the amount of inoculum best used, the time point of inoculation should also be elucidated. The persistence of the fungus in the environment should also be considered (Franken, 2012; Andrade-Linares et al., 2013). The final product should be easy for use and storage by farmers. Moreover, possible quantitative changes of treated crops such as fruit size and total production should be monitored to ensure profits are maintained.

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APPENDICES

Appendix I-A: ITS sequencing results of fungi isolated from *Caladenia* spp.

Source	Sequence results from AGRF
Caladenia caerulea	GTGACCTGCGGAGGATCATTAACGAGGTACAAGTCGGTCGACAGTGCTGGCGGAGACG CACGTGCACGTCGGTCGCAAACCAATCCACACACCTGTGAACGTATGGCCTTTGGGTCT CACGACTCGGGGGCAAACCTTTTTTACCCACTCTGTCTGTAAAGGAATGTCTATGTGCTC AAAGCGCAAAGCAAACAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAA CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTG AACGCACCTTGCACCCTTTGGTATTCCGAAGGGTACGCCCGTTTGAGTGTCATTGTAATC TCACCCCCGGAGACTTGTTTTCCGGGAGTGGACTTGGACGTTGCCGTGACTCCGGCTCGT CTCGAATGTCTCAGTGTACCCCGATCATCGGCGTCAACAGTGTGATATGTATCTTCACTG TGAGTCTCTCCGGAGGCGCGCTCTCGAACGTGGGCATATGCTGCCAACCGTCTTCGGAC AATCTCTGACAATTTGACCTCAAATCGGGTGGGACTACCCGCTGAACTTAAGCATATCA
Caladenia picta	GACTGCGGAGGATCATTAACGAGGTTACAAGTCGGTCGACAGTGCTGGCGGAAACGCA CGTGCACGTCGGTCGCAAACCAATCCACACACCTGTGAACGTATGGCCTTTGGGTCTCA CGACCCGGGGCAAACATTTTTTACCCACTCTGTTTGTAAAGGAATGTCTATGTGCTCAAA GCGCAAAGCAAACAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGC AGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAAC GCACCTTGCACCCTTTGGTATTCCGAAGGGTACGCCCGTTTGAGTGTCATTGTAATCTCA CCTCCAAAGACTTTGTTCTTTGGGAGTGGACTTGGACGTTGCCGTGACTCCGGCTCGTCT CGAATGTCTCAGTGTACCCCGATCTTCGGCGTCAACAGTGTGATATGTATCTTCACTGTC AGTCTCTTCGGAGGCGCGCTCTCGAACGTGGGCCTATGCTGCCAACCGTCTTCGGACAA TTTCTGACAATTTGACCTCAAATCGGGTGGGACTACCCGCTGAACTTAAGCATATCAATA AGNCGGAGGAANNNAAAG
Caladenia fuscata	CGTAAGGTGAACCTGCGGAAGGATCATTAACGAGGTACAAGTCGGTCGACAGTGCTGG CGGAGACGCACGTGCACGTCGGTCGCAAACCAATCCACACACCTGTGAACGTATGGCCT TTGGGTCTCACGACTCGGGGGCAAACCTTTTTTACCCACTCTGTCTGTAAAGGAATGTCT ATGTGCTCAAAGCGCAAAGCAAACAACTTTCAACAACGGATCTCTTGGCTCTCGCATCG ATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATC GAATCTTTGAACGCACCTTGCACCCTTTGGTATTCCGAAGGGTACGCCCGTTTGAGTGTC ATTGTAATCTCACCCCCGGAGACTTGTTTTCTGGGAGTGGACTTGGACGTTGCTGTGACT CCGGCTCGTCTCGAATGTCTCAGTGTACCCCGATCCTCGGCGTCAACAGTGTGATATGTA TCTTCACTGTG
Caladenia sp	AGGTTTCGTAAGGTGAACCTGCGGAAGGATCATTAATGAATCGTAAGTCGGTCGACCGT GCTGGCGGCAACGCACGTGCACGTCGGTCGCAAACCAATCCACACACCTGTGAACGTAT GGCCTCTCGAGTCCTTTTGGACTCGGGGCAAAACCCATTTTTACTCTGATCGTAAAGGAA TGTCTTTGCCTAAAGCGCAAAAGCAAACAACTTTCAACAACGGATCTCTTGGCTCTCGC ATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAAT CATCGAATCTTTGAACGCACCTTGCACCCTTTGGTATTCCGAAGGGTACGCCCGTTTGAG TGTCATTGTAATCTCACCCCCGGAATCTTTTCTGGGGAGTGGACTTGGACGTTGCCGTGT CACGGCTCGTCTGGAATGTCTCAGTGCTACCCCGTCTGTCGGCGTATACAGTGTGATAAG TATCTTCACTGGTCAGCTTCCTCGAGGCGCGCTCTCGGACGGATCGGTGTGCTGCCAACC GTCTTCGGACAATACTG
Caladenia filamentosa	GGTTTCGTAAGGTGAACCTGCGGAAGGATCATTAACGAATTTCCAAGTCGGTCGACCGT GCTGGCGGAAACGCACGTGCACGTCGATCGCAAACCAATCCACACACCCGTGAACGTAT GGCCTCTCGGGTCTTTGACTCGGGGGCAAACCATTTTTCGCACTCTGATAGTAAAGGAAT GTTCTTTGCCTAATACGCAAAAACAAACAACTTTCAACAACGGATCTCTTGGCTCTCGCA TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATC ATCGAATCTTCGAACGCACCTTGCACCCTTTGGTATTCCGAAGGGTACGCCCGTTTGAGT GTCATTGTAATCTCACCCCCGAAATCTTTTTTTTGGGAGTGGACTTGGACGCTGCCGTGT CACGGCTCGTCTCGAACGTCTCAGTGTACCCCGCCGTCGGCGTCAAACAGTGTGATAAG TATCTTCACTGGTTAGTCTCTCCGGAGGCGCGCTCTCGGATTGGTGGTGTGCTGCCAACC GTCCTCGGACAATACTCTGACAATTTGACCTCAAATCGGGTGGGACTACCCGCTGAACT TAAGCATATCATAA

Caladenia gracillimum	AGGTCCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGTTTCCAACTCACAACCCAAT GTGAACATACCTACGTTGCTTCGGCGGCATCCGGCCCCAGGCCGCGCCGCCGGAGACCC AAACTCTTTGTTTTCCAATGTGGTTACTTCTGAGTATTCTTGAAATAAATCAAAACCTTC AACAACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGAAAAGTAA TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCTGGA ACTCCGGCGGGCACGCCTGTCCGAGCGTCATTTCAACCCTCAGGCCCCCCTTTCGGGGG CGGGCCTGGTGTTGGGGCGCGGCCGTCCTCACCGGCGGCCGGCCTCCAAATTCAGTGGC GGTCACGCCGCAATCCCTTGCGTAGTAATATCACCTCGCACTGGAGAGCGACGCGGTCC ACGCCGTGAAACCCCAACTTTTCAATGGTTGACCTCGGATCAGGTGGGAATACCCGCTG AACTTAAGCATATCATAA
Caladenia catenata	AAAGGTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGTTTCCAACTCACAACCCAA TGTGAACATACCTACGTTGCTTCGGCGGCATCCGGCCCCAGGCCGCGCCGCCGGAGACC CAAACTCTTTGTTTTCCAATGTGGTTACTTCTGAGTATTCTTGAAATAAATCAAAACCTT CAACAACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGAAAAGTA ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCTGG AACTCCGGCGGGCACGCCTGTCCGAGCGTCATTTCAACCCTCAGGCCCCCCTTTCGGGG GCGGGCCTGGTGTTGGGGCGCGGCCGTCCTCACCGGCGGCCGGCCTCCAAATTCAGTGG CGGTCACGCCGCAATCCCTTGCGTAGTAATATCACCTCGCACTGGAGAGCGACGCGGTC CACGCCGTGAAACCCCAACTTTTCAATGGTTGACCTCGGATCAGGTGGGAATACCCGCT GAACTTAAGCATATCAATAA

Appendix I-B: ITS sequencing results from fungi isolated from *Bulbophyllum* spp.

Source	Sequence results from AGRF
Bulbophyllum bracteatum	CATTCACTAGAAAGGAACCGATTTGAGGTCAATTGTCAAGATGCTTGTCCAAACAGGAC GGTTCGCAGCACAGAGCCCACAGAGCAGACGTGTCCCAAAGGGACTTGTTCCAGTGAAG ATGCTTATCACACTGAAGACGCCGCCGAAGCAGGGTGCACTCATGCATTTGAGACTGGT CGTCATTACACGACAGAGTCCAAGTCCACACCCAACCACGACAAAGTGTTTGGGGTGAG ATTACAATGACACTCAAACGGGTGTACCTTTCGGAATACCAAAAGGTGCAAGGTGCGTT CAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGT TCTTCATCGGTGCGAGAGCCAAGAGATCCGTTGTTGAAAGTTGTATTTGTATGCATTATG CACAGATACGTTCCATTACATATCAGAGTGTGTAAAAATACTCTGAGATCCAGACCGAC AGCGAAGCCAGCCTGCTGCGAGTCGGATGAACCGACGGGCGAATCCCAGAGTCATAAG TGCACAGGTGTGTGGATTTGCGATCGACGTGCACATGCGTTGCCACCAGCACAGACGAC CGACTTTAGATTCGTTAATGATCCTTCCGCAGGTTCACCTACGGAACATTTATATTGCAT AAGCATCAGAGTACTTGTACCCAACCTCTCGGTTGGGACTCGACCATATCTTAAGCACTT GAAGTGCCCACTACCATTTGGCCTGTGAACTGCACCCCACGACAAGGGGCTTGGCTGCG GATTGTCCCTGTTCCATGTGCTGTTACCATACCCAAGGTGATTAGCCTGGTGCTGCGCTA CATCTTTCGATGAGCCGCGGTGACATGGACTTGACAGGATTTTCCCGCAATTTGATAGTG TCGCCTCTCATGAGAGACACTAGCGTTATACTCCCNGTGTGTGTATAGACTNCGGGGTNT CACTTTTTGGGGCACACTAAGGTTTGTGCATGTGCACTGTGCNGNGAGAATTGTGGTGCT CCTCATACACGTGGAATCTACAGACCCTGTTATGACATTTTCTTTCCATAAAAATAACCA GAANAAAAAA
Bulbophyllum schillerianum	TCAAGGTTGGAATCCAACCGATTTGAGGTCAATTGTCAAAGGTTGTCCGGAGACGGTTC GACAGCACAGAGCCNACAACGCATGCGTGTCCCAAAGGACTTTGTTCCAGTGAAGATGC TTATCACACTGAAGACGCTGCAACAGCAGGGTGCACTCATTCATTTGAGACCAGCCGTT GTGACACGACAGGGTCCAAGTCCACGTCCGACAGCGACAAAGCTGAAGGAGTGAGATT ACAATGACACTCAAACGGGTGTACCCTTCGGAATACCAAAGGGTGCAAGGTGCGTTCAA AGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTT CATCGATGCGAGAGCCAAGAGATCCATTGTTGAAAGTTGTATTTTTGCGCGTTATGCACA TGTACATTCCATTACATTCAGAGTGTGTAAAAATACTCCAAGATCCAGGCCAACCTAAG TCGACCGGCTGCAAGTCGGACAAGCCAACAGGCAAATCTAAGAGTCAAAGGTTCACAG

	GTGTGTGGATTTGCGACCAACGTGCACAATGCGTCTCCGCCAGCACAGATGACCGACTT GTAATTCATTAATGATCCTTCCGCAGGTTCACCTACGGAACATTTGCATTTCGTACAGAG TACTTGTACCCAACTTTCCAATTGGGATTCGACCATATCTTAAGCATCGAGCGATGCCCA CTACCATCTGGCCTGTGAACTGCACCCCACAGAGGAGGGGCTTGGCTGCAGATTCTCCC TATTCCACATGCTGTTACCATACCCAAGGTTGTTAGCCTGGTGCTGACGCTGCACCTTTC GGTGAGCCGCAGTGATGTGGACATGACAGGATCTTCCCGCAATTTGATAGTGTTGCCTC CACAAAGGAGACTAGCGTTTAATCCACCTGGTTGTTTAGAGTCGCTGGTGAAACCCTTTC GGGCCACTGAGGCTTCTGCAAGTGTACTCTGCGGAAGGAACATTGCTGTTCTTCGTAGA CTTTTGCTACTGAAACCTTGTTACGACTTTTACTTCCNCAAAAAAAAGACANAGAAAA
Bulbophyllum shepherdii	ACCCTTTGGCACCACCCCGTGAGGTCTGTCAAGAAGCCGCATCCGCCGGAGCGGACCAT TTGATCCAGTGAACGTGGCAGAGGATCTCGCACTCGACGTTCGCAAGCAGACCTCGACA TTTATGACAAGGTCACCGCGTGCGTCGGTGCAACCATATATCTGAGGAAGCGCGACACA TAGGGTCGTGAATCCCGAACCTGGACAGGCCACATAAAGTGACAAGTCGCAAAGACAT AATGACGCTCAATGGGGCGTATCGTTCCTGGATAAGGCTCGATGCAGTGCGTTCAACAA TTCGATGGTTCACGTATAAGTTTTGGACTTGCATATCACACCACTTATCGCATTTTGCAA CGGTCTTCATCGAATGACGTGCCAAGGGATCCAACGCTACTGGTTGTGTTATCTCTCGGT TTAAGGTAGGCATGACACGGATTACAATGGTTTGTCAAGATCCCGAGGGGACCAAGACT AGCGCCTGGAGAGGCGCATTGCATCCAGAGTGTGATGAGGGGCCCGTGAAGGCTGACCT CTCCATAACTATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGACTTACTTCC TCTAATGGAACCAAGAAA
Bulbophyllum minutissimum	CGTGGACTGCGGAGGATCATTATCGTGGGGCTTCGGCCCTAGTCGAGATAGAACCCTTG CCTTTTTGAGTACCCTTTCTTGTTTCCTCGGCGGGCCTGCCCGCCGATGGGGACCACCAA AAACGCTTTGCAGTACCTGTAACAGTCTGATAAACAAACAAAATAATCAAAACTTTCAA CAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGT GAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTC CTTAGGGCATGCCTGTTCGAGCGTCATTTAAACCTTCAAGCTCAGCTTGGTGTTGGGTGA CTGTCCGCCGCCCCCGGGCGCGGACTCGCCCCAAATTCATTGGCGGCCGGTACGTAGGC TTCGAGCGCAGCAGAAACGCGAACTCGGGCCCGCGGTGTCGGCTCCCAGAAGCTATCTT CACAATTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAANG NCGGAGGAAGCN
Bulbophyllum exiguum	TCATTGGTTCTACCTGATCCGAGGTCAACCACTAGAAAAAAAATAGAGGTTTATGGCGA TAGTTAGGCTACAATAAAGCGAATAAAATTTACTACGCTTAGGGTGAGACCGTAACCCT GCCACTGACTTTGAGGAGTTACCAGACGGTAATGCTCCCAACGCTAAGCAACTAAGGCT TAATGGTCGTAATGACGCTCGAACAGGCATGCCCACTAGAATACTAATGGGCGCAATGT GCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGC TGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGACTTATTTAT ATATGTAACTCAAATGCGCCACACAGAAACAAGAGTTTAGTGAGTCCTTCGGCGGTCCG CAGGCTACCGGGTAGCTACAGTGTAGCTCCAGGGTAGGAGACTACAGGGTAGCTACCGG GTAGCTCCAGGGTAGGTATGCCTAGTAGGCACCCTGGATGTGCGATCACCGCCGAGGCA ACACGGGTATGTTCACATGGGTTTGGAGTTTGATAACTCAGTAATGATCCCTCCGCTGGT TCACCAACGGAAACCTTGTTACGACTTTTACTTCCCAATTNNNNNNCCNNNNA

Appendix II

Appendix II: LSU sequencing results from fungi isolated from *Bulbophyllum* spp.

Source	Sequence results
Bulbophyllum bracteatum	GTACCACACAAAACAGCATCGTGCCAGGCTTCTTCACCGACCTCCACG CCTGCCTACTCGTCAGCGCGTCACAAGAACGCTGACGGCGAGGTATGG GTAACACGCTTGAGCGCCATCCATTTTCAGGGCTAGTTCATTCGGCAGG TGAGTTGTTACACACTCCTTAGCGGGTTCCGACTTCCATGGCCACCGTC CTGCTGTCTAGATGAACTAACACCTTTTGTGGTGTCTGATGAGCGTGTA TTCCGGCACCTTAACCTCGCGATCGGTTCATCCCGCATCGCCAGTTCTG CTTACCAAAAATGGCCCACTAGAAACTCTCACCCAGAGAAGAGTCCAA TCAAGTGACAATTCTGTCTTGCACATTTAGAGTTTGAGAATAGGTTAAG GTTGTTTCAACCCCAAGGCCTCTAATCATTCGCTTTACCGCACAAGGCT GATAATGAGTTTCTGCTATCCTGAGGGAAACTTCGGCAGGAACCAGCT ACTAGATGGTTCGATTAGTCTTTCGCCCCTATACCCAAATTTGACGATC GATTTGCACGTCAGAATCGCTACGAGCCTCCACCAGAGTTTCCTCTGGC TTCACCCTATTCAGGCATAGTTCACCATCTTTCGGGTCCCAACATATAC GCTCTACCGCGGATGCGTCACAGAAGGTCTGCTCCGGGCGTCGGTGCA CAAGTACATGATCCCGACCTTTCACTTTCATTACGCGCCCAGGTTTGAC ACCTAAACACTCGCGCACATGTTAGACTCCTTGGTCCGTGTTTCAAGAC GGGTCGCTTAAAGCCATTATGCCAGCATCCTAAGCACGTACCGAGGGC GAACCCCGGCCATAAGGCGTGCTGCGTTCCTCAGTCCCAACCGAGACA TACAACGAGGGGCTATAACACTGCCCGAAGACAGCCACATTCCCCAAG CCTTTCTCCCTCGATCGAAACTGACGCTGACCCATTTGCCAGAAAGTAC ACCAGGCAGAAGCCAGGCTGAATTCCGACCAATGTGACTGACTTCAAA CGCTTCCCTTTCAACAATTTCACGTACTGTTTCACTCTCTTTCCAAAGGG CTTTTCATCTTTTCCCTCCCGGGAATTGGTTCGCTATCGGGCTCTCGCCA AAATTTAACTTTTAAAGGGAATTTACCCCCCCCTTTTTGAGTGGGATTC CCAAACAAATCGACCCCTCCAAAAGGGTTTCTCAAAGACCTTGGGGGT CCCACCCCCAAAAGGGGATT
Bulbophyllum schillerianum	AGACATAAAAACCAGCATGCTGCCAGGCTTCTTCACCAACCTCCACGC CTGCCTACTCGTCAACGCGTCACAAGAACGCTGACGGCGAGGTATGGG TAACACGCTTGAGCGCCATCCATTTTCAGGGCTAGTTCATTCGGCAGGT GAGTTGTTACACACTCCTTAGCGGGTTCCGACTTCCATGGCCACCGTCC TGCTGTCTAGATGAACTAACACCTTTTGTGGTGTCTGATGAGCGTGTAT TCCGGCACCTTAACCTCGCGATCGGTTCATCCCGCATCGCCAGTTCTGC TTACCAAAAATGGCCCACTAGAAACTCTCACCCACAGAGAAGTCCAAT CAAGTGACAACCCTATCTTGCACATTTAGAGTTTGAGAATAGGTTAAG GTTGTTTCAACCCCAAGGCCTCTAATCATTCGCTTTACCGCACAAGGCT GATAATGAGTTTCTGCTATCCTGAGGGAAACTTCGGCAGGAACCAGCT ACTAGATGGTTCGATTAGTCTTTCGCCCCTATACCCAAATTTGACGATC GATTTGCACGTCAGAATCGCTACGAGCCTCCACCAGAGTTTCCTCTGGC TTCACCCTATTCAGGCATAGTTCACCATCTTTCGGGTCCCAACATATAC GCTCTACCTCGGATGCGTCACAGAAGGTCTGCTCCGGGCGTCGGTGCA CAAGTACATGATCCCGACCTTTCACTTTCATTACGCGCCCAGGTTTGAC ACCTAAACACTCGCGCACATGTTAGACTCCTTGGTCCGTGTTTCAAGAC GGGTCGCTTGAAGCCATTATGCCAGTGTCCTAAGCACGTACCGAGGGC GCGAACCCCGGCCAGAAAGCGTGCTGCGTTCCTCAATCCCAACCGAGA

CATACAACAAGAGGCTATAACACTGCCCGAAGACAGCCACATTCCCCA
AGTCTTTCTCTCTCGATCAAAATTGACACTGACCCATTTGCCAGAAAAT
ACACCAGGCAGAAGCCAGGCTGAGTTCCGACCAATGCGACTGACTTCA
AACGCTTCCCTTTCACAATTTCACGTACTGGTTTCATCTCTTTCCAAAAT
GCTTTTCATCTTTCCTCCCGGGACTTGTTCGCTATCGGTCTCTCGCCATA
TTTACTTTTAAAGGGAATTACCACCCCTTTTGAGCTGCATTCCCAACAA
CCCCACCCCCTTAAAAGGGTTTAAAGAAACCAAGGGTGTCCCCCCCCA
AACGGGATTCTTCCCCCTTTAAAC

Appendix III

Appendix III-A: Values of plant height, root length, shoot length and fresh weight between in *Ser*+/*Ser*- treatments (3 ml inoculum) in the two experiments.

Serendipita+ (Exp 1)				
No.	Total Length (cm)	Shoot Length (cm)	Root Length (cm)	Fresh Weight (g)
1	23	7	16	0.5342
2	22.5	7.5	15	0.5619
3	19	9	10	0.7944
4	18	4	14	0.5846
5	15	5	10	0.7109
6	17.5	5	12.5	0.5342
7	15	5	10	0.6645
8	25	12	13	0.786
9	28.5	10.5	18	1.1808
10	21.5	8	13.5	0.4869
11	17	5.5	11.5	0.7803
12	14	4	10	0.6211
13	20.5	6.5	14	0.5217
14	17.5	8.5	9	0.6391
15	15	4.5	10.5	0.5894
16	20	10	10	0.566
17	20	7.5	12.5	0.3841
18	22.5	7	15.5	0.4218
19	19	5	14	0.4259
20	21.5	5.5	16	0.5897

Serendipita- (Exp 1)				
No.	Total Length (cm)	Shoot Length (cm)	Root Length (cm)	Fresh weight (g)
1	22	11	11	0.705
2	25	9	16	0.3073
3	18	6	12	0.2603
4	28	10	18	0.8628
5	21	6.5	14.5	0.6892
6	28	9	19	0.7031
7	20.5	8	12.5	0.5912
8	21.5	9	12.5	0.6568

9	23	9	14	0.6512
10	18	9.5	8.5	0.4702
11	24.5	7.5	17	0.4457
12	17	8	9	0.4555
13	23.5	11.5	12	1.127
14	25	13.5	11.5	0.5224
15	21	9	12	0.3913
16	15	7.5	7.5	0.2712
17	26	9.5	16.5	0.5614
18	28	10	18	1.1067
19	23	9.5	13.5	0.5293
20	20	7	13	0.4384

Serendipita+ (Exp 2)				
No.	Total Length (cm)	Shoot Length (cm)	Root Length (cm)	Fresh Weight (g)
1	19	9	10	0.64
2	20	8.5	11.5	0.6372
3	19	10.5	8.5	0.8123
4	15	9.5	5.5	0.5488
5	18	9	9	0.447
6	19	6.5	12.5	0.452
7	19.5	10.5	9	1.16
8	21	11.5	9.5	0.7962
9	20	6.5	13.5	0.765
10	13	6	7	0.3625
11	21	8.5	12.5	0.643
12	9	4	5	0.3066
13	16	4.5	11.5	0.5236
14	19.5	6.5	13	0.7056
15	18	9.5	8.5	0.4933
16	14	9	5	0.5984
17	21	10	11	1.144
18	20	11	9	0.6561
19	22	13	9	0.733
20	10	5	5	0.2465

Serendipita- (Exp 2)				
No.	Total Length (cm)	Shoot Length (cm)	Root Length (cm)	Fresh weight (g)

1	22	11	11	0.705
2	25	9	16	0.3073
3	18	6	12	0.2603
4	28	10	18	0.8628
5	21	6.5	14.5	0.6892
6	28	9	19	0.7031
7	20.5	8	12.5	0.5912
8	21.5	9	12.5	0.6568
9	23	9	14	0.6512
10	18	9.5	8.5	0.4702
11	24.5	7.5	17	0.4457
12	17	8	9	0.4555
13	23.5	11.5	12	1.127
14	25	13.5	11.5	0.5224
15	21	9	12	0.3913
16	15	7.5	7.5	0.2712
17	26	9.5	16.5	0.5614
18	28	10	18	1.1067
19	23	9.5	13.5	0.5293
20	20	7	13	0.4384

Appendix III-B: Values of plant height, root length, shoot length and fresh weight between in *Ser*+/*Ser*- treatments (1.5 ml inoculum) in the three experiments.

Serendipita+ (Exp 1)				
No.	Total Length (cm)	Shoot Length (cm)	Root Length (cm)	Fresh Weight (g)
1	21	8.5	12.5	0.786
2	24	14	10	0.92
3	22	14	8	0.907
4	24	9.5	14.5	0.668
5	27	15	12	1.006
6	24	13	11	0.811
7	19	7.5	11.5	0.667
8	20	8	12	0.416
9	24	9	15	0.575
10	23	9	14	0.625
11	26	17	9	1.327
12	28	17	11	3.088
13	20	9	11	0.764
14	23	10	13	0.718
15	25	19	6	1.323
16	30	15	15	1.949

17	27	11	16	0.716
18	25	18	7	1.378
19	31	17	14	2.207
20	32	11	21	0.749

Serendipita- (Exp 1)				
No.	Total Length (cm)	Shoot Length (cm)	Root Length (cm)	Fresh weight (g)
1	28	13	15	0.828
2	28	8	20	0.624
3	28	14	14	0.928
4	33	13.5	19.5	2.334
5	30	11	19	0.508
6	21	13	8	0.834
7	24	14	10	1.234
8	30	10	20	0.477
9	29	7.5	21.5	0.832
10	25	6	19	0.281
11	30	10	20	0.57
12	25	10	15	0.644
13	28	10	18	0.477
14	26	12.5	13.5	0.933
15	20	9.5	10.5	0.734
16	27	10.5	16.5	0.732
17	27	9	18	0.354
18	20	9	11	0.414
19	24	15	9	0.746
20	22	11	11	0.724

Serendipita+ (Exp 2)				
No.	Total Length (cm)	Shoot Length (cm)	Root Length (cm)	Fresh Weight (g)
1	21.5	5.5	16	0.411
2	14	9	5	0.417
3	25	9.5	15.5	0.48
4	19	8	11	0.501
5	24	15.5	8.5	0.861
6	25	9	16	0.722
7	13	7	6	0.395
8	21.5	12	9.5	1.12
9	26	15	11	0.877

10	20	14	6	1.173
11	26	14	12	0.976
12	28	10	18	0.8
13	24	10	14	0.623
14	21	9.5	11.5	0.609
15	20	9	11	0.483
16	30	9	21	0.62
17	20	8	12	0.336
18	30	7	23	0.265
19	12	5	7	0.176
20	10	5	5	0.195

Serendipita- (Exp 2)				
No.	Total Length (cm)	Shoot Length (cm)	Root Length (cm)	Fresh weight (g)
1	22	8	14	0.325
2	18.5	8.5	10	0.52
3	21	10.5	10.5	0.639
4	25	8	17	0.355
5	24	13	11	0.708
6	23	11	12	0.76
7	22	13	9	0.742
8	21	12.5	8.5	0.582
9	21	6.5	14.5	0.384
10	22	10.5	11.5	0.493
11	24	16	8	0.707
12	24	12	12	0.535
13	28	8.5	19.5	0.274
14	36	18.5	17.5	0.966
15	21	11.5	9.5	1.11
16	14	7	7	0.16
17	20	5	15	0.381
18	29	11	18	0.8
19	29	8	21	0.854
20	20	6.5	13.5	0.695

Serendipita+ (Exp 3)				
No.	Total Length (cm)	Shoot Length (cm)	Root Length (cm)	Fresh Weight (g)
1	21	13	8	0.615
2	17	6	11	0.333

3	23.5	10.5	13	0.518
4	21	10.5	10.5	0.558
5	20	12	8	0.54
6	18	9	9	0.504
7	25	14	11	0.853
8	20	12	8	0.515
9	16	9	7	0.524
10	17	9	8	0.565
11	27	9	18	0.438
12	17	8	9	0.435
13	14	9	5	0.532
14	23	8	15	0.55
15	14	8	6	0.515
16	20	5.5	14.5	0.365
17	17	11	6	0.529
18	17	8	9	0.3
19	22	8.5	13.5	0.437
20	21	7	14	0.45

Serendipita- (Exp 3)				
No.	Total Length (cm)	Shoot Length (cm)	Root Length (cm)	Fresh weight (g)
1	26.5	7.5	19	0.373
2	19	7	12	0.387
3	22	7	15	0.368
4	25	5	20	0.225
5	30	6	24	0.485
6	22	7.5	14.5	0.343
7	21	5.5	15.5	0.414
8	18	10	8	0.576
9	17	8	9	0.376
10	20	7.5	12.5	0.411
11	18	11	7	0.577
12	27	8	19	0.491
13	23	6.5	16.5	0.362
14	25	7	18	0.233
15	21	5.5	15.5	0.231
16	22	6	16	0.227
17	25	6	19	0.533
18	23	6.5	16.5	0.23
19	16	6	10	0.211
20	23	7	16	0.31

Appendix IV

Appendix IV: Values of plant height, root length, shoot length and fresh weight between in *Ser*+/PM+ & *Ser*-/PM+ treatments in the three experiments.

Serendipita+/PM+ (Exp 1)				
No.	Total Length (cm)	Shoot Length (cm)	Root Length (cm)	Fresh Weight (g)
1	22	12.5	9.5	1.166
2	23.5	14	9.5	1.085
3	24	12	12	1.106
4	24	15	9	1.109
5	22	11	11	1.283
6	27.5	11.5	16	1.116
7	26	11.5	14.5	1.209
8	25	14	11	1.127
9	26	9.5	16.5	0.612
10	30	11	19	1.133
11	23	13	10	0.866
12	24	12	12	0.986
13	20	12	8	0.703
14	22	13	9	1.388
15	20	10	10	1.247
16	22	9.5	12.5	0.838
17	25	15	10	1.31
18	22	9	13	0.612
19	24	13	11	0.981
20	20	8	12	0.683

Serendipita-/PM+ (Exp 1)				
No.	Total Length (cm)	Shoot Length (cm)	Root Length (cm)	Fresh weight (g)
1	21	9.5	11.5	0.644
2	20	12.5	7.5	0.639
3	21	10.5	10.5	0.615
4	26	12.5	13.5	0.888
5	24	11	13	0.585
6	24	10	14	0.572
7	26	10	16	0.452
8	25	10.5	14.5	1.01
9	27	11.5	15.5	0.803
10	21	10.5	10.5	0.606
11	20	12	8	0.56
12	24	13	11	0.93

13	26	11	15	0.926
14	20	9	11	0.814
15	20	11	9	0.656
16	20	11	9	0.781
17	21	12	9	1.22
18	22	13	9	0.809
19	21.5	10	11.5	0.432
20	25	10.5	14.5	0.89

Serendipita+/PM+ (Exp 2)				
No.	Total Length (cm)	Shoot Length (cm)	Root Length (cm)	Fresh Weight (g)
1	19	10.5	8.5	0.625
2	22	15.5	6.5	0.864
3	23	11.5	11.5	0.812
4	14	6	8	0.487
5	20.5	12.5	8	0.84
6	21	11.5	9.5	0.748
7	25	13	12	0.494
8	24	12	12	0.766
9	22	9	13	1.035
10	26	13	13	0.897
11	24	14	10	1.331
12	23	12	11	1.059
13	27	14	13	0.783
14	30	14	16	0.979
15	26	14	12	1.067
16	27	12	15	1.107
17	25	12	13	1.369
18	27	13	14	0.981
19	21	14	7	1.333
20	25	15	10	1.06

Serendipita-/PM+ (Exp 2)				
No.	Total Length (cm)	Shoot Length (cm)	Root Length (cm)	Fresh weight (g)
1	22	12	10	0.782
2	24	12	12	0.608
3	23	11.5	11.5	0.614
4	22.5	11.5	11	0.74
5	22	9	13	0.322
6	30	11	19	0.63
7	21	11	10	0.745

8	19.5	9.5	10	0.446
9	25	10	15	0.65
10	24	10	14	0.721
11	30	10	20	0.535
12	26	13	13	1.1
13	20	10	10	0.634
14	20	11	9	0.87
15	24	11	13	0.838
16	20	9	11	0.537
17	27	12	15	1.13
18	24	8	16	0.681
19	22	11	11	0.921
20	26	11	15	1.01

Serendipita+/PM+ (Exp 3)				
No.	Total Length (cm)	Shoot Length (cm)	Root Length (cm)	Fresh Weight (g)
1	18	8	10	0.537
2	19	4.5	14.5	0.15
3	25.5	8	17.5	0.673
4	25	7.5	17.5	0.518
5	17	8	9	0.364
6	26	11	15	0.877
7	19.5	6.5	13	0.245
8	17	6	11	0.234
9	19	5	14	0.303
10	17	7	10	0.327
11	16	5.5	10.5	0.28
12	22	5.5	16.5	0.335
13	16	6	10	0.417
14	13.5	4.5	9	0.257
15	13	6	7	0.183
16	27	6.5	20.5	0.351
17	15	5	10	0.435
18	13	4.5	8.5	0.207
19	19	7	12	0.315
20	20	6	14	0.286

Serendipita-/PM+ (Exp 3)				
No.	Total Length (cm)	Shoot Length (cm)	Root Length (cm)	Fresh weight (g)
1	14	5	9	0.308
2	14	7	7	0.192

3	19	8	11	0.282
4	21	5	16	0.285
5	22	5	17	0.293
6	22	6	16	0.352
7	19	9	10	0.816
8	23	5	18	0.252
9	24	7	17	0.32
10	18.5	7.5	11	0.31
11	21	7	14	0.213
12	23	5	18	0.313
13	20	5.5	14.5	0.295
14	23	8	15	0.372
15	24	8.5	15.5	0.276
16	17	6	11	0.163
17	21	7	14	0.322
18	20	7	13	0.272
19	17	7.5	9.5	0.236
20	23	7	16	0.178

Appendix V

Appendix V-A: statistical analysis of growth experiment variables between *Ser+*/*Ser-* treatments (3 ml inoculum) in the three experiments using jamovi 2.2.5 statistical software.

Results of growth experiment 1 (3 ml)

Independent Samples T-Test

Independent Samples T-Test

		Statistic	df	p
Total Length (cm)	Student's t	-2.388	38.0	0.022
	Welch's t	-2.388	38.0	0.022
Shoot Length (cm)	Student's t	-3.339	38.0	0.002
	Welch's t	-3.339	35.7	0.002
Root Length (cm)	Student's t	-0.703	38.0	0.486
	Welch's t	-0.703	36.1	0.487
Fresh Weight (g)	Student's t	0.475	38.0	0.638
	Welch's t	0.475	35.0	0.638

Assumptions

Normality Test (Shapiro-Wilk)

	W	p
Total Length (cm)	0.984	0.823
Shoot Length (cm)	0.952	0.086
Root Length (cm)	0.979	0.664
Fresh Weight (g)	0.908	0.003

Note. A low p-value suggests a violation of the assumption of normality

Homogeneity of Variances Test (Levene's)

	F	df	df2	p
Total Length (cm)	0.0218	1	38	0.883
Shoot Length (cm)	2.3647	1	38	0.132
Root Length (cm)	0.7474	1	38	0.393

Homogeneity of Variances Test (Levene's)

	F	df	df2	p
Fresh Weight (g)	1.7640	1	38	0.192

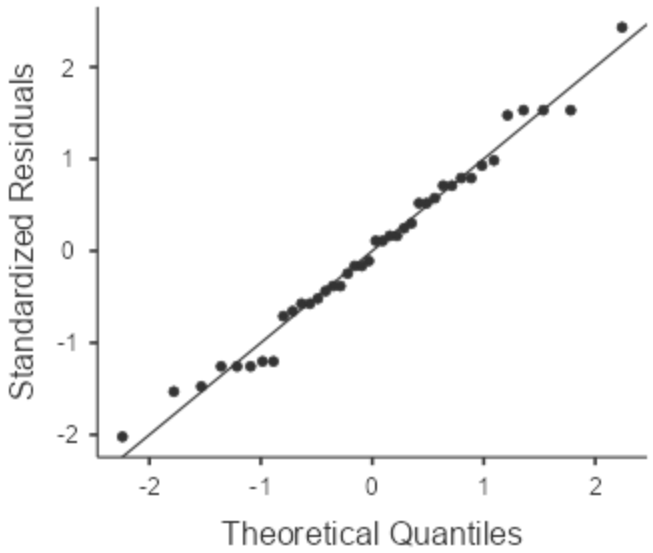
Note. A low p-value suggests a violation of the assumption of equal variances

Group Descriptives

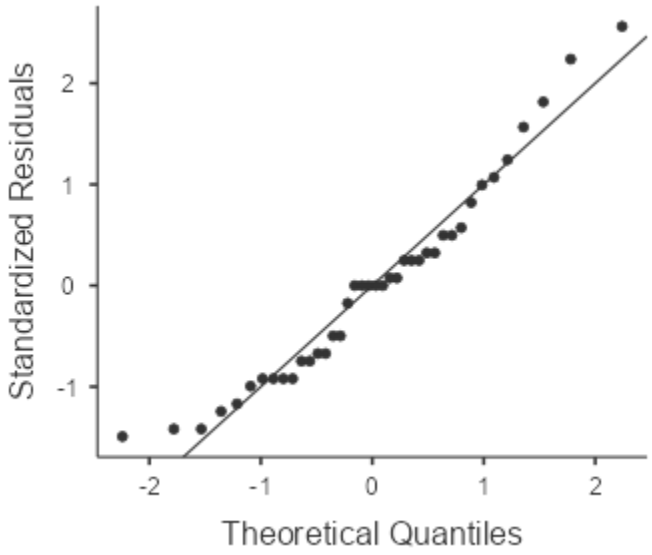
	Group	N	Mean	Median	SD	SE
Total Length (cm)	Serendipita +	20	19.600	19.500	3.691	0.8253
	Serendipita -	20	22.400	22.500	3.726	0.8332
Shoot Length (cm)	Serendipita +	20	6.850	6.750	2.277	0.5093
	Serendipita -	20	9.000	9.000	1.762	0.3940
Root Length (cm)	Serendipita +	20	12.750	12.750	2.568	0.5741
	Serendipita -	20	13.400	12.750	3.243	0.7251
Fresh Weight (g)	Serendipita +	20	0.619	0.587	0.177	0.0395
	Serendipita -	20	0.587	0.545	0.239	0.0535

Plots

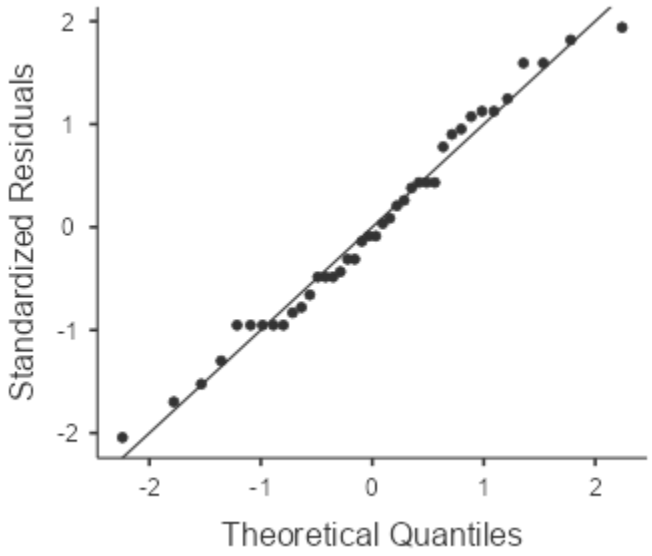
Total Length (cm)



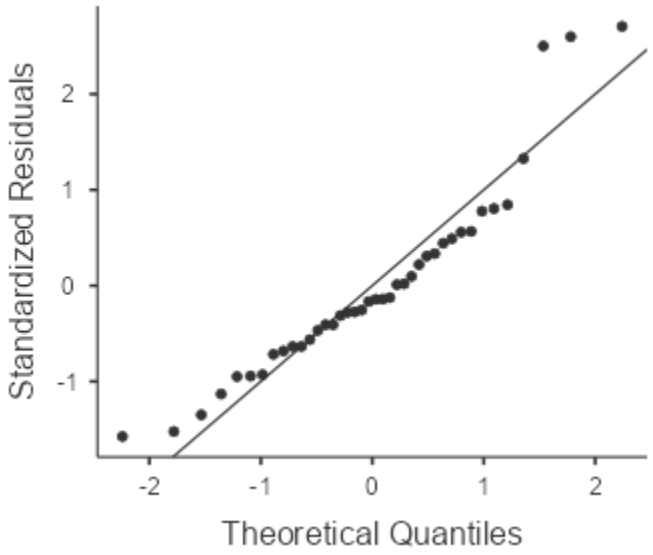
Shoot Length (cm)



Root Length (cm)



Fresh Weight (g)



Descriptives

Descriptives

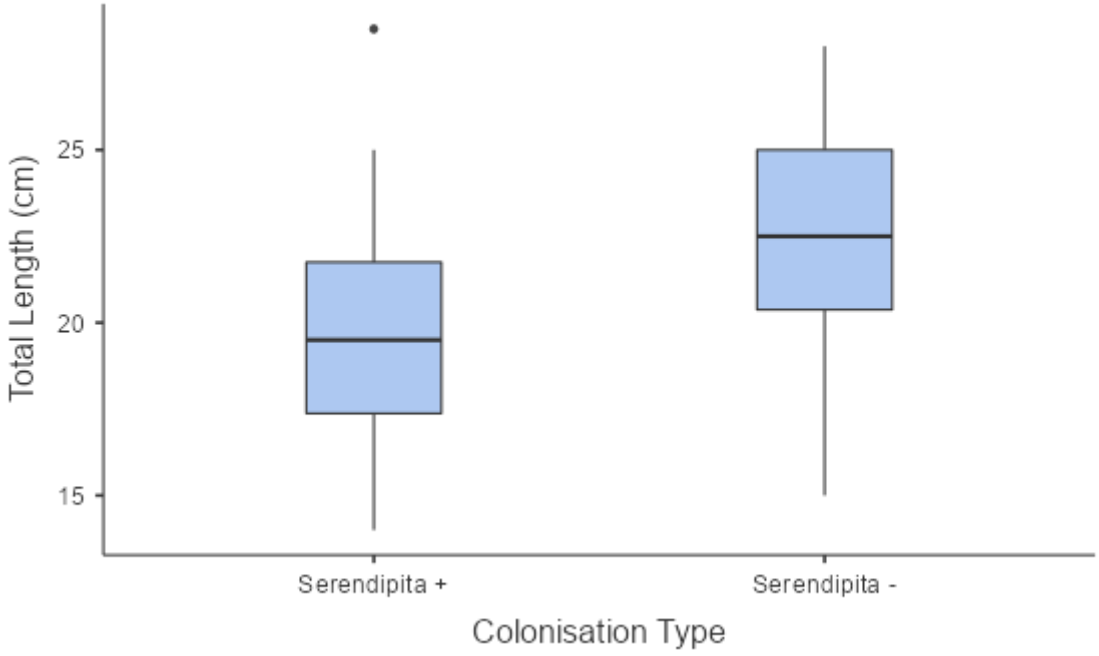
	Colonisation Type	Total Length (cm)	Root Length (cm)	Shoot Length (cm)	Fresh Weight (g)
N	Serendipita +	20	20	20	20
	Serendipita -	20	20	20	20
Missing	Serendipita +	0	0	0	0
	Serendipita -	0	0	0	0
Mean	Serendipita +	19.6	12.8	6.85	0.619
	Serendipita -	22.4	13.4	9.00	0.587
Median	Serendipita +	19.5	12.8	6.75	0.587
	Serendipita -	22.5	12.8	9.00	0.545
Standard deviation	Serendipita +	3.69	2.57	2.28	0.177
	Serendipita -	3.73	3.24	1.76	0.239
Minimum	Serendipita +	14.0	9.00	4.00	0.384
	Serendipita -	15.0	7.50	6.00	0.260
Maximum	Serendipita +	28.5	18.0	12.0	1.18
	Serendipita -	28.0	19.0	13.5	1.13
Shapiro-Wilk W	Serendipita +	0.964	0.937	0.930	0.863
	Serendipita -	0.967	0.963	0.956	0.919

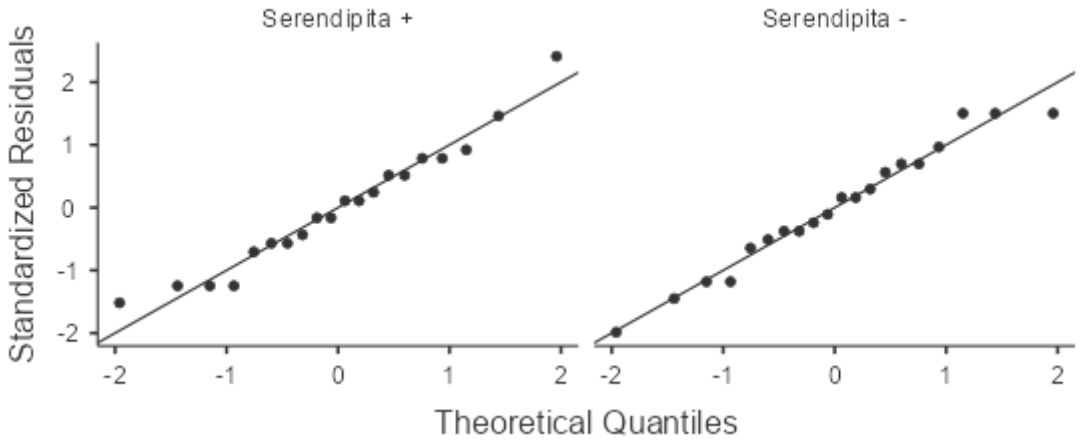
Descriptives

	Colonisation Type	Total Length (cm)	Root Length (cm)	Shoot Length (cm)	Fresh Weight (g)
Shapiro-Wilk p	Serendipita +	0.616	0.206	0.157	0.009
	Serendipita -	0.689	0.614	0.476	0.095

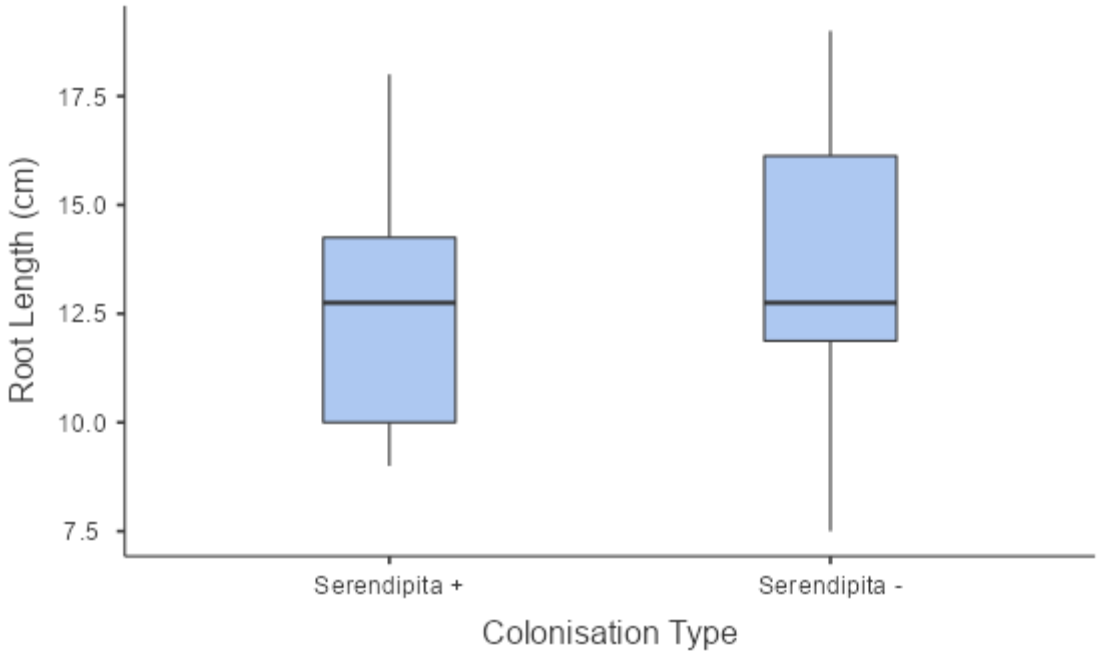
Plots

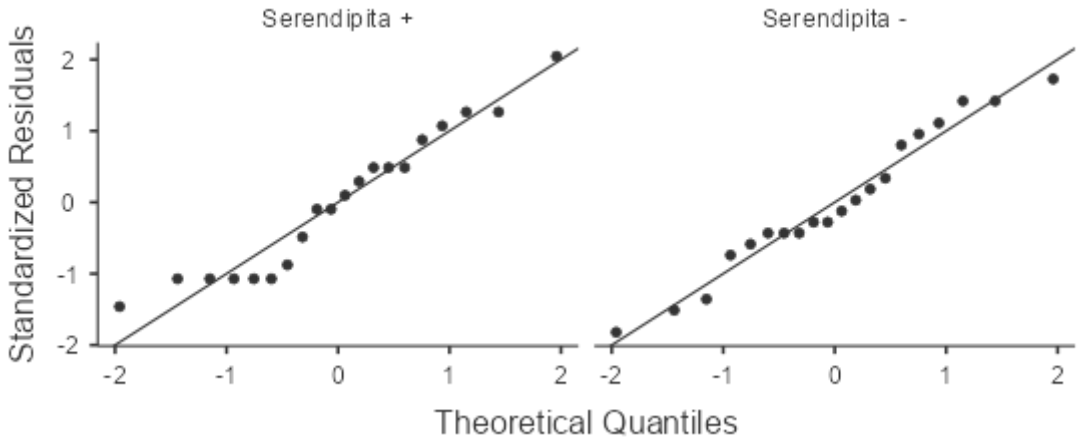
Total Length (cm)



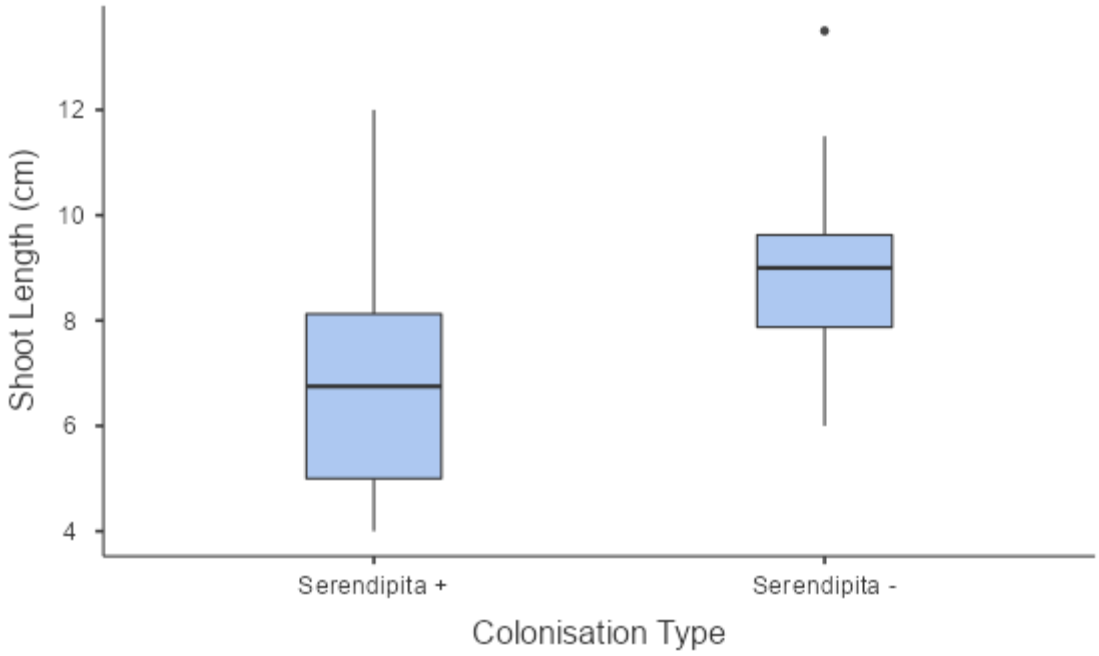


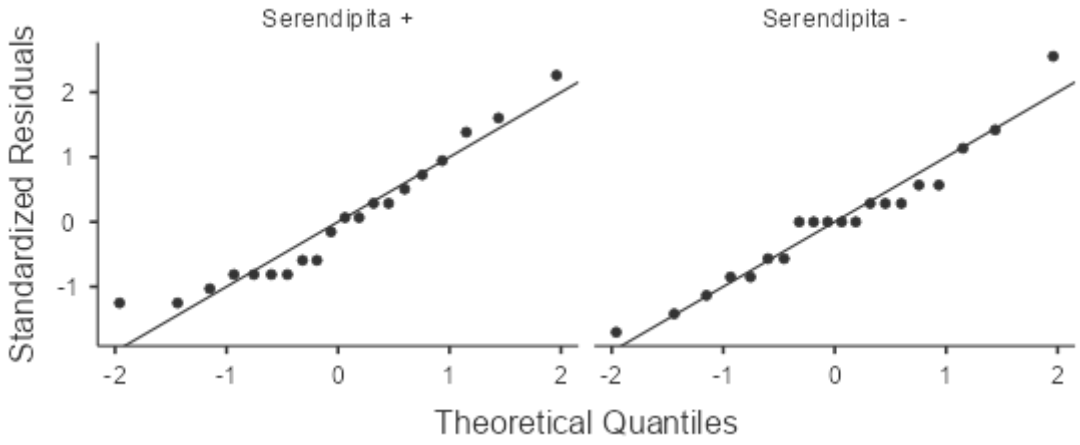
Root Length (cm)



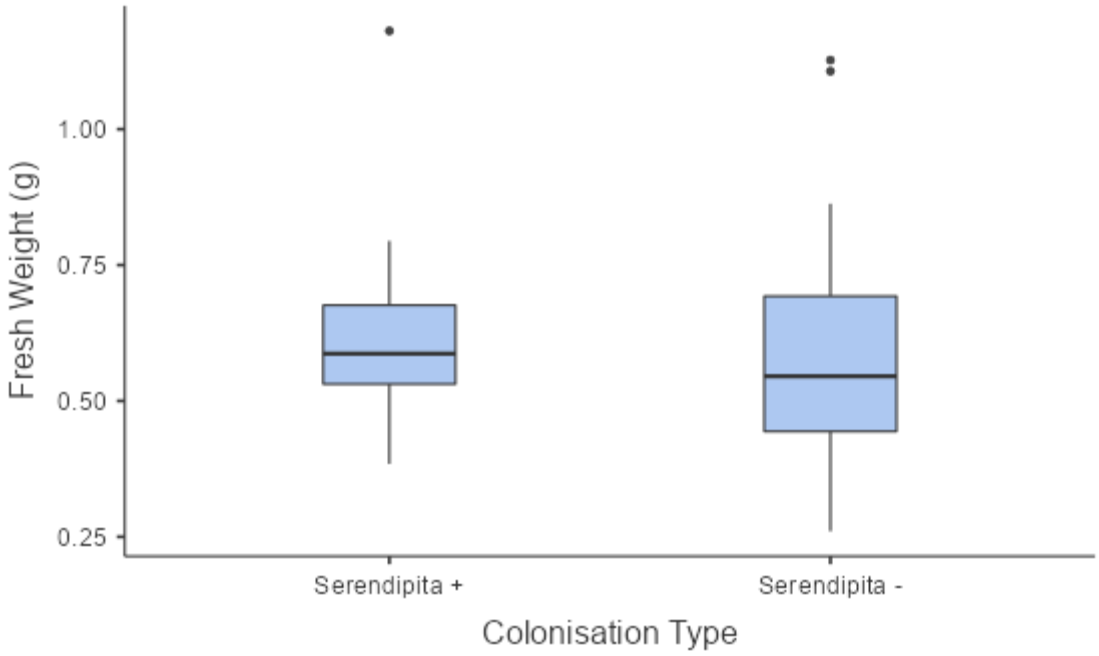


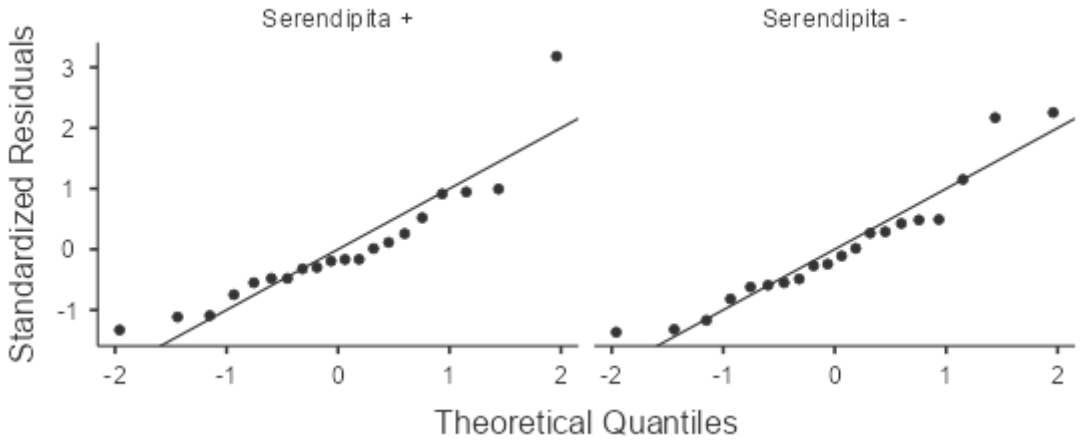
Shoot Length (cm)





Fresh Weight (g)





Results of growth experiment 2 (3 ml)

Independent Samples T-Test

Independent Samples T-Test

		Statistic	df	p
Total Length (cm)	Student's t	-4.004	38.0	< .001
	Welch's t	-4.004	38.0	< .001
Shoot Length (cm)	Student's t	-0.849	38.0	0.401
	Welch's t	-0.849	34.4	0.402
Root Length (cm)	Student's t	-4.351	38.0	< .001
	Welch's t	-4.351	36.9	< .001

Independent Samples T-Test

		Statistic	df	p
Fresh Weight (g)	Student's t	0.614	38.0	0.543
	Welch's t	0.614	38.0	0.543

Assumptions

Normality Test (Shapiro-Wilk)

	W	p
Total Length (cm)	0.949	0.071
Shoot Length (cm)	0.979	0.665
Root Length (cm)	0.971	0.385
Fresh Weight (g)	0.930	0.016

Note. A low p-value suggests a violation of the assumption of normality

Homogeneity of Variances Test (Levene's)

	F	df	df2	p
Total Length (cm)	0.0141	1	38	0.906
Shoot Length (cm)	3.3656	1	38	0.074
Root Length (cm)	0.6341	1	38	0.431
Fresh Weight (g)	0.0295	1	38	0.865

Note. A low p-value suggests a violation of the assumption of equal variances

Group Descriptives

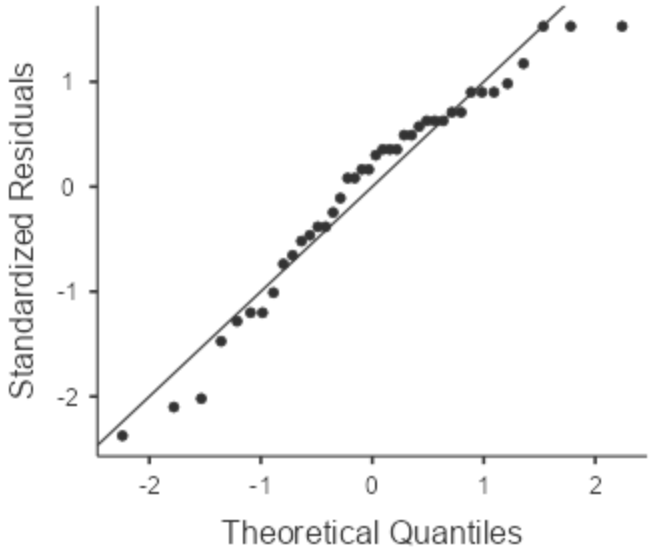
	Group	N	Mean	Median	SD	SE
Total Length (cm)	Serendipita +	20	17.700	19.000	3.697	0.8267
	Serendipita -	20	22.400	22.500	3.726	0.8332
Shoot Length (cm)	Serendipita +	20	8.425	9.000	2.462	0.5504
	Serendipita -	20	9.000	9.000	1.762	0.3940
Root Length (cm)	Serendipita +	20	9.275	9.000	2.731	0.6107
	Serendipita -	20	13.400	12.750	3.243	0.7251
Fresh Weight (g)	Serendipita +	20	0.634	0.639	0.237	0.0530

Group Descriptives

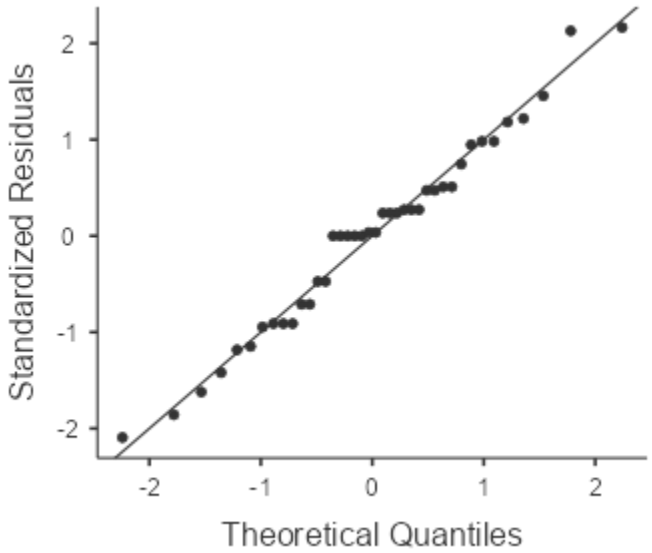
	Group	N	Mean	Median	SD	SE
	Serendipita -	20	0.587	0.545	0.239	0.0535

Plots

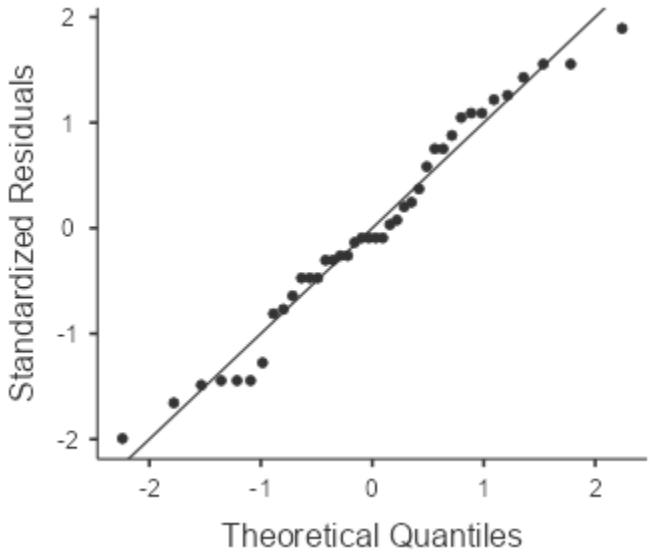
Total Length (cm)



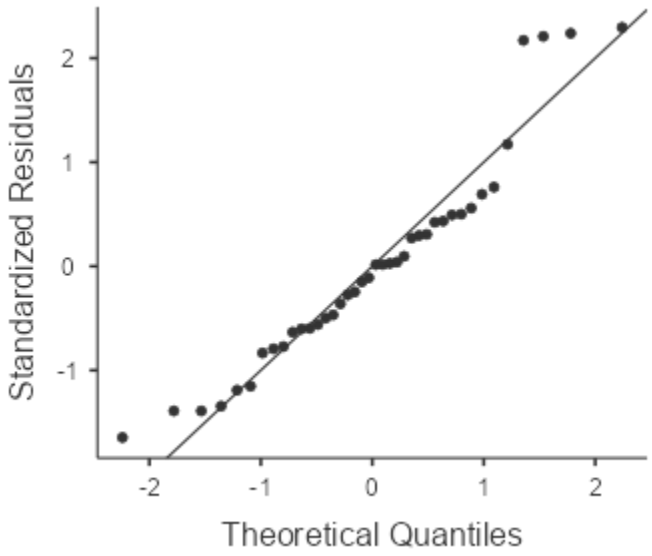
Shoot Length (cm)



Root Length (cm)



Fresh Weight (g)



Descriptives

Descriptives

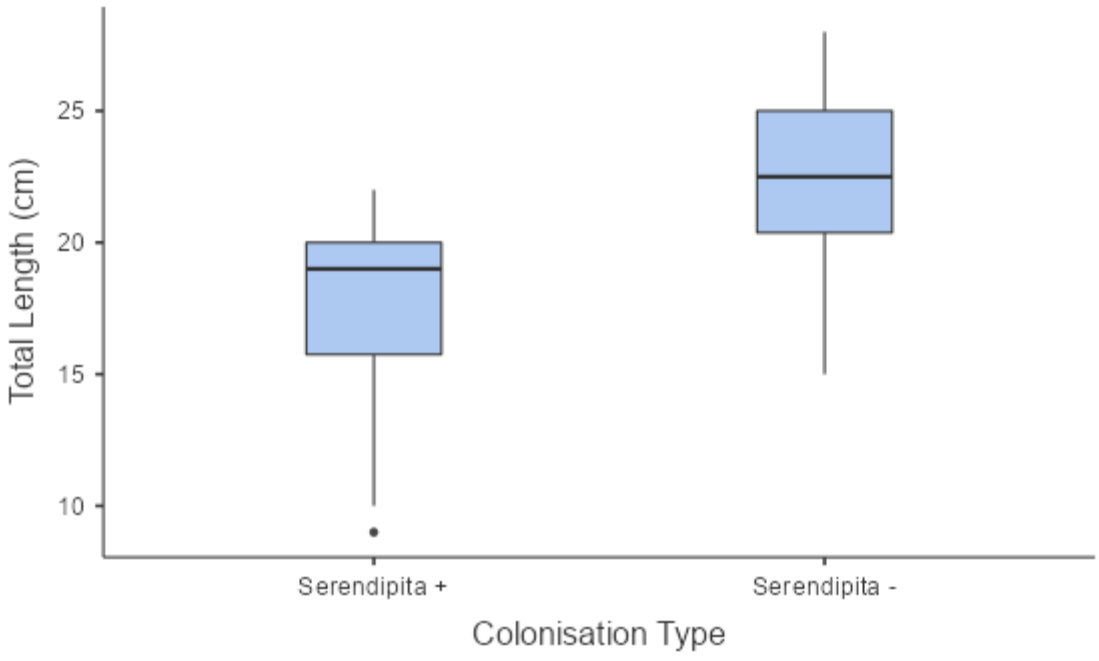
	Colonisation Type	Total Length (cm)	Shoot Length (cm)	Root Length (cm)	Fresh Weight (g)
N	Serendipita +	20	20	20	20
	Serendipita -	20	20	20	20
Missing	Serendipita +	0	0	0	0
	Serendipita -	0	0	0	0
Mean	Serendipita +	17.7	8.43	9.28	0.634
	Serendipita -	22.4	9.00	13.4	0.587

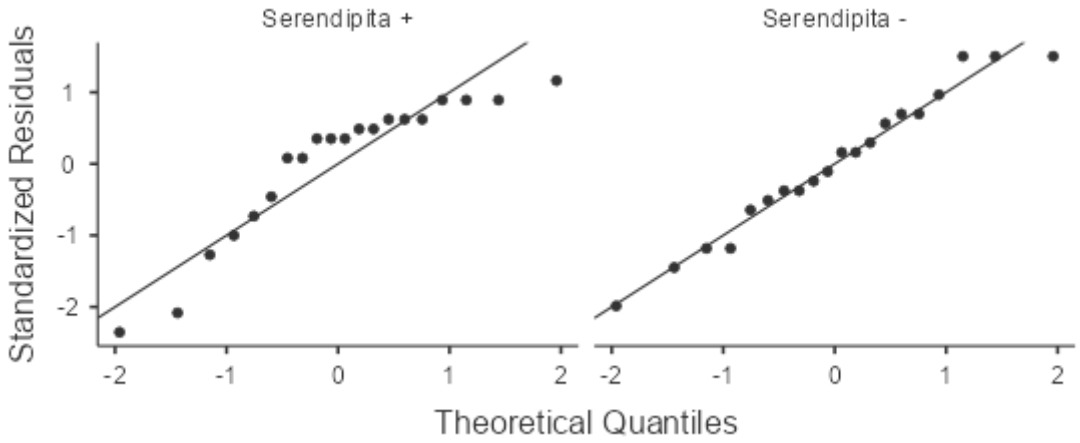
Descriptives

	Colonisation Type	Total Length (cm)	Shoot Length (cm)	Root Length (cm)	Fresh Weight (g)
Median	Serendipita +	19.0	9.00	9.00	0.639
	Serendipita -	22.5	9.00	12.8	0.545
Standard deviation	Serendipita +	3.70	2.46	2.73	0.237
	Serendipita -	3.73	1.76	3.24	0.239
Minimum	Serendipita +	9.00	4.00	5.00	0.246
	Serendipita -	15.0	6.00	7.50	0.260
Maximum	Serendipita +	22.0	13.0	13.5	1.16
	Serendipita -	28.0	13.5	19.0	1.13
Shapiro-Wilk W	Serendipita +	0.855	0.962	0.930	0.942
	Serendipita -	0.967	0.956	0.963	0.919
Shapiro-Wilk p	Serendipita +	0.007	0.579	0.152	0.257
	Serendipita -	0.689	0.476	0.614	0.095

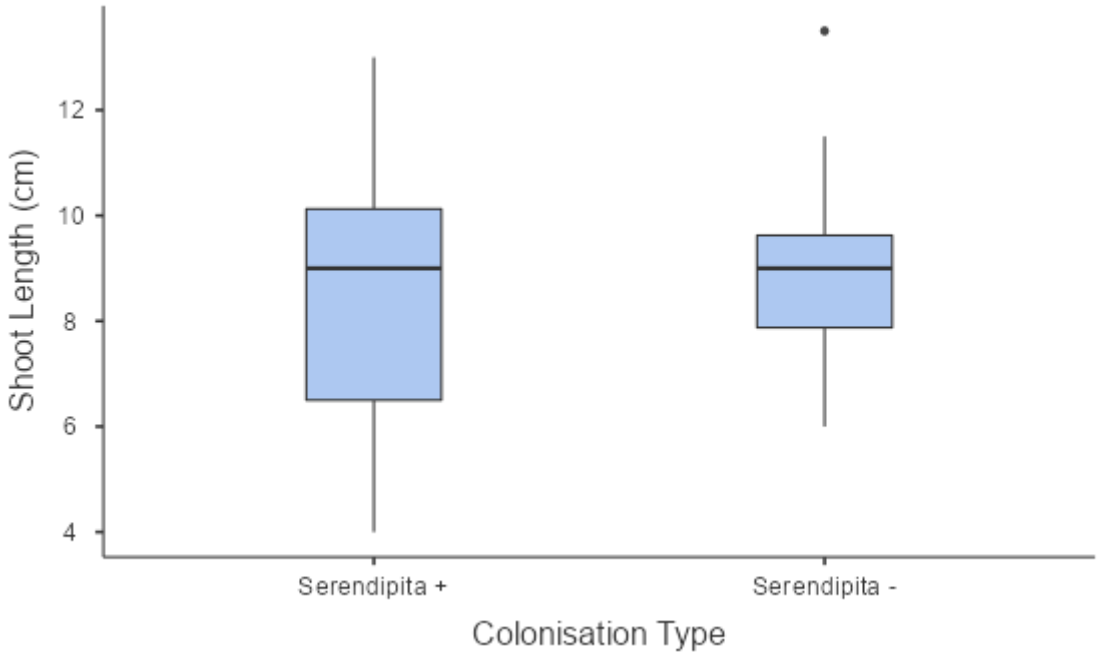
Plots

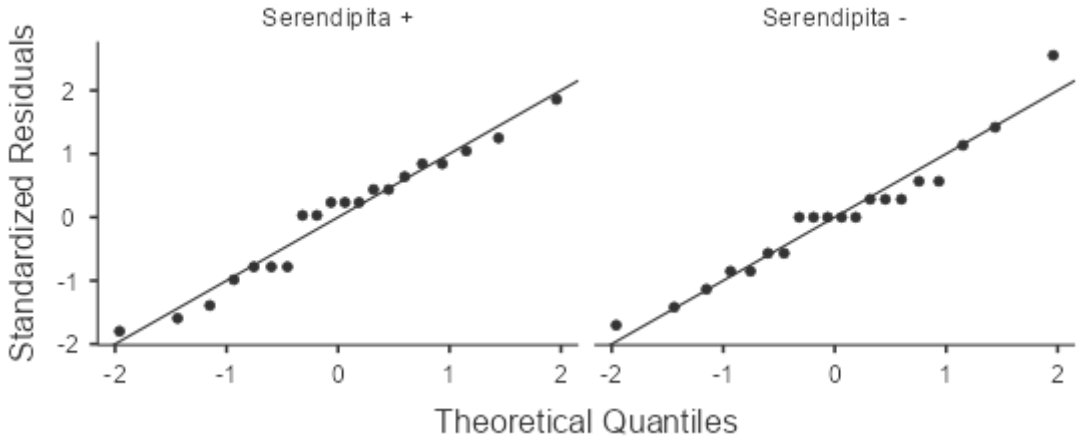
Total Length (cm)



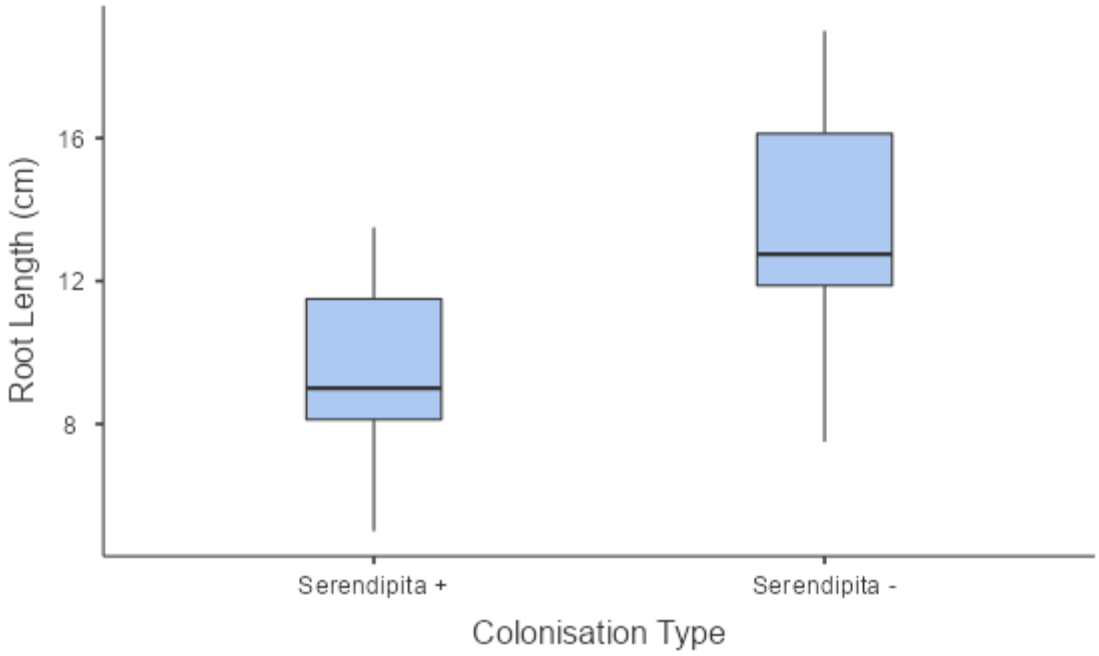


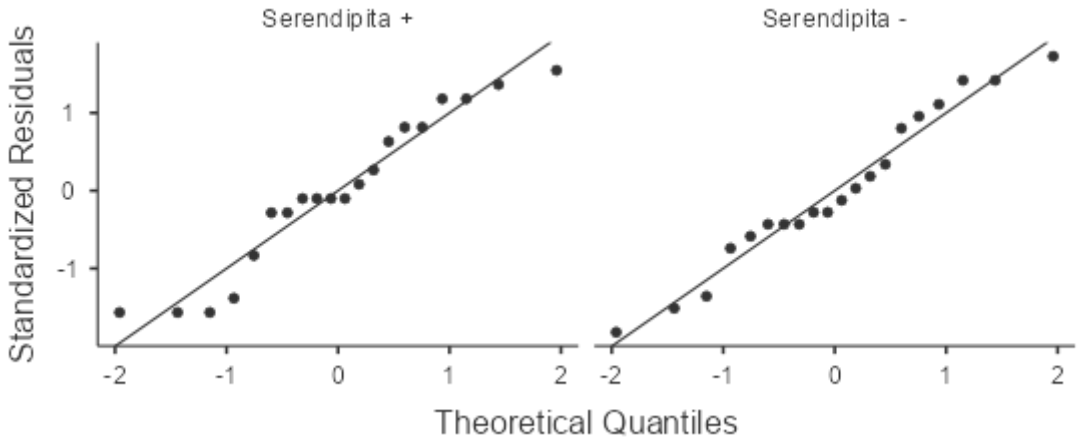
Shoot Length (cm)



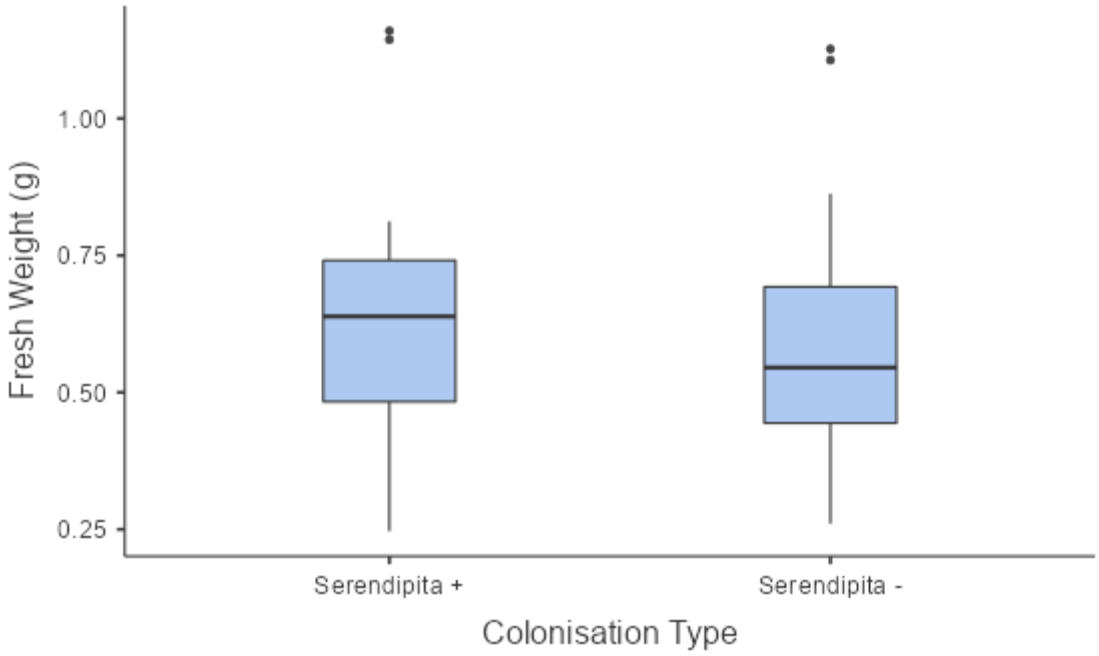


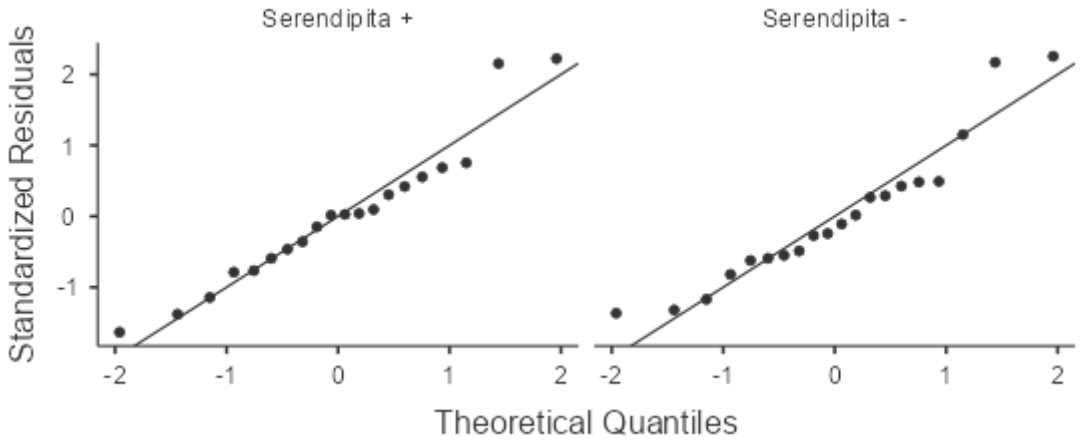
Root Length (cm)





Fresh Weight (g)





Appendix V-B: statistical analysis of growth experiment variables between *Ser+*/*Ser-* treatments (1.5 ml inoculum) in the three experiments using jamovi 2.2.5 statistical software.

Results of growth experiment 1 (1.5 ml)

Independent Samples T-Test

Independent Samples T-Test

		Statistic	df	p
Total Length (cm)	Student's t	-1.31	38.0	0.197
	Welch's t	-1.31	38.0	0.197

Independent Samples T-Test

		Statistic	df	p
Shoot Length (cm)	Student's t	1.75 [a]	38.0	0.088
	Welch's t	1.75	32.4	0.089
Root Length (cm)	Student's t	-2.64	38.0	0.012
	Welch's t	-2.64	36.2	0.012
Fresh Weight (g)	Student's t	1.81	38.0	0.077
	Welch's t	1.81	32.9	0.079

[a] Levene's test is significant ($p < .05$), suggesting a violation of the assumption of equal variances

Assumptions

Normality Test (Shapiro-Wilk)

	W	p
Total Length (cm)	0.977	0.586
Shoot Length (cm)	0.965	0.239
Root Length (cm)	0.978	0.605
Fresh Weight (g)	0.777	< .001

Note. A low p-value suggests a violation of the assumption of normality

Homogeneity of Variances Test (Levene's)

	F	df	df2	p
Total Length (cm)	0.0231	1	38	0.880
Shoot Length (cm)	8.7702	1	38	0.005
Root Length (cm)	3.1633	1	38	0.083
Fresh Weight (g)	3.1250	1	38	0.085

Note. A low p-value suggests a violation of the assumption of equal variances

Group Descriptives

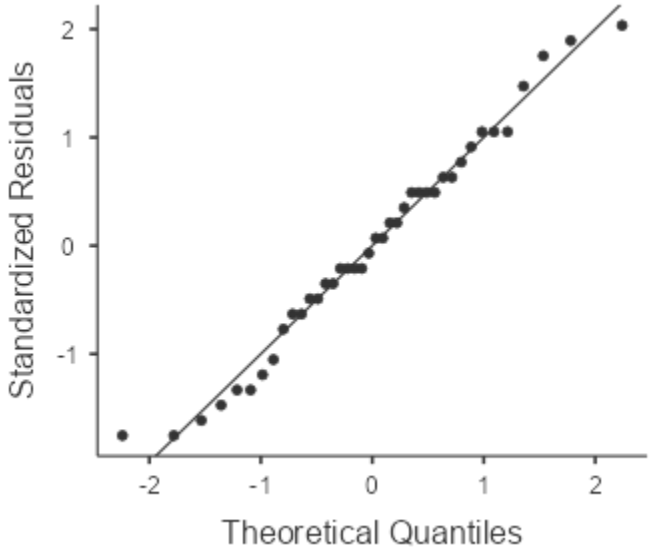
	Group	N	Mean	Median	SD	SE
Total Length (cm)	Serendipita +	20	24.75	24.000	3.626	0.811
	Serendipita -	20	26.250	27.000	3.596	0.8042

Group Descriptives

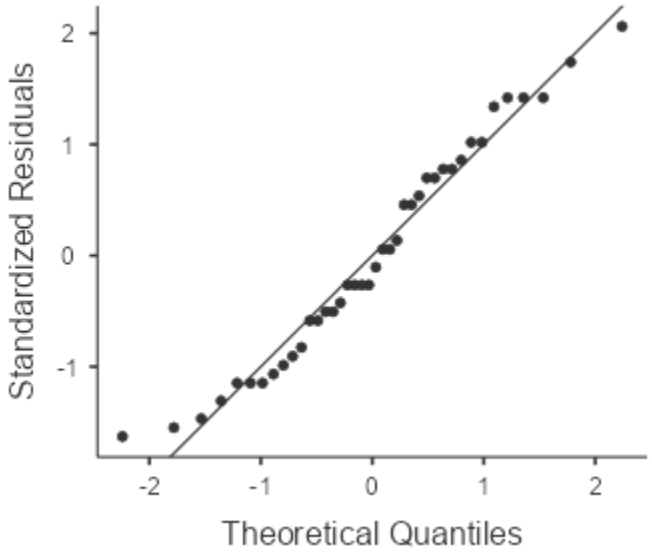
	Group	N	Mean	Median	SD	SE
Shoot Length (cm)	Serendipita +	20	12.57	12.000	3.757	0.840
	Serendipita -	20	10.825	10.250	2.413	0.5396
Root Length (cm)	Serendipita +	20	12.18	12.000	3.427	0.766
	Serendipita -	20	15.425	15.750	4.302	0.9620
Fresh Weight (g)	Serendipita +	20	1.08	0.798	0.657	0.147
	Serendipita -	20	0.760	0.728	0.434	0.0971

Plots

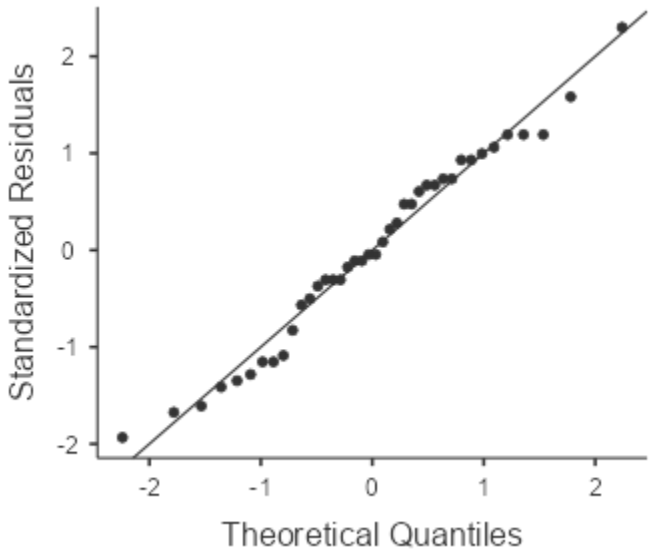
Total Length (cm)



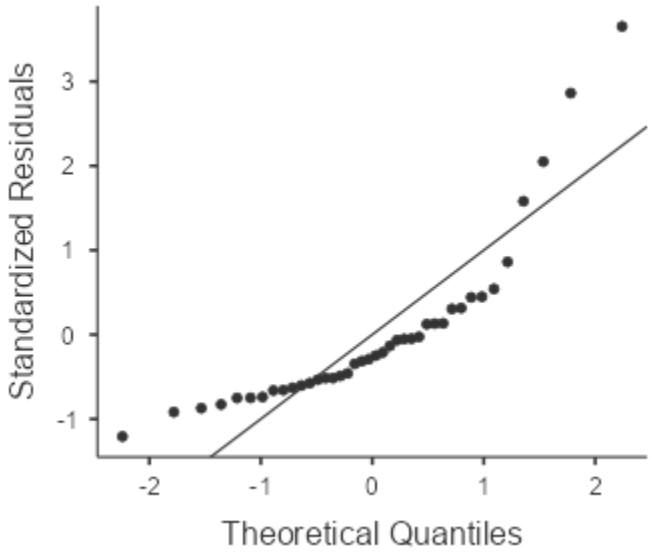
Shoot Length (cm)



Root Length (cm)



Fresh Weight (g)



Descriptives

Descriptives

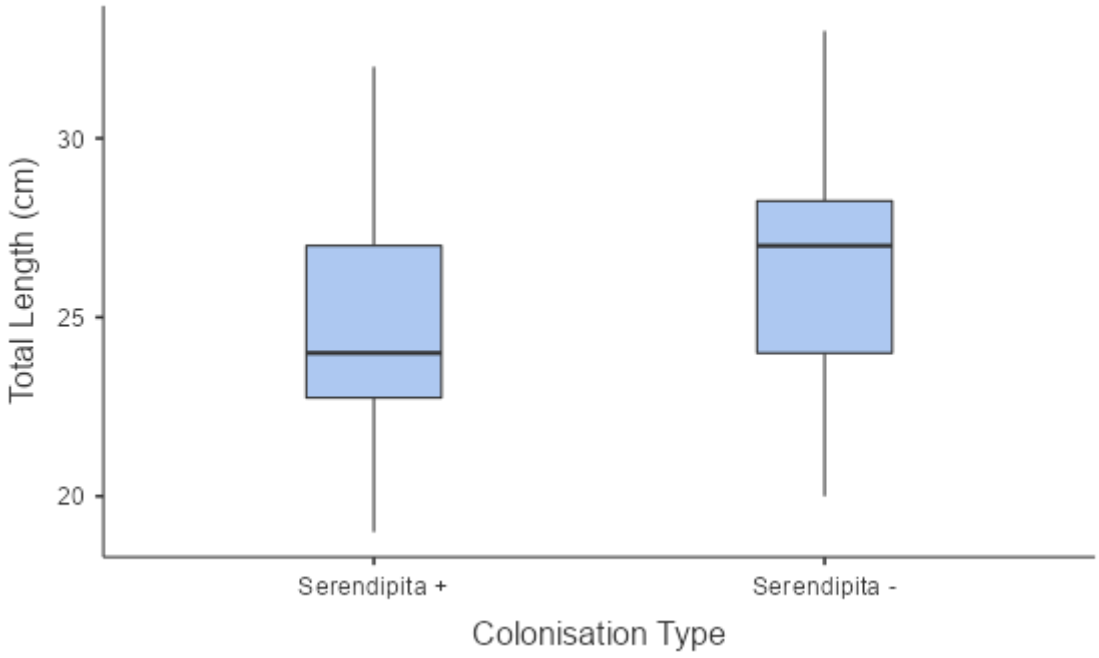
	Colonisation Type	Total Length (cm)	Shoot Length (cm)	Root Length (cm)	Fresh Weight (g)
N	Serendipita +	20	20	20	20
	Serendipita -	20	20	20	20
Missing	Serendipita +	0	0	0	0
	Serendipita -	0	0	0	0
Mean	Serendipita +	24.8	12.6	12.2	1.08
	Serendipita -	26.3	10.8	15.4	0.760
Median	Serendipita +	24.0	12.0	12.0	0.798
	Serendipita -	27.0	10.3	15.8	0.728
Standard deviation	Serendipita +	3.63	3.76	3.43	0.657
	Serendipita -	3.60	2.41	4.30	0.434
Minimum	Serendipita +	19	7.50	6.00	0.416
	Serendipita -	20	6.00	8.00	0.281
Maximum	Serendipita +	32	19.0	21.0	3.09
	Serendipita -	33	15.0	21.5	2.33
Shapiro-Wilk W	Serendipita +	0.962	0.913	0.966	0.772
	Serendipita -	0.955	0.966	0.917	0.736
Shapiro-Wilk p	Serendipita +	0.592	0.074	0.678	< .001

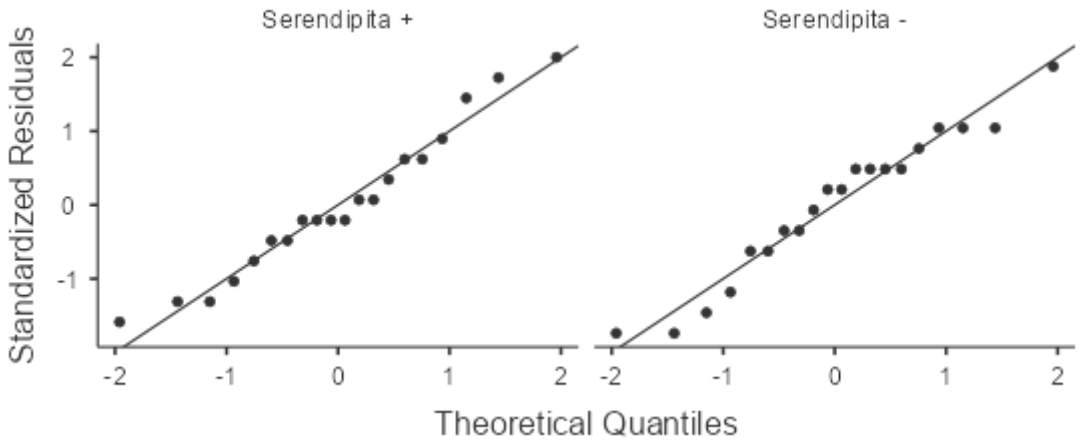
Descriptives

	Colonisation Type	Total Length (cm)	Shoot Length (cm)	Root Length (cm)	Fresh Weight (g)
	Serendipita -	0.448	0.674	0.087	< .001

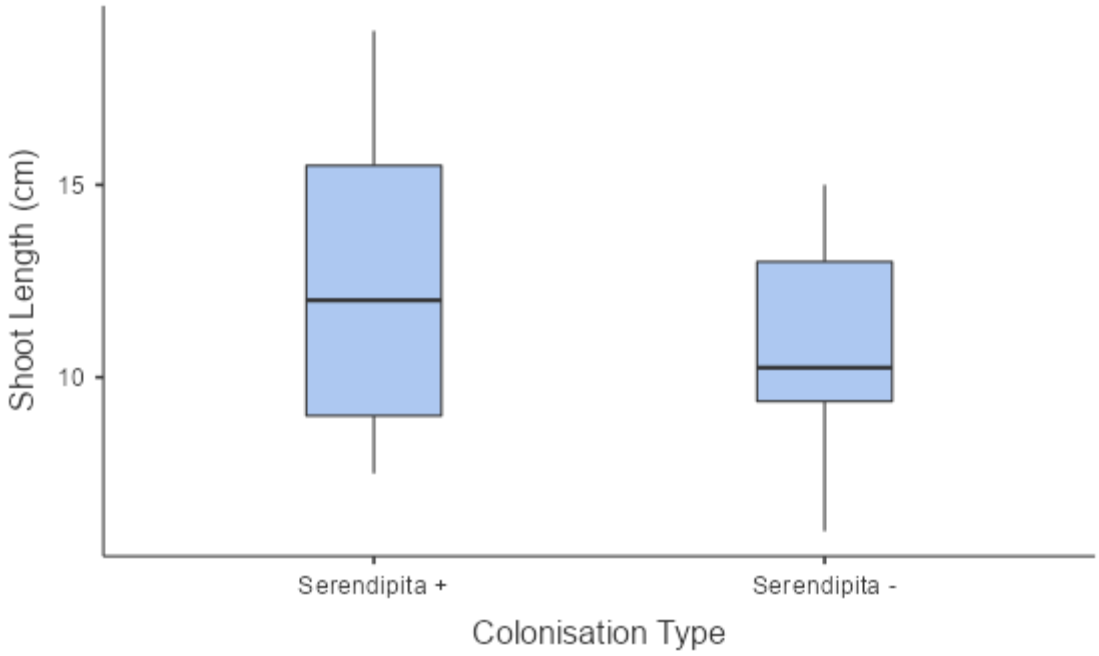
Plots

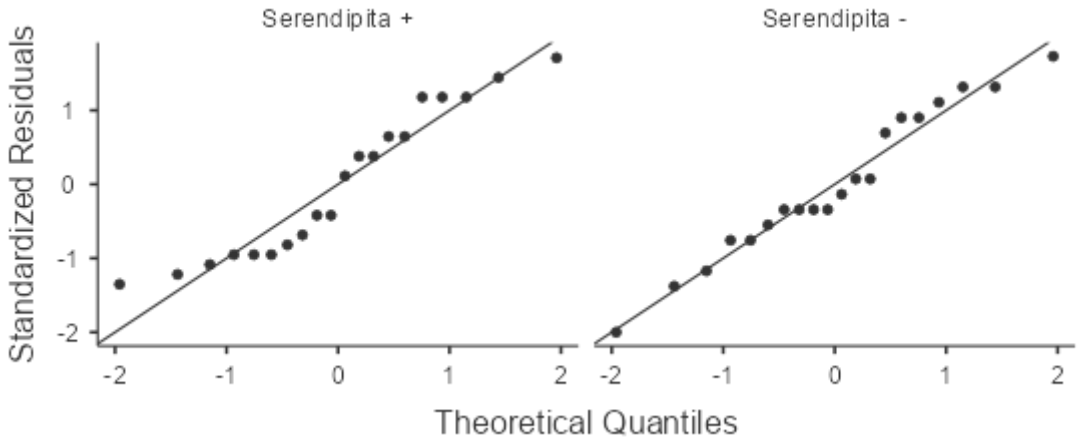
Total Length (cm)



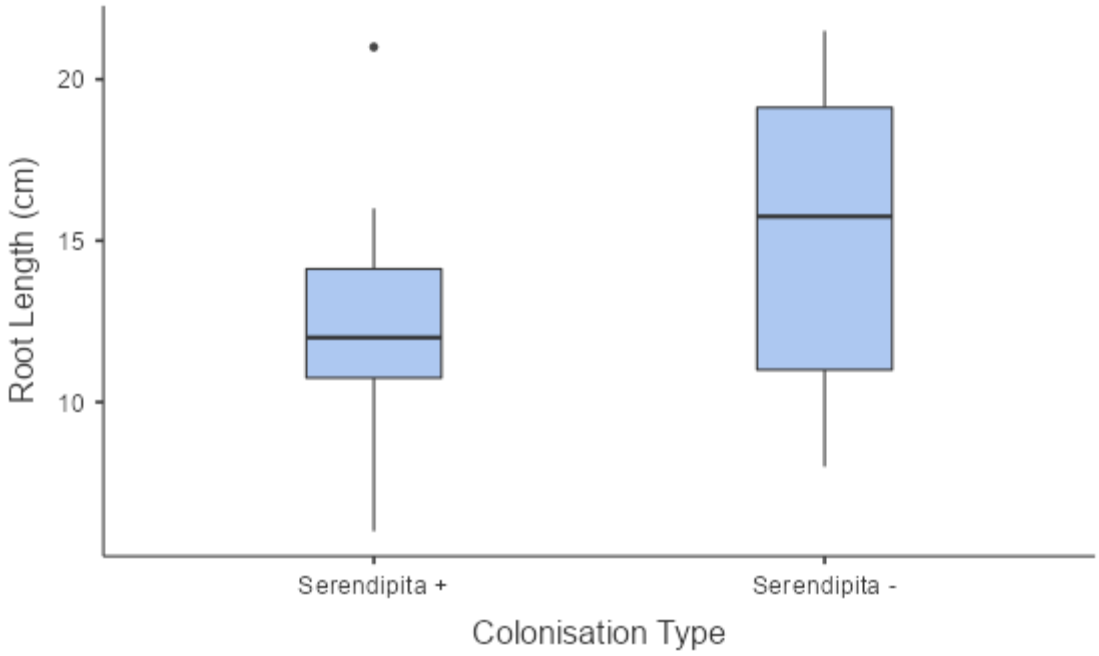


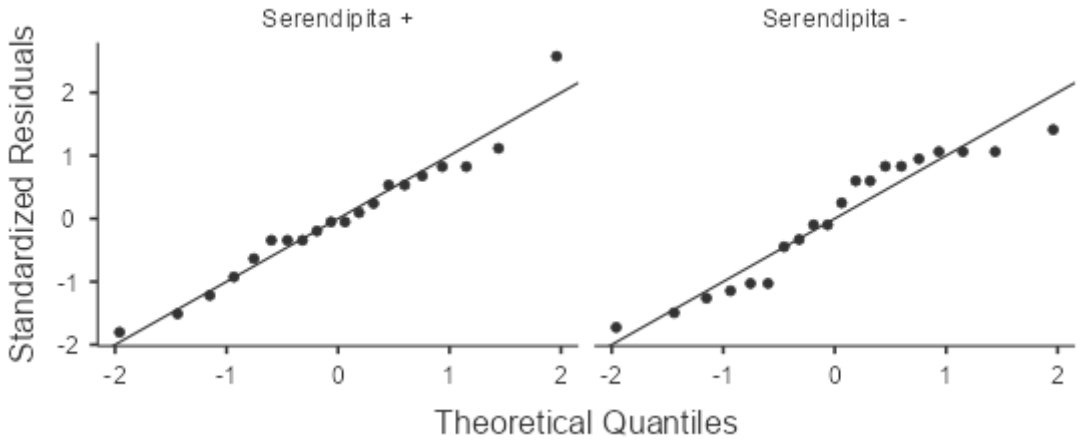
Shoot Length (cm)



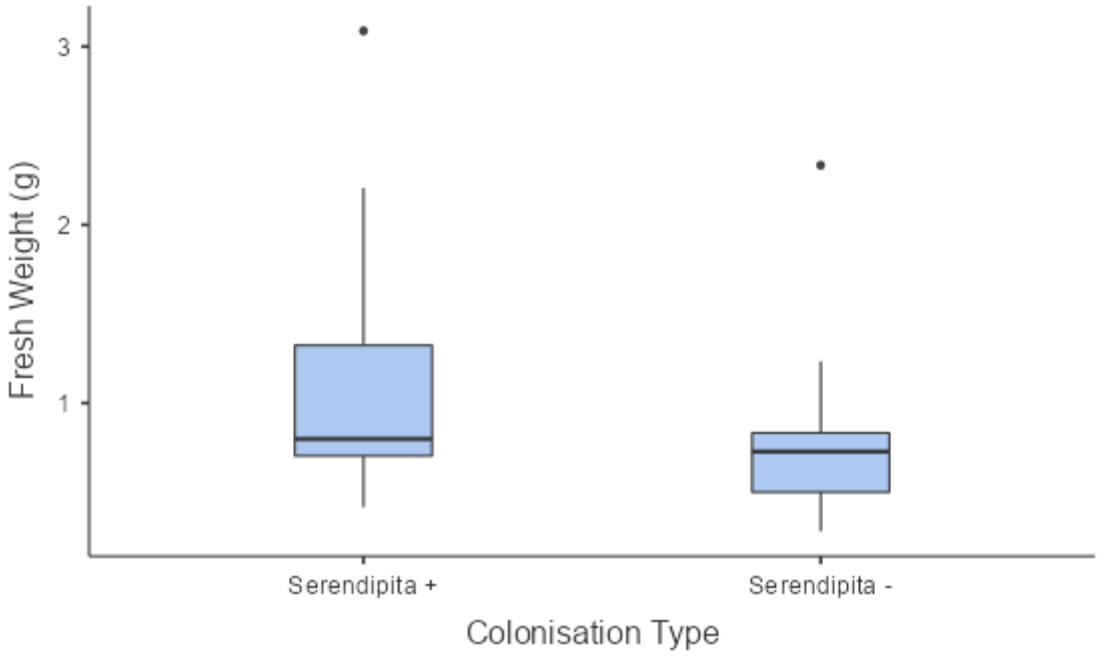


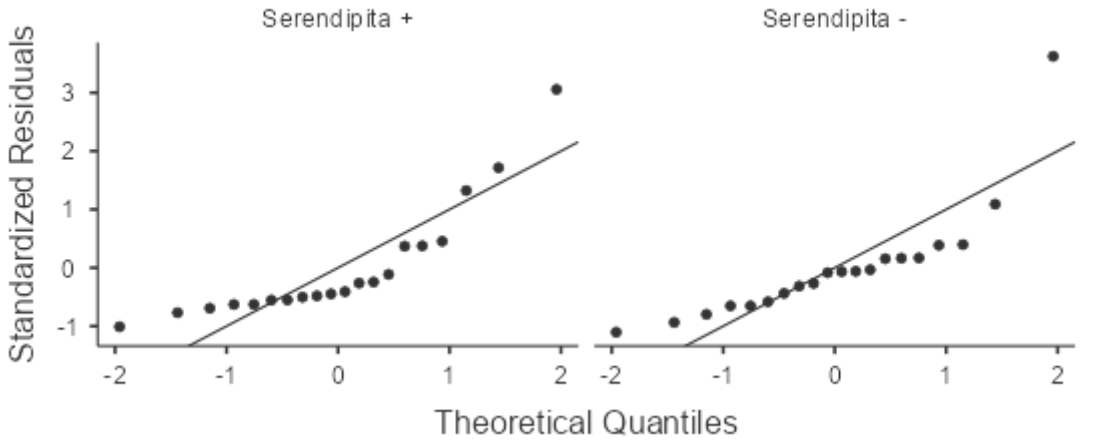
Root Length (cm)





Fresh Weight (g)





Results of growth experiment 2 (1.5 ml)

Independent Samples T-Test

Independent Samples T-Test

		Statistic	df	p
Total Length (cm)	Student's t	-1.0433	38.0	0.303
	Welch's t	-1.0433	36.4	0.304
Shoot Length (cm)	Student's t	-0.7043	38.0	0.486
	Welch's t	-0.7043	37.8	0.486
Root Length (cm)	Student's t	-0.6846	38.0	0.498

Independent Samples T-Test

		Statistic	df	p
Fresh Weight (g)	Welch's t	-0.6846	35.9	0.498
	Student's t	0.0294	38.0	0.977
	Welch's t	0.0294	36.9	0.977

Assumptions

Normality Test (Shapiro-Wilk)

	W	p
Total Length (cm)	0.976	0.553
Shoot Length (cm)	0.963	0.208
Root Length (cm)	0.965	0.245
Fresh Weight (g)	0.973	0.431

Note. A low p-value suggests a violation of the assumption of normality

Homogeneity of Variances Test (Levene's)

	F	df	df2	p
Total Length (cm)	1.149	1	38	0.290
Shoot Length (cm)	0.201	1	38	0.656
Root Length (cm)	0.599	1	38	0.444
Fresh Weight (g)	0.634	1	38	0.431

Note. A low p-value suggests a violation of the assumption of equal variances

Group Descriptives

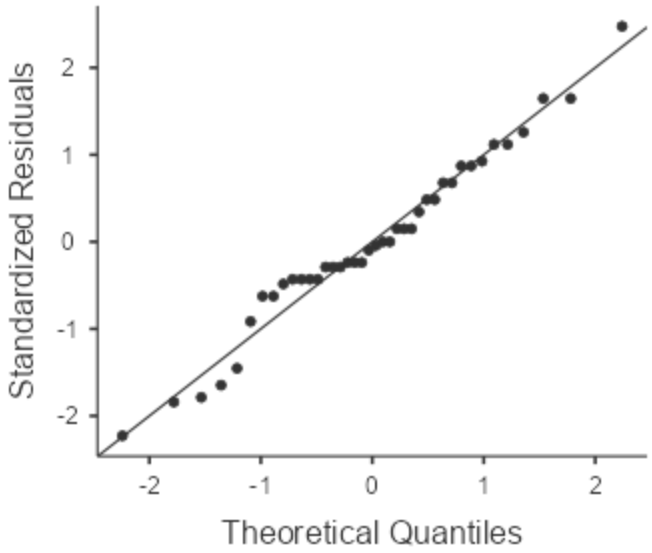
	Group	N	Mean	Median	SD	SE
Total Length (cm)	Serendipita +	20	21.500	21.500	5.751	1.2861
	Serendipita -	20	23.225	22.000	4.647	1.0390
Shoot Length (cm)	Serendipita +	20	9.550	9.000	3.145	0.7033
	Serendipita -	20	10.275	10.500	3.362	0.7517
Root Length (cm)	Serendipita +	20	11.950	11.250	5.145	1.1505
	Serendipita -	20	12.950	12.000	4.026	0.9002

Group Descriptives

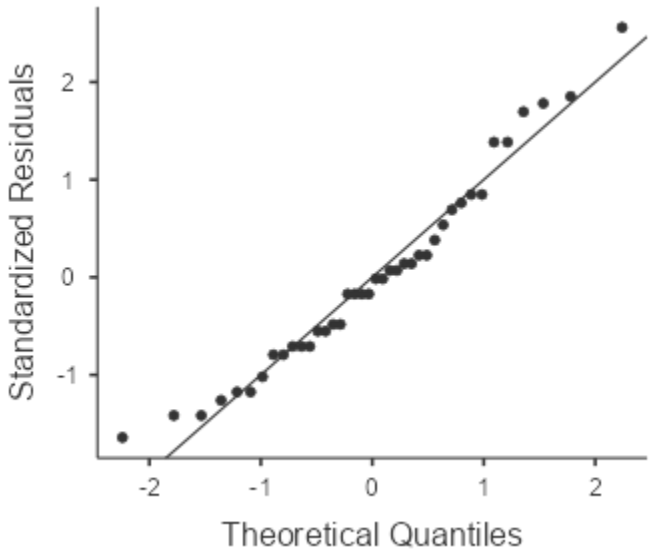
	Group	N	Mean	Median	SD	SE
Fresh Weight (g)	Serendipita +	20	0.602	0.555	0.292	0.0652
	Serendipita -	20	0.600	0.611	0.244	0.0545

Plots

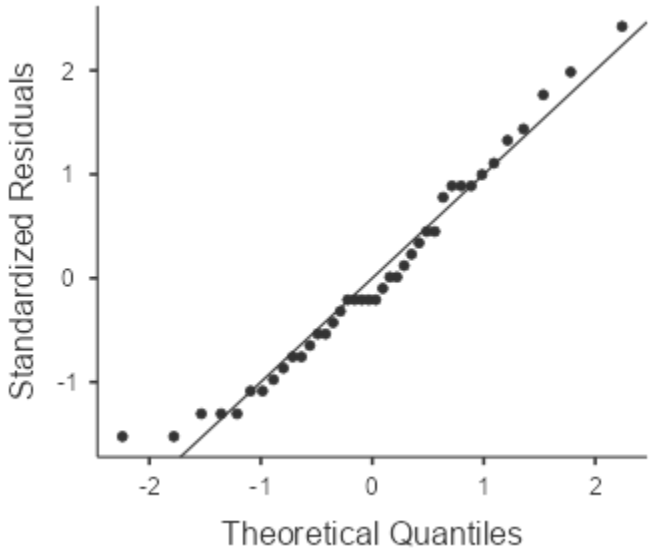
Total Length (cm)



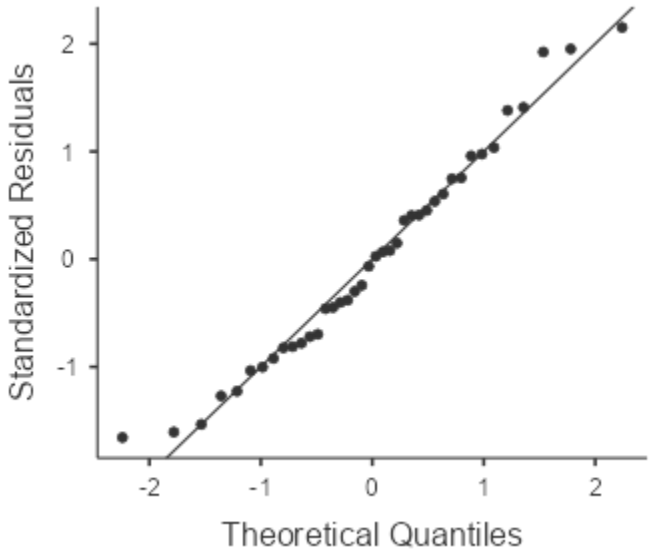
Shoot Length (cm)



Root Length (cm)



Fresh Weight (g)



Descriptives

Descriptives

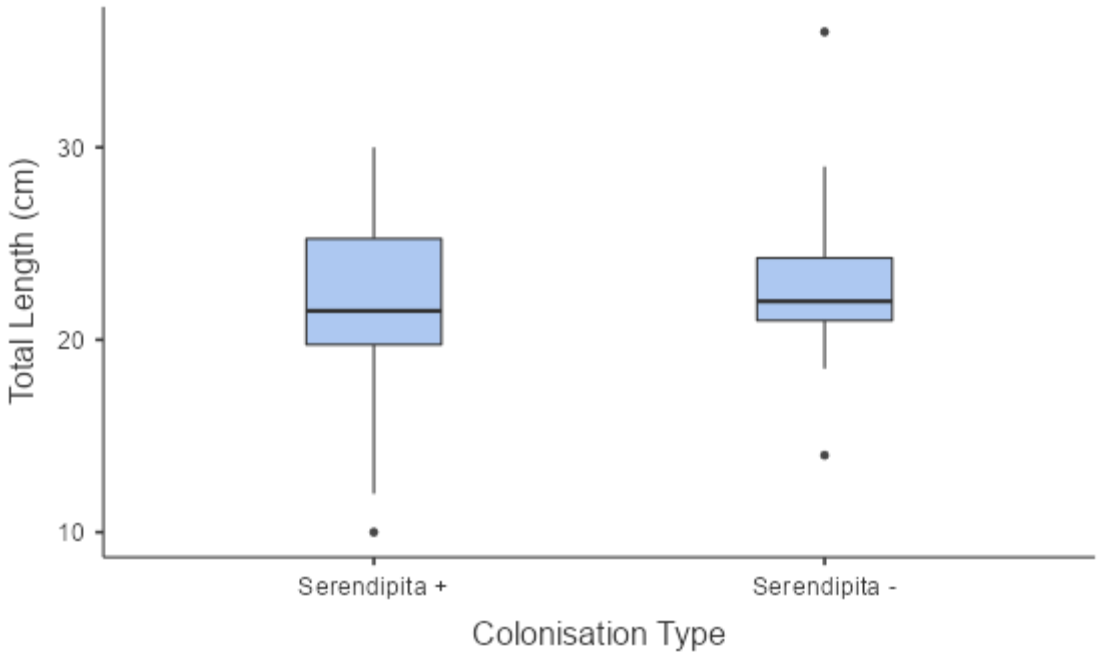
	Colonisation Type	Total Length (cm)	Shoot Length (cm)	Root Length (cm)	Fresh Weight (g)
N	Serendipita +	20	20	20	20
	Serendipita -	20	20	20	20
Missing	Serendipita +	0	0	0	0
	Serendipita -	0	0	0	0
Mean	Serendipita +	21.5	9.55	11.9	0.602
	Serendipita -	23.2	10.3	12.9	0.600

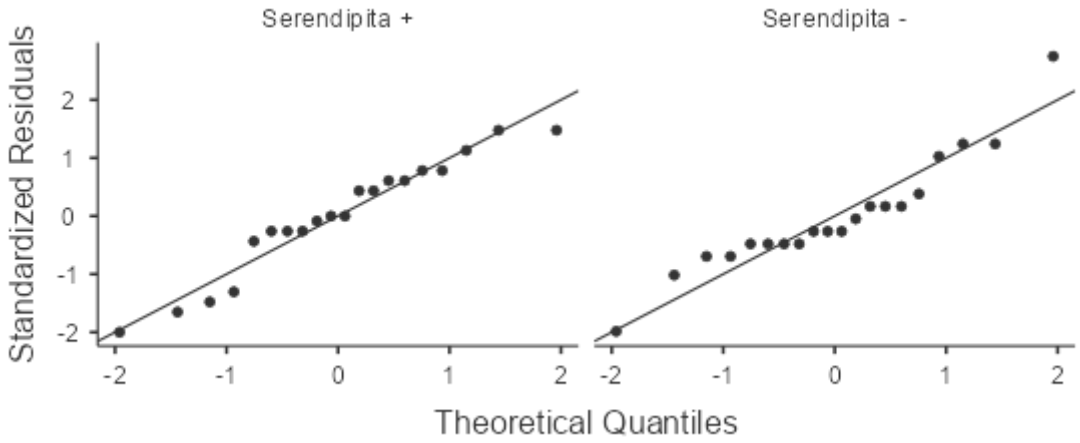
Descriptives

	Colonisation Type	Total Length (cm)	Shoot Length (cm)	Root Length (cm)	Fresh Weight (g)
Median	Serendipita +	21.5	9.00	11.3	0.555
	Serendipita -	22.0	10.5	12.0	0.611
Standard deviation	Serendipita +	5.75	3.15	5.15	0.292
	Serendipita -	4.65	3.36	4.03	0.244
Minimum	Serendipita +	10.0	5.00	5.00	0.176
	Serendipita -	14.0	5.00	7.00	0.160
Maximum	Serendipita +	30.0	15.5	23.0	1.17
	Serendipita -	36.0	18.5	21.0	1.11
Shapiro-Wilk W	Serendipita +	0.944	0.926	0.949	0.956
	Serendipita -	0.917	0.951	0.957	0.984
Shapiro-Wilk p	Serendipita +	0.288	0.127	0.355	0.474
	Serendipita -	0.087	0.384	0.479	0.975

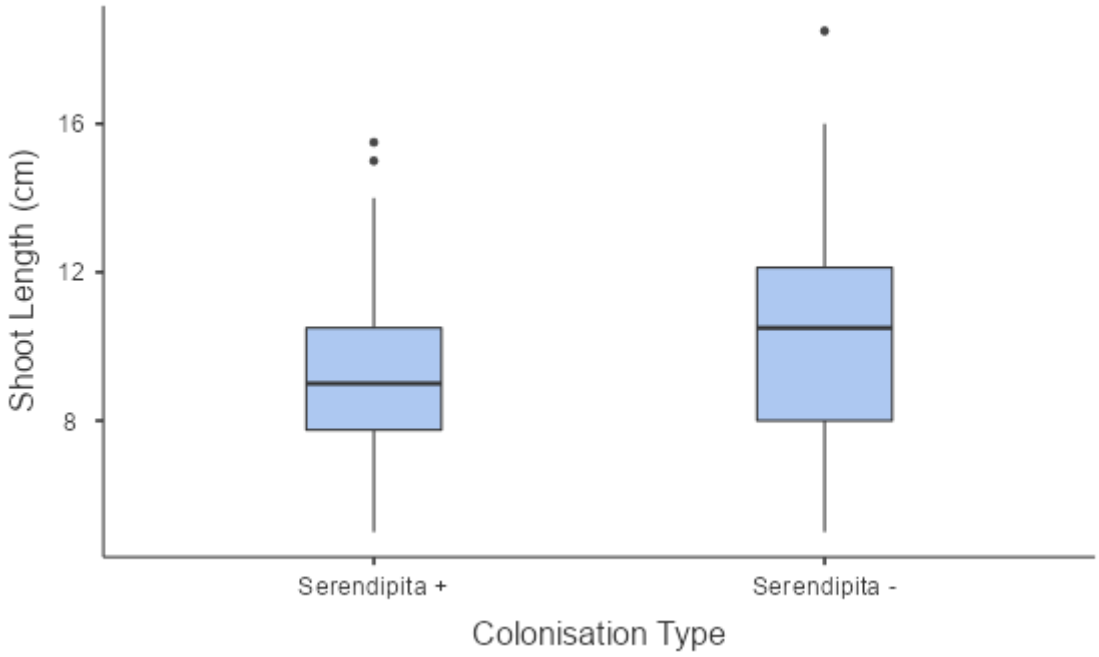
Plots

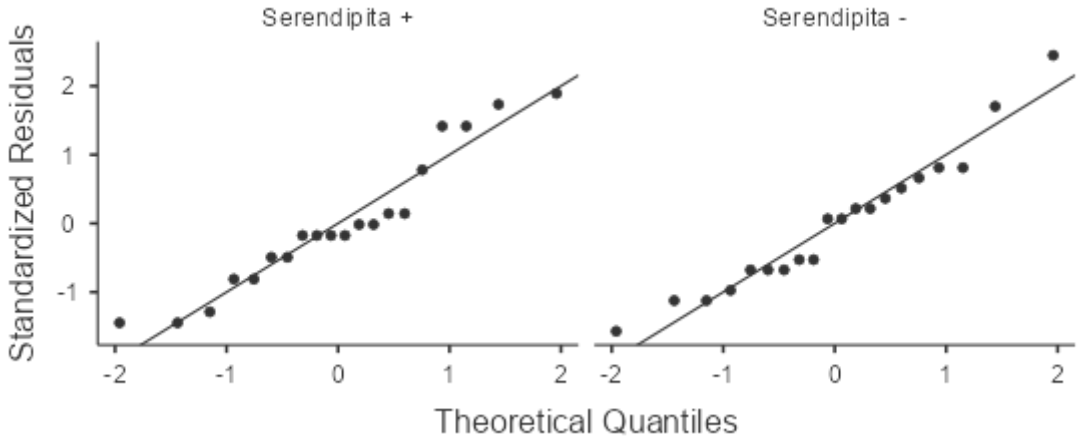
Total Length (cm)



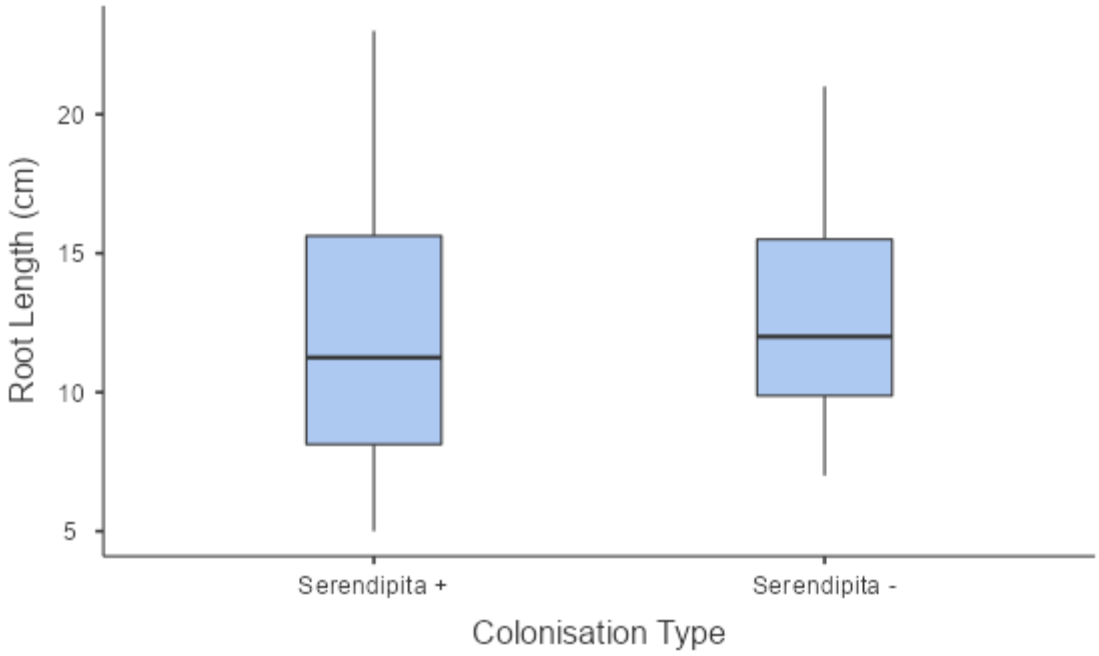


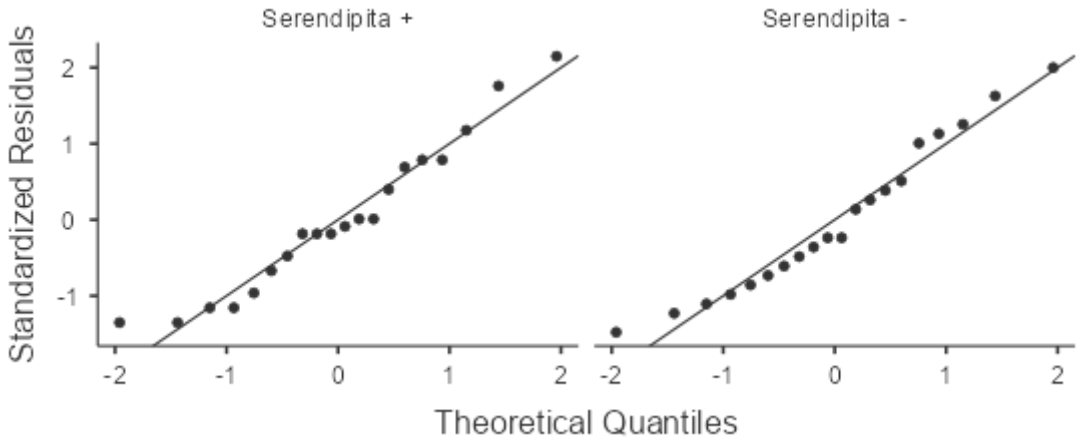
Shoot Length (cm)



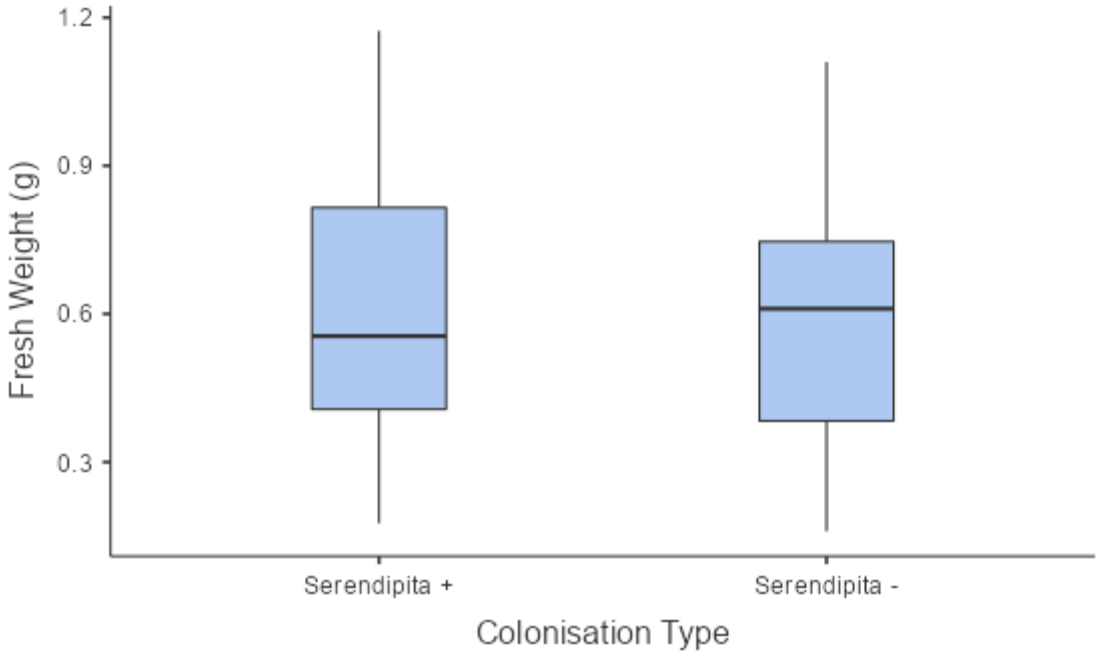


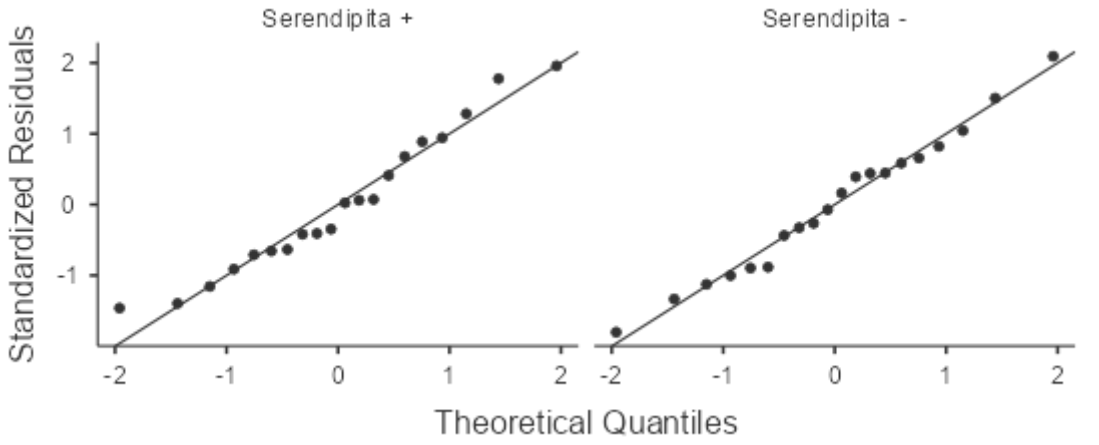
Root Length (cm)





Fresh Weight (g)





Results of growth experiment 3 (1.5 ml)

Independent Samples T-Test

Independent Samples T-Test

		Statistic	df	p
Total Length (cm)	Student's t	-2.35	38.0	0.024
	Welch's t	-2.35	38.0	0.024
Shoot Length (cm)	Student's t	3.90	38.0	< .001
	Welch's t	3.90	32.8	< .001
Root Length (cm)	Student's t	-3.99	38.0	< .001

Independent Samples T-Test

		Statistic	df	p
Fresh Weight (g)	Welch's t	-3.99	36.4	< .001
	Student's t	3.65	38.0	< .001
	Welch's t	3.65	37.9	< .001

Assumptions

Normality Test (Shapiro-Wilk)

	W	p
Total Length (cm)	0.978	0.624
Shoot Length (cm)	0.951	0.083
Root Length (cm)	0.987	0.925
Fresh Weight (g)	0.949	0.072

Note. A low p-value suggests a violation of the assumption of normality

Homogeneity of Variances Test (Levene's)

	F	df	df2	p
Total Length (cm)	0.0152	1	38	0.903
Shoot Length (cm)	3.7880	1	38	0.059
Root Length (cm)	0.3397	1	38	0.563
Fresh Weight (g)	0.4936	1	38	0.487

Note. A low p-value suggests a violation of the assumption of equal variances

Group Descriptives

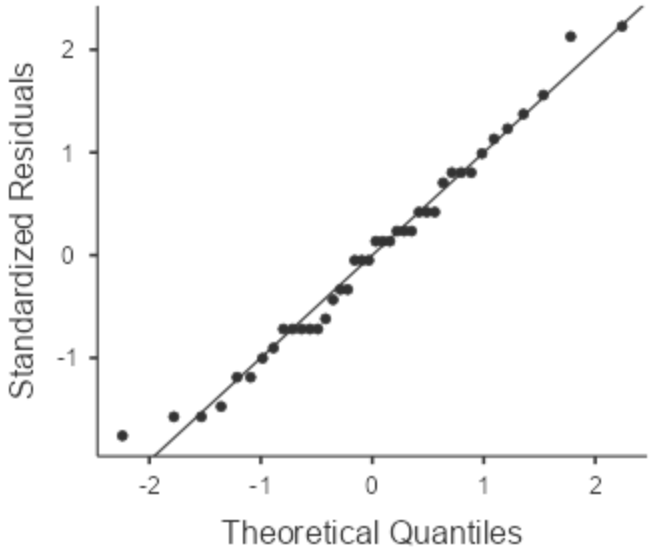
	Group	N	Mean	Median	SD	SE
Total Length (cm)	Serendipita +	20	19.525	20.000	3.515	0.7860
	Serendipita -	20	22.175	22.000	3.603	0.8056
Shoot Length (cm)	Serendipita +	20	9.350	9.000	2.231	0.4988
	Serendipita -	20	7.025	7.000	1.464	0.3274
Root Length (cm)	Serendipita +	20	10.175	9.000	3.503	0.7833
	Serendipita -	20	15.150	15.750	4.347	0.9720

Group Descriptives

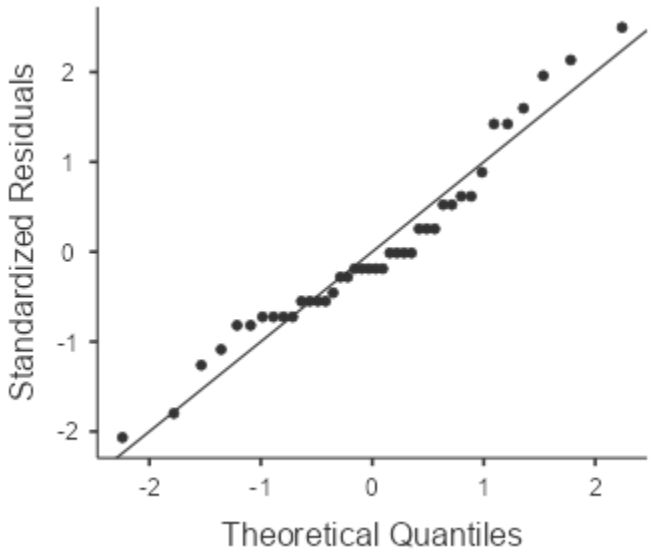
	Group	N	Mean	Median	SD	SE
Fresh Weight (g)	Serendipita +	20	0.504	0.516	0.115	0.0258
	Serendipita -	20	0.368	0.370	0.120	0.0268

Plots

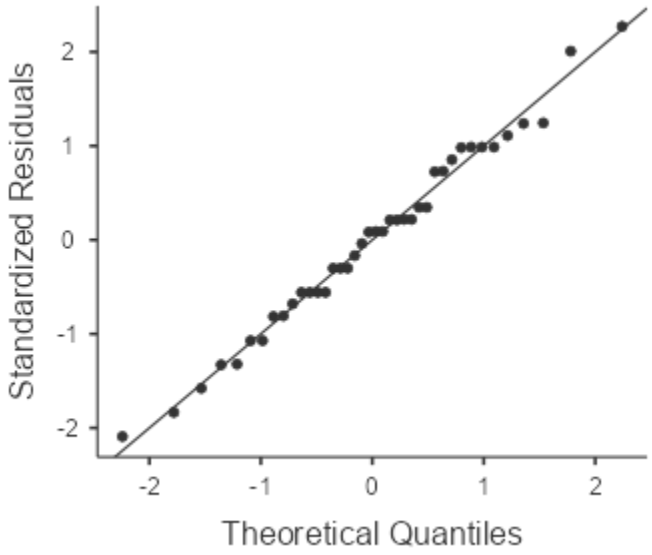
Total Length (cm)



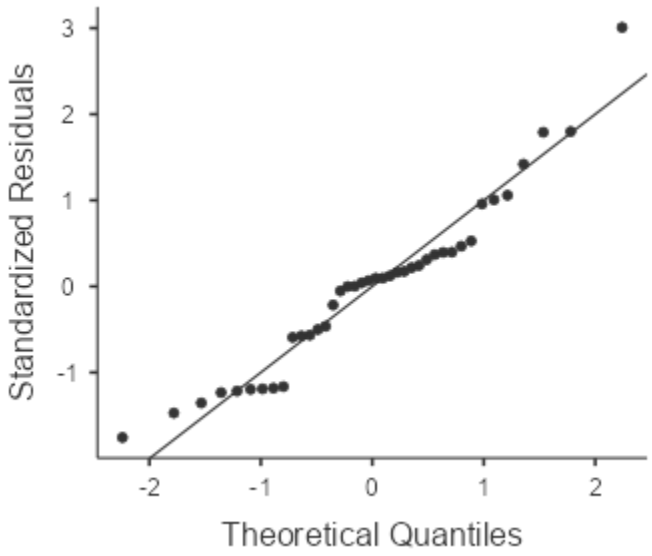
Shoot Length (cm)



Root Length (cm)



Fresh Weight (g)



Descriptives

Descriptives

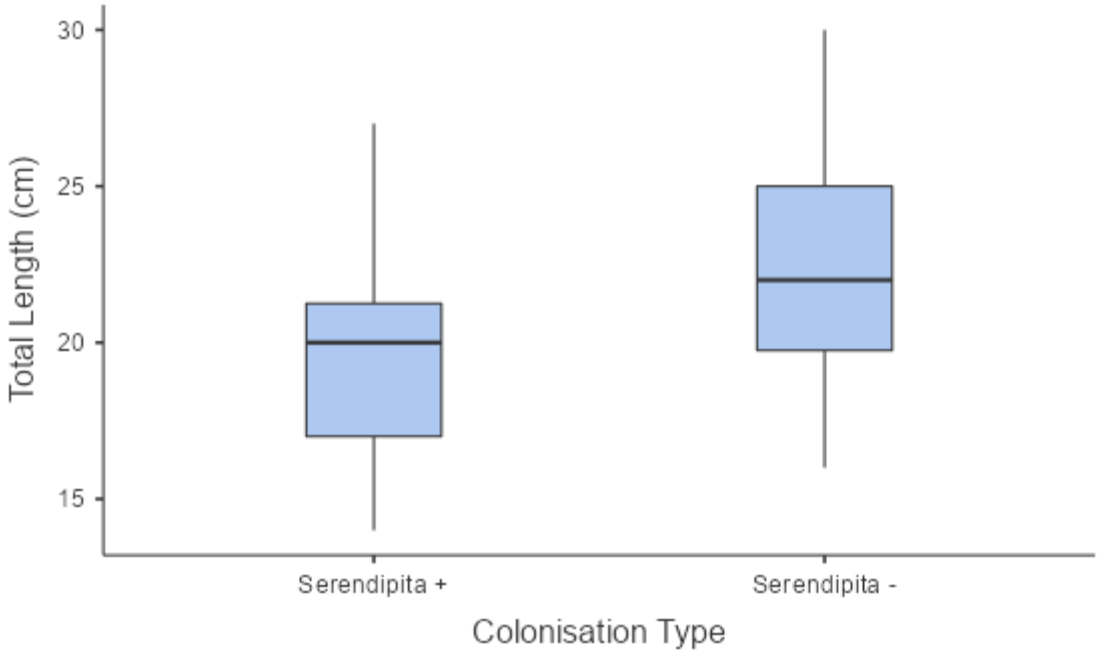
	Colonisation Type	Total Length (cm)	Shoot Length (cm)	Root Length (cm)	Fresh Weight (g)
N	Serendipita +	20	20	20	20
	Serendipita -	20	20	20	20
Missing	Serendipita +	0	0	0	0
	Serendipita -	0	0	0	0
Mean	Serendipita +	19.5	9.35	10.2	0.504
	Serendipita -	22.2	7.03	15.2	0.368

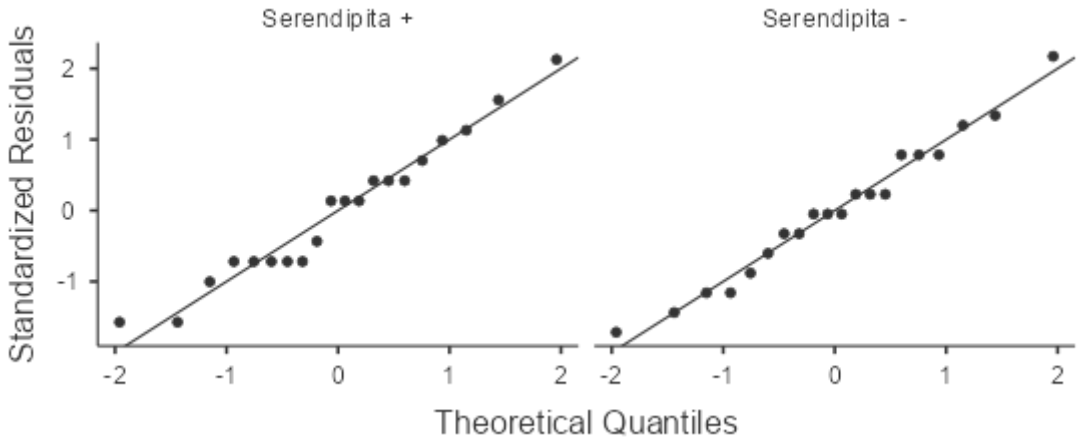
Descriptives

	Colonisation Type	Total Length (cm)	Shoot Length (cm)	Root Length (cm)	Fresh Weight (g)
Median	Serendipita +	20.0	9.00	9.00	0.516
	Serendipita -	22.0	7.00	15.8	0.370
Standard deviation	Serendipita +	3.51	2.23	3.50	0.115
	Serendipita -	3.60	1.46	4.35	0.120
Minimum	Serendipita +	14.0	5.50	5.00	0.300
	Serendipita -	16.0	5.00	7.00	0.211
Maximum	Serendipita +	27.0	14.0	18.0	0.853
	Serendipita -	30.0	11.0	24.0	0.577
Shapiro-Wilk W	Serendipita +	0.960	0.958	0.947	0.882
	Serendipita -	0.980	0.886	0.963	0.917
Shapiro-Wilk p	Serendipita +	0.549	0.502	0.322	0.019
	Serendipita -	0.928	0.022	0.601	0.087

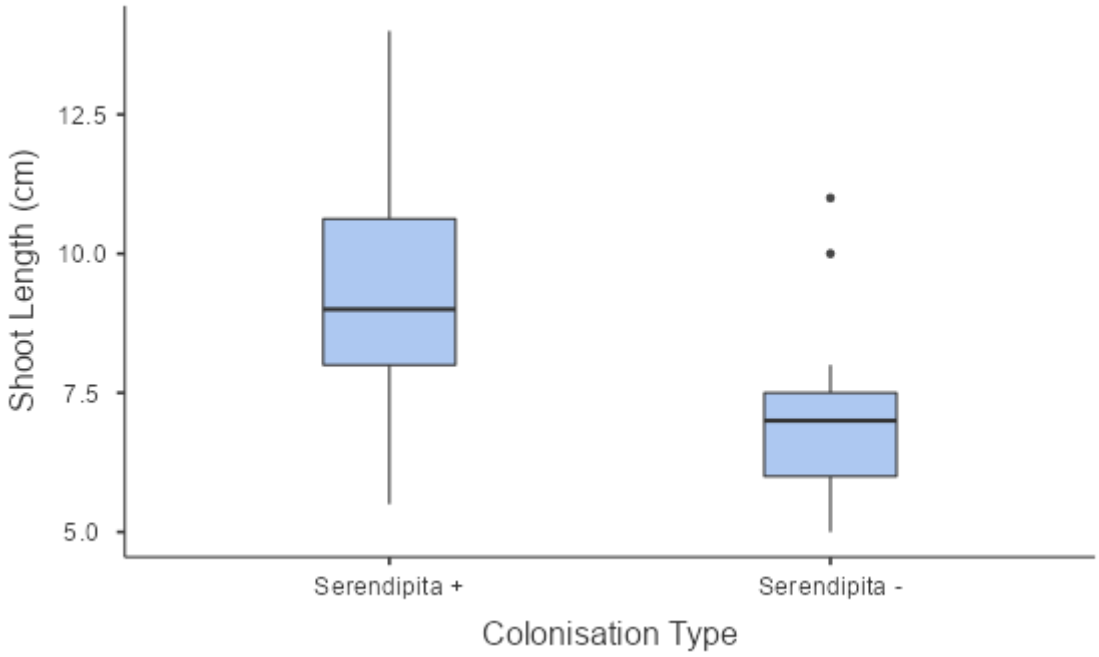
Plots

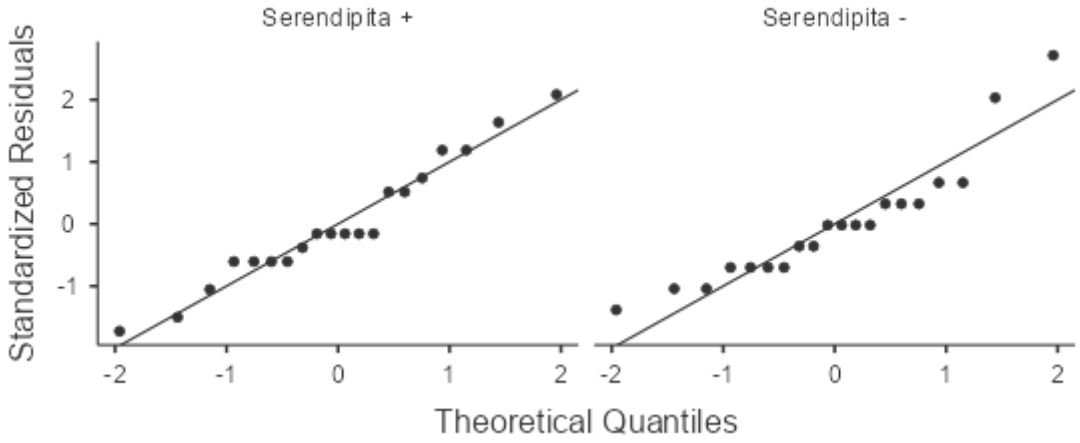
Total Length (cm)



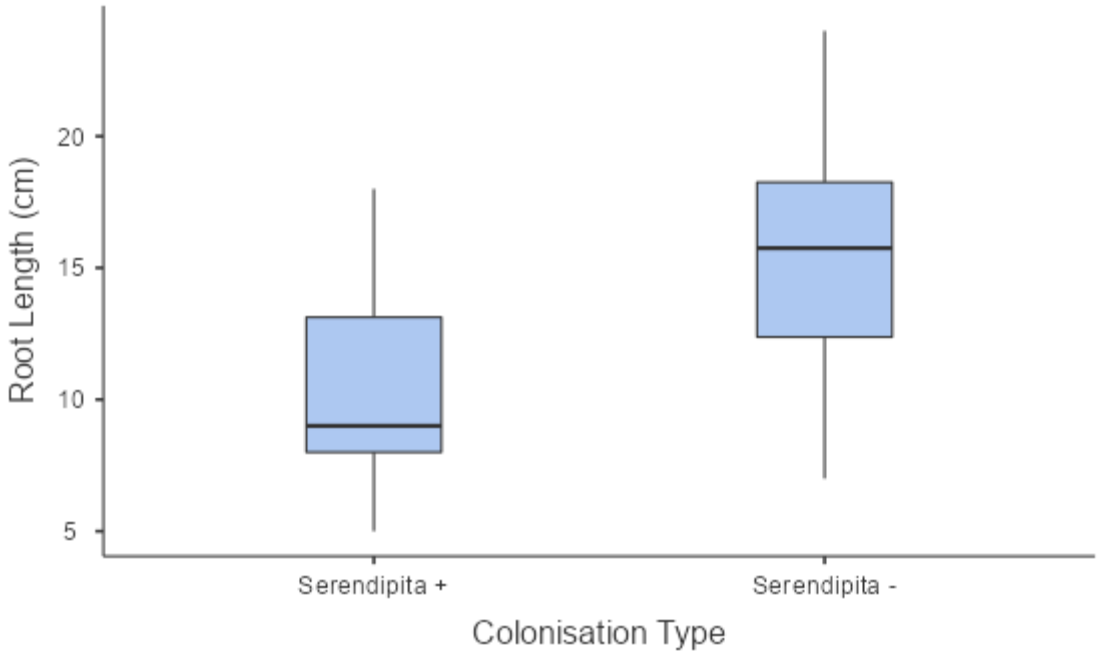


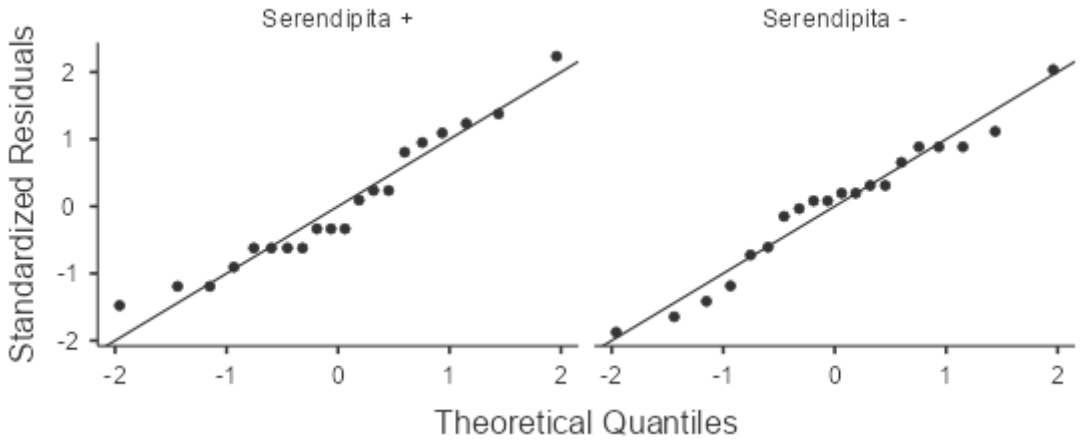
Shoot Length (cm)



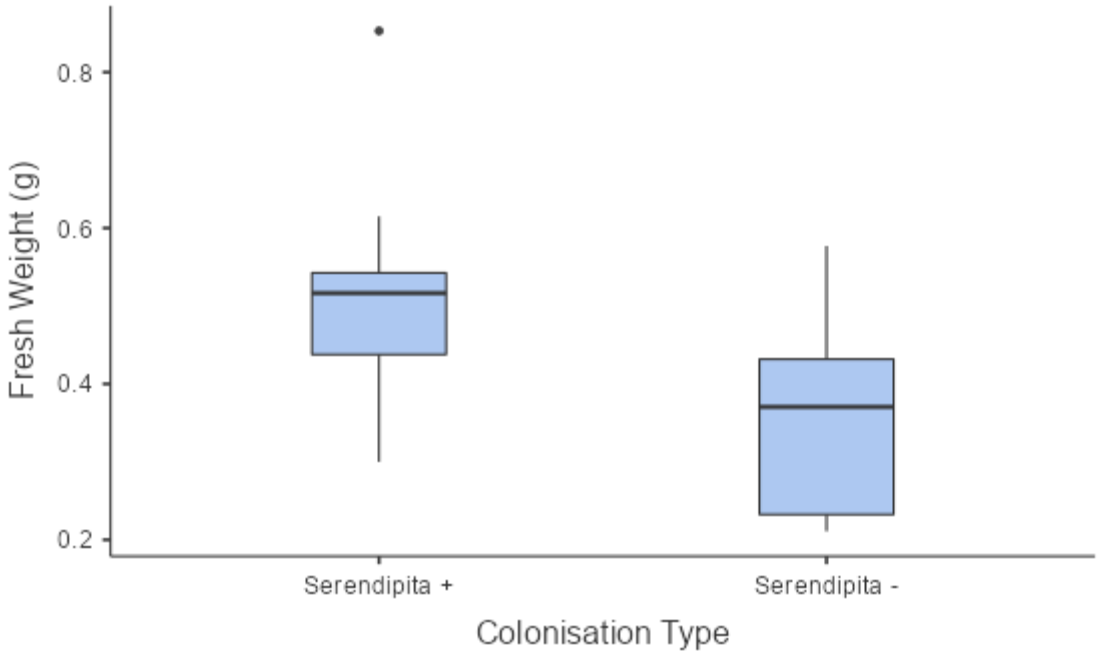


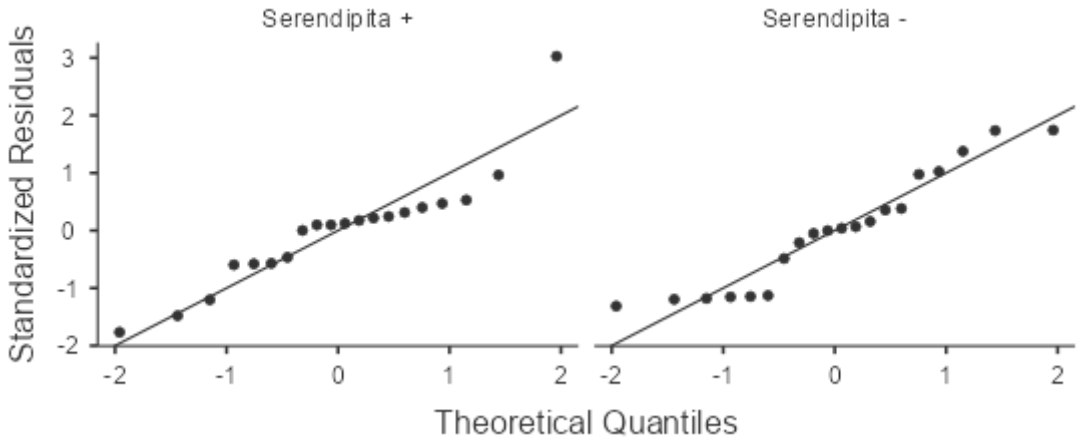
Root Length (cm)





Fresh Weight (g)





Appendix VI

Appendix VI: statistical analysis of bioprotection experiment variables between *Ser*+/PM+ and *Ser*-/PM+ treatments (1.5 ml inoculum) in the three experiments using jamovi 2.2.5 statistical software.

Results of bioprotection experiment 1

Independent Samples T-Test

Independent Samples T-Test

		Statistic	df	p
Total Length (cm)	Student's t	1.099	38.0	0.279
	Welch's t	1.099	38.0	0.279
Shoot Length (cm)	Student's t	1.529 [a]	38.0	0.134
	Welch's t	1.529	30.7	0.136
Root Length (cm)	Student's t	0.115	38.0	0.909
	Welch's t	0.115	37.8	0.909
Fresh Weight (g)	Student's t	4.147	38.0	< .001
	Welch's t	4.147	37.0	< .001

[a] Levene's test is significant (p < .05), suggesting a violation of the assumption of equal variances

Assumptions

Normality Test (Shapiro-Wilk)

	W	p
Total Length (cm)	0.948	0.066
Shoot Length (cm)	0.988	0.933
Root Length (cm)	0.951	0.080
Fresh Weight (g)	0.977	0.580

Note. A low p-value suggests a violation of the assumption of normality

Homogeneity of Variances Test (Levene's)

	F	df	df2	p
Total Length (cm)	0.5258	1	38	0.473
Shoot Length (cm)	4.4991	1	38	0.040

Homogeneity of Variances Test (Levene's)

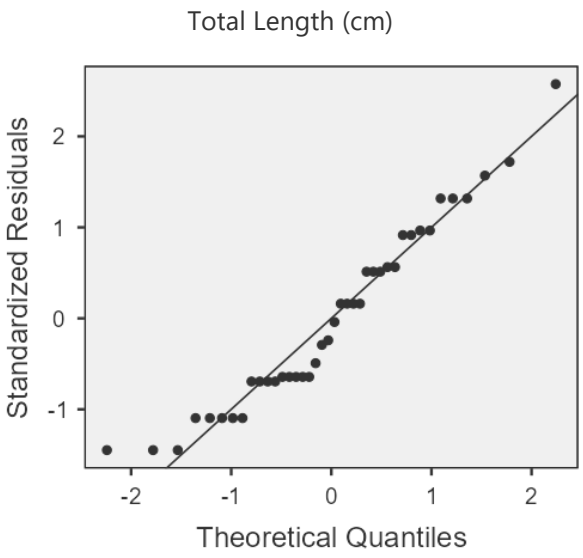
	F	df	df2	p
Root Length (cm)	0.0474	1	38	0.829
Fresh Weight (g)	0.6188	1	38	0.436

Note. A low p-value suggests a violation of the assumption of equal variances

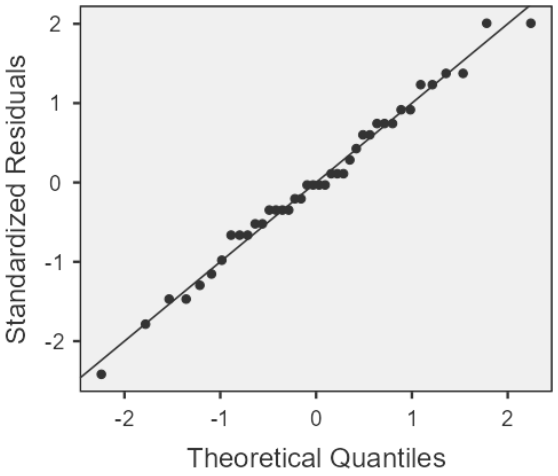
Group Descriptives

	Group	N	Mean	Median	SD	SE
Total Length (cm)	Serendipita +/ PM+	20	23.60	23.75	2.558	0.5719
	Serendipita -/ PM+	20	22.725	21.750	2.479	0.5543
Shoot Length (cm)	Serendipita +/ PM+	20	11.82	12.00	1.955	0.4372
	Serendipita -/ PM+	20	11.050	11.000	1.146	0.2562
Root Length (cm)	Serendipita +/ PM+	20	11.78	11.00	2.840	0.6350
	Serendipita -/ PM+	20	11.675	11.250	2.657	0.5941
Fresh Weight (g)	Serendipita +/ PM+	20	1.03	1.11	0.236	0.0527
	Serendipita -/ PM+	20	0.742	0.719	0.200	0.0446

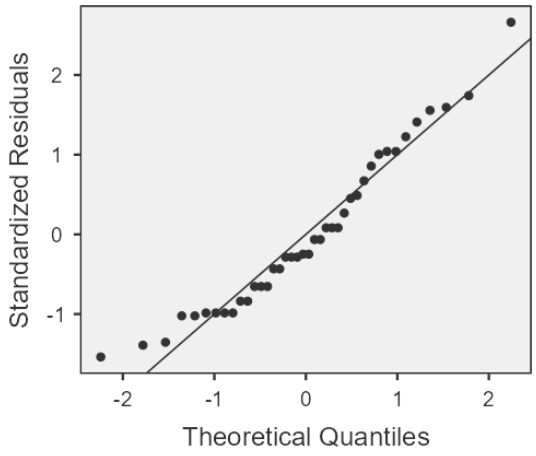
Plots



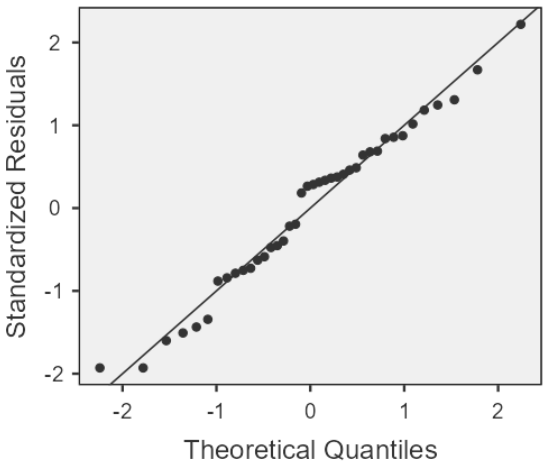
Shoot Length (cm)



Root Length (cm)



Fresh Weight (g)



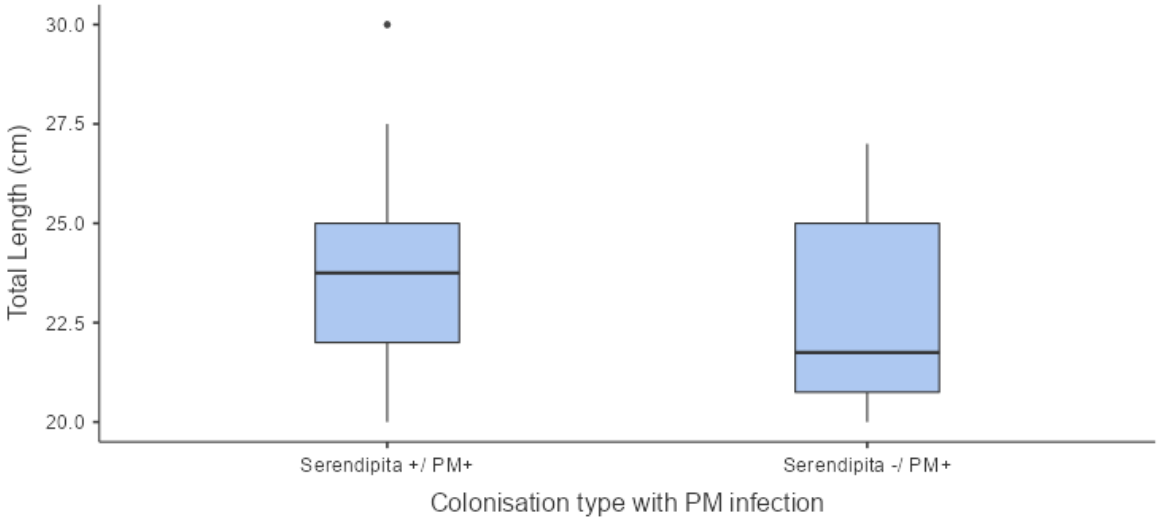
Descriptives

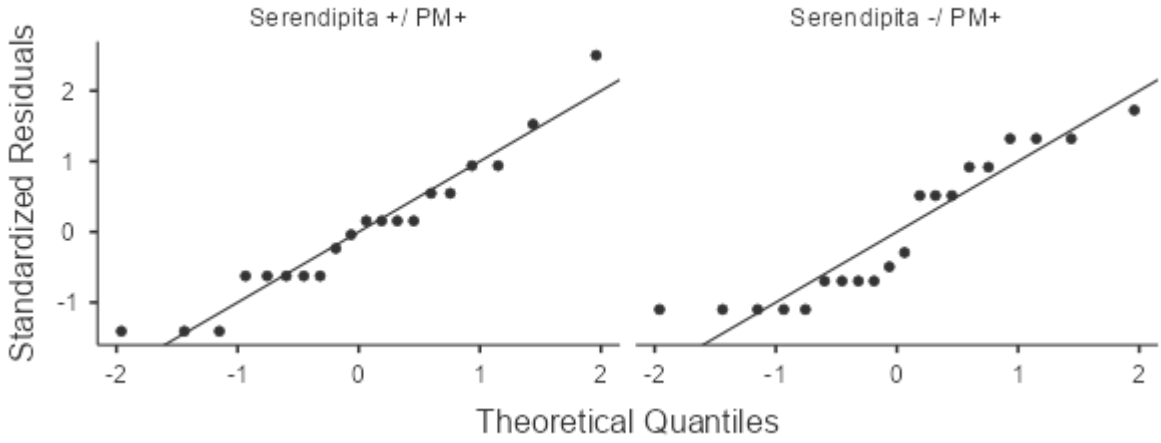
Descriptives

	Colonisation type with PM infection	Total Length (cm)	Shoot Length (cm)	Root Length (cm)	Fresh Weight (g)
N	Serendipita +/ PM+	20	20	20	20
	Serendipita -/ PM+	20	20	20	20
Missing	Serendipita +/ PM+	0	0	0	0
	Serendipita -/ PM+	0	0	0	0
Mean	Serendipita +/ PM+	23.6	11.8	11.8	1.03
	Serendipita -/ PM+	22.7	11.1	11.7	0.742
Median	Serendipita +/ PM+	23.8	12.0	11.0	1.11
	Serendipita -/ PM+	21.8	11.0	11.3	0.719
Standard deviation	Serendipita +/ PM+	2.56	1.96	2.84	0.236
	Serendipita -/ PM+	2.48	1.15	2.66	0.200
Minimum	Serendipita +/ PM+	20.0	8.00	8.00	0.612
	Serendipita -/ PM+	20.0	9.00	7.50	0.432
Maximum	Serendipita +/ PM+	30.0	15.0	19.0	1.39
	Serendipita -/ PM+	27.0	13.0	16.0	1.22
Shapiro-Wilk W	Serendipita +/ PM+	0.941	0.971	0.903	0.925
	Serendipita -/ PM+	0.867	0.953	0.939	0.957
Shapiro-Wilk p	Serendipita +/ PM+	0.251	0.786	0.047	0.124
	Serendipita -/ PM+	0.010	0.410	0.229	0.477

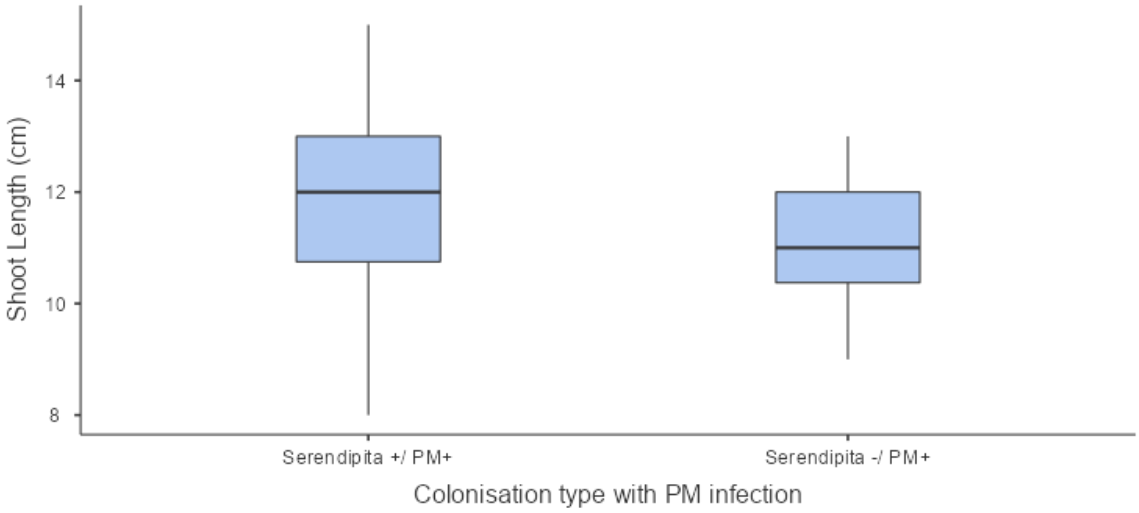
Plots

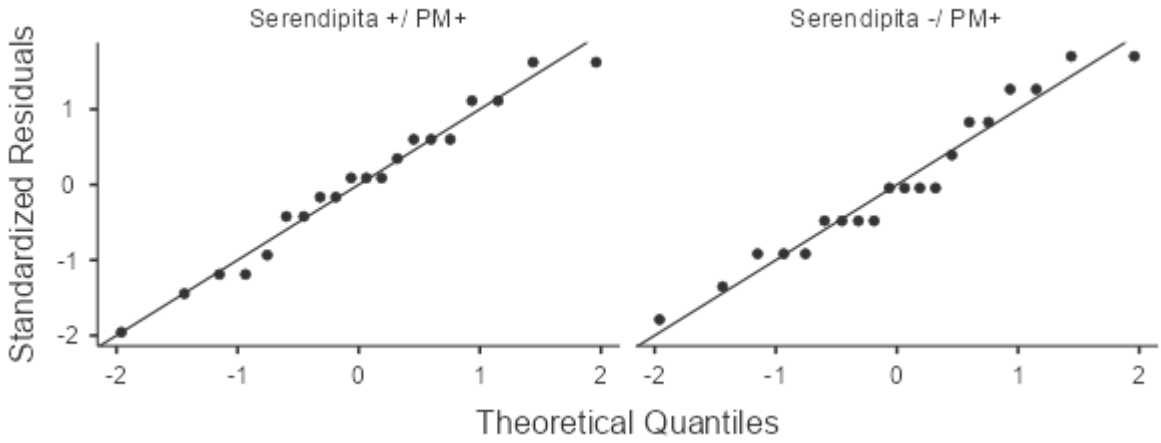
Total Length (cm)



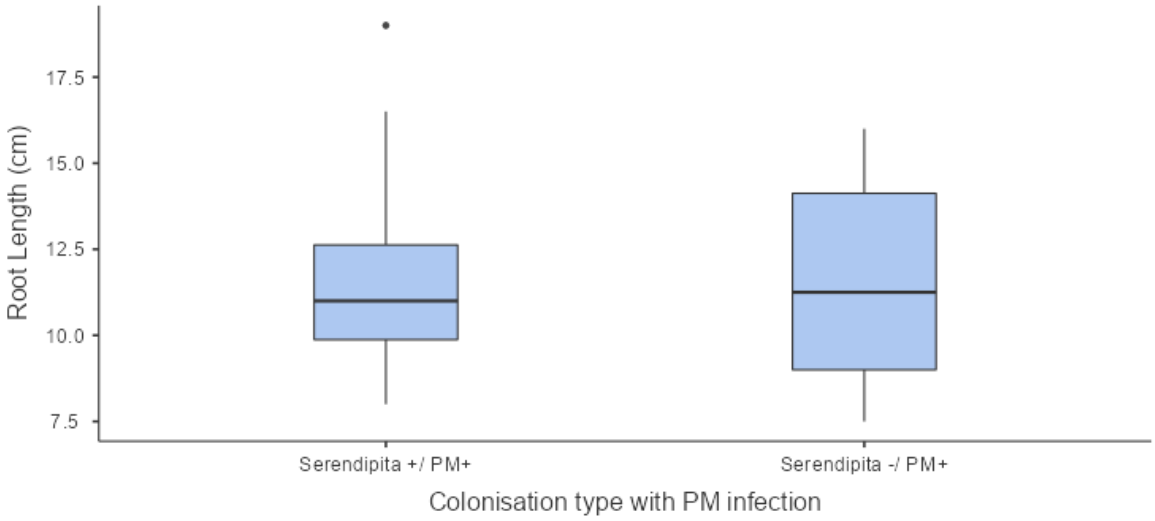


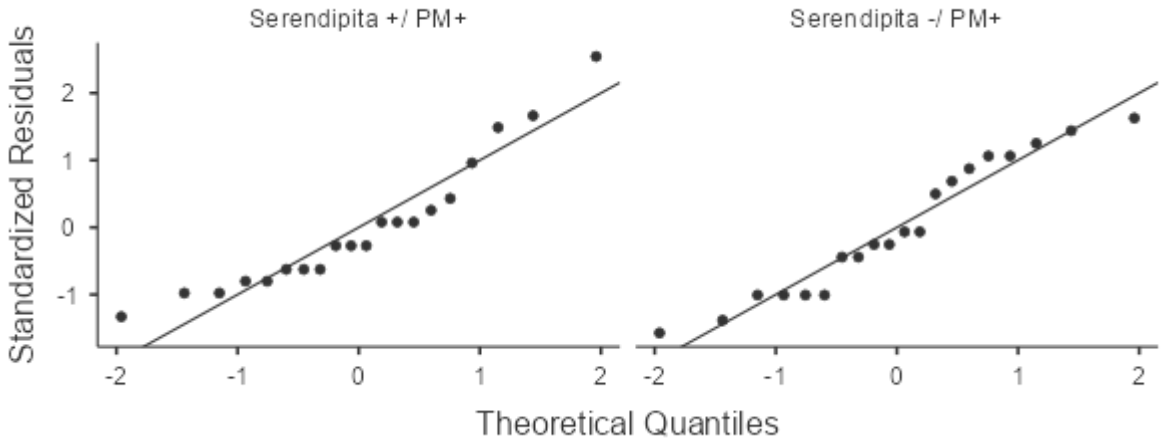
Shoot Length (cm)



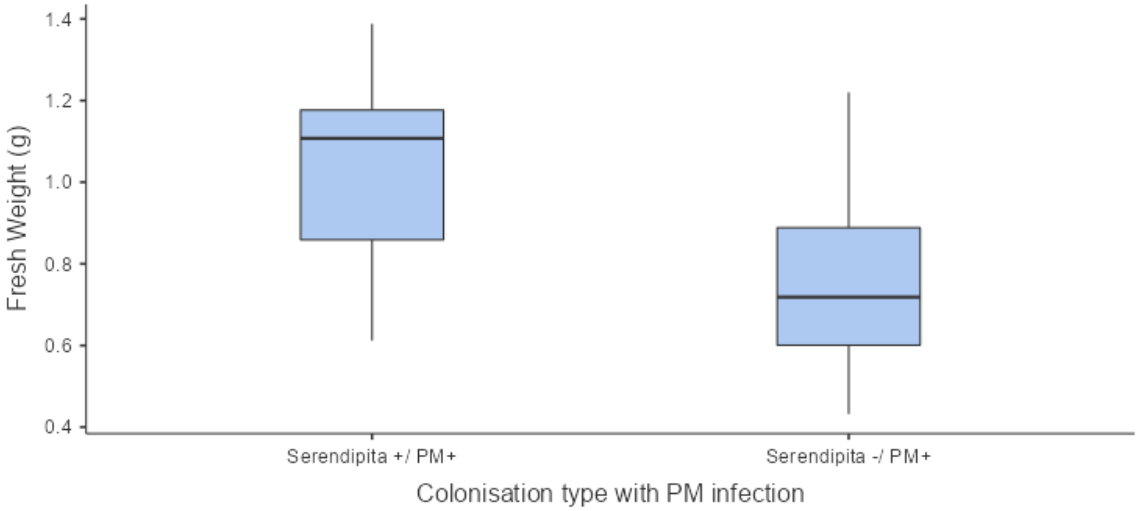


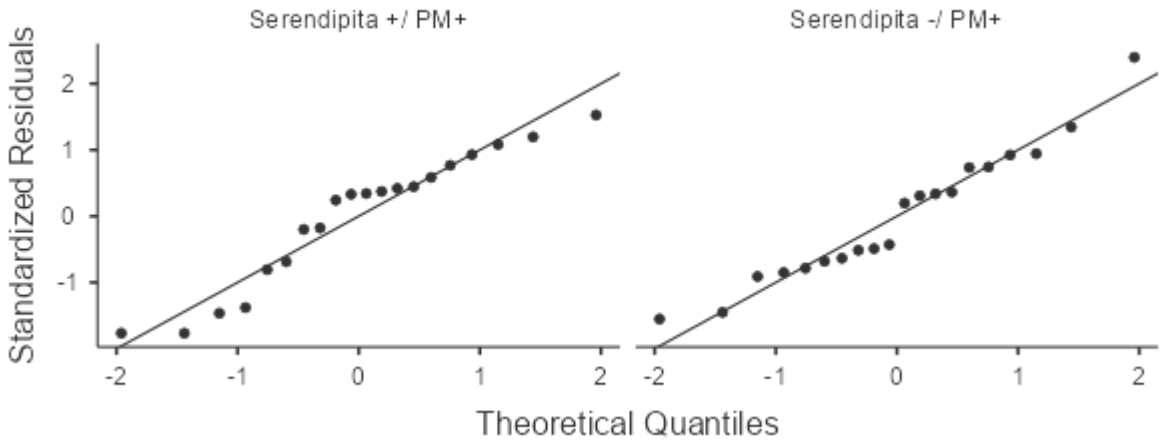
Root Length (cm)





Fresh Weight (g)





Results of bioprotection experiment 2

Independent Samples T-Test

Independent Samples T-Test

		Statistic	df	p
Total Length (cm)	Student's t	-0.0239	38.0	0.981
	Welch's t	-0.0239	37.3	0.981
Shoot Length (cm)	Student's t	3.1501	38.0	0.003
	Welch's t	3.1501	30.1	0.004
Root Length (cm)	Student's t	-1.9726	38.0	0.056
	Welch's t	-1.9726	37.4	0.056
Fresh Weight (g)	Student's t	2.8215	38.0	0.008
	Welch's t	2.8215	36.7	0.008

Assumptions

Normality Test (Shapiro-Wilk)

	W	p
Total Length (cm)	0.967	0.298
Shoot Length (cm)	0.914	0.005
Root Length (cm)	0.965	0.241
Fresh Weight (g)	0.969	0.342

Note. A low p-value suggests a violation of the assumption of normality

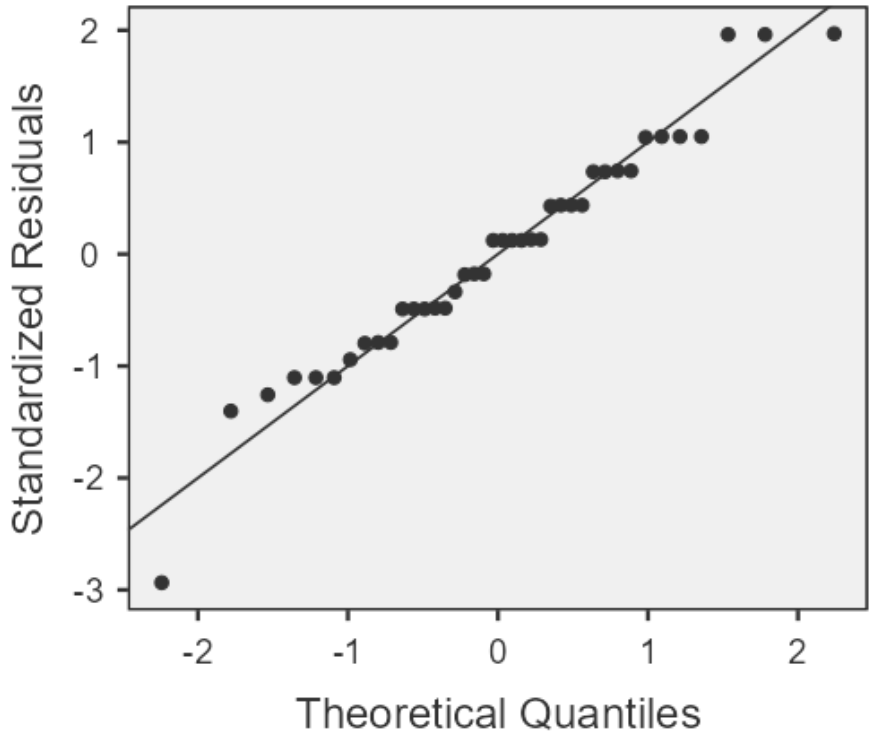
Homogeneity of Variances Test (Levene's)

	F	df	df2	p
Total Length (cm)	0.173	1	38	0.680
Shoot Length (cm)	2.206	1	38	0.146
Root Length (cm)	0.138	1	38	0.712
Fresh Weight (g)	0.845	1	38	0.364

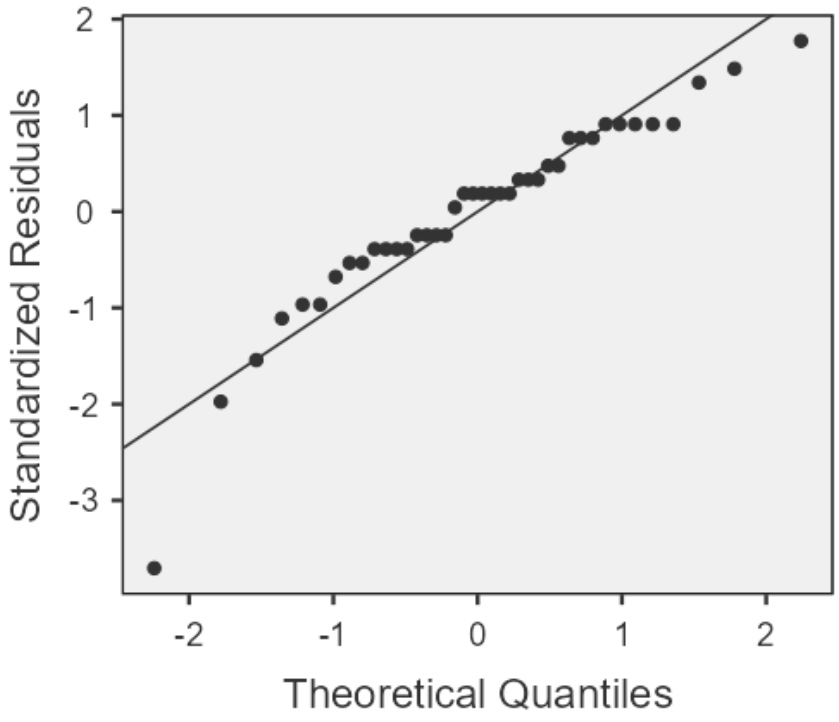
Note. A low p-value suggests a violation of the assumption of equal variances

Plots

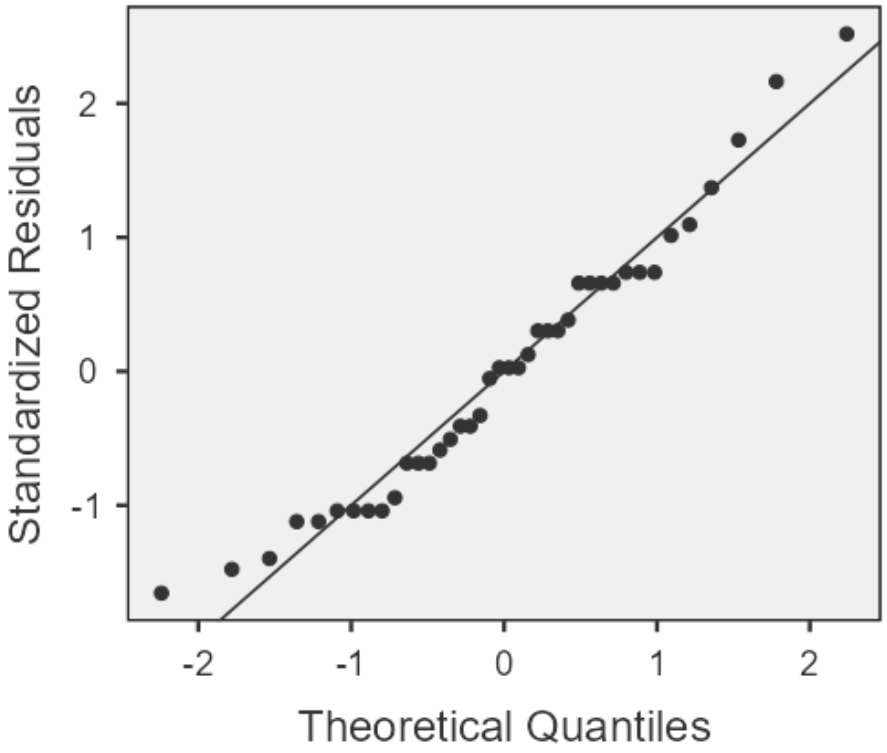
Total Length (cm)



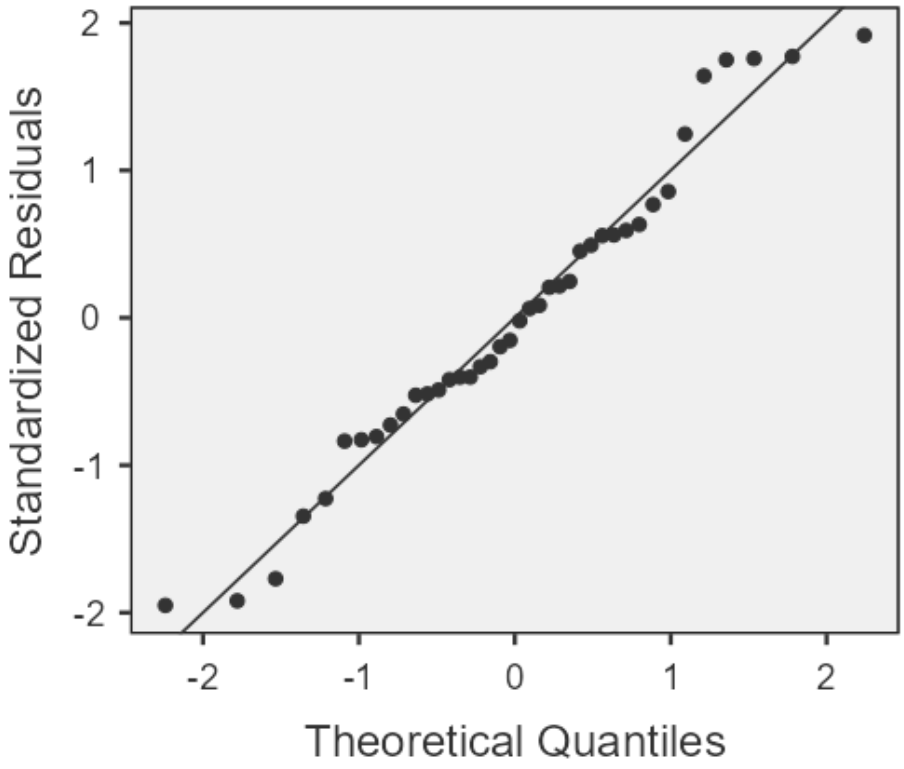
Shoot Length (cm)



Root Length (cm)



Fresh Weight (g)



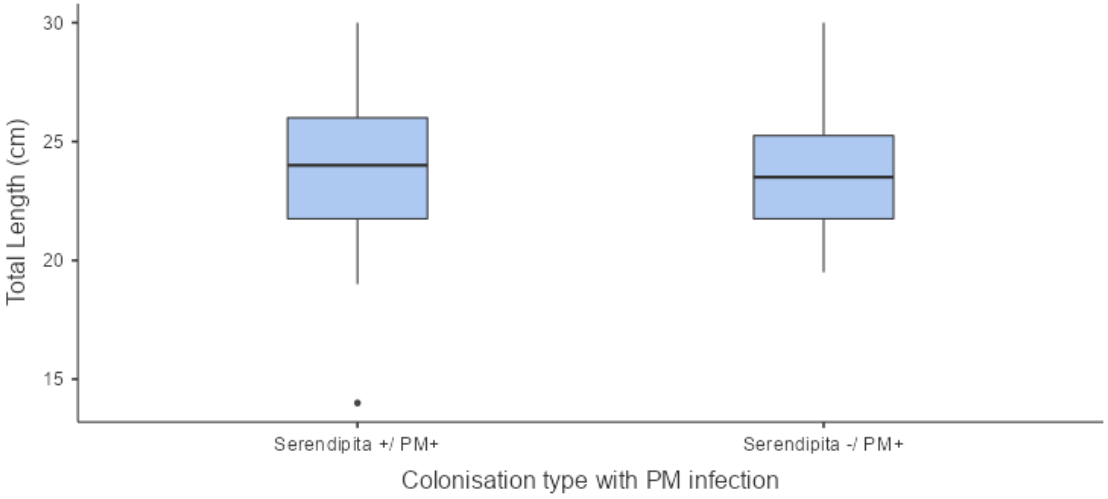
Descriptives

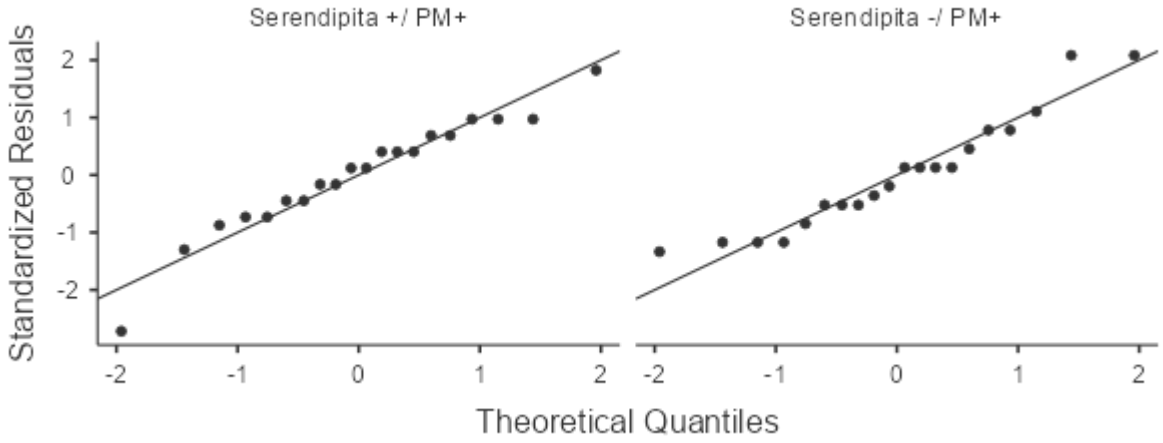
Descriptives

	Colonisation type with PM infection	Total Length (cm)	Shoot Length (cm)	Root Length (cm)	Fresh Weight (g)
N	Serendipita +/ PM+	20	20	20	20
	Serendipita -/ PM+	20	20	20	20
Missing	Serendipita +/ PM+	0	0	0	0
	Serendipita -/ PM+	0	0	0	0
Mean	Serendipita +/ PM+	23.6	12.4	11.2	0.932
	Serendipita -/ PM+	23.6	10.7	12.9	0.726
Median	Serendipita +/ PM+	24.0	12.8	11.8	0.938
	Serendipita -/ PM+	23.5	11.0	12.5	0.701
Standard deviation	Serendipita +/ PM+	3.52	2.16	2.65	0.252
	Serendipita -/ PM+	3.07	1.23	3.03	0.208
Minimum	Serendipita +/ PM+	14.0	6.00	6.50	0.487
	Serendipita -/ PM+	19.5	8.00	9.00	0.322
Maximum	Serendipita +/ PM+	30.0	15.5	16.0	1.37
	Serendipita -/ PM+	30.0	13.0	20.0	1.13
Shapiro-Wilk W	Serendipita +/ PM+	0.951	0.885	0.968	0.961
	Serendipita -/ PM+	0.927	0.961	0.909	0.974
Shapiro-Wilk p	Serendipita +/ PM+	0.380	0.022	0.709	0.561
	Serendipita -/ PM+	0.137	0.563	0.061	0.835

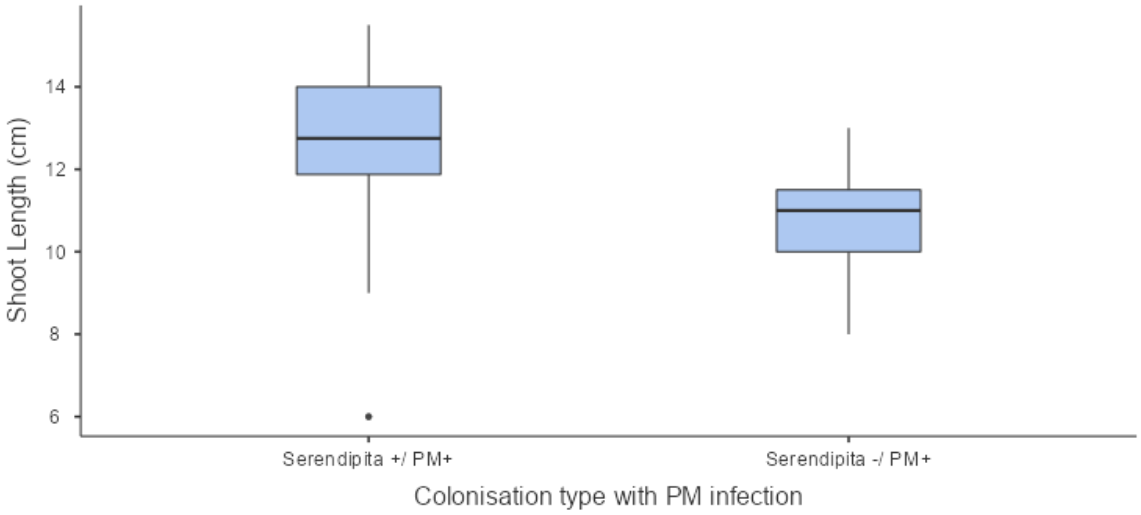
Plots

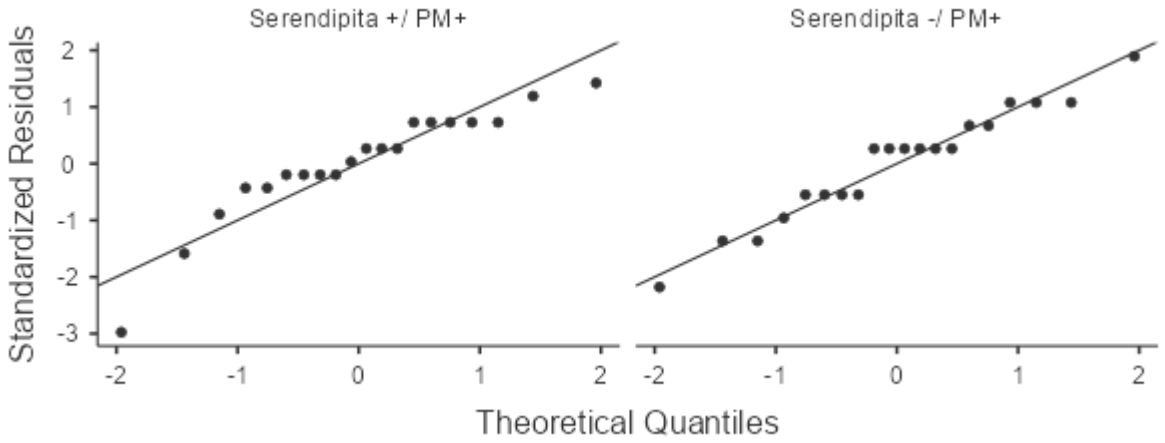
Total Length (cm)



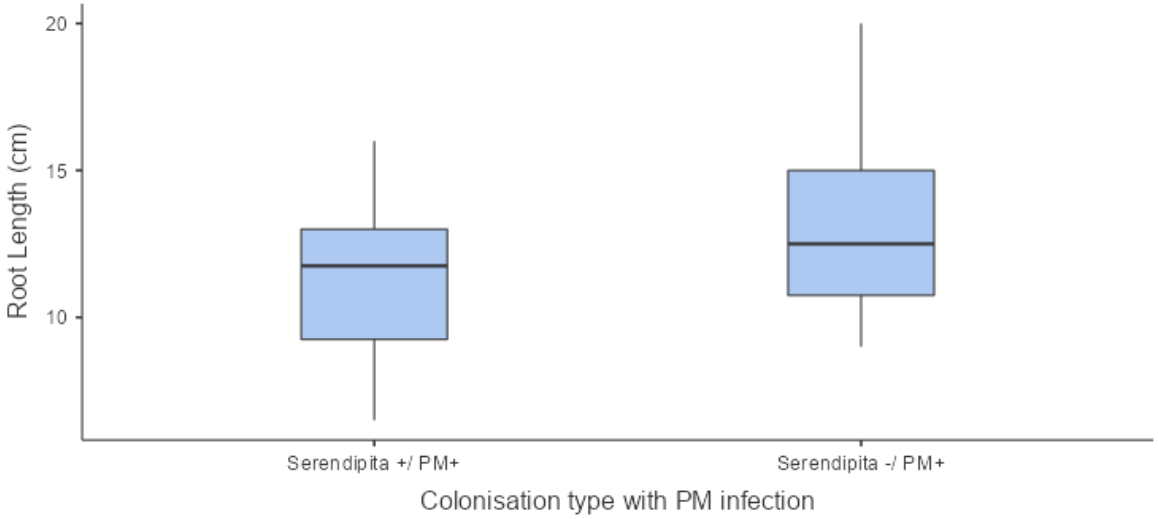


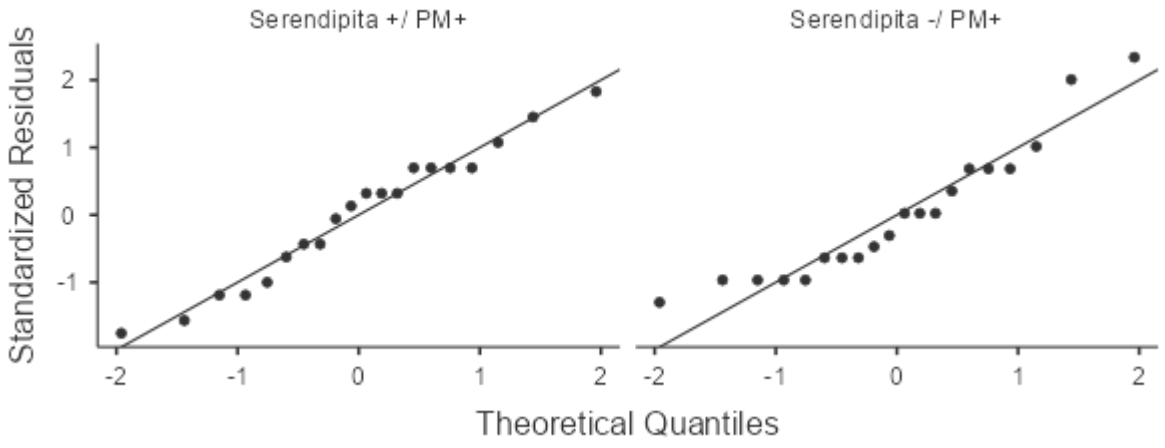
Shoot Length (cm)



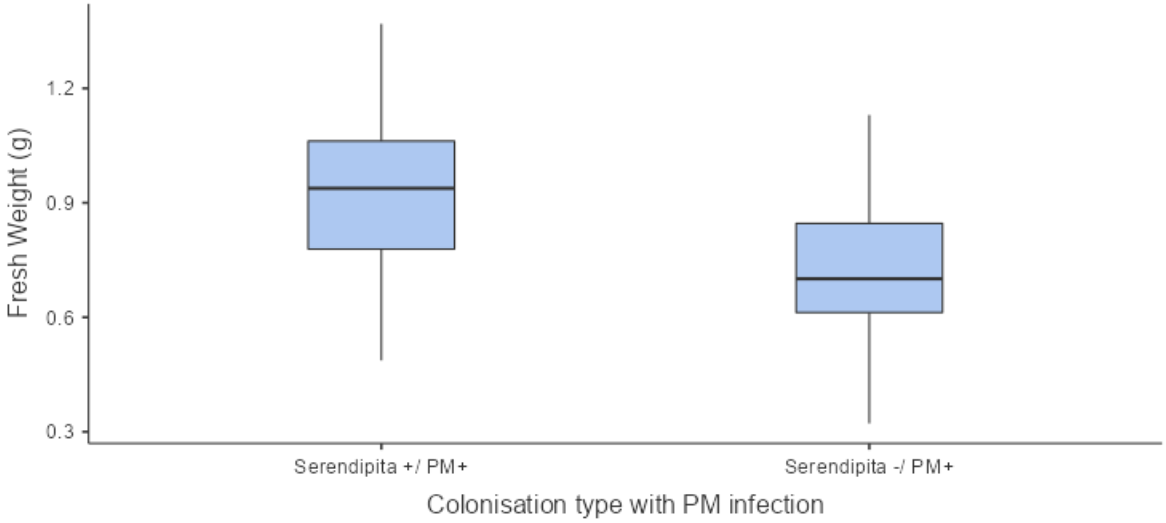


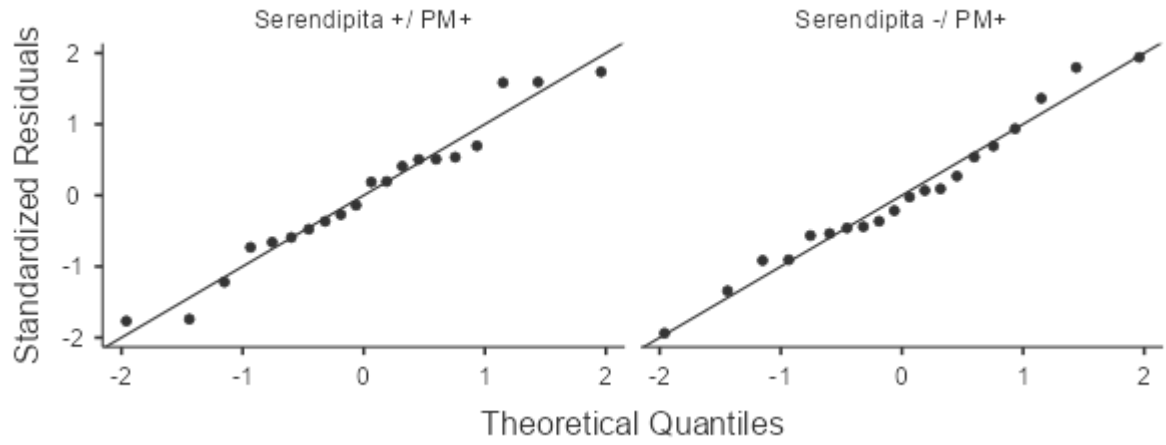
Root Length (cm)





Fresh Weight (g)





Results of bioprotection experiment 3

Independent Samples T-Test

Independent Samples T-Test

		Statistic	df	p
Total Length (cm)	Student's t	-1.194	38.0	0.240
	Welch's t	-1.194	34.0	0.241
Shoot Length (cm)	Student's t	-0.551	38.0	0.585
	Welch's t	-0.551	36.2	0.585
Root Length (cm)	Student's t	-1.059	38.0	0.296
	Welch's t	-1.059	37.6	0.296
Fresh Weight (g)	Student's t	1.261	38.0	0.215

Independent Samples T-Test

		Statistic	df	p
	Welch's t	1.261	35.4	0.215

Assumptions

Normality Test (Shapiro-Wilk)

	W	p
Total Length (cm)	0.974	0.465
Shoot Length (cm)	0.933	0.020
Root Length (cm)	0.973	0.457
Fresh Weight (g)	0.794	< .001

Note. A low p-value suggests a violation of the assumption of normality

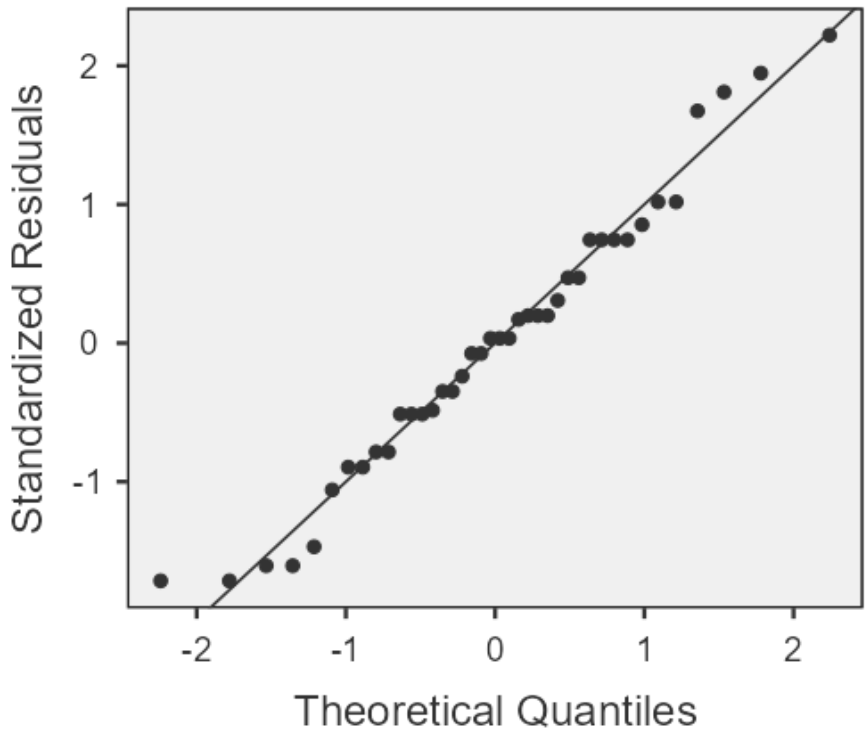
Homogeneity of Variances Test (Levene's)

	F	df	df2	p
Total Length (cm)	1.757	1	38	0.193
Shoot Length (cm)	0.202	1	38	0.656
Root Length (cm)	0.247	1	38	0.622
Fresh Weight (g)	2.490	1	38	0.123

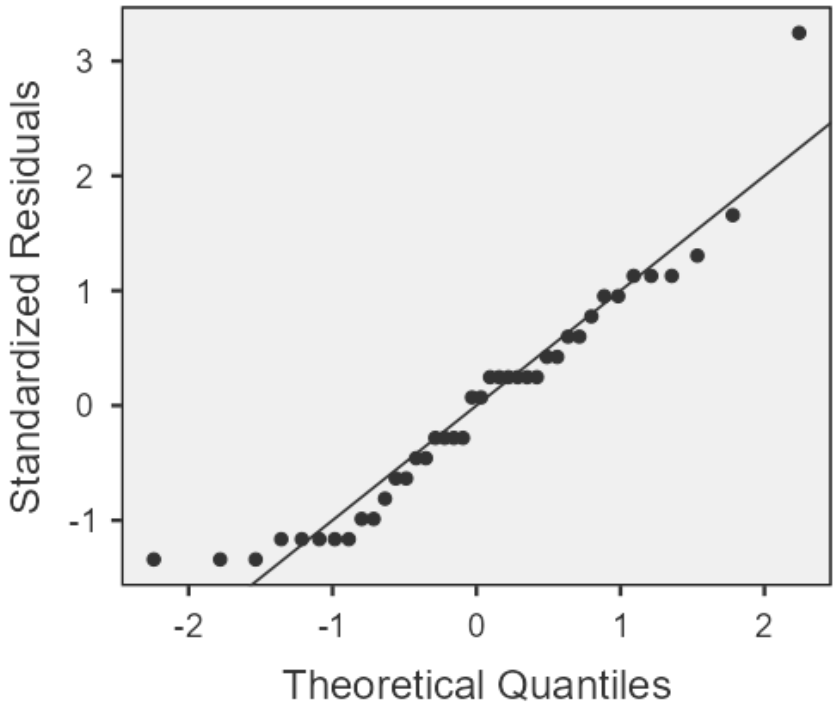
Note. A low p-value suggests a violation of the assumption of equal variances

Plots

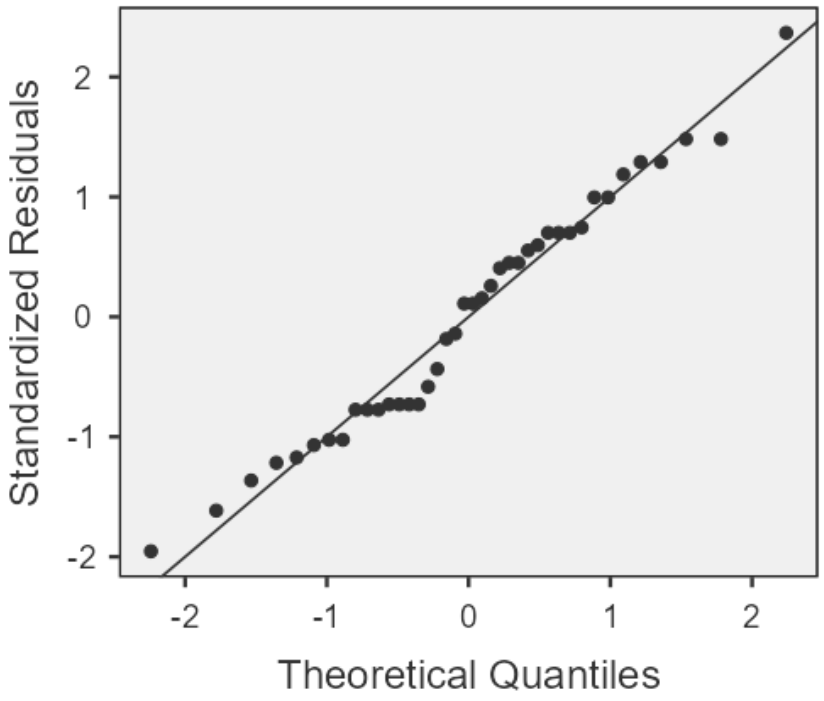
Total Length (cm)



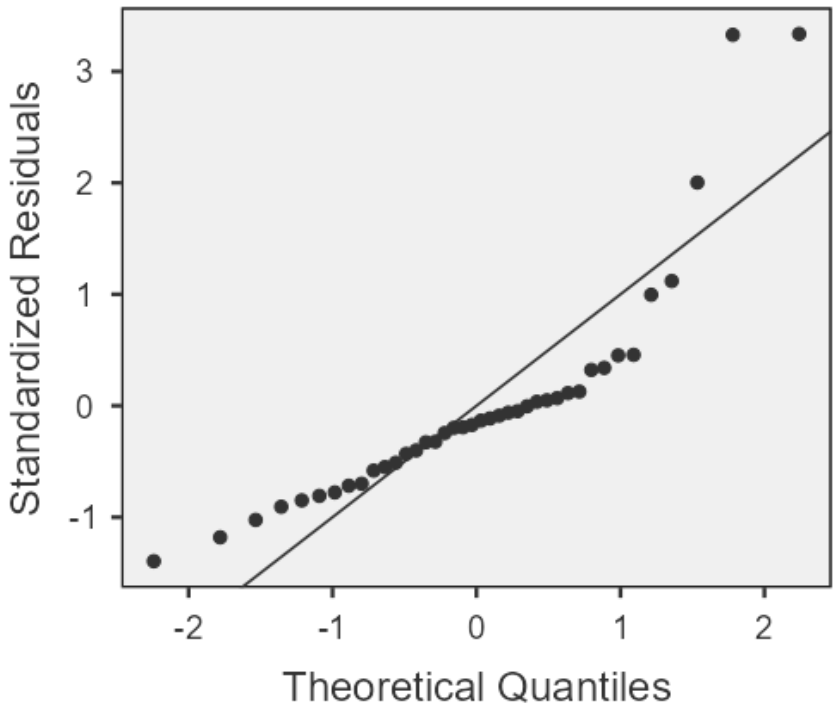
Shoot Length (cm)



Root Length (cm)



Fresh Weight (g)



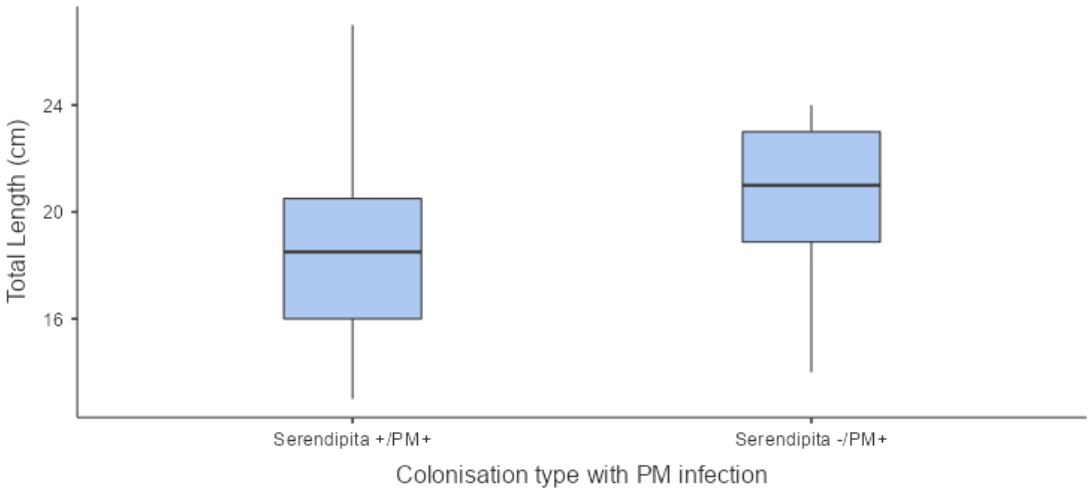
Descriptives

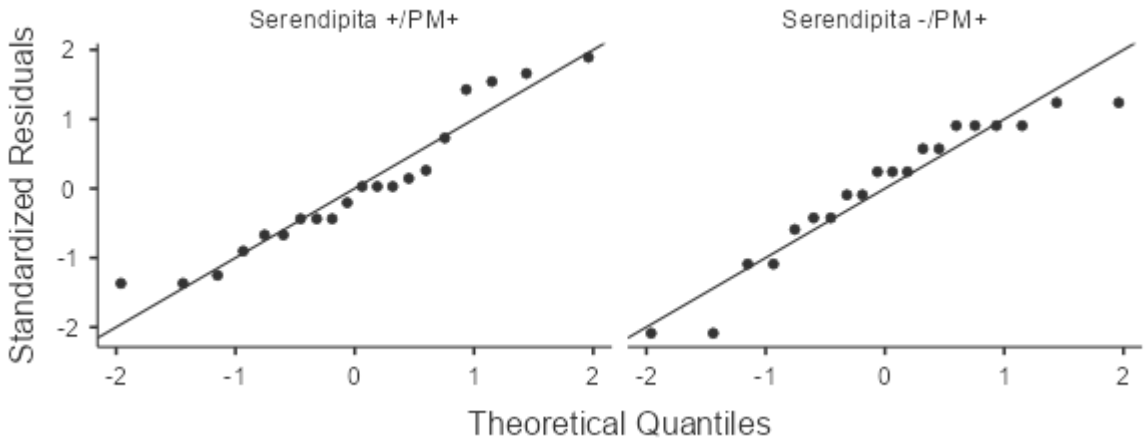
Descriptives

	Colonisation type with PM infection	Total Length (cm)	Shoot Length (cm)	Root Length (cm)	Fresh Weight (g)
N	Serendipita +/PM+	20	20	20	20
	Serendipita -/PM+	20	20	20	20
Missing	Serendipita +/PM+	0	0	0	0
	Serendipita -/PM+	0	0	0	0
Mean	Serendipita +/PM+	18.9	6.40	12.5	0.365
	Serendipita -/PM+	20.3	6.65	13.6	0.302
Median	Serendipita +/PM+	18.5	6.00	11.5	0.321
	Serendipita -/PM+	21.0	7.00	14.3	0.289
Standard deviation	Serendipita +/PM+	4.29	1.59	3.61	0.176
	Serendipita -/PM+	3.01	1.27	3.25	0.133
Minimum	Serendipita +/PM+	13.0	4.50	7.00	0.150
	Serendipita -/PM+	14.0	5.00	7.00	0.163
Maximum	Serendipita +/PM+	27.0	11.0	20.5	0.877
	Serendipita -/PM+	24.0	9.00	18.0	0.816
Shapiro-Wilk W	Serendipita +/PM+	0.925	0.899	0.944	0.866
	Serendipita -/PM+	0.912	0.910	0.937	0.640
Shapiro-Wilk p	Serendipita +/PM+	0.122	0.039	0.284	0.010
	Serendipita -/PM+	0.071	0.064	0.214	< .001

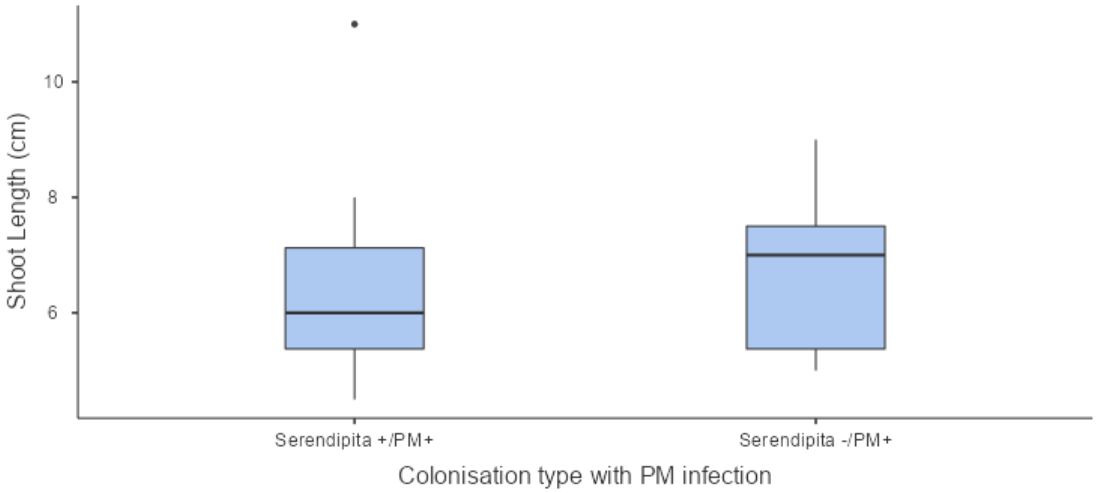
Plots

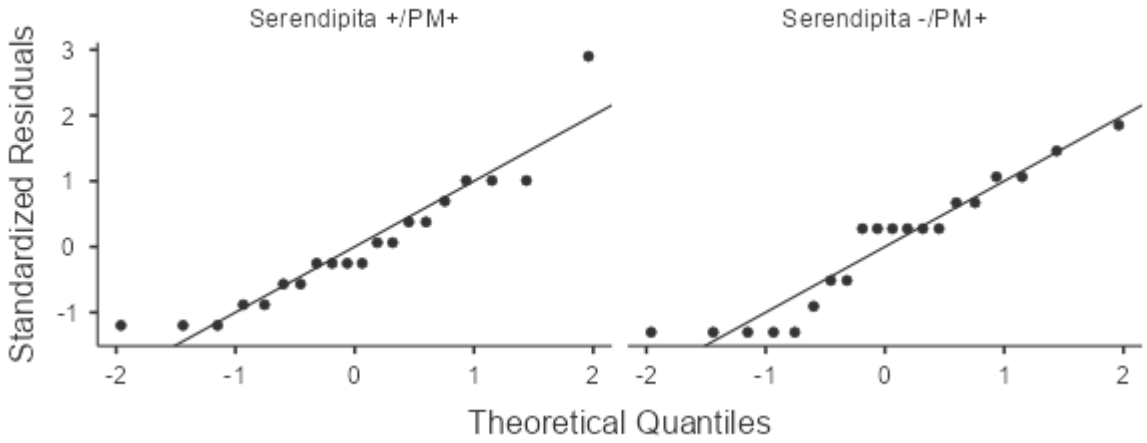
Total Length (cm)



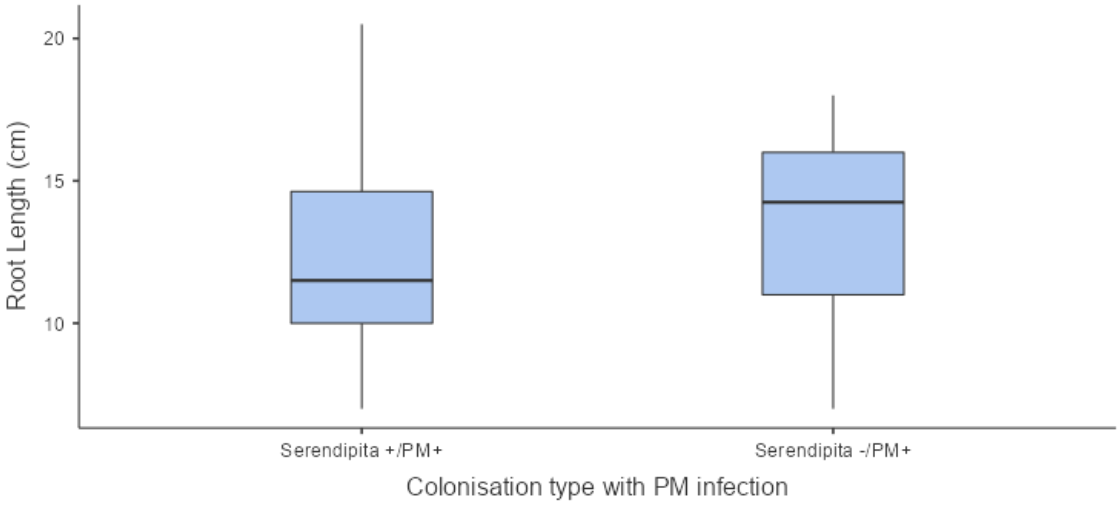


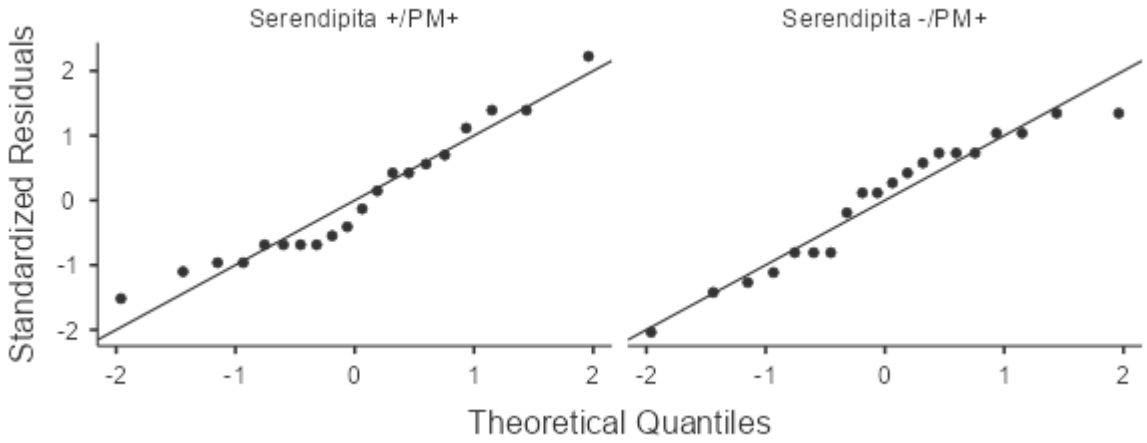
Shoot Length (cm)



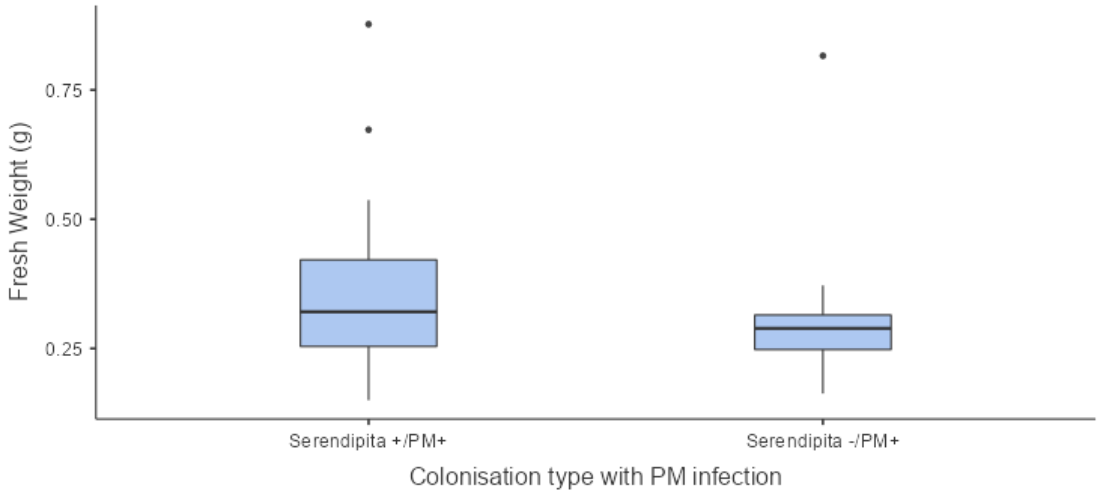


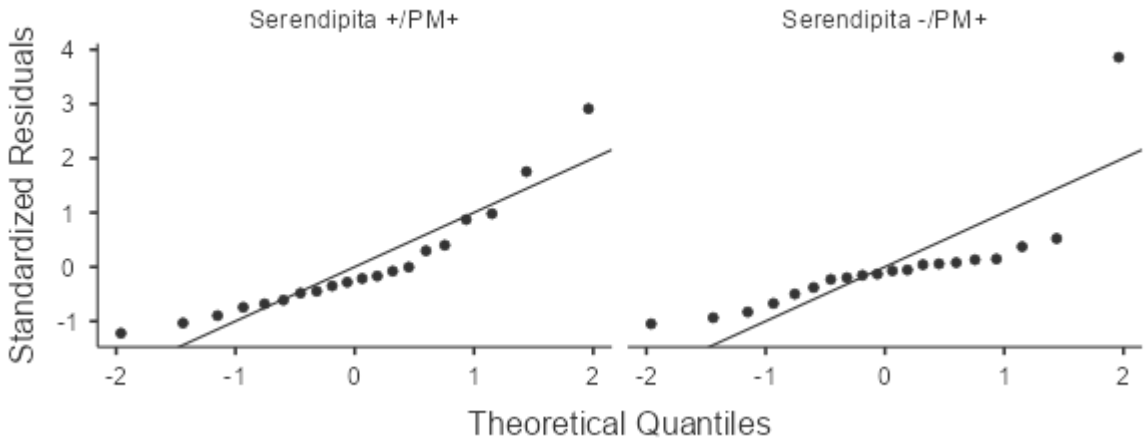
Root Length (cm)





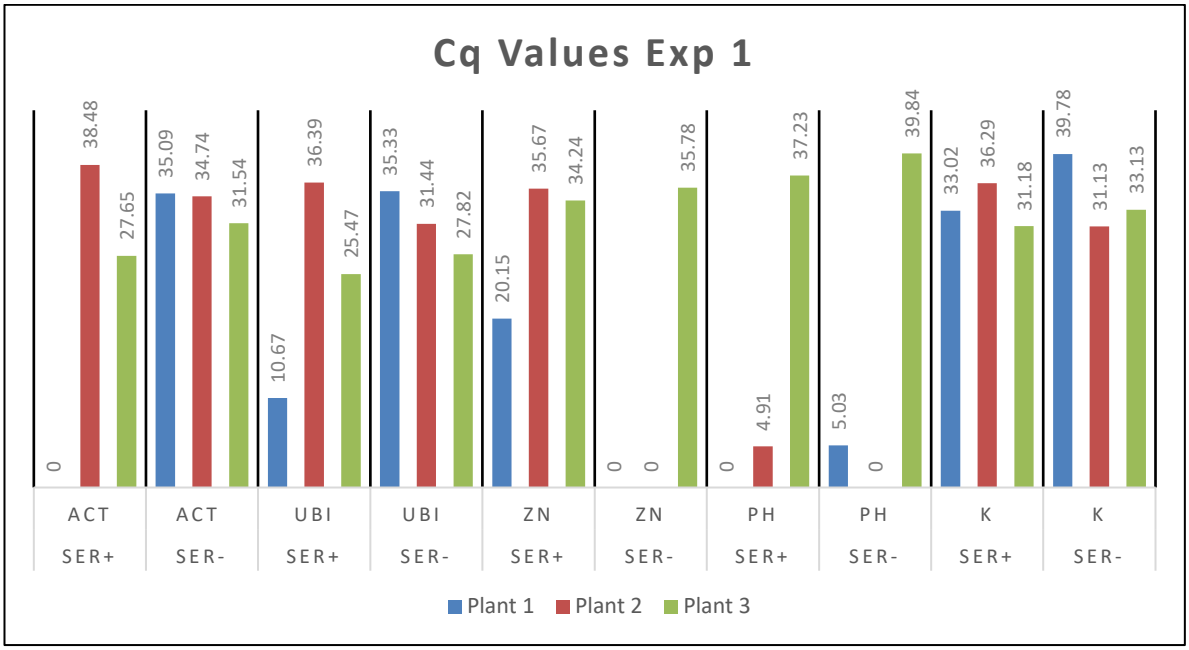
Fresh Weight (g)

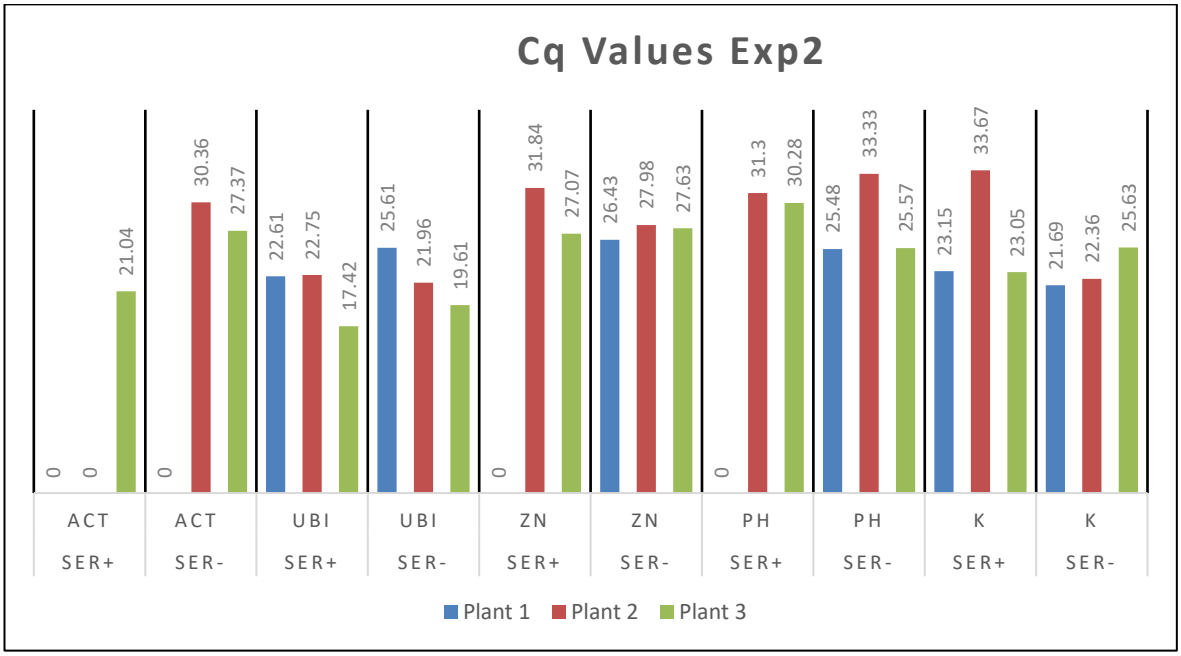




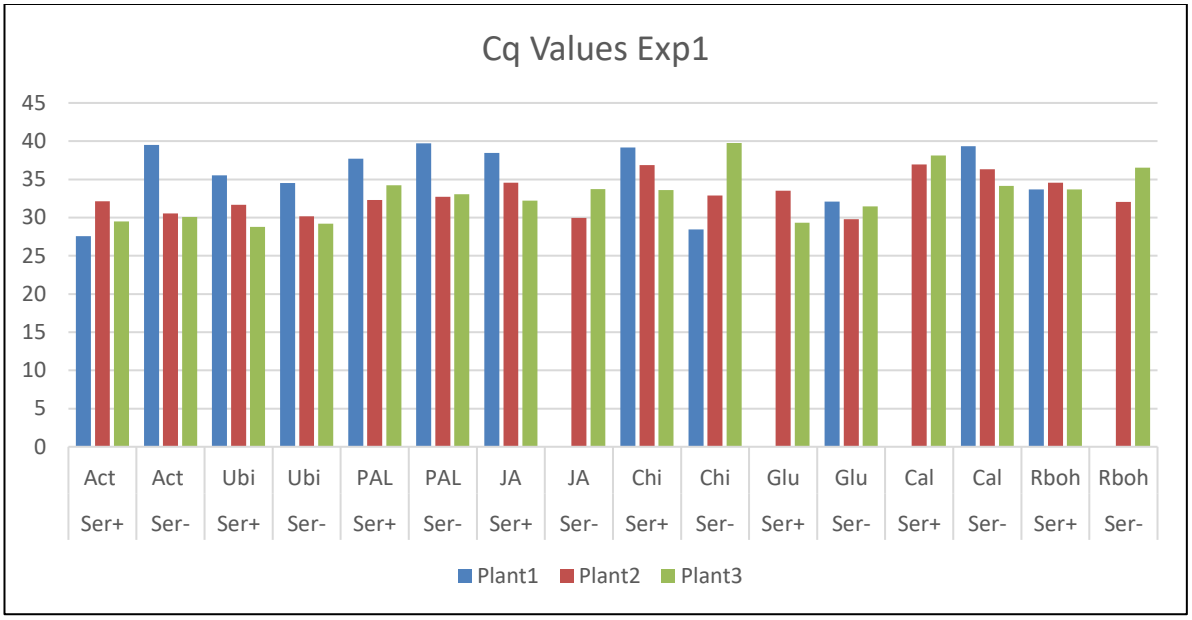
Appendix VII

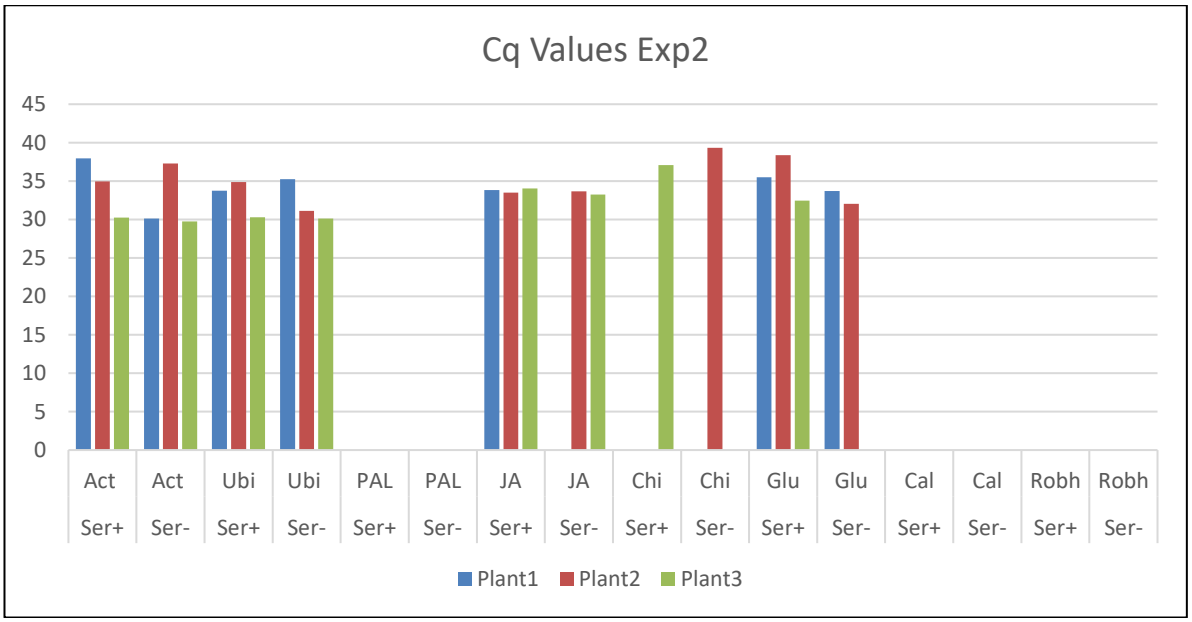
Appendix VII-A: The results of Cq values in growth experiment 1 and 2. Cq values of ubiquitin, actin, zinc transporter, potassium transporter and phosphate transporter in *Ser+*/*Ser-* treatments.





Appendix VII-B: The results of Cq values in bioprotection experiment 1 and 2. Cq values of ubiquitin, actin, PAL, JA-Lipoxygenase, chitinase, β-1,3-glucanase, callose 11 synthase and Robh (NADPH oxidase) genes in *Ser*+/PM+ and *Ser*-/PM+ treatments.





Appendix VII

Appendix VII: statistical analysis of the number of infected plants experiment (three repeats) in *Ser*+/PM+ and *Ser*-/PM+ treatments using jamovi 2.2.5 statistical software.

Results of number of infected plants experiment 1

Contingency Tables

Contingency Tables

		Infection Status		
Colonisation Type		**Infected**	**Not infected**	**Total**
Serendipita +/ PM +	Observed	6	14	20
	% within row	30.0 %	70.0 %	100.0 %
	% within column	27.3 %	77.8 %	50.0 %
Serendipita -/PM+	Observed	16	4	20
	% within row	80.0 %	20.0 %	100.0 %
	% within column	72.7 %	22.2 %	50.0 %
Total	Observed	22	18	40
	% within row	55.0 %	45.0 %	100.0 %
	% within column	100.0 %	100.0 %	100.0 %

χ^2 Tests

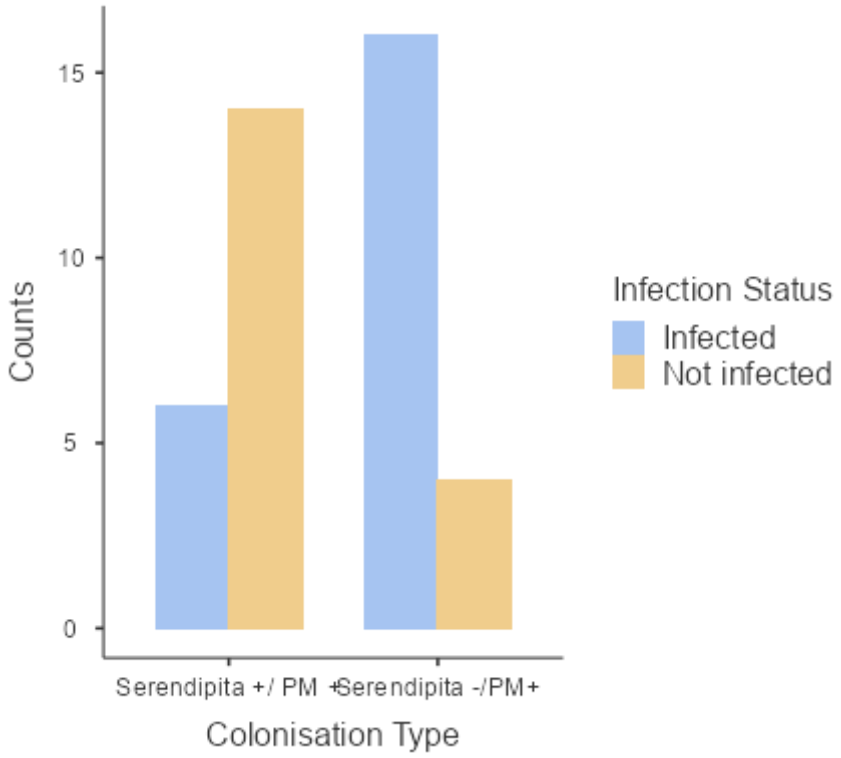
	Value	**df**	**p**
χ^2	10.1	1	0.001
Fisher's exact test			0.004
N	40		

Comparative Measures

		95% Confidence Intervals	
	Value	**Lower**	**Upper**
Difference in 2 proportions	-0.500 [a]	-0.767	-0.233

[a] rows compared

Plots



Results of number of infected plants experiment 2

Contingency Tables

Contingency Tables

		Infection Status		
Colonisation Type		**Infected**	**Not infected**	**Total**
Serendipita +/PM+	Observed	8	12	20
	% within row	40.0 %	60.0 %	100.0 %
	% within column	34.8 %	70.6 %	50.0 %
Serendipita -/PM+	Observed	15	5	20
	% within row	75.0 %	25.0 %	100.0 %
	% within column	65.2 %	29.4 %	50.0 %
Total	Observed	23	17	40
	% within row	57.5 %	42.5 %	100.0 %
	% within column	100.0 %	100.0 %	100.0 %

χ^2 Tests

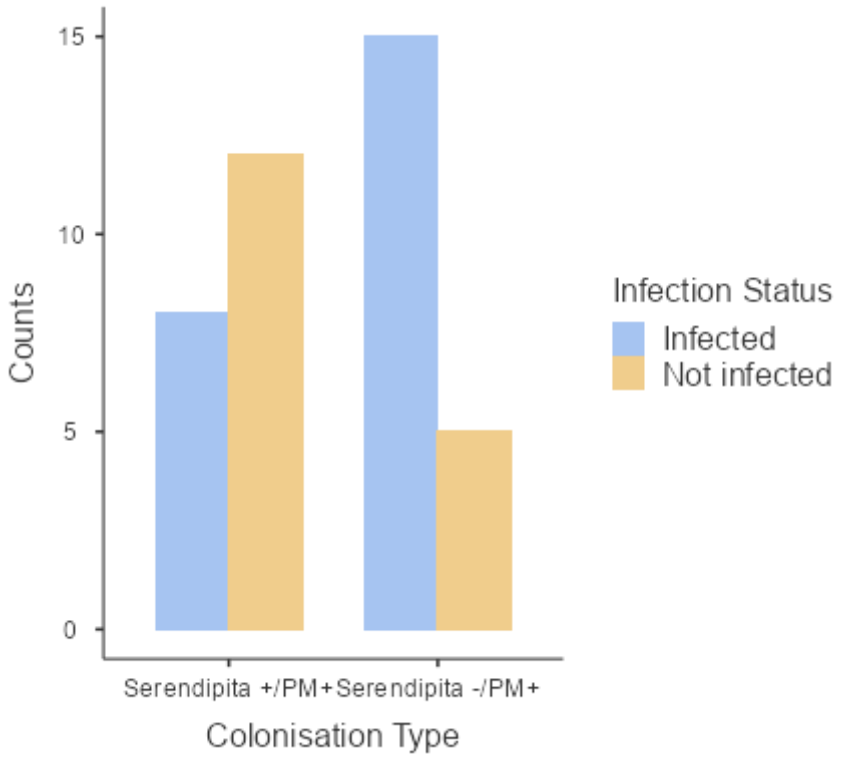
	Value	df	p
χ^2	5.01	1	0.025
Fisher's exact test			0.054
N	40		

Comparative Measures

		95% Confidence Intervals	
	Value	Lower	Upper
Difference in 2 proportions	-0.350 [a]	-0.637	-0.0634

[a] rows compared

Plots



Results of number of infected plants experiment 3

Contingency Tables

Contingency Tables

		Infection Status		
Colonisation Type		**Infected**	**Not infected**	**Total**
Serendipita +/PM+	Observed	9	11	20
	% within row	45.0 %	55.0 %	100.0 %
	% within column	36.0 %	73.3 %	50.0 %
Serendipita -/PM+	Observed	16	4	20
	% within row	80.0 %	20.0 %	100.0 %
	% within column	64.0 %	26.7 %	50.0 %
Total	Observed	25	15	40
	% within row	62.5 %	37.5 %	100.0 %
	% within column	100.0 %	100.0 %	100.0 %

χ^2 Tests

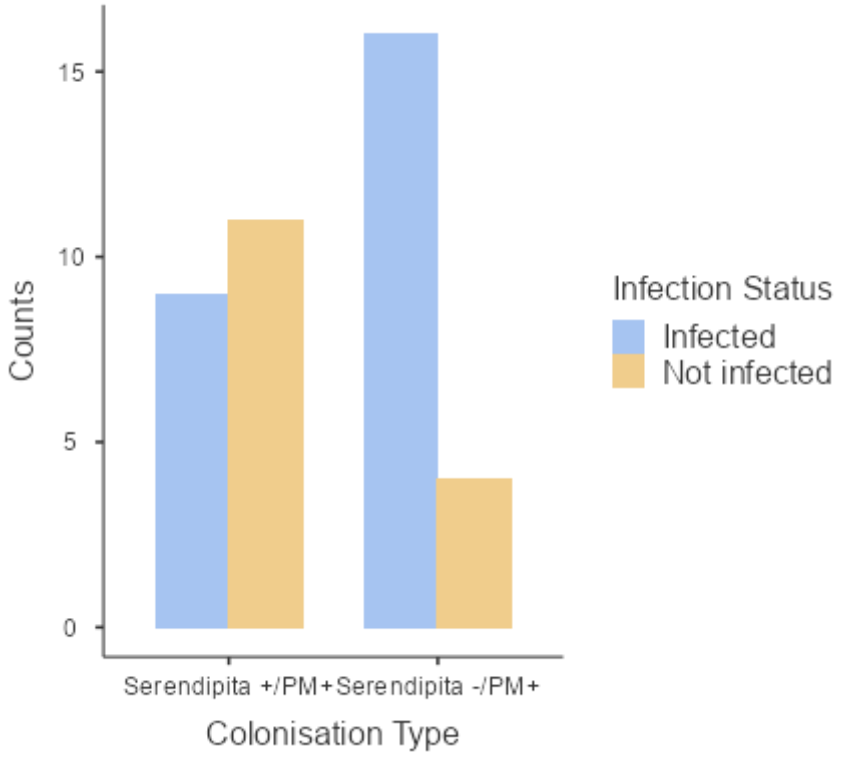
	Value	**df**	**p**
χ^2	5.23	1	0.022
Fisher's exact test			0.048
N	40		

Comparative Measures

		95% Confidence Intervals	
	Value	**Lower**	**Upper**
Difference in 2 proportions	-0.350 [a]	-0.630	-0.0702

[a] rows compared

Plots


15
10
5
0
Counts
Serendipita +/PM+
Serendipita -/PM+
Colonisation Type
Infection Status
Infected
Not infected