

**Interactions between symbiotic and pathogenic fungi
in the invasive weed, *Impatiens glandulifera*
(Himalayan balsam)**

By

Nadia binti Ab Razak

Thesis submitted for the Degree of Philosophy

School of Biological Sciences

Royal Holloway, University of London

April 2019

DECLARATION OF AUTHORSHIP

I, Nadia binti Ab Razak, hereby declare that the research, field works, lab works and data analysis for this thesis was conducted by myself with the exception of the following:

1. Brian C. Sutton identified endophyte fungi morphologically in Chapter 3, 4, 5, 6 and 7.
2. CABI Molecular Identification Services identified endophyte fungi by molecular technique in Chapter 4 and 5.
3. Neil Morley (Royal Holloway) assisted on the preparation of PDA and PCA plates in Chapter 3, 4, 5, 6 and 7.



Nadia binti Ab Razak

ACKNOWLEDGEMENTS

Syukur, Alhamdulillah. Finally, I made it! First of all, I thank Allah SWT for giving me strength, blessing and great health to complete my study. The path towards this dissertation has been circuitous. Its completion is thanks in large part to the special people who challenged, supported and stuck with me along the way.

First and foremost, I would like to render my gratitude to the Majlis Amanah Rakyat (MARA) for the generous funding. Next, my special thanks go to my supervisor, Prof. Alan Gange for his continuous support, enthusiastic guidance and patience throughout my study. Your words soothe my days. I would also like to thank my internal advisor, Prof. Julia Koricheva and external advisor, Dr. Carol Ellison from CABI Bioscience for their thoughtful comments and suggestions towards my research.

I would also like to thank Royal Holloway staff, especially Neil Morley (Lab Technician) and Mark Levy (Glasshouse Technician) for always being there when I needed them. My appreciation also goes to the Gange lab group especially Dr. Amanda Currie, Dr. Ruth Chitty and Francesca Baylis for their kindness, laughter and generally making this experience immeasurably better. A huge thank you also to CABI Bioscience staff especially Dr. Rob Tanner, Dr. Daisuke Kurose, Kate Pollard, Sonal Varia, Suzy Wood and Nikolai for helping me out with endophyte and rust related works. Thank you also to Dr. Brian Sutton for helping me out in fungi identification.

I would not have contemplated this road if not for my family. A special thank you to my parents, Ab Razak Yusof and Foziah Yaacob for their constant *doa*, unconditional love, emotional support and timeless availability for video calls eventhough they are thousands of miles away. Thank you too to my siblings, nephews, nieces, uncles and aunties for believing in me to achieve success in this study. Not to forget, my parents in-law and in-law family's members for their love and support throughout this incredible journey. Many thanks also to my friends in Malaysia for their endless encouragement. Thank you also to my Malaysian friends in the UK who always shared laughter and sorrow in this foreign country.

Words and numbers could not represent the amount I owe to the man in my life, Mr Perfect Husband. Dear Mohd Syafiq Japar, thank you for being a fantastic and wonderful husband for the past four years. Thank you for the hugs and kisses every time I got depressed and for calming words during my difficult times. Your jokes wiped away my exhausted days. Without your love and sacrifices, this PhD would not be possible for me. I dedicate this thesis to you and wish you all the best in your PhD journey. Let's enjoy and embrace our next adventure with our little angel!

ABSTRACT

Impatiens glandulifera or Himalayan balsam is an invasive plant species which is commonly found in riparian habitats and may threaten the biodiversity in the UK by suppressing other native plants, blocking drainage systems and potentially, leading to soil erosion during winter. Between 2006 and 2010, a rust fungus, *Puccinia komarovii* var. *glanduliferae* was first identified infecting balsam in its native range (Western Himalayas). Subsequently, the rust was released for the first time in Europe in August 2014 as a classical biological control (CBC) agent on balsam. This study was initiated to observe the effectiveness of the rust on balsam growth and to determine the factors that affect its impact in the field particularly insects presence, arbuscular mycorrhizal fungi (AMF) and endophytic fungi. An investigation on the interactions of fungi and insects on balsam was conducted. It was found that the combination of aphids, mycorrhizas and endophytes affected plant performance, dependent upon the fungal identities. The interactions between balsam and associated microbial communities through plant soil feedback (PSF) experiment was examined too. In contrast to a previous study of balsam, a negative PSF was recorded whereby plants were shorter, lighter and had lower AMF colonization in a soil that had previously supported balsam, compared to control soil. The competition between balsam and two native plant species was examined in the next chapter, to determine the effect of AMF on balsam competitive ability. It was discovered that mycorrhizas reduced balsam biomass when it was grown singly but tended to increase when it was grown in both inter- and intra-specific competition. Finally, the last experimental chapter examined how AMF and endophytic fungi influence the efficacy of the rust fungus, as a CBC agent. It is suggested that both fungi negatively affected plant growth and rust development.

TABLE OF CONTENTS

DECLARATION OF AUTHORSHIP	2
ACKNOWLEDGEMENTS	3
ABSTRACT	5
TABLE OF CONTENTS	6
LIST OF TABLES.....	9
LIST OF FIGURES	11
LIST OF ABBREVIATIONS	16
LIST OF AUTHORITIES –FUNGAL SPECIES	16
LIST OF AUTHORITIES –PLANT SPECIES	17
LIST OF AUTHORITIES –INSECT HERBIVORES	19
CHAPTER 1 GENERAL INTRODUCTION.....	21
1.1 Invasive Species.....	21
1.2 The study species: <i>Impatiens glandulifera</i>	23
1.2.1 The introduction and spread of <i>Impatiens glandulifera</i> in the UK	26
1.2.2 Impact of <i>Impatiens glandulifera</i> infestation	27
1.3 Biological Control.....	30
1.3.1 CBC management of <i>Impatiens glandulifera</i>	32
1.3.2 Rust fungus, <i>Puccinia komarovii</i> var. <i>glanduliferae</i>	33
1.4 Arbuscular Mycorrhizal Fungi (AMF)	38
1.5 Endophytic Fungi.....	39
1.6 Multiple Fungal Interactions.....	42
1.6.1 Interaction of AM Fungi and Plant Pathogens	42
1.6.2 Interactions of AM and Endophytic Fungi.....	44
1.6.3 Interactions of Endophytic Fungi and Plant Pathogens	46
1.6.4 Interaction of AM, Endophytic Fungi and Plant Pathogens	47
1.7 Aims, Objectives and Hypotheses	47
CHAPTER 2 GENERAL METHODS	51
2.1 Seed Germination.....	51
2.2 Endophyte Inoculation	53
2.3 Leaf Surface Sterilisation.....	54
2.4 Root Staining	59
2.5 Rust Fungus Infection and Harvesting.....	60
2.6 Plant Harvesting	61
2.7 Statistical Analysis	62

CHAPTER 3 PRELIMINARY EXPERIMENTS	64
3.1 Fungi on Himalayan balsam in the field	66
3.1.1 Method	67
3.1.2 Statistical Analysis	68
3.1.3 Results and Discussion	68
3.1.3.1 Plant growth and AMF colonisation	68
3.1.3.2 Endophytic fungal communities	70
3.2 Endophyte inoculation in a controlled environment.....	72
3.2.1 Method	73
3.2.2 Results and Discussion	74
3.2.2.1 Plant growth and AMF colonisation	74
3.2.2.2 Endophytic fungal communities	76
3.3 Endophyte inoculation in an insect-free environment	78
3.3.1 Method	78
3.3.2 Results and Discussion	79
3.4 Arbuscular mycorrhizal fungal 'spore-wash'	80
3.4.1 Method	81
3.4.2 Results and Discussion	82
3.5 Seed-borne fungal endophytes.....	84
3.5.1 Method	85
3.5.2 Results and Discussion	86
CHAPTER 4 PLANT-FUNGI-INSECT INTERACTIONS	89
4.1 Methods.....	94
4.1.1 Statistical Analysis	96
4.2 Results.....	97
4.2.1 Plant growth and AMF colonisation.....	97
4.2.2 Insect attributes.....	103
4.2.3 Endophytic fungal communities.....	106
4.3 Discussion	115
4.4 Conclusion.....	118
CHAPTER 5 PLANT-SOIL FEEDBACKS.....	121
5.1 Methods.....	123
5.1.1 Statistical Analysis	128
5.2 Results.....	129
5.2.1 Plant growth	129
5.2.2 AMF colonisation.....	135
5.2.3 Endophytic fungal communities.....	137

5.3 Discussion	145
5.4 Conclusion	150
CHAPTER 6 MYCORRHIZAL EFFECTS ON BALSAM COMPETITIVE ABILITY	152
6.1 Methods	156
6.1.1 Statistical Analysis	159
6.2 Results	160
6.2.1 Plant growth	160
6.2.2 AMF colonisation	162
6.2.3 Endophytic fungal communities	163
6.3 Discussion	166
6.4 Conclusion	170
CHAPTER 7 ASSEMBLING THE FUNGAL COMMUNITY: INTERACTIONS BETWEEN MYCORRHIZAS, ENDOPHYTES AND THE RUST	172
7.1 Methods	175
7.1.1 Statistical Analysis	177
7.2 Results	178
7.2.1 Plant growth	178
7.2.2 AMF colonisation	180
7.2.3 Endophytic fungal communities	181
7.2.4 Rust Fungus Infection	185
7.3 Discussion	187
7.4 Conclusion	194
CHAPTER 8 GENERAL DISCUSSION	197
8.1 Summary of Outcomes	197
8.2 Multiple fungi effect on the classical biological control of Himalayan balsam	199
8.3 Final Conclusions and Impact of this Study	203
REFERENCES	205

LIST OF TABLES

Table 1.1	The proposed life cycle of <i>Puccinia komarovii</i> var <i>glanduliferae</i> (Tanner, 2011)	37
Table 3.1	Isolation frequency (% of plants) mean of each endophyte species across all treatments. <i>n</i> =10 for all treatments.	71
Table 3.2	Isolation Frequency (IF) mean of isolated endophyte across treatments. <i>n</i> =5 for all treatments. +/-AS were <i>Acremonium strictum</i> inoculated/free plants, +/-CA were <i>Colletotrichum acutatum</i> inoculated/free plants and +/-G were <i>Geniculosporium spp.</i> inoculated/free plants.	77
Table 3.3	The Isolation Frequency mean (% of plants) of each endophyte species across treatments with <i>n</i> =5 for each treatments. CA were <i>Co. acutatum</i> inoculated plants, CS were <i>Cl. sphaerospermum</i> inoculated plants, CACS were combination of both species and C were control plants.	80
Table 3.4	Mycorrhizal species composition in Plantworks and Symbio inoculum.	84
Table 3.5	Isolation Frequency (IF) % mean recorded from non sterilised and non squashed seeds.....	87
Table 4.1	Four-way ANOVA factorial analysis results across (a) Plantworks and (b) Symbio treatments. Degrees of Freedom for F values = 1, 64 and <i>n</i> = 5 for each treatment. (Note: IN: Insects, CA: <i>Colletotrichum acutatum</i> and CS: <i>Cladosporium sphaerospermum</i> . Bold indicated significant values).	100
Table 4.2	Four-way factorial analysis of insect number across treatments. Degrees of Freedom for F values = 1,64 and <i>n</i> =5 in all treatments. (Note: IN: Insecticide application, CA: <i>Colletotrichum acutatum</i> and CS: <i>Cladosporium sphaerospermum</i> . Bold indicated significant values).	104
Table 4.3	Isolation Frequency (%) mean of each endophyte species across plants attacked by insects. <i>n</i> = 5 for each treatment. (Note: +PW: Plantworks plants, +SY: Symbio plants, +CA: <i>Colletotrichum acutatum</i> inoculated plants, +CS: <i>Cladosporium sphaerospermum</i> inoculated plants; +CACS: dual endophyte inoculated plants and C: Control plants. Bold numbers indicted the highest IF mean in that particular treatments.....	107
Table 4.4	Isolation Frequency (%) mean of each endophyte species across insects free plants. <i>n</i> = 5 for each treatment. (Note: +PW: Plantworks plants, +SY: Symbio plants, +CA: <i>Colletotrichum acutatum</i> inoculated plants, +CS: <i>Cladosporium sphaerospermum</i> inoculated plants; +CACS: dual endophyte inoculated plants and C: Control plants. Bold numbers indicted the highest IF mean in that particular treatments.	109
Table 4.5	Four way factorial analysis of isolation frequency mean of (a) <i>Cladosporium sphaerospermum</i> and (b) <i>Colletotrichum acutatum</i> across	

treatments. Degrees of Freedom for F values = 1,65 and $n=5$ in all treatments. AM: Mycorrhizal treatments. Legend as in Table 4.1..... 113

Table 5.1 One-way factorial analysis results across treatments. Degrees of Freedom for F values = 1, 18 and $n = 10$ for each treatment. (a) Plants were grown in the Inocula Soil were compared with the plants grown in the Control Soil, (b) Plants were grown in the Conditioned Soil were compared with the plants grown in the Conditioned Control Soil, (c) Plants were grown in the Inocula Soil –Phase 1 were compared with the plants grown in the Conditioned Soil –Phase 2 while (d) Plants were grown in the Conditioned Soil –Phase 2 were compared with the plants grown in the Clean Soil –Phase 2. Bold indicated significant values. 133

Table 5.2 The Isolation Frequency (%) mean of each endophyte species across both phases. $N = 10$ for each treatment. Bold values indicate the highest IF. (Note: +PW: Plantworks Soil, -PW: Plantworks Control Soil, +SY: Symbio Soil, -SY: Symbio Control Soil, +CPW: Conditioned Plantworks soil, +C-PW: Conditioned Plantworks Control soil, +CSY: Conditioned Symbio Soil, +C-SY: Conditioned Symbio Control Soil, -C-PW: Clean Plantworks Control Soil and –C-SY : Clean Symbio Control Soil..... 138

Table 6.1 Two-way factorial analysis of balsam parameters, testing for effects of AMF and competition. Degrees of Freedom for F values = 1, 36 and $n=5$ in all treatments. Bold indicates significant values. 160

Table 6.2 One-way factorial analysis of native plants species, testing for effects of AMF. Degrees of Freedom for F values = 1, 8 and $n=5$ in all treatments. Bold indicates significant values. 160

Table 6.3 Isolation Frequency (%) mean of endophyte species in native plants. +/-AM were mycorrhizal present/absent plants. 164

Table 6.4 Isolation Frequency (IF) mean of each endophyte species in Himalayan balsam across treatments. +PL referred to competition with *Plantago lanceolata* while +HL competition with *Holcus lanatus*. +/-AM were mycorrhizas present/absent plants. 165

Table 7.1 Three-way factorial analysis of plant parameters, testing for effects of AM, endophyte and rust. Degrees of Freedom for F values = 1, 32 and $n=5$ in all treatments. Bold indicated significant values..... 178

Table 7.2 Isolation Frequency (IF) of each endophyte species across treatments. +/-R: Rust present/absent, +/-AM: Mycorrhizal present/absent and +/-CA: Leaves were inoculated with *Colletotrichum acutatum*/endophyte free leaves. 182

Table 7.3 Two-way ANOVA factorial analysis of the effects of AM and endophyte on the pustule density. Degrees of Freedom for F values = 1, 16 and $n=5$ in all treatments. Bold indicated significant values..... 186

Table 8.1 Summary of objectives and outcomes of the study. 197

LIST OF FIGURES

- Figure 1.1** (a) Himalayan balsam population near river bank. (b) Plant height that can exceed 2m tall, (c) adventitious root system, (d) oppositely arranged and in whorls 3-5 leaves and (e) purple pink zygomorphic flower and seed capsules. All photos except (b) were courtesy from CABI staff –Carol Ellison. 25
- Figure 1.2** (a) Aecial cups infection on hypocotyls and (b) their close-up image. (c) Transverse section of aecial cups under microscope. The photos were courtesy of CABI staff. Two former photos were from Nobert Maczey while the latter was from Carol Ellison. (d) Urediniospores infection on the abaxial leaf surface. 36
- Figure 2.1** (a) Size of balsam seed (no 1) compared to 5 pence and other native plant seeds (*Plantago lanceolata* is number 2 and *Holcus lanatus* is number 3). (b) Germinating balsam seeds in a sealed Petri plate. (c) Germinating seeds were sown in the seed trays covered with a propagator hood and (d) removed the hood a week after. Different commercial mycorrhizal features, (e) Symbio inoculum in a powder form while (f) Plantworks inoculum in a granular form. 52
- Figure 2.2** (a) Balsam seedling at three whorl stage that was ready to be inoculated. (b) Balsam plants were left in a Perspex box for 48 hours to allow spore germination. (c) *Colletotrichum acutatum* culture grown on a PDA plate and (d) under dissecting microscope at 40x magnification. 54
- Figure 2.3** (a) Two leaf discs from each leaf were placed abaxial surface on a PDA plate and (b) the same fragments were used for leaf press. The clean plate showed effective endophyte isolation technique. (c) Fungal cultures grown on PDA plates were transferred to PCA plates to eliminate overlapping growth and encourage sporulation –front and (d) back of PDA plate. The samples of fungi stock in a PCA plate (e) is *Colletotrichum acutatum* while (f) is *Cladosporium sphaerospermum*. 58
- Figure 2.4** Arbuscular mycorrhizal fungi (AMF) colonisation on root sections under microscope at 40x magnification containing (a) arbuscules, (b) hyphae and vesicles. 60
- Figure 2.5** Rust fungus spores at 40x magnification under compound microscope. 61
- Figure 2.6** (a) Plant before flowering and ready to be harvested. (b) Plant shoots were kept in an envelope and placed in a drying cabinet for two weeks. 62
- Figure 3.1** Himalayan balsam (a) seedlings grown in a glasshouse and (b) mature plants grown in grow bags in the field. 68

Figure 3.2 Mean of (a) leaf number and (b) shoot biomass between Symbio-treated plants and control plants. $n=10$ in all treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, $*p < 0.05$ 70

Figure 3.3 Himalayan balsam grown in a Controlled Environment room. 74

Figure 3.4 Mean of (a) plant height, (b) shoot biomass and (c) AMF colonisation across treatments. $n=5$ in all treatments. Error bars are one SE. AS was *Acremonium strictum* plants, CA was *Colletotrichum acutatum* plants while GS was *Geniculosporium sp.* plants. Asterisks above bars indicate significant pairwise differences between means, $*p < 0.05$ 75

Figure 3.5 Mean of (a) plant height, (b) shoot biomass and (c) mycorrhizal colonisation across treatments. SY is Symbio inoculum while PW is Plantworks inoculum. $n=5$ across treatments. Error bars are one SE. 83

Figure 3.6 Three treatments with 10 seeds each in three different plates. (a) Clean plate in +St-Sq seeds and (b) 'juicy' liquid diffused out from +St+Sq seeds, meanwhile (c) Fungi dominance grown from -St-Sq seeds. 86

Figure 4.1 The plants were grown outside in the field to allow for natural insect attack. 95

Figure 4.2 Mean of plant height across (a) *Colletotrichum acutatum* inoculated plants, (b) *Cladosporium sphaerospermum* inoculated plants and (c) dual endophytes inoculated plants. +/-PW for Plantworks inoculum present/absent, +/-SY for Symbio inoculum present/absent and +/-E for endophytes present/absent. $n=5$ in all treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, $*p < 0.05$ and $**p < 0.001$ 98

Figure 4.3 Mean of shoot biomass across (a) *Colletotrichum acutatum* inoculated plants, (b) *Cladosporium sphaerospermum* inoculated plants and (c) dual endophytes inoculated plants. +/-PW for Plantworks inoculum present/absent, +/-SY for Symbio inoculum present/absent and +/-E for endophytes present/absent. $n=5$ in all treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, $*p < 0.05$ and $**p < 0.001$ 99

Figure 4.4 Mean of mycorrhizal colonisation across (a) *Colletotrichum acutatum* inoculated plants, (b) *Cladosporium sphaerospermum* inoculated plants and (c) dual endophytes inoculated plants. +/-PW for Plantworks inoculum present/absent, +/-SY for Symbio inoculum present/absent and +/-E for endophytes present/absent. $n=5$ in all treatments. Error bars are one SE. 102

Figure 4.5 Mean of *Aphis fabae* per plant across (a) *Colletotrichum acutatum* inoculated plants, (b) *Cladosporium sphaerospermum* inoculated plants and (c) dual endophytes inoculated plants. +/-PW for Plantworks

inoculum present/absent, +/-SY for Symbio inoculum present/absent and +/-E for endophytes present/absent. $n=5$ in all treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, $*p < 0.05$. 105

Figure 4.6 Isolation Frequency mean of *Cladosporium sphaerospermum* on (a) PW and (b) SY treated plants, and *Colletotrichum acutatum* on (c) PW and (d) SY treated plants. Legend shows the leaves were inoculated with *C. acutatum* (CA), *C. sphaerospermum* (CS), dual species (CACS) and control (C). +/-I for insects present/absent. $n=5$ in all treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, $*p < 0.05$ 111

Figure 4.7 Endophyte communities in plants that were attacked by insects and their controls across (a) PW-treated and (b) SY-treated plants. Please note difference in scales for visual clarity. Vertical axis most likely represents isolation frequency of endophyte communities while horizontal axis likely represents separation by insect presence. 114

Figure 5.1 Photos show (a) a pot was wrapped with a nylon mesh in order to reduce contamination from external mycorrhizal fungi, (b) soils were air dried in a polytunnel and (c) how the plants were sunk into field soil..... 126

Figure 5.2 Diagram showing how the treatments were set up for each phase; (a) conditioning soil in 2016 and (b) conditioned soil in 2017. +/-PW shows presence/absence of Plantworks inoculum, +/-SY shows presence/absence of Symbio inoculum and +/-C indicates conditioned/'clean' soil (see text). 127

Figure 5.3 Mean of (a) plant height and (b) shoot biomass between treated plants and their control in both phases. Blue bars represent mycorrhizas present while green bars represent mycorrhizas absent. PW is Plantworks soil and SY is Symbio soil in Phase 1. +CPW is Conditioned Plantworks soil and +CSY is Conditioned Symbio soil in Phase 2. $n=10$ in all treatments. Error bars are one SE. 130

Figure 5.4 Mean of (a) plant height and (b) shoot biomass in different soil conditions –both from the plants grown in Phase 2. PW is Plantworks soil and SY is Symbio soil. $n=10$ in all treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, $*p < 0.05$ and $**p < 0.001$ 131

Figure 5.5 Mean of (a) plant height and (b) shoot biomass of plants grown without AM in conditioning soil in phase 1 and clean soil in phase 2. -PW is without Plantworks inoculum and -SY is without Symbio inoculum. $n=10$ in all treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, $**p < 0.001$ 132

Figure 5.6 AM colonisation of treated plants and their controls in both phases. PW is Plantworks soil and SY is Symbio soil in Phase 1. +CPW is Conditioned Plantworks soil and +CSY is Conditioned Symbio soil in Phase 2.

$n=10$ in all treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, $**p < 0.001$ 136

Figure 5.7 AM colonisation in different soil conditions. Both were from the plants grown in Phase 2. PW is Plantworks soil and SY is Symbio soil. $n=10$ in all treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, $**p < 0.001$ 136

Figure 5.8 Inocula effect on endophyte fungal communities within and between the two phases of (a) Plantworks plants and (b) Symbio plants. +/-PW: Plantworks present/absent, +/-SY: Symbio present/absent and +C: Conditioned soils). Please note difference in scales for visual clarity. Vertical axis most likely represents isolation frequency of endophyte communities while horizontal axis likely shows separation by soil conditioning. 142

Figure 5.9 Conditioning of soil by balsam in the absence of (a) Plantworks and (b) Symbio affect endophyte communities. +/-PW: Plantworks present/absent, +/-SY: Symbio present/absent and +/-C: conditioned/clean soils). Please note difference in scales for visual clarity. Vertical axis most likely represents isolation frequency of endophyte communities while horizontal axis likely shows separation by soil conditioning. 143

Figure 5.10 Both inocula effect on endophyte communities and the different in the both phases. +/-PW: Plantworks present/absent, +/-SY: Symbio present/absent and +C: conditioned soils). Please note difference in scales for visual clarity. Vertical axis most likely represents isolation frequency of endophyte communities while horizontal axis likely shows separation of by soil conditioning. 144

Figure 6.1 Similar size of Himalayan balsam and co-occurring native plant species in a pot, (a) *Plantago lanceolata* and (b) *Holcus lanatus*. Photos of (c) the former plant species (Source from Online Atlas of the British and Irish Flora: <http://www.brc.ac.uk/plantatlas/plant/plantago-lanceolata>) and (d) the latter plant species (Source from Online Atlas of the British and Irish Flora: <http://www.brc.ac.uk/plantatlas/plant/holcus-lanatus>) in a field. 158

Figure 6.2 Mean of (a) height and (b) shoot biomass of balsam across treatments. Blue bars represent mycorrhizas present while green bars represent mycorrhizas absent. +HB was intraspecific competition while +PL and +HL were interspecific competition with *P. lanceolata* and *H. lanatus* respectively. 'None' indicates a single balsam with no competition as a control. $n=5$ across treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, $**p < 0.001$ 161

Figure 6.3 Shoot biomass mean of (a) *P. lanceolata* and (b) *H. lanatus* across treatments. -AM was mycorrhizal absent while +AM was mycorrhizal present. $n=5$ across treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, $**p < 0.001$ 161

Figure 6.4 AM colonisation mean of balsam across treatments. Blue bars represent mycorrhizas present while green bars represent mycorrhizas

absent. +HB was intraspecific competition while +PL and +HL were interspecific competition with *P. lanceolata* and *H. lanatus* respectively. 'None' indicates a single balsam with no competition as a control. $n=5$ across treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, $**p < 0.001$ 162

Figure 6.5 AMF colonisation mean of (a) *P. lanceolata* and (b) *H. lanatus* in interspecific competition. -AM was mycorrhizal absent while +AM was mycorrhizal present. $n=5$ across treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, $*p < 0.05$ and $**p < 0.001$ 163

Figure 7.1 Mean of (a) plant height, (b) leaf number and (c) shoot biomass across treatments. Blue bars indicate rust-treated plants while green bars indicate non-rust plants. +/-AM indicates mycorrhizal presence/absence while +/-E were *Colletotrichum acutatum* present/absent. $n=5$ in all treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, $*p < 0.05$ and $**p < 0.001$ 179

Figure 7.2 Mean of arbuscular mycorrhizal colonisation across treatments. Blue bars indicate rust-treated plants while green bars indicate non-rust plants. +/-AM indicates mycorrhizal presence/absence while +/-E were *Colletotrichum acutatum* present/absent. $n=5$ in all treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, $*p < 0.05$ and $**p < 0.001$ 180

Figure 7.3 Endophyte species communities between (a) rust, (b) endophyte and (c) AMF treated plants and their controls. Please note difference in scales for visual clarity. Vertical axis most likely represents isolation frequency of endophyte communities while horizontal axis likely shows separation by fungi infection. 184

Figure 7.4 Isolation Frequency mean of *Colletotrichum acutatum* across treatments. Legend shows the leaves were inoculated with *Co. acutatum* (+CA), or the control (-CA). +/-R represents rust was present/absent and +/-AM represents mycorrhiza was present/absent. $n=5$ in all treatments. Error bars are one SE. 185

Figure 7.5 Mean of uredinia pustule number/cm² on balsam leaves on rust-infected plants. +/-AM were mycorrhiza present/absent plants while +/-E were *Colletotrichum acutatum* present/absent plants. $n=5$ in all treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, $*p < 0.05$ and $**p < 0.001$ 186

LIST OF ABBREVIATIONS

AMF	Arbuscular Mycorrhizal Fungi
ANOVA	Analysis of Variance
CABI	Centre of Agricultural and Bioscience International
IF	Isolation Frequency
PCA	Potato Carrot Agar
PDA	Potato Dextrose Agar
PW	Plantworks
SY	Symbio

LIST OF AUTHORITIES –FUNGAL SPECIES

<i>Acremonium incoloratum</i> (Sukapure & Thirum.) W. Gams
<i>Acremonium murorum</i> (Corda) W. Gams
<i>Acremonium strictum</i> W. Gams
<i>Acrodontium hydnicola</i> (Peck) de Hoog
<i>Alternaria alternata</i> (Fr.) Keissl.
<i>Alternaria infectoria</i> E.G. Simmons
<i>Archaeospora trappei</i> (R.N. Ames & Linderman) J.B. Morton & D. Redecker
<i>Aureobasidium pullulans</i> (de Bary & Löwenthal) G. Arnaud
<i>Chaetomium cochliodes</i> Palliser
<i>Chaetomium elatum</i> Kunze
<i>Chaetomium globosum</i> Kunze
<i>Cladosporium cladosporioides</i> (Fresen.) G.A. de Vries
<i>Cladosporium oxysporum</i> Berk. & M.A. Curtis
<i>Cladosporium sphaerospermum</i> Penz.
<i>Claroideogloium claroideum</i> (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüßler
<i>Claroideogloium etunicatum</i> (W.N. Becker & Gerd.) C. Walker & A. Schüßler
<i>Clonostachys rosea</i> (Link) Schroers, Samuels, Seifert & W. Gams
<i>Colletotrichum acutatum</i> J.H. Simmonds
<i>Colletotrichum destructivum</i> O'Gara
<i>Colletotrichum gloeosporioides</i> (Penz.) Penz. & Sacc.
<i>Cronartium ribicola</i> J.C. Fisch.
<i>Didymella macrostoma</i> (Mont.) Qian Chen & L. Cai
<i>Epichloe coenophiala</i> (Morgan-Jones & W. Gams) C.W Bacon & Schardl
<i>Epichloe elymi</i> Schardl & Leuchtm.
<i>Epichloe occultans</i> (C.D. Moon, B. Scott & M.J. Chr.) Schardl
<i>Funneliformis geosporus</i> (T.H. Nicolson & Gerd.) C. Walker & A. Schüßler
<i>Funneliformis mosseae</i> (T.H. Nicolson & Gerd.) C. Walker & A. Schüßler
<i>Fusarium acuminatum</i> Ellis & Everh.

<i>Fusarium equiseti</i> (Corda) Sacc.
<i>Fusarium fujikuroi</i> Nirenberg
<i>Fusarium oxysporum</i> Schldl.
<i>Gigaspora margarita</i> W.N. Becker & I.R. Hall
<i>Gloeosporium orbiculare</i> (Berk.) Berk.
<i>Gloesporium orbiculare</i> (Berk.) Berk.
<i>Glomus deserticola</i> Trappe, Bloss & J.A. Menge
<i>Glomus monosporus</i> Gerd. & Tappe
<i>Mycosphaerella polygoni-cuspidati</i> Hara
<i>Neotyphodium lolii</i> (Latch, M.J. Chr. & Samuels) Glenn, C.W. Bacon & Hanlin
<i>Nigrospora oryzae</i> (Berk. & Broome) Petch
<i>Paraglomus brasilianum</i> (Spain & J. Miranda) J.B. Morton & D. Redecker
<i>Passalora fulva</i> (Cooke) U. Braun & Crous
<i>Phialophora cyclaminis</i> J.F.H. Beyma
<i>Phytophthora nicotianae</i> var. <i>parasitica</i> (Dastur) G.M. Waterh.
<i>Prospodium tuberculatum</i> (Kuntze) Arthur
<i>Puccinia araujiae</i> Lév.
<i>Puccinia graminis</i> Pers. F. sp. <i>tritici</i>
<i>Puccinia komarovii</i> var. <i>glanduliferae</i> R.A. Tanner, C.A. Ellison, L. Kiss & H.C. Evans
<i>Puccinia polygoni-amphibii</i> var. <i>tovariae</i> Arthur
<i>Puccinia spegazzinii</i> De Toni
<i>Rhizoctonia solani</i> J.G. Kühn
<i>Rhizoglomus microaggregatum</i> (Koske, Gemma & P.D. Olexia) Sievard., G.A. Silva & Oehl
<i>Rhizophagus aggregatus</i> (N.C. Schenck & G.S. Sm) C. Walker
<i>Rhizophagus clarus</i> (T.H. Nicolson & N.C. Schenck) C. Walker & A. Schüßler
<i>Rhizophagus intraradices</i> (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüßler
<i>Rhizophagus irregularis</i> (Błaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler
<i>Scutellospora calospora</i> (T.H. Nicolson & Gerd.) C. Walker & F.E. Sanders
<i>Sordaria fimicola</i> (Roberge ex Desm.) Ces. & De Not.
<i>Stemphylium botryosum</i> Wallr.
<i>Trichoderma viride</i> Pers.

LIST OF AUTHORITIES –PLANT SPECIES

<i>Acacia caven</i> (Molina) Molina
<i>Acer negundo</i> L.
<i>Agrostis stolonifera</i> L.
<i>Allium cepa</i> L.

<i>Araujia hortorum</i> E.Fourn.
<i>Betula pendula</i> Roth
<i>Bidens pilosa</i> L.
<i>Brachypodium pinnatum</i> (L.) P.Beauv.
<i>Bromus diandrus</i> Roth
<i>Buddleja davidii</i> Franch.
<i>Cabralea canjerana</i> (Vell.) Mart.
<i>Calotropis gigantean</i> (L.) W.T.Aiton
<i>Cardiospermum grandiflorum</i> Sw.
<i>Centaurea maculosa</i> Eichw.
<i>Centaurea solstitialis</i> L.
<i>Centaurea stoebe</i> Ten.
<i>Cirsium arvense</i> (L.) Scop.
<i>Citrullus lanatus</i> (Thunb.) Matsum. & Nakai
<i>Cucumis sativus</i> L.
<i>Dactylis glomerata</i> L.
<i>Elymus hystrix</i> M.E. Jones
<i>Eragrostis pilosa</i> (L.) P.Beauv.
<i>Fallopia japonica</i> (Houtt.) Ronse Decr.
<i>Festuca arundinacea</i> Schreb.
<i>Festuca idahoensis</i> Elmer
<i>Festuca ovina</i> L.
<i>Gossypium hirsutum</i> L.
<i>Heracleum mantegazzianum</i> Sommier & Levier
<i>Heracleum sphondylium</i> Pall.
<i>Holcus lanatus</i> L.
<i>Impatiens amphorata</i> Edgew.
<i>Impatiens amplexicaulis</i> Edgew.
<i>Impatiens brachycentra</i> Kar. & Kir.
<i>Impatiens edgeworthii</i> Hook.f.
<i>Impatiens glandulifera</i> Royle
<i>Impatiens parviflora</i> DC.
<i>Impatiens racemose</i> Hook.f. & Thomson
<i>Impatiens radiata</i> Hook.f.
<i>Impatiens thomsonii</i> Hook.f.
<i>Impatiens urticifolia</i> Wall.
<i>Lafoensia pacari</i> A.St.-Hil.
<i>Lantana camara</i> L.
<i>Leucanthemum vulgare</i> Lam.
<i>Lolium multiflorum</i> Lam.
<i>Lolium perenne</i> L.
<i>Lotus corniculatus</i> L.

Lycopersicon esculentum Mill.

Mikania micrantha Kunth

Oryza sativa L.

Paspalum distichum L.

Phaseolus vulgaris L.

Picea abies (L.) H. Karst

Pinus monticola Douglas ex D. Don

Pisum sativum L.

Plantago lanceolata L.

Poa annua Cham. & Schlttdl.

Populus nigra L.

Prunella vulgaris L.

Rubus caesius L.

Salix alba L.

Setaria glauca (L.) P. Beauv.

Stipa pulchra Hitchc.

Theobroma cacao L.

Tragopogon dubius Scop.

Tragopogon pratensis L.

Trifolium pratense L.

Trifolium subterraneum L.

Triticum aestivum L.

Urtica dioica Vell

Vicia faba L.

LIST OF AUTHORITIES –INSECT HERBIVORES

Altica himensis (LeConte)

Aphis fabae De Geer

Cassida rubiginosa Muller, O.F.

Chelymorpha alternans (Fabricius)

Helicoverpa armigera (Hubner)

Junonia coenia Hubner

Larinus minutus Gyllenhal

Mamestra brassicae

Phlogophora meticulosa (Linnaeus)

Poeciloceris pictus F.

Sitobion avenae (Fabricius)

Sitona lineatus (Linnaeus)

Taeniothrips inconsequences (Uzel)

CHAPTER 1

General Introduction

CHAPTER 1 GENERAL INTRODUCTION

1.1 Invasive Species

The main causes of increasing spread of non-native invasive plant species across the world over recent decades are trade liberalization and rapid globalization (Perrings et al. 2002). These authors claimed that human behaviour or natural range extensions (McGinley 2010) are likely to be the main point to the establishment and infestation of invasive species.

There are many inconsistent terms used to describe and define invasive species which might lead to the confusion within the field of invasive species science (Richardson et al. 2000; Ricciardi & Cohen 2007). Aliens, weeds, non-native species and invasive alien species (IAS) are usually the terms used to refer to an introduced species which poses damage, threats and negative effects on the biodiversity and ecosystem in regions outside their native range (Richardson et al. 2000; Brundu 2014; Jeschke et al. 2014).

Richardson et al. (2000) define the following terms of invasive ecology:

- Alien species: Plant taxa presence in an area due to human activity either intentionally or accidentally introduced. They are also known as 'non-native' or 'introduced species'.
- Naturalised plants: Alien plants which grow yearly without distraction to human activity or ecosystems.
- Invasive plants: Naturalised plants which produce a large number of offspring and grow far apart from parent plants.

- Weeds: Plants (not necessarily alien) that grow in unwanted areas and are likely to pose negative economic and environmental impacts. They are also known as plant pests or harmful species.
- Transformers: A subset of invasive plants that have clear ecosystem impacts. They are excessive users of resources and donors of limited resources.
- Introduced range: An area where a species has spread by human assisted intervention and is limited due to geographical barriers.
- Native range: An area where a species has naturally occurred with or without human assisted intervention.

Ricciardi & Cohen (2007) suggested the term 'invasive' species should not be used to define a species that poses a threat to the ecosystem and biodiversity. They found there is no evidence and correlation between rapid colonisation and negative impact on biodiversity. However, Mack et al. (2005) defined an invasive alien species (IAS) as one that grows and spreads rapidly in a new area and is detrimental to the environment. This fact was supported by McNeely et al. (2001), in that the impacts of IAS population and infestation on the environment and economy are significant and diverse in nature. Therefore, since balsam was brought into the UK and has exhibited negative impacts on ecosystems (Tickner et al. 2001; Hulme & Brenner, 2006; Tanner 2011), 'IAS' or 'invasive species' terms were used throughout this research and thesis to denote the introduced species.

1.2 The study species: *Impatiens glandulifera*

Impatiens glandulifera (Family: Balsaminaceae), usually known as Himalayan balsam is native to the foothills at altitudes of 2,000m above sea level (ASL) up to the timberline of the Western Himalayas (India and Pakistan) (Tanner et al. 2014) which has become established and invasive throughout UK (Beerling & Perrins 1993), mainland Europe (Pysek & Prach 1995), temperate North America (Toney et al. 1998; Clements et al. 2008), and temperate Asia (Tanner 2007). It is regarded as one of the top 20 invasive plants in the UK (Crawley 1987; Cockel & Tanner 2011).

Impatiens glandulifera is found mainly in riparian habitats such as canal band, river banks, waste ground, lake edges, damp woodland and occasionally mires (Beerling & Perrins 1993; Andrews et al. 2005; Tanner 2007) (Figure 1.1a). In an Environment Agency 2010 report, this non-native annual plant species occupied over 13% of river systems in England and Wales, and could grow up to 3m in deciduous woodland (Andrews et al. 2005) making it the tallest European annual plant (Beerling & Perrins 1993) (Figure 1.1b). *I. glandulifera* is an attractive plant with erect, usually hollow, sappy, fleshy and brittle green stems with a reddish tinge early in the year and which in summer turn from pink to red. The thick stem bases are often fringed with fleshy adventitious root at the lowest nodes (Figure 1.1c). The leaves may have a reddish midrib, arranged oppositely or in whorls of 3 – 5 (Figure 1.1d), lanceolate to lanceolate-elliptic shape and serrated margins. The inflorescences consist of 2 – 14 flowers, from purple-pink (Figure 1.1e) to (rarely) white in colour with markings and spots inside. This species has a

short adventitious root system with a distinctive structure. Flowers are zygomorphic; their lowest sepal pouch-like forming a sac that ends in a straight spur and the upper petal forming a broad two lobed standard. Each flower can grow up to 4cm long, is sweetly scented, trumpet shaped with wide petals and usually flowering from June to October. Its seed capsule is approximately 2.5cm long, hanging on red stalks and contains up to 16 seeds (Beerling & Perrins 1993). Each plant can produce up to 2500 seeds and can propel the seeds up to 10m from the parent plant through exploding seed capsules (Chapman & Gray 2012). The seeds are black in colour at maturity, large in size (Tanner 2011) and have a short life span which remain within the seed bank for up to two years (Beerling & Perrins 1993) though Mumford (1990) stated under artificial conditions and following a period of stratification at 4°C, seeds can remain viable for several years and germinate successfully.

Seed germination occurs in February and March and the last stage of cotyledon occurs in April, depending on a mild winter. From April onwards, rapid growth is seen in their maximum shoot length and total leaf area. Flowering develops from July to October and from mid-July onwards, the plants start to set seeds (Beerling & Perrins 1993). In contrast, Tanner (2011) observed seeds do not always germinate as early as February while plants flower as early as mid-June. Seed set then occurs from late July to early October until the plants are killed by the first frost.

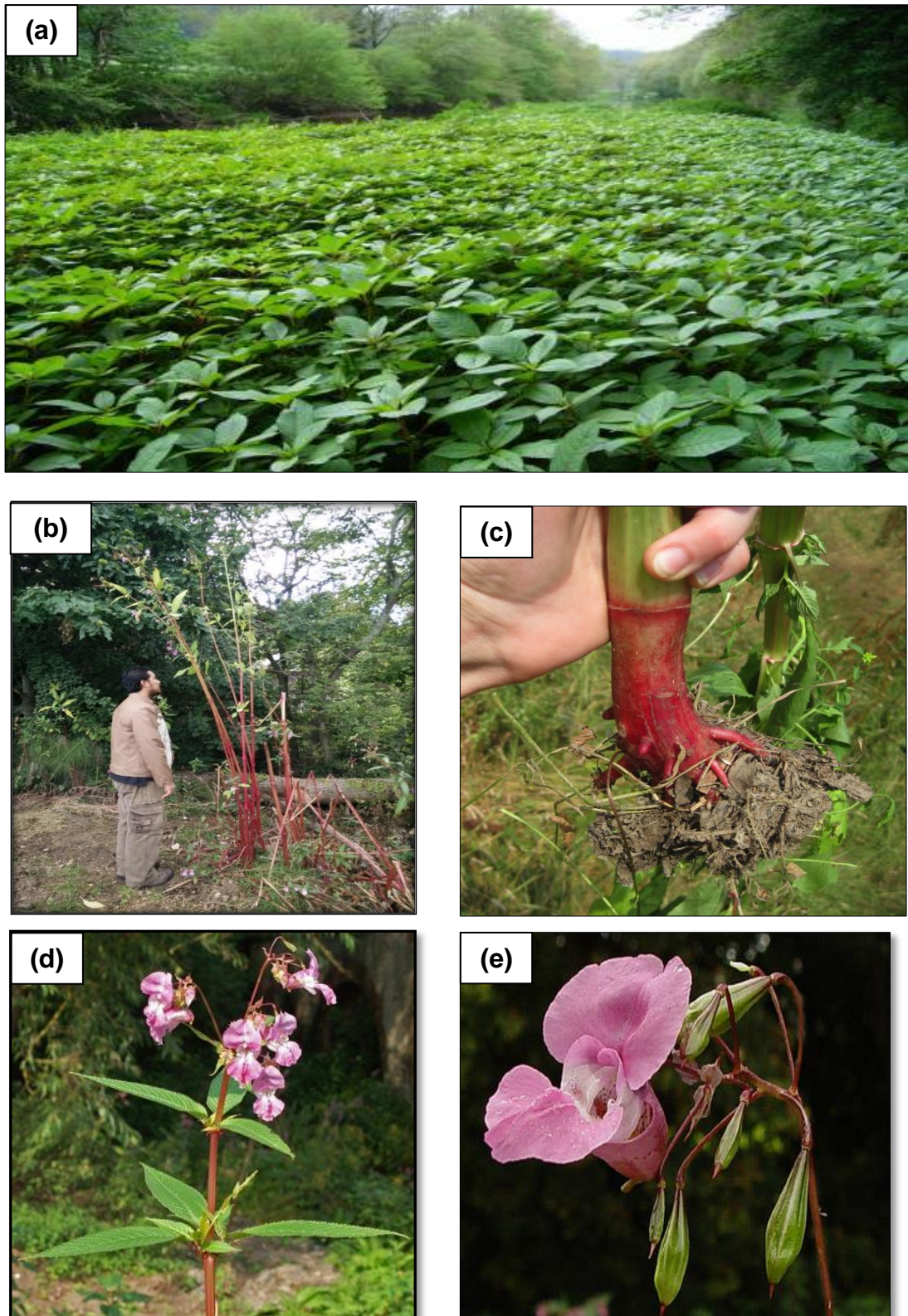


Figure 1.1 (a) Himalayan balsam population near river bank. (b) Plant height that can exceed 2m tall, (c) adventitious root system, (d) oppositely arranged and in whorls 3-5 leaves and (e) purple pink zygomorphic flower and seed capsules. All photos except (b) were courtesy from CABI staff –Carol Ellison.

1.2.1 The introduction and spread of *Impatiens glandulifera* in the UK

Impatiens glandulifera was first recorded and introduced due to its floristic purposes and aesthetic appeal in Europe by John Forbes Royle from India when he was in the United Kingdom (UK) in 1839 (Tanner 2007). However, Tanner (2011) pointed out this was probably inaccurate, as Royle also visited the UK in 1837. He stated that the seeds of *Impatiens* species (although it did not describe *I. glandulifera* clearly) were imported to England by the East India Company and the seeds were widely distributed by The Royal Horticultural Society (RHS). There are varying opinions on the year of when *I. glandulifera* was first introduced in the UK. However, Tanner (2011) then believed it is feasible that *I. glandulifera* seeds were first introduced in England in 1839 by the Horticultural Society of London (later RHS) after finding the plant in the Botanical register from 1840.

Thereupon, by 1855, seeds of *I. glandulifera* were spread out at the large Victoria gardens and unusual growth occurred leading to high populations in non-urban areas (Kent 1975). Extremely high growth rates and high seed production lead to its active infestation and distribution. However, it is restricted to high moisture habitats only (Beerling & Perrins 1993; Pysek & Prach 1993). Beerling & Perrins (1993) found that this herbaceous annual plant had infested most parts of Great Britain and Ireland as well as isolated areas such as the Isles of Scilly, Shetland and Orkney. Besides the UK, they also indicated that *I. glandulifera* can also be found along the river banks of Sweden, Czechoslovakia, Switzerland and Southern Russia (Beerling & Perrins 1993).

Perring (1970) referred to British rivers as 'balsam highways' since there are only a few river banks that have not been covered by *I. glandulifera* and that infestation had occupied over 50% of the UK's 10 x 10km recording squares (Preston et al. 2002). According to Dawson & Holland (1999), *I. glandulifera* commonly invades medium to small sized rivers with poor water quality at lower altitude. These authors stated that the seeds of *I. glandulifera* can be dispersed by water currents, as they become negatively buoyant and are carried downstream, which leads to seed germination at the bottom of the water body and successful growth in disturbed ground (Trewick & Wade 1986). Thus, this action may inhibit the seeds from spreading in the upper catchment areas which have critical efficient control (Dawson & Holland 1999). They also found other dispersal mechanisms of this non-native plant species seeds are by air turbulence, movements by animals such as cattle, human disruption either deliberately or accidentally such as recreational activities or by vehicles, and also regular vegetation maintenance by hand or machinery. These natural or non-natural distribution processes of plant seeds lead to high infestation and populations of *I. glandulifera* and may pose a major impact to the biodiversity and ecosystems (Dawson & Holland 1999).

1.2.2 Impact of *Impatiens glandulifera* infestation

According to Jeschke et al. (2014), the 'impact' term should be defined clearly and the term falls into 4 categories: (i) directionality (unidirectional or bidirectional changes), (ii) classification and measurement, (iii) ecological or socio-economic changes and (iv) scale (spatial or temporal). These authors

maintained that the term is referring to the changes caused by the non-native plant species to the area where they are introduced.

Himalayan balsam is likely to pose a major risk to the biodiversity as the plant may outcompete the native plants in order to obtain nutrient and light supply especially in accessible habitats such as national parks (Tanner 2011) leading to unidirectional changes (possibly to reduce species diversity) as mentioned by Jeschke et al. (2014). For example, balsam in damp woodland habitats may suppress other native plants (Perrins et al. 1993) and diminish species diversity and richness by up to 25% in the introduced area (Hulme & Bremner 2006). In contrast, Pyšek et al. (2012) found that due to the invasion of non-native plant species, native plant species richness and diversity are likely to be reduced whereas soil biota richness and soil nutrients as well as water concentrations tended to increase, which can be considered as bidirectional changes (possibly an increase or decrease in species diversity) as documented by Jeschke et al. (2014).

The impacts of Himalayan balsam on native plant species may occur naturally or through human disruption in an area (Jeschke et al. 2014). In a study of insect effects on plant species, Chittka & Schürkens (2001) showed that *I. glandulifera* produces a higher rate of sugar nectar production (0.47 ± 0.12 mg per flower per hour) than other European plants enabling it to lure pollinators away from native species and resulting in a decrease of as much as 50% of pollinator visits to the native plant species. Meanwhile, Hulme & Bremner (2006) claimed that the after effect of invasion by *I. glandulifera* in a human-disrupted environment is likely to result in the loss of as many as up to 15

species m⁻² of native species in an area. However, these authors still maintained there is only a small threat of this non-native plant species to other vegetation in an area.

It is estimated that £1.7 billion is spent annually on non-native species such as plant pathogens, arthropods, mammals and plant species in the British economy, with £1 million from the total cost spent on *I. glandulifera* management (Williams et al. 2010). In addition, this annual herbaceous plant may increase debris intake into the river system as the plant dies and is killed by the frost during autumn, leaving riverbanks exposed and likely leading to soil erosion (Tanner 2011). As a result, habitat niches used by invertebrates and spawning grounds for fish will potentially diminish which in turn may have negative effects on the ecology and invertebrate biodiversity (Tanner 2011). This has shown the impact of balsam infestation ecologically and economically in an introduced range.

Finally, in terms of the scale (spatial and temporal) category, Malíková & Prach (2010) reported that *I. glandulifera* has occupied up to 76.8% of the length of Czech Republic rivers since 1990. These authors focused on the invasion of *I. glandulifera* at four rivers of comparable size from the time they were first recorded and as a result, there were no changes on the balsam abundance along the river banks. However, since balsam can spread and invade an area at a rapid rate, this annual species is able to populate and dominate continuously and quickly within 100 years, from the first localities downstream to large areas of the Czech Republic river banks; externally and internally as well as upstream areas (Malíková & Prach 2010).

Additionally, in multi-site removal and addition experiments, native plant species were not affected whether balsam was removed or added in the field (Prowse 2001), however, balsam did reduce the growth and occurrence of *Urtica dioica* in a riparian system in the UK (Tickner et al. 2001). In mature woodland in the north of England, balsam successfully outcompeted the native plants, including tree seedlings, which potentially may inhibit the next generation of woodlands (Maule et al. 2000). However, in a woodland area in Germany, there were no impacts of balsam on established tree seedlings of silver Birch, *Betula pendula* and Norway spruce, *Picea abies* (Ammer et al. 2011). This has shown that effective management is urgently needed to control the aggressive populations and infestation of *I. glandulifera* to ensure the ecosystems and biodiversity are in a good state.

1.3 Biological Control

According to Driesche et al. (2008), there are several definitions of biological control which depend on their purposes and intention. Classical biological control (CBC) is likely to be applied permanently on a non-native invasive plant species in a large area for ecological changes, especially against pests of natural areas (forests, wetlands), urban areas and agricultural areas. This is carried out by attacking the non-native plant species using a new species of natural enemy (fungi, pathogens, insects or herbivores) which are found in the site of origin of the pest or weed. This method must be conducted for the advantage of the public rather than for individuals (Driesche et al. 2008).

Driesche et al. (2008) identified that a conservation biological control strategy is feasible to temporarily kill the plant pests on either native plants or non-native plants in crop production areas. This approach is only applicable to a certain and specific location and time depending on the target plant's population. This method begins with natural control by preserving any natural enemies in the field to suppress the pests which may be strengthened by the soils, crops or other vegetation. If this method is still insufficient, augmentation biological control may be applied by providing the right and suitable natural enemy species to suppress the pest population. Biopesticides which contain pathogens may be applied on crops too if there are any additional pests (Driesche et al. 2008).

The advantage of using biological control over chemical control is that the biological control agents can be target according to the genetic variation in the plant host (Evans 1998) compared to weed resistance problems when using pesticides (Holt & Hochberg 1997). Biological control of invasive plant species using fungal pathogens has been well documented and widely used (Trujillo 2005; Ellison et al. 2006; Ellison et al. 2008; Tanner, Ellison, et al. 2015; Anderson et al. 2016; Fourie & Wood 2018) as a weed management strategy. Plant-fungal interactions in a biological control approach are described further in the next section and Chapter 7.

Throughout this study, CBC method was selected by using a rust fungus, *Puccinia komarovii* var. *glanduliferae* to control *I. glandulifera* growth. It is probably impossible to kill all balsam populations in the UK since we could not apply and spread herbicide that might inadvertently kill native plants and

aquatic organisms. However, effective ways to control and suppress their growth are required since the plant infestation and population is expanding year by year (Malíková & Prach 2010).

1.3.1 CBC management of *Impatiens glandulifera*

In recent years, there has been much interest in applying CBC against *I. glandulifera* (Shaw & Tanner 2008) due to time consuming, labour intensive and geographically limited access to conventional techniques (manually; cutting and hand pulling) (Tanner 2007). In addition, cutting the target plants must be done carefully below the lowest node to prevent seed set (Howell 2002). Furthermore, applying chemical control may damage the environment. Although Glyphosate® application is effective against Himalayan balsam, it will also kill other plants in the surrounding areas (Stensones & Garnett 1994), while 2,4-D amine can only be applied when the plant is at the rosette stage in early spring (Environment Agency 2003). Therefore due to these factors, studies on biological control programmes against this non-native plant species have been conducted since 2006 (Tanner et al. 2011).

Himalayan balsam should be susceptible to a CBC strategy since this species has few specific natural enemies in its introduced range (Shaw 2003). However, to date, there are few studies on CBC for the management of *I. glandulifera*. Research conducted by Tanner et al. (2015b) proposed a rust fungus, *P. komarovii* var. *glanduliferae* as a potential CBC agent which is likely to inhibit balsam growth. Detailed studies that have led this rust pathogen becoming the most suitable CBC agent are described further in Chapter 7.

1.3.2 Rust fungus, *Puccinia komarovii* var. *glanduliferae*

Classical biological control of invasive plant species in Europe is still in the early stages compared to the other geographical regions such as Australia, South Africa and North America (Cock et al. 2010). However, UK Government departments (Shaw 2003) and European funding bodies (Cock & Seier 2007) interested in CBC have increased in recent decades (Cortat et al. 2010). It is suggested that the reason why CBC using fungal species is slow is because of the concern of transportation of plant pathogens between countries (Evans et al. 2001) and the lack of clear procedures for licencing fungal biological control agents (Seier 2005; Sheppard et al. 2006). Therefore, this has favoured scientists and researchers to use arthropods biological control agents instead.

An understanding of biogeographical range, life cycle and reviewing herbarium samples of the target species, and also in-country support is essential to provide detailed information and successful delivery of the biological control programme (Tanner 2011). Thus, Centre of Agriculture and Bioscience International (CABI) Egham, UK have collaborated with the National Bureau of Plant Genetic Resources, New Delhi Under a MoU titled 'The study of biological control of invasive plant species and Indian natural enemies' in order to conduct biological surveys in India and to provide a clear understanding of *P. komarovii* in the native range (Tanner 2011).

Puccinia komarovii was first identified on *Impatiens parviflora* and *Impatiens amphorata* in their native range, in Central Asia and the northern Himalayas respectively in 1904 (Piskorz & Klimko 2006; Tanner et al. 2015a). Since then,

the number of *P. komarovii* hosts on *Impatiens* species are increasing, namely: *Impatiens amplexicaulis*, *Impatiens urticifolia* (Zhuang & Wei 1994); *Impatiens brachycentra* (Afshan et al. 2012); *Impatiens edgeworthii*, *Impatiens racemosa*, *Impatiens radiata* (Iqbal & Khalid 1996) and *Impatiens thomsonii* (Arthur & Cummins 1933). In addition, there are a few unknown *Impatiens* species found in the foothills of the Himalayas which have been identified as hosts of *P. komarovii* (Arthur & Cummins 1933; Iqbal & Khalid 1996).

In July 2008, *P. komarovii* was first found infecting *I. glandulifera* populations in their native (Himalayas) (Tanner et al. 2015b). At the beginning, the authors only found a few plants which had been infected by an aecial stage of rust in high altitude areas. Following an in-depth observation, under favourable climate, they found a large number of patches of the aecial stage infecting *I. glandulifera* seedlings (6 cm – 18 cm) resulting in more than 50% of the plants being infected in June and July between 2009 – 2010. In 2010, rust infected plants were imported by CABI into the UK by a high-level quarantine facility under the Department of Environment, Food and Rural Affairs (DEFRA). They were either live seedlings or as dried material preserved for evaluation and following detailed study on *P. komarovii* as a CBC agent, the fungus was released for the first time in the introduced range in August 2014 (Tanner et al. 2015a).

Previously, *P. komarovii* was known as an autoecious and host specific rust which infected only *I. parviflora*. However after host range testing, based on molecular and cross-inoculation studies by Tanner et al. (2015b), the rust fungus on *I. glandulifera* was separated at the varietal level. The authors

indicated there is clear separation of two rust strains of *I. glandulifera* (from India) and *I. parviflora* (from Hungary and China) in analysis of the nrDNA ITS and 28S (LSU) regions. In addition, *I. glandulifera* indicated immunity in cross inoculations of *P. komarovii* ex *I. parviflora* and vice versa in establishing the presence of species-specific rust pathotypes. Therefore, based on these two factors, the authors proposed the rust on *I. glandulifera* as *Puccinia komarovii* var. *glanduliferae*, an autoecious and fully-cycled (macrocyclic) rust (Tanner et al. 2015b). This is consistent with the study by Tanner (2011) who established the life cycle of rust fungus *P. komarovii* var. *glanduliferae* (Table 1.1), based on the field observations in the native range and the experiments conducted under quarantine conditions. For a few weeks after seed germination, the infected plants which are in aecial stage probably face premature death due to no setting of seeds and the reduction of their height, hence they are smaller and less healthy than uninfected plants. Aecia-infected plants will probably collapse due to secondary infections and split open hypocotyls, which are hard to find in late July and August (Figure 1.2).

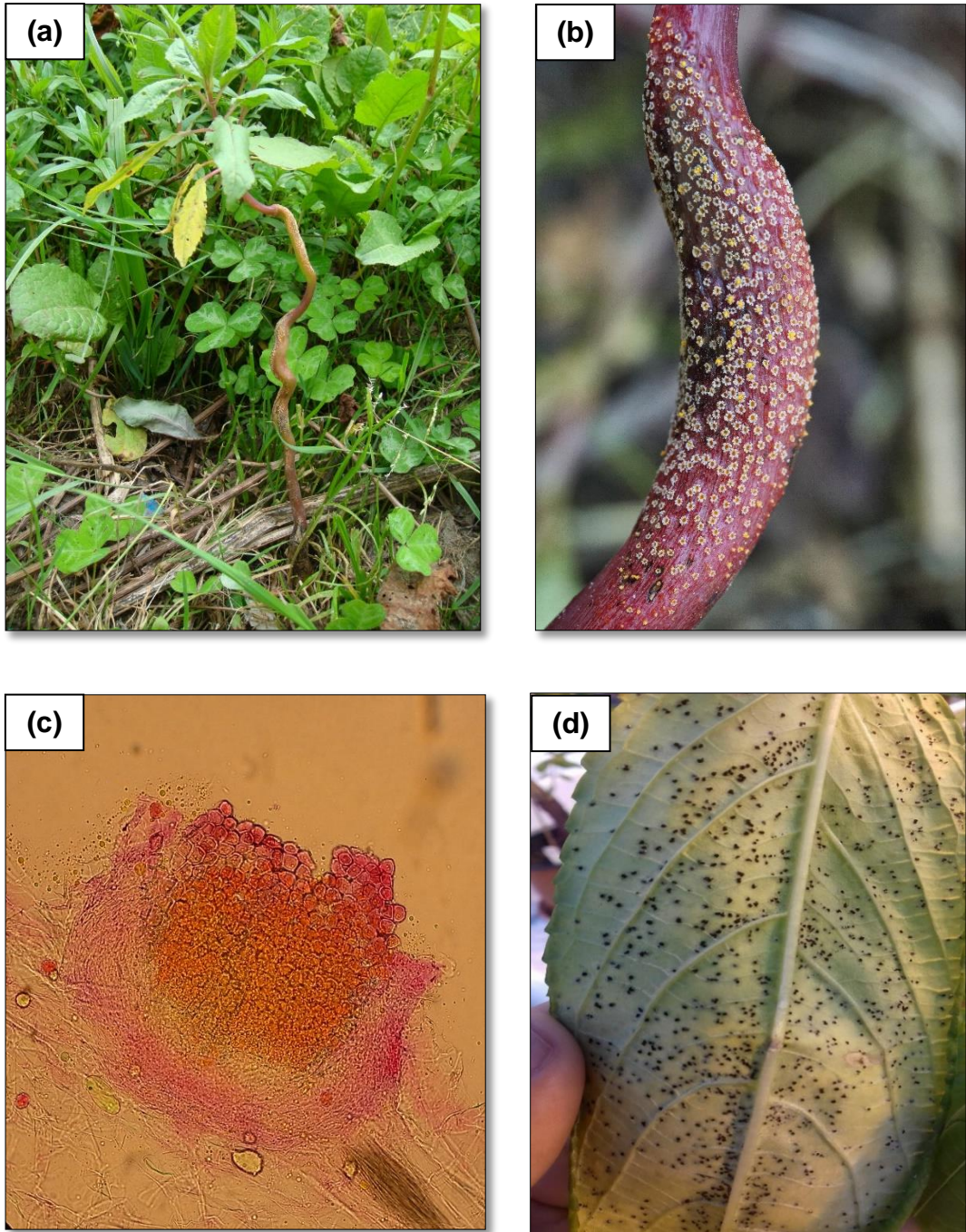


Figure 1.2 (a) Aecial cups infection on hypocotyls and (b) their close-up image. (c) Transverse section of aecial cups under microscope. The photos were courtesy of CABl staff. Two former photos were from Nobeert Maczey while the latter was from Carol Ellison. (d) Urediniospores infection on the abaxial leaf surface.

Table 1.1 The proposed life cycle of *Puccinia komarovii* var *glanduliferae* (Tanner, 2011)

Period (Season)	Life cycle of <i>Puccinia komarovii</i> on <i>Impatiens glandulifera</i> growth
Oct – March (Winter)	Teliospores and balsam seeds are in dormancy state below the soil surface and probably under snow.
April – May (Spring)	From previous season’s leaf litter infected with teliospores, seedlings, as well as teliospores emerge and grow as the increase of temperature and light, snow starts to melt, providing sufficient water for germination. Subsequently, the release of basidiospores infect the hypocotyls of young seedlings. These infected plants produce spermagonia and later aecia on the stems of young plants.
June – July (Summer)	Aecial cups which appear on the surface containing aeciospores , are dispersed by wind current and rain splash. They infect the leaves and encourage the hypocotyl to grow longer and taller than those uninfected plants, resulting in the boost up of the aecia above the canopy. This would support the dispersion of aeciospores to other individuals. Later on, there is an incubation period within the leaves followed by production of urediniospores by uredinia at the under surface of the leaves. Urediniospores spread locally to infect leaves of other individuals within populations and its dispersal is enhanced by wind currents. Within this crucial cycling stage, there is more than one generation involved and if the rust is close to epidermis area and in patchy forms, it probably may injure the plants and bring detrimental impact on the infected plants.
Aug – Sept (Autumn)	The formation of teliospores could be developed and they are enhanced by the cool temperature and potential chemical reaction in the aging leaves. Teliospores are released as the plant begins to die and drop the leaves with telia attached to it or discharged from the telia into the soil. <i>Impatiens glandulifera</i> release and spread the seeds to the entire population.

1.4 Arbuscular Mycorrhizal Fungi (AMF)

Plant species consume and obtain essential resources from the microbial diversity in soil which in turn can successfully improve plant development and increase the diversity and composition of above and below-ground invertebrate and fungal communities (van der Putten et al. 2009). Arbuscular mycorrhizal fungi (AMF) within the root system form a mutualistic relationship with plants through arbuscules, a branching tree-like structure which helps plants to absorb water and phosphate from the soil (Jakobsen et al. 1992; Smith & Read 1997). In return, carbon is transferred from plants to AMF, mostly across the arbuscular membrane and with some across the hyphal wall within the root (Smith & Read 1997; Brundrett 2002). Vesicles, globular or round segmented structures form in a root and are thought to be food storage organs, being full of lipid (Smith, & Read 1997).

The infestation of non-native plant species that are associated with and dependent on AMF in a new habitat may lead to a competition with native plant species as reported by Harner et al (2010). These authors claimed there is high colonisation and establishment of a non-native species, *Centaurea stoebe* in a riparian system in the United States whereas Marler et al (1999) showed that AMF presence enabled *Centaurea maculosa* to grow well and compete with the native plant species, *Festuca idahoensis* in rangelands in Western North America. In contrast, there are also non-native plant species that have a low dependency on AMF and this probably reduces the network penetration of AMF hyphae within the invaded areas compared to the uninvaded areas (Vogelsang et al. 2006) especially during plant colonisation

in a new habitat (Harley & Harley 1987). *I. glandulifera* is a non-native plant species that has been reported as being sparsely colonised by AMF (Beerling & Perrins 1993). Since *I. glandulifera* has sparse associations with AMF, the plant is likely to infest areas where degradation of AMF has occurred naturally, or prior to invasion of other plant species with non or low mycorrhizal dependency (Reinhart & Callaway 2006). These authors also believed that an AMF network, which is potentially poor and imperfect, develops when *I. glandulifera* forms monocultures, which in turn reduces the chances of native plant species colonisation. As a result, *I. glandulifera* might invade and dominate the areas due to the lack of competition (Reinhart & Callaway 2006).

On the other hand, AMF colonisation may reduce *I. glandulifera* growth since there appears to be a negative relationship between mycorrhizal colonisation and plant height and total leaf area in the introduced range (Tanner et al. 2014). This may simply be due to the cost of the association of *I. glandulifera* with new and unsuitable AMF (Tanner et al. 2014). To the best of our knowledge, no previous study had been carried out on the relationship between AMF colonisation and balsam growth and plant size. Thus, this study was initiated to identify the effect of AMF colonisation on plant growth.

1.5 Endophytic Fungi

Endophytes are usually referred as fungi or bacteria that inhabit the interior part of living tissues of plants and present symptomless infections on plant hosts (Rodriguez et al. 2009). To date, fungal endophytes are well reported in grass (Latch 1993; Rudgers et al. 2009; Rudgers & Orr 2009; Larimer et al.

2012) and forb species (Gange et al. 2007; Wearn et al. 2012; Aschehoug et al. 2014; Hodgson et al. 2014), since the fungi are capable of protecting plants from herbivores and other plant pathogens, which are important in economic and ecological aspects. Endophytes of Balansiaceae that are present in grasses, sedges and rushes are vertically transmitted from the parent generation to the offspring through seeds, whereas endophytes in non-woody, herbaceous and forb species are commonly transmitted by air-borne spores, known as horizontal transmission (Currie et al. 2014).

Endophytes found in forbs seem to show a lack of host specificity with the same fungal species colonising all plants that grow closely in the same population (Petrini 1986). However, this contrasts with the study by Gange et al. (2007) who found the fungus, *Cladosporium cladosporioides* had higher isolation frequency in *Cirsium arvense* plants, compared to the closely related, *Leucanthemum vulgare* when plants were growing adjacent to each other. This is probably due to the excellent performance of fungal growth within *C. arvense* or that the fungus is more able to colonise this plant species. However, the opposite situation seems to occur for *L. vulgare*, which was colonised frequently by *Acremonium murorum*. Perhaps the fungi penetrate plants through the damaged tissues caused by insect feeding or through epidermis and stomata which are the most feasible entry routes (Gange et al. 2007). These authors also suggested that the structure of the endophyte assemblage and interactions with other organisms are determined by the order of their colonisation within a plant species.

The endophytic fungus *Alternaria alternata* showed higher isolation frequency in gall material than leaves in dry and moist habitats, showing its tendency to increase the growth of gall tissues (Gange et al. 2002b). These authors suggested it is possible that the fungus suppresses other fungal species in the gall tissues. In addition, a study showed that *Chaetomium cochliodes* is capable of producing secondary metabolites which move systemically in the plant and spread from inoculated leaves to new leaves (Hartley et al. 2015). The impact on foliar chemical composition and infection was better in newly colonised leaves compared to the old leaves, shown by significant changes in ten metabolite compounds. However, the greater infection in new leaves was possibly because the plants were in defensive mode towards endophytes in old leaves (Hartley et al. 2015).

To our current knowledge, there are no studies on the effects and chemical changes of endophytic fungi on balsam growth. Recent studies have established that balsam generates positive plant soil feedbacks which alter nutrient levels as well as influencing and manipulating soil microbial and fungal endophytic communities in its invaded range (Pattison et al. 2016). In return, plants could possibly be more resistant and well defended against natural enemies and any control agents. Therefore, throughout this study, single or combinations of endophytes into balsam leaves were conducted in order to examine their effects on plant growth and to study whether these fungi enhance rust fungus effectiveness.

1.6 Multiple Fungal Interactions

1.6.1 Interaction of AM Fungi and Plant Pathogens

Arbuscular mycorrhizal fungi are well documented as being able to protect their hosts against plant pathogens (Linderman 1994; Azcon-Aguilar & Barea 1996; Fusconi et al. 1999; Borowicz 2001; Sikes et al. 2009; Wang et al. 2018). For example, a study has shown that the AMF, *Funneliformis mosseae* could protect tomato, *Lycopersicon esculentum* against infection by *Phytophthora nicotianae* var *parasitica* (*P. parasitica*) by recording smaller necrosis percentage when mycorrhizas and pathogen were added compared to the AMF-free plants (Fusconi et al. 1999). Similar results were found when *F. mosseae* protected tomato against the air-borne fungal pathogen, *Passalora fulva* by maintaining photosynthetic activity compared to the control (Wang et al. 2018).

A single inoculation of *F. mosseae* inhibited the development of the airborne pathogen, *Gloesporium orbiculare* in cucumber, *Cucumis sativus* and was more effective when combined with the plant growth-promoting fungus (PGPF) *Fusarium equiseti* by increasing the plant weight (Saldajeno & Hyakumachi 2011). However, Chandanie et al. (2006) found no significant effect on disease development and growth rate in cucumber when *F. mosseae* was added alone compared to the non-treated plants although the leaf disease symptoms were increased. However, a combined inoculation of AM with PGPF, *Phoma* sp. isolate (GS8-2) was effective in suppressing the disease symptoms. The reason for contrasting results with the usage of the same mycorrhiza are unknown although both studies used the same commercial inoculum which is

from Japan, and the plants were challenged with the same pathogen when four weeks old.

Interestingly, plant and fungal identity can be important factors in determining AMF-mediated host tolerance to a pathogen (Newsham et al. 1995; Sikes et al. 2009) instead of AMF species richness (Lewandowski et al. 2013). Furthermore, a study showed that a plant with a complex root system, *Setaria glauca*, is more susceptible to pathogen infection by *Fusarium oxysporum* compared to a plant with a simple root system, *Allium cepa*. There were no differences of pathogen infection on *A. cepa* roots irrespective of whether the pathogen was added alone or when the pathogen was combined with AM from Glomeraceae and Gigasporaceae family. In contrast, there were severe infections in *S. glauca* roots when the pathogen was added alone and in combination with members of Gigasporaceae, but the plant was protected when combined with the members of Glomeraceae (Sikes et al. 2009). Similarly, the AMF, *Rhizophagus clarus* protected a daisy, *Leucanthemum vulgare* from a plant root pathogen, *Rhizoctonia solani* whether single or in the mixture, while other mycorrhizal species, *Claroideoglossum etunicatum* and *Rhizophagus intraradices* did not reduce the deleterious effects. It is also interesting to note that the presence of the pathogen increased the number of arbuscules in the daisy (Lewandowski et al. 2013).

This has shown that network penetration of AMF hyphae and their identities plays an important role in plant growth and plant communities as well as soil health and associated native species (Gange & Ayres 1999; Gange et al. 1993) as up to 80% of plant species are thought to be dependent on

mycorrhizal fungi (Jeffries et al. 2003). The occurrence of AMF generally exerts positive effects on the host against plant pathogens, and has made these fungi potential biological control agents in crop plantations such as tomato, pea, pepper and cucumber (Siddiqui & Akhtar 2008) via effective bioprotection mechanisms whether working separately and/or together (Harrier & Watson 2004).

Currently, no study has been conducted on the interactions between mycorrhizal fungi and a plant pathogen in Himalayan balsam. Therefore, to support the finding and examine the effectiveness of the rust fungus, *Puccinia komarovii* var. *glanduliferae* as a CBC agent of balsam (Tanner et al. 2015b), it is important to understand their interactions to provide information to reinforce the management of this invasive species.

1.6.2 Interactions of AM and Endophytic Fungi

Plants associate with multiple microbial communities throughout their growth and development. Although the interactions between endophytic and mycorrhizal fungi are likely to be common in nature, only a few studies have been documented in grasses (Chu-Chou et al. 1992; Omacini et al. 2006; Mack & Rudgers 2008; Larimer et al. 2012) while more limited studies have been conducted in forb species (Eschen et al. 2010). *Funneliformis mosseae* enhanced endophyte, *Epichloe elymi* growth by increasing tiller production and in return, the latter fungus increased AMF colonisation in a host grass, *Elymus hystrix* (Larimer et al. 2012). This has shown that the host may be unable to accommodate both symbionts simultaneously and that the

endophyte effect varies between AMF species as the endophyte reduced *Claroideoglossum claroideum* colonisation (Larimer et al. 2012). Similarly in forbs, endophyte species can positively or negatively affect the abundance of arbuscules in *Cirsium arvense*, while the mycorrhizal fungi can alter the composition of endophyte fungal communities through nutrient availability (Eschen et al. 2010).

Epichloe coenophiala endophytes have been shown to suppress mycorrhizal colonisation in tall fescue, *Festuca arundinacea* plants (Chu-Chou et al. 1992; Mack & Rudgers 2008). This is probably because of a few mechanisms which were; (a) endophyte density could alter nutrient requirement by host plants by increasing root surface area for nutrient absorption that indirectly affected AMF, (b) as endophytes inhabit shoots, it gave spatial priority to this fungus to gain more carbon compared to AMF (c) endophyte gains a temporal priority as it is vertically transmitted compared to horizontally transmitted mycorrhizal fungi (Mack & Rudgers 2008) and (d) because of toxic metabolites (secondary metabolites and flavonoids) produced by the endophytic fungus that reduce the AM fungal establishment (Chu-Chou et al. 1992). Similarly, in a monoculture of endophyte-inoculated (+E) cool-season grass species, *Lolium multiflorum*, a fungal endophyte, *Epichloe occulta* negatively affected the mycorrhizal colonisation (Omacini et al. 2006). However, when in a mixture of +E and endophyte-free (-E) plants, the former plants increased the AMF colonisation in neighbouring plants. This was probably because of the resistance on systemic induction in +E plants by diverting the resources to the symbiotic endophyte that improved host growth rather than to the other competitors for resources, mycorrhizal fungi (Omacini et al. 2006).

To date, no published study has examined the manipulation of both fungi and their interactions in balsam. Therefore, to provide an important understanding of microbial community interactions in balsam, a study on their interactions with presence and absence of both fungi was conducted as described in Chapters 4 and 7.

1.6.3 Interactions of Endophytic Fungi and Plant Pathogens

Endophytes are known to benefit host plant growth and defend against herbivores (Gange et al. 2007; Gange et al. 2012) and pathogens (Busby et al. 2015). Unlike the relationship between mycorrhizas and plant pathogens that were well documented (Linderman 1994; Azcon-Aguilar & Barea 1996; Fusconi et al. 1999; Borowicz 2001; Sikes et al. 2009; Wang et al. 2018), the interactions between fungal endophytes and plant pathogens is more poorly known (Gao et al. 2010), especially in forb and invasive species. Therefore, to understand the efficacy of the rust fungus as a CBC agent on balsam (Tanner et al. 2015b), the interactions of endophytic fungal communities and the plant pathogen is important to explore.

One study found that endophyte species (*Colletotrichum sp*, *Xylaria sp* and *Fusarium sp.*) have the ability to protect cacao, *Theobroma cacao* by decreasing leaf necrosis percentage and leaf mortality when the seedlings were challenged with a pathogen, *Phytophthora sp* (Arnold et al. 2003). The protection was greater in mature leaves than young leaves and *in vitro* studies suggested that leaf chemistry mediated the protection by direct interactions of endophyte with foliar pathogens (Arnold et al. 2003). Fungal endophytes from

Pinus monticola mediated host resistance and survival against white pine blister rust, *Cronartium ribicola* (Ganley et al. 2008) and similarly, endophytes were suggested to suppress pathogens in cereal production (O'Hanlon et al. 2012). Gao et al. (2010) has suggested there were direct and indirect effects that have triggered endophyte inhibition of plant pathogens. For example, endophytic fungi directly produced antibiotics and lytic enzyme which strongly suppressed the growth of other microorganisms including pathogens (Gunatilaka 2012) in host plants, while the endophytic fungi also indirectly activated plant defence mechanisms to resist infection of pathogen (Gao et al. 2010).

1.6.4 Interaction of AM, Endophytic Fungi and Plant Pathogens

To date, to the best of my knowledge, no published study has been conducted on the interactions of AMF, endophytes and a plant pathogen in a host plant. Therefore, to achieve the main objective of this research which was studying the factors that affect rust fungus as a CBC agent, a study using mycorrhizas, an endophyte (*Colletotrichum acutatum*) and the rust fungus in balsam was conducted as described in Chapter 7.

1.7 Aims, Objectives and Hypotheses

The main aims of this research were to determine the effect of insects and multiple fungal interactions on Himalayan balsam growth and performance and to examine the factors that may affect the biological control of this invasive

plant. The objectives of this research along with the associated hypotheses tested are detailed below:

Objective 1: The interaction between AMF, endophyte and invertebrates in Himalayan balsam.

In Chapter 4, the main objective was to study how insects, mycorrhizas and endophytes affect balsam performance. The second objective was to evaluate how the foliar endophyte communities in balsam plants were influenced by insects. The hypothesis to be tested in this chapter was that the multiple interactions would impact plant growth and the composition of the foliar endophytic communities, but that interactions would depend upon the identity of the fungal species.

Objective 2: The interaction between balsam and associated microbial communities through plant soil feedbacks.

In Chapter 5, the main objective was to study the effect of mycorrhizas on balsam performance in the presence of commercial inocula, compared with plants that were grown in the soil that had previously supported the plant. The second objective was to study the differences of foliar endophyte communities in plants that grew in 'clean' soil compared with that which had previously supported balsam plants. The hypothesis to be tested in this study was that Himalayan balsam grown in a balsam soil may show different plant performance and would display different above and below ground microbial communities, through the process of plant-soil feedback.

Objective 3: Mycorrhizal fungal effect on balsam competitive interactions.

In Chapter 6, the main objective was to determine how AMF affect balsam performance in competitive environments and how this impacts on foliar fungal communities. The hypothesis to be tested was that mycorrhizal colonisation would reduce balsam growth and fungal communities when in a monoculture, but that this may differ when it co-occurs with native plant species.

Objective 4: Multiple fungal effect on classical biological control of Himalayan balsam

In Chapter 7, the main objective was to study the effect of the interactions between AMF, endophytic fungi and the rust fungus on balsam growth. The second objective was to determine the effect of multiple fungal infections on above and below ground communities. The hypothesis was that the fungal interactions would seriously impact on rust effectiveness as a CBC agent on balsam. This is because of induced chemical changes in the host plant.

CHAPTER 2

General Methods

CHAPTER 2 GENERAL METHODS

2.1 Seed Germination

Ripe *Impatiens glandulifera* seeds (Figure 2.1a) were collected from wild populations at Harmondsworth Moor, Middlesex, UK: 51°29 '58.2N, 000° 29.02.3"E in September and October yearly (2015-2018). The seeds were air dried in a tray for one week in the laboratory then stored in a sealed bag in a 4°C refrigerator for nine months until used in the experiments.

At the start of each experiment, the seeds were soaked in 5% ethanol (5% v/v: 5ml ethanol in 100ml distilled water) for 15 minutes to soften the seed capsule for rapid germination and to eliminate pathogenic fungi. These seeds were sown on moistened filter paper with sterile distilled water in a 9cm diameter Petri plate and the lids were sealed in position with parafilm. The plates were placed in the 4°C refrigerator for 8 weeks to allow germination. Later, the germinating seeds (Figure 2.1b) were planted in seed trays containing non-sterile John Innes No. 2 compost (Keith Singleton, Egremont, UK) covered with a propagator hood (Figure 2.1c) in a glasshouse to enhance propagation. After one week, when the hypocotyls were emerging, the hoods were removed (Figure 2.1d).

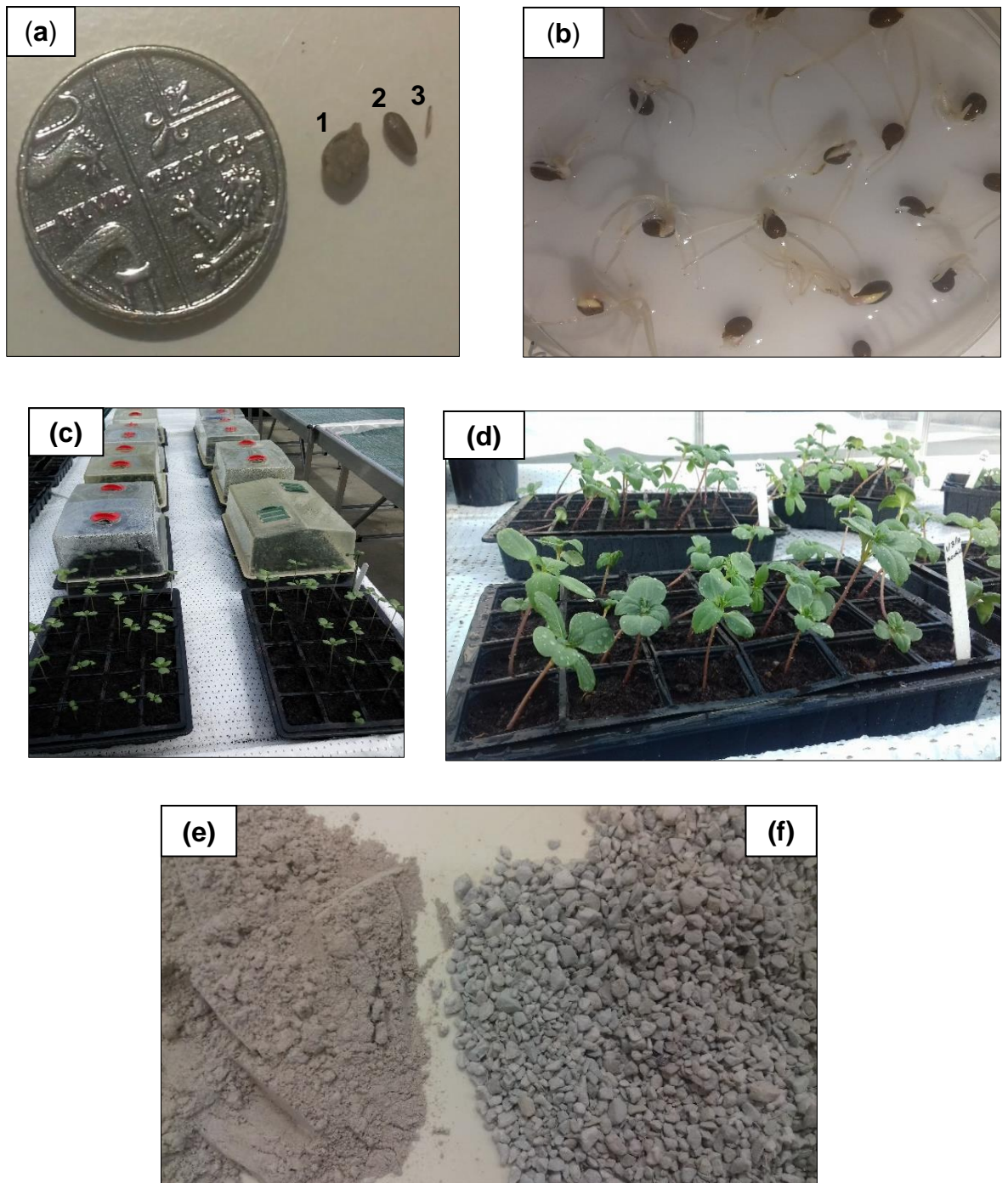


Figure 2.1 (a) Size of balsam seed (no 1) compared to 5 pence and other native plant seeds (*Plantago lanceolata* is number 2 and *Holcus lanatus* is number 3). (b) Germinating balsam seeds in a sealed Petri plate. (c) Germinating seeds were sown in the seed trays covered with a propagator hood and (d) removed the hood a week after. Different commercial mycorrhizal features, (e) Symbio inoculum in a powder form while (f) Plantworks inoculum in a granular form.

After 4 weeks when the seedlings were strong and producing true leaves, they were potted into 2L pots containing John Innes Type 2 compost and the roots were surrounded by live mycorrhizal inoculum treatment, as appropriate. As recommended by the companies, 7.5g of Plantworks inoculum (Sittingbourne, Kent) and 2g of Symbio product (Wormley, Surrey) were added in each 1L pot (Figure 2.1e and 2.1f) while sterile products were autoclaved at 121°C for 30 minutes and used for control plants.

2.2 Endophyte Inoculation

The seedlings were selected for inoculation when the plants were at the three whorl leaf stage (Figure 2.2a) and of a similar height. The endophyte spores were harvested from the culture plate (Figure 2.2c and 2.2d) using a sterile needle in a laminar flow cabinet and the concentration was adjusted using a haemocytometer to approximately 1.5×10^5 spores ml^{-1} in 0.05% Tween 80. The leaves were inoculated by spraying the spores suspension on the abaxial side of the leaves (9-11 leaves per plant), in two strokes (ca. 550 μl /leaf) using a handheld bottle sprayer. Control plants were inoculated with 0.05% Tween 80 only. After spraying the spore suspension, the plants were placed in a Perspex box (100cm x 100cm x 100cm) (Figure 2.2b) for 48h to provide high humidity environment and allow the spores to germinate. Between each treatment, the Perspex box was cleaned thoroughly using a 100% ethanol to avoid contamination from the previous test. After 48h, the plants were removed from the box and were placed in the glasshouse.

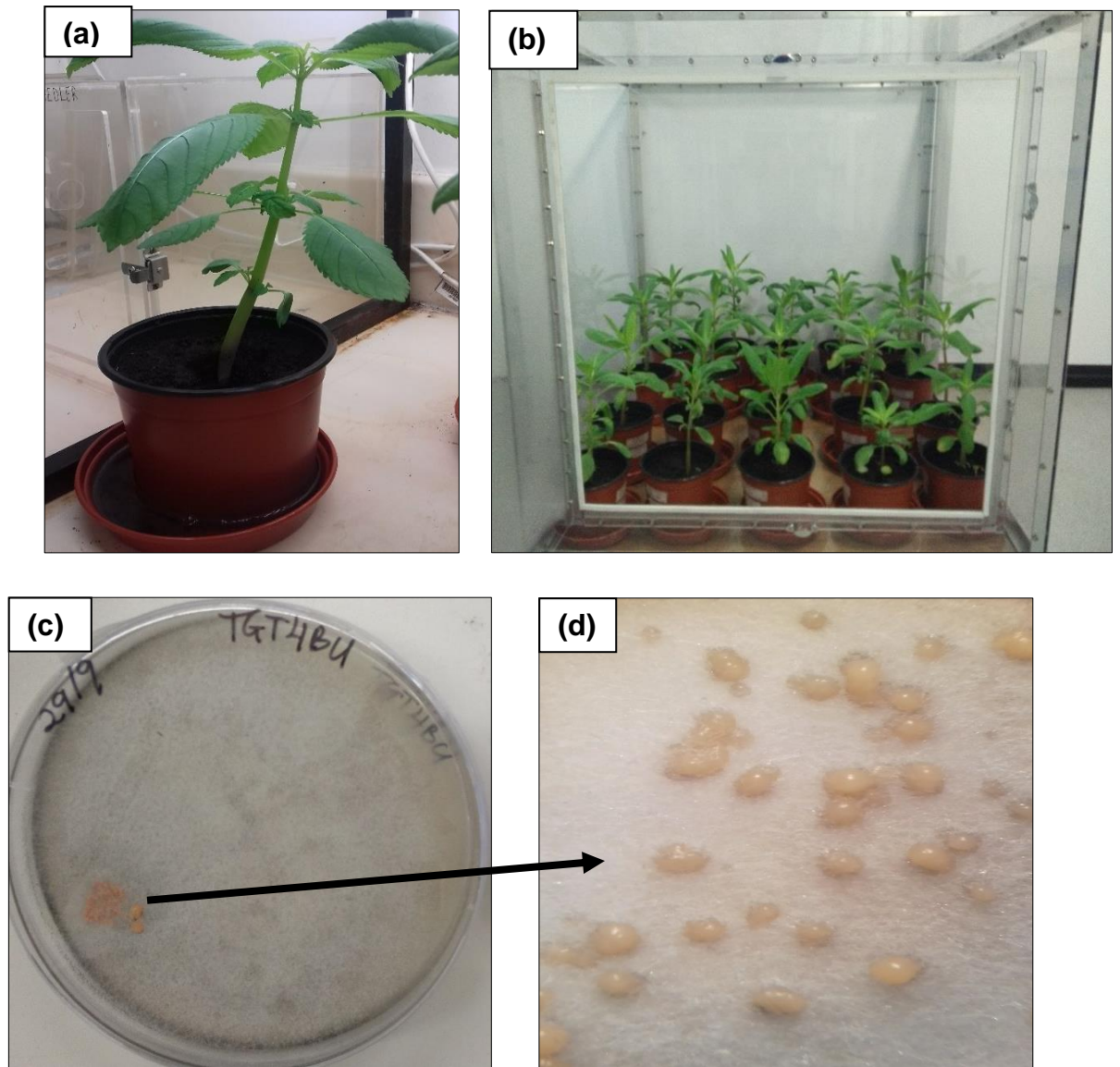


Figure 2.2 (a) Balsam seedling at three whorl stage that was ready to be inoculated. (b) Balsam plants were left in a Perspex box for 48 hours to allow spore germination. (c) *Colletotrichum acutatum* culture grown on a PDA plate and (d) under dissecting microscope at 40x magnification.

2.3 Leaf Surface Sterilisation

Three leaves (bottom, middle and top) from each plant in each treatment were harvested and isolated for endophytes. The isolations were conducted using fresh leaves to avoid changes in the fungal communities within the tissues.

Two round discs, each approximately 6mm diameter were cut from each leaf using a sterilised hole punch and surface-sterilised following method III of Schulz et al. (1993) with slight modifications. The discs were immersed in 100% ethanol for 30 sec, washed in sterile distilled water, immersed in 4.7% sodium hypochlorite (NaOCl) (4.7% v/v: 4.7ml NaOCl in 100ml distilled water) for one min, immersed in ethanol for a further 30 sec and followed by four separate washes in sterile distilled water. The fragments were placed abaxial surface downwards (Figure 2.3a) onto potato dextrose agar (PDA) plate with 80mg L⁻¹ streptomycin sulphate and 60mg L⁻¹ penicillin G added to inhibit bacterial contamination. The same discs were pressed onto PDA as control plate in order to examine the efficacy of surface sterilization to remove epiphytic fungi (Figure 2.3b). The plates were sealed with Parafilm to prevent contamination and stored in a storage box at room temperature.

To eliminate confusion through over-growth on the plate, all fungal colonies growing on PDA plates were removed before overlapping on each other and transferred onto potato carrot agar (PCA) plates to induce sporulation and to allow for identification (Figure 2.3c and 2.3d). After at least one month, the fungal structures on PCA were identified and retained as separate pure cultures (Figure 2.3e and 2.3f). Fungal identification was made in two ways. First, the fungi were placed in Erythrosin stain on slides for morphologically identification by Dr. Brian C. Sutton (ex-CABI).

Second, if the cultures contained sterile mycelium and could not be identified through visualisation, there were sent to the Microbial Identification Service, Centre of Agriculture and Biosciences International (CABI), for molecular

identification. The molecular analysis was run following their in-house methods. All samples were checked for purity and molecular assays were carried out on each sample using nucleic acid as a template. A propriety formulation [microLYSIS®-PLUS (MLP), Microzone, UK] was subjected to the rapid heating and cooling of a thermal cycler, to lyse cells and release deoxyribunocleic acid (DNA). Once DNA was extracted, Polymerase Chain Reaction (PCR) was employed to amplify copies of the rDNA in vitro. The quality of the PCR product was assessed by undertaking gel electrophoresis. PCR purification step was carried out to remove unutilised dNTPs, primers, polymerase and other PCR mixture compounds and obtained a highly purified DNA template for sequencing. This procedure also allowed concentration of low yield amplicons. Sequencing reactions were undertaken using BigDye® Terminator v3.1 kit from Applied Biosystems (Life Technologies, UK) which utilised fluorescent labelling of the chain terminator ddNTPs, to permit sequencing. Removal of excess unincorporated dye terminators was carried out to ensure a problem-free electrophoresis of fluorescently labelled sequencing reaction products on the capillary array AB 3130 Genetic Analyzer (DS1) DyeEx™ 2.0 (Qiagen, UK). Modules containing prehydrated gel-filtration resin were optimised for clean-up sequencing reactions containing BigDye® terminators. Dye removal was followed by suspension of the purified products in highly deionised formamide Hi-Di™ (Life Technologies, UK) to prevent rapid sample evaporation and secondary structure formation. Samples were loaded onto the AB 3130 Genetic Analyzer and sequencing was undertaken to determine the order of the nucleotide bases, adenine, guanine, cytosine and thymine in the DNA oligunocleotide. Following the

sequencing, identifications were undertaken by comparing the sequence obtained with those available from the European Molecular Biology Laboratory (EMBL) database via the European Bioinformatics Institute (EBI).

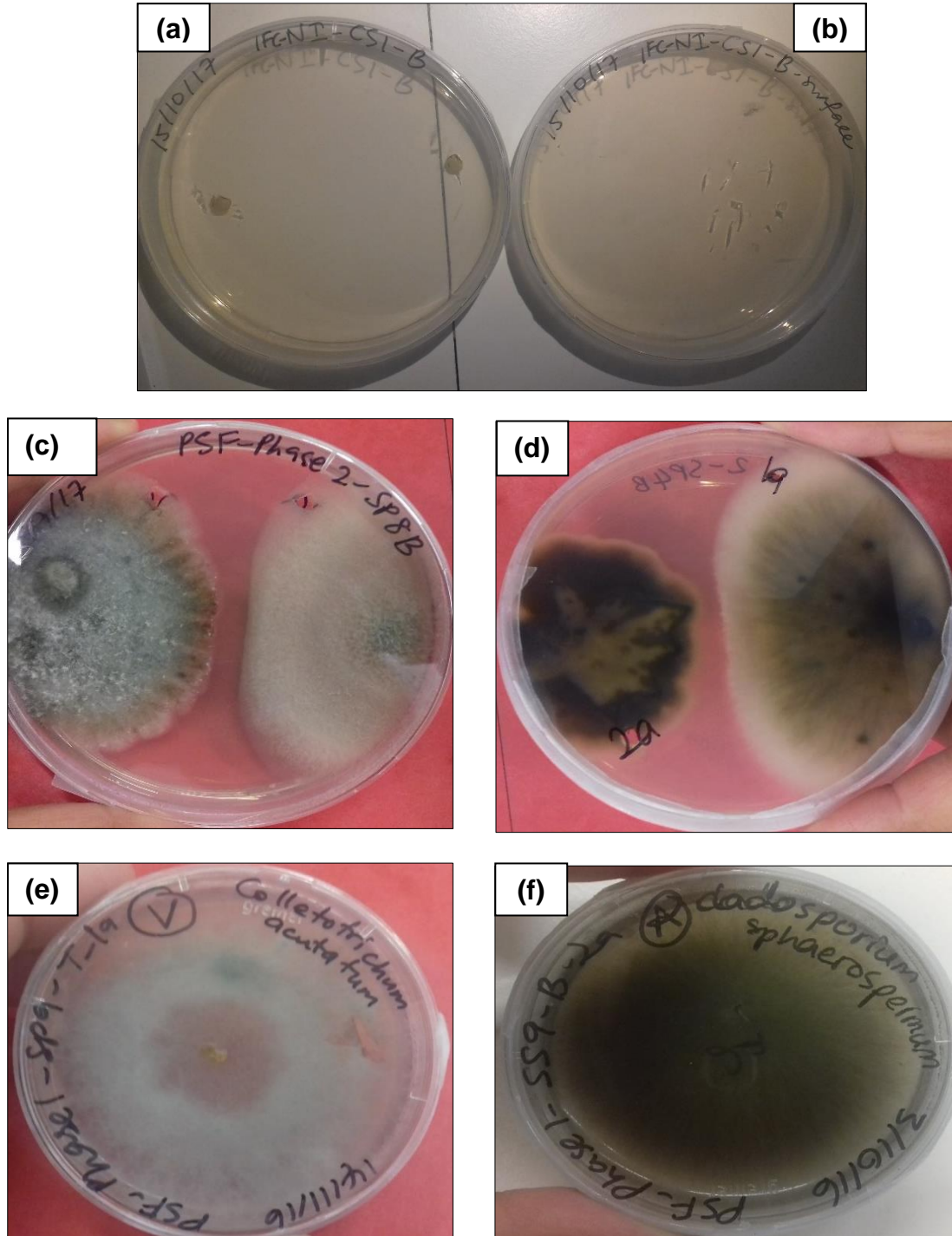


Figure 2.3 (a) Two leaf discs from each leaf were placed abaxial surface on a PDA plate and (b) the same fragments were used for leaf press. The clean plate showed effective endophyte isolation technique. (c) Fungal cultures grown on PDA plates were transferred to PCA plates to eliminate overlapping growth and encourage sporulation –front and (d) back of PDA plate. The samples of fungi stock in a PCA plate (e) is *Colletotrichum acutatum* while (f) is *Cladosporium sphaerospermum*.

2.4 Root Staining

Roots were extracted from the soil and washed under running water until all soil particles were removed. The roots were cut into pieces approximately 1 cm in length and were immersed in 70% ethanol and were stored in vials until used for root staining. The root staining method (Vierheilig et al. 1998) was begun by washing the roots under running water and removing any ethanol excess. Then the roots were placed in a biopsy processing cassette (Thermos Fisher scientific, Waltham, USA), immersed in a beaker containing 10% potassium hydroxide solution (KOH) (10% w/v: 10g KOH in 100ml distilled water) and placed in a water bath at 80°C for 25 min. Then, the cassettes containing roots were washed under running water for a period of 5 min until the KOH solution was completely removed. The cassette was immersed in a beaker containing staining solution (84.4:15:0.6, distilled water: 1% hydrochloric acid: Quink blue pen ink) and placed back into the water bath for a period of 30 min.

Upon removal from the stain solution, the roots were placed evenly on a slide that containing distilled water to prevent the roots from drying out and the cover slip was sealed using nail varnish. The roots were analysed under compound microscope at 40x magnification. The percentage arbuscular mycorrhizal fungi (AMF) colonisation of each root sample was analysed with the cross-hair eyepiece method of McGonigle et al. (1990). The percentage of root length colonised by AMF was evaluated by using click counter where 100 root sections was recorded for the presence and absence of hyphae, vesicles and arbuscules (Figure 2.4). This process was repeated for all root samples.

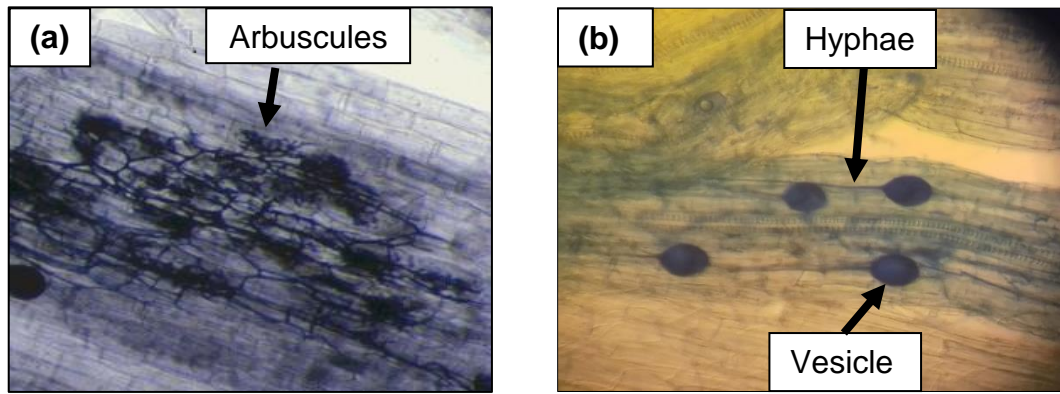


Figure 2.4 Arbuscular mycorrhizal fungi (AMF) colonisation on root sections under microscope at 40x magnification containing (a) arbuscules, (b) hyphae and vesicles.

2.5 Rust Fungus Infection and Harvesting

Twenty Himalayan balsam plants were grown in the glasshouse in order to bulk up rust fungus inoculum, which was provided by CABI. The seedlings were infected with the rust at the three or four leaf whorl stage. A 1:50 ratio of spores to talc was prepared fresh and mixed in a 9cm diameter Petri plate and applied to the abaxial surface of leaves, as described by Tanner et al. (2015a).

The spore/talc mix was evenly spread on to the lower leaf surface using a camel hair-brush (Humbro Senator, number 3) and was sprayed with distilled water to enhance an efficient infection. Following inoculation, the plants were placed in a dew chamber (Mercia Scientific) for 48h and set at 15°C. After 48h, the plants were placed separately from uninfected control plants in the glasshouse. Between each treatment, the dew chamber was cleaned thoroughly using 100% ethanol to avoid contamination from the previous test. The remainder of the spore/talc mix was tapped into a Petri plate containing

tap water medium in order to examine the efficacy of rust fungus inoculation and development. The plates were then placed in a dew chamber.

Upon rust fungus development and completion of the life cycle, urediniospores were harvested (Figure 2.5) from the plants using a sterile needle or by tapping infected leaves or stems over a Petri plate and stored in a 4°C refrigerator until used in next experiment.



Figure 2.5 Rust fungus spores at 40x magnification under compound microscope.

2.6 Plant Harvesting

Plants were harvested (Figure 2.6a) after being grown for nine weeks to maturity and ensuring the plant did not flower (Himalayan balsam is listed under Schedule 9 of the Wildlife and Countryside Act, 1981). Plant height, leaf number and rust spores were measured and recorded. The shoots were collected, kept in an envelope individually and dried in a drying cabinet for two weeks (Figure 2.6b). Upon drying, the shoots were measured for dry shoot biomass. The leaves and roots were collected for fungi assessment as detailed in section 2.3 and 2.4.

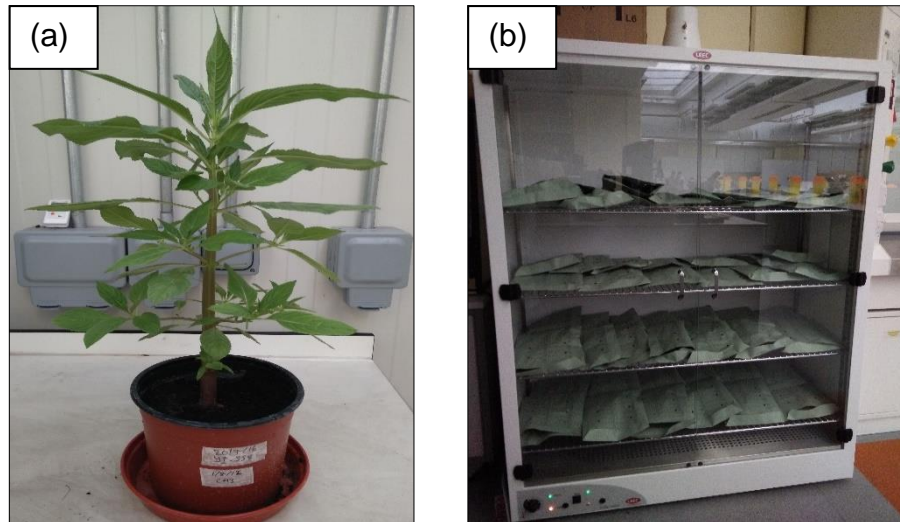


Figure 2.6 (a) Plant before flowering and ready to be harvested. (b) Plant shoots were kept in an envelope and placed in a drying cabinet for two weeks.

2.7 Statistical Analysis

All of the statistical analysis was conducted using R Studio version 1.1.383. The normality of the data and equality of variance were evaluated prior to analysis. Percentage data were arcsine transformed to meet the assumptions of the test beforehand. All data that violated the assumptions were transformed with square-root or logarithmic transformations. Endophyte Isolation Frequency (IF) was calculated for each fungal species by dividing the number of isolations of a fungal species per plant by the total number of isolations of all fungal species in that plant (Gange et al. 2007). Endophyte species richness and species abundance were examined across the treatments. Differences of endophyte community composition between treatments were analysed by analysis of similarities (ANOSIM) using the Community Analysis Package (CAP5) and the results shown by non-metric multidimensional scaling (NMDS).

CHAPTER 3

Preliminary Experiments

CHAPTER 3 PRELIMINARY EXPERIMENTS

This chapter describes five small experiments to underpin the main experiments as described in Chapters 4, 5, 6 and 7. A summary of each experiment is given as below:

- **Section 3.1: Fungi on Himalayan balsam in the field**

This section describes the outcomes of mycorrhizal presence on plant growth in the field, using two commercial inocula, sold by Plantworks (PW) and Symbio (SY). It was found that SY inoculum showed a promising effect in weakening plant growth. This experiment also has shown that there were ten endophyte species present in the environment and mycorrhizas influence the endophyte community composition in balsam leaves.

- **Section 3.2 : Endophyte inoculation in a controlled environment**

This study investigated the effectiveness of the endophyte inoculation technique and examined the inoculation effect on balsam growth. It was found that spraying endophytes on to balsam leaves in a perspex box was successful and endophytic *Colletotrichum acutatum* had the greatest capacity to reduce balsam growth. This study also showed that the identity of individual endophyte species may be a deciding factor in plant growth and the microbial communities within the leaves.

- **Section 3.3 : Endophyte inoculation in an insect-free environment**

This study was conducted to investigate whether an insecticide (Deltamethrin) has any direct effect on plant growth. It was found that the insecticide treatment did not affect plant growth and did not have

any fungicidal effect on the endophytes within balsam. Therefore, the insecticide could be used as an experimental tool in balsam-fungi-insect interactions study as described details in Chapter 4.

- **Section 3.4 : Arbuscular mycorrhizal fungi ‘spore-wash’**

This study investigated the most appropriate non-mycorrhizal controls for the two mycorrhizal inoculants used –PW and SY. This study found no differences in plant performance between sterilised inoculum and filtrate for both inocula, and mycorrhizal colonisation was only found with the live inocula. Therefore, sterilised inoculum was selected to be used as the control in the main experiments when live inoculum was involved.

- **Section 3.5 : Seed-borne fungal endophytes**

This study investigated endophyte fungal transmission in balsam plants by establishing three treatments (sterilised and squashed seeds, sterilised intact seeds and finally non-sterilised but intact seeds). It was found that the seed sterilisation technique was effective and so was used as the primary method in all main experiments in order to eliminate the confounding effects of seed surface fungi. It also suggested that all fungi found in the balsam leaves were transmitted horizontally from the environment as no endophytes were found within the seeds.

3.1 Fungi on Himalayan balsam in the field

Arbuscular mycorrhizal fungi (AMF) are soil fungi that may influence plant nutrition and growth by enhancing the uptake of over 50% of phosphorus and other nutrients to the plant through hyphal networks (Jakobsen et al. 1992; Smith & Read 1997). However, plant species do not profit equally from AMF and vary in growth response which depends on the fungal composition and identity, that differ between locations (Helgason et al. 1999; Husband et al. 2002). Specific plant-AMF combinations may influence the result of shoot biomass and phosphorus uptake of the plant (Ravnskov & Jakobsen 1995; Klironomos 2000; Castelli & Casper 2003; Smith et al. 2004; Streitwolf-Engel et al. 2010). Thus, it is ecologically relevant to study the effect of AMF identity on plant community composition (van der Heijden et al. 2003; Stampe & Daehler 2003; Vogelsang et al. 2006).

Foliar endophytic fungi in herbaceous plants are ubiquitous in nature and known to be diverse in plant communities. It has been thought that the fungi infect the leaves in their hosts most commonly by air-borne spores (termed 'horizontal transmission') but also via vertical transmission through the seeds (Hodgson et al. 2014). Endophyte communities are well documented from the field sites used in this thesis (Wearn et al. 2012; Hodgson et al. 2014) but to date, only one published paper exists on the endophytes found in balsam (Pattison et al. 2016). Thus, this study was conducted to examine the 'background' endophytes communities that exist in *I. glandulifera* in the field sites. Additionally, two commercial mycorrhizal inocula were applied in this

study, to determine their effect on plant growth and the interaction with endophyte fungal communities.

3.1.1 Method

The study was conducted with three treatments; Plantworks (PW) inoculum, Symbio (SY) inoculum and control with 10 replicates each as described detail in section 2.1. Balsam seeds were sown and grown in a glasshouse for four weeks (Figure 3.1a). Later, when the seedlings were sturdy and had strong stems, there were potted in a 2L pot that were filled with John Innes Type 2 compost with addition of 15g of PW or 2g of SY. Control plants were grown with autoclaved inoculum only. The pots were placed in a grow bag of compost in order to avoid fungal uptake from the field (Figure 3.1b). The plants were grown outside at a CABI field site for eight weeks (July-August 2015) in a randomised block design and watered with 250ml water daily. Before flowering, the plants were harvested and plant parameters (height, leaf number and shoot biomass) were recorded. Three leaves and root samples from each plants were collected for AM and endophyte fungi assessment as described in sections 2.3 and 2.4. Two species were identified by molecular identification: *Colletotrichum acutatum* (GenBank accession number: MH428675) and a member of the Chaetomiaceae (GenBank accession number: MH428676), while the remaining species were identified morphologically by Dr. Brian C. Sutton as mentioned above in section 2.3.

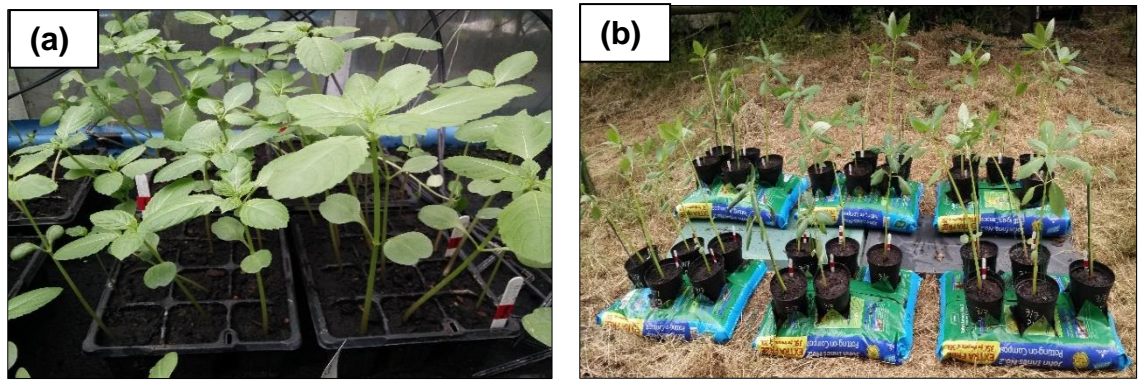


Figure 3.1 Himalayan balsam (a) seedlings grown in a glasshouse and (b) mature plants grown in grow bags in the field.

3.1.2 Statistical Analysis

To analyse the effect and interaction of mycorrhizas on balsam performance and Isolation Frequency (IF) and differences of each endophyte species between treatments, a one-way factorial ANOVA was performed with mycorrhizal presence as main effect. The analysis was done separately for each inoculum, as the controls for each differed in the nature of the carrier medium (Figure 2.1). Species abundance and species richness were examined and differences in endophyte community composition between treatments were examined with NMDS.

3.1.3 Results and Discussion

3.1.3.1 Plant growth and AMF colonisation

No effect of mycorrhizal colonisation were found on plant height irrespective of inoculum type. However, SY plants bore fewer leaves ($F_{1,18} = 5.293$, $p < 0.05$) (Figure 3.2a) and had lower shoot biomass ($F_{1,18} = 7.27$, $p < 0.05$)

(Figure 3.2b) compared to the control plants. PW inoculum produced twice the amount of AM colonisation compared to the SY plants. There was no colonisation in control plants, showing that the compost was free from natural fungi. The reason why the two inocula produced different levels of colonisation is currently unknown, but it is likely because the species in each inoculum differed, suggesting that different mycorrhizal species resulted in a different plant performance and AM colonisation. This is similar to a previous study that showed AM species specificity in affecting the growth of *Plantago lanceolata* and plant tolerance against a specialist lepidopteran herbivore, *Junonia coenia* (Bennett & Bever 2007). That study showed that all effects are possible, of the three different mycorrhizal fungi tested; *Archaeospora trappei* promoted both plant growth and plant tolerance, *Scutellospora calospora* did not affect plant growth and reduced plant tolerance to herbivory while *Glomus* white and the combination of these multiple fungi produced similar results which increased the plant growth but did not alter plant response against herbivory (Bennett & Bever 2007).

The most interesting result was that SY inoculum showed a promising effect in weakening the plants even though the root colonisation was much lower than that of PW inoculum. This is contradictory to the suggestion that AM may promote growth of invasive plant species (Chmura & Gucwa-Przepióra 2012). It is possible that the Symbio product caused growth depression of balsam plants through poor development of fungal structures or a lower rate of nutrient transfer per unit area which resulted in providing no or only few nutrients to the plant (Jin et al. 2017). Similar to this result, Tanner et al. (2014) found a negative relationship between AMF colonisation and balsam growth in the

introduced range likely because it is the cost of the association of the plant with incompatible mycorrhizal species. Thus, this has highlighted the importance of the identity of mycorrhizal species in determining plant germination and development. Therefore, it was sensible to apply SY inoculum in the main experiments in this thesis in order to achieve the main objective which was to reduce balsam growth and performance.

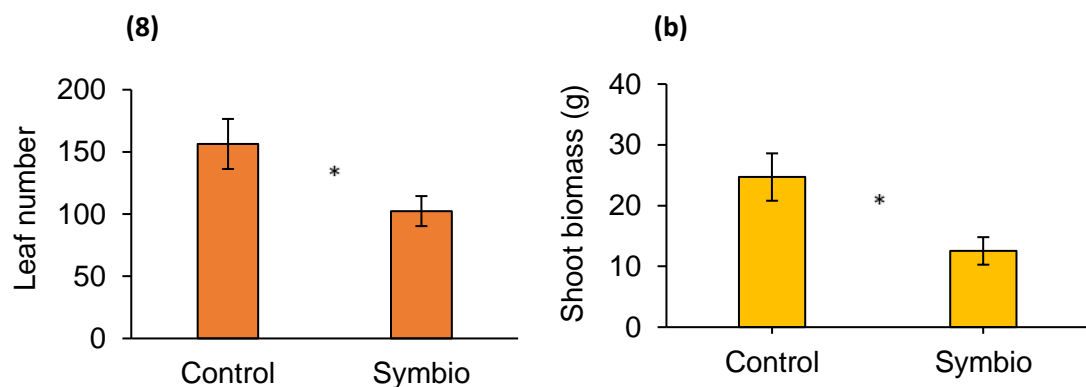


Figure 3.2 Mean of (a) leaf number and (b) shoot biomass between Symbio-treated plants and control plants. $n=10$ in all treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, $*p < 0.05$.

3.1.3.2 Endophytic fungal communities

Ten endophyte species were isolated from the plants across all treatments (Table 3.1). The highest Isolation Frequency (IF) was shown by *Cladosporium sphaerospermum* (30.56%) while the rarest species was *Chaetomium globosum* (0.83%) where the latter and *Geniculosporium sp.* were isolated from SY plants only. *Sordaria fimicola* was isolated from PW plants only, while the species that were found only in control plants were *Acrodontium hydnicola* and *Stemphylium botryosum*. It is interesting to note that a *Beauveria sp.* was isolated from PW and control plants only while Chaetomiaceae was isolated

from SY and control plants. This suggests that the identity of mycorrhizal species in each inoculum may affect the community of endophytes in the leaves through their impact on nutrient availability as shown by Eschen et al. (2010). Two common species –*Acremonium strictum* and *Colletotrichum acutatum* and one rare species –*Geniculosporium sp.* were selected to be inoculated into balsam leaves in a controlled experiment as described further in section 3.2. These species were selected in order to observe their effects on balsam performance since they had different IF percentages, but also to monitor the effectiveness of endophyte inoculation techniques. Although *Cl. sphaerospermum* was the commonest and *Ch. globosum* was the rarest species, these species were not selected because of spore constraints in the ‘stock’ cultures.

Table 3.1 Isolation frequency (% of plants) mean of each endophyte species across all treatments. $n=10$ for all treatments.

Endophyte species	Plantworks	Symbio	Control
<i>Acremonium strictum</i>	26.67	21.67	35
<i>Acrodonium hydnicola</i>	0	0	5
<i>Beauveria sp</i>	5	0	5
Chaetomiaceae	0	2.5	5
<i>Chaetomium globosum</i>	0	2.5	0
<i>Cladosporium sphaerospermum</i>	36.67	35	20
<i>Colletotrichum acutatum</i>	8.33	15	5
<i>Geniculosporium sp</i>	0	3.33	0
<i>Sordaria fimicola</i>	8.33	0	0
<i>Stemphylium botryosum</i>	0	0	5

There were no differences in IF, species abundance and species richness of endophytes between the AM treatments. However, the NMDS ordination clearly separated the endophyte fungal communities between PW-treated plants compared to the control plants (ANOSIM $R = 0.152$, $p < 0.05$) and also

between SY plants and control plants (ANOSIM $R = 0.132$, $p < 0.05$). This suggests that AM presence influence the endophyte community composition in balsam shoots and Symbio inoculum potentially reduced balsam performance. Therefore, the Symbio inoculum was chosen for further study and its interactions with other fungi (endophytes and the plant pathogen) and their effect on the plant's growth.

3.2 Endophyte inoculation in a controlled environment

Endophyte fungi have been found living in the leaves of all vascular tissues symptomlessly (Petrini 1991) and comprise diverse communities. Effects of endophytes on plant performance range from beneficial in protecting plants from abiotic stress (Rodriguez et al. 2009), against herbivores (Jallow et al. 2004; Gange et al. 2012) and pathogens (Gao et al. 2010), to antagonistic relationships (Jaber & Vidal 2009; Gange et al. 2007). However, there have been few studies of the interactions between endophytes and mycorrhizal colonisation in forbs (Eschen et al. 2010; Wearn et al. 2012) and only one in Himalayan balsam (Pattison et al. 2016). Therefore, throughout this thesis, the interaction of multiple fungal effects on balsam performance by manipulating the fungal presence was conducted. Thus, this preliminary study was conducted to investigate the effectiveness of the endophyte inoculation technique and to study the inoculation effect on balsam growth in a controlled environment. Three endophyte species were selected to be inoculated; *Acremonium strictum*, *Colletotrichum acutatum* and *Geniculosporium sp.* as the two former species were common while the latter was the rarest species but which also produced abundant spores as described in section 3.1.

3.2.1 Method

Balsam seeds were sown and the plants were grown in a Controlled Environment (CE) facility (16h light/8h dark at $23 \pm 1^\circ\text{C}$, 35% relative humidity) with six treatments and five replicates each. When the seedlings reached the three leaf whorl stages, the abaxial surface were inoculated with spore suspensions in two strokes (*ca.* 550 μl /leaf) of *Acremonium strictum* (+AS), *Colletotrichum acutatum* (+CA) and *Geniculosporium sp.* (+GS) while control plants were inoculated with 0.05% Tween 80 only. The spore concentration was adjusted using a haemocytometer to approximately 1.5×10^5 spores ml^{-1} (in 0.05% Tween 80). The detail of the inoculation method was given in section 2.2. The plants were grown in a 1.3L pot containing John Innes Type 2 compost in the CE room for six weeks after inoculation (Figure 3.3). The plants were harvested before flowering and plant parameters (height, leaf number, shoot biomass) were recorded. Leaf material was collected for endophyte assessment as explained in section 2.3. The fungal cultures that grew on PCA plates were placed on a slide with Erythrosine stains and were identified morphologically by Dr. Brian C. Sutton. To analyse the effect of endophyte on balsam performance, a one-way factorial ANOVA was performed with endophytes presence as main effect. The analysis was done individually between each endophyte and the control plants.



Figure 3.3 Himalayan balsam grown in a Controlled Environment room.

3.2.2 Results and Discussion

3.2.2.1 Plant growth and AMF colonisation

Colletotrichum acutatum and *A. strictum* were successfully recovered from the inoculated plants with *C. acutatum* inoculated plants being significantly shorter ($F_{1,8} = 5.293$, $p < 0.05$) (Figure 3.4a) and lighter ($F_{1,8} = 10.01$, $p < 0.05$) (Figure 3.4b) compared to the control while *A. strictum* did not significantly affect the plant growth. It was also interesting to note although *Geniculosporium sp.* was not recovered, the plants were significantly shorter than their controls ($F_{1,8} = 10.28$, $p < 0.05$). Clearly, this fungus is not easily recovered from foliar tissues, which may explain its apparent rarity in section 3.1.3.2 above. These data also suggest that it is not a good choice for manipulative experiments as it cannot be recovered even from the sprayed leaf. However, it is intriguing that the inoculation clearly had an effect on plant growth, although the fungus was not recovered. It is likely that the fungus has extremely restricted growth within a leaf, similar to other endophytes (Yan et al. 2015). However, there must be some interaction (perhaps biochemical) with the host, as growth of the latter was impaired.

The most encouraging result was that AMF colonisation was found in all endophyte-inoculated plants and differed from their controls though the percentage root length colonised was low (Figure 3.4c). There was no mycorrhizal colonization in any endophyte-free plant roots showing that there is a possibility of using endophytes to increase AMF colonisation.

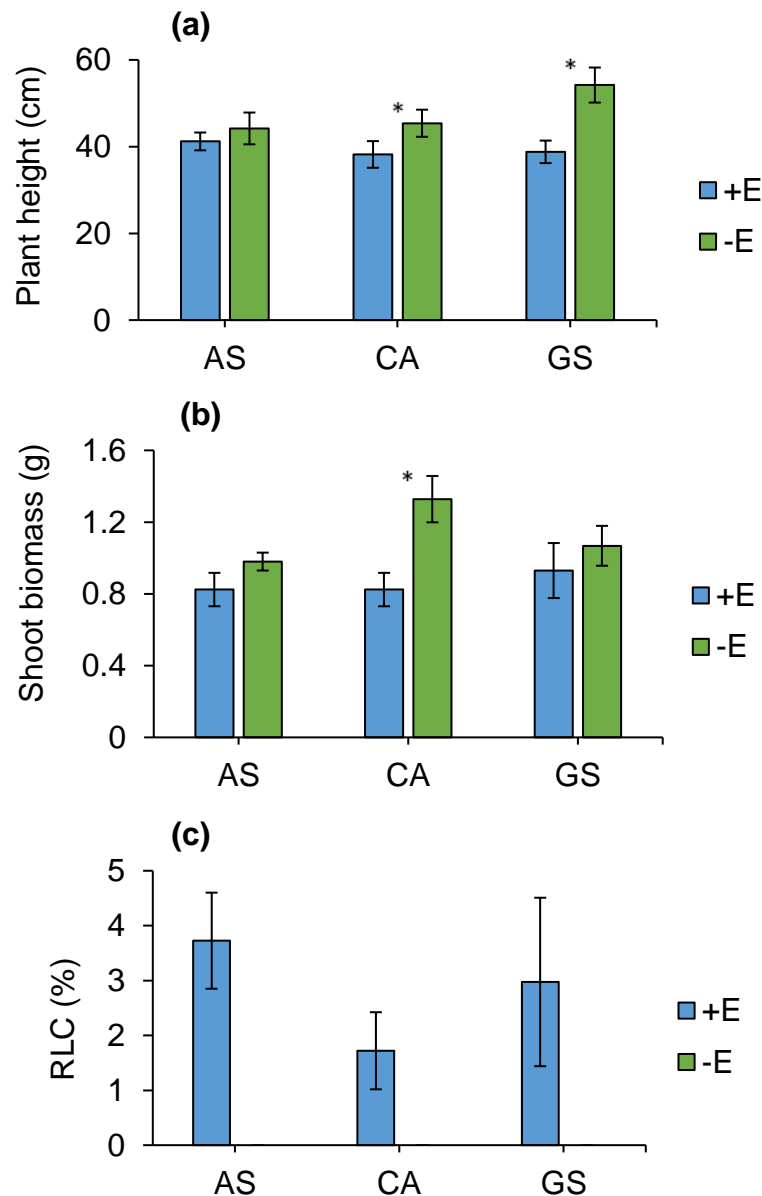


Figure 3.4 Mean of (a) plant height, (b) shoot biomass and (c) AMF colonisation across treatments. $n=5$ in all treatments. Error bars are one SE. AS was *Acremonium strictum* plants, CA was *Colletotrichum acutatum* plants while GS was *Geniculosporium sp.* plants. Asterisks above bars indicate significant pairwise differences between means, $*p < 0.05$.

3.2.2.2 Endophytic fungal communities

There were six endophyte species that were isolated in this study (Table 3.2). The highest IF mean was *Cladosporium sphaerospermum* which most likely entered the leaves from the environment as the fungus was not inoculated into the leaves. This fungus was isolated from +GS inoculated plants but not from the other treatments. *Acremonium strictum* and *C. acutatum* were isolated from plants onto which they were inoculated and both seemed to prevent the entry of other endophyte species. The most likely explanation for the differences is because of antagonistic interactions between the endophytes wherein the host plant's defence mechanism was activated, resulting in systemic movement of chemicals compound through the plant (Yan et al. 2015), thereby preventing the 'background' endophytes entering the foliar leaves. Similar results of antagonistic endophytes interactions were recorded in *Cirsium arvense* involving *Cladosporium cladosporioides* (Gange et al. 2007) and *Alternaria alternata* (Gange et al. 2012). This was supported by the fact that *Cl. sphaerospermum* and *Chaetomium globosum* were isolated from *Geniculosporium sp.* inoculated plants when the inoculated fungus was not recovered.

Table 3.2 Isolation Frequency (IF) mean of isolated endophyte across treatments. $n=5$ for all treatments. +/-AS were *Acremonium strictum* inoculated/free plants, +/-CA were *Colletotrichum acutatum* inoculated/free plants and +/-G were *Geniculosporium spp.* inoculated/free plants.

Endophyte species	+AS	-AS	+CA	-CA	+GS	-GS
<i>Acrodontium hydnicola</i>	0	20	0	0	0	0
<i>Acremonium strictum</i>	20	0	0	0	0	0
<i>Chaetomium globosum</i>	0	20	0	0	6.67	0
<i>Cladosporium sphaerospermum</i>	0	0	0	0	73.33	0
<i>Colletotrichum acutatum</i>	0	0	20	0	0	0
<i>Geniculosporium sp</i>	0	0	0	0	0	0

In conclusion, the endophyte inoculation technique was successful and showed that it is important to choose the correct fungi to inoculated, so that they can be recovered. In this study, *C. acutatum* had the greatest capacity to reduce balsam growth which did not happen in the other treatments, yet, this fungus is known to cause high mortality in strawberry plants (Freeman & Katan 1997). The capability to reduce balsam size was ecologically required in order to control its growth and ultimately the populations in the field. Additionally, adding endophytes into balsam leaves potentially increased AMF colonisation and appeared to suppress the entry of other endophyte species. This also suggested that the identity of individual endophyte species is important and may be a deciding factor on balsam growth and the microbial communities too, as each endophyte species may produce different allelochemical reactions.

Therefore, in order to achieve the main aim of this research; to study the effectiveness of biological control of Himalayan balsam, *C. acutatum* was selected in order to study the interactions between this fungus with mycorrhizas and the plant pathogen on balsam growth and to investigate the interaction between them as explained detail in Chapter 7.

3.3 Endophyte inoculation in an insect-free environment

Endophyte fungi are known for having a potential interactions with herbivores (Hartley & Gange 2009; Jaber & Vidal 2009; Gange et al. 2012; Hammer & van Bael 2015), yet no study has been conducted on their interactions in balsam and whether these affect plant growth. Therefore, the balsam-insect interaction study as described in detail in Chapter 4 was carried out, while this preliminary study was conducted to investigate whether insecticide has any direct effect on plant growth or fungi effect in the balsam leaves.

3.3.1 Method

The experiment was conducted in a Controlled Environment (CE) facility (16h light/8h dark at $23 \pm 1^{\circ}\text{C}$, 35% relative humidity) for eight weeks with eight treatments resulting in 40 plants in total. The plants were sprayed with 20ml of insecticide (Provado ultimate bug killer with active ingredient –Deltamethrin) while the control plants were sprayed with distilled water. After a week, the leaves were inoculated with a spore suspension (1.5×10^5 spores ml^{-1}) of *Colletotrichum acutatum*, *Cladosporium sphaerospermum* and a combination of both species. Control plants were inoculated with 0.05% Tween 80 only.

Details of endophyte inoculation procedures are given in section 2.2. The plants were harvested after eight weeks and plant parameters (height and shoot biomass) were recorded. Leaf materials were collected for endophyte assessment as described in section 2.3. Fungal slides were prepared with Erythrosine stain and were identified morphologically by Dr. Brian C. Sutton. Only one species was identified by molecular identification: *Colletotrichum destructivum* (GenBank Accession number: MH665647). To analyse the effect of insecticide treatment on Isolation Frequency (IF) of inoculated endophytes between treatments and on balsam performance, a one-way factorial ANOVA was performed with insecticide presence as the main effect.

3.3.2 Results and Discussion

Seven endophyte species were recorded in this study (Table 3.3) and there were no differences in Isolation Frequency (IF) mean of each endophyte species between insecticide-treated plants and the control plants (all $p > 0.05$). There were also no differences in plant height and biomass of insecticide-treated plants compared to the control (all $p > 0.05$). This indicates that the insecticide treatment did not affect plant growth and did not have direct fungicidal effect on endophytes within balsam. This result is important as it showed that the insecticide could be used as an experimental tool (Chapter 4) with no unintended effects on non-target organisms.

Table 3.3 The Isolation Frequency mean (% of plants) of each endophyte species across treatments with $n=5$ for each treatments. CA were *Co. acutatum* inoculated plants, CS were *Cl. sphaerospermum* inoculated plants, CACS were combination of both species and C were control plants.

Endophyte Species	With Insecticide				No Insecticide			
	CA	CS	CA CS	C	CA	CS	CA CS	C
<i>Acremonium incoloratum</i>	0	0	0	0	0	0	0	26.67
<i>Chaetomium elatum</i>	0	0	0	0	0	0	6.67	0
<i>Cladosporium oxysporum</i>	0	0	0	40	0	0	0	0
<i>Cladosporium sphaerospermum</i>	0	0	30	0	0	0	0	0
<i>Colletotrichum acutatum</i>	55	0	70	0	42	0	43	0
<i>Colletotrichum destructivum</i>	45	0	0	0	0	60	0	0
<i>Exophiala spp.</i>	0	0	0	20	0	0	20	6.67

3.4 Arbuscular mycorrhizal fungal ‘spore-wash’

Arbuscular mycorrhizal (AM) fungi within the root system form a symbiotic relationship, generally regarded as a mutualistic interface with the plant through an extensive hyphal network which helps plant to absorb water and nutrients (mainly phosphorus and nitrogen) from the soil (Jakobsen et al. 1992). Root colonisation with AMF also enhances plant protection particularly against soil-borne pathogenic fungi (Wang et al. 2018) and mediates on plant interspecific competition and community structure (Stampe & Daehler 2003; Lin et al. 2015).

Comparing the growth of mycorrhizal plants with the control (non-mycorrhizal) plants is important in order to observe if the mycorrhizal association has any

effects on the host. It is critical to determine the effect of the mycorrhizal fungi only (Abbott & Robson 1984) as the physiology of the plant will be affected by the presence of other soil microorganisms (Gryndler et al. 2018). Previous studies have demonstrated the most suitable comparison between infected and uninfected plants were between washed spores from a sandbased inoculum and soil that had grown uninfected plants (Koide & Li 1989). However, the efficacy of commercial products are variable and unknown in this regard, as no one has ever tested them before. Commercial products containing mutualistic AM fungi are now widely available and are sold as plant growth promoters and 'biofertilizers'. This study used two market leading products –Plantworks (PW) and Symbio (SY), with and without either product, to determine the most appropriate non-mycorrhizal controls for commonly used mycorrhizal inoculants. It is suspected that the products contain an abundant non-mycorrhizal microbial community, therefore the inoculum were filtered and compared its application to that of the product itself.

3.4.1 Method

The plants were grown in a Controlled Environment (CE) facility (16h light/8h dark at $23 \pm 1^{\circ}\text{C}$, 35% relative humidity) for eight weeks with six treatments resulting in 30 plants in total. The seedlings were potted into 1.3L pot and watered with 100ml daily. Five plants were grown with addition of each live inoculum of 9.75g of PW and 1.3g of SY as recommended by the companies (7.5g/L of Plantworks and 1g/L of Symbio). Five plants were potted with each of sterile (autoclaved in 121°C for 30 minutes) inoculum as control plants. Meanwhile the remaining five plants were grown with 15ml microbial filtrate of

each inoculum. To produce filtrate, each inoculum was added to 1L of sterile water and left for 24 hours in a 4°C refrigerator. Then, the mixture was filtered through a 38µm membrane, to remove all mycorrhizal propagules (Koide & Li 1989). After eight weeks, the plants were harvested and plant parameters (height, leaf and biomass) were recorded. Root materials were collected for mycorrhizal colonisation assessment. To examine the effect of different mycorrhizal treatments on balsam performance, a one-way factorial ANOVA was performed with AM type (live, sterile and filtrate) as main effect. The analysis was done separately for each inoculum, because of the differences in their physical structure (Figure 2.1).

3.4.2 Results and Discussion

There were no differences in plant performance between treatments for both inocula (all $p > 0.05$) (Figure 3.5). The dissimilar results in plant height and weight between PW and SY might be explained by the different origin and species composition of these inocula, as the former originated from the United Kingdom and consisted of five species meanwhile the latter was manufactured in the United States of America and was composed of nine mycorrhizal species (Table 3.4).

For both inocula, mycorrhizal colonisation was only achieved when live inoculum was added (Figure 3.6c). This suggests that mycorrhizal spores only occurred in a live inoculum while spores in the sterilised inoculum were eliminated in the high temperature of the autoclave and successfully removed in the filtration process. This finding supports previous results showing that

mycorrhizal colonisation was high when spores were used (Klironomos & Hart 2002). Of most importance was the fact that sterile inoculum plants were shown to be suitable to use as controls in subsequent experiments when involving live inoculum as treatment.

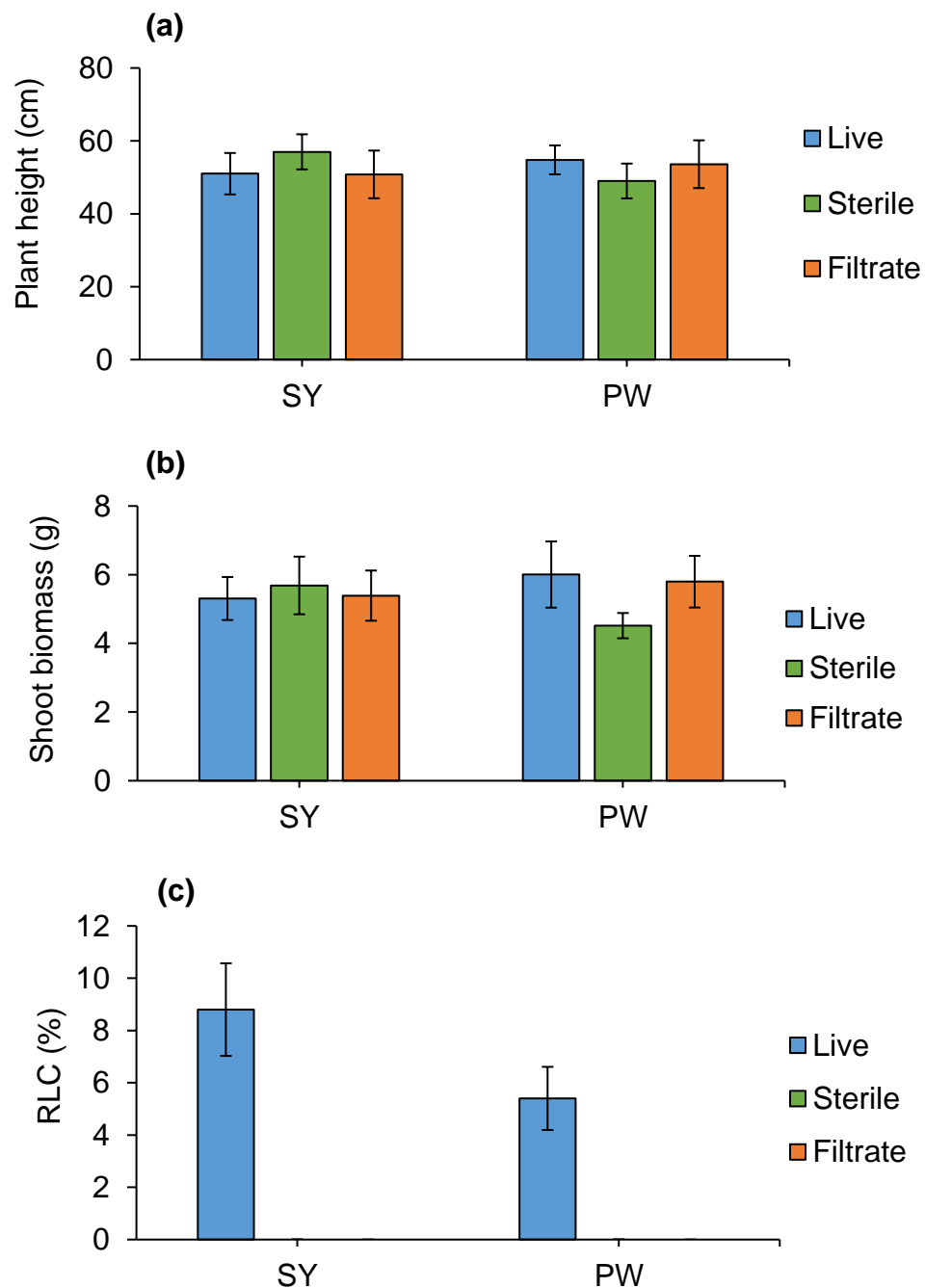


Figure 3.5 Mean of (a) plant height, (b) shoot biomass and (c) mycorrhizal colonisation across treatments. SY is Symbio inoculum while PW is Plantworks inoculum. $n=5$ across treatments. Error bars are one SE.

Table 3.4 Mycorrhizal species composition in Plantworks and Symbio inoculum.

Plantworks	Symbio
<i>Claroideoglomus claroideum</i>	<i>Claroideoglomus etunicatum</i>
<i>Funneliformis geosporus</i>	<i>Funneliformis mosseae</i>
<i>Funneliformis mosseae</i>	<i>Gigaspora margarita</i>
<i>Rhizophagus irregularis</i>	<i>Glomus monosporus</i>
<i>Rhizophagus microaggregatum</i>	<i>Glomus deserticola</i>
	<i>Paraglomus brasilianum</i>
	<i>Rhizophagus aggregatus</i>
	<i>Rhizophagus clarum</i>
	<i>Rhizophagus irregularis</i>

3.5 Seed-borne fungal endophytes

Seeds are important in a plant's life cycle developing into a new plant when the conditions for germination are met (Nelson 2004). Seed-borne fungal endophytes are vital in understanding seed establishment as the fungal are passed to the next generation via vertical transmission which ensuring their presence in the next seedling (Cope-Selby et al. 2017; Shade et al. 2017). This transmission enhances plant survival and defence (Rudgers et al. 2009) and also provide valuable endosymbionts to the offspring (Shade et al. 2017).

However, foliar endophytes in forbs are known to be horizontally transmitted (John et al. 2015) as many of the common endophytes (e.g. *Alternaria*, *Cladosporium* and *Epicoccum*) are saprophytic (Hayes 1979), abundant in spore rain populations (Marchisio & Airaudi 2001) and incompetent for vertical transmission (Sanchez Marquez et al. 2012). Interestingly, although these species seem not to be host specific (Rodriguez et al. 2009), they were suggested to enhance plant protection from insect herbivores (Gange et al.

2012) and pathogens (Gao et al. 2010). Therefore, this raises a question whether endophyte species are in a mutualistic relationship with their hosts which then may lead to vertical transmission. To date, there is little knowledge of the vertical transmission of endophyte species in forbs, yet, it has been suggested it may be a widespread phenomenon (Hodgson et al. 2014). Therefore, the aim of this study was to examine whether the balsam seed coat was a fungal barrier and to investigate the endophyte fungal transmission in balsam plants.

3.5.1 Method

There were three treatments with 10 seeds each (Figure 3.6). First treatment was sterilised and squashed seeds (+St+Sq). The seeds were sterilised in 5% bleach for 30 minutes and were macerated using a sterile hole punch in a laminar flow cabinet. The second treatment was sterilised but not squashed seeds (+St-Sq) and finally, non sterilised and not squashed seeds (-St-Sq) were the control. The seeds were embedded in potato dextrose agar (PDA) plates and kept in a storage box. After one week, any fungal cultures grown in the plates were transferred into potato carrot agar (PCA) plate to encourage sporulation. After 4 weeks, the fungal cultures from PCA plates were placed on slides with Erythrosine stain and were identified morphologically by Dr. Brian C. Sutton. Isolation Frequency (IF) of endophytes was determined.

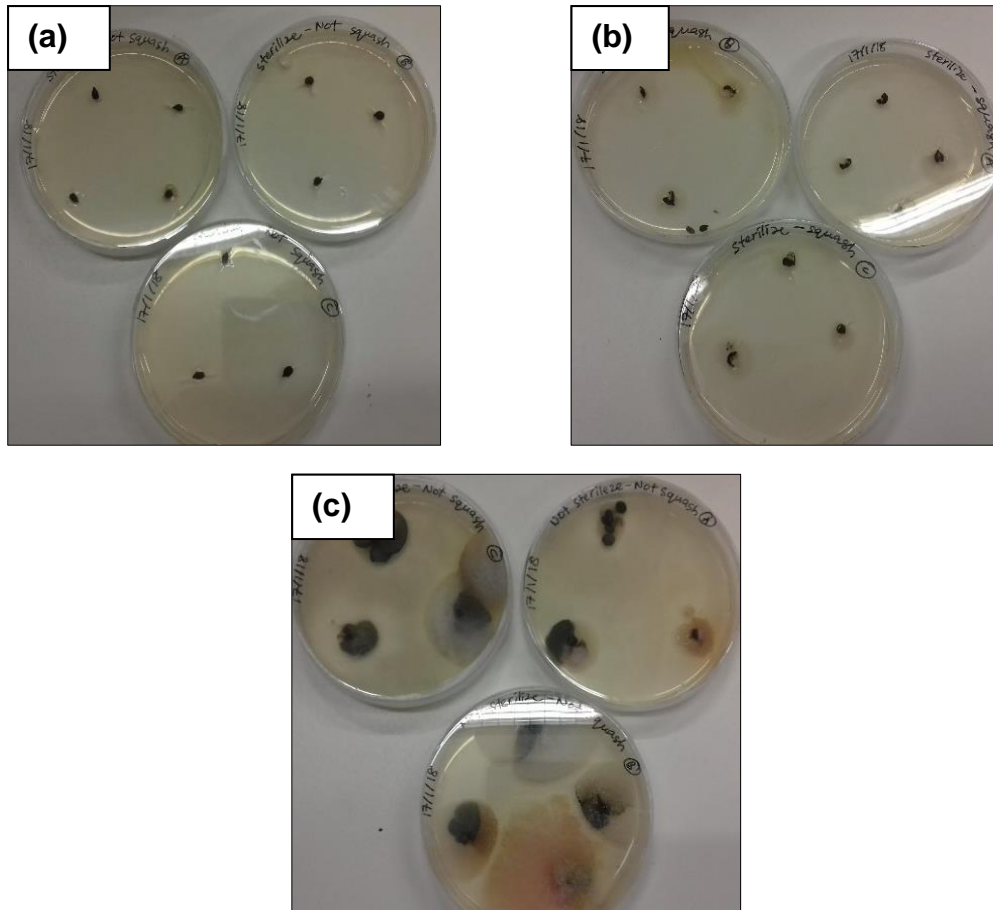


Figure 3.6 Three treatments with 10 seeds each in three different plates. (a) Clean plate in +St-Sq seeds and (b) 'juicy' liquid diffused out from +St+Sq seeds, meanwhile (c) Fungi dominance grown from –St-Sq seeds.

3.5.2 Results and Discussion

There were six endophyte species recorded from –St-Sq seeds (Table 3.5) while no species were isolated from the other treatments (+St+Sq and +St-Sq). It is also interesting to note that the endophytes fungi in this small study (*Acremonium sp.*, *Alternaria alternata* and *Cladosporium cladosporioides*) were also found in the main experiments (Chapter 4-7). However, the former was found from the seeds while the latter was isolated from the leaves. This suggested that seed coat was not a fungal barrier and endophyte fungi were potentially vertically transmitted from the seed coat into the balsam leaves.

The transmission may have occurred externally as it was isolated from the seed coat while no fungi were isolated from +St+Sq seeds showing that endosperms were free from fungi. This also showed that the seed sterilisation technique was extremely effective. Thus, seed surface sterilisation was used as the very first method in all of further experiments to eliminate the entrance of saprophytic fungi. The fact that nothing is transmitted within the seeds strongly suggests that all fungi found in the balsam leaves must have come from the environment.

Table 3.5 Isolation Frequency (IF) % mean recorded from non sterilised and non squashed seeds.

Endophyte species	IF mean (%)
<i>Acremonium sp.</i>	10
<i>Alternaria alternata</i>	25
<i>Aureobasidium pullulans</i>	15
<i>Cladosporium cladosporioides</i>	30
<i>Didymella macrostoma</i>	10
<i>Fusarium fujikuroi</i>	10

CHAPTER 4

Plant-Fungi-Insect Interactions

CHAPTER 4 PLANT-FUNGI-INSECT INTERACTIONS

Associations between fungi, host plants and insect herbivores are well studied when involving arbuscular mycorrhizal (AM) fungi (Wamberg et al. 2003; Gange 2007; Babikova et al. 2014; Williams et al. 2014; Gilbert & Johnson 2015) and endophytic fungi (Russo et al. 1997; Hartley & Gange 2009; Jaber & Vidal 2009; Bahar et al. 2011; Gange et al. 2012) but less clearly understood when both fungi are combined in herbaceous plants (Vicari et al. 2008). Previous studies mainly investigated bottom-up effects of AM fungi on aphids (Gange et al. 2005; Bennett & Bever 2007; Gange 2007; Babikova et al. 2014; Williams et al. 2014), but few have examined the top-down effects of aphids on AM fungi. It appears that the effects of herbivory on AM are considerable, for example the colonisation was reduced by 40% after 15 weeks of insect attack in *Plantago lanceolata* (Gange & Brown 2002). However, there is also a suggestion that insect herbivory may increase AM colonisation (Wamberg et al. 2003). Feeding by the beetle *Sitona lineatus* increased the colonisation of annual pea, *Pisum sativum*, however this was dependent on the age of the plant and the duration of the insect feeding. Pea plants that were 15-days-old and had 10 days of herbivory showed an increase in colonisation of 45%, compared to the plants that were not eaten (Wamberg et al. 2003). However, despite the reported positive and negative instances of insect attack, a meta analysis showed that most of the time, herbivory does not alter mycorrhizal colonisation in many types of plants including both grasses and forbs (Barto & Rillig 2010).

Some studies have found that the presence of AM has positive effects on insect herbivore growth rate and mortality (Gange 2007) through increased plant host quality and increased phloem size (Koricheva et al. 2009). Similarly, mycorrhizal fungi affected wheat, *Triticum aestivum* resistance to the English Grain aphid, *Sitobion avenae* by enhancing aphid development and reproductive success (Simon et al. 2017). Moreover, mycorrhizal colonisation increased the size of vascular bundles and this is likely to be a reason why aphid feeding success was greater on mycorrhizal-inoculated plants (Simon et al. 2017). Additionally, sucking and specialist insects were positively affected when AM was added to host plants, while chewing and generalist insects were negatively affected (Gange et al. 2005; Bennett & Bever 2007). This is because nutrients and carbon compounds are passed from plants to insects for conversion into their own biomass and so might be a competitor with mycorrhizal fungi (Gange 2007), explaining why insects negatively affect mycorrhizal colonisation. There are significant interactions between aphids and AM fungi likely because of the physiological changes in the condition of the shared host plant. For example, aphid infestation can reduce mycorrhizal development through C removal, whereas AM fungi alter volatile organic compound (VOC) emissions, making the plant less attractive to aphids (Babikova et al. 2014). Herbivore-plant-mycorrhizal interactions are complex, as the herbivory effect on a plant host can be transmitted to other plants through fungal networks below-ground (Gilbert & Johnson 2015). Mycorrhizal functioning may be affected by insect presence when the supply of recent photosynthate is diverted from plants to AM while in return, mycorrhizas can

alter nutrition and plant defence signalling pathways against insect herbivores (Gilbert & Johnson 2015).

In forbs, insect feeding and plant growth rates may also be affected by endophytic fungal presence (Gange et al. 2012). In that study, the endophytic fungus *Chaetomium cochliodes* in *Cirsium arvense* leaves increased the growth rate of a specialist feeder, *Cassida rubiginosa* and reduced growth rate of the generalist insect *Mamestra brassicae*. This is likely because of the changes in plant chemistry either in defence or nutrient mode since the fungi produce antifungal chemicals (Kang et al. 1999) which affect the production of defensive chemicals in the leaves. Thus, these authors suggested the production of chemical changes in the hosts by *C. cochliodes* is beneficial to specialist insects, while being detrimental to the generalist species (Gange et al. 2012). However, there were no effects on a generalist beetle, *Chelymorpha alternans* feeding on a tropical vine that was inoculated with *Colletotrichum gloeosporioides*, yet larvae that were fed with the endophyte had lower fecundity when adult (van Bael et al. 2009). It is also interesting to note that endophyte-inoculated plants may affect plant physiology in response to insect herbivory which reduces *Aphis fabae* fecundity (Jaber & Vidal 2009; Akello & Sikora 2012). In addition, a study was conducted on the interaction of cotton plants, *Gossypium hirsutum* with four different endophytes and showed reduced growth rate of larvae of the generalist lepidopteran, *Helicoverpa armigera* (McGee 2002). Meanwhile a weevil, *Larinus minutus* was attracted to *Fusarium* sp. inoculated leaves and avoided *Alternaria* sp. and *Epicoccum* sp. on knapweed, *Centaurea stoebe* (Newcombe et al. 2009). However, *Co. gloeosporioides* inoculated leaves of milkweed, *Calotropis gigantea* plants had

no effect on grasshopper, (*Poecilocerus pictus*) feeding (Devarajan & Suryanarayanan 2006). These outcomes show that the identity of the fungal species and insect herbivores involved were important in determining whether the outcome of the interaction was positive or negative.

Both endophytes and mycorrhizal fungi can alter plant growth and insects feeding development, but to date, no one has examined the effects of the presence of both fungi on insects in a forb species. One paper has explored the interaction of the foliar endophyte, *Neotyphodium lolii*, a mycorrhizal fungus, *Funneliformis mosseae* and the noctuid moth, *Phlogophora meticulosa* on ryegrass, *Lolium perenne* (Vicari et al. 2008). It was found that AM reduced the insect resistance effect of the endophyte in the host plant, whereas in the absence of endophyte, antagonistic effects of AM were seen. The above study was conducted with a grass species and some effects were additive and some nonadditive, with interactions between the fungi. This emphasized the importance of performing similar experiments in forb plants.

A survey of the natural enemies of *Impatiens glandulifera* in its native range was conducted in 2006 by Tanner et al. (2008). The authors found damaged leaves indicative of arthropod attack in the area surveyed, from Kashmir to Garhwal (Pakistan and India). Species identified were *Taeniothrips inconsequens* and a flea beetle *Altica himensis*, together with many unidentified arthropods in the Coleoptera, Hemiptera and Lepidoptera. The two former species were regarded as possible candidates for the biocontrol of balsam while the other arthropods were the least likely candidates as there were far too rare (Tanner et al. 2008). Meanwhile, *Aphis fabae* has frequently

been found associated with balsam in the introduced range in the UK (Beerling & Perrins 1993) and the Czech Republic (Starý et al. 2014). *A. fabae* is generally known as a plant pest and significantly reduces the growth rate of faba bean, *Vicia faba* especially during the seedling stage (Shannag & Ababneh 2007). Therefore, a study on fungi-insect interactions on balsam was conducted with *A. fabae* as the focal species, as it has the potential to cause damage to balsam in the UK. The knowledge and information found from this association should provide a better understanding of the nature of fungal-insect interactions and their effects on Himalayan balsam growth.

This experiment was designed to investigate the interactions of mycorrhizal and endophyte fungi with insect herbivores. To determine the role of AM and endophytes in an integrated pest management of Himalayan balsam, the compatibility of an endophytic isolate of *Colletotrichum acutatum*, *Cladosporium sphaerospermum*, and the combination of both species with natural and reduced levels of insect attack, in the presence of commercial inoculum ('Plantworks inoculum' hereby after referred as PW, and 'Symbio inoculum' referred to as SY) was used against the aphid, *A. fabae* in a field experiment. The first objective of this experiment was to study the interactions between fungi and insects and also their effect on balsam performance. The second objective was to study the foliar endophyte communities in balsam plants that were attacked by insects. The hypothesis of this study was, there would be interactions between the endophytes, mycorrhizas and insect that may lead to altered plant performance and microbial communities and these interactions would depend upon the identity of the fungal species.

4.1 Methods

There were 32 different treatments with five replicate plants in each treatment giving a total of 160 plants overall. The plants were grown in the glasshouse for seven weeks from seedling establishment, in 2L pots with the presence of two commercial inoculum treatments –15g of Plantworks (PW) and 2g of Symbio (SY), while sterile PW and sterile SY were autoclaved at 121°C for 30 minutes and used for controls. Details of plant germination and propagation were given in section 2.1. After five weeks, when the plants were at the three whorl leaf stage, half of all plants were sprayed with insecticide. The plants were sprayed (two strokes) with Provado ultimate bug killer (with active ingredient Deltamethrin) while the control plants were sprayed once with two strokes of distilled water. A preliminary study showed there were no direct effects of the insecticide on plant and fungal growth in insect-free conditions, as demonstrated in section 3.3. Then, a week after, five insecticide sprayed and control plants from each inoculum were inoculated with *Colletotrichum acutatum* (CA), *Cladosporium sphaerospermum* (CS) and a combination of both species (CACS) with $1.5 \times 10^5 \text{ ml}^{-1}$ spores concentration, while control plants were sprayed with 0.05% Tween 80 only. Details of the endophyte inoculation technique was described in section 2.2. These two endophyte species were selected as both of them were common in balsam leaves when the plants were grown in the field, as described in section 3.1.

The plants were grown in the field (Figure 4.1) for four weeks (July – August 2016) to allow for natural levels of insect attack, in a randomised block design and were watered with 250ml water twice daily. The plants were harvested

when mature, but before first flowering and plant height, leaf number and dry shoot biomass were recorded. Leaf and root samples from each plant were collected for fungal identification and analysis. Leaf surface sterilisation and root staining techniques were conducted as described in sections 2.3 and 2.4. Two endophyte species appeared to be sterile in culture and were identified by molecular methods: *Lecanicillium* sp. (GeneBank accession number: MH428682) and *Peniophora* sp. (GeneBank accession number: MH428683), while the remaining species were identified morphologically by Dr Brian C. Sutton as described in section 2.3. Insect numbers were recorded weekly using a click counter and the total of insects per plant were recorded. Insects were identified using a 40x magnification binocular microscope.



Figure 4.1 The plants were grown outside in the field to allow for natural insect attack.

4.1.1 Statistical Analysis

All of the statistical analysis was conducted using R Studio version 1.1.383 as described in section 2.7. Percentage data were arcsine transformed to meet the assumptions of normality beforehand. All data that violated the assumptions were transformed with square-root or logarithmic transformations. To analyse the effects and interactions of fungi on balsam performance, a four-way factorial ANOVA was performed with mycorrhizas, endophytes (*Colletotrichum acutatum* and *Cladosporium sphaerospermum*) and insect presence as main effects. The analysis was done separately for each inoculum treatment. Mycorrhizal colonisation data for both inocula were examined with a three-way ANOVA with endophytes (*Co. acutatum* and *Cl. sphaerospermum*) and insect as main effects because all uninoculated plants showed zero colonisation. Differences in endophyte isolation frequency (IF) of each fungal species between treatments were examined with a similar analytical design (four-way ANOVA) and the percentage data were transformed. Differences in species abundance and species richness were examined across treatments and endophyte community composition was compared with NMDS as described in section 2.7.

4.2 Results

4.2.1 Plant growth and AMF colonisation

Plants were taller when attacked by insects, but that only happened in the PW experiment (Table 4.1). Addition of *Cl. sphaerospermum* resulted in shorter (Figure 4.2) and lighter (Figure 4.3) plants in both inocula. Meanwhile addition of PW lowered shoot biomass, but this did not happen in SY plants. Insect attack reduced plant height and biomass when *Cl. sphaerospermum* was present irrespective of inoculum type, leading to significant interaction terms (IN x CS) in the analysis (Table 4.1). Similarly, insect attack reduced plant height and biomass of PW plants when both endophytes were present. No mycorrhizal colonisation was recorded in AM-free plants (Figure 4.4). The presence of endophytes and insects did not affect mycorrhizal colonisation from either inoculum. PW-treated plants showed twice the colonisation level of SY plants irrespective of whether endophytes were added. The highest colonisation was recorded when AM was added in insect-free plants and in the absence of endophytes.

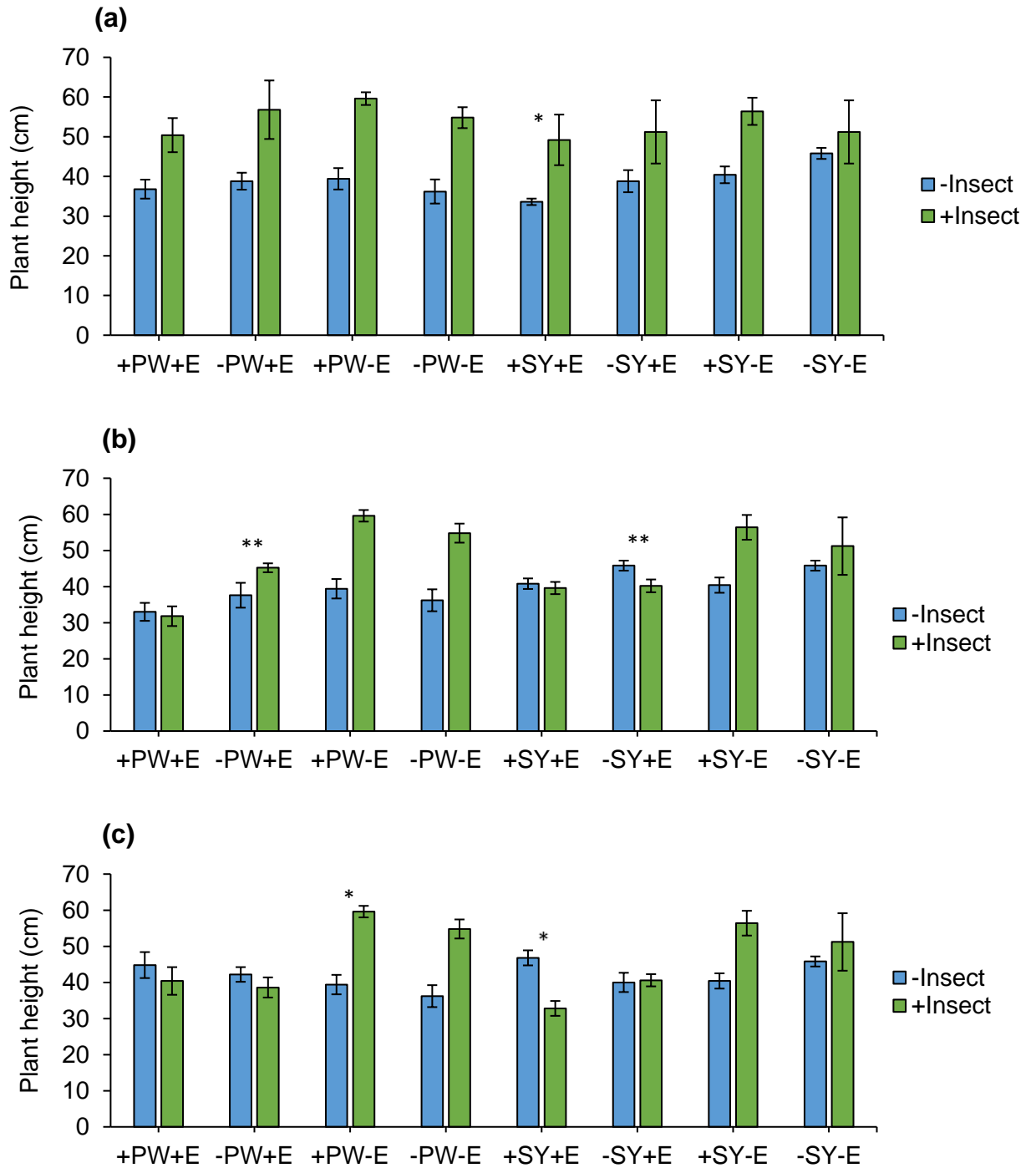


Figure 4.2 Mean of plant height across (a) *Colletotrichum acutatum* inoculated plants, (b) *Cladosporium sphaerospermum* inoculated plants and (c) dual endophytes inoculated plants. +/-PW for Plantworks inoculum present/absent, +/-SY for Symbio inoculum present/absent and +/-E for endophytes present/absent. $n=5$ in all treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, * $p < 0.05$ and ** $p < 0.001$.

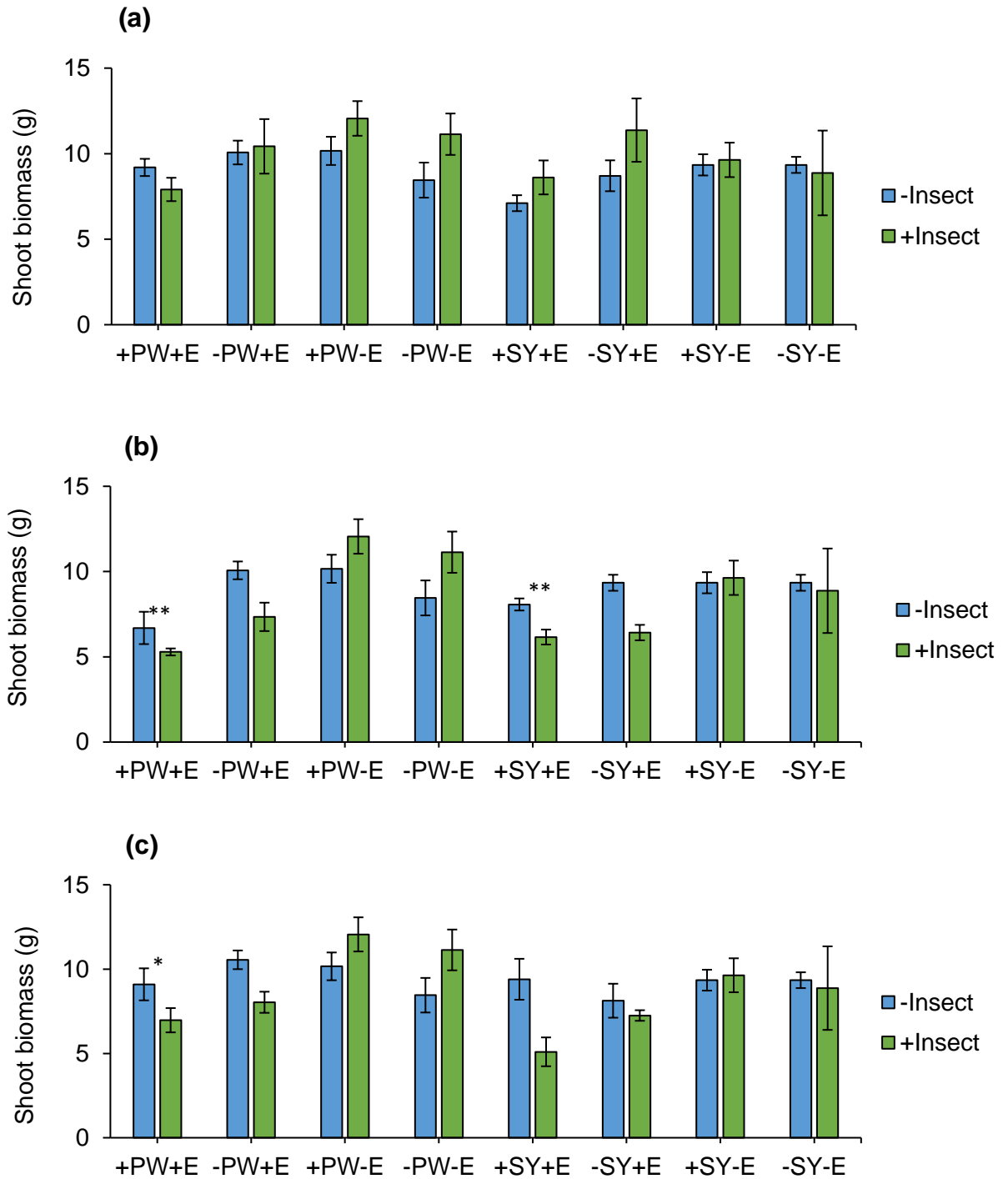


Figure 4.3 Mean of shoot biomass across (a) *Colletotrichum acutatum* inoculated plants, (b) *Cladosporium sphaerospermum* inoculated plants and (c) dual endophytes inoculated plants. +/-PW for Plantworks inoculum present/absent, +/-SY for Symbio inoculum present/absent and +/-E for endophytes present/absent. $n=5$ in all treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, * $p < 0.05$ and ** $p < 0.001$.

Table 4.1 Four-way ANOVA factorial analysis results across (a) Plantworks and (b) Symbio treatments. Degrees of Freedom for F values = 1, 64 and n = 5 for each treatment. (Note: IN: Insects, CA: *Colletotrichum acutatum* and CS: *Cladosporium sphaerospermum*. Bold indicated significant values).

(a) Plantworks-treated plants	Height		Leaf number		Shoot biomass	
	F	<i>p</i>	F	<i>p</i>	F	<i>p</i>
IN	24.616	<0.001	10.492	0.001	2.192	0.143
PW	1.281	0.261	4.714	0.033	6.334	0.014
CA	0.908	0.344	0.918	0.341	0.091	0.763
CS	18.480	<0.001	6.663	0.012	19.695	<0.001
IN x PW	0.908	0.344	0.000	0.983	0.044	0.833
IN x CA	2.947	0.090	0.448	0.505	3.021	0.087
IN x CS	27.212	<0.001	24.549	<0.001	12.888	<0.001
PW x CA	0.394	0.532	0.807	0.372	0.807	0.372
PW x CS	1.389	0.242	6.529	0.013	4.317	0.041
CA x CS	4.201	0.044	5.329	0.024	7.493	0.008
IN x PW x CA	0.145	0.704	3.589	0.062	0.267	0.606
IN x PW x CS	0.353	0.554	0.046	0.831	1.431	0.236
IN x CA x CS	0.311	0.579	0.007	0.932	2.084	0.153
PW x CA x CS	8.570	0.004	2.722	0.103	6.664	0.012
IN x PW x CA x CS	0.916	0.342	0.076	0.783	0.000	0.983

(b) Symbio-treated plants	Height		Leaf number		Shoot biomass	
	F	<i>p</i>	F	<i>p</i>	F	<i>p</i>
IN	3.321	0.073	1.656	0.202	2.100	0.152
SY	2.058	0.156	3.289	0.074	2.100	0.152
CA	3.579	0.063	8.369	0.005	0.134	0.715
CS	7.036	0.010	10.712	<0.001	9.992	0.002
IN x SY	0.002	0.961	0.097	0.756	0.459	0.500
IN x CA	0.193	0.662	1.933	0.169	0.929	0.338
IN x CS	25.463	<0.001	17.111	<0.001	11.369	<0.001
SY x CA	0.517	0.474	2.055	0.156	1.161	0.285
SY x CS	0.052	0.820	0.024	0.876	0.081	0.776
CA x CS	0.430	0.514	0.009	0.924	0.094	0.759
IN x SY x CA	6.100	0.016	5.599	0.021	2.344	0.130
IN x SY x CS	3.106	0.082	3.945	0.051	0.235	0.629
IN x CA x CS	2.467	0.121	1.092	0.299	1.282	0.261
SY x CA x CS	1.413	0.239	0.659	0.419	1.938	0.168
IN x SY x CA x CS	0.404	0.527	0.631	0.429	0.367	0.546

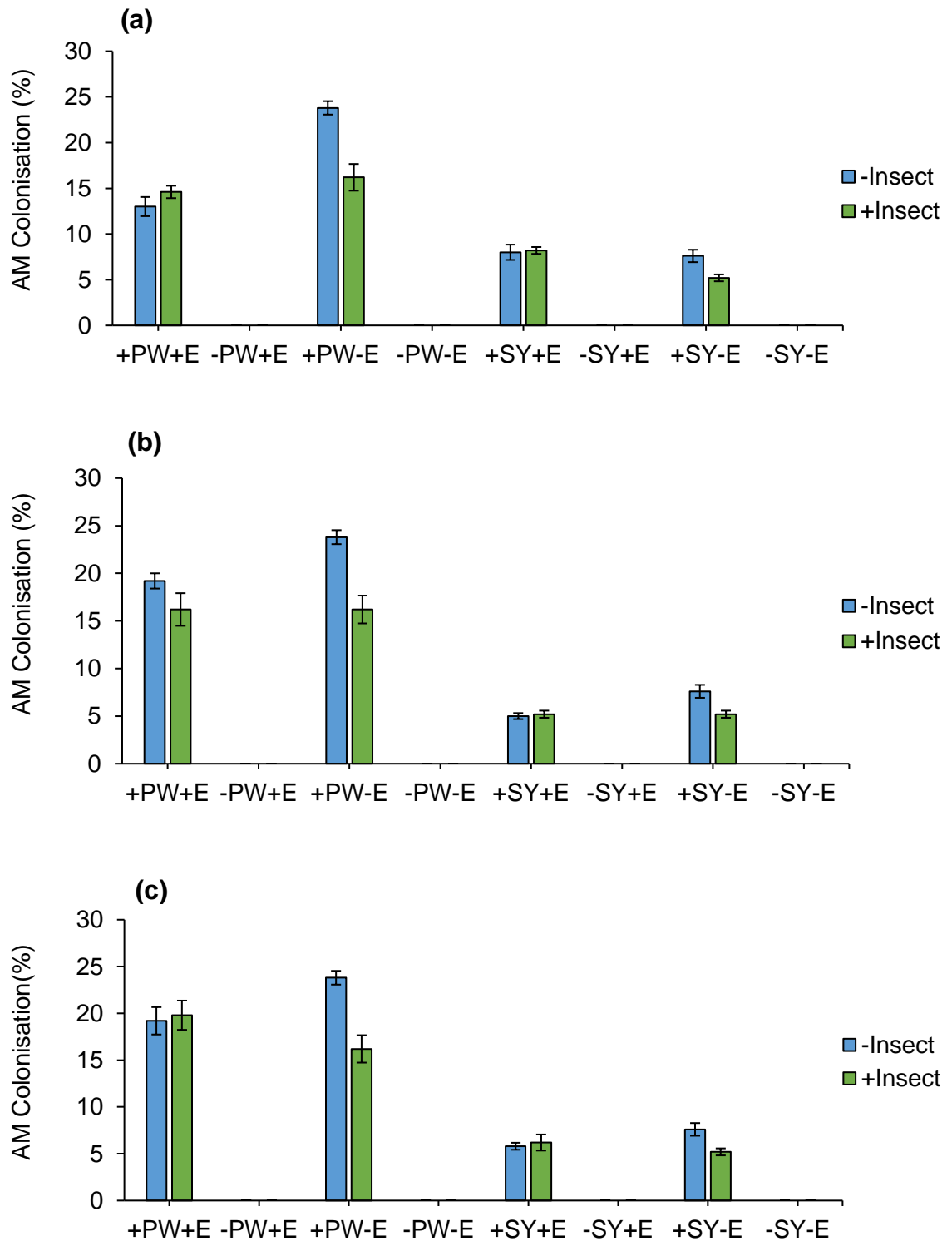


Figure 4.4 Mean of mycorrhizal colonisation across (a) *Colletotrichum acutatum* inoculated plants, (b) *Cladosporium sphaerospermum* inoculated plants and (c) dual endophytes inoculated plants. +/-PW for Plantworks inoculum present/absent, +/-SY for Symbio inoculum present/absent and +/-E for endophytes present/absent. $n=5$ in all treatments. Error bars are one SE.

4.2.2 Insect attributes

Insect herbivores that were recorded across treatments were the black bean aphid, *Aphis fabae* and a specialist green aphid, *Impatiensium balsamines*. Thus, the former insect was selected as the latter was too rare to be statistically analysed. This study has shown that the application of the chemical successfully reduced insect numbers across the treatments (Table 4.2). Addition of PW increased aphid numbers but adding *Cl. sphaerospermum* reduced aphids on PW plants. Aphid infestation was reduced when *Co. acutatum* was added in SY-treated plants (Figure 4.5). The two-way interactions between AM and *Cl. sphaerospermum* were a reflection of reduced aphid numbers, but only when the AM was absent, which did not happen with *Co. acutatum*. Similarly, the mycorrhizas increased aphid numbers when *Cl. sphaerospermum* was present, but not when the endophyte was absent. Again, this did not happen with *Co. acutatum*. The interactions between insect, AM and *Cl. sphaerospermum* resulted from the fact that the endophyte reduced aphid numbers only when PW was absent which did not happen with SY inoculum or addition of *Co. acutatum*.

Table 4.2 Four-way factorial analysis of insect number across treatments. Degrees of Freedom for F values = 1,64 and $n=5$ in all treatments. (Note: IN: Insecticide application, CA: *Colletotrichum acutatum* and CS: *Cladosporium sphaerospermum*. Bold indicated significant values).

	Plantworks-treated plants		Symbio-treated plants	
	F	<i>p</i>	F	<i>p</i>
IN	81.379	<0.001	56.923	<0.001
AM	4.306	0.042	1.279	0.262
CA	0.037	0.848	3.960	0.050
CS	5.338	0.024	2.091	0.153
IN:AM	0.704	0.404	0.046	0.830
IN:CA	0.182	0.671	4.075	0.047
IN:CS	0.439	0.510	3.306	0.073
AM:CA	0.078	0.780	1.551	0.217
AM:CS	5.475	0.022	4.075	0.047
CA:CS	0.049	0.826	0.074	0.786
IN:AM:CA	0.329	0.568	2.260	0.137
IN:AM:CS	5.754	0.019	0.312	0.578
IN:CA:CS	0.520	0.473	1.092	0.299
AM:CA:CS	0.026	0.871	0.312	0.578
IN:AM:CA:CS	0.478	0.491	1.215	0.274

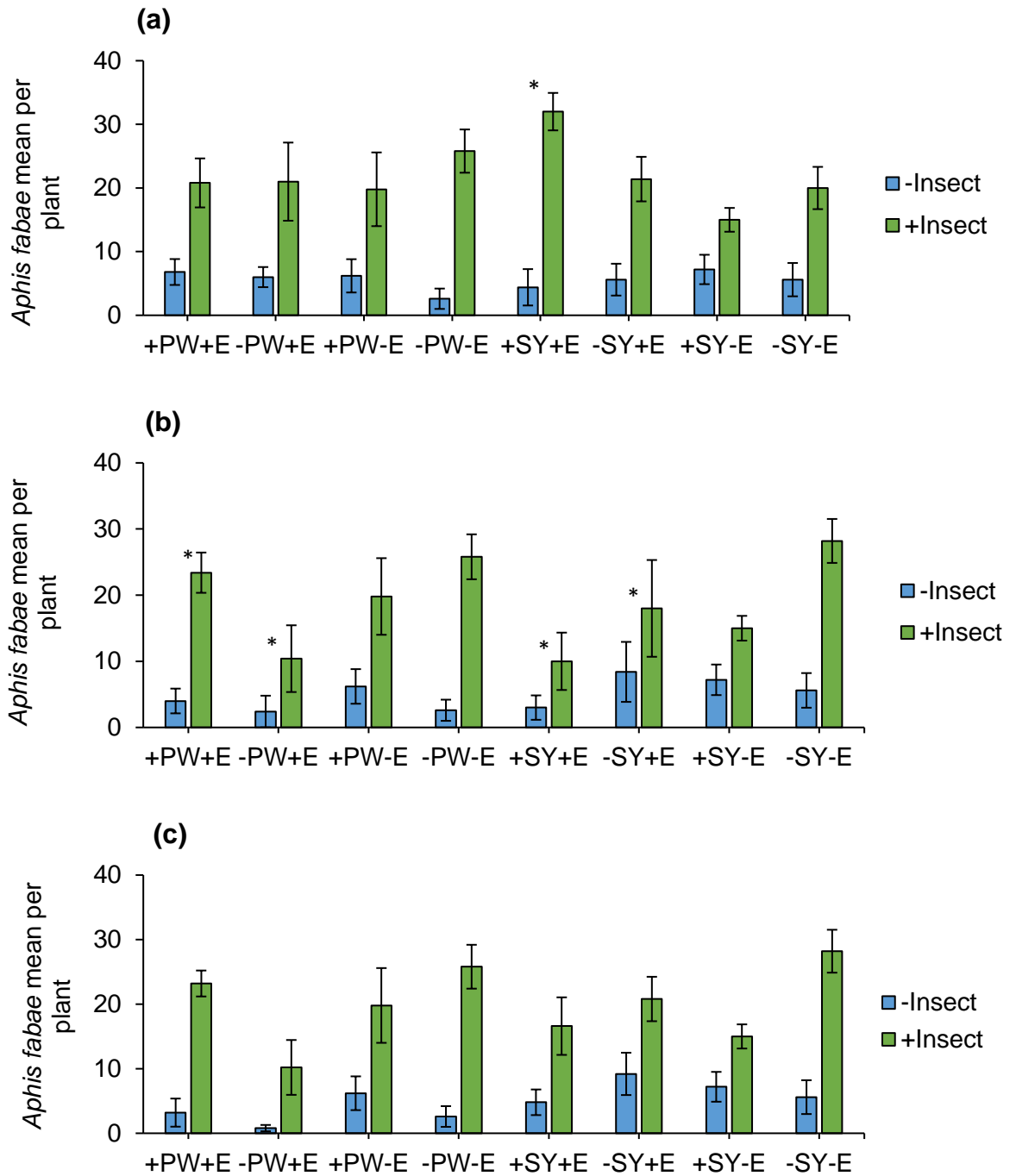


Figure 4.5 Mean of *Aphis fabae* per plant across (a) *Colletotrichum acutatum* inoculated plants, (b) *Cladosporium sphaerospermum* inoculated plants and (c) dual endophytes inoculated plants. +/-PW for Plantworks inoculum present/absent, +/-SY for Symbio inoculum present/absent and +/-E for endophytes present/absent. $n=5$ in all treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, * $p < 0.05$.

4.2.3 Endophytic fungal communities

There were 12 endophyte species recorded across plants attacked by insects (Table 4.3) while 11 species were isolated from insect-free plants (Table 4.4). *Clonostachys rosea*, *Nigrospora oryzae* and *Peniophora spp.* were isolated from the former plants only, while *Penicillium sp.* and *Sordaria fimicola* were recorded from the latter plants only. The remaining species were found in both treatments.

Cladosporium sphaerospermum was successfully recovered from its inoculated leaves regardless of whether insects or PW were present (Figure 4.6a). This fungus was found from dual endophyte inoculated plants too, apart from when insects and PW were absent. In contrast, this fungus was recovered from plants which received dual inoculation irrespective whether insects and SY were added (Figure 4.6b). Meanwhile *Colletotrichum acutatum* was recovered from the leaves where it was inoculated when insects were absent, irrespective whether PW was present (Figure 4.6c). However, when insects and PW were present, this effect seemed to diminish. This fungus was not recovered from single and dual endophyte inoculated leaves. Meanwhile in SY-treated plants, *Co. acutatum* was recovered from the plants in which it was inoculated when both insects and SY were present or absent (Figure 4.6d). However, this fungus was not found when insects were present in the absence of SY, but it was found when insects were absent and SY was present.

Table 4.3 Isolation Frequency (%) mean of each endophyte species across plants attacked by insects. $n = 5$ for each treatment. (Note: +PW: Plantworks plants, +SY: Symbio plants, +CA: *Colletotrichum acutatum* inoculated plants, +CS: *Cladosporium sphaerospermum* inoculated plants; +CACS: dual endophyte inoculated plants and C: Control plants. Bold numbers indicated the highest IF mean in that particular treatments.

Endophyte species	+PW				-PW				+SY				-SY			
	+CA	+CS	+CA CS	C	+CA	+CS	+CA CS	C	+CA	+CS	+CA CS	C	+CA	+CS	+CA CS	C
<i>Acremonium strictum</i>	0	16.67	0	0	0	0	0	0	6.67	20	0	0	6.67	6.67	0	13.33
<i>Chaetomium cochliodes</i>	0	16.67	10	0	0	0	5	0	0	0	0	0	0	0	6.67	0
<i>Cladosporium cladosporioides</i>	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0
<i>Cladosporium oxysporum</i>	0	20	0	0	16.67	6.67	0	0	0	6.67	0	0	0	11.67	38.33	0
<i>Cladosporium sphaerospermum</i>	0	23.33	20	31.67	5	46.67	10	20	40	23.33	20	25	50	43.33	40	43.33
<i>Clonostachys rosea</i>	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0

<i>Colletotrichum acutatum</i>	0	0	0	11.67	6.67	10	5	5	13.33	0	0	11.67	0	5	5	0
<i>Geniculosporium spp</i>	0	0	0	0	0	0	0	0	0	20	0	20	6.67	0	0	0
<i>Lecanicillium sp.</i>	80	13.33	10	40	71.67	26.67	54.17	43.33	33.33	10	50	43.33	33.33	38.33	5	26.67
<i>Nigrospora oryzae</i>	0	0	0	0	0	0	5	0	0	0	10	0	0	0	0	0
<i>Peniophora sp.</i>	0	10	0	5	0	10	11.67	0	6.67	0	0	0	0	0	0	16.67
<i>Trichoderma viride</i>	20	0	0	11.67	0	0	0	11.67	0	0	0	0	0	0	0	0

Table 4.4 Isolation Frequency (%) mean of each endophyte species across insects free plants. $n = 5$ for each treatment. (Note: +PW: Plantworks plants, +SY: Symbio plants, +CA: *Colletotrichum acutatum* inoculated plants, +CS: *Cladosporium sphaerospermum* inoculated plants; +CACS: dual endophyte inoculated plants and C: Control plants. Bold numbers indicated the highest IF mean in that particular treatments.

Endophyte species	+PW				-PW				+SY				-SY			
	+CA	+CS	+CA CS	-C	+CA	+CS	+CA CS	-C	+CA	+CS	+CA CS	-C	+CA	+CS	+CA CS	-C
<i>Acremonium strictum</i>	6.67	0	0	20	0	0	0	3.33	18.33	10.67	0	0	0	0	6.67	0
<i>Chaetomium cochliodes</i>	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Cladosporium cladosporioides</i>	6.67	0	0	0	6.67	0	0	0	0	0	20	0	10	20	0	0
<i>Cladosporium oxysporum</i>	0	0	0	0	0	0	10	0	0	0	0	0	0	6.67	0	0
<i>Cladosporium sphaerospermum</i>	31.67	10	30	10	13.33	10	0	33.33	13.33	4	13.33	16.67	13.33	5	66.67	25
<i>Colletotrichum acutatum</i>	21.67	50	30	20	16.67	43.33	66.67	15	26.67	41.33	6.67	0	16.67	0	0	18.33

<i>Geniculosporium spp</i>	11.67	0	0	6.67	33.33	0	0	6.67	0	0	0	6.67	0	5	0	0
<i>Lecanicillium sp.</i>	21.67	6.67	10	36.67	36.67	0	0	25	36.67	6.67	13.33	70	4	40	0	51.67
<i>Penicillium sp.</i>	0	33.33	10	0	0	33.33	8.33	0	0	10.67	6.67	0	0	10	6.67	0
<i>Sordaria fimicola</i>	0	0	0	0	0	6.67	0	0	0	0	0	0	0	0	0	0
<i>Trichoderma viride</i>	0	0	0	6.67	0	6.67	0	16.67	0	0	0	6.67	20	0	0	0

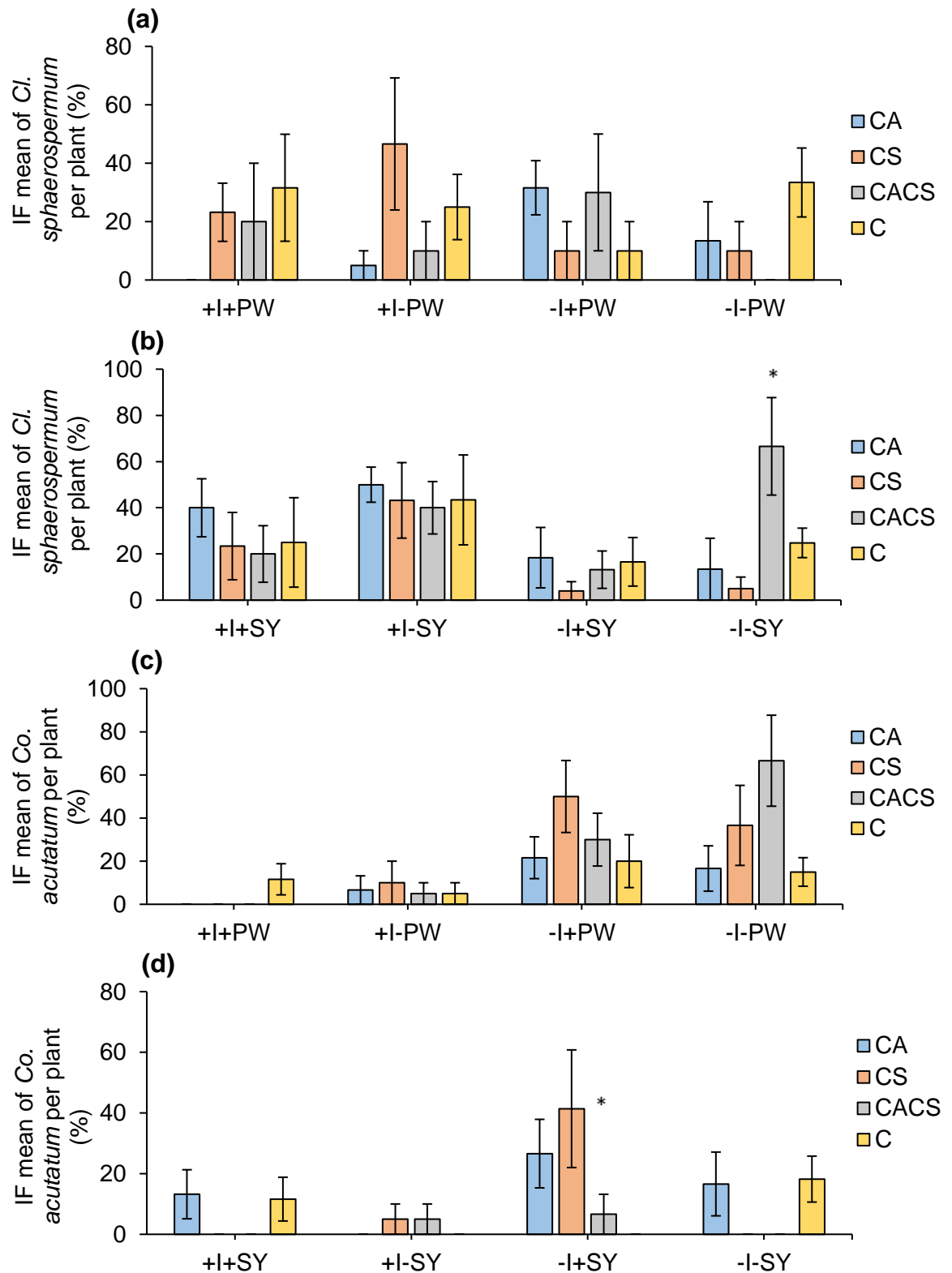


Figure 4.6 Isolation Frequency mean of *Cladosporium sphaerospermum* on (a) PW and (b) SY treated plants, and *Colletotrichum acutatum* on (c) PW and (d) SY treated plants. Legend shows the leaves were inoculated with *C. acutatum* (CA), *C. sphaerospermum* (CS), dual species (CACS) and control (C). +/-I for insects present/absent. $n=5$ in all treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, $*p < 0.05$.

Insect herbivores increased the IF mean of *Cl. sphaerospermum* in both inocula and IF mean of *C. acutatum* in SY-treated plants, but reduced it in PW plants (Table 4.5). Application of PW increased *Cl. sphaerospermum* frequency and reduced frequency of *Co. acutatum*, however, the opposite result was found when SY was added. It is also interesting to note that addition of *Co. acutatum* increased the IF mean of *Cl. sphaerospermum* in SY plants, but reduced it in other treatments. In contrast, addition of *Cl. sphaerospermum* increased its frequency in PW, but reduced it in SY plants. Dual endophyte application increased IF mean of *Cl. sphaerospermum* but reduced that *Co. acutatum* in SY plants. Insect attack and addition of *Co. acutatum* reduced IF mean of this fungus only when PW was present and SY was absent, leading to the significant interaction terms (IN x CA) in the analysis (Table 4.5b).

The NMDS ordination clearly separated the endophyte fungal communities between insect attacked plants and their controls in PW plants (ANOSIM $R = 0.2027$, $p < 0.05$) and SY plants (ANOSIM $R = 0.0593$, $p < 0.05$) (Figure 4.7). Meanwhile, addition of *Cl. sphaerospermum* reduced endophyte species richness ($F_{1,65} = 14.400$, $p < 0.001$) of PW plants and SY plants ($F_{1,65} = 9.422$, $p < 0.05$) which did not happen when *Co. acutatum* was added.

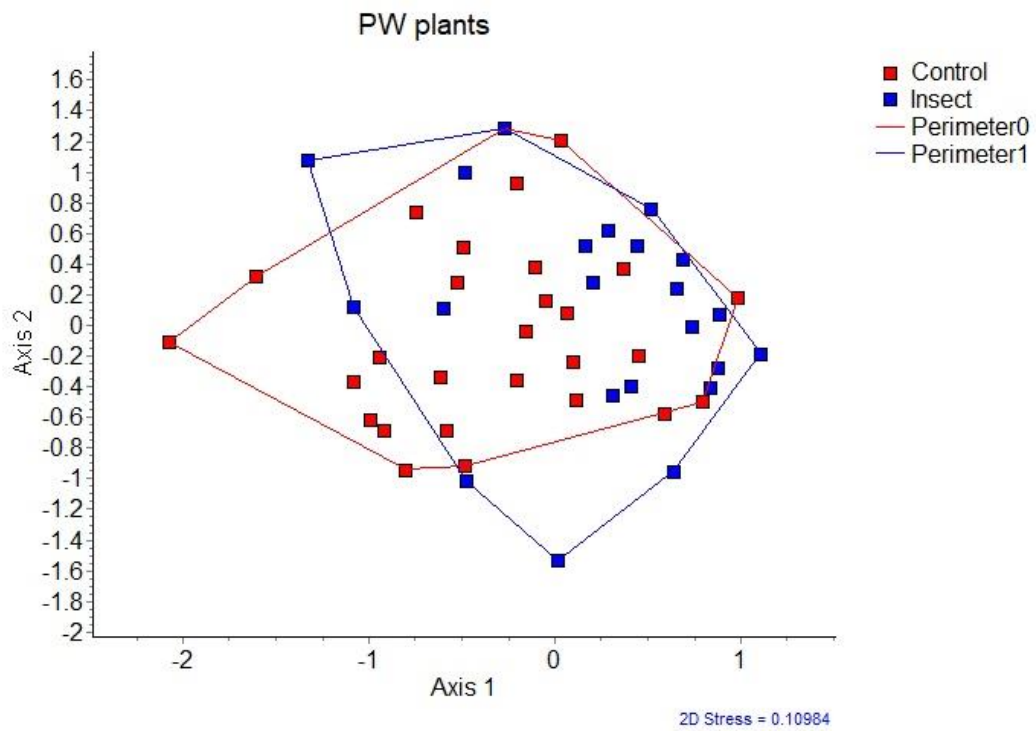
Table 4.5 Four way factorial analysis of isolation frequency mean of (a) *Cladosporium sphaerospermum* and (b) *Colletotrichum acutatum* across treatments. Degrees of Freedom for F values = 1,65 and $n=5$ in all treatments. AM: Mycorrhizal treatments.

Legend as in Table 4.1.

(a) IF mean of CS	Plantworks-treated plants		Symbio-treated plants	
	F	<i>p</i>	F	<i>p</i>
IN	22.928	<0.001	331.427	<0.001
AM	22.928	<0.001	430.804	<0.001
CA	547.355	<0.001	11.271	<0.001
CS	9362.708	<0.001	5590.963	<0.001
IN:AM	0.004	0.9480	0.020	0.8879
IN:CA	0.171	0.6807	0.065	0.7988
IN:CS	2.013	0.1607	3.553	0.0639
AM:CA	0.009	0.9262	0.113	0.7377
AM:CS	1.903	0.1725	0.732	0.3954
CA:CS	0.005	0.9433	5.520	0.0218
IN:AM:CA	2.183	0.1444	1.023	0.3156
IN:AM:CS	0.858	0.3577	0.062	0.8045
IN:CA:CS	5.664	0.0203	10.217	0.0021
AM:CA:CS	0.306	0.5820	0.063	0.8031

(b) IF mean of CA	Plantworks-treated plants		Symbio-treated plants	
	F	<i>p</i>	F	<i>p</i>
IN	2440.261	<0.001	756.982	<0.001
AM	62.166	<0.001	399.743	<0.001
CA	7919.896	<0.001	12991.573	<0.001
CS	0.067	0.7963	0.862	0.3566
IN:AM	0.736	0.3940	6.537	0.0129
IN:CA	20.161	<0.001	71.630	<0.001
IN:CS	0.186	0.6673	0.124	0.7255
AM:CA	0.102	0.7502	31.190	<0.001
AM:CS	0.372	0.5443	0.135	0.7144
CA:CS	3.897	0.0526	14.003	0.0003
IN:AM:CA	2.361	0.1292	0.052	0.8196
IN:AM:CS	1.773	0.1877	0.239	0.6262
IN:CA:CS	0.880	0.3518	10.103	0.0022
AM:CA:CS	4.992	0.0289	0.897	0.3472

(a)



(b)

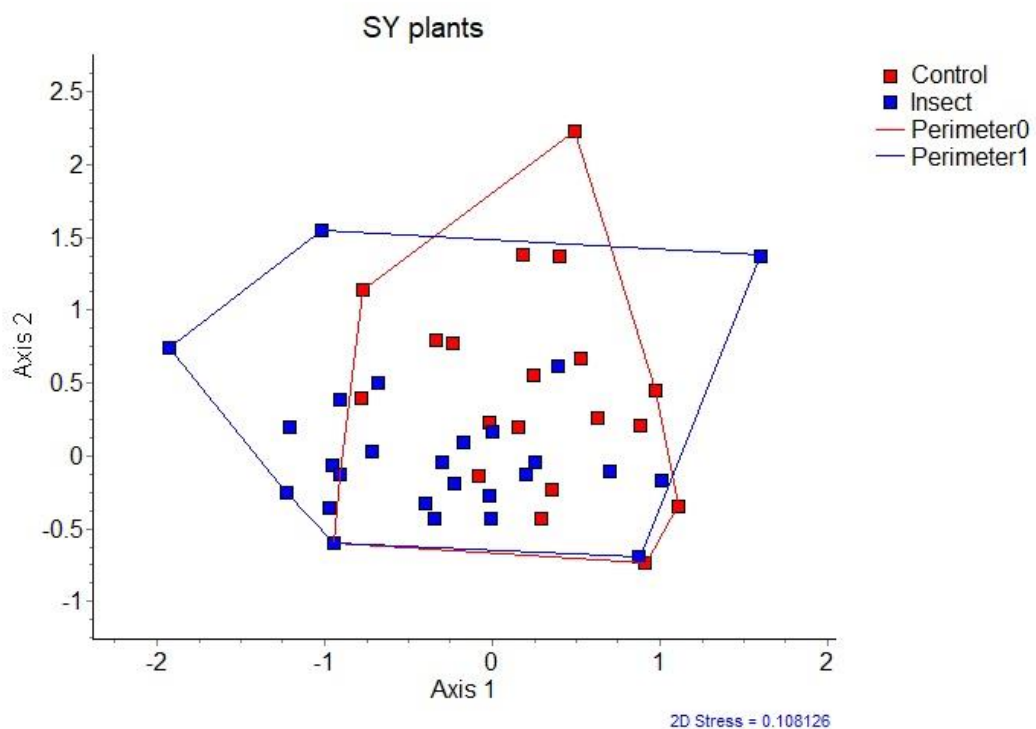


Figure 4.7 Endophyte communities in plants that were attacked by insects and their controls across (a) PW-treated and (b) SY-treated plants. Please note difference in scales for visual clarity. Vertical axis most likely represents isolation frequency of endophyte communities while horizontal axis likely represents separation by insect presence.

4.3 Discussion

These results clearly show that combinations of insects and fungi affected plant performance, but the outcome depends upon the identity of the mycorrhizas and endophytes. To date, there have been no studies that have explored the interactions between these particular endophyte species and aphids in forbs, however, there has been some work on the fungi in isolation. A *Cladosporium* sp. has been recorded to show pathogenicity towards an aphid and may kill them externally by being toxic or internally through penetration of hyphae into the body segment (Bahar et al. 2011). Therefore, *Cladosporium* sp. was chosen as a biocontrol agent of aphids in Egypt (Abdel-Baky & Abdel-Salam 2003).

This is consistent with the finding here that *Cl. sphaerospermum* reduced aphid numbers when the AM was absent. In addition, a study showed that a plant pathogen, *Passalora fulva* did not affect AMF colonisation in tomato and suggested that mycorrhizal colonisation provided a protection against it (Wang et al. 2018). However, in this experiment, *Cl. sphaerospermum* reduced plant growth with both AM inocula, when insects were present. This suggests that the presence of *Cl. sphaerospermum* and aphids, directly inhibited plant performance while *Cl. sphaerospermum* suppressed aphid infestation in balsam.

Meanwhile there were no differences of watermelon, *Citrullus lanatus* plant size, but fewer lesion areas on the leaves when aphid attacked plants that were inoculated with the fungal pathogen, *Gloeosporium orbiculare* compared to the control plants that were infected with pathogen only (Russo et al. 1997).

This suggests that aphid feeding initiated a plant protection mechanism, resulting in fewer lesions, while the fungus reduced insect attack on the plants (Russo et al. 1997) through chemicals (phytohormones and jasmonic acid) induced by the fungi and chewing insects (Pineda et al. 2013). Similarly, the aboveground fungal pathogen, *Colletotrichum gloeosporioides* promoted plant defence mechanisms that indirectly inhibited belowground percentage of roots colonised by AMF in bean plants, *Phaseolus vulgaris* (Ballhorn et al. 2014). However, endophyte presence in this study did not affect mycorrhizal colonisation.

In contrast, in this study, application of *Co. acutatum* reduced plant size and leaf numbers of plants that were attacked by insects, but only when SY was present. However, this endophytic fungus increased aphid number on SY plants when it was inoculated singly but reduced aphid infestation in the dual endophyte treatments. This suggests that *Co. acutatum* elicits chemical changes in the host, which are different in single and dual inoculations and which have different effects on insects. Additionally, there was also a suggestion that *Co. acutatum* reduces AM colonisation, however, the opposite may occur when this fungus is inoculated with another in the presence of PW inoculum. This suggests that dual endophyte infections were having a dramatic effect on the plants, and that the multiple attack by fungi and insects, initiated secondary metabolite production and induction of systemic plant resistance to inhibit aphid infestation which as result, reduced its growth and also mycorrhizal colonisation. Mycorrhizal identity was also vital in determining whether aphid attack was enhanced or suppressed, as the different inocula contained different species as described in section 3.4.2 and it seems likely

that the PW inoculum encouraged aphid infestation in all situations. This is consistent with the previous studies that show AM can have positive or negative effect on the insect growth and development depending upon their identities (Gehring & Whitham 2002; Gange et al. 2005).

In this study, insect herbivory, AM and endophytes influenced endophyte infection of *Cl. sphaerospermum* in the plants. The isolation frequency of this fungus was reduced when aphids, dual endophytes and SY were present together, but increased when PW was present. This suggests that AM may be a determining factor in the recovery of endophytes from the inoculated leaves (Eschen et al. 2010). Moreover, *Cl. sphaerospermum* addition reduced endophyte species richness in both AM treatments which suggests that interactions between the endophytes themselves in plants may be negative (Gange et al. 2007), since they are very often antagonistic *in vivo* (Chagas et al. 2013).

Aphids, AM and *Co. acutatum* affected the recovery rate of *Co. acutatum* in both AM plants, but addition of *Cl. sphaerospermum* did not affect the recovery of *Co. acutatum*. In spite of the fact that *Colletotrichum sp.* is commonly found in many plant hosts as a generalist among endophyte communities (Brown et al. 1998; Kumar & Hyde 2004) and is common in the spore rain (Fröhlich et al. 2000), it was successfully recovered from the inoculated leaves when only aphids and PW were absent or either were applied. This likely shows that when insects and PW were present together, secondary metabolite compounds, most likely phenolic acids and flavonoids were activated in response to their presence. These compounds have antimicrobial and

antioxidant activities which may prevent the inoculated fungus from entering the foliar tissues (Szewczyk et al. 2016; Szewczyk et al. 2018).

This suggests that it is possible that insect herbivores indirectly affect endophyte communities in balsam and that insects may have interfered with the entry of endophytes into balsam leaves. So this implies that one is always likely to get different results in lab v. field conditions, unless researchers control for insects in the field studies. This may also suggest that it is possible for the mycorrhizas to affect the ability of the endophyte to infect the plant in different ways. This adds a whole new level of complexity to the system –not only that there are interactions between the endophytes, mycorrhizas and insects, but that these interactions depend upon the identity of the fungal species.

4.4 Conclusion

Overall, the combination of AM and *Cl. sphaerospermum* encouraged aphid infestation, and the combined effect of insects, AM and this endophyte reduced plant growth. Moreover, interactions between aphids, both endophytes and PW resulted in reduced plant performance also. In addition, the presence of insects and PW virtually prevented *Co. acutatum* from infecting plants, but seemed to have the opposite effect with *Cl. sphaerospermum* and increased infection. Meanwhile when SY was present, it seemed to encourage *Co. acutatum* and suppressed *Cl. sphaerospermum* from entering into the leaves. This shows the positive association of SY and *Co. acutatum* and interestingly, their interaction increased aphid infestation

and possibly reduced plant growth which might be helpful for biological control of balsam. Thus, an experiment consisting of SY, *Co. acutatum* and the rust fungus was conducted in order to examine their interactions on plant performance as described in Chapter 7.

CHAPTER 5

Plant-Soil Feedbacks

CHAPTER 5 PLANT-SOIL FEEDBACKS

Soil characteristics such as physical, chemical and biological properties are important in determining plant growth, productivity and reproductive success of individual plants, neighbouring plants and community composition as well (van der Putten et al. 2013). A plant-soil feedback (PSF) is when a plant may influence and alter soil properties which lead to the altered performance of individuals of either the same species or other plant species in a subsequent generation (Bever et al. 1997; Ehrenfeld et al. 2005; Kulmatiski & Kardol 2008).

Direct, intraspecific or conspecific PSF occurs when an individual plant species has an impact on the performance of itself or other individuals from the same species. Meanwhile, indirect, interspecific or heterospecific PSF occurs when different plant species influence each other (Mccarthy-Neumann & Kobe 2010; van de Voorde et al. 2011). Positive PSF occurs when a given plant species has a subsequent enhancing effect on the same plant species growth, whereas negative PSF occurs when the soil is reduced in its ability to support the plant growth, which may result in the death of a plant and may promote the coexistence of other plant species (van der Putten et al. 2013). Changes in populations of antagonistic or mutualistic soil microbes and their effects on plant growth and performance may determine the direction of the feedback as either positive or negative.

Plant-Soil Feedbacks have been widely investigated over the past two decades in invasive species performance and fitness (Klironomos 2002;

Callaway et al. 2004; van Grunsven et al. 2007; van der Putten et al. 2013; Pattison et al. 2016). Generally, invasive plants tend to establish positive PSF that promote invasion in the introduced range and inversely in their native range, possibly because of enemy release, mutualism interaction or biogeographical differences in soil biotic communities (Reinhart & Callaway 2006; Callaway et al. 2011; Gundale et al. 2014).

Recent studies showed that Himalayan balsam exhibits a positive feedback, as it grew at a faster rate and produced more leaves and shoot biomass when grown in soil that had previously supported the species. It also displayed an ability to manipulate above and below-ground microbial assemblages by showing lower arbuscular mycorrhizal (AM) fungi root colonisation, greater endophyte species richness and also changing the level of nutrients in the soil (Pattison et al. 2016). Although it has been widely documented that balsam is weakly dependent on AM for phosphate uptake (Beerling & Perrins 1993), mycorrhizal fungi still need carbon from hosts to grow, which can lead to a negative effect on plant performance at high levels of colonisation (Tanner et al. 2014). Any AM fungal root colonisation above an optimum level may result in altering the association to become parasitic (Gange & Ayres 1999) resulting in depressed plant growth (Jin et al. 2017). It is believed that, AMF have an antagonistic relationship with balsam and it has been suggested that the changes in mycorrhizal levels and/or microbial species in the soil may in turn influence the foliar endophyte community composition (Pattison et al. 2016).

The interaction between balsam, soil feedback and the associated microbial community deserves further investigation as there are limited studies on this

subject. It is also critical to determine if these interactions might promote or discourage the rust fungus as a biological control agent against this species (Tanner et al. 2015a). Therefore, this study was initiated. The objectives were to study how different commercial inocula affect the degree of soil conditioning by balsam. The second objective was to study the differences in foliar endophyte communities of plants grown in soil that had (termed 'conditioned' soil) or had not (termed 'clean' soil) previously supported balsam. The hypothesis to be tested in this study was that Himalayan balsam grown in a conditioned soil may show altered plant performance and foliar endophyte communities, but that this would depend on the type of commercial mycorrhizal inoculum used.

5.1 Methods

The plants grown in this experiment had two growth phases that were conducted over two years, following the recommended procedure of Kulmatiski & Kardol (2008). Phase I involved conditioning the soil and was conducted in summer 2016, meanwhile Phase II, consisting of evaluating balsam performance and fungal interactions in conditioned soil, and was conducted in summer 2017. Phase I was performed by growing balsam in pots in the field, to 'condition' the soil. Soil without a balsam plant was the control in this study. Then, the conditioned and control soils from Phase I were tested by cultivating balsam in both soils. Plant performance was measured and interaction with fungi were examined which then was referred to as Phase II.

In Phase I, all pots were filled with Number 2 John Innes compost and the plants were colonised with one of two different inocula Plantworks (PW) and Symbio (SY), with a respective control for each inoculum. Himalayan balsam seeds were sown in both inocula treatments, which are hereafter termed 'PW soil' (+PW) and 'SY soil' (+SY). Half of the control inocula which were sown with seeds were called 'PW control soil' (-PW) and 'SY control soil' (-SY) while the remaining control inocula containing compost and autoclaved inocula without seeds was referred to as 'Clean PW Soil' (-C-PW) and 'Clean SY Soil' (-C-SY). There were six treatments altogether with 10 replicates resulting in 40 plants and 20 compost-only pots (Figure 5.2a).

For the first phase of the experiment, the external base of each pot was wrapped with a nylon mesh of 34 μ m aperture size by using duct tape (Figure 5.1a). Then, the pots were buried into the soil which were surrounded with thick sand and were rotated weekly to minimise the possibility of fungal hyphae from the environment entering the pot. The plants were placed in the field in a randomised block design and were watered with 250ml water daily. After eight weeks, the plants were harvested and the plant parameters (height, leaf number and shoot biomass) were recorded. Leaf and root samples were collected for all fungal assessments as described in sections 2.3 and 2.4. All soils were left to air dry for three weeks (Figure 5.1b) and were stored in an envelope individually for 11 months in a cold and dark room for Phase II experiment usage.

A year later, Phase II was conducted. The soils from Phase I were taken from the room and put in the same size pot as above, and the pots were wrapped

with the nylon mesh individually. These were labelled as 'Conditioned PW Soil' (+C+PW), 'Conditioned SY Soil' (+C+SY), 'Conditioned PW Control Soil' (+C-PW), 'Conditioned SY Control Soil' (+C-SY), 'Clean PW Soil' (-C-PW) and 'Clean SY Soil' (-C-SY) (Figure 5.2b). Two balsam seeds were sown in each soil and after 14 days, the weaker seedling was removed. Then, the pots were placed in a controlled environment facility (16h light/8h dark at $23 \pm 1^{\circ}\text{C}$, 35% relative humidity) for 5 weeks before placing outside, to ensure seedling establishment. There were 6 treatments and 10 replicates for each, resulting in 60 plants which were grown in the same field site for eight weeks (Figure 5.1c). Before flowering, the plants were harvested and plant parameters were recorded. Leaf and root materials were collected for mycorrhizal and endophyte fungal assessment as explained in sections 2.3 and 2.4. Three endophyte species were identified by molecular identification: *Colletotrichum destructivum* (GeneBank accession number: MH665647), *Didmyellaceae* (GeneBank accession number: MH665648) and *Pleosporales* (GeneBank accession number: MH665646), while the remaining species were identified morphologically by Dr. Brian C. Sutton as described in section 2.3.

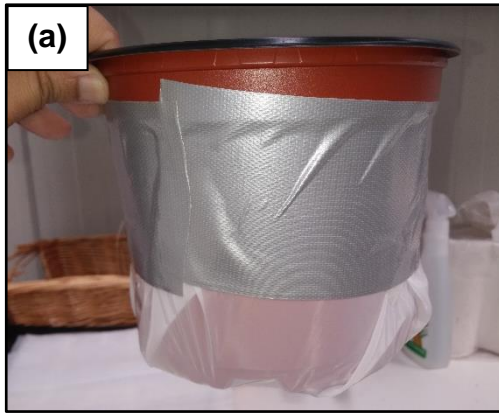
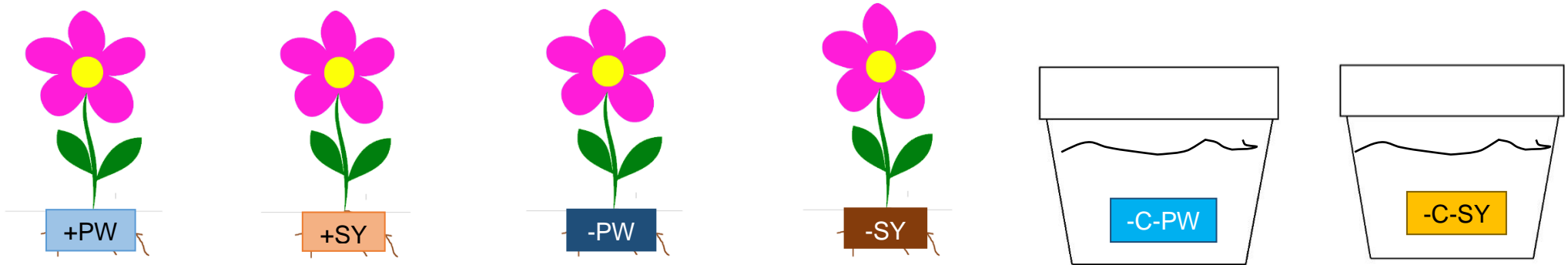


Figure 5.1 Photos show (a) a pot was wrapped with a nylon mesh in order to reduce contamination from external mycorrhizal fungi, (b) soils were air dried in a polytunnel and (c) how the plants were sunk into field soil.

(a) Phase I (conditioning soil)



(b) Phase II (conditioned soil)

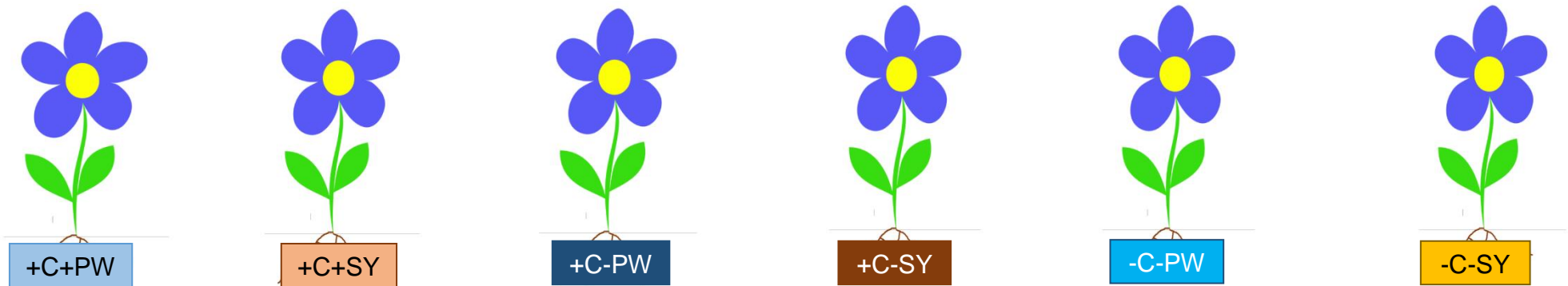


Figure 5.2 Diagram showing how the treatments were set up for each phase; (a) conditioning soil in 2016 and (b) conditioned soil in 2017. +/-PW shows presence/absence of Plantworks inoculum, +/-SY shows presence/absence of Symbio inoculum and +/-C indicates conditioned/'clean' soil (see text).

5.1.1 Statistical Analysis

All of the statistical analysis was conducted using R Studio version 1.1.383 as described in section 2.7. In the Phase I experiment, to analyse the effect of mycorrhizas on balsam performance, a one-way ANOVA was performed with mycorrhizal presence as the main effect. Meanwhile in Phase II, again, one-way ANOVA was performed to analyse the effect of mycorrhizas on the soil conditioning by balsam with AM presence as a factor. To examine the effect of soil with AM on plants, one-way ANOVA was conducted between plants that were grown in inoculated and conditioned soils with soil treatment as the main effect. Finally, to examine the effect of soil treatments, one way ANOVA was conducted with soil presence as main effect between plants that were grown in conditioned and clean soils. The analysis was done separately for each inoculum, because of the physical structure differences in the carriers (Figure 2.1).

Endophyte Isolation Frequency (IF) differences of species between treatments were examined as described in section 2.7 with a similar analytical design and the percentage data were arcsine transformed. Species abundance and species richness were examined and differences in endophyte community composition between treatments were examined with NMDS.

5.2 Results

5.2.1 Plant growth

In phase 1, there were no effects of mycorrhizas on any plant growth parameter compared to the control soil (Table 5.1a). Similarly, there were no effects of mycorrhizas on plant growth between conditioned soil (+C+PW and +C+SY) and conditioned control soil (+C-PW and +C-SY) in Phase 2 (Table 5.1b). However, plants in conditioned soil (+C+PW and +C+SY) were shorter (Figure 5.3a) and were lighter (Figure 5.3b) compared to the plants in inocula soil (+PW and +SY) (Table 5.1c). Interestingly, plants in the conditioned soil (+C+PW and +C+SY) were shorter (Figure 5.4a) and lighter (Figure 5.4b) than those grown in the clean soil (-C-PW and -C-SY) (Table 5.1d). In the absence of mycorrhizas, PW plants grown in the clean soil (-C-PW) were shorter ($F_{1,18} = 24.91$, $p < 0.001$) and lighter ($F_{1,18} = 82.44$, $p < 0.001$) than plants grown in phase 1 (-PW) (Figure 5.5a). Similar differences were seen between SY plants grown in the clean soil (-C-SY) were shorter ($F_{1,18} = 22.91$, $p < 0.001$) and lighter ($F_{1,18} = 129.7$, $p < 0.001$) than plants grown in the phase 1 (-SY) (Figure 5.5b).

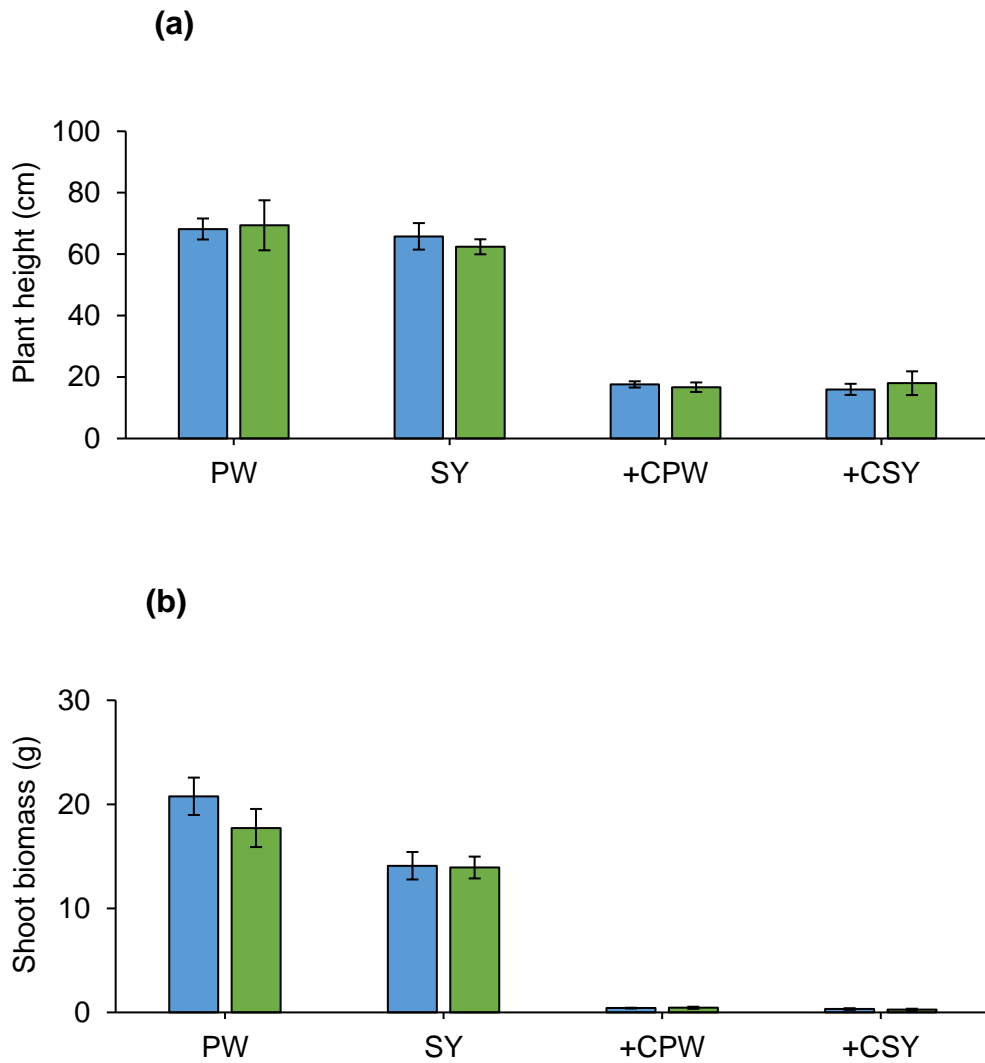


Figure 5.3 Mean of (a) plant height and (b) shoot biomass between treated plants and their control in both phases. Blue bars represent mycorrhizas present while green bars represent mycorrhizas absent. PW is Plantworks soil and SY is Symbio soil in Phase 1. +CPW is Conditioned Plantworks soil and +CSY is Conditioned Symbio soil in Phase 2. $n=10$ in all treatments. Error bars are one SE.

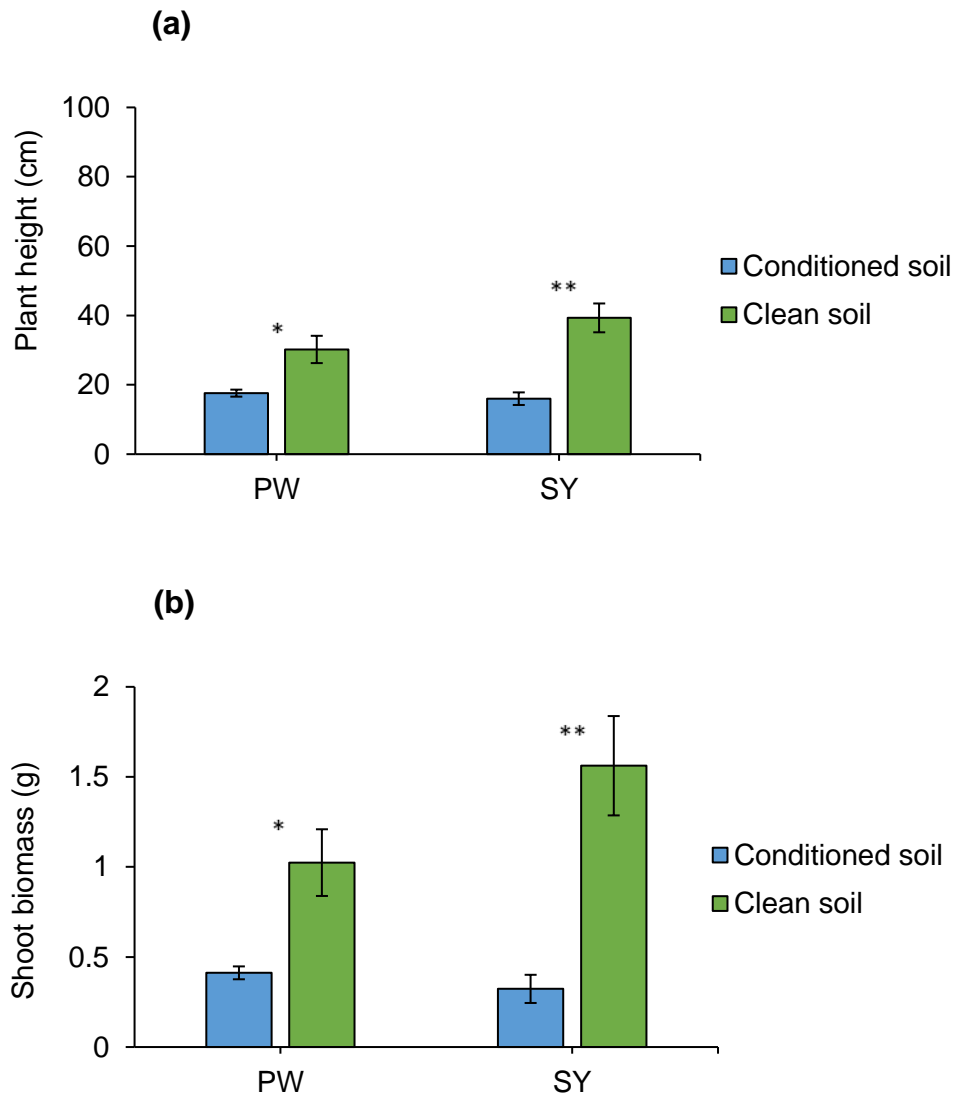


Figure 5.4 Mean of (a) plant height and (b) shoot biomass in different soil conditions –both from the plants grown in Phase 2. PW is Plantworks soil and SY is Symbio soil. $n=10$ in all treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, * $p < 0.05$ and ** $p < 0.001$.

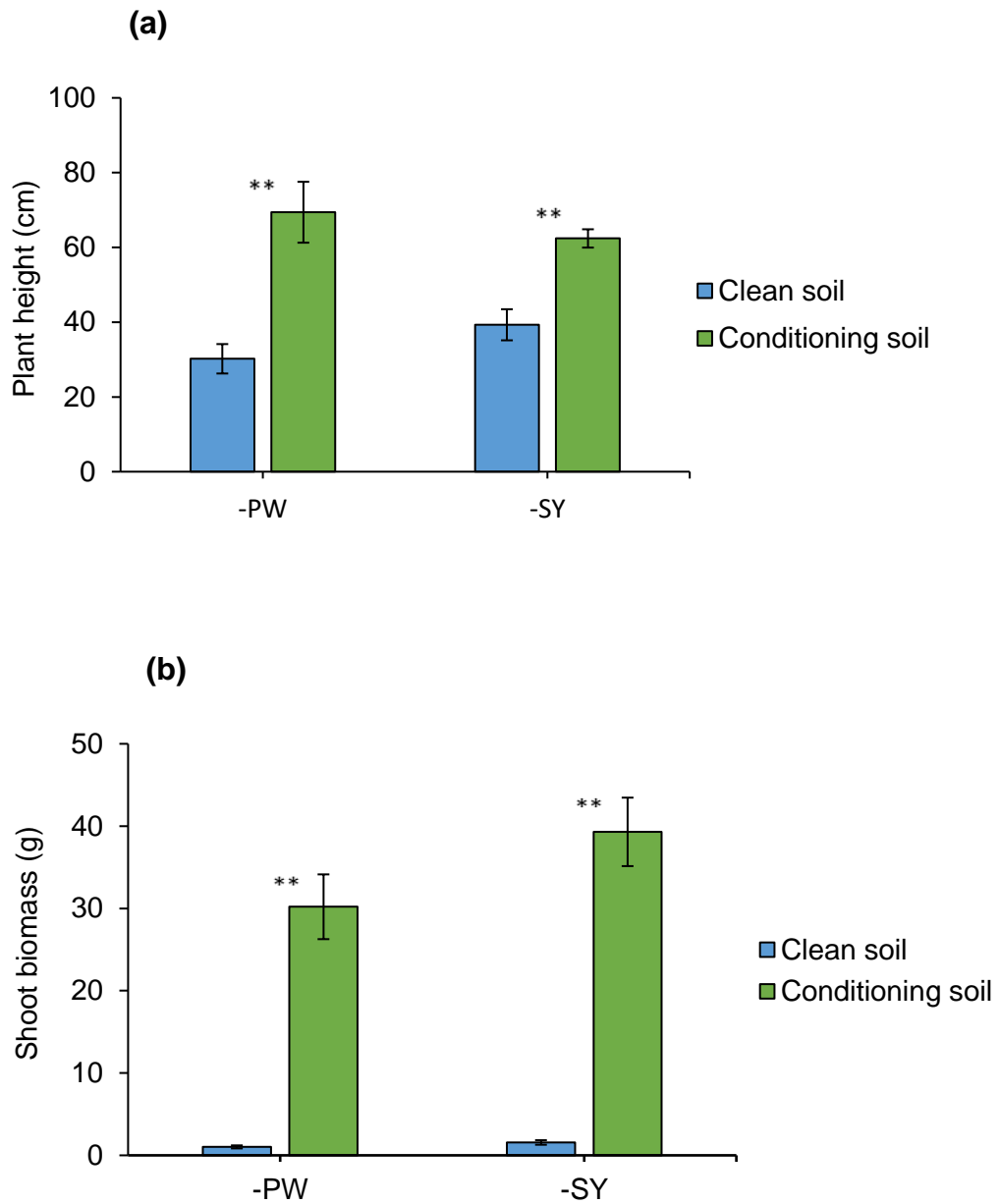


Figure 5.5 Mean of (a) plant height and (b) shoot biomass of plants grown without AM in conditioning soil in phase 1 and clean soil in phase 2. -PW is without Plantworks inoculum and -SY is without Symbio inoculum. $n=10$ in all treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, ** $p < 0.001$.

Table 5.1 One-way factorial analysis results across treatments. Degrees of Freedom for F values = 1, 18 and $n = 10$ for each treatment. (a) Plants were grown in the Inocula Soil were compared with the plants grown in the Control Soil, (b) Plants were grown in the Conditioned Soil were compared with the plants grown in the Conditioned Control Soil, (c) Plants were grown in the Inocula Soil –Phase 1 were compared with the plants grown in the Conditioned Soil –Phase 2 while (d) Plants were grown in the Conditioned Soil –Phase 2 were compared with the plants grown in the Clean Soil –Phase 2. Bold indicated significant values.

(a) Phase I –Conditioning phase (Inocula Soil vs Control Soil)	Height		Leaf number		Shoot biomass		RLC	
	F	<i>p</i>	F	<i>p</i>	F	<i>p</i>	F	<i>p</i>
AM –Plantworks	0.018	0.893	1.816	0.194	1.412	0.250	191.7	<0.001
AM –Symbio	0.469	0.502	1.781	0.920	0.010	0.920	98.35	<0.001

(b) Phase II –Feedback phase (Conditioned Soil vs. Conditioned Control Soil)	Height		Leaf number		Shoot biomass		RLC	
	F	<i>p</i>	F	<i>p</i>	F	<i>p</i>	F	<i>p</i>
AM –Plantworks	0.236	0.633	1.429	0.248	0.134	0.719	9.634	<0.001
AM –Symbio	0.038	0.847	0.710	0.410	1.035	0.322	2.338	0.144

(c) Both phases (Inocula Soil vs Conditioned Soil)	Height		Leaf number		Shoot biomass		RLC	
	F	<i>p</i>	F	<i>p</i>	F	<i>p</i>	F	<i>p</i>
Soil -Plantworks	201.4	<i>p<0.001</i>	243.2	<i>p<0.001</i>	128.5	<i>p<0.001</i>	51.75	<i>p<0.001</i>
Soil -Symbio	113.1	<i>p<0.001</i>	174.2	<i>p<0.001</i>	108.4	<i>p<0.001</i>	18.7	<i>p<0.001</i>

(d) Phase II (Conditioned Soil vs Clean Soil)	Height		Leaf number		Shoot biomass		RLC	
	F	<i>p</i>	F	<i>p</i>	F	<i>p</i>	F	<i>p</i>
Soil -Plantworks	9.615	0.006	16.09	<i>p<0.001</i>	10.53	0.004	18.55	<i>p<0.001</i>
Soil -Symbio	26.38	<i>p<0.001</i>	14.27	<i>p<0.001</i>	18.67	<i>p<0.001</i>	2.025	0.172

5.2.2 AMF colonisation

Balsam grown in PW soil showed twice the amount of AM colonisation than plants grown in SY soil in both growth phases (Figure 5.6). Plants grown in the inoculated soil (+PW and +SY) showed higher AM colonisation than plants grown in the control soil (-PW and -SY) (Table 5.1a). Similarly, plants grown in the conditioned PW soil (+C+PW) had higher colonisation compared to the plants without PW inoculum in conditioned soil (+C-PW), however, no difference in SY-treated plants was found (Table 5.1b).

In contrast, plants grown in the conditioned soils (+C+PW and +C+SY) had lower AM colonisation compared to the inocula soils (+PW and +SY) (Table 5.1c). However, plants grown in the conditioned soil with PW inoculum (+C+PW) recorded higher colonisation compared to the plants in the clean soil (-C-PW) (Figure 5.7), while no difference was found in SY plants (Table 5.1d).

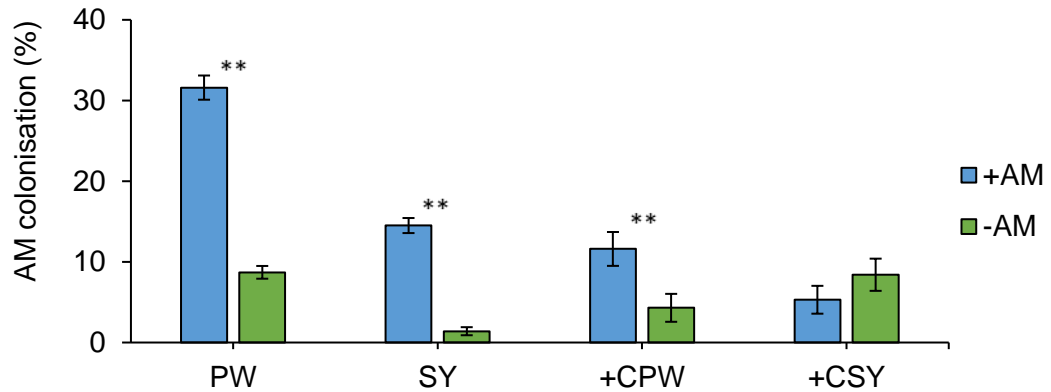


Figure 5.6 AM colonisation of treated plants and their controls in both phases. PW is Plantworks soil and SY is Symbio soil in Phase 1. +CPW is Conditioned Plantworks soil and +CSY is Conditioned Symbio soil in Phase 2. $n=10$ in all treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, $**p < 0.001$.

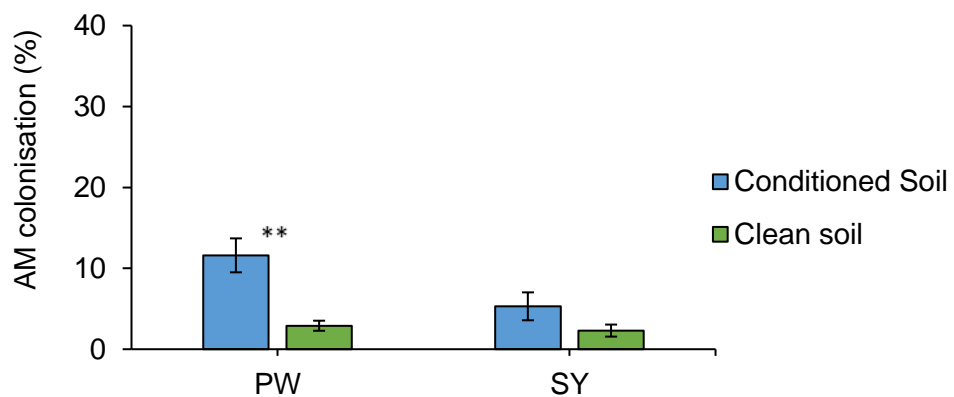


Figure 5.7 AM colonisation in different soil conditions. Both were from the plants grown in Phase 2. PW is Plantworks soil and SY is Symbio soil. $n=10$ in all treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, $**p < 0.001$.

5.2.3 Endophytic fungal communities

Eleven endophyte species were isolated from plants in the conditioning soil phase while 16 species were recorded from the second phase (Table 5.2). Five species were found from conditioning soil plants only –*Chaetomium cochliodes*, *Lecanicillium sp.*, *Nigrospora oryzae*, *Peniophora sp.*, and *Phialophora cyclaminis*. Ten endophyte species were only isolated from conditioned soil plants –*Acremonium incoloratum*, *Alternaria alternata*, *Chaetomium elatum*, *Colletotrichum destructivum*, Didmyellaceae, *Exophiala sp.*, *Fusarium acuminatum*, *Geniculosporium sp.*, Pleosporales and *Alternaria infectoria*. Six endophyte species were found in both phases –*Acremonium strictum*, *Cladosporium cladosporioides*, *Cladosporium oxysporum*, *Cladosporium sphaerospermum*, *Colletotrichum acutatum* and *Penicillium sp.*

Cl. sphaerospermum recorded the highest average Isolation Frequency (IF) value across conditioning soil plants but was isolated with a low frequency from feedback plants. This fungus recorded higher IF mean in inocula PW (+PW) plants ($F_{1,18} = 26.61$, $p < 0.05$) compared to the conditioned PW plants (+C+PW), but showed no difference in SY-treated plants. Meanwhile *A. alternata* recorded higher IF mean in conditioned PW (+C+PW) plants ($F_{1,18} = 11.91$, $p < 0.05$) compared to the plants grown in inoculum (+PW) soil. Furthermore, the IF of *A. alternata* was higher in conditioned SY plants (+C+SY) ($F_{1,18} = 10.85$, $p < 0.05$) compared to the inoculum (+SY) plants.

Table 5.2 The Isolation Frequency (%) mean of each endophyte species across both phases. $N = 10$ for each treatment. Bold values indicate the highest IF. (Note: +PW: Plantworks Soil, -PW: Plantworks Control Soil, +SY: Symbio Soil, -SY: Symbio Control Soil, +CPW: Conditioned Plantworks soil, +C-PW: Conditioned Plantworks Control soil, +CSY: Conditioned Symbio Soil, +C-SY: Conditioned Symbio Control Soil, -C-PW: Clean Plantworks Control Soil and -C-SY : Clean Symbio Control Soil.

Endophyte species	Conditioning Soil Phase (Phase 1)					Evaluating Feedback Phase (Phase 2)						
	+PW	-PW	+SY	-SY	Total	+C+PW	+C-PW	+C+SY	+C-SY	-C-PW	-C-SY	Total
<i>Acremonium incoloratum</i>	0	0	0	0	0	14.14	17.9	9.17	11.23	23.03	22.07	16.26
<i>Acremonium strictum</i>	2	0	0	0	0.5	1.43	0	0	1.3	0	0	0.46
<i>Alternaria alternata</i>	0	0	0	0	0	33.1	37.5	24.1	12.07	34.53	29.23	28.42
<i>Alternaria infectoria</i>	0	0	0	0	0	2.5	1.43	0	0	0	0	0.66
<i>Chaetomium cochliodes</i>	0	0	3.33	0	0.83	0	0	0	0	0	0	0
<i>Chaetomium elatum</i>	0	0	0	0	0	1.11	8.1	4.2	4.3	6.51	8.5	5.45
<i>Cladosporium cladosporioides</i>	0	12.5	0	4.5	4.25	0	0	0	0	0	0	1.33
<i>Cladosporium oxysporum</i>	0	0	2	4.5	1.63	0	4.7	3.33	3.33	0	3.9	2.54
<i>Cladosporium sphaerospermum</i>	39.03	58.67	32.85	27.17	39.43	0	2	10	0	3	3.4	3.07
<i>Colletotrichum acutatum</i>	13.33	10.33	2.5	6.5	8.17	2.54	8.45	1.67	2.68	8.17	11.43	5.82
<i>Colletotrichum destructivum</i>	0	0	0	0	0	0	3.75	5.83	0	7.5	4	3.51
<i>Didmyellaceae</i>	0	0	0	0	0	0	0	0	0	0	5.5	0.92

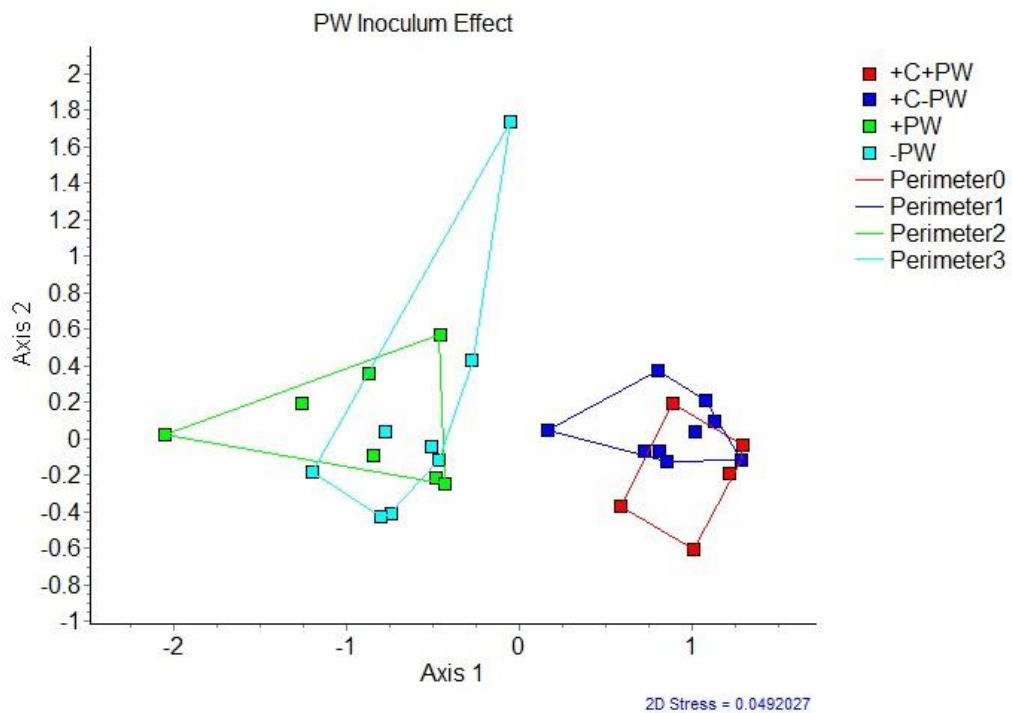
Endophyte species	Conditioning Soil Phase (Phase 1)					Evaluating feedback Phase (Phase 2)						
	+PW	-PW	+SY	-SY	Total	+CPW	+C-PW	+CSY	+C-SY	-C-PW	-C-SY	Total
<i>Exophiala sp.</i>	0	0	0	0	0	0	0	21.67	5	9	8.93	7.43
<i>Fusarium acuminatum</i>	0	0	0	0	0	1.43	1.25	3.33	0	1.11	1	1.35
<i>Geniculosporium sp.</i>	0	0	0	0	0	1.11	0	0	0	0	0	0.19
<i>Lecanicillium sp.</i>	12.33	2	35.33	10.5	15.04	0	0	0	0	0	0	0
<i>Nigrospora oryzae</i>	1.67	4	2	9	4.17	0	0	0	0	0	0	0
<i>Penicillium sp.</i>	5	2.5	0	0	1.88	1.43	0	0	2.92	4.36	2	1.79
<i>Peniophora sp.</i>	15	10	0	2.5	6.88	0	0	0	0	0	0	0
<i>Phialophora cyclaminis</i>	1.67	0	10	5.33	4.25	0	0	0	0	0	0	0
<i>Pleosporales</i>	0	0	0	0	0	1.25	4	0	6.01	2.92	0	2.36

The differences between the communities in the different treatments within a year were recorded too. IF mean of *Chaetomium elatum* was lower in plants grown with PW inoculum in conditioned soil (+C+PW) compared to the plants without inoculum in conditioned soil (+C-PW) ($F_{1,18} = 4.984, p < 0.05$) while *Lecanicillium sp.* was higher in SY inoculum soil (+SY) compared to the control (-SY) ($F_{1,18} = 4.51, p < 0.05$). In addition, endophyte species richness per plants of plants grown in the conditioned soils with PW (+C+PW) was lower than that of their controls (+C-PW) ($F_{1,18} = 4.457, p < 0.05$) and to the plants grown in the clean soils without PW (-C-PW) plants ($F_{1,18} = 5.968, p < 0.05$). Similarly, species richness of plants grown in the conditioned soils with SY (+C+SY) was lower compared to those grown in the clean soil when SY was absent (-C-SY plants) ($F_{1,18} = 5.188, p < 0.05$)

The presence of inocula had an effect on endophyte communities within and between the phases of PW inoculum (+PW) plants, their controls (-PW), plants grown in the conditioned soil with PW (+C+PW) and their controls (+C-PW) (ANOSIM $R = 0.5680, p < 0.001$) (Figure 5.8a). Similar differences were seen between SY inoculum (+SY) plants, their controls (-SY), SY inoculum plants in the conditioned soil (+C+SY) and their controls (+C-SY) (ANOSIM $R = 0.5034, p < 0.001$) (Figure 5.8b). In addition, conditioning of soil by balsam in the absence of mycorrhizas also affected endophyte communities, between plants grown in the conditioned soil without PW (+C-PW) and plants grown in the clean soil without PW (-C-PW) (ANOSIM $R = 0.2007, p < 0.001$) (Figure 5.9a). There were also differences in communities within plants grown in the conditioned soil without SY (+C-SY) and those grown in the clean soil without SY (-C-SY) (ANOSIM $R = 0.5529, p < 0.001$) (Figure 5.9b). Interestingly, both

inocula differed in the effects on endophyte communities where plants grown in the PW inoculum (+PW), SY inoculum (+SY), plants grown in the conditioned soil with PW (+C+PW) and with SY (+C+SY) were further separated in the two phases (ANOSIM $R = 0.5040$, $p < 0.001$) (Figure 5.10).

(a)



(b)

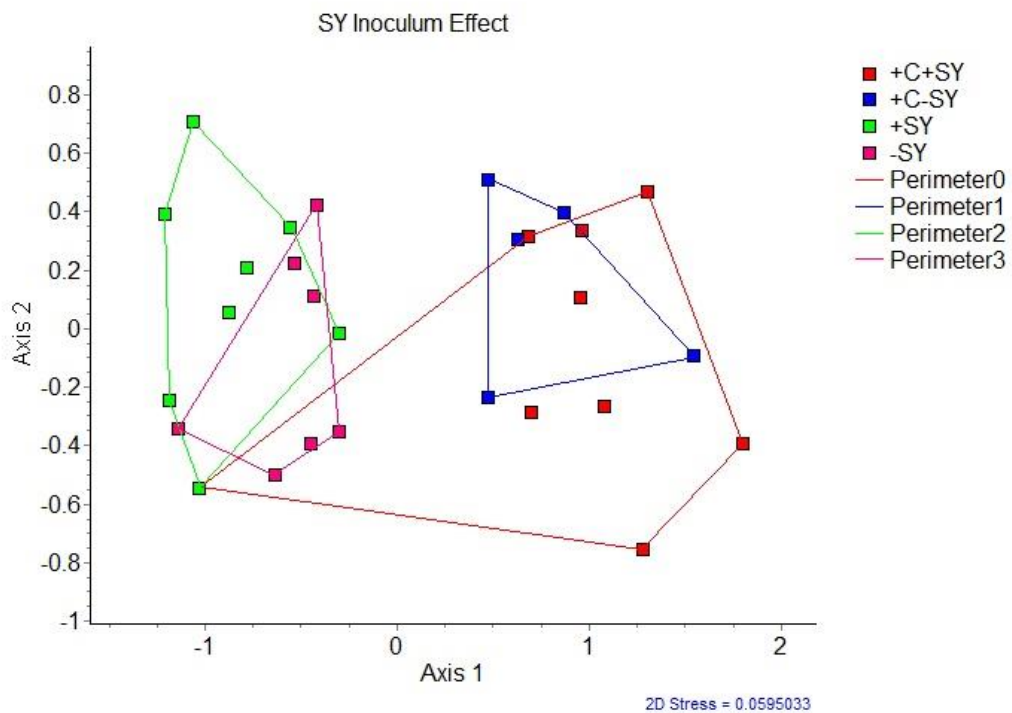
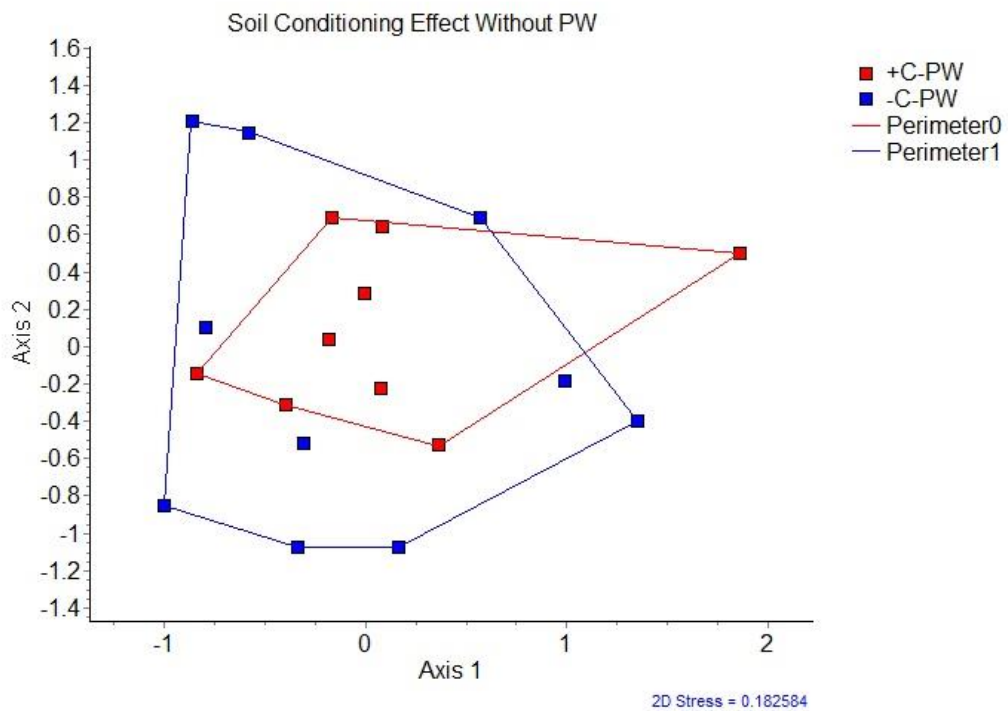


Figure 5.8 Inocula effect on endophyte fungal communities within and between the two phases of (a) Plantworks plants and (b) Symbio plants. +/-PW: Plantworks present/absent, +/-SY: Symbio present/absent and +C: Conditioned soils). Please note difference in scales for visual clarity. Vertical axis most likely represents isolation frequency of endophyte communities while horizontal axis likely shows separation by soil conditioning.

(a)



(b)

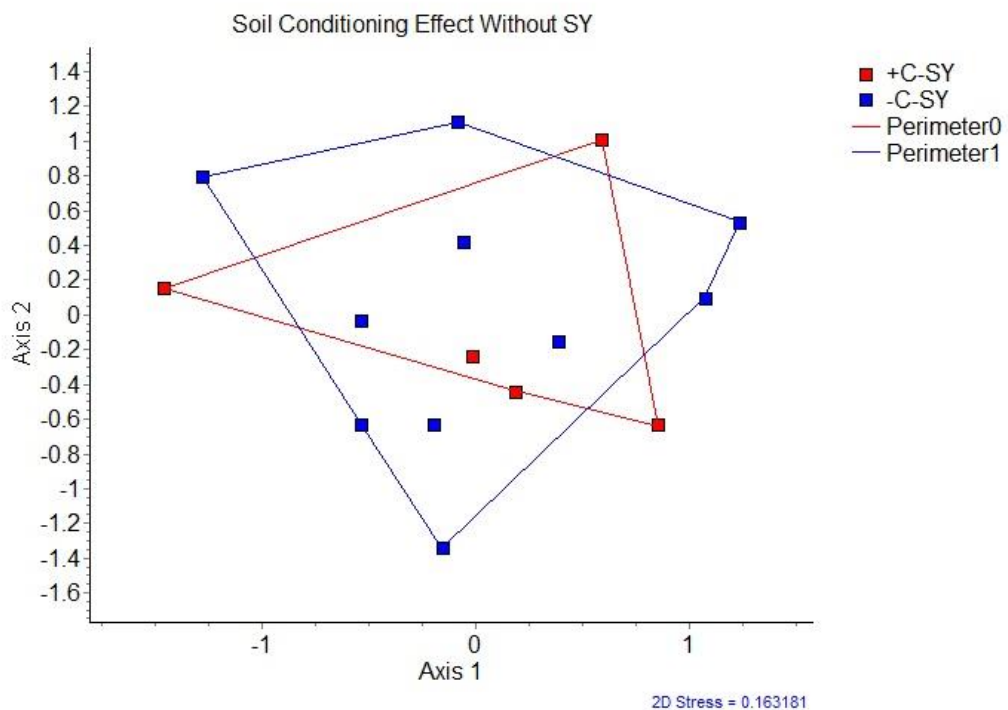


Figure 5.9 Conditioning of soil by balsam in the absence of (a) Plantworks and (b) Symbio affect endophyte communities. +/-PW: Plantworks present/absent, +/-SY: Symbio present/absent and +/-C: conditioned/clean soils). Please note difference in scales for visual clarity. Vertical axis most likely represents isolation frequency of endophyte communities while horizontal axis likely shows separation by soil conditioning.

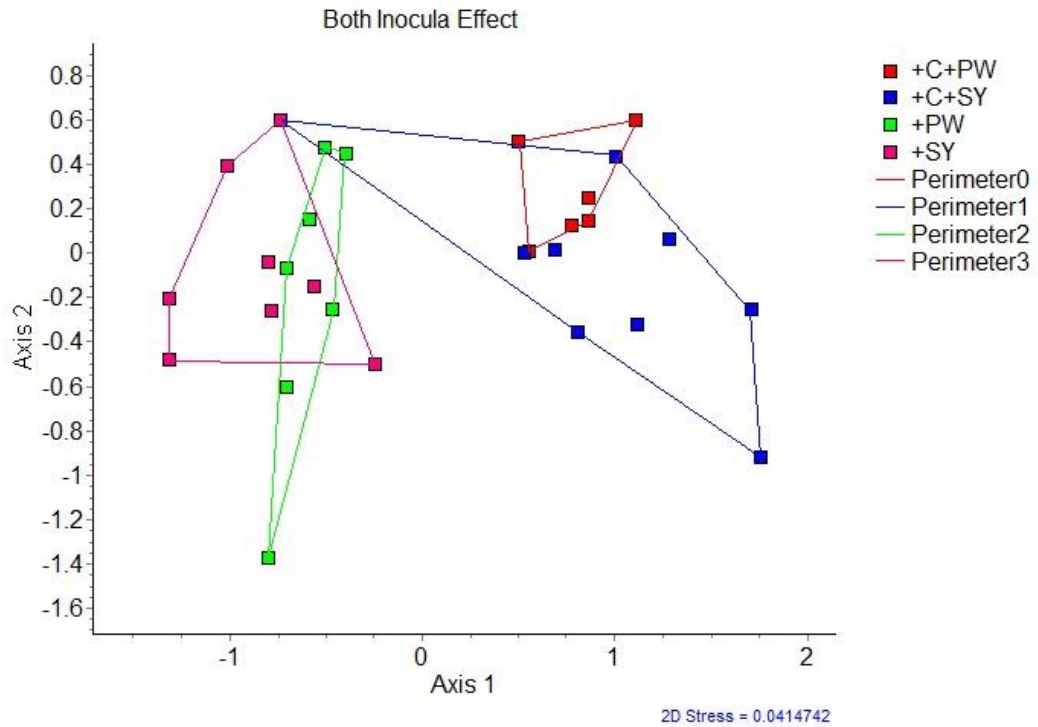


Figure 5.10 Both inocula effect on endophyte communities and the different in the both phases. +/-PW: Plantworks present/absent, +/-SY: Symbio present/absent and +C: conditioned soils). Please note difference in scales for visual clarity. Vertical axis most likely represents isolation frequency of endophyte communities while horizontal axis likely shows separation of by soil conditioning.

5.3 Discussion

Many studies have been reported on PSF of invasive species especially in grasses (van Grunsven et al. 2007; Schittko et al. 2016) yet only few studies have focused on forb species (Kos et al. 2015), and only one on Himalayan balsam (Pattison et al. 2016). Therefore this study was conducted to contribute to the body of knowledge on Himalayan balsam soil feedback with mycorrhizas and soil type as the main factors and to examine whether commercial mycorrhizal inocula produced the same effects as natural colonisation. The results indicate that both mycorrhizal identity and soil type are critical in determining the outcome of the feedback.

Himalayan balsam plants grown in a conditioned soil were shorter, bore fewer leaves, with lower shoot biomass and less AM colonisation than plants in the inoculated soils irrespective of inoculum type, suggesting that it displayed a negative PSF. Similarly, the conditioned plants were shorter and lighter than those in phase 1, in the absence of inoculum (-PW and -SY). The difference in plant growth in the two years was noticeable and this may be because the second year was much warmer than the first. However, plants grown in the conditioned soil with the presence of PW (+C+PW) showed higher AM colonisation compared to the balsam grown in the clean soil without PW (-C-PW), while no difference was seen in SY-treated plants. This finding suggests that addition of PW promoted mycorrhizal colonisation. It is interesting to note that, while higher AMF colonisation was seen in the plants grown in the conditioned soils with PW inoculum (+C+PW), this did not result in greater vegetative growth, suggesting that the inoculum was not beneficial to the

plants despite colonisation being high. It has been well documented that AMF enhanced the uptake of phosphate to plants (Richardson et al. 2009) while in return hosts provided carbon to AMF, which may weaken the plant growth at high level of colonisation (Gange & Ayres 1999).

Interestingly, PW inoculum plants had double the colonisation amount of the SY inoculum plants in both phases which was similar to the plant-fungi-insect interaction experiment described in section 4.2.1. This may be explained by the different mycorrhizal species composition of the two inocula as explained in section 3.4.2, as the possibility of fungal entry from the environment was minimised by a thick sand 'wall' made around the pots together with pots being rotated weekly. In addition, SY inoculum consists of spores only, while the PW is made of spores, pieces of hyphae and colonised roots and previous studies have shown that the colonisation was variable between inocula that have different propagule types (Klironomos & Hart 2002; Faye et al. 2013; Herrmann & Lesueur 2013). This has shown that not only are AMF important in determining plant performance in native and invaded areas, but it also depends on their identities. In addition, AMF are known to show species specificity, where the same AMF species can occur in different plant species and produce different effects on the plant growth (Klironomos 2000; Edwards 2017).

In this study, balsam plant biomass in a conditioned soil was 25g lighter, 50cm shorter and AM colonisation was 10% reduced compared to the recent study which recorded a positive PSF (Pattison et al. 2016). There were differences between the recent study (Pattison et al. 2016) and this study where the former

conditioned field soil had not been cultivated for over 30 years and was dominated by native species (*Urtica dioica*) and an invasive species (*Impatiens parviflora*) whereas this work used a commercial compost (John Innes) with the addition of commercial mycorrhizal inoculum. This may explain the differences in the findings as both studies begin with very different soil and nutrient composition. In addition, a recent study demonstrated that balsam induced alteration in soil chemical properties including increasing ammonium (NH_4^+) and decreasing nitrate (NO_3^-) levels (Majewska et al. 2018). Whether similar changes occurred in the present experiment is unknown, but this is another likely explanatory factor for the difference seen here and in the results of Pattisson et al. (2016). Many studies have been performed in commercial composts because they are supposed to be more uniform than field soils, thus reducing variability (Jeffries et al. 2003; Edwards 2017). However, it is clear that they do not mimic field soils in any way. Furthermore, this has demonstrated that the outcome of a PSF can be reversed by using a different soil type.

This is similar to previous studies when *Bromus diandrus* in a monoculture experienced a positive feedback in soil that was collected from an abandoned citrus agriculture site (Hilbig & Allen 2015) while three invasive plant species (*Heracleum mantegazzianum*, *Tragopogon dubius* and *Eragrostis pilosa*) grown in a soil with live inoculum produced a negative feedback (being shorter and less biomass) compared to native plant species (*Heracleum sphondylium*, *Tragopogon pratensis* and *Poa annua*) (van Grunsven et al. 2007). The negative feedbacks are most likely because of the accumulation of pathogenic fungi in the soil resulting in weakened plants and reduced AM colonisation

(van Grunsven et al. 2007). Although this results produced a similar outcome, which is negative feedback, to my knowledge, this is the first study conducted on PSF in balsam with the addition of mycorrhizal inoculum. Therefore, this finding could provide important insights into our understanding on the fungal community interactions in the invaded areas and these may contribute to establishing their role in the biological control of balsam by using the rust fungus (Tanner et al. 2015a).

PSF have generally focused on plant parameters and the effect on soil microbial communities, however, the effects on above-ground communities have generally been ignored. The commonest endophyte species identified in this study were *Cl. sphaerospermum* in the conditioning soil and *Al. alternata* in the conditioned soil. Both species are known to be ubiquitous in nature, yet still showed differences in infection levels between treatments. It also should be noted that the endophyte community within plants in the inocula soil in Phase 1 and in plants grown in the conditioned soil in Phase 2 were different, with only three similar species occurring in both treatments. This is because it was two different years and one would expect the spore rain, climate and nutrient deplicit to be different in both years. However, it is also interesting to note that the communities in the different treatments within a year also differed. This suggests that AMF were important in determining the structure of the foliar endophyte communities (Eschen et al. 2010) in balsam leaves and to date, to my knowledge, this is the first study that has demonstrated the effect of mycorrhizal on the endophyte communities in balsam. Effects of endophytes on mycorrhizal colonisation are well established (Chu-Chou et al.

1992; Larimer et al. 2012; Zhou et al. 2018) but, little is known for the opposite effects.

Furthermore, endophyte species richness was lower in plants grown in the conditioned soil compared to the plants grown in the clean soil and there was clear separation of endophyte fungal communities between treatments. This likely showed that Himalayan balsam generated a negative PSF on fungal endophyte communities and suggested AMF and soil conditions may have had a significant effect on foliar endophyte communities, as the plants that grew in the balsam soils were less susceptible to endophyte infection. Changes in soil conditions may lead to changes in the foliar fungal communities in the perennial forb *Cirsium arvense* (Eschen et al. 2010) and balsam (Pattison et al. 2016).

Thus, these findings with Himalayan balsam suggest that differences in mycorrhizal composition and soil conditions may affect the plant performance and ultimately the ecological impact of foliar fungal endophytes, such as protection against herbivores or pathogens by inducing phytoalexins (Gao et al. 2010). Therefore, it is important to understand their interactions as it could have important consequences for biological control of this invasive species.

5.4 Conclusion

In conclusion, this study showed that balsam grown in the conditioned soils produced a negative PSF and also altered foliar microbial communities, differing from the previous study (Pattison et al. 2016). Furthermore, commercial inocula do not seem to give the same result as natural AMF and commercial compost is not a good mimic of field soil and can reverse the PSF. Finally, the continuous stands of balsam may harbour reduced endophyte communities in the leaves.

CHAPTER 6

Mycorrhizal effects on balsam competitive ability

CHAPTER 6 MYCORRHIZAL EFFECTS ON BALSAM COMPETITIVE ABILITY

Arbuscular mycorrhizal fungi (AMF) have been shown to provide direct benefits to plants by promoting plant growth (Gange & Ayres 1999), protection from air-borne fungal pathogens (Wang et al. 2018) and also maintaining soil health (Jeffries et al. 2003). There is also great interest in understanding how the relationship between plants and AM fungi can influence plant community structure and productivity (Stampe & Daehler 2003; Yang et al. 2014) as well as interspecific competition (Danieli-Silva et al. 2010; Wagg et al. 2011; Emery & Rudgers 2012; Zhang et al. 2017).

Plant competition is an important determining factor in the structure of plant communities (Aerts 1999), which is mediated by AMF (Lin et al. 2015). However, it is not only the presence of AMF that has an effect on plant competition, but also the identity of mycorrhizal species composition and the competing plants and whether they are AMF-dependent or non dependent (Scheublin et al. 2007). These authors have shown that the AMF-dependent legume *Lotus corniculatus* was strongly affected by the presence of AMF and outcompeted the less AMF-dependent grass, *Festuca ovina* and a mycorrhizal-dependent forb, *Plantago lanceolata* (Scheublin et al. 2007). Similarly, AMF also indirectly promoted the competitive effects of the AMF-dependent invasive plant species, *Centaurea maculosa* on native grasslands, composed of *Festuca idahoensis* in western North America (Marler et al. 1999). In terms of mycorrhizal identity, a study showed that the composition of AMF communities is vital to enable plants to co-exist and distribute soil

nutrients between plant species (van der Heijden et al. 2003). These authors also showed that the perennial forb, *Prunella vulgaris* was outcompeted by the grass, *Brachypodium pinnatum*, when *Glomus* sp. isolate BEG 19 was added compared to when *Glomus* sp. isolate BEG 21 was present. In addition, the latter AMF taxon was likely to be ineffective in phosphorus acquisition, as it had lower P content than plants inoculated with *Glomus* sp. isolate Basle Pi (van der Heijden et al. 2003).

Meanwhile, mycorrhizal colonisation positively affected shoot biomass of the deciduous shrub, *Acacia caven* while negatively affecting an annual forb species, *Bidens pilosa* when the species were grown in isolation. However, these effects disappeared when the plants were grown at higher densities (Pérez & Urcelay 2009). This suggests there is an interaction between mycorrhizal colonisation in the roots and plant density (Pérez & Urcelay 2009) and the presence of common mycelial networks (Workman & Cruzan 2016) which deserves more attention and may affect the outcome of plant competition and invasion success.

On top of plant density, range-based (native or introduced range) differences can strongly affect mycorrhizal responsiveness too. For example, AMF has a suppressive effect on the biomass of an annual invasive species, *Centaurea solstitialis* when grown in competition against a North American native bunchgrass, *Stipa pulchra* but the effect was much stronger in a native range genotype compared with one from the introduced range (Waller et al. 2016). This has shown that different plant genotypes have different AMF responsiveness, which may in turn influence plant competition (Waller et al.

2016). Besides, mycorrhizal diversity also has a large impact on the interspecific competition as the more diverse AMF can increase plant performance and plant productivity. For example, growth of the legume, *Trifolium pratense* was suppressed by the presence of high AMF richness when in competition with the grass, *Lolium multiflorum* which did not occur when AMF richness was low (Wagg et al. 2011).

Several studies have shown that mycorrhizal presence may influence intraspecific competition in grasses (West 1996; Watkinson & Freckleton 1997) and forbs (Facelli et al. 1999; Facelli & Facelli 2002). For example, mycorrhizal colonisation increased shoot biomass of *Holcus lanatus* and *Dactylis glomerata* when they were experiencing intraspecific interactions (West 1996) compared with competing plants without the mycorrhiza. Similarly, mycorrhizal inoculation benefited the growth of *Trifolium subterraneum* in a monoculture and also enhanced P uptake (Facelli & Facelli 2002). However, the positive effects reduced as the plant density increased (Facelli et al. 1999). In addition to mycorrhizal colonisation, the characteristics of plant species may have an influence on plant competitive interactions. This can be seen when AMF exhibited parasitic effects on monocultures of the tropical shrub *Cabralea canjerana* while having a symbiotic effect on similar monocultures of *Lafoensia pacari*. The former species has very large leaves which may have increased the ability to obtain resources and intensify the intraspecific competition, while the latter species has small leaves, meaning less intense intraspecific competition and allowing mycorrhizas to show positive effects on plant growth (Danieli-Silva et al. 2010).

Himalayan balsam is known to be a strong competitor due to its rapid growth, coupled with high reproductive output and ability to outcompete native flora (Andrews et al. 2005). These features may enable it to become a more serious threat to nature in the future (Prach 1994; Pysek & Prach 1995). A study has shown that Himalayan balsam was the best competitor by producing high aboveground biomass when competing with the native species, *Salix alba* and *Urtica dioica*. In addition, balsam produced greater biomass when in intraspecific competition compared to four other invasive species (*Acer negundo*, *Buddleja davidii*, *Fallopia japonica* and *Paspalum distichum*) and five native (*Agrostis stolonifera*, *Populus nigra*, *Rubus caesius*, *S. Alba*, *U. dioica*) species (Bottollier-Curtet et al. 2013). Interestingly, balsam was able to outcompete the perennial herbaceous native species, *Urtica dioica* (Bottollier-Curtet et al. 2013; Gruntman et al. 2014) and the superior competitive ability of this invasive species was suggested to be promoted by its strong allelopathic effect on the native species (Gruntman et al. 2014). This has shown the potential of balsam to become an aggressive and better competitor in self-replicating stands after several growing seasons by displaying a positive plant-soil feedback (Pattison et al. 2016) and this may be a reason why it forms monocultures in the field.

Despite the fact that Himalayan balsam is a vigorous annual invasive plant species and likely to suppress native plant species growth in the introduced range (Tanner & Gange 2013), the effect of mycorrhizas on balsam-native plant competition still remains unknown. Thus, this study was conducted to examine the mycorrhizal effect on the competitive interactions between Himalayan balsam and the most dominant co-occurring native species in the

introduced range, *P. lanceolata* and *H. lanatus*. These species form a pronounced association with mycorrhizas compared to balsam (Harley & Harley 1987; Wearn et al. 2012). In this study, mycorrhizal effect on balsam and native plant performance in a competitive environment and also above and below ground microbial communities between plants were conducted. The tested hypothesis was that mycorrhizal colonisation may reduce balsam growth while the native plants are expected to be better competitors when AM is present, as they are AM-dependent. Furthermore, addition of AM should increase colonisation levels, but reduce endophyte fungal communities in the plants as described in sections 4.2 and 5.2.

6.1 Methods

The two native plants species in this study were ribwort plantain, *Plantago lanceolata* (PL) (Figure 6.1a) and Yorkshire fog grass, *Holcus lanatus* (HL) (Figure 6.1b). The former plant is a perennial forb from Plantaginaceae family which can flower in its first year from seed in spring and grow until late in the growing season. The plants can survive during overwintering stage as a small rosette of leaves (Wearn et al. 2012). The mature plants have a good root association with mycorrhizal fungi and short thick rhizome (Wearn et al. 2012) and can often be attacked by a range of generalist insects that can reduce AM colonisation in this strongly mycotrophic plant (Gange et al. 2002a) (Figure 6.1c). The latter species, *H. lanatus* is a perennial grass from Poaceae family that grows in humid environments (Hubbard 1984), and is most frequently found on fertile soils (Grime et al. 1989) in meadows, pastures and rough grassland (Hubbard 1985). This species is also known to show extensive

mycorrhizal colonisation (Harley & Harley 1987) and flowers between May and August (Hubbard 1985) (Figure 6.1d).

A completely randomised design with all the possible pairs of native plant species and balsam were conducted allowing the study of interspecific competition –Himalayan balsam with *P. lanceolata* (HBxPL) and Himalayan balsam with *H. lanatus* (HBxHL). Two balsam plants per unit were grown as an intraspecific competition (HBxHB) treatment, as well as balsam grown singly, without competition (HB). Two plants per pot were selected for competition treatments, to mimic a density per m² that is found in the field. Similar sized plants (4 weeks old) were grown in a 2L pot with and without Symbio inoculum as described in section 2.1 and placed in a glasshouse for nine weeks. There were 8 treatments with 5 replicates for each, resulting in 40 plants in total. The plants were watered twice daily with 300ml of water. Before flowering, the plants were harvested and plant parameters (plant height and shoot biomass) of balsam and biomass of native species were recorded. Leaves and root samples were collected for fungal assessment as described in sections 2.3 and 2.4. The endophyte fungal species were identified morphologically by Dr Brian C. Sutton as described in section 2.3 and no molecular identification was required, as all cultures produced spores.



Figure 6.1 Similar size of Himalayan balsam and co-occurring native plant species in a pot, (a) *Plantago lanceolata* and (b) *Holcus lanatus*. Photos of (c) the former plant species (Source from Online Atlas of the British and Irish Flora: <http://www.brc.ac.uk/plantatlas/plant/plantago-lanceolata>) and (d) the latter plant species (Source from Online Atlas of the British and Irish Flora: <http://www.brc.ac.uk/plantatlas/plant/holcus-lanatus>) in a field.

6.1.1 Statistical Analysis

All of the statistical analysis was conducted using R Studio version 1.1.383 as described in section 2.7. All data that violated the assumptions were transformed with square-root or logarithmic transformations while percentage data were arcsine transformed to meet the assumptions of the test beforehand. To analyse the effect and interaction of mycorrhizas and the competition on balsam performance, a two-way factorial ANOVA was performed with mycorrhizal and competition as main effects. Balsam performance was defined as the height, weight and RLC percentage of the plant in each pot and the mean of similar parameters was calculated if there were two plants in each pot. A one-way ANOVA was performed to examine the native plants performance (shoot biomass and RLC percentage) with mycorrhizal presence as the main effect.

Differences in endophyte isolation frequency (IF) of each fungal species between treatments were examined with a similar analytical design which were two-way ANOVA for balsam and one-way ANOVA for native plants. Differences in species richness across treatments were examined too.

6.2 Results

6.2.1 Plant growth

Balsam plants with mycorrhizal inoculum were taller compared to the AMF-free plants (Table 6.1). It is interesting to note that AM single balsam were taller, but had lower biomass. Perhaps of most interest was the fact that addition of mycorrhizas tended to decrease the biomass of balsam when it was grown singly, but increased it when grown in monoculture (Table 6.1, $p = 0.08$; Figure 6.2b). Mycorrhizal-inoculated balsam that competed with *P. lanceolata* were the tallest (Figure 6.2a) and heaviest (Figure 6.2b) compared to the other treatments. Mycorrhizas had no effect on *P. lanceolata* biomass while addition of AMF reduced biomass of *H. lanatus* (Table 6.2, Figure 6.3).

Table 6.1 Two-way factorial analysis of balsam parameters, testing for effects of AMF and competition. Degrees of Freedom for F values = 1, 36 and $n=5$ in all treatments. Bold indicates significant values.

	Height		Shoot biomass		RLC	
	F	p	F	p	F	p
AMF	8.706	<0.001	0.049	0.826	38.155	<0.001
Competition	0.431	0.515	0.499	0.484	6.269	0.017
AM x Competition	2.563	0.118	3.053	0.089	1.464	0.234

Table 6.2 One-way factorial analysis of native plants species, testing for effects of AMF. Degrees of Freedom for F values = 1, 8 and $n=5$ in all treatments. Bold indicates significant values.

	<i>Plantago lanceolata</i>				<i>Holcus lanatus</i>			
	Shoot biomass		RLC		Shoot biomass		RLC	
	F	p	F	p	F	p	F	p
AMF	0.016	0.902	10.45	0.012	22.71	<0.001	16.52	<0.001

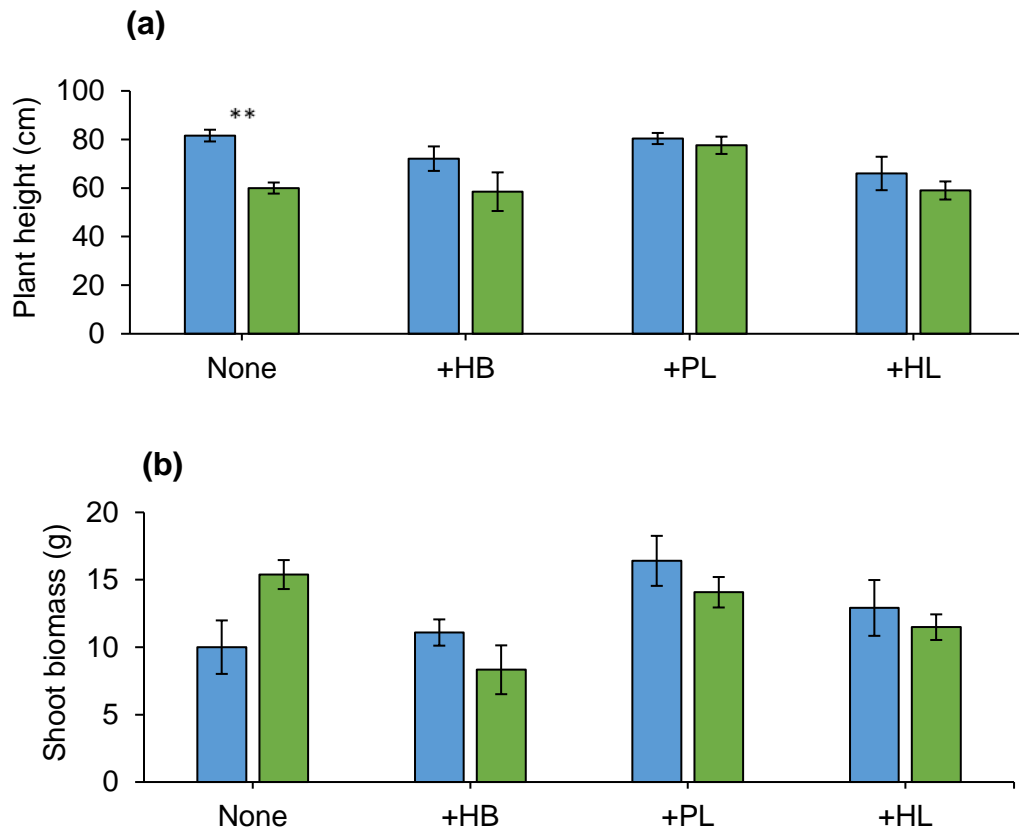


Figure 6.2 Mean of (a) height and (b) shoot biomass of balsam across treatments. Blue bars represent mycorrhizas present while green bars represent mycorrhizas absent. +HB was intraspecific competition while +PL and +HL were interspecific competition with *P. lanceolata* and *H. lanatus* respectively. 'None' indicates a single balsam with no competition as a control. $n=5$ across treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, $**p < 0.001$.

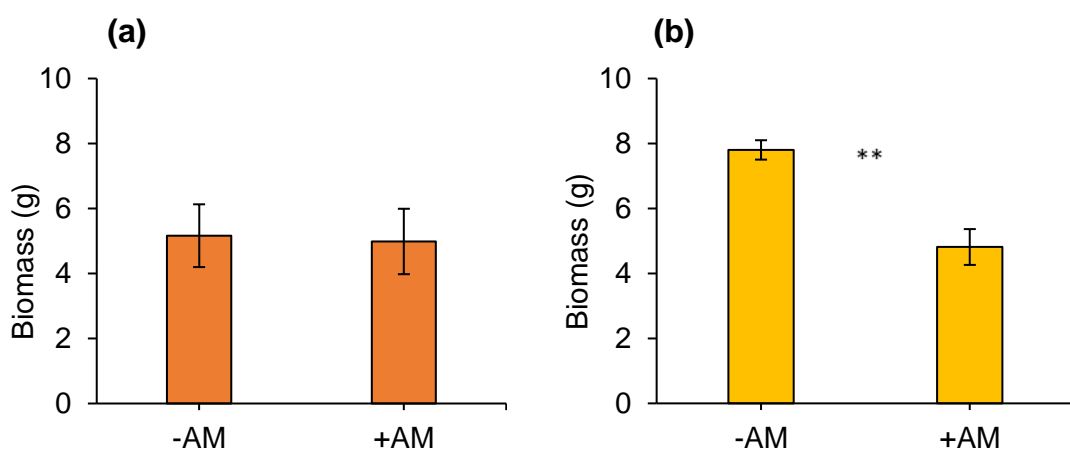


Figure 6.3 Shoot biomass mean of (a) *P. lanceolata* and (b) *H. lanatus* across treatments. -AM was mycorrhizal absent while +AM was mycorrhizal present. $n=5$ across treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, $**p < 0.001$.

6.2.2 AMF colonisation

Addition of inoculum significantly increased colonisation levels, although some colonisation was seen in uninoculated plants (Table 6.1, Figure 6.4). Balsam grown alone had similar colonisation to the plants experiencing interspecific competition but was double that of plants that experienced intraspecific competition (Table 6.1, Figure 6.4). No arbuscules were recorded across the treatments and no colonisation was recorded from AMF-free plants when grown in an intraspecific competition. Mycorrhizal inoculum increased the colonisation levels of *P. lanceolata* and *H. lanatus* (Table 6.2, Figure 6.5).

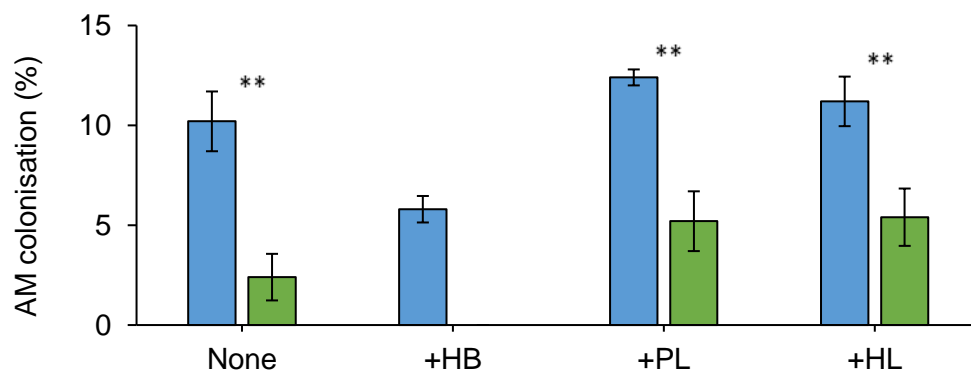


Figure 6.4 AM colonisation mean of balsam across treatments. Blue bars represent mycorrhizas present while green bars represent mycorrhizas absent. +HB was intraspecific competition while +PL and +HL were interspecific competition with *P. lanceolata* and *H. lanatus* respectively. 'None' indicates a single balsam with no competition as a control. $n=5$ across treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, $**p < 0.001$.

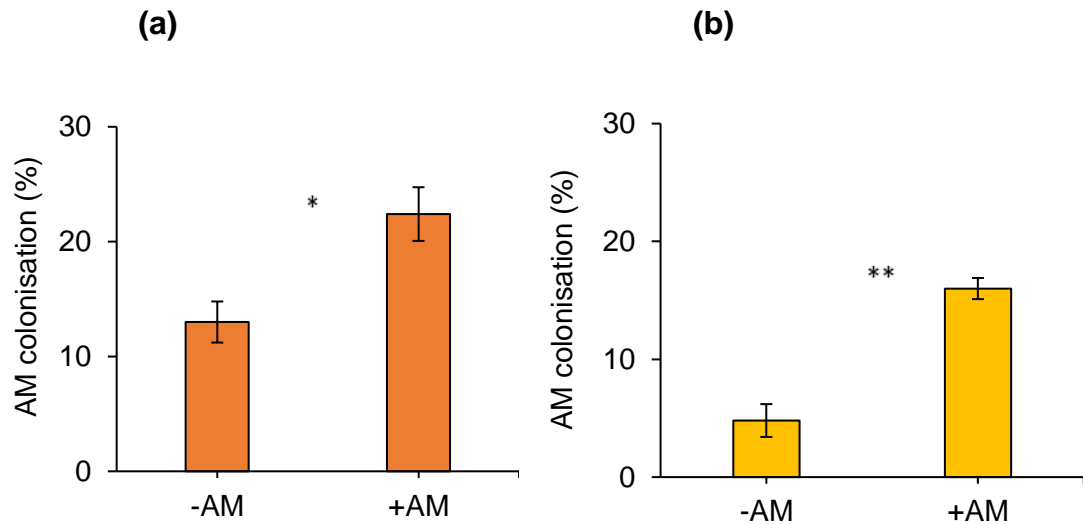


Figure 6.5 AMF colonisation mean of (a) *P. lanceolata* and (b) *H. lanatus* in interspecific competition. –AM was mycorrhizal absent while +AM was mycorrhizal present. $n=5$ across treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, * $p < 0.05$ and ** $p < 0.001$.

6.2.3 Endophytic fungal communities

There were less endophyte species recorded with low total of IF mean in this study compared to the other experiments (sections 4.2.3, 5.2.3 and 7.2.3). This is probably because of the time this study was conducted which was in late spring while the rest were in summer. The weather was dry and unfavourable for endophytes to enter the leaves as these fungi do prefer humid conditions. Therefore, future research on competitive interactions between balsam and native plants should be conducted in summer to encourage the entrance of more endophyte species.

Six endophyte species were recorded from native plants species with *Exophiala sp* being the commonest (Table 6.3), while eight species (none of which were *Exophiala sp*.) were recorded in balsam with *Colletotrichum acutatum* being the dominant (Table 6.4). *Acremonium incoloratum* was the

rarest across treatments. Only one species (*Co. acutatum*) was recorded from balsam that grew alone, while none were recorded when balsam grew in competition with *P. lanceolata*. However, five endophyte species were recorded in *P. lanceolata*, four when AM were absent and only one (*Exophiala* sp) when AM were present. *Cladosporium cladosporioides*, *Cladosporium oxysporum* and a species of *Pencillium* were recorded from balsam when in competition with *H. lanatus*, but only when AM were absent. No endophytes were isolated when AM was added. In contrast, *H. lanatus* yielded two endophyte species irrespective of whether AM were present. It is interesting to note, that two endophyte species (*Ac. incoloratum* and *Alternaria alternata*) were recorded when AM-inoculated balsam in intraspecific competition and were not found in other treatments. Similarly, three endophyte species were isolated from *P. lanceolata* when AM were absent, but were not recorded in *H. lanatus*. There was no difference in the IF mean of each endophyte fungal species and no difference in species richness of endophyte fungal communities across the treatments.

Table 6.3 Isolation Frequency (%) mean of endophyte species in native plants. +/-AM were mycorrhizal present/absent plants.

Endophyte species	<i>P. lanceolata</i>		<i>H. lanatus</i>	
	+AM	-AM	+AM	-AM
<i>Acremonium incoloratum</i>	0	0	5	0
<i>Arthirinium</i> state <i>Apiospora montagnei</i>	0	8.33	0	0
<i>Cladosporium oxysporum</i>	0	8.33	0	5
<i>Cladosporium sphaerospermum</i>	0	8.33	0	0
<i>Colletotrichum acutatum</i>	0	8.33	0	0
<i>Exophiala</i> sp.	20	0	15	15

Table 6.4 Isolation Frequency (IF) mean of each endophyte species in Himalayan balsam across treatments. +PL referred to competition with *Plantago lanceolata* while +HL competition with *Holcus lanatus*. +/-AM were mycorrhizas present/absent plants.

Endophyte species	Single		Intraspecific		Interspecific +PL		Interspecific +HL	
	+AM	-AM	+AM	-AM	+AM	-AM	+AM	-AM
<i>Acremonium incoloratum</i>	0	0	6.67	0	0	0	0	0
<i>Alternaria alternata</i>	0	0	20	0	0	0	0	0
<i>Chaetomium elatum</i>	0	0	0	20	0	0	0	0
<i>Cladosporium cladosporioides</i>	0	0	0	0	0	0	0	20
<i>Cladosporium oxysporum</i>	0	0	0	0	0	0	0	10
<i>Cladosporium sphaerospermum</i>	0	0	0	20	0	0	0	0
<i>Colletotrichum acutatum</i>	20	0	3.33	0	0	0	0	0
<i>Penicillium spp.</i>	0	0	10	0	0	0	0	10

6.3 Discussion

This study has shown that mycorrhizas reduced balsam biomass when it was grown singly but tended to increase when it was grown in both inter- and intra-specific competition. The other surprising result is that AMF had no effect on *Plantago lanceolata* biomass, but reduced that of *Holcus lanatus*. This suggested that the Symbio inoculum failed to form an extensive mycelial network when balsam was grown alone, and thereby caused a growth depression through poor development of fungal structures such as arbuscules or lower rate of nutrient transfer (Jin et al. 2017). A similar, consistent detrimental effect of mycorrhizas on balsam growth was seen in the insect-interaction study (section 4.2) and when balsam was grown in the field (section 3.1.3). Therefore, as this inoculum appears to consistently weaken the plant, it was chosen to enhance rust fungus attack as a biological control agent of balsam and their interactions, as described in section 7.2.

Mycorrhizal colonisation apparently did not influence interspecific interactions because the growth of balsam was similar when competing with both native species, irrespective of whether AM was present. Although balsam competed with mycorrhizal-dependent plants, the AMF did not appear to favour the native plants. Therefore, these data did not support the hypothesis that AMF may mediate plant dominance in the competition of invasive and native plants (Zobel & Moora 1995). However, AM presence seemed to increase balsam size when experiencing intraspecific competition. This has shown that mycorrhizas benefited the plants that experienced intraspecific competition when the opposite effect was seen when balsam grown alone. Single (or

sparse field population) plants of balsam may reduce the development of the AMF network in soil which subsequently costs the AMF-dependent native plant species and decreases the biomass of native plants within the stands (Tanner & Gange 2013), especially in highly invaded areas (Pattison et al. 2016). Therefore, this may be a reason why balsam forms monocultures in the field and displays a better competitive ability in self-replicating stands (Pattison et al. 2016). Since AMF-inoculated balsam experiencing intraspecific competition produced lower biomass than AMF-free grown alone balsam and balsam in interspecific competition, these fungi might be exploited for biological control of balsam. Future research should be conducted using mycorrhizal inoculation on balsam in the field, to examine the effect on intraspecific competition and whether these interactions promote or suppress the effectiveness of the rust fungus as a CBC agent.

Addition of AMF had no effect on balsam when competing with *P. lanceolata* and there was no mycorrhizal effect on *P. lanceolata* biomass. Previous study showed that different AMF had different effects on *P. lanceolata* growth (Bennett & Bever 2007). These authors revealed that *Glomus* white promoted plant growth, AMF *Archaespora trappei* provided less growth promotion while the fungus *Scutellospora calospora* did not enhance plant growth. Although these AMF were absent in the Symbio product, it still did not affect the plant growth. Similar results were found when mycorrhizas had no effect on *P. lanceolata* biomass when experiencing intraspecific competition (Ayres et al. 2006) and also when in interspecific competition with the legume, *Lotus corniculatus* (Scheublin et al. 2007). However, when balsam competed with *H. lanatus*, the mycorrhizas negatively affected the native plants. This suggests

that the mycorrhizal effect on interspecific competition was strongly influenced by the plant functional type (Lin et al. 2015). In addition, both native plant species recorded lower biomass than the balsam that they competed with. This has shown that when balsam competes with the native species, the plants grow aggressively and exhibit competitive dominance resulting in fast growth for light and nutrients (Čuda et al. 2015). This was consistent with the study conducted by Gruntman et al. (2014) that showed balsam genotypes exert competitive superiority effects on the neighbouring plant species despite the fact that the competitor was a perennial herb, *U. dioica*. Similarly in this study, balsam exhibited greater plant performance than the native plants and this could be enhanced by the balsam's strong allelopathic effects on the co-occurring native plants (Gruntman et al. 2014). In addition, a recent study showed that Himalayan balsam induced alteration in soil chemical properties such as ammonium (NH_4^+) and nitrate (NO_3^-) that affected the biomass and performance of co-existing native species, which were species-specific (Majewska et al. 2018).

In addition, although balsam is thought to form a sparse association with AMF (Beerling & Perrins 1993) and mycorrhizas reduced balsam performance within the introduced range (Tanner et al. 2014), all plants in this study showed increased colonisation with the addition of mycorrhizas. Balsam in interspecific competition recorded twice the mycorrhizal colonisation than plants experiencing intraspecific competition. However, both native plants recorded higher colonisation compared to the balsam with *P. lanceolata* the highest as it is a strongly mycorrhizal-dependent plant (Gange et al. 2002a). This suggests that when balsam grew with the native plants, its competitive ability

for soil resources was greater and the mycorrhizas can distribute nutrients between co-occurring plant species (van der Heijden et al. 2003). This is supported by the fact that balsam grown with intraspecific competition had lower root length colonisation when compared to balsam existing with the native species.

Very few foliar fungal endophytes were recorded with very low IF in this study compared to the others (chapters 3, 4, 5 and 7). This is probably because of the plants being grown in late spring and in the glasshouse which probably prevented the entrance of air-borne fungal species into the leaves. This can be supported by the fact that plenty of endophyte species with high IF were recorded when balsam was grown in the summer and in the field as described in sections 3.1, 4.2.3 and 5.2.3. Therefore, it may be suggested that changing environment conditions can influence foliar fungal communities in the leaves (Ahlholm et al. 2002; Currie et al. 2014).

Moreover, *Co. acutatum* was the dominant species recorded across the treatments and was found in mycorrhizal-inoculated balsam when grown alone and when in intraspecific competition. It is also interesting to note that these plants recorded lower biomass compared to the balsam that co-existed with the native plants. This was consistent with the other studies when balsam and this fungus were interacting with insects as described in section 4.3 and the pathogen in section 7.3. Thus, it is possible that this fungus may have weakened balsam performance as low biomass and plant size were seen. It is also interesting to note that, in intraspecific competition, endophyte species that were recorded when AM was present disappeared when AM was absent.

Furthermore, *Exophiala* sp. was recorded from AMF-inoculated native species only, while *A. alternata* was found in AMF-inoculated balsam in intraspecific competition only. This supports the suggestion that mycorrhizas influence the structure of endophyte communities in plants (Eschen et al. 2010) and that endophyte communities are plant species-specific (Gange et al. 2007).

6.4 Conclusion

In conclusion, mycorrhizal colonisation increased balsam growth when the plants experienced intraspecific competition, although plant performance was poorer than balsam experiencing interspecific competition. Meanwhile, there were no direct effects of mycorrhizas on balsam during interspecific competition. However, mycorrhizas negatively affected single balsam performance and *Co. acutatum* was the only fungus that was found from these plants and therefore, this could potentially be exploited for biological control of balsam in the field. Thus, both fungi were added to rust-infected balsam as described in chapter 7, to examine their interactions and whether they suppressed or enhanced the effectiveness of rust fungus as CBC agent of balsam. In addition, similar research should be conducted in the monocultures of balsam in the field to examine how the fungal interactions weaken the plant growth.

CHAPTER 7

Assembling the fungal
community: Interactions
between mycorrhizas,
endophytes and the rust

CHAPTER 7 ASSEMBLING THE FUNGAL COMMUNITY: INTERACTIONS BETWEEN MYCORRHIZAS, ENDOPHYTES AND THE RUST

Note: Part of this chapter has been published as Gange et al. (2018).

Classical Biological Control (CBC) involves the introduction of natural enemies, such as insect herbivores or pathogenic fungi that were collected from the plant's origin, and released against invasive plant species in invaded areas. CBC has been applied worldwide as a management tool against weed species for over 120 years, where at least 165 pests and weed species have been targeted, involving 7,000 introductions of CBC agents using 2,700 species (Cock et al. 2010). Although 380 CBC agents that have been released originated from European countries (Tanner 2011), only two were released against invasive plant species in the UK. The first was the psyllid, *Aphalara itadori* against Japanese knotweed, *Fallopia japonica* (Shaw et al. 2009; Shaw et al. 2011) and the second was the rust fungus, *Puccinia komarovii* var. *glanduliferae* against Himalayan balsam, *Impatiens glandulifera* (Tanner et al. 2015a; Tanner et al. 2015b). Following the successful approval for the release of the psyllid in the former study, a fungal pathogen, *Mycosphaerella polygoni-cuspidati* was studied as a promising second CBC agent against *F. japonica* due to its noticeable host specificity on the plant (Seier et al. 2014). Meanwhile the latter study was relevant to this chapter and *P. komarovii* history and the background was described in detail in section 1.3.

Other studies have investigated the use of rust fungi as CBC agents against invasive alien plant species. One example is that of the neotropical rust pathogen, *Prospodium tuberculatum* that was released in Australia in 2001

against the weed, *Lantana camara* (Ellison et al. 2006). Interestingly, this fungus can cycle continuously through the urediniospore stage in all seasons as there is no environmental pressure to produce overwintering stages (teliospores) in tropical habitats and lowland ranges. However, at higher altitudes and extreme areas such as mountainous ranges, uredinia were found to be replaced with the overwintering stage, teliospores, when frost and occasionally snow occur (Ellison et al. 2006). The second example was the rust fungus *Puccinia spegazzinii* that released in India in 2005 against the weed, *Mikania micrantha* (Ellison et al. 2008). Unlike *P. tuberculatum*, only teliospores and basidiospores of *P. spegazzinii* were found in the field, with spermogonia, aecia and uredinia being unknown (Evans & Ellison 2005). Meanwhile, two other rust fungi are being considered as biological control agents; *Puccinia araujiae* against the moth plant, *Araujia hortorum* in New Zealand (Anderson et al. 2016) and *P. arechavaletae* against balloon vine, *Cardiospermum grandiflorum* in South Africa (Fourie & Wood 2018). Both fungi have yet to be released in the field and these studies were carried out in the glasshouse. The former rust fungus completes its life cycle on the host (Anderson et al. 2016) while the latter agent has a microcyclic life cycle with basidiospores being a crucial stage that optimally developed at 20°C (Fourie & Wood 2018). These studies show that different rust fungus species have different life cycles and conditions needed for plant infection and ultimately reducing plant growth and development.

Endophytic, mycorrhizal and pathogenic fungi can all coexist in plant tissues, however, little is known about the effect of their interactions on plant growth and microbial communities. Species in five endophytic genera; *Colletotrichum*,

Pestalotiopsis, *Phoma*, *Phomopsis* and *Alternaria* have been found to have a diverse array of interactions with the rust fungus *Puccinia polygoni-amphibii* var. *tovariae* in *Fallopia japonica* (Kurose et al. 2012). This rust is a further potential CBC agent for *F. japonica* (Kurose et al. 2009). These genera were selected to be inoculated into the leaves as they were the dominant fungi in the plant. A *Phomopsis* sp. endophyte had a synergistic effect with the rust, thus enhancing its potential as a CBC agent against *F. japonica*. In contrast, *Alternaria* and *Phoma* spp. suppressed rust colonisation while *Colletotrichum* and *Pestalotiopsis* isolates were neutral on rust development (Kurose et al. 2012). This shows that endophyte species produce very different responses whether as promoting fungi, suppressing fungi or neutral fungi, dependent upon their identities.

A recent study has demonstrated that the presence of *Trichoderma* sp. can protect wheat plants from the stem rust infection (*Puccinia graminis* Pers. f. sp. *tritici*), but the synergistic effect was greater with the combination of mycorrhizal fungi and *Trichoderma* sp when challenging the rust fungus, in improving plant growth and yield (El-Sharkawy et al. 2018). The plant's immune system was triggered against the pathogen by the direct inhibitory effect of endophytic fungi whether singly or in combination and their induction of phenolic acids and defence-related enzyme production (El-Sharkawy et al. 2018). These studies show that it is important to understand not only the interaction of fungi and host plants, but also, the interaction of endophytes and pathogenic fungi, the influence of mycorrhizal fungi and pathogens as well as multiple fungi interactions in determining the success or failure of the CBC agents. If endophytes and mycorrhizas have a potential role in supporting a

rust fungus in the host plant, this interaction could be highlighted and would give a vital understanding for the biological control of weeds. To date, there are no studies conducted on the interaction of AM and a rust pathogen and endophytes within a host plant, therefore the present study was carried out to investigate the effect of multiple fungal attack on balsam performance. In addition, it is important to examine the effect of multiple fungal attack on above and below ground microbial communities. The hypothesis of this study was, there is an interactions between the fungi in plants that may lead to altered plant performance, and microbial communities which in return may enhance or suppressed the efficacy of the rust as a CBC agent of Himalayan balsam.

7.1 Methods

There were eight treatments, with five replicates of each, producing 40 plants in total. The plants were grown in 2L pots containing John Innes compost Number 2, with and without commercial Symbio inoculum and the leaves were inoculated with and without the endophyte, *Colletotrichum acutatum*. This fungus was chosen as it recorded the highest isolation frequency percentage in balsam leaves when the plants were grown in the field as described in section 3.1. A week after endophyte application, the leaves were infected with the rust fungus, *Puccinia komarovii* var. *glanduliferae*. Control plants received sprays of the spore carrier only that did not contain the respective fungi. Details of endophyte inoculation and rust infection procedures were given in sections 2.2 and 2.5 respectively. Similar techniques were applied in this experiment for bulking up rust fungus stocks as described in section 2.5. The only difference in this study was that, the leaves were inoculated using a

suspension of rust fungus in distilled water with 0.05% Tween 80, as it was easier to quantify and inoculate a large number of plants, compared to the spore/talc mix. The spore concentration was adjusted using a haemocytometer to approximately 1.5×10^5 spores ml^{-1} and the inoculated plants placed in a Perspex box for 48 hours. The boxes were cleaned thoroughly with 100% ethanol between each treatment to avoid contamination. The abaxial leaves were sprayed with two strokes of spore suspension (ca. 550 μl /leaf) using a handheld bottle sprayer. The remaining spore suspension was sprayed on a Petri dish containing tap water medium to determine the viability of the rust infection by assessing germination rate and the plates also were placed in the inoculation box.

The plants were put in a glasshouse for nine weeks in a randomised block design and were watered with 250ml water twice daily. Before flowering, the plants were harvested and plant parameters (height, leaf number and shoot biomass) were recorded. Leaves and root samples were collected for endophyte and mycorrhizal assessment as described in sections 2.3 and 2.4. Infected leaves from each plant in each treatment were collected and an acetate grid (of squares 1cm x 1cm) was placed on the leaves and number of rust pustules per cm^2 in five randomly selected squares were counted. This was done in the second and third week post rust inoculation.

7.1.1 Statistical Analysis

All of the statistical analysis was conducted using R Studio version 1.1.383 as described in section 2.7. All data that violated the assumptions were transformed with square-root or logarithmic transformations while percentage data were arcsine transformed to meet the assumptions of the test beforehand. To analyse the effect and interaction of fungi on balsam performance, a three-way factorial ANOVA was performed with mycorrhizal, endophyte and rust presence as main effects.

Differences in endophyte isolation frequency (IF) of each fungal species between treatments were examined with a similar analytical design. Differences of endophyte community composition was compared with NMDS and differences in species abundance and species richness across treatments were examined too.

7.2 Results

7.2.1 Plant growth

Plants with rust were significantly shorter, bore fewer leaves and had lower shoot biomass compared to the control plants (Table 7.1). It appeared that AM and endophyte together could be enhancing rust in reducing the plant performance, because the greatest reductions were seen when rust was applied with either or both fungi (Figure 7.1). There was a suggestion that adding endophyte to rust-treated plants, may reduce plant size (Table 7.1). The rust alone significantly reduced plant biomass, but not height or leaf number (Figure 7.1). However, no significant interactions were found between the fungi (Table 7.1).

Table 7.1 Three-way factorial analysis of plant parameters, testing for effects of AM, endophyte and rust. Degrees of Freedom for F values = 1, 32 and $n=5$ in all treatments. Bold indicated significant values.

	Height		Leaf number		Shoot biomass		RLC	
	F	<i>p</i>	F	<i>p</i>	F	<i>p</i>	F	<i>p</i>
AM	1.577	0.218	5.908	0.020	0.000	0.993	23.238	<0.001
Endophyte	4.083	0.052	0.639	0.429	0.000	0.988	0.029	0.866
Rust	8.813	0.006	56.026	<0.001	12.267	0.001	1.343	0.255
AM x Endophyte	1.619	0.212	2.034	0.163	0.436	0.514	1.447	0.238
AM x Rust	2.576	0.118	1.134	0.295	0.338	0.565	0.547	0.465
Endophyte x Rust	0.598	0.445	2.319	0.137	0.351	0.558	3.012	0.092
AM x Endophyte x Rust	0.180	0.674	2.034	0.163	1.217	0.278	0.038	0.847

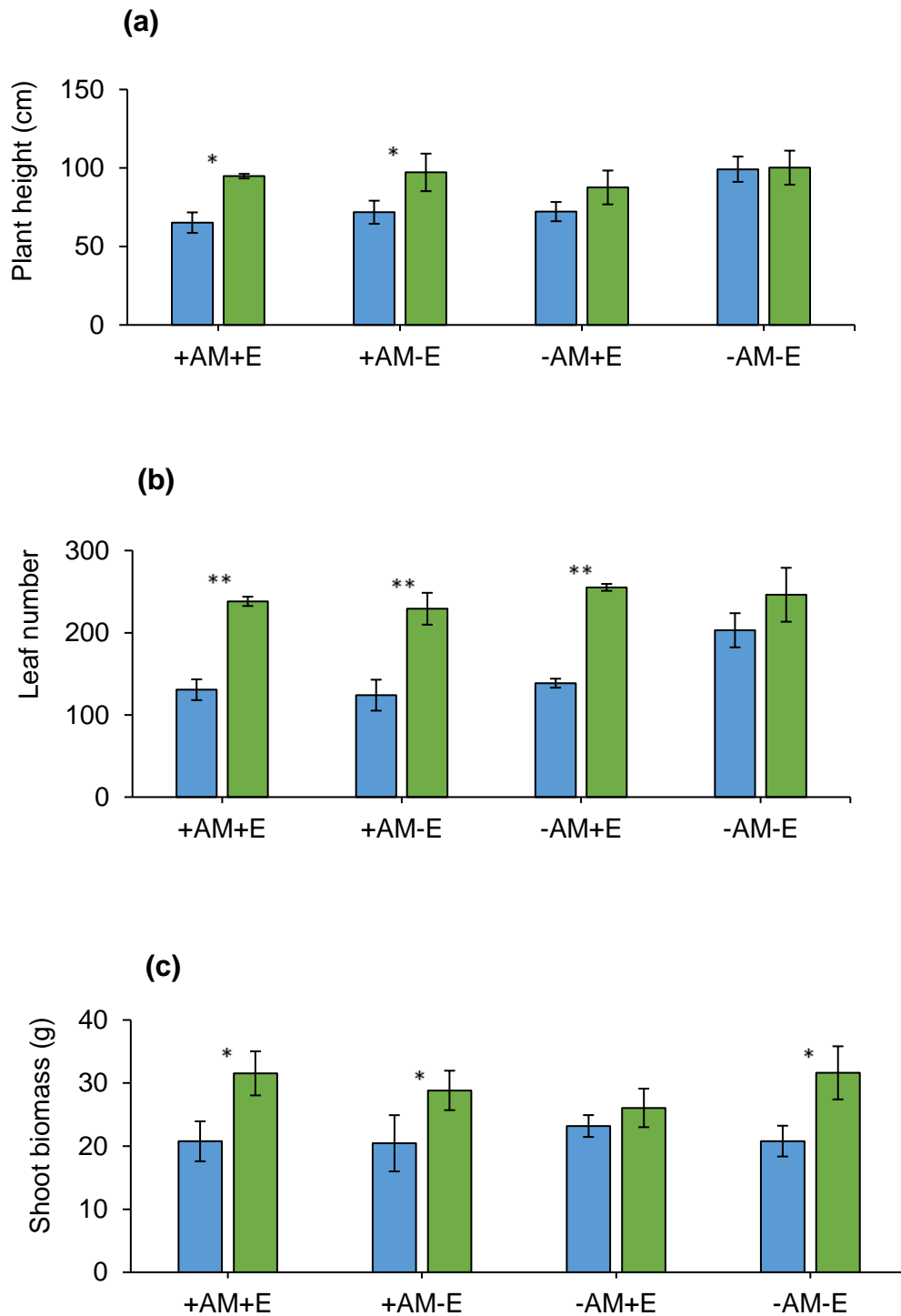


Figure 7.1 Mean of (a) plant height, (b) leaf number and (c) shoot biomass across treatments. Blue bars indicate rust-treated plants while green bars indicate non-rust plants. +/-AM indicates mycorrhizal presence/absence while +/-E were *Colletotrichum acutatum* present/absent. $n=5$ in all treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, * $p < 0.05$ and ** $p < 0.001$.

7.2.2 AMF colonisation

Rust-treated plants had higher AM colonisation than controls when both AM and endophyte were present (Figure 7.2), but this effect disappeared when the endophyte was absent. Mycorrhizal addition increased the level of root colonisation (Table 7.1) though some colonisation was seen in uninoculated controls in this experiment (Figure 7.2). No arbuscules were recorded.

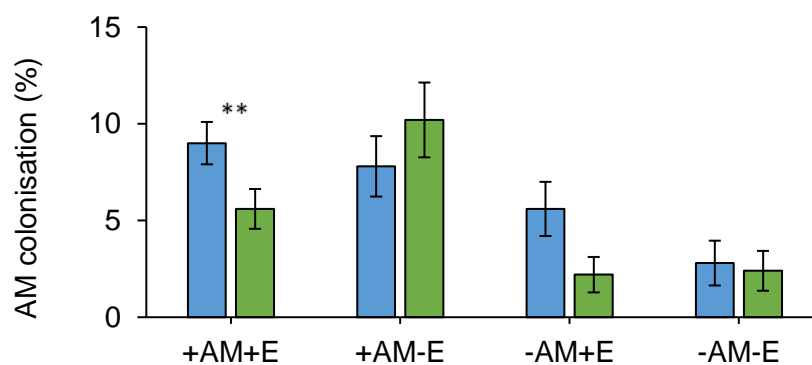


Figure 7.2 Mean of arbuscular mycorrhizal colonisation across treatments. Blue bars indicate rust-treated plants while green bars indicate non-rust plants. +/-AM indicates mycorrhizal presence/absence while +/-E were *Colletotrichum acutatum* present/absent. $n=5$ in all treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, * $p < 0.05$ and ** $p < 0.001$.

7.2.3 Endophytic fungal communities

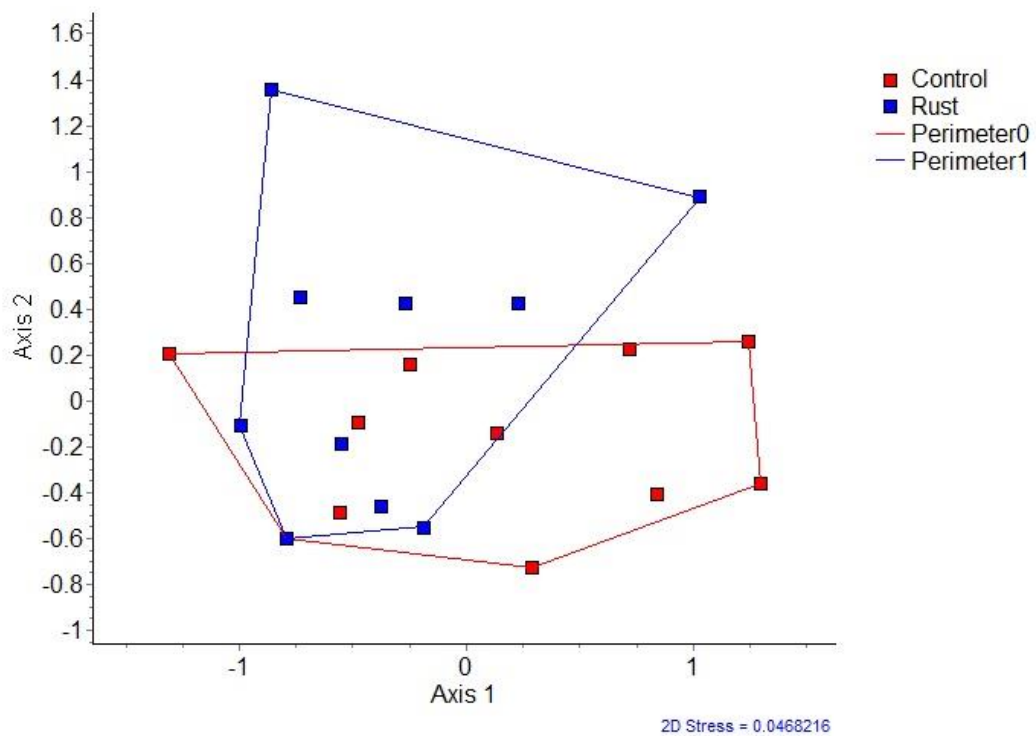
Ten endophyte species were recorded across the treatments (Table 7.2). Two species were only found in rust infected plants – *Cladosporium oxysporum* and *Geniculosporium spp.*, while *Chaetomium elatum* and *Penicillium spp* were isolated from control plants only. The remaining six endophyte species were found in both treatments. *Cladosporium sphaerospermum* displayed a high IF in rust-treated plants, but was rarest in controls. Addition of AMF and endophyte on rust-treated plants decreased IF mean of *Alternaria alternata* ($F_{1,32} = 4.911, p < 0.05$) and *Exophiala sp.* ($F_{1,32} = 11.622, p < 0.05$) compared to the controls.

Table 7.2 Isolation Frequency (IF) of each endophyte species across treatments. +/-R: Rust present/absent, +/-AM: Mycorrhizal present/absent and +/-CA: Leaves were inoculated with *Colletotrichum acutatum*/endophyte free leaves.

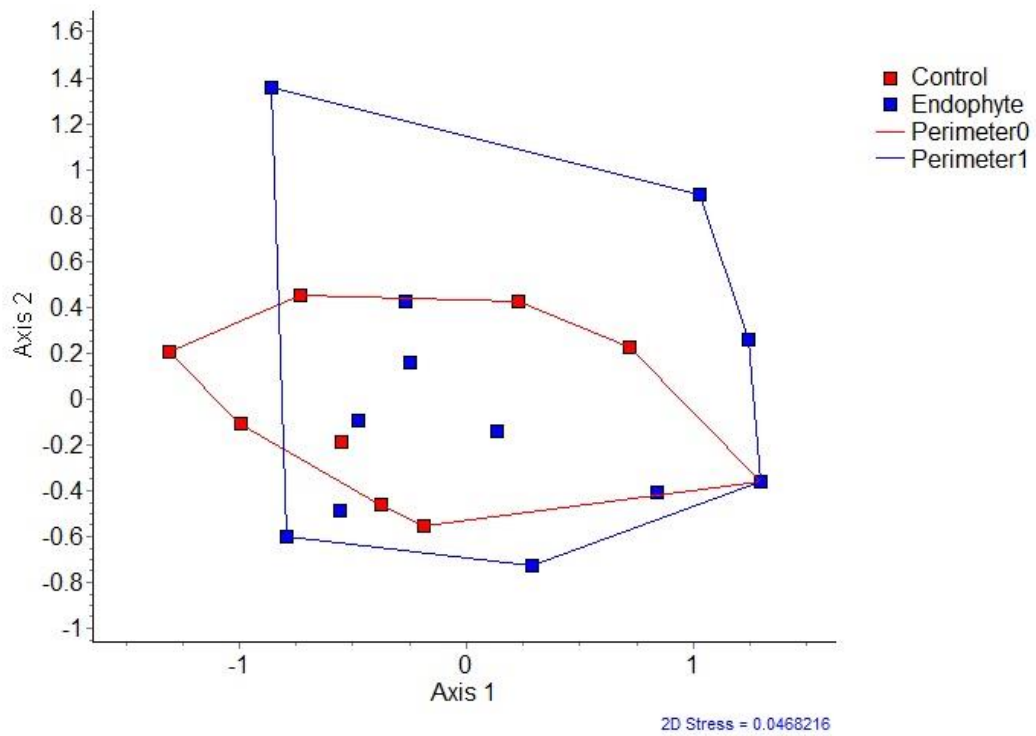
Endophyte species	+R				-R			
	+AM		-AM		+AM		-AM	
	+CA	-CA	+CA	-CA	+CA	-CA	+CA	-CA
<i>Alternaria alternata</i>	0	0	0	23.4	0	40	0	0
<i>Arthrinium</i> state of <i>Apiospora montagnei</i>	0	0	0	9.8	16.8	0	20	0
<i>Chaetomium elatum</i>	0	0	0	0	0	0	0	20
<i>Cladosporium cladosporioides</i>	0	0	0	5.8	0	0	20	0
<i>Cladosporium oxysporum</i>	0	10	0	4	0	0	0	0
<i>Cladosporium sphaerospermum</i>	20	5	20	0	10	0	0	0
<i>Colletotrichum acutatum</i>	0	5	20	11.6	6.6	0	20	0
<i>Exophiala</i> sp.	20	0	0	40.6	0	0	40	0
<i>Geniculosporium</i> sp.	20	0	0	5	0	0	0	0
<i>Penicillium</i> sp.	0	0	0	0	46.6	20	0	20

In addition, multiple fungal attack decreased species richness ($F_{1,32} = 7.538$, $p < 0.05$) of endophyte communities in the plants. The NMDS ordination clearly separated the endophyte fungal communities between rust-treated plants and the controls (ANOSIM $R = 0.234$, $p < 0.05$) (Figure 7.3a), between endophyte inoculated plants and the control (ANOSIM $R = 0.202$, $p < 0.05$) (Figure 7.3b) and also between AM-treated plants and the control plants (ANOSIM $R = 0.266$, $p < 0.05$) (Figure 7.3c).

(a)



(b)



(c)

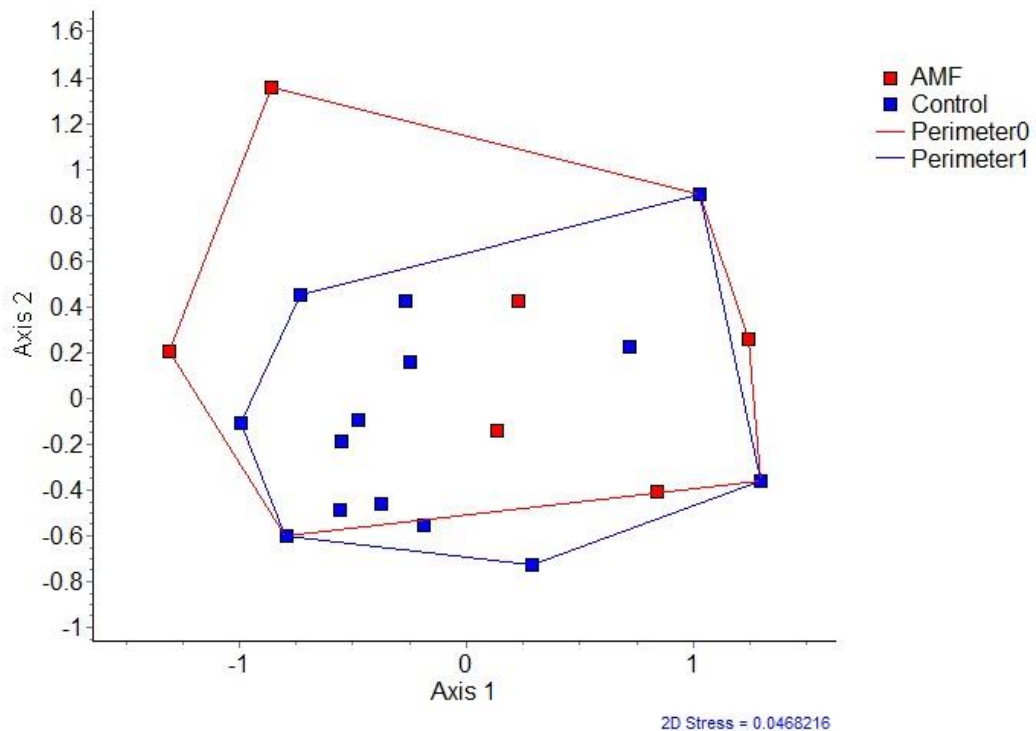


Figure 7.3 Endophyte species communities between (a) rust, (b) endophyte and (c) AMF treated plants and their controls. Please note difference in scales for visual clarity. Vertical axis most likely represents isolation frequency of endophyte communities while horizontal axis likely shows separation by fungi infection.

In the presence of multiple fungi, *Co. acutatum* was not recovered although the leaves were inoculated compared to the plants when both or either fungi was absent (Figure 7.4). The recovery rate was the highest when AM was absent, irrespective of whether the rust was present. It is also interesting to note that the endophyte was isolated from the uninoculated plants, but only when rust was present. However, this did not happen on free-rust plants.

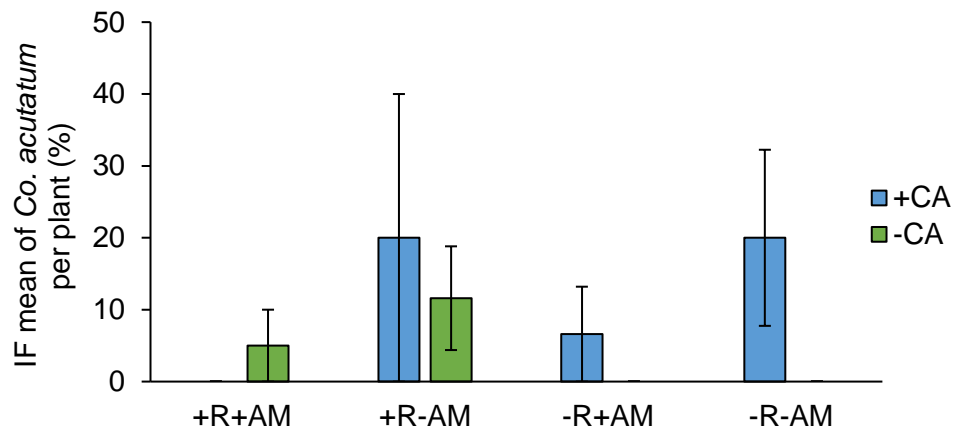


Figure 7.4 Isolation Frequency mean of *Colletotrichum acutatum* across treatments. Legend shows the leaves were inoculated with *Co. acutatum* (+CA), or the control (-CA). +/-R represents rust was present/absent and +/-AM represents mycorrhiza was present/absent. $n=5$ in all treatments. Error bars are one SE.

7.2.4 Rust Fungus Infection

The presence of *Co. acutatum* and mycorrhizal fungi reduced pustule number in rust infected plants (Figure 7.5). There was a significant interaction between AM and the endophyte, as addition of *Co. acutatum* greatly reduced rust infection when the mycorrhiza was absent (Table 7.3).

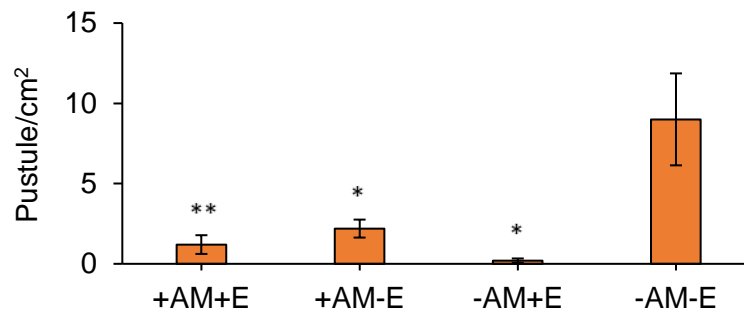


Figure 7.5 Mean of uredinia pustule number/cm² on balsam leaves on rust-infected plants. +/-AM were mycorrhiza present/absent plants while +/-E were *Colletotrichum acutatum* present/absent plants. $n=5$ in all treatments. Error bars are one SE. Asterisks above bars indicate significant pairwise differences between means, * $p < 0.05$ and ** $p < 0.001$.

Table 7.3 Two-way ANOVA factorial analysis of the effects of AM and endophyte on the pustule density. Degrees of Freedom for F values = 1, 16 and $n=5$ in all treatments. Bold indicated significant values.

	Pustule	
	F	p
AM	2.082	0.168
Endophyte	26.469	<0.001
AM x Endophyte	10.354	0.005

7.3 Discussion

To date, this is the first study where the effects of multiple fungi were experimentally tested on Himalayan balsam. In this study, rust-treated plants demonstrated poor plant performance compared to their controls as the uredinia and telia pustules densely covered the abaxial leaf surfaces reducing the available area for photosynthesis (Murray & Walters 1992) which curtailed plant growth. In this study, it is reasonable to suggest that the presence of multiple fungi weakened the plant's performance, rust development and also affected above and below ground fungi communities.

It was interesting to note that *Cl. sphaerospermum* recorded the highest IF in the presence of rust but the least in the control plants. This species is commonly found in forbs likely because it is a common member of the spore rain (Marchisio & Airaudi 2001) and may well have been present in the atmosphere or from vertical transmission via seed from mother plants (Hodgson et al. 2014). However, in this case, it is suggested that rust fungus addition with mycorrhizas and *Co. acutatum* may play a big role by providing an entry route for *Cl. sphaerospermum* into the foliar tissues. Although *Colletotrichum* sp. had no effect on rust colonisation in *Fallopia japonica* (Kurose et al. 2012), suppressive effects of *Co. acutatum* in this study may have induced phytoalexin production (Gao et al. 2010) and decreased the level of rust development. This could have indirectly allowed the entry of *Cl. sphaerospermum* into the balsam leaves as the plant had reduced resources available for additional defence mechanisms allowing the entry of a 'background' endophyte. This suggestion is supported by the fact that *Cl.*

sphaerospermum was not isolated when AMF and *Co. acutatum* were absent although the rust was present. Interestingly, this did not happen with other endophyte species. This finding also shows that mycorrhizas may affect the invasion of *Cl. sphaerospermum* into the leaves as shown previously when insects were present, in section 4.3.

Colletotrichum acutatum was successfully recovered from most of the treatments and displayed the highest IF on rust-treated plants. Surprisingly, this fungus was not recovered when multiple fungi were present. However, *Co. acutatum* was isolated from plants even when the leaves were not inoculated, something which did not happen in rust-free plants. It is known that Himalayan balsam contains secondary metabolites including flavones, caffeic acid derivatives and naphthoquinones (Lobstein et al. 2001; Šerá et al. 2005). Previous studies showed that naphthoquinones exhibited inhibitory effects on fungal spore germination (Foote et al. 1949; Yang et al. 2001). In addition, endophytes may induce host defences such as phenolic metabolites against pathogens (Dingle & McGee 2003; Hartley et al. 2015). It is possible that the presence of the pathogen and mycorrhizas may have altered plant defences by inducing naphthoquinones within the leaves (Ruckli et al. 2014) and other secondary metabolites, which indirectly prevented the inoculated fungus from entering the leaves. This suggests that the combination of rust and mycorrhiza may have prevented *Co. acutatum* infection and that the rust alone causes plants to be more susceptible to air-borne endophytes. This was supported by the fact that *Co. acutatum* was isolated from uninoculated endophyte plants, but only when the rust was applied. In addition to that, seven endophyte species were recorded from rust-treated plants when mycorrhizas and *Co.*

acutatum were absent which was higher than in the other treatments. Clearly, these complex interactions require further study, involving the use of metabolomics to try and understand the mechanism by which fungi interact within and with the plant.

Many studies have reported that not only the identity of plant, but the identity of mycorrhizal fungal species are important in determining plant growth (Newsham et al. 1995; Helgason et al. 1999; Husband et al. 2002; Scheublin et al. 2007; Sikes et al. 2009). This can also be supported by the findings from Lewandowski et al. (2013) who showed that AMF species richness alone was less of an important factor than mycorrhiza identity when considering plant tolerance against pathogen attack. The benefits of mycorrhizas forming an association with plants depend on the fungal species involved whether as a plant protection or phosphorus (P) uptake (Maherali & Klironomos 2007; Sikes et al. 2009). Maherali & Klironomos (2007) found that AMF from the Family Glomeraceae that concentrated in the hyphae outside the plant root were better in plant protection by reducing infection of root pathogen, *Fusarium oxysporum* and *Phythium* sp. in *Plantago lanceolata* compared to the fungi from the Family Gigasporaceae. In contrast, the latter family have hyphae growing within the root, so promoted P uptake and increased the shoot biomass. Similarly, *Setaria glauca* was protected from the root pathogen, *F. oxysporum* when AMF from the Family Glomeraceae were added, while severe infections were recorded when the members of Gigasporaceae were present (Sikes et al. 2009). The Symbio inoculum used in this study was composed of nine fungal species that are mainly from the Family Glomeraceae with only one member of Gigasporaceae as described in section 3.4.2. The

differences species within the inoculum may be the reason why this inoculum weakened the rust development in the balsam leaves. The 'Symbio effect' not only reduced the pathogenic effect of the rust, but also reduced plant size and biomass. This has shown that although fungal species in the Symbio inoculum may protect the host plant against a pathogen, it also reduces the plant performance. The fact that addition of this inoculum reduced pathogen incidence means that unfortunately it could not be used to assist in the biological control of balsam using the rust fungus.

Arbuscular mycorrhizal fungi are known to form symbiotic associations with plants and dependent on them for their carbon supply in order to complete their life-cycle. Almost 80% of terrestrial flowering plants are colonised by AMF and there are approximately 150 species of AMF known (Harrier & Watson 2004). This enables each mycorrhizal species to colonise a wide host range and it is important to select the most suitable AMF species to inoculate the plant, in order to optimise the benefits of the mutual relationships. Many studies have demonstrated positive mycorrhizal effects on plant growth against pathogens. For example, mixtures of AMF strains (*Rhizophagus intraradices*, *Glomus hoi*, *Gigaspora margarita* and *Scutellospora gigantea*) enhanced common bean, *Phaseolus vulgaris* growth when infected by the pathogen, *Fusarium solani* due to strengthening the plant immune system as phenols and flavonoids contents increased (Eke et al. 2016). In addition, the AMF *Rhizophagus irregularis* enabled stronger defensive response of wheat, *Triticum aestivum* against pathogens by maximising nutrients and water uptake into the plants (Pérez-De-Luque et al. 2017). Moreover, a recent study on wheat also showed that the mycorrhizal fungus, *Funneliformis mosseae*

activated a broad-spectrum defence response in inoculated plants that enabled the plants to induce a pathogen-specific defence response when challenged by *Xanthomonas translucens*, which led to a stronger productivity and more effective protection (Fiorilli et al. 2018). However, a study showed that AMF colonised rice plants, *Oryza sativa*, were more susceptible to sheath blight, *Rhizoctonia solani*, by increasing lesion length and numbers compared to the controls (Bernaola et al. 2018). These authors suggested that the mycorrhizas altered defence-related pathways and reduced broad-spectrum defence in inoculated plants (Bernaola et al. 2018). These studies provide evidence that mycorrhizal fungi do not always protect plants against pathogens, due to AMF mediated changes in the plant defences via modulation of jasmonic acid and salicylic acid dependent pathways (Pozo & Azcón-Aguilar 2007; Koricheva et al. 2009; Jung et al. 2012; Bernaola et al. 2018). Interestingly, the results reported in this thesis, suggest that mycorrhizas may have altered the defence related mechanism which reduced plant performance and also rust density. This has shown that mycorrhizal fungi may influence and reduce rust development and infection in the field, which could have serious consequences for the effectiveness of biological control of balsam. Field colonisation by mycorrhizas may therefore partly explain patchy rust efficacy between release sites (Gange et al. 2018).

The outcome of the complex interactions between plant, rust pathogen and AMF, was a significant reduction in disease severity. Evidence suggests that when plants were attacked by rust, the plants may have induced the defence signalling pathway and triggered jasmonic acid production. Jasmonic acid is expressed in arbuscules (Pozo & Azcón-Aguilar 2007) and effective against

necrotrophs (Ton et al. 2002; Glazebrook 2005). However in this study, few or no arbuscules were found in mycorrhizal-treated plants when the plants were challenged by the rust fungus. Despite the absence of arbuscules, the addition of mycorrhizas still weakened plant growth and reduced rust fungus infection. Overall, it is possible that induction of jasmonic acid may have resulted in the low levels of arbuscular formation. Meanwhile, other studies showed that naphthoquinones were released by balsam roots, which could be growth inhibitors of fungi (Ruckli et al. 2014). The concentrations of naphthoquinones in the balsam were higher than those in the native plant, *Impatiens noli-tangere* (Lobstein et al. 2001). This suggests that balsam might have released naphthoquinones in response to rust attack, especially as the concentrations are naturally high in seedlings (Ruckli et al. 2014). Interestingly, Himalayan balsam is only susceptible to infection by the uredinia when seedlings have reached the three leaf whorl stage and to infection by the basidiospores when the seeds are just germinating. It may be that the resistance of plants to rust infection between these stages may be due to high naphthoquinones in the seedlings. Therefore, this may suggest that naphthoquinones were active in plant protection against rust fungus infection at certain plant growth stages. Measurement of these biochemical changes was beyond the scope of this thesis, but this is clearly an important avenue for future research.

Furthermore, multiple fungal presence reduced the species richness of endophytes. The causes and mechanism(s) of this phenomenon are unknown. However, it may be that when the plants suffer rust attack and mycorrhizal inoculation, they might mobilise naphthoquinones against the pathogen and indirectly prevent the 'background' endophytes from entering into the foliar

tissues. This can be supported as rust-treated plants without AMF and endophytes had greater endophyte species communities compared with when the fungi were present. This is because, when *Co. acutatum* became established in the plants, it may induce systemic defences which repel the entry of other species (van Hulten et al. 2006). It is important to study the fungal endophyte communities in the target weed in order to determine their role in classical biological control (Evans 2008). Plants may respond to endophyte infection in the same way as they do with pathogenic fungi, by producing arabidopsides and other oxylipin esters. However, the chemicals produced may be insufficient to hinder the endophyte from establishing within the plant tissues (Hartley et al. 2015).

In addition, both AMF and fungal endophytes depend on their plant host for nutrients and their survival. Mycorrhizal fungi can enhance endophyte growth (Larimer et al. 2012) and alter endophyte communities through nutrient availability (Eschen et al. 2010). However, in the combination of both fungi and the rust pathogen, mycorrhizas seemed to prevent the entry of the inoculated endophyte suggesting that the host may be unable to accommodate multiple fungi concurrently. The mechanism of the interactions between fungi is still unknown in forbs, however, mycorrhizal fungi were reported not to affect the endophyte when they occurred together in grasses (Mack & Rudgers 2008). Interestingly, in balsam, higher mycorrhizal colonisation was seen when the rust and endophyte were present, suggesting that these fungi had benefited the mycorrhiza, but in return negatively impacting the inoculated endophyte.

A study showed that endophytic fungi (*Chaetomium sp.* and *Phoma sp.*) reduced the density of pustules of *Puccinia triticana* in wheat plants, *Triticum aestivum*, but the endophyte had no effect on the plant growth (Dingle & McGee 2003). This was consistent with the current study in that while both mycorrhizal and endophyte fungi reduced rust infection, this did not detract from the rust's ability to reduce balsam growth. This suggests that attack by multiple fungi weakened the plant, causing it to use resources on defence, rather than growth (Wang et al. 2018).

This study has demonstrated the effectiveness of the rust fungus as a classical biological control agent on balsam (Tanner et al. 2015a) and it also appeared to suggest that patchy rust infection on balsam in the field (Gange et al. 2018) may be influenced by the existence of AMF and *Co. acutatum* in the plants. The rust is most effective when the mycorrhiza and endophyte are absent, though both fungi were also effective in reducing plant performance. Therefore it would be sensible to try to reduce AMF and endophyte communities in the field in order to enhance the rust in weakening the plant's growth as balsam with rust in the absence of mycorrhizal and *Co. acutatum* were shorter than controls and exhibited the highest pathogen density.

7.4 Conclusion

Overall, complex interactions exist between mycorrhizas, endophyte and rust fungi in balsam, that weakened the plant growth, influenced rust density and also affected above and below ground fungal communities. This study provided insight knowledge into why classical biological control with fungi may

be impaired by other naturally fungi in the system and interacting with the biocontrol agent. Mycorrhizal fungi and *Co. acutatum* presence greatly reduced rust development, probably by inducing host defences which might affect the classical biological control aspect of this invasive plant. Thus, further studies on reducing mycorrhizal and endophyte communities in balsam populations in the field should be conducted urgently in order to examine how this affects rust establishment. A full understanding of the system will only be achieved by a consideration of the biochemical nature of these interactions.

CHAPTER 8

General Discussion

CHAPTER 8 GENERAL DISCUSSION

8.1 Summary of Outcomes

The main aims of this PhD were firstly to find out the effect of insect and multiple fungal interactions on plant performance and secondly to examine the factors that may have affected the efficacy of the rust fungus, *Puccinia komarovii* var. *glanduliferae* as a classical biological control agent of Himalayan balsam. A summary of the objectives and the outcomes of the study are given below (Table 8.1):

Table 8.1 Summary of objectives and outcomes of the study.

Objectives	Outcomes
Chapter 4:	
1. To study the interactions between mycorrhizas, endophytes and insects and also their effect on balsam growth.	<ul style="list-style-type: none">• Combinations of insect and fungi affected plant performance, but depended upon the fungal species.• Aphids, mycorrhizal fungi and <i>Cladosporium sphaerospermum</i> directly reduced plant growth.• Insects and Plantworks mycorrhizal inoculum prevented <i>Colletotrichum acutatum</i> from infecting the plants, but the opposite effect occurred with <i>Cl. sphaerospermum</i> increasing the infection. However, when Symbio inoculum was present, the effects were reversed.
2. To study the foliar endophyte communities in balsam plants that were attacked by insects.	<ul style="list-style-type: none">• The positive association of mycorrhizal and endophyte reduced plant growth which may be useful for biological control of balsam.

Chapter 5:

1. To study how different commercial mycorrhizal inocula affect the degree of soil conditioning by balsam.
2. To study the differences in foliar endophyte communities of plants that grew in soil that had or had not previously supported balsam.

- A negative Plant-Soil-Feedback (PSF) of balsam was recorded with shorter, lighter plants, bearing fewer leaves when grown in a soil that had previously supported balsam, compared to clean soil.
- Commercial inocula do not seem to give the same result as natural AMF and subsequently reversed the PSF.
- Continuous stands of balsam may reduce endophyte communities which may enable the rust to be more effective in reducing plant growth in older stands.

Chapter 6:

1. To study mycorrhizal effect on balsam and native plant performance in a competitive environment.
2. To study above and below ground communities between plants.

- Mycorrhizas reduced balsam biomass when it was grown singly but tended to increase when it was grown in both inter- and intra-specific competition.
- *Co. acutatum* was the dominant endophyte species but was mostly found in AM-inoculated balsam when in intraspecific competition and therefore, this fungus probably contributed to weakening the plant performance.

Chapter 7:

To investigate the effect of multiple fungal attack on balsam performance and also on above and below ground microbial communities.

- Complex three way interactions exist between endophytes, mycorrhizal fungi and the rust, which weakened the plant growth and rust density. Above and below ground fungal communities were also affected by the fungal presence.
- AMF and *Co. acutatum* did not enhanced rust fungus effectiveness as a CBC agent of Himalayan balsam since it reduced rust development.

8.2 Multiple fungi effect on the classical biological control of Himalayan balsam

The results from this thesis show there are complex multiple interactions between fungi, insect and Himalayan balsam. Despite the fact that commercial inocula in this study did not resemble the natural AMF, the presence of mycorrhizal fungi reduced plant growth when insects and rust were present. The combined effect of each mycorrhizal inoculum (Plantworks/Symbio) and *Cladosporium sphaerospermum* promoted aphid infestation which lead to the reduction of plant growth. Although AMF increased balsam growth that experienced intraspecific competition, the plant biomass was still lower than that of balsam experiencing interspecific competition. Meanwhile, the presence of *Cl. sphaerospermum* and *Co. acutatum* irrespective of whether they were in single or dual inoculations, also reduced balsam growth. Similarly, the combination of Symbio and *Co. acutatum* reduced plant performance, but the rust fungus was most effective in the absence of AMF and *Co. acutatum*. This is because, endophytic fungi may have activated defence mechanisms which limited the pathogen's ability to infect and replicate (Dingle & McGee 2003; Kurose et al. 2012). Meanwhile the presence of mycorrhizal fungi may also have activated a broad-spectrum defence response in the roots and leaves of that switched to a pathogen-specific defence upon pathogen attack (Fiorilli et al. 2018) leading to the reduction of rust infection in balsam. Therefore, it is recommended to search for ways to reduce mycorrhizas and endophytes in monocultures of balsam in the field, in order to encourage rust fungus development and infection, and thereby weaken the plant's performance.

It is important to study the fungal endophyte communities in the target weed in order to determine their role in biological control (Evans 2008). This study has shown that the endophyte, *Co. acutatum* was antagonistic to the rust development. Therefore, steps should be taken to try to prevent or reduce the infection of this fungus into balsam plants in order to maximise the effectiveness of rust fungus. The occurrence of *Co. acutatum* as an anthracnose disease is much more frequent in agricultural crops such as strawberry (Freeman & Katan 1997; Denoyes-Rothan et al. 2003; Racedo et al. 2013) and cucumber (Chandanie et al. 2006). It is very rare to find these crop plants grow nearby balsam, thus eliminating the opportunity of *Co. acutatum* to infect balsam leaves. In the present study, although *Co. acutatum* was found from balsam grown in the field (section 3.1.3.2) and in intraspecific competition (section 6.2.3), the infection frequencies were very low. To further support this fact, *Co. acutatum* infection frequency was reduced in balsam that was grown in a soil that had previously supported balsam compared to the balsam in clean soil. A similar result was recorded when balsam was grown in mycorrhizal inoculum, which recorded lower *Co. acutatum* infection frequency compared to the balsam in clean soil (section 5.2.3). In addition, a previous study also showed that *Co. acutatum* was not found in plants from the conditioned soil (Pattison et al. 2016). The most interesting result was the fact that *Co. acutatum* was antagonistic to the pathogen, while the rust infection was the highest when rust was present alone. The commonest endophyte species recorded from these plants were *Exophiala sp.* and *Alternaria alternata* which were not found from rust-free plants. Although *A. alternata* inhibited rust colonisation in *Fallopia japonica* (Kurose et al. 2012) and

protected grapevines from pathogen *Plasmopara viticola* attack (Musetti et al. 2007), further study should be conducted to inoculate both endophytes into rust-treated balsam to examine their interactions and effects. Furthermore, as *Plantago lanceolata* was a common co-existing native plant with balsam in the field and contained high isolation frequency of *A. alternata* (Hodgson et al. 2014), it is may be beneficial to grow this plant nearby balsam populations. This is because, if *A. alternata* successfully supports the growth of rust fungus, the endophyte can be transmitted horizontally from *P. lanceolata* to balsam and increased infection might encourage rust growth.

Similar to the addition of *Co. acutatum*, the presence of commercial mycorrhizal inoculum in this study shown a negative effect on rust development in the balsam leaves. This raises questions as to whether AM fungi may hinder the establishment of the rust in field populations (Gange et al. 2018). However, balsam is known to form a sparse association with mycorrhizal fungi (Beerling & Perrins 1993) and most commonly infests disturbed areas (river banks) or communities in which other plant species that (e.g. *Rumex* spp.) have a low mycorrhizal dependency (Reinhart & Callaway 2006). Thus, this may reduce the potential of mycorrhizal fungi to jeopardise rust fungus efficacy. Nevertheless, future work should be conducted to examine the effect and interactions of local mycorrhizal communities and rust infection in the plants as some soils in the field may be colonised by diverse communities of mycorrhizal fungi (Koch et al. 1997; Koch et al. 2011). Therefore, a better understanding of the biology of the local AMF community and its interaction with the rust fungus could be useful in aiding habitat restoration of area previously supporting dense Himalayan balsam stands,

where the rust is having an impact. In addition, if mycorrhizal species that are compatible with rust infection can be found, then it is ecologically sensible to propose application of these fungi in the field as they will benefit native plants and aid in the restoration of floristic diversity (Berruti et al. 2016). However, this approach might only be successful after the rust has become established and had some effect on the population of balsam (Bieberichid et al. 2018).

If mycorrhizas are to be applied in the field, then inoculation in spring is preferable to ensure the best chance for successful association with the plants (Koziol et al. 2017). There are several ways to inoculate AMF in the field such as broadcasting, tilling, drilling, hydroseeding, seed inocula pellets and inoculated seed plants (Hayman et al. 1981; Koziol et al. 2017). Hayman et al. (1981) found that tilling and applying inoculum in a slurry produced the greatest mycorrhizal colonisation while broadcasting and pellets showed opposite effects. Therefore, application of mycorrhizal fungi in a slurry into the field together with the seeds of native wildflowers could be trialled.

Overall, this study has demonstrated the importance of examining the factors that may affect the virulence and effectiveness of the pathogen against invasive species. If the AMF inoculation in the field together with native plant seed is successful, following the implementation of CBC and a suitable endophyte was found to promote rust development, similar approaches could be considered with other invasive plant species such as *F. japonica*. Furthermore, this study has provided better awareness and understanding to landowners on the best way to prevent balsam growth from widely infesting their land. Rust fungus infection and addition of mycorrhizal-dependent

wildflowers near to balsam populations could be applied by the landowners in order to manage populations of the weed. Complete eradication of Himalayan balsam is not a realistic option, since weed is too widespread in the UK. CBC using the rust fungus together with habitat restoration, potentially aided by the seeding of habitats including suitable mycorrhizal, post rust impact, offers a sustainable solution to this weed. This is attainable, if scientists, practitioners and landowners work together to reduce the impact of Himalayan balsam on native biodiversity.

8.3 Final Conclusions and Impact of this Study

Invasive species are one of the greatest challenges for most ecosystems and can lead to a huge economic loss. Overall, this study demonstrated that incorporation of plant-fungi-insect interactions is an important aspect within the development of a biocontrol strategy for invasive weed management. This work has provided evidence of the effect of endophytes and mycorrhizal fungi on Himalayan balsam, which affected not only plant growth, but also both above and below ground fungal communities. Another important result was that both mycorrhizas and endophytes could potentially disrupted the efficacy of the rust fungus as a CBC agent of balsam. Therefore, urgent work is needed to improve and support the effectiveness of the pathogen. Overall, it has been shown that the establishment of a plant pathogenic biocontrol agent may be dependent on the presence of other fungi in the roots and shoots of the plant, as well insect presence on the leaves. For biological control to be more predicatable in the future, one needs to consider not just the virulence of the pathogen, but also how this virulence is affected by the plant's microbiome.

REFERENCES

REFERENCES

- Abbott, L.K., & Robson, A.D., 1984. The effect of VA mycorrhizae on plant growth. In C. L. Powell & D. J. Bagyaraj, eds. *VA Mycorrhiza*. Boca Raton, Florida: CRC Press, pp. 113–130.
- Abdel-Baky, N.F., & Abdel-Salam, A.H., 2003. Natural incidence of *Cladosporium* spp. as a bio-control agent against whiteflies and aphids in Egypt. *Journal of Applied Entomology*, 127(4), pp.228–235.
- Aerts, R., 1999. Interspecific competition in natural plant communities : mechanisms , trade-offs and plant-soil feedbacks. *Journal of Experimental Botany*, 50(330), pp.29–37.
- Afshan, N.S., Khalid, A.N., & Niazi, A.R., 2012. Some new rust fungi (Uredinales) from Fairy Meadows, Northern Areas, Pakistan. *Journal of Yeast and Fungal Research*, 3(5), pp.65–73.
- Ahlholm, J.U., Helander, M., Henriksson, J., Metzler, M., & Saikkonen, K., 2002. Environmental conditions and host genotype direct genetic diversity of *Venturia ditricha*, a fungal endophyte of birch trees. *Evolution*, 56(8), pp.1566–1573.
- Akello, J., & Sikora, R., 2012. Systemic acropetal influence of endophyte seed treatment on *Acyrtosiphon pisum* and *Aphis fabae* offspring development and reproductive fitness. *Biological Control*, 61(3), pp.215–221.
- Ammer, C., Schall, P., Wördehoff, R., Lamatsch, K., & Bachmann, M., 2011. Does tree seedling growth and survival require weeding of Himalayan balsam (*Impatiens glandulifera*)? *European Journal of Forest Research*, 130(1), pp.107–116.
- Anderson, F.E., Santos López, S.P., Sánchez, R.M., Reinoso Fuentealba, C.G., & Barton, J., 2016. *Puccinia araujiae*, a promising classical biocontrol agent for moth plant in New Zealand: Biology, host range and hyperparasitism by *Cladosporium uredinicola*. *Biological Control*, 95, pp.23–30.
- Andrews, M., Maule, H.G., Raven, J.A., & Mistry, A., 2005. Extension growth of *Impatiens glandulifera* at low irradiance: Importance of nitrate and potassium accumulation. *Annals of Botany*, 95(4), pp.641–648.
- Antunes, P.M., Koch, A.M., Dunfield, K.E., Hart, M.M., ... Klironomos, J.N., 2009. Influence of commercial inoculation with *Glomus intraradices* on the structure and functioning of an AM fungal community from an agricultural site. *Plant and Soil*, 317(1–2), pp.257–266.
- Arnold, A.E., Mejia, L.C., Kyllö, D., Rojas, E.I., ... Herre, E.A., 2003. Fungal endophytes limit pathogen damage in a tropical tree. In *Proceedings of the National Academy of Sciences*. pp. 15649–15654.

- Arthur, J.C., & Cummins, G.B., 1933. Rusts of the Northwest Himalayas. *Mycologia*, 25, pp.397–406.
- Aschehoug, E.T., Callaway, R.M., Newcombe, G., Tharayil, N., & Chen, S., 2014. Fungal endophyte increases the allelopathic effects of an invasive forb. *Oecologia*, 175(1), pp.285–291.
- Ayres, R.L., Gange, A.C., Aplin, D.M., Ayres, R.L., ... Aplin, D.M., 2006. Interactions between arbuscular mycorrhizal fungi and intraspecific competition affect size and size inequality of *Plantago lanceolata* L. *Journal of Ecology*, 94(2), pp.285–294.
- Azcon-Aguilar, C., & Barea, J.M., 1996. Arbuscular mycorrhizas and biological control of soilborne plant pathogens - an overview of the mechanisms involved. *Mycorrhiza*, 6, p.457–464.
- Babikova, Z., Gilbert, L., Bruce, T., Dewhurst, S.Y., ... Johnson, D., 2014. Arbuscular mycorrhizal fungi and aphids interact by changing host plant quality and volatile emission. *Functional Ecology*, 28(2), pp.375–385.
- van Bael, S.A., Valencia, M.C., Rojas, E.I., Gómez, N., ... Windsor, D.M., 2009. Effects of foliar endophytic fungi on the preference and performance of the Leaf Beetle *Chelymorpha alternans* in Panama. *Biotropica*, 41(2), pp.221–225.
- Bahar, M.H., Backhouse, D., Gregg, P.C., & Mensah, R., 2011. Efficacy of a *Cladosporium* sp. fungus against *Helicoverpa armigera* (Lepidoptera: Noctuidae), other insect pests and beneficial insects of cotton. *Biocontrol Science and Technology*, 21(12), pp.1387–1397.
- Ballhorn, D.J., Younginger, B.S., & Kautz, S., 2014. An aboveground pathogen inhibits belowground rhizobia and arbuscular mycorrhizal fungi in *Phaseolus vulgaris*. *BMC Plant Biology*, 14(1), pp.1–13.
- Barto, E.K., & Rillig, M.C., 2010. Does herbivory really suppress mycorrhiza? A meta-analysis. *Journal of Ecology*, 98(4), pp.745–753.
- Beerling, D.J., & Perrins, J.M., 1993. *Impatiens glandulifera* Royle (*Impatiens roylei* Walp.). *Journal of Ecology*, 81(2), pp.367–382.
- Bennett, A.E., & Bever, J.D., 2007. Mycorrhizal species differentially alter plant growth and response to herbivory. *Ecology*, 88(1), pp.210–218.
- Bernaola, L., Cosme, M., Schneider, R.W., & Stout, M., 2018. Belowground inoculation with arbuscular mycorrhizal fungi increases local and systemic susceptibility of rice plants to different pest organisms. *Frontiers in Plant Science*, 9, pp.1–16.
- Berruti, A., Lumini, E., Balestrini, R., & Bianciotto, V., 2016. Arbuscular mycorrhizal fungi as natural biofertilizers: Let's benefit from past successes. *Frontiers in Microbiology*, 6, pp.1–13.

- Bever, J.D., Westover, K.M., & Antonovics, J., 1997. Incorporating the soil community into plant population dynamics : The utility of the feedback approach. *Journal of Ecology*, 85(5), pp.561–573.
- Bieberichid, J., Lauerer, M., Drachsler, M., Heinrichs, J., ... Feldhaar, H., 2018. Species- And developmental stage-specific effects of allelopathy and competition of invasive *Impatiens glandulifera* on cooccurring plants. *PLoS ONE*, 13(11), pp.1–19.
- Borowicz, V.A., 2001. Do arbuscular mycorrhizal fungi alter plant-pathogen relations? *Ecology*, 82(11), pp.3057–3068.
- Bottollier-Curtet, M., Planty-Tabacchi, A.M., & Tabacchi, E., 2013. Competition between young exotic invasive and native dominant plant species: Implications for invasions within riparian areas. *Journal of Vegetation Science*, 24(6), pp.1033–1042.
- Brown, K.B., Hyde, K.D., & Guest, D.I., 1998. Preliminary studies on endophytic fungal communities of *Musa acuminata* species complex in Hong Kong and Australia. *Fungal Diversity*, 1, pp.27–51.
- Brundrett, M.C., 2002. Coevolution of roots and mycorrhizas of land plants. *New Phytologist*, 154(2), pp.275–304.
- Brundu, G., 2014. Plant invaders in European and Mediterranean inland waters: profiles, distribution, and threats. *Hydrobiologia*, 746, pp.61–79.
- Busby, P.E., Ridout, M., & Newcombe, G., 2015. Fungal endophytes: modifiers of plant disease. *Plant Molecular Biology*, (November).
- Callaway, R.M., Thelen, G.C., Rodriguez, A., & Holben, W.E., 2004. Soil biota and exotic plant invasion. *Nature*, 427(6976), pp.731–733.
- Callaway, R.M., Bedmar, E.J., Reinhart, K.O., Silvan, C.G., & Klironomos, J., 2011. Effects of soil biota from different ranges on “Robinia” invasion : acquiring mutualists and escaping pathogens. *Journal of Ecology*, 92(5), pp.1027–1035.
- Castelli, J.P., & Casper, B.B., 2003. Intraspecific AM fungal variation contributes to plant-fungal feedback in a serpentine grassland. *Ecology*, 84, pp.323–336.
- Ceccarelli, N., Curadi, M., Martelloni, L., Sbrana, C., ... Giovannetti, M., 2010. Mycorrhizal colonization impacts on phenolic content and antioxidant properties of artichoke leaves and flower heads two years after field transplant. *Plant and Soil*, 335(1), pp.311–323.
- Chagas, F.O., Dias, L.G., & Pupo, M.T., 2013. A mixed culture of endophytic fungi increases production of antifungal polyketides. *Journal of Chemical Ecology*, 39(10), pp.1335–1342.
- Chandanie, W.A., Kubota, M., & Hyakumachi, M., 2006. Interactions

- between plant growth promoting fungi and arbuscular mycorrhizal fungus *Glomus mosseae* and induction of systemic resistance to anthracnose disease in cucumber. *Plant and Soil*, 286(1–2), pp.209–217.
- Chapman, D.S., & Gray, A., 2012. Complex interactions between the wind and ballistic seed dispersal in *Impatiens glandulifera* (Royle). *Journal of Ecology*, 100(4), pp.874–883.
- Chittka, L., & Schürkens, S., 2001. Successful invasion of a floral market: An exotic Asian plant has moved in on Europe's river-banks by bribing pollinators. *Nature*, 411(6838), p.653.
- Chmura, D., & Gucwa-Przepióra, E., 2012. Interactions between arbuscular mycorrhiza and the growth of the invasive alien annual *Impatiens parviflora* DC: A study of forest type and soil properties in nature reserves (S Poland). *Applied Soil Ecology*, 62, pp.71–80.
- Chu-Chou, M., Guo, B., An, Z.Q., Hendrix, J.W., ... Burrus, P.B., 1992. Suppression of mycorrhizal fungi in fescue by the *Acremonium coenophialum* endophyte. *Soil Biology and Biochemistry*, 24(7), pp.633–637.
- Clements, D.R., Feenstra, K.R., Jones, K., & Staniforth, R., 2008. The biology of invasive alien plants in Canada. 9. *Impatiens glandulifera* Royle. *Canadian Journal of Plant Science*, 88(2), pp.403–417.
- Cock, M.J.W., van Lenteren, J.C., Brodeur, J., Barratt, B.I.P., ... Parra, J.R.P., 2010. Do new access and benefit sharing procedures under the Convention on Biological Diversity threaten the future of biological control? *BioControl*, 55(2), pp.199–218.
- Cock, M.J.W., & Seier, M.K., 2007. The scope for biological control of giant hogweed, *Heracleum mantegazzianum*. In P. Pysek et al., eds. *Ecology and Management of Giant Hogweed*. CABI Publishing, Wallingford, UK., pp. 225–227. P. Pysek et al., eds.
- Cockel, C.P. & Tanner, R.A., 2011. *Impatiens glandulifera* Royle (Himalayan balsam). In R. A. Francis, ed. *A Handbook of Global Freshwater Invasive Species*. London, UK.: Earthscan, pp. 67–77. R. A. Francis, ed.
- Cope-Selby, N., Cookson, A., Squance, M., Donnison, I., ... Farrar, K., 2017. Endophytic bacteria in *Miscanthus* seed: implications for germination, vertical inheritance of endophytes, plant evolution and breeding. *GCB Bioenergy*, 9(1), pp.57–77.
- Cortat, G., Shaw, R., Tanner, R., Eschen, R., ... Pratt, C., 2010. Potential solutions for the control of riparian and aquatic invasive weeds : A review on the progress of classical biological control programmes in the UK. In *AFPP – Twenty-First Columa Conference International Meeting On Weed Control*. Dijon.

- Crawley M.J., 1987. What makes a community invasible? In C. M. J. & E. P. J. Gray A. J., ed. *Colonization, Succession and Stability*. Oxford, Great Britain: Blackwell Scientific Publications, pp. 429–453.
- Čuda, J., Skálová, H., Janovský, Z., & Pyšek, P., 2015. Competition among native and invasive *Impatiens* species: The roles of environmental factors, population density and life stage. *AoB PLANTS*, 7(1), pp.1–12.
- Currie, A.F., Wearn, J., Hodgson, S., Wendt, H., ... Jin, L., 2014. Foliar fungal endophytes in herbaceous plants: A marriage of convenience? In V. C. Verma & A. C. Gange, eds. *Advances in Endophytic Research*. Springer India, pp. 63–80.
- Danieli-Silva, A., Uhlmann, A., Vicente-Silva, J., & Stürmer, S.L., 2010. How mycorrhizal associations and plant density influence intra- and inter-specific competition in two tropical tree species: *Cabralea canjerana* (Vell.) Mart. and *Lafoensia pacari* A.St.-Hil. *Plant and Soil*, 330(1), pp.185–193.
- Dawson, F.H., & Holland, D., 1999. The distribution in bankside habitats of three alien invasive plants in the U.K. in relation to the development of control strategies. *Hydrobiologia*, 415, pp.193–201.
- Denoyes-Rothan, B., Guérin, G., Délye, C., Smith, B., ... Freeman, S., 2003. Genetic diversity and pathogenic variability among isolates of *Colletotrichum* species from strawberry. *Phytopathology*, 93(2), pp.219–228.
- Devarajan, P.T., & Suryanarayanan, T.S., 2006. Evidence for the role of phytophagous insects in dispersal of non-grass fungal endophytes. *Fungal Diversity*, 23, pp.111–119.
- Dingle, J., & McGee, P.A., 2003. Some endophytic fungi reduce the density of pustules of *Puccinia recondita* f. sp. *tritici* in wheat. *Mycological Research*, 107(3), pp.310–316.
- Douds, D.D., Nagahashi, G., Wilson, D.O., & Moyer, J., 2011. Monitoring the decline in AM fungus populations and efficacy during a long term bare fallow. *Plant and Soil*, 342(1–2), pp.319–326.
- Driesche, R.V., Hoddle, M., & Center, T., 2008. *Control of Pests and Weeds by Natural Enemies: An Introduction to Biological Control*, Oxford, UK: Blackwell Publishing.
- Edwards, L.C., 2017. The improvement of plant performance in reduced peat growing media using arbuscular mycorrhizal fungi. PhD Thesis. Royal Holloway, University of London.
- Ehrenfeld, J.G., Ravit, B., & Elgersma, K., 2005. Feedback in the plant-soil system. *Annual Review of Environment and Resources*, 30(1), pp.75–115.

- Eke, P., Chatue Chatue, G., Wakam, L.N., Kouipou, R.M.T., ... Boyom, F.F., 2016. Mycorrhiza consortia suppress the fusarium root rot (*Fusarium solani* f. sp. *Phaseoli*) in common bean (*Phaseolus vulgaris* L.). *Biological Control*, 103, pp.240–250.
- El-Sharkawy, H.H.A., Rashad, Y.M., & Ibrahim, S.A., 2018. Biocontrol of stem rust disease of wheat using arbuscular mycorrhizal fungi and *Trichoderma* spp. *Physiological and Molecular Plant Pathology*, 103, pp.84–91.
- Ellison, C.A., Pereira, J.M., Thomas, S.E., Barreto, R.W., & Evans, H.C., 2006. Studies on the rust *Prospodium tuberculatum*, a new classical biological control agent released against the invasive alien weed *Lantana camara* in Australia. 1. Life-cycle and infection parameters. *Australasian Plant Pathology*, 35(3), pp.309–319.
- Ellison, C.A., Evans, H.C., Djeddour, D.H., & Thomas, S.E., 2008. Biology and host range of the rust fungus *Puccinia spegazzinii*: A new classical biological control agent for the invasive, alien weed *Mikania micrantha* in Asia. *Biological Control*, 45(1), pp.133–145.
- Emery, S.M., & Rudgers, J.A., 2012. Impact of competition and mycorrhizal fungi on growth of *Centaurea stoebe*, an invasive plant of sand dunes. *The American Midland Naturalist*, 167(2), pp.213–222.
- Environment Agency, 2003. *Guidance for the control of invasive weeds in or near fresh water.*, Environment Agency, London, UK.
- Environment Agency, 2010. *Our river habitats: the state of river habitats in England, Wales and the Isles of Man: a snap shot.*, Environment Agency, London.
- Eschen, R., Hunt, S., Mykura, C., Gange, A.C., & Sutton, B.C., 2010. The foliar endophytic fungal community composition in *Cirsium arvense* is affected by mycorrhizal colonization and soil nutrient content. *Fungal Biology*, 114(11–12), pp.991–8.
- Evans, H., 2008. The endophyte release hypothesis: implications for classical biological control and plant invasions. In R. B. Julien MH, Sforza R, Bon MC, Evans HC, Hatcher PE, Hinz HL, ed. *Proceedings of the XII International Symposium on Biological Control of Weeds*. La Grande Motte, France: CABI Publishing, Wallingford, pp. 20–26.
- Evans, H., & Ellison, C., 2005. The biology and taxonomy of rust fungi associated with the neotropical vine *Mikania micrantha*, a major invasive weed in Asia. *Mycologia*, 97(4), pp.935–947.
- Evans, H.C., 1998. The Safe Use of Fungi for Biological Control of Weeds. *Phytoprotection*, 79(4), p.67.
- Evans, H.C., Fröhlich, J., & Shamoun, S.F., 2001. Biological control of weeds. In S. B. Pointing & K. D. Hyde, eds. *Bio- Exploitation of*

- Filamentous Fungi*. Hong Kong, China: Fungal Diversity Press, pp. 349–401.
- Facelli, E., & Facelli, J.M., 2002. Soil phosphorus heterogeneity and mycorrhizal symbiosis regulate plant intra-specific competition and size distribution. *Oecologia*, 133(1), pp.54–61.
- Facelli, E., Facelli, J.M., Smith, S.E., & McLaughlin, M.J., 1999. Interactive effects of arbuscular mycorrhizal symbiosis , intraspecific competition and resource availability on *Trifolium sub terraneum*. *New Phytologist*, 141(3), pp.535–547.
- Faye, A., Dalpé, Y., Ndung'u-Magiroy, K., Jefwa, J., ... Lesueur, D., 2013. Evaluation of commercial arbuscular mycorrhizal inoculants. *Canadian Journal of Plant Science*, 93(6), pp.1201–1208.
- Fiorilli, V., Vannini, C., Ortolani, F., Garcia-Seco, D., ... Bonfante, P., 2018. Omics approaches revealed how arbuscular mycorrhizal symbiosis enhances yield and resistance to leaf pathogen in wheat. *Scientific Reports*, 8(1), pp.1–18.
- Foote, M.W., Little, J.E., & Sproston, T.J., 1949. On naphthoquinones as inhibitors of spore germination of fungi. *Journal Biological Chemistry*, 181, pp.481–487.
- Fourie, A., & Wood, A.R., 2018. The rust fungus *Puccinia arechavaletae*, a potential biological control agent of balloon vine (*Cardiospermum grandiflorum*) in South Africa . I : Biology. *Australian Plant Pathology*, 47, pp.379–387.
- Freeman, S., & Katan, T., 1997. Identification of *Colletotrichum* species responsible for anthracnose and root necrosis of strawberry in Israel. *Phytopathology*, 87(5), pp.516–521.
- Fröhlich, J., Hyde, K., & Petrini, O., 2000. Endophytic fungi associated with palms. *Mycological Research*, 104, pp.1202–1212.
- Fusconi, A., Gnani, E., Trotta, A., & Berta, G., 1999. Apical meristems of tomato roots and their modifications induced by arbuscular mycorrhizal and soilborne pathogenic fungi. *New Phytologist*, 142(3), pp.505–516.
- Gange, A.C., 2007. Insect – mycorrhizal interactions : patterns , processes , and consequences. In T. Ohgushi, T. P. Craig, & P. W. Price, eds. *Ecological Communities: Plant Mediation in Indirect Interaction Webs*. New York: Cambridge University Press, pp. 124–144.
- Gange, A., & Ayres, R.L., 1999. On the relation between arbuscular mycorrhizal colonization and plant “benefit.” *Oikos*, 87(3), pp.615–621.
- Gange, A., Currie, A., Ab Razak, N., Ellison, C., ... Rabiey, M., 2018. The threat of invasive species to biodiversity: Biological control of Himalayan balsam. *Open Access Government*, May 2018, pp.364–365.

- Gange, A.C., Bower, E., & Brown, V.K., 2002a. Differential effects of insect herbivory on arbuscular mycorrhizal colonization. *Oecologia*, 131(1), pp.103–112.
- Gange, A.C., & Brown, V.K., 2002. Actions and interactions of soil invertebrates and arbuscular mycorrhizal fungi in affecting the structure of plant communities. *Mycorrhizal Ecology*, 157, pp.321–344.
- Gange, A.C., Brown, V.K., & Aplin, D.M., 2005. Ecological specificity of arbuscular mycorrhizae: Evidence from foliar- and seed-feeding insects. *Ecology*, 86(3), pp.603–611.
- Gange, A.C., Brown, V.K., & Sinclair, G.S., 1993. Vesicular-arbuscular mycorrhizal fungi: a determinant of plant community structure in early succession. *Functional Ecology*, 7(5), pp.616–622.
- Gange, A.C., Croft, R., & Wu, W., 2002b. Gall insect and endophytic fungal co-occurrence in a xeric and mesic site. *Ecological Entomology*, 27(3), pp.362–365.
- Gange, A.C., Eschen, R., Wearn, J.A., Thawer, A., & Sutton, B.C., 2012. Differential effects of foliar endophytic fungi on insect herbivores attacking a herbaceous plant. *Oecologia*, 168, pp.1023–1031.
- Gange, A.C., Dey, S., Currie, A.F., & Sutton, B.C., 2007. Site- and species-specific differences in endophyte occurrence in two herbaceous plants. *Journal of Ecology*, 95(4), pp.614–622.
- Ganley, R.J., Sniezko, R.A., & Newcombe, G., 2008. Endophyte-mediated resistance against white pine blister rust in *Pinus monticola*. *Forest Ecology and Management*, 255(7), pp.2751–2760.
- Gao, F.K., Dai, C.C., & Liu, X.Z., 2010. Mechanisms of fungal endophytes in plant protection against pathogens. *African Journal of Microbiology Research*, 4(13), pp.1346–1351.
- Gehring, C.A., & Whitham, T.G., 2002. Mycorrhizae-herbivore interactions: population and community consequences. In M. G. A. van der Heijden & I. R. Sanders, eds. *Mycorrhizal ecology*. Berlin, Germany: Springer-Verlag, pp. 295–320.
- Gilbert, L., & Johnson, D., 2015. Plant-mediated “apparent effects” between mycorrhiza and insect herbivores. *Current Opinion in Plant Biology*, 26, pp.100–105.
- Glazebrook, J., 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology*, 43(1), pp.205–227.
- Grime, J.P., Hodgson, J.G., & Hunt, R., 1989. *Comparative Plant Ecology. A Functional Approach to Common British Species.*, London, UK.: Unwin Hyman Ltd.

- van Grunsven, R.H.A., van der Putten, W.H., Bezemer, T.M., Tamis, W.L.M., ... Veenendaal, E.M., 2007. Reduced plant-soil feedback of plant species expanding their range as compared to natives. *Journal of Ecology*, 95(5), pp.1050–1057.
- Gruntman, M., Pehl, A.K., Joshi, S., & Tielbörger, K., 2014. Competitive dominance of the invasive plant *Impatiens glandulifera*: Using competitive effect and response with a vigorous neighbour. *Biological Invasions*, 16(1), pp.141–151.
- Gryndler, M., Šmilauer, P., Püschel, D., Bukovská, P., ... Jansa, J., 2018. Appropriate nonmycorrhizal controls in arbuscular mycorrhiza research: a microbiome perspective. *Mycorrhiza*, 28(5), pp.1–16.
- Gunatilaka, A.A.L., 2012. Natural products from plant-associated microorganisms: Distribution, structural diversity, bioactive and implications of their occurrence. *Journal of Natural Products*, 69(3), pp.509–526.
- Gundale, M.J., Kardol, P., Nilsson, M.C., Nilsson, U., ... Wardle, D.A., 2014. Interactions with soil biota shift from negative to positive when a tree species is moved outside its native range. *New Phytologist*, 202(2), pp.415–421.
- Hammer, T.J., & Van Bael, S.A., 2015. An endophyte-rich diet increases ant predation on a specialist herbivorous insect. *Ecological Entomology*, 40(3), pp.316–321.
- Harley, J.L., & Harley, E.L., 1987. A check-list of mycorrhiza in the British Flora-Second Addenda and Errata. *New Phytologist*, 107(4), pp.741–749.
- Harner, M.J., Mummey, D.L., Stanford, J.A., & Rillig, M.C., 2010. Arbuscular mycorrhizal fungi enhance spotted knapweed growth across a riparian chronosequence. *Biological Invasions*, 12(6), pp.1481–1490.
- Harrier, L.A., & Watson, C.A., 2004. The potential role of arbuscular mycorrhizal (AM) fungi in the bioprotection of plants against soil-borne pathogens in organic and/or other sustainable farming systems. *Pest Management Science*, 60(2), pp.149–157.
- Hartley, S.E., & Gange, A.C., 2009. Impacts of plant symbiotic fungi on insect herbivores: Mutualism in a multitrophic context. *Annual Review of Entomology*, 54(1), pp.323–342.
- Hartley, S.E., Eschen, R., Horwood, J.M., Gange, A.C., & Hill, E.M., 2015. Infection by a foliar endophyte elicits novel arabidopside-based plant defence reactions in its host, *Cirsium arvense*. *New Phytologist*, 205(2), pp.816–827.
- Hayes, A.J., 1979. The microbiology of plant litter decomposition. *Science Progress*, 66(261), pp.25–42.

- Hayman, D., Morris, E., & Page, R., 1981. Methods for inoculating field crops with mycorrhizal fungi. *Annals of Applied Biology*, 99(3), pp.247–253.
- van der Heijden, M.G.A., Wiemken, A., & Sanders, I.R., 2003. Different arbuscular mycorrhizal fungi alter coexistence and resource distribution between co-occurring plant. *New Phytologist*, 157(3), pp.569–578.
- Helgason, T., Fitter, A.H., & Young, J.E.W., 1999. Molecular diversity of arbuscular mycorrhizal fungi colonising *Hyacinthoides non-scripta* (bluebell) in a seminatural woodland. *Molecular Ecology*, (8), pp.659–666.
- Herrmann, L., & Lesueur, D., 2013. Challenges of formulation and quality of biofertilizers for successful inoculation. *Applied Microbiology and Biotechnology*, 97(20), pp.8859–8873.
- Hilbig, B.E., & Allen, E.B., 2015. Plant-soil feedbacks and competitive interactions between invasive *Bromus diandrus* and native forb species. *Plant and Soil*, 392, pp.191–203.
- Hodgson, S., Cates, C., Hodgson, J., Morley, N.J., ... Gange, A.C., 2014. Vertical transmission of fungal endophytes is widespread in forbs. *Ecology and Evolution*, 4, pp.1199–208.
- Holt, R.D., & Hochberg, M.E., 1997. When is biological control evolutionary stable (or is it)? *Ecology*, 78(6), pp.1673–1683.
- Howell, B., 2002. Control and eradication of Himalayan balsam. *Quarterly Journal of Forestry*, 96, pp.125–127.
- Hu, J.L., Lin, X.G., Wang, J.H., Shen, W.S., ... Mao, T.T., 2010. Arbuscular mycorrhizal fungal inoculation enhances suppression of cucumber fusarium wilt in greenhouse soils. *Pedosphere*, 20(5), pp.586–593.
- Hubbard, C.E., 1985. *Grasses.*, Middlesex: Penguin Books Ltd.
- Hubbard, C.E., 1984. *Grasses. A guide to their structure, identification, uses and distribution in the British Isles.*, London, UK.: Penguin.
- Hulme, P.E., & Bremner, E.T., 2006. Assessing the impact of *Impatiens glandulifera* on riparian habitats: Partitioning diversity components following species removal. *Journal of Applied Ecology*, 43(1), pp.43–50.
- van Hulten, M., Pelsler, M., van Loon, L.C., Pieterse, C.M.J., & Ton, J., 2006. Costs and benefits of priming for defense in *Arabidopsis*. *Proceedings of the National Academy of Sciences*, 103(14), pp.5602–5607.
- Husband, R., Herre, E.A., Turner, S.L., Gallery, R., & Young, J.P.W., 2002. Molecular diversity of arbuscular mycorrhizal fungi and patterns of host association over time and space in a tropical forest. *Molecular Ecology*, 11, pp.2669–2678.

- Iqbal, S.H., & Khalid, A.N., 1996. Material for the fungus flora of Pakistan. II. An updated check list of rust fungi (Uredinales) of Pakistan. *Sultania*, 1, pp.39–67.
- Jaber, L.R., & Vidal, S., 2009. Interactions between an endophytic fungus, aphids and extrafloral nectaries: Do endophytes induce extrafloral-mediated defences in *Vicia faba*? *Functional Ecology*, 23(4), pp.707–714.
- Jakobsen, I., Abbott, L.K., & Robson, A.D., 1992. External hyphae of vesicular-arbuscular mycorrhizal fungi associated with *Trifolium subterraneum* L. 1. Spread of hyphae and phosphorus inflow into roots. *New Phytologist*, 120(3), pp.371–380.
- Jallow, M.F.A., Dugassa-Gobena, D., & Vidal, S., 2004. Indirect interaction between an unspecialized endophytic fungus and a polyphagous moth. *Basic and Applied Ecology*, 5(2), pp.183–191.
- Jeffries, P., Gianinazzi, S., Perotto, S., Turnau, K., & Barea, J.M., 2003. The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. *Biology and Fertility of Soils*, 37, pp.1–16.
- Jeschke, J.M., Bacher, S., Blackburn, T.M., Dick, J.T.A., ... Kumschick, S., 2014. Defining the impact of non-native species. *Conservation Biology*, 28(5), pp.1188–1194.
- Jin, L., Wang, Q., Wang, Q., Wang, X., & Gange, A.C., 2017. Mycorrhizal-induced growth depression in plants. *Symbiosis*, pp.1–8.
- John, J., Lundholm, J., Kernaghan, G., Rudgers, J.A., ... Emery, S.M., 2015. Fungal symbiont effects on dune plant diversity depend on precipitation. *Journal of Ecology*, 103(1), pp.219–230.
- Jung, S.C., Martinez-Medina, A., Lopez-Raez, J.A., & Pozo, M.J., 2012. Mycorrhiza-induced resistance and priming of plant defenses. *Journal of Chemical Ecology*, 38(6), pp.651–664.
- Kang, J.G., Kim, K.K., & Kang, K.Y., 1999. Antagonism and structural identification of antifungal compound from *Chaetomium cochliodes* against phytopathogenic fungi. *Agricultural Chemistry Biotechnology*, (42), pp.146–150.
- Kent, D.H., 1975. *The Historical Flora of Middlesex.*, London, UK.: Ray Society.
- Klironomos, J.N., 2002. Feedback with soil biota contributes to plant rarity and invasiveness in communities. *Nature*, 417(6884), pp.67–70.
- Klironomos, J.N., 2000. Host specificity and functional diversity among arbuscular mycorrhizal fungi. In M. B. & P. J.-G. C.R. Bell, ed. *8th International Symposium on Microbial Ecology*. Society for Microbial

Ecology, Halifax, UK. pp. 845–851.

- Klironomos, J.N., & Hart, M.M., 2002. Colonization of roots by arbuscular mycorrhizal fungi using different sources of inoculum. *Mycorrhiza*, 12(4), pp.181–184.
- Koch, A.M., Antunes, P.M., Barto, E.K., Cipollini, D., ... Klironomos, J.N., 2011. The effects of arbuscular mycorrhizal (AM) fungal and garlic mustard introductions on native AM fungal diversity. *Biological Invasions*, 13(7), pp.1627–1639.
- Koch, M., Tanami, Z., Bodani, H., Winingar, S., & Kapulnik, Y., 1997. Field application of vesicular-arbuscular mycorrhizal fungi improved garlic yield in disinfected soil. *Mycorrhiza*, 7(1), pp.47–50.
- Koide, R.T., & Li, M.G., 1989. Appropriate controls for vesicular arbuscular mycorrhiza Research. *New Phytologist*, 111(1), pp.35–44.
- Koricheva, J., Gange, A.C., & Jones, T., 2009. Effects of mycorrhizal fungi on insect herbivores: A meta-analysis. *Ecology*, 90(8), pp.2088–2097.
- Kos, M., Tuijl, M.A.B., de Roo, J., Mulder, P.P.J., & Bezemer, T.M., 2015. Species-specific plant-soil feedback effects on above-ground plant-insect interactions. *Journal of Ecology*, 103(4), pp.904–914.
- Kozioł, L., Schultz, P.A., Bever, J.D., House, G., ... Middleton, E., 2017. *A Practical Guide to Inoculation with Arbuscular Mycorrhizal Fungi in Ecological Restoration.*, United States of America
- Kulmatiski, A., & Kardol, P., 2008. Getting plant–soil feedbacks out of the greenhouse : Experimental and conceptual approaches. *Progress in Botany*, 69(4), pp.449–472.
- Kumar, D.S.S., & Hyde, K.D., 2004. Biodiversity and tissue-recurrence of endophytic fungi in *Tripterygium wilfordii*. *Fungal Diversity*, 17(1994), pp.69–90.
- Kurose, D., Furuya, N., Tsuchiya, K., Tsushima, S., & Evans, H.C., 2012. Endophytic fungi associated with *Fallopia japonica* (Polygonaceae) in Japan and their interactions with *Puccinia polygoni-amphibii* var. *tovariae*, a candidate for classical biological control. *Fungal Biology*, 116(7), pp.785–791.
- Kurose, D., Furuya, N., Matsumoto, M., Djeddour, D.H., ... Tsuchiya, K., 2009. Evaluation of a *Puccinia* rust as a potential biological control agent of *Fallopia japonica*. *Journal of the Faculty of Agriculture, Kyushu University*, 54(1), pp.59–64.
- Larimer, A.L., Bever, J.D., & Clay, K., 2012. Consequences of simultaneous interactions of fungal endophytes and arbuscular mycorrhizal fungi with a shared host grass. *Oikos*, 121(12), pp.2090–2096.

- Latch, G.C.M., 1993. Physiological interactions of endophytic fungi and their hosts. Biotic stress tolerance imparted to grasses by endophytes. *Agriculture, Ecosystems & Environment*, 44(1–4), pp.143–156.
- Lee, K., Pan, J.J., & May, G., 2009. Endophytic *Fusarium verticillioides* reduces disease severity caused by *Ustilago maydis* on maize. *FEMS Microbiology Letters*, 299(1), pp.31–37.
- Lewandowski, T.J., Dunfield, K.E., & Antunes, P.M., 2013. Isolate identity determines plant tolerance to pathogen attack in assembled mycorrhizal communities. *PLoS ONE*, 8(4), pp.2–8.
- Lin, G., McCormack, M.L., & Guo, D., 2015. Arbuscular mycorrhizal fungal effects on plant competition and community structure. *Journal of Ecology*, 103, pp.1224–1232.
- Linderman, R.G., 1994. Role of VAM fungi in biocontrol. In L. R. G. Pfleger F. L., ed. *Mycorrhizae and plant health*. St. Paul, MN, USA: APS Press, pp. 1–25.
- Lobstein, A., Brenne, X., Feist, E., Metz, N., ... Anton, R., 2001. Quantitative determination of naphthoquinones of *Impatiens* species. *Phytochemical Analysis*, 12(3), pp.202–205.
- Mack, K.M.L., & Rudgers, J.A., 2008. Balancing multiple mutualists: Asymmetric interactions among plants, arbuscular mycorrhizal fungi, and fungal endophytes. *Oikos*, 117(2), pp.310–320.
- Mack, R.N., Simberloff, D., Lonsdale, M.W., & Evans, H., 2005. Biotic Invasions: Causes, Epidemiology, Global Consequences and Control. *Bulletin of the Ecological Society of America*, 86(4), pp.249–250.
- Maherali, H., & Klironomos, J.N., 2007. Influence of phylogeny on fungal community assembly and ecosystem functioning. *Science*, 316(5832), pp.1746–1748.
- Majewska, M.L., Rola, K., Stefanowicz, A.M., Nobis, M., ... Zubek, S., 2018. Do the impacts of alien invasive plants differ from expansive native ones? An experimental study on arbuscular mycorrhizal fungi communities. *Biology and Fertility of Soils*, 54(5), pp.631–643.
- Malíková, L., & Prach, K., 2010. Spread of alien *Impatiens glandulifera* along rivers invaded at different times. *Ecohydrology and Hydrobiology*, 10(1), pp.81–85.
- Marchisio, V.F., & Airaudi, D., 2001. Temporal trends of the airborne fungi and their functional relations with the environment in a suburban site. *Mycologia*, 93(5), pp.831–840.
- Marler, M.J., Zabinski, C. a., & Callaway, R.M., 1999. Mycorrhizae indirectly enhance competitive effects of an invasive forb on a native bunchgrass. *Ecology*, 80(4), pp.1180–1186.

- Maule, H., Andrews, M., Watson, C., & Cherrill, A., 2000. Distribution, biomass and effect on native species of *Impatiens glandulifera* in a deciduous woodland in northeast England. *Aspects of Applied Biology*, 58, pp.31–38.
- Mccarthy-neumann, S., & Kobe, R.K., 2010. Conspecific and heterospecific plant-soil feedbacks influence survivorship and growth of temperate tree seedlings. *Journal of Ecology*, 98(2), pp.408–418.
- McGee, P.A., 2002. Reduced growth and deterrence from feeding of the insect pest *Helicoverpa armigera* associated with fungal endophytes from cotton. *Australian Journal of Experimental Agriculture*, 42, pp.995–999.
- McGinley, M., 2010. Invasive species. *Encyclopedia of Earth*, 3(1), pp.1–4.
- McGonigle, T.P., Miller, M.H., Evans, D.G., Fairchild, G.L., & Swan, J.A., 1990. A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. *New Phytologist*, 115(3), pp.495–501.
- McNeely, J.A., Mooney, H.A., Neville, L.E., Schei, P., & Waage, J.K., 2001. *Global strategy on invasive alien species*, IUCN.
- Mumford, P.M., 1990. Dormancy break in seeds of *Impatiens glandulifera* Royle. *New Phytologist*, 115(1), pp.171–175.
- Mummey, D.L., Antunes, P.M., & Rillig, M.C., 2009. Arbuscular mycorrhizal fungi pre-inoculant identity determines community composition in roots. *Soil Biology and Biochemistry*, 41(6), pp.1173–1179.
- Murray, D.C., & Walters, D.R., 1992. Increased photosynthesis and resistance to rust infection in upper, uninfected leaves of rusted broad bean (*Vicia faba* L.). *New Phytologist*, 120(2), pp.235–242.
- Musetti, R., Polizzotto, R., Vecchione, A., Borselli, S., ... Pertot, I., 2007. Antifungal activity of diketopiperazines extracted from *Alternaria alternata* against *Plasmopara viticola*: An ultrastructural study. *Micron*, 38(6), pp.643–650.
- Nelson, E.B., 2004. Microbial dynamics and interactions in the spermosphere. *Annual Review of Phytopathology*, 42(1), pp.271–309.
- Newcombe, G., Shipunov, A., Eigenbrode, S.D., Raghavendra, A.K.H., ... Schwarzländer, M., 2009. Endophytes influence protection and growth of an invasive plant. *Communicative and Integrative Biology*, 2(1), pp.29–31.
- Newsham, K.K., Fitter, A.H., & Watkinson, A.R., 1995. Multi-functionality and biodiversity in arbuscular mycorrhizas. *Trends in Ecology & Evolution*, 10(10), pp.407–411.

- O'Hanlon, K.A., Knorr, K., Jørgensen, L.N., Nicolaisen, M., & Boelt, B., 2012. Exploring the potential of symbiotic fungal endophytes in cereal disease suppression. *Biological Control*, 63(2), pp.69–78.
- Omacini, M., Eggers, T., Bonkowski, M., Gange, A.C., & Jones, T.H., 2006. Leaf endophytes affect mycorrhizal status and growth of co-infected and neighbouring plants. *Functional Ecology*, 20(2), pp.226–232.
- Ortas, I., 2010. Effect of mycorrhiza application on plant growth and nutrient uptake in cucumber production under field conditions. *Spanish Journal of Agricultural Research*, 8(S1), pp.116–122.
- Pattison, Z., Rumble, H., Tanner, R.A., Jin, L., & Gange, A.C., 2016. Positive plant-soil feedbacks of the invasive *Impatiens glandulifera* and their effects on above-ground microbial communities. *Weed Research*, 56(3), pp.198–207.
- Pellegrino, E., Turrini, A., Gamper, H.A., Cafà, G., ... Giovannetti, M., 2012. Establishment, persistence and effectiveness of arbuscular mycorrhizal fungal inoculants in the field revealed using molecular genetic tracing and measurement of yield components. *New Phytologist*, 194(3), pp.810–822.
- Pérez-De-Luque, A., Tille, S., Johnson, I., Pascual-Pardo, D., ... Cameron, D.D., 2017. The interactive effects of arbuscular mycorrhiza and plant growth-promoting rhizobacteria synergistically enhance host plant defences against pathogen. *Scientific Reports*, 7(1), pp.1–10.
- Pérez, M., & Urcelay, C., 2009. Differential growth response to arbuscular mycorrhizal fungi and plant density in two wild plants belonging to contrasting functional types. *Mycorrhiza*, 19(8), pp.517–523.
- Perring, F.H., 1970. The last seventy years. The flora of a changing Britain. In F.H. Perring, ed. *Botanical Society of the British Isles*. Cambridge, UK., pp. 128–135.
- Perrings, C., Williamson, M., Barbier, E.B., Delfino, D., ... Watkinson, A., 2002. Biological invasion risks and the public good: An economic perspective. *Ecology and Society*, 6(1).
- Perrins, J., Fitter, A., & Williamson, M., 1993. Population biology and rates of invasion of 3 introduced *Impatiens* species in the British-Isles. , 20(1), pp.33–44.
- Petrini, O., 1991. Fungal endophytes of tree leaves. In J. H. Andrews & S. S. Hirano, eds. *Microbial Ecology of Leaves*. New York: Springer Verlag, pp. 179–197.
- Petrini, O., 1986. Taxonomy of endophytic fungi of aerial plant tissues. In N.J. Fokkema & J. van den Heuvel, ed. *Microbiology of the Phyllosphere*. Cambridge, UK.: Cambridge University Press, pp. 175–187.

- Pineda, A., Dicke, M., Pieterse, C.M.J., & Pozo, M.J., 2013. Beneficial microbes in a changing environment: Are they always helping plants to deal with insects? *Functional Ecology*, 27(3), pp.574–586.
- Piskorz, R., & Klimko, M., 2006. The effect of *Puccinia komarovii* Tranzsch. infection on characters of *Impatiens parviflora* DC. in Galio sylvatici-Carpinetum (R. Tx. 1937) Oberd. 1957 forest association. *Acta Societatis Botanicorum Poloniae*, 75(1), pp.51–59.
- Pozo, M.J., & Azcón-Aguilar, C., 2007. Unraveling mycorrhiza-induced resistance. *Current Opinion in Plant Biology*, 10(4), pp.393–398.
- Prach, K., 1994. Seasonal dynamics of *Impatiens glandulifera* in two riparian habitats in central England. In L. C. de Waal, L. E. Child, & M. Wade, eds. *Ecology and Management of Invasive Riverside Plants*. Chichester, UK: John Wiley and Sons, pp. 127–133.
- Preston, C.D., Pearman, D.A., & Dines, T.D., 2002. *New Atlas of the British and Irish Flora. An Atlas of the vascular plants of Britain, Ireland, the Isle of Man and the Channel Islands*, Oxford, UK.: Oxford University Press.
- Prowse, A., 2001. *Ecological effects of the invasion of native plant communities by the alien Himalayan balsam (Impatiens Gladulifera Royle)*. PhD Thesis. Royal Holloway, University of London
- van der Putten, W.H., Bardgett, R.D., De Ruiter, P.C., Hol, W.H.G., ... Wardle, D.A., 2009. Empirical and theoretical challenges in aboveground-belowground ecology. *Oecologia*, 161(1), pp.1–14.
- van der Putten, W.H., Bardgett, R.D., Bever, J.D., Bezemer, T.M., ... Wardle, D.A., 2013. Plant-soil feedbacks: The past, the present and future challenges. *Journal of Ecology*, 101(2), pp.265–276.
- Pyšek, P., & Prach, K., 1995. Invasions dynamics of *Impatiens glandulifera*- A century of spreading reconstructed. *Biological Conservation*, 74, pp.41–48.
- Pyšek, P., & Prach, K., 1993. Plant invasions and the role of riparian habitats: a comparison of four species alien to central Europe. *Journal of Biogeography*, 20(4), pp.413–420.
- Pyšek, P., Jarošík, V., Hulme, P.E., Pergl, J., ... Vilà, M., 2012. A global assessment of invasive plant impacts on resident species, communities and ecosystems: The interaction of impact measures, invading species' traits and environment. *Global Change Biology*, 18(5), pp.1725–1737.
- Racedo, J., Salazar, S.M., Castagnaro, A.P., & Díaz Ricci, J.C., 2013. A strawberry disease caused by *Acremonium strictum*. *European Journal of Plant Pathology*, 137(4), pp.649–654.
- Ravnskov, S., & Jakobsen, I., 1995. Functional compatibility in arbuscular mycorrhizas measured as hyphal P transport to the plant. *New*

- Phytologist*, 129(4), pp.611–618.
- Reinhart, K.O., & Callaway, R.M., 2006. Soil biota and invasive plants. *New Phytologist*, 170(3), pp.445–457.
- Ricciardi, A., & Cohen, J., 2007. The invasiveness of an introduced species does not predict its impact. *Biological Invasions*, 9(3), pp.309–315.
- Richardson, A.E., Barea, J.M., McNeill, A.M., & Prigent-Combaret, C., 2009. Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. *Plant and Soil*, 321(1–2), pp.305–339.
- Richardson, D.M., Pyšek, P., Rejmánek, M., Barbour, M.G., ... West, C.J., 2000. Naturalization and invasion of alien plants: Concepts and definitions. *Diversity and Distributions*, 6(2), pp.93–107.
- Rodriguez, A., & Sanders, I.R., 2015. The role of community and population ecology in applying mycorrhizal fungi for improved food security. *ISME Journal*, 9(5), pp.1053–1061.
- Rodriguez, R.J., White, J.F., Arnold, A.E., & Redman, R.S., 2009. Fungal endophytes: diversity and functional roles. *The New Phytologist*, 182(2), pp.314–330.
- Ruckli, R., Hesse, K., Glauser, G., Rusterholz, H.P., & Baur, B., 2014. Inhibitory potential of naphthoquinones leached from leaves and exuded from roots of the invasive plant *Impatiens glandulifera*. *Journal of Chemical Ecology*, 40(4), pp.371–378.
- Rudgers, J.A., Afkhami, M.E., Rúa, M.A., Davitt, A.J., ... Huguet, V.M., 2009. A fungus among us: Broad patterns of endophyte distribution in the grasses. *Ecology*, 90(6), pp.1531–1539.
- Rudgers, J.A., & Orr, S., 2009. Non-native grass alters growth of native tree species via leaf and soil microbes. *Journal of Ecology*, 97(2), pp.247–255.
- Russo, V.M., Russo, B.M., Peters, M., Perkins-Veazie, P., & Cartwright, B., 1997. Interaction of *Colletotrichum orbiculare* with thrips and aphid feeding on watermelon seedlings. *Crop Protection*, 16(6), pp.581–584.
- Saldajeno, M.G.B., & Hyakumachi, M., 2011. The plant growth-promoting fungus *Fusarium equiseti* and the arbuscular mycorrhizal fungus *Glomus mosseae* stimulate plant growth and reduce severity of anthracnose and damping-off diseases in cucumber (*Cucumis sativus*) seedlings. *Annals of Applied Biology*, 159(1), pp.28–40.
- Sanchez Marquez, S.G.F., Bills, N.H., & Zabalgoitia, I., 2012. Non-systemic fungal endophytes of grasses. *Fungal Ecology*, 5, pp.289–297.
- Scheublin, T.R., Van Logtestijn, R.S.P., & Van Der Heijden, M.G.A., 2007.

- Presence and identity of arbuscular mycorrhizal fungi influence competitive interactions between plant species. *Journal of Ecology*, 95(4), pp.631–638.
- Schittko, C., Runge, C., Strupp, M., Wolff, S., & Wurst, S., 2016. No evidence that plant–soil feedback effects of native and invasive plant species under glasshouse conditions are reflected in the field. *Journal of Ecology*, 104(5), pp.1243–1249.
- Schulz, B., Wanke, U., Draeger, S., & Aust, H.J., 1993. Endophytes from herbaceous plants and shrubs: effectiveness of surface sterilization methods. *Mycological Research*, 97(12), pp.1447–1450.
- Schwartz, M.W., Hoeksema, J.D., Gehring, C.A., Johnson, N.C., ... Pringle, A., 2006. The promise and the potential consequences of the global transport of mycorrhizal fungal inoculum. *Ecology Letters*, 9(5), pp.501–515.
- Seier, M.K., 2005. Fungal pathogens as classical biological control agents for invasive alien weeds- are they a viable concept for Europe? In W. Nentwig, ed. *Biological Invasions-From Ecology to Control*. Berlin, Germany: Neobiota, pp. 165–175.
- Seier, M.K., Pollard, K.M., Thomas, S.E., & Kurose, D., 2014. Assessing pathogen specificity - the case of the *Mycosphaerella* leaf-spot for control of Japanese knotweed in the United Kingdom. In *XIV International Symposium on Biological Control of Weeds*. Kruger National Park, South Africa, p. 116.
- Šerá, B., Vrchotová, N., & Tříška, J., 2005. Phenolic compounds in the leaves of alien and native Impatiens plants. In D. V. Alford & G. F. Backhaus, eds. *Plant protection and plant health in Europe: introduction and spread of invasive species*. Alton: British Crop Production Council, pp. 281–282.
- Shade, A., Jacques, M.A., & Barret, M., 2017. Ecological patterns of seed microbiome diversity, transmission, and assembly. *Current Opinion in Microbiology*, 37, pp.15–22.
- Shannag, H.K., & Ababneh, J.A., 2007. Influence of Black Bean Aphid, *Aphis fabae* Scopoli. on Growth Rates of Faba Bean. *World Journal of Agricultural Sciences*, 3(3), pp.344–349.
- Shaw, D., & Tanner, R., 2008. Weed like to see less of them. *Biologist*, 55(4), pp.208–214.
- Shaw, R.H., 2003. Biological control of invasive weeds in the UK: Opportunities and challenges. In P.W. and M.W.J. Child, H. Brock, G. Brundu, K. Prach, P. Pysek, eds. *Plant Invasions, Ecological Threats and Management Solutions*. Leiden, Netherlands.: Backhuys Publishers, pp. 337–354.

- Shaw, R.H., Bryner, S., & Tanner, R., 2009. The life history and host range of the Japanese knotweed psyllid, *Aphalara itadori* Shinji: Potentially the first classical biological weed control agent for the European Union. *Biological Control*, 49(2), pp.105–113.
- Shaw, R.H., Tanner, R., Djeddour, D., & Cortat, G., 2011. Classical biological control of *Fallopia japonica* in the United Kingdom - lessons for Europe. *Weed Research*, 51(6), pp.552–558.
- Sheppard, A., Shaw, R.H., & Sforza, R., 2006. Top 20 environmental weeds for classical biological in Europe. *Weed Research*, 46, pp.93–117.
- Siddiqui, Z.A., & Akhtar, M.S., 2008. Arbuscular mycorrhizal fungi as potential bioprotectants against plant pathogens. In Z. A. Siddiqui, M. S. Akhtar, & K. Futai, eds. *Mycorrhizae: Sustainable Agriculture and Forestry*. Springer, pp. 73–98.
- Sikes, B.A., Cottenie, K., & Klironomos, J.N., 2009. Plant and fungal identity determines pathogen protection of plant roots by arbuscular mycorrhizas. *Journal of Ecology*, 97(6), pp.1274–1280.
- Simon, A.L., Wellham, P.A.D., Aradottir, G.I., & Gange, A.C., 2017. Unravelling mycorrhiza-induced wheat susceptibility to the English grain aphid *Sitobion avenae*. *Scientific Reports*, 7, pp.1–11.
- Smith, S.E. & Read, D.J., 1997. *Mycorrhizal Symbiosis* 2nd ed., Academic Press, London.
- Smith, S.E., Smith, F.A., & Jakobsen, I., 2004. Functional diversity in arbuscular mycorrhizal (AM) symbioses: The contribution of the mycorrhizal P uptake pathway is not correlated with mycorrhizal responses in growth or total P uptake. *New Phytologist*, 162(2), pp.511–524.
- Stampe, E.D., & Daehler, C.C., 2003. Mycorrhizal species identity affects plant community structure and invasion : A microcosm study. *Oikos*, 100(2), pp.362–372.
- Starý, P., Rakhshani, E., Tomanović, Ž., Kavallieratos, N.G., ... Havelka, J., 2014. Aphid-parasitoid associations on the impatiens plants in Central Europe (Hemiptera, Aphididae; Hymenoptera, Braconidae, Aphidiinae). *Journal of the Entomological Research Society*, 16(3), pp.33–43.
- Stenones, A., & Garnett, R.P., 1994. Controlling invasive weeds using glyphosate. In L. C. De Waal et al. *Ecology and Management of Invasive Riverside Plants*. Chichester, UK: John Wiley & Sons, pp. 183–188.
- Streitwolf-Engel, R., Boller, T., Wiemken, A., & Sanders, I. R., 2010. Clonal growth traits of two *Prunella* species are determined by co-occurring arbuscular mycorrhizal fungi from a calcareous grassland. *New Phytologist*, 85(2), pp.181–191.

- Szewczyk, K., Sezai Cicek, S., Zidorn, C., & Granica, S., 2018. Phenolic constituents of the aerial parts of *Impatiens glandulifera* Royle (Balsaminaceae) and their antioxidant activities. *Natural Product Research*, pp.1–5.
- Szewczyk, K., Zidorn, C., Biernasiuk, A., Komsta, Ł., & Granica, S., 2016. Polyphenols from *Impatiens* (Balsaminaceae) and their antioxidant and antimicrobial activities. *Industrial Crops and Products*, 86, pp.262–272.
- Tanner, R., 2007. A review on the potential for the biological control of the invasive weed , *Impatiens glandulifera* in Europe. *Plant Invasions*, 12(23), pp.1–12.
- Tanner, R., 2011. *An ecological assessment of Impatiens glandulifera in its introduced and native range and the potential for its classical biological control*. PhD Thesis. Royal Holloway, University of London.
- Tanner, R., Ellison, C., Evans, H., & Berezky, Z., 2011. The potential for the biological control of Himalayan balsam using the rust pathogen *Puccinia cf. komarovii*: Opportunities for Europe and North America. In *XIII International Symposium on Biological Control of Weeds*.
- Tanner, R., Ellison, C., Shaw, R., Evans, H., & Gange, A., 2008. Losing patience with impatiens: Are natural enemies the solution? *Outlooks on Pest Management*, 19(2), pp.86–91.
- Tanner, R.A., Pollard, K.M., Varia, S., Evans, H.C., & Ellison, C.A., 2015a. First release of a fungal classical biocontrol agent against an invasive alien weed in Europe: biology of the rust, *Puccinia komarovii* var. *glanduliferae*. *Plant Pathology*, pp.1–10.
- Tanner, R.A., Ellison, C.A., Seier, M.K., Kovács, G.M., & Evans, H.C., 2015b. *Puccinia komarovii* var. *glanduliferae* var. nov.: a fungal agent for the biological control of Himalayan balsam (*Impatiens glandulifera*). *European Journal Plant Pathology*, 141, pp.247–266.
- Tanner, R.A., Jin, L., Shaw, R., Murphy, S.T., & Gange, A.C., 2014. An ecological comparison of *Impatiens glandulifera* Royle in the native and introduced range. *Plant Ecology*, 215(5).
- Tanner, R.A., & Gange, A.C., 2013. The impact of two non-native plant species on native flora performance: Potential implications for habitat restoration. *Plant Ecology*, 214(3), pp.423–432.
- Tickner, D.P., Angold, P.G., Gurnell, A.M., Mountford, J.O., & Sparks, T., 2001. Hydrology as an influence on invasion: Experimental investigations into competition between the alien *Impatiens glandulifera* and the native *Urtica dioica* in the UK. In G. Burundu & J. Brock, eds. *Plant Invasions: Species Ecology and Ecosystem Management*.
- Ton, J., Van Pelt, J.A., Van Loon, L.C., & Pieterse, C.M.J., 2002. Differential effectiveness of salicylate-dependent and jasmonate/ethylene-

- dependent induced resistance in *Arabidopsis*. *Molecular Plant-Microbe Interactions*, 15(1), pp.27–34.
- Toney, J.C., Rice, P.M., & Forcella, F., 1998. Exotic plant records in the northwest United States 1950-1996: An ecological assessment. *Northwest Science*, 72(3), pp.198–213.
- Trewick, S. & Wade, P.M., 1986. The distribution and dispersal of two alien species of *Impatiens*, waterway weeds in the British Isles. In *Proceedings 7th International European Weed Research Society/Association of Applied Biologists Symposium on Aquatic Weeds*. Loughborough, UK., pp. 351–356.
- Trujillo, E.E., 2005. History and success of plant pathogens for biological control of introduced weeds in Hawaii. *Biological Control*, 33(1), pp.113–122.
- Vicari, M., Hatcher, P.E., Ayres, P.G., & Sep, N.O., 2008. Combined effect of foliar and mycorrhizal endophytes on an insect herbivore. *Ecological Society of America*, 83(9), pp.2452–2464.
- Vierheilig, H., Coughlan, A.P., Wyss, U.R.S., & Recherche, C. De, 1998. Ink and vinegar, a simple staining technique for arbuscular-mycorrhizal fungi. *Applied and Environmental Microbiology*, 64(12), pp.5004–5007.
- Vogelsang, K.M., Reynolds, H.L., & Bever, J.D., 2006. Mycorrhizal fungal identity and richness determine the diversity and productivity of a tallgrass prairie system. *New Phytologist*, 172(3), pp.554–562.
- van de Voorde, T.F.J., van der Putten, W.H., & Martijn Bezemer, T., 2011. Intra- and interspecific plant-soil interactions, soil legacies and priority effects during old-field succession. *Journal of Ecology*, 99(4), pp.945–953.
- Wagg, C., Jansa, J., Stadler, M., Schmid, B., & van der Heijden, M.G.A., 2011. Mycorrhizal fungal identity and diversity relaxes plant – plant competition. *Ecology*, 92(6), pp.1303–1313.
- Waller, L.P., Callaway, R.M., Klironomos, J.N., Ortega, Y.K., & Maron, J.L., 2016. Reduced mycorrhizal responsiveness leads to increased competitive tolerance in an invasive exotic plant. *Journal of Ecology*, 104(6), pp.1599–1607.
- Wamberg, C., Christensen, S., & Jakobsen, I., 2003. Interaction between foliar-feeding insects, mycorrhizal fungi, and rhizosphere protozoa on pea plants. *Pedobiologia*, 47(3), pp.281–287.
- Wang, Y.-Y., Yin, Q.-S., Qu, Y., Li, G.-Z., & Hao, L., 2018. Arbuscular mycorrhiza-mediated resistance in tomato against *Cladosporium fulvum*-induced mould disease. *Journal of Phytopathology*, 166(1), pp.67–74.
- Watkinson, A.R., & Freckleton, R.P., 1997. Quantifying the impact of

- arbuscular mycorrhiza on plant competition. *Journal of Ecology*, 85(4), pp.541–545.
- Wearn, J.A., Sutton, B.C., Morley, N.J., & Gange, A.C., 2012. Species and organ specificity of fungal endophytes in herbaceous grassland plants. *Journal of Ecology*, 100, pp.1085–1092.
- West, H.M., 1996. Influence of arbuscular mycorrhizal infection on competition between *Holcus Lanatus* and *Dactylis Glomerata*. *Journal of Ecology*, 84(3), pp.429–438.
- Williams, A., Birkhofer, K., & Hedlund, K., 2014. Above- and below-ground interactions with agricultural management: Effects of soil microbial communities on barley and aphids. *Pedobiologia*, 57(2), pp.67–74.
- Williams, F., Eschen, R., Harris, A., Djeddour, D., ... Murphy, S.T., 2010. The economic cost of invasive non-native species on Great Britain. *CABI report*, (November), pp.1–199.
- Workman, R.E., & Cruzan, M.B., 2016. Common mycelial networks impact competition in an invasive grass. *American Journal of Botany*, 103(6), pp.1041–1049.
- Yan, J.F., Broughton, S.J., Yang, S.L., & Gange, A.C., 2015. Do endophytic fungi grow through their hosts systemically? *Fungal Ecology*, 13, pp.53–59.
- Yang, G., Liu, N., Lu, W., Wang, S., ... Chen, Y., 2014. The interaction between arbuscular mycorrhizal fungi and soil phosphorus availability influences plant community productivity and ecosystem stability. *Journal of Ecology*, 102(4), pp.1072–1082.
- Yang, X., Summerhurst, D.K., Koval, S.F., Ficker, C., ... Bernards, M.A., 2001. Isolation of an antimicrobial compound from *Impatiens balsamina* L. using bioassay-guided fractionation. *Phytotherapy Research*, 15(8), pp.676–680.
- Zhang, F.J., Li, Q., Chen, F.X., Xu, H.Y., ... Wan, 2017. Arbuscular mycorrhizal fungi facilitate growth and competitive ability of an exotic species *Flaveria bidentis*. *Soil Biology and Biochemistry*, 115, pp.275–284.
- Zhou, Y., Li, X., Gao, Y., Liu, H., ... Ren, A.Z., 2018. Plant endophytes and arbuscular mycorrhizal fungi alter plant competition. *Functional Ecology*, 32(5), pp.1168–1179.
- Zhuang, J.Y., & Wei, S.X., 1994. An annotated checklist of rust fungi from the Mt. Qomolangma region (Tibetan Everest Himalaya). *Mycosystema*, 7, pp.37–87.
- Zobel, M., & Moora, M., 1995. Interspecific competition and arbuscular mycorrhiza: Importance for the coexistence of two calcareous grassland

species. *Folia Geobotanica*, 30(2), pp.223–230.