**Three-way fungal interactions affect the potential biological control of Himalayan balsam, *Impatiens glandulifera***

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# CONFLICT OF INTEREST

The authors declare that this work was conducted without any commercial or proprietary relationships in the mycorrhizal products that could be considered conflicts of interest.

# DATA AVAILABILITY STATEMENT

The raw data sets are available from the corresponding author upon reasonable request.

# Abstract

Himalayan balsam (*Impatiens glandulifera)* is one of the most invasive weeds across Europe. The rust fungus, *Puccinia komarovii* var. *glanduliferae* has been introduced as a biological control agent, but success has been patchy. Here, we investigated whether mycorrhizal and endophytic fungi can affect rust efficacy and plant growth. Over three experiments we found that AM fungi and the rust alone or together consistently reduced plant growth, but this depended on the identity of species in the AM inoculum. Meanwhile, AM fungi increased infection frequency of the endophyte *Colletotrichum acutatum.* Rust inoculation had no detrimental effects on mycorrhizal colonisation or *C. acutatum* infection, but the latter two fungi reduced rust sporulation. However, plant size was reduced when all three fungal types were present, suggesting that a combined fungal inoculum offers a promising approach for the control of this weed.

# KEYWORDS

Arbuscular mycorrhizal fungi, endophyte, invasive species, plant pathogen, rust fungus

# 1 | INTRODUCTION

Plants contain an internal microbiome with positive or negative consequences for the host plant in terms of establishment, growth and protection from pathogens and herbivores (Compant et alet al*.,* 2019). The most widely studied plant beneficial parts of the microbiome include bacteria, arbuscular mycorrhizal (AM) fungi in the roots and foliar fungal endophytes in the shoots. Recent interest has begun to focus on parts of the microbiome in the biological control of invasive weeds, because agents such as insects or pathogenic fungi can be greatly affected by the presence of AM fungi or endophytes (Trognitz et al*.,* 2016). For example, previous studies have shown the protective effect of AM fungi against plant pathogens (Borowicz, 2001). Indeed, these fungi have been suggested as potential biological control agents of root-borne plant diseases (Weng et al*.,* 2022), mostly through the activation of chemical defence systems in the host. Given that AM fungi can also elicit defence changes in the foliar parts of a plant also (Benjamin et al*.,* 2022), this could represent a potential barrier to the success of a pathogenic fungus introduced for the biological control of a weed.

*Impatiens glandulifera* Royle is an invasive weed in the UK and across large parts of Europe (Coakley & Petti, 2021). It is a fast-growing annual, reaching heights of up to 4 m when growing in a partly shaded, nutrient-rich environments such as woodland edges near riverbanks (Tanner & Gange, 2020). This species has been the subject of a biological control programme, in which the pathogenic rust fungus *Puccinia komarovii var. glanduliferae* R. Tanner, C.A. Ellison, L. Kiss & H.C. Evans has been introduced in an attempt to control it (Pollard et al*.,* 2022). However, success has been patchy, due to there being different biotypes of the weed (Pollard et al*.,* 2021) as well as a suggestion of antagonistic interactions between the rust and other fungi in the leaves (Currie et al*.,* 2020).

It is intriguing that AM fungi do not always have positive effects on plants (Jin et al*.,* 2017). If nutrient conditions are not limiting or if the balance of carbon-nutrient exchange is uneven, then mycorrhizal presence can result in reduced plant growth (Jin et al*.,* 2017). Furthermore, it has been suggested that such negative growth effects could be exploited in attempt to control weeds biologically, particularly if the weed in question is a fast-growing annual, with little or no reliance on the mycorrhizal association (Veiga et al*.,* 2011). Such an approach requires careful research, because negative growth effects are very much dependent on the species of AM fungi involved (Li et al*.,* 2019). However, the rewards are potentially high, as the same fungi can produce positive growth effects in the native vegetation, allowing for a reduction of weed prevalence through interspecific competition (El Omari & El Ghachtouli, 2021). Evidence suggests that *I. glandulifera* may be antagonised by AM fungi, with a negative relation found between root colonisation levels and plant size (Tanner et al*.,* 2014). This plant seems to actively manipulate soil microbial communities in its favour, reducing fungal abundance but increasing that of bacteria (Pattison et al*.,* 2016). Therefore, one aim of this study was to examine whether inoculation of plants with mycorrhizal fungi would affect the plant growth. We compared two commercial fungal inoculants, containing different sets of fungal species with plants being grown in controlled conditions.

Unlike AM fungi, there are very few studies on the interactions between foliar endophytes and plant pathogenic fungi in forbs, as most work has focused on crop plants or trees. Given that foliar endophytic fungi produce and/or induce a wide variety of chemical defences in foliar tissues, it is highly likely that they interact with a fungal biological control agent, and most likely impair its growth and efficacy (De Silva et al*.,* 2019). Furthermore, although endophytes and AM fungi are widespread in herbaceous plants, their interactions are little understood within these plants (Dastogeer et al*.,* 2020). An antagonistic relationship was found between AMF and endophytes in *Cirsium arvense* (L.) and *Plantago lanceolata* (L.), whichmay have been due to competition for resources between the symbionts in the host plants (Wearn et al*.,* 2012). In turn, through processes of nutrient allocation, AM fungi may alter the community composition of endophytes in foliar tissues (Eschen et al*.,* 2010). Meanwhile, in *I. glandulifera,* there is some evidence that AM fungi can influence colonisation of plant tissues by some endophytes (Ab Razak & Gange, 2021). The rich chemical defences associated with endophyte colonisation likely has a significant role to play in mediating interactions between these fungi, AM fungi and biological control agents such as plant pathogens (Nisa et al*.,* 2015).

To date, no studies have been conducted on multiple fungal (arbuscular mycorrhizal, foliar fungal endophyte and plant pathogen) interactions in forbs although a few studies have been attempted with the ecologically and economically important endophytes in grasses (Vandegrift et al*.,* 2015). If fungal biological control of *I. glandulifera* and other weeds is to become more predictable and consistent, then it is critical to conduct complex three-way interaction experiments between the different parts of the plant microbiome (Garbelotto et al*.,* 2019). Here, we report on three experiments, designed to investigate the effects of combinations of these fungi on the growth of *I. glandulifera.* In the first, we sought to examine how two commercial mycorrhizal inoculants influence the effect of the rust pathogen on its host. We hypothesised that the presence of AM fungi would antagonise the rust, but that this might differ between inoculants varying in mycorrhizal species. In the second experiment, we examined the interactions between AM fungi and two commonly-occurring endophytes in this plant, *Colletotrichum acutatum* and *Cladosporium oxysporum.* We hypothesised that AM fungi would increase endophyte infection and that the increased carbon demand by these fungi within the plants would weaken growth. Finally, we examined the interactions between AM fungi, an endophyte and the rust, where we hypothesised that the combination of all three fungal types would ultimately lead to smaller plants. This would be due to the microbial mediation of trade-offs between growth and defence that every plant experiences (Karasov et al*.,* 2017).

# 2. | MATERIALS AND METHODS

# 2.1 | Experiment One: Interactions between AM fungi and rust fungus, *Puccinia komarovii* var. *glanduliferae*

Ripe *I. glandulifera* seeds were collected from a wild population at Harmondsworth Moor, Middlesex, UK (51.48 oN, -0.48 oW) in autumn and stored for 6 months at 4oC. Seeds were surface-sterilised in 5% sodium hypochlorite, washed in sterile water and germinated on moist filter paper at 4oC. Seedlings were planted in 2L pots containing John Innes Type 3 compost. Use of the AM inocula replicated that in Ab Razak and Gange (2021) in which two commercial mycorrhizal inoculants were used, provided by PlantWorks (Sittingbourne, Kent, UK) and Symbio (Wormley, Surrey, UK). Hereafter we refer to these two inoculants as ‘PlantWorks’ and ‘Symbio’ respectively. The species composition of each inoculant is given in Ab Razak & Gange (2021). We used the recommended rate of inoculation for herbaceous plants wherein 15 g of PlantWorks or 2g of Symbio were applied as a layer in a hole prepared for seedling planting. One seedling was planted per pot. As the species composition and carrier material differed between the products, we sterilised each separately at 120oC for 30 minutes. Therefore, there was a different control for each product, consisting of sterilised PlantWorks or Symbio. In addition, the controls received 15 ml of microbial filtrate, consisting of live inoculum in sterile water, passed through a 38-μm membrane, to remove all mycorrhizal propagules (Koide & Li, 1989). When the plants had reached the three whorl stage they were infected with the rust fungus, *Puccinia komarovii* var. *glanduliferae* (here onwards referred to as rust)in an identical manner to that described by Currie et al*.* (2020). Leaves were sprayed on the abaxial surface with a spore suspension at a rate of approximately 1.5x10 5 spores ml-1 in 0.05% Tween 80 (*ca.* 550μl/leaf). Rust material was of the Indian strain and its production is fully described in Pollard et al*.* (2022). After inoculation, plants were placed in a 1m3 Perspex dew chamber for 24h to facilitate spore germination. The viability of the rust spores was checked by spraying a small amount of spore solution onto a Petri dish containing tap water agar which was placed into the Perspex box at the same time as the plants. When plants were removed the Petri dishes were examined for spore germination. One day after inoculation, the plants were placed in a glasshouse in a randomised block design, consisting of the eight treatments (+ PlantWorks with/without rust, + Symbio with/without rust) with six replicates of each, giving a total of 48 plants. Plants were grown for 9 weeks and were harvested before flowering, so as not to contravene the terms of the license for growing this plant from the Animal and Plant Health Agency, in which the escape of seeds into the wild must be prevented. At harvest, we measured plant height, leaf number and dry shoot biomass. Leaf and root samples were collected for fungal assessments as described in 2.4 and 2.5 below.

# 2.2 | Experiment Two: Interactions between AM fungi and the endophytes *Colletotrichum acutatum* and *Cladosporium oxysporum.*

Results from Experiment 1 showed that the Symbio inoculum offered the most promising results in terms of the greater growth reduction of plants and increased sporulation of the rust. This inoculum was therefore used in experiments 2 and 3. In previous work by Currie et al*.* (2020), the two most common foliar fungal endophytes in field isolations were *Colletotrichum acutatum* and *Cladosporium oxysporum.* Material of each pure culture was identified by molecular methods as described fully in Currie et al. (2020) and deposited in GenBank with accession numbers MH428675 and MH428677, respectively, as well as the CABI culture collection (accession numbers IMI505519 and IMI505553, respectively). The material used here was identical to that in Currie et al*.* (2020) and Ab Razak & Gange (2021).

Seeds originated from the same stock as in Experiment 1 and were germinated in the same way. The resulting plants were grown in a glasshouse for nine weeks, in 2L pots containing John Innes Type 3 compost with addition of Symbio inoculum as described above. Control plants were grown with the sterilised inoculum. When the plants were at the three whorl leaf stage, the leaves were inoculated with either *C. acutatum, Cl. oxysporum* or a combination of both species. The spores were harvested from a culture stock plate using a sterile needle in a laminar flow cabinet, and the spore concentration was adjusted using a haemocytometer to approximately 1.5x105 spores ml-1 in 0.05% Tween 80. The leaves were inoculated by spraying the spore suspension on the abaxial side of the leaves (9-11 leaves per plant), in two strokes (*ca.* 550μl/leaf) using a handheld bottle sprayer. Control plants were sprayed with 0.05% Tween 80 only. Next, the plants were placed in the 1m3 perspex dew chamber for 48 hours to provide a high humidity environment for the spores to germinate. The remaining spore suspension was sprayed onto a potato carrot agar (PCA) plate and also placed in the box in order to examine the efficacy of endophyte inoculation. Between each treatment, the box was cleaned thoroughly using a 100% ethanol to avoid contamination from the previous test. After 48 hours, the plants were removed and the 2l pots were arranged in a randomised block design in a glasshouse and watered with 250ml water twice daily. Overall, there were 40 plants, comprised of five replicates of the eight treatments (i.e. with and without Symbio, with and without *C. acutatum* and with and without *Cl. oxysporum).* The plants were harvested after 9 weeks, but before first flowering. The plant height, leaf number and dry shoot biomass were recorded for each plant. Leaf and root samples from each plant were collected for fungal identification and analysis as described in 2.4 and 2.6 below.

# 2.3 | Experiment Three: Interactions between the endophyte, *C. acutatum,* AM fungi and rust fungus, *P. komarovii* var. *glanduliferae*

Seeds were germinated in the same manner as above. Eight treatments, with five replicates of each, producing 40 plants in total were established. Plants were grown in 2L pots containing John Innes No 3 compost with the addition of Symbio mycorrhizal inoculum (as above) while the control plants were grown with sterilised AMF (as described in 2.1 above). One seedling was planted in each pot. When the plants reached the three whorl stage, the leaves were inoculated with a spore suspension of the endophyte, *C. acutatum,* as described in section 2.2*.* A week after the endophyte application, the leaves were infected with rust fungus, *P. komarovii* var. *glanduliferae* as described in section 2.1*.* Control plants received sterile sprays that did not contain the respective fungi. The plants were then grown in a glasshouse for nine weeks. Plant parameters (height, leaf number and dry shoot biomass) were recorded while leaf and root samples were collected for rust, endophyte and AMF colonisation assessments respectively, as described in 2.4, 2.5 and 2.6 below.

# 2.4 | Arbuscular Mycorrhizal Fungi (AMF) Colonisation

Root material was collected from each plant for AM fungal colonisation assessment. Roots were extracted from the compost and washed under running water until all soil particles were removed. The roots were cut into pieces approximately 10mm in length and were stored in 70% ethanol for root staining at a later date. The root staining method followed Vierheilig et al*.* (1998). Roots were rinsed in tap water to remove the ethanol and each sample was placed into an individual Biopsy processing cassette. Cassettes were immersed in a beaker containing 10% potassium hydroxide (KOH) and placed in a water bath at 80oC for 25 mins. The cassettes were then removed and rinsed under running tap water for a period of 10 mins. The cassettes were immersed in a beaker containing staining solution (84.4:15:0.6, sterile distilled water (SDW): 1% hydrochloric acid: Quink blue pen ink) and placed back into the water bath for a period of 30 mins. Cassettes were then rinsed to remove excess stain, the roots were mounted on a slide in SDW, sealed using nail polish and were analysed under microscope. AMF colonisation was quantified using the cross-hair eyepiece method of McGonigle et al*.* (1990). The percentage root length colonisation (% RLC) was evaluated by counting 100 intersections with root sections where the presence of intraradical hyphae, vesicles and arbuscules was recorded, at a magnification of x400. This process was repeated for all root samples.

# 2.5 | Rust Fungus Infection

Ten rust-infected leaves were picked at random from each plant in the respective treatments and an acetate grid consisting of 10 x 10 mm squares placed upon each leaf. The number of rust pustules in five randomly selected squares was counted and a mean calculated for each leaf and then for each plant, following Currie et al*.* (2020).

# 2.6 | Endophyte Isolation

Three leaves (bottom, middle and top) from each plant in each treatment were harvested for endophyte evaluation. Two round fragments, each approximately 6mm in diameter were cut from each leaf using a sterilised hole punch and followed a modified surface sterilisation using method III in Schulz et al*.* (1993). The fragments were immersed in 100% ethanol for 30 sec, washed in SDW, immersed in 4.7% sodium hypoclorite (NaOCl) (4.7% v/v: 4.7ml NaOCl in 100ml SDW) for one min, immersed in 100% ethanol for a further 30 sec and followed by four separate rinses with SDW. The fragments were placed abaxial surface downwards onto potato dextrose agar (PDA) amended with 80 mg L-1 streptomycin sulphate and 60 mg L-1 penicillin G to inhibit bacterial contamination. Before plating, the fragments were pressed onto several PDA plates to examine the efficacy of the surface sterilisation procedure. The 90 mm plates were sealed with parafilm to prevent contamination and stored in a plastic box at room temperature. Single isolations of each endophyte growing from the sterilised leaf were set up on potato carrot agar (PCA) plates. Once sporulation had occurred, fungal material including conidia, conidiophores and mycelia were mounted in Erythrosin stain on slides for morphological identification by B. C. Sutton. A sample of the isolates of *C. acutatum* and *Cl. oxysporum* on PCA was sent to the Microbial Identification Service, Centre for Agriculture and Bioscience International (CABI), for molecular confirmation. 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).

# 2.7 | Statistical Analysis

All of the statistical analyses were conducted using R 4.0.2. The normality was tested prior to analysis and plots of residuals examined. In experiment 1, the data sets involving addition of PlantWorks or Symbio were analysed separately, due to the differences in the controls (2.1, above). Count data (number of pustules per unit area) were analysed with a Poisson generalised linear model structure, using a log link function, having checked for overdispersion, and employing mycorrhizal and rust addition (experiment 1) or mycorrhizal addition and the rust and the endophyte (experiment 3) as main effects. Percentage data were subjected to the logit transformation prior to analysis (Warton & Hui, 2011). All plant growth data that violated the assumptions were transformed with square-root or logarithmic transformations. To analyse the effect and interaction of fungi on balsam height and biomass, a two-way factorial ANOVA was performed with mycorrhizal and rust presence as main effects in experiment one, while three-way factorial ANOVA was conducted with mycorrhizal and the two endophytes as main effects in experiment two. Meanwhile three-way factorial ANOVA was performed with AM fungi, endophyte and rust presence as main effects in experiment three. Infection frequency of endophyte species between treatments were examined with three-way ANOVA for experiments two and three. Particular fungi that were absent in treatments were excluded from these analyses.

# 3 | RESULTS

# 3.1 | Experiment One

Inoculation with PlantWorks had no effect on plant height (Figure 1a) or biomass, but in this part of the experiment, the rust fungus reduced final dry shoot biomass (*F1,20* = 6.4, *p* < 0.05) (Figure 1b). Overall, inoculation with Symbio and the rust resulted in shorter plants (Symbio: *F1,20* = 16.9, *p* < 0.001; rust: *F1,20* = 14.7, *p* < 0.001), but there was a strong interaction term found between the AM fungi and rust (*F1,20* = 15.9, *p* < 0.001), because the reduction in height was most obvious when both fungal inocula were applied (Figure 1a). The reduction in height translated into a reduction in biomass, with smallest plants produced when both fungal types were applied (Figure 1b).

*Figure 1 near here*

No AM fungal colonisation was found in the control plants grown with the sterile inocula. Rust presence had no effect on root colonisation by either AM fungal product (Figure 1c) but both AM fungal inocula increased the sporulation of the rust, as measured by pustule density (PlantWorks: *z* = 5.8, df = 1, *p* < 0.05; Symbio: *z* = 10.9, *p* < 0.01) (Figure 1d).

# 3.2 | Experiment Two

Infection with *Cl. oxysporum* reduced plant height (*F1,32* = 22.08, *p <* 0.001) (Figure 2a) and dry biomass (*F1,32* = 13.78, *p <* 0.001) (Figure 2b). While inoculation with Symbio AM fungi or *C. acutatum* had no effect on plant size, the smallest plants were produced when all three fungi were inoculated (Figure 2). No AM fungal colonisation was found in plants given sterile inoculum. However, infection with *C. acutatum* significantly increased the % RLC (*F1,16* = 13.8, *p* < 0.01) by AM fungi, while *Cl. oxysporum* had no effect on AM colonisation. The highest rate of AM fungal colonisation (8.2 ± 0.37%) was found in plants infected with both endophytes, while the lowest (5.1 ± 0.3%) was recorded in control plants without either endophyte.

*Figure 2 near here*

Recovery of both endophytes was inconsistent across treatments (Figure 3), but to an extent this was because AM fungal inoculation significantly affected recovery. AM fungi increased infection by *C. acutatum* (*F1,32* = 7.2, *p* < 0.05), and this fungus was successfully recovered from the inoculated and control leaves only when AMF was also present (Figure 3a). Meanwhile, AM fungal inoculation appeared to reduce infection by *Cl. oxysporum* (*F1,32* = 6.3, *p* < 0.05) (Figure 3b). In both cases, the endophytes were also found in uninoculated plants, showing that there was a background community of endophyte species infecting the plants also. Eight endophyte species were recorded across the AMF-treated plants while there were nine species present in AMF-free plants. *Cladosporium cladosporioides* was only found in mycorrhizal plants, while *Chaetomium cochlioides* and *Nigrospora oryzae* were only present in mycorrhizal-free plants. Other species were found across all treatments, and were *Acremonium strictum, Cl. sphaerospermum, Geniculosporium* spp*., Lecanicillium* spp.and *Peniophora* spp.

*Figure 3 near here*

# 3.3 | Experiment Three

Plants infected with rust were significantly shorter (*F1,32* = 8.81, *p* < 0.01) (Figure 4a), and had lower aboveground dry biomass (*F1,32* = 12.27, *p* < 0.01) (Figure 4b). Overall, in this study, AM fungal addition had no effect on biomass, but there was a three-way interaction between AM fungi, endophyte and rust (*F1,32* = 4.5, *p* < 0.05). This was because the rust reduced biomass whenever AM fungi were present, but not when the endophyte was inoculated without AM fungi (Figure 4b).

*Figure 4 near here*

Some AM fungal colonisation was observed in plants treated with sterile inoculum (Figure 5a) but addition of inoculum greatly increased colonisation levels (*F1,32* = 28.2, *p* < 0.001). Neither the rust nor endophyte affected AM colonisation overall, but there was a significant interaction between them (*F1,32* = 5.67, *p* < 0.05). Rust infection increased AM colonisation when the endophyte was present, but not when it was absent (Figure 5a). Recovery of the endophyte was highest in plants inoculated with this fungus (Figure 5b). However, in other treatments, *C. acutatum* was only found when the rust was also inoculated. It was encouraging that no rust infection was found in uninoculated plants, indicating no cross-contamination (Figure 5c). Rust infection was highest when both AM fungi and the endophyte were absent (Figure 5c) but the overall effect of AM fungi was weak (*z* = 3.69, df = 1, *p* = 0.09). However, inoculation with the endophyte significantly reduced sporulation by the rust (*z* = 10.82, df =1, *p* < 0.01).

*Figure 5 near here*

Nine background endophyte species were recorded across all treatments (Table S1). One species were found in rust infected plants only –*Geniculosporium* spp.Meanwhile, *Chaetomium elatum* and *Penicillium* spp.were only recorded when both the mycorrhizal fungi and rust were absent. It is clear that addition of the mycorrhizal fungi, endophyte and the rust all affected the species composition of the background endophyte community.

# 4 | DISCUSSION

In these experiments, we found that various components of the plant microbiome can significantly affect the efficacy of a weed biological control agent. AM fungi seemed to be compatible with the rust fungus, often increasing its effect on plant growth. While AM fungi tended to increase endophyte infection, mycorrhizal presence mitigated any negative effects of the endophytes on the rust. Our results also highlighted the potential importance of the naturally-occurring constituents of the microbiome, with the background endophyte community likely to be critical in affecting the outcome of any weed biological control attempt involving plant pathogens. Perhaps the most noteworthy feature of these results is that the majority of them run counter to the previously reported interactions between these fungal types, and between them and their host plant.

For the majority of plants, cultivated or otherwise, colonisation by AM fungi is regarded as beneficial (Tawaraya, 2003). However, it has long been known that the outcome of any plant-mycorrhizal interaction can either be positive or negative, dependent upon the identity of the fungi, the host plant and the prevailing environmental conditions (Gange & Ayres 1999; Johnson & Graham, 2013). In the current study, the role of AM species identity was highlighted, as inoculation with the Symbio product consistently reduced growth of *I. glandulifera* while PlantWorks inoculum had little effect. Symbio contains twice as many species as PlantWorks, with only two in common, and this higher diversity of fungi might account for the negative effects seen with this product (Lee et al*.,* 2013). However, one must not conclude from this study that Symbio has detrimental effects on a range of plants. Indeed, previous studies have used the same product and seen positive growth effects in plants across a range of native and ornamental (Eschen et al*.,* 2010; Rumble et al*.,* 2022). The PlantWorks product also has a beneficial effect on growth of crops such as strawberry *(Fragaria ananassa)* and soybean (*Glycine max)* (Boyer et al*.,* 2016; Adeyemi et al*.,* 2020).

Clearly, the identity of the plant is also important, and in this respect one might describe *I. glandulifera* as a facultatively mycorrhizal plant (Smith et al*.,* 2009). Field studies have shown a negative relation between AM colonisation and plant size (Tanner et al*.,* 2014) and the fact that inoculating plants with AM fungi reduced their size confirms the antagonism of the plant by these fungi (Ab Razak & Gange, 2021). The most likely reason is that the balance of carbon – nutrient ‘trade’ is tipped heavily in favour of the fungi (Jin et al*.,* 2017). *Impatiens glandulifera* has a remarkable growth rate in the invaded range, suggesting that it possesses an extremely efficient root system for nutrient acquisition (Tanner et al*.,* 2014; Coakely & Petti, 2021). If the plant is rarely nutrient limited then inoculating it with a consortium of mycorrhizal species could mean a significant carbon drain, resulting in smaller plants (Jin et al*.,* 2017).

Therefore, these controlled experiments suggest that AM fungi could represent a potentially useful tool in the biological control of this weed, by antagonising the target, while having null or positive effects on the native vegetation (Veiga et al*.,* 2011). As Li et al*.* (2019) suggest, such an approach requires careful consideration of the mycorrhizal species involved. However, it would appear that the Symbio product contains the ‘right’ species to enable this effect. This situation is not unique; control of the invasive fast-growing weed *Poa annua* in golf putting greens can be achieved by the addition of AM fungi (Gange et al*.,* 1999). The mechanism is similar to the one we propose for *I. glandulifera*; *P. annua* has excellent nutrient acquisition ability and a very high growth rate, meaning that AM fungi reduce its growth through carbon demand, while simultaneously increasing the growth of the desirable grass species in a putting green with which it competes (Gange, 1998). Control of the weed is thus achieved through a combination of differential nutrient gains and interspecific competition. We suggest that a similar strategy could be employed in areas invaded by *I. glandulifera*; control of the weed could be improved by direct action of AM fungi, coupled with altered interspecific competition, thereby leading to increased plant diversity (Van der Heijden et al*.,* 2008). A similar approach has been used in American prairie grasslands with good success (Koziol et al*.,* 2022). The Symbio product contains a number of native fungal species, but extensive field testing is required to ensure that these fungi can establish sustainable populations, since some commercial products have been shown to have little persistence in field soils (Berruti et al., 2017). Furthermore, it is important to ascertain whether addition of inoculum has any effects on resident mycorrhizal communities (Basiru & Hijri, 2022).

In addition to direct growth effects on plants, AM fungi can also protect their hosts against pathogen attack (Borowicz, 2001). Indeed, it has been suggested that by increasing growth and disease resistance, these fungi can enable some weeds to become invasive (Chen et al*.,* 2021). In foliar tissues, the mechanism of resistance is thought to be a chemical one; for example increased levels of phenolics in wheat leaves resulting from inoculation with AM fungi resulted in reduced incidence of the rust pathogen *Puccinia graminis* f. sp. *tritici* (El-Sharkawy et al*.,* 2018). In the present work, we found that AM fungi had little effect on the rust in one experiment and increased rust incidence in another, thereby failing to uphold our first hypothesis. Furthermore, the rust had no antagonistic effects on AM fungal colonisation, instead there was a tendency to increase the colonisation. While we currently do not have an explanation for these positive interactions, we suggest that the explanation may also lie in the chemical ecology of this plant. The principal defence compounds are phenolic acids, flavonoids and naphthoquinones (Ruckli et al*.,* 2014; Szewczyk et al*.,* 2016), all relatively large carbon-based molecules. If the AM fungi are, in effect, carbon parasites then colonisation may result in the plant becoming carbon-limited with defence production sacrificed at the expense of growth (Jin et al*.,* 2017). Lowered defence may enable the rust to infect more extensively, resulting in smallest plants when both fungal types were present.

Throughout these studies, the rust fungus had a consistent negative effect on plant growth, as one would expect from a biological control agent (Pollard et al*.,* 2022). However, in field conditions, the efficacy of the rust has been patchy, due to the existence of *I. glandulifera* biotypes with varying degrees of pathogen resistance (Pollard et al*.,* 2021). Our results suggest that AM fungi could offer a potential aid to reduce such resistance, by weakening the plant and allowing the rust fungus to exert a greater effect. We consistently found that plants were smaller when colonised by the Symbio AM fungi and the rust, rather than the rust alone. However, the generality of this result needs to be tested by examining the interactions between AM fungi and different *I. glandulifera* biotypes and also the different rust strains that have been introduced (Pollard et al*.,* 2021).

Endophyte fungi present a further barrier to the success of the rust in reducing balsam growth (Currie et al*.,* 2020). These unspecialised endophytes (described and defined in Gange et al*.,* 2019) often protect plants against diseases, by modifying the chemistry of host tissues (Busby et al*.,* 2016). There is very little known on how AM fungal colonisation affects endophyte infection in foliar tissues, with both negative and positive effects being recorded (Wearn et al*.,* 2012, Ab Razak & Gange, 2021). Here, we found that AM fungi increased infection of leaves by *C. acutatum*, upholding our second hypothesis. Furthermore, the smallest plants were produced when inoculated with both endophytic and AM fungi. *Colletotrichum acutatum* is a prominent member of the indigenous background community of endophytes in this plant (Currie et al*.,* 2020; Ab Razak & Gange, 2021) and so this might represent a further advantage of inoculating plants with the Symbio product in the field. Adding AM fungi may facilitate endophyte infection such as *C. acutatum* and the increased carbon demand by this fungal consortium reduced plant growth, upholding our third hypothesis. This was despite the fact that the rust incidence appeared to be reduced by the endophyte. Interestingly, rust infection did not have an effect on plant growth when *I. glandulifera* was inoculated with only *C. acutatum,* but it did when AM fungi were also present. We suspect that this is also a result of carbon limitation in the plant; when the AM fungi are present, the defence system elicited by the endophyte is less pronounced, enabling the effect of rust infection to be more clearly seen. Meanwhile, infection by the rust increased the presence of *C. acutatum* even when the endophyte had not been inoculated. Similar results have been seen before (De Souza et al*.,* 2017), in which a rust fungus increased endophyte growth in the weed *Euphorbia hirta*. This shows the importance of considering the background community of endophytes in studies such as this, something which is very rarely done. Indeed, consideration of the multitrophic interactions between soil and foliar fungi has been minimal in biological control research, but this is something that is likely to be a crucial factor in determining the efficacy of a fungal control agent (de la Cruz et al., 2018).

Compared with other weeds, the endophyte community of *I. glandulifera* is relatively impoverished (Currie et al*.,* 2020). The two commonest species, *C. acutatum* and *Cl. oxysporum* both seem to have antagonistic effects on plant growth, here and elsewhere (Ab Razak & Gange 2021). To date, the indigenous endophyte community of the target weeds has received very little attention when considering the success of introduced pathogenic control agents (Morin, 2020). It is likely that the composition of a plant’s microbiome determines the success of a biological control agent establishing (Currie et al*.,* 2020). Given that the constituents of the foliar microbiome are influenced by the identity of the host and a wide range of environmental factors, it is perhaps not surprising that biological control of weeds using pathogens has on occasions seen variation in success in both space and time (Schwarzlander et al*.,* 2018). We therefore recommend that consideration of the plant’s overall microbiome and how it may react to the inoculation of a control agent should be a fundamental part of future studies in weed biological control. While challenging, such an approach offers great potential in making such control programmes more effective (Orozco-Mosqueda et al*.,* 2018). Furthermore, it is now accepted that successful biological programmes require simultaneous habitat restoration, in which the root microbiome could play a fundamental role (Van Der Heijden et al*.,* 2008; De la Cruz et al., 2018).

# 5 | **CONCLUSIONS**

*Impatiens glandulifera* appears to be a facultatively mycorrhizal plant and its antagonism by certain AM fungi could be exploited in future biological control programmes. AM fungi can increase the efficacy of the rust pathogen *P. komarovii* var *glanduliferae* with plants being smallest when both the AM fungi and rust were inoculated simultaneously. Furthermore, AM fungi can enhance the infection of plants by unspecialised endophytes, which in turn can reduce plant size even if the rust is absent. These interactions are different to those previously reported with other plants, and likely stem from the carbon demand by AM fungi which compromises the plant’s defence system. Furthermore, the use of AM fungal inocula in the field could have additional benefits, by increasing plant diversity, through a combination of positive direct growth effects and interspecific competition tipped in favour of the native plants.

# AUTHOR CONTRIBUTIONS

All authors contributed to the design of the experiments, which were conducted by NAR and AFC. BCS identified all fungi. NAR and ACG conducted data analyses and wrote the paper, with substantial contributions from all authors.

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# FIGURE 1

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# FIGURE 2



# FIGURE 3



# FIGURE 4



# FIGURE 5

# FIGURE LEGENDS

**FIGURE 1** Mean of plant height (A), shoot biomass (B), mycorrhizal colonisation (C) and rust pustule incidence (D) of *Impatiens glandulifera* when inoculated with two different mycorrhizal fungal products (AM) and/or the rust fungus *Puccinia komarovii* var *glanduliferae.*‘C’ indicates sterilized inoculum control. Vertical bars represent ± one standard error.

**FIGURE 2** Mean of height (A) and shoot biomass (B) of *Impatiens glandulifera* when inoculated with Symbio mycorrhizal fungi or sterilised inoculum and the endophytes *Colletotrichum acutatum*, *Cladosporium oxysporum* or both fungi. ‘Control’ indicates no endophyte. Vertical bars represent ± one standard error.

**FIGURE 3** Infection frequency of *Impatiens glandulifera* by *Colletotrichum acutatum* (A) and *Cladosporium oxysporum* (B) when inoculated with Symbio mycorrhizal fungi or sterilised inoculum and either *C. acutatum*, *Cl. oxysporum* or both endophytes. ‘Control’ indicates no endophyte. Vertical bars represent ± one standard error.

**FIGURE 4** Mean of height (A) and shoot biomass (B) of *Impatiens glandulifera* when inoculated with the rust fungus *Puccinia komarovii* var. *glanduliferae* or not and/or Symbio mycorrhizal fungi (AMF) and/or the endophyte *Colletotrichum acutatum*. ‘Control’ indicates no AMF or endophyte. Vertical bars represent ± one standard error.

**FIGURE 5** Mean of mycorrhizal colonisation (A), infection frequency of *Colletotrichum acutatum* (B) and rust pustule incidence (C) of *Impatiens glandulifera* when inoculated with the rust fungus *Puccinia komarovii* var. *glanduliferae* or not and/or Symbio mycorrhizal fungi (AM) and/or the endophyte *Colletotrichum acutatum* (C. ac). ‘C’ indicates no AMF or endophyte. Vertical bars represent ± one standard error.