Endophytic fungi in the invasive weed *Impatiens glandulifera*: a barrier to classical biological control?

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Summary

The rust fungus, *Puccinia komarovii* var. *glanduliferae*, has been introduced into the UK for biological control of the invasive weed, *Impatiens glandulifera* (Himalayan balsam). However, establishment of the pathogen has differed across the country, which may be partly explained by variation in plant genotype. The aim of this study was to examine whether there is a further layer of phenotypic resistance, provided by indigenous foliar endophytic fungi. Culturable endophytes were isolated from a number of different balsam populations, and the commonest species were inoculated into ‘clean’ balsam plants, to test their interactions with the rust. We found that endophyte communities within balsam are low in diversity and become more dissimilar with increasing distance between populations. Three endophytes (*Colletotrichum acutatum*, *Alternaria alternata* and *Cladosporium oxysporum*) were common and appeared to be antagonistic to the rust, reducing pustule number and mitigating the effect of the pathogen on plant biomass. *I. glandulifera* thus partially conforms to the endophyte-enemy release hypothesis, in that as an introduced species, it has an impoverished endophyte complement, acquired from the local environment. However, these endophytes represent a potential barrier to effective biological control and future weed control strategies need to find strains of rust that can overcome plant genetic resistance and the overlaying phenotypic resistance, conferred by endophytes. Future classical biological control programmes of weeds must therefore take into account the fungal bodyguards that invasive species may acquire in their introduced ranges.

Keywords: endophyte, invasive species, pathogen, *Puccinia komarovii* var. *glanduliferae*, resistance, rust fungus.

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Introduction

*Impatiens glandulifera* Royle (Himalayan balsam) is an annual herb, native to the foothills of the Western Himalayas, in northern India and Pakistan. It was first introduced into the UK in 1839, since when it has become one of the most invasive plants, with a distribution that now covers most of the British Isles (Cockel & Tanner, 2012). Since 1839, it has also become established in 27 European countries, where it is widespread in 18 and invasive in at least 12 (EPPO: https://gd.eppo.int/taxon/IPAGL/distribution). It is also regarded as invasive in America, Canada, Japan, Russia and New Zealand (Cockel & Tanner, 2012).

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Although widely distributed, it is mostly restricted to wetland areas, being most common in riverine habitats and damp woodlands (Varia et al., 2016).

Numerous factors contribute to the invasion success of *I. glandulifera*. In Europe, it is the tallest growing annual herb (up to 4 m in height) and the fact that it is considerably taller than native herbaceous species enables it to be a strong competitor. Furthermore, it is tolerant of shading and has remarkably high N assimilation efficiency, enabling it to invade woodland and other areas, where nutrients might otherwise be limiting (Andrews et al., 2009). As expected from a large plant, seed production is very high (up to 2500 per plant and 30 000 per m²) and dispersal is highly efficient, up to 7 m from the parent plant, aided by a ballistic mechanism (Cockel & Tanner, 2012). *I. glandulifera* often forms continuous monocultures, in an arrested succession, wherein secretion of chemicals into soil causes allelopathic effects, reducing growth of native plant species (Tanner & Gange, 2013). Furthermore, the plant exhibits a positive plant–soil feedback (Pattison et al., 2016), showing enhanced performance in soil that has previously supported the species. This is thought to be due to its ability to manipulate soil microbial communities to its own advantage, changing the abundance of fungi and bacteria (Pattison et al., 2016).

Invasion of plant communities and the formation of balsam monocultures have detrimental effects on a range of native species. *Impatiens glandulifera* is a strong competitor, directly reducing native plant growth and diversity and also indirectly, through a reduction in mycorrhizal fungi that benefit native plants (but not *I. glandulifera*; Tanner & Gange, 2013). Presence of the plant severely reduces invertebrate diversity and abundance, both above and below ground (Tanner et al., 2013). Particular attention has been paid to pollinating insects, as the plant is extremely attractive to pollinators (contributing to its fecundity), luring these away from native plants, thereby reducing their seed production (Davis et al., 2018).

Due to these problems, much attention has been focused on controlling populations of the plant in the UK. Chemical control is problematic, due to the proximity of its growth to water courses, and so manual labour, often using volunteers is commonly used (Varia et al., 2016). This approach is time-consuming and costly, and requires continual engagement to be successful. However, as a non-native species, with few or no natural antagonists, it should be amenable to a classical biological control programme (Tanner et al., 2008).

An extensive survey of natural enemies was undertaken from 2006 to 2010 in the plant’s native range, in India and Pakistan (Varia et al., 2016). This resulted in the identification of a rust fungus (*Puccinia komarovi* var. *glanduliferae* R.A. Tanner, C.A. Ellison, L. Kiss & H.C. Evans) that is host-specific (Tanner et al., 2015a) and which was first released in the UK in summer 2014 (Tanner et al., 2015b). Although highly damaging in the native range, establishment across UK populations of the weed has been patchy (Varia et al., 2016). Molecular analysis has shown that the plant was introduced at least several times to the UK from several different regions in India and Pakistan (Nagy & Korpeälainen, 2015), and so different populations of balsam vary genetically. It is therefore highly likely that the original strain of the rust fungus, which originated from India, has limited ability to infect plants originating from other parts of the range. Indeed, trials with a strain of the rust from Pakistan have proven successful in areas where the Indian strain has been less effective (Varia et al., 2016). The relatively low, but persistent, genetic diversity means that phenotypic plasticity (and resistance to the pathogen) is likely to contribute to the spread of this plant (Hagenblad et al., 2015).

It is becoming increasingly clear that the community of microbes within a plant, collectively termed the ‘microbiome’ contributes greatly to the phenotypic resistance of a plant to invading pathogens (Busby et al., 2019). A major component of the foliar microbiome is endophytic fungi; those species that inhabit the living tissues of plants, while causing no signs of disease (Rodriguez et al., 2009). Endophytes have been recorded from virtually every plant species ever examined and comprise a diverse set of species with varying lifestyles, including saprotrophs, latent pathogens, pathogens of other hosts and entomopathogenic species (Currie et al., 2014). These fungi have dramatic effects on the chemistry of their host plants, producing and/or inducing an array of metabolites that have activity against insects and fungal pathogens (Nisa et al., 2015). Indeed, in a recent review, Busby et al. (2016) found that endophyte presence was antagonistic to plant pathogens in 71% of studies, with 25% showing no effect and only 4% showing facilitation of the pathogen. However, effects were context-dependent and seemed to differ between wild, agricultural and invasive plants, though independent sample sizes were small. In the UK, Himalayan balsam is known to harbour a community of fungal endophytes in the leaves, which appears to be similar from year to year (Pattison et al., 2016). It may also harbour bacterial endophytes, but this is currently unknown (Ab Razak, 2019). We therefore hypothesised that endophytic fungi may be a cause of the environmental resistance within balsam populations to the rust fungus, separate and
Materials and methods

Inter-site similarities in endophyte communities

A transect of length 125 km through south-west England was used to sample mature plants of *I. glandulifera* at eight different locations, on 8 August 2016. The locations were as follows: (i) campus of Royal Holloway University of London (51.43°N, −0.56°W), campus of the University of Reading (51.44°N, −0.94°W), River Loddon, Basingstoke, Hampshire (51.28°N, −1.04°W), River Itchen, Winchester, Hampshire (51.08°N, −1.27°W), River Dun, West Dean, Salisbury, Wiltshire (51.03°N, −1.63°W), River Ebbll, Broadchalke, Salisbury, Wiltshire (51.04°N, −1.94°W), River Tarrant, Tarrant Crawford, Blandford Forum, Dorset (50.84°N, −2.11°W) and River Stour, Zeals, Wiltshire (51.08°N, −2.31°W).

At each location, 10 asymptomatic plants (i.e. showing no signs of disease) were selected, each separated by at least 2 m, and five intact leaves (i.e. showing no sign of invertebrate attack) were removed sequentially up the plant, from the lower, middle and upper sections. Leaves were placed on ice and used to culture endophytes within 24 h. Three round discs, each 6 mm in 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 s, washed in sterile distilled water, immersed in 4.7% sodium hypochlorite (NaOCl) for one minute and immersed in ethanol for a further 30 s, followed by four separate washes in sterile water. The discs were placed abaxial surface downwards onto potato dextrose agar (PDA) plates, containing 80 mg L⁻¹ streptomycin sulphate and 60 mg L⁻¹ penicillin G to inhibit bacterial contamination. Similar discs were pressed onto PDA plates to examine the efficacy of the surface sterilisation method. The plates were sealed with Parafilm™ to prevent contamination and stored in the light at 20°C. To eliminate confusion through overgrowth on the plate, all fungal colonies growing on PDA plates were removed before overlapping on each other and transferred onto PCA plates to induce sporulation and to allow for identification. After 10 weeks growth on PCA, all fungal colonies were identified by B.C. Sutton, using macro and microscopic features. Non-sporulating mycelia were subject to a minimum of 2 weeks under UV light and/or changes in temperature to induce spore production.

Experiment 1: Interactions between the endophyte *Colletotrichum acutatum* and *Puccinia komarovii var. glanduliferae*

In the field study above, and in subsequent field experiments (Ab Razak, 2019), the majority of plants contained one or two endophyte species. In 67% of ‘single endophyte’ isolations, the fungus was attributed to *C. acutatum* J.H. Simmonds. As this is part of a species complex (Damm et al., 2012), material was subjected to a molecular analysis.

In brief, a proprietary formulation (microLYSIS®-PLUS (MLP); Microzone) was subjected to rapid heating and cooling in a thermal cycler, to lyse cells and release DNA, which was amplified using PCR. The quality of the PCR product was assessed by undertak- ing gel electrophoresis. A PCR purification step was carried out to remove unutilised dNTPs, primers, polymerase and other PCR mixture compounds, to obtain a highly purified DNA template for sequencing. This procedure also allowed concentration of low yield amplicons. Sequencing reactions were undertaken using a BigDye® Terminator v3.1 kit from Applied Biosystems (Life Technologies) which used 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) DyeExTM 2.0 (Qiagen). Modules containing pre-hydrated 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-DiTM (Life Technologies) to prevent rapid sample evaporation and secondary structure formation. Samples were loaded onto the AB 3130 Genetic Analyzer for sequencing, and sequences compared with those available from the European Molecular Biology Laboratory (EMBL) database via the European Bioinformatics Institute (EBI). The sequence was identified as *C. acutatum* (with 100% match) and deposited in GenBank, with accession number MH428675.

The experimental design consisted of four treatments with 10 replicates of each. Plants of *I. glandulifera* were grown in a glasshouse for 7 weeks, in 2 L pots containing John Innes number three compost (Westland Horticulture). When the plants were at the three whorl leaf stage, the leaves of 20 plants were

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inoculated with a spore suspension containing approximately $1.5 \times 10^5$ spores mL$^{-1}$ of *C. acutatum* in 0.05% Tween 80. Control plants were sprayed with 0.05% Tween 80 only. Each leaf was inoculated by spraying the spores on the abaxial surface, in two strokes (ca. 550 μL per leaf) using a handheld bottle sprayer. After spraying, the plants were placed in a dew chamber (100 x 100 x 100 cm) for 24 h to provide high humidity environment, facilitating spore germination.

One week after endophyte application, the leaves of 20 plants (10 that had received endophyte and 10 that had not) were infected with the rust fungus, *P. komarovii* var. *glanduliferae*. The inoculation method followed that of Tanner et al. (2015b) in that a 1:50 ratio of spores to talc was prepared fresh and mixed in a 9 cm diameter Petri dish. An aliquot of 0.135 μg was evenly spread on to the abaxial leaf surface with a camel hair brush, resulting in a concentration of about $1.7 \times 10^5$ spores per leaf. Leaves were sprayed with sterile distilled water to enhance infection. Control plants were dusted with talc only and plants were placed in the dew chamber for a further 24 h.

Plants were arranged in a randomised block design in a greenhouse and grown for nine weeks, with 250 mL water given daily. At this point, flower buds were forming, so harvesting occurred, as the permit for growing this invasive species from the Animal and Plant Health Agency does not allow for escape of seed into the wild. Plants were severed at ground level, and shoot biomass (fresh and dry) recorded. Rust-infected leaves from each plant in each treatment were collected and an acetate grid (of squares 1 x 1 cm) was placed on the leaves and the number of rust pustules per cm$^2$ in five randomly selected squares counted.

**Experiment 2: Interactions between the endophytes Alternaria alternata, Cladosporium oxysporum and Puccinia komarovii var. glanduliferae**

The most commonly occurring ‘two endophyte’ combination in field plants and in Ab Razak (2019) was that of *A. alternata* (FR.) Keissl. and *C. oxysporum* Berk. & M.A. Curtis, found in 54% of these plants. This experiment was designed to investigate how the presence of these two fungi, singly and in combination, affects infection by the rust.

An identical design to that above was used, with 10 replicates of eight treatments. These treatments were with and without *A. alternata* (applied at a rate of $2.375 \times 10^5$ spores mL$^{-1}$ of 0.05% Tween 80), with and without *C. oxysporum* (applied at a rate of $5.025 \times 10^5$ spores mL$^{-1}$ of 0.05% Tween 80) and with and without *P. komarovii* var. *glanduliferae* (applied at a rate of $1.7 \times 10^5$ spores per leaf). Spores were applied as described above, and plants retained within the dew chamber for 24 h after each inoculation. Harvesting took place after 9 weeks’ growth in a glasshouse, whereupon foliar biomass and rust pustule abundance were measured.

**Experiment 3: Interactions between rust and endophytes in the field**

This study was conducted on the banks of the River Mole, within the private land owned by London Gatwick Airport (51.15°N, −02.1°W). A natural population of *I. glandulifera* was used to establish $5 \times 5$ m quadrats in spring 2017. Spores of *P. komarovii* var. *glanduliferae* were applied in 500 mL of solution ($4 \times 10^4$ spores per mL in 0.05% Tween 80 with sterile water) which was sprayed evenly across the quadrat on 3 July. This procedure was repeated in the same quadrats in 2018. In August of each year, when plants were mature, 10 individual plants were chosen at random from the rust-inoculated and control quadrats, excavated and shoot biomass (fresh and dry) recorded. Endophyte fungi were cultured from each of three leaves on each plant, using the procedure described above and rust pustule abundance measured, as above.

**Data analysis**

In the inter-site study, isolation frequency of each fungal species was calculated by dividing the total number of isolations (individual colonies) of each species in a plant by the total number of all fungal isolations for that plant. Adjusted quantitative Sørensen indices of similarity between all possible site pairs were then calculated with the ‘SpadeR’ package in R 3.6.0. A linear regression was used to examine the relation between inter-site distance and inter-site similarity, using all possible pairs of sites.

For experiments 1, 2 and 3, all biomass data sets were tested for normality and plots of residuals examined. Differences in dry shoot biomass were examined with two (experiment 1) or three (experiment 2) factor analysis of variance, employing the different fungal species as main effects. In experiment 3, differences in dry shoot biomass were examined with an inverse Gaussian generalised linear model, using a log link function. Differences in pustule numbers were examined with a Poisson generalised linear model structure, using a log link function, having checked for overdispersion in R 3.6.0.

For experiment 3, we compared the frequency distributions of endophytes in rusted and non-rusted plants with a simple two sample chi-squared test. We
also examined the variability in size of plants, by comparing coefficients of variation of biomass and calculating Lorenz asymmetry coefficients using the ‘Hmisc’ and ‘ineq’ packages in R. Briefly, the asymmetry coefficient shows whether a population structure is biased by containing many small individuals or a few large ones. A full description of these statistics and tests for their comparison is given in Gange and Gadhave (2018).

**Results**

Himalayan balsam populations that were relatively close to each other harboured communities of endophytes that were more similar than did populations that were far apart (Fig. 1A, $F_{1,26} = 7.94, P < 0.01$). Indeed, the populations that were separated by over 105 km had no endophytes in common. Overall, a total of 14 endophyte species were recovered, but the majority of plants contained one or two species only (Fig. 1B).

There was a remarkable degree of uniformity across sites, with five (Reading, Winchester, West Dean, Broadchalke and Tarrant Crawford) yielding an average of 1.25 ± 0.16 endophyte species per plant, while Royal Holloway yielded 1.125 ± 0.22, Zeals 1.375 ± 0.18 and Basingstoke 1.875 ± 0.29. Overall, across all sites there were 1.32 ± 0.08 endophyte species per plant.

In experiment 1, addition of the rust to plants grown in the glasshouse resulted in a significant reduction in their dry biomass (Fig. 2A; $F_{1,36} = 15.71, P < 0.01$). Presence of *C. acutatum* also reduced biomass, but of most interest was that the fact that the effect of the rust only occurred when the endophyte was absent, leading to a significant interaction term in the analysis ($F_{1,36} = 7.71, P < 0.05$). The presence of the endophyte clearly interfered with the action of the rust, shown also by the fact that rust pustule production was almost absent in plants that were infected with the endophyte (Fig. 2B; $z = 3.76, P < 0.001$).

In experiment 2, addition of the rust to plants grown in the glasshouse produced only a weak reduction in their dry biomass in the overall analysis (Fig. 3A; $F_{1,72} = 3.56, P = 0.06$). However, this was mainly a result of the significant interactions between the rust and *A. alternata* ($F_{1,72} = 10.12, P < 0.01$) and *C. oxysporum* ($F_{1,72} = 77.43, P < 0.001$). In both cases, presence of the endophyte interfered with the efficacy of the rust. This was particularly noticeable in the treatment where both endophytes were inoculated, where addition of the rust resulted in plants that were equal in size to the controls, with no fungi added (Fig. 3A).

It is intriguing that in this experiment, the interactions between the endophytes and the rust were not
necessarily manifest in rust pustule number (Fig. 3B). Addition of *C. oxysporum* did not affect pustule production, but addition of *A. alternata* reduced this by over 60% ($z = 2.51$, $P < 0.05$). This reduction was seen irrespective of whether *C. oxysporum* was present or not (Fig. 3B).

In experiment 3, all sampled plants inoculated with rust in the field had developed pustules. However, inoculation of plants in the field with rust changed the endophyte profile of those plants in both 2017 ($\chi^2 = 100.5$, df = 4, $P < 0.001$) and 2018 ($\chi^2 = 51.67$, d.f. = 4, $P < 0.001$; Fig. 4A,B).

In both years, addition of rust resulted in more plants with higher numbers of endophytes and plants yielding zero or one endophyte species were absent in the rusted treatment. Rust infection did not reduce the size of infected plants in either year, but there was a dramatic increase in the variability of those plants in 2017 (CV test: $z = 2.38$, $P < 0.01$) and in 2018 (CV test: $z = 1.92$, $P < 0.05$; Fig. 4C,D). This was despite the fact that 2018 plants were smaller overall, due to the dry summer. In 2017, the Lorenz asymmetry coefficient of dry biomass for control plants was 1.44, while that for rust-infected plants was 0.96. In 2018, these values were 1.38 and 0.91 respectively. Both of these scenarios indicate that there was a greater number of smaller plants in the rust-treated plots, but the lack of a significant difference in overall size was caused by the fact that some plants in the rust treatments were large and apparently unaffected by the rust.

**Discussion**

It is clear that *I. glandulifera* harbours a variety of fungal endophytes within its leaves and that the presence of some species can lessen the impact and reduce sporulation by *P. komarovii* var. *glanduliferae*, upholding our original hypothesis. This is the first demonstration of asymptomatic endophytic fungi being antagonistic towards a rust fungus introduced as a classical biological control agent. We also show that endophyte communities vary from place to place and that endophyte presence in the field may explain why different results were found in glasshouse and natural conditions.

Endophyte communities within *I. glandulifera* decreased in similarity with increasing distance between plant populations. Such a negative relation between similarity and inter-site distance has been noted before in the perennial forb *Cirsium arvense* (L.) Scop. (Gange *et al*., 2007). In that study, another forb species, *Leucanthemum vulgare* Lam. showed no such relation, leading Gange *et al*., (2007) to suggest that the latter species exerted a degree of control over the endophytes that colonised it, while the former species did not, with inter-site differences arising from differences in the

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**Fig. 3** (A) Dry foliar biomass of Himalayan balsam plants, infected with the rust pathogen and/or the endophytes *Alternaria alternata* and *Cladosporium oxysporum*. Grey bars indicate addition of the rust. (B) Rust pustule abundance in plants with and without *A. alternata* and *C. oxysporum*. Bars represent means ± one standard error.

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availability of inoculum. A combination of biotic filtering by the host and dispersal-limitation over regional geographic distances has been proposed as the drivers of endophyte community composition (David et al., 2016), and it would appear that both mechanisms are true for *I. glandulifera*. One remarkable finding in the current study was the degree of uniformity in endophyte species richness across plant populations. At an average 1.3 species per plant, it was also remarkably low compared with other native British annual plants. For example, Hodgson et al. (2014) sampled six different native annual forbs with an identical isolation technique and recorded a mean of 11.5 ± 1.7 fungal species per plant. Thus, while *I. glandulifera* can be colonised by several endophyte species, fungal diversity is much lower than in native forbs, suggesting that many local fungi have difficulty in colonising this alien plant. A similar situation has been reported with the invasive *Centaurea stoebe* L. in North America, where endophyte communities in the native range are much richer than those in the invasive range (Shipunov et al., 2008).

The loss of endophyte diversity by a plant in its invaded range has been formalised as the endophyte-enemy release hypothesis (E-ERH; Evans, 2008), which proposes that some invasive plants are introduced with their co-evolved endophytes, which afford them protection against any local antagonists, and without their natural enemies also, have a distinct competitive advantage over native species (e.g. Aschehoug et al., 2012). Alternatively, some introduced plants may lose some or all of their co-evolved endophytes, which would have afforded them protection against antagonists (insects and pathogens) in their native range. The E-ERH predicts that there is a cost to this natural protection, manifest as lower growth and fecundity in the native range. Thus, when released from co-evolved endophytes, plants grow with increased vigour, but reduced defences. These endophyte-deficient plants might then be highly susceptible to an introduced (co-evolved) biological control agent, explaining some of the remarkable successes that have been seen in classical biological control programmes of invasive non-native plants (Evans, 2008).

*Impatiens glandulifera* would appear to partially conform to the latter part of the hypothesis. It is thought that the species was introduced to Europe by seed (Tanner, 2011) but in an extensive study, Ab Razak (2019) failed to isolate any endophytes from over 200 seeds. This shows that vertical transmission
of endophytes does not occur and that the fungi must be acquired from the local environment. Furthermore, both Pattison et al. (2016) and Ab Razak (2019) found that the endophyte community within plants was similar from year to year in any one locality. Such environmentally determined endophyte colonisation is therefore a strong determinant of the inter-site differences recorded (David et al., 2016).

Despite the fact that *I. glandulifera* contains a sparse endophyte community, the fungi within it appeared to have strong interactions with the introduced rust pathogen, *P. komarovi* var. *glanduliferae*. The presence of three endophyte species, *Colletotrichum acutatum*, *A. alternata* and *Cladosporium oxysporum*, singly or in combination, reduced the detrimental effect of the rust on plant biomass, in glasshouse conditions. This may not fit the endophyte-impooverished part of the E-ERH, but it could partially explain why establishment of the rust has been patchy across different field sites, if particular endophytes or combinations of fungi occurred in those sites (Varia et al., 2016). It would be particularly instructive to examine the endophytic fungal community of *I. glandulifera* in its native range and its interactions with the rust.

Antagonistic effects of endophytes on plant pathogens have been noted before in a variety of host plants (Busby et al., 2016), though effects can range from negative to positive, depending on the identity of the plant and the endophyte species (Kurose et al., 2012). Ghorbanpour et al. (2018) describe the various possible mechanisms, which include competition between the fungi for space and nutrients, antagonism and induced systemic resistance. Competition for space is unlikely to occur in leaf tissue, as extensive growth of the endophytes does not occur (Yan et al., 2015). The two latter chemical explanations are more likely, as many endophytes produce and induce an array of antimicrobial compounds within plants (Nisa et al., 2015). Chemical changes induced by endophytes are known to be systemic and to have detrimental effects on some insect herbivores, leading to their recent description as ‘plant bodyguards’ (Gange et al., 2019). There is no reason why this definition should not be extended to include protection against plant pathogens also (Busby et al., 2016).

A further explanation for pathogen antagonism, discounted by Ghorbanpour et al. (2018) is myco-parasitism. Many species of *Cladosporium* are hyper-parasites of rust fungi and Anderson et al. (2016) suggested that biological control of moth plant in New Zealand (*Araujia hortorum* E. Fourn.) could be compromised by hyperparasitism of the rust control agent (*Puccinia arauciae* Lév) by *C. uredinicola* Spel. Furthermore, Zheng et al. (2017) found that a strain of *A. alternata* could hyperparasitise *Puccinia striiformis* Westend f. *sp. tritici* Eriks, the causal agent of wheat stripe rust. Meanwhile, species of *Colletotrichum* appear not to exist as hyperparasites; instead, some species are necrotrophic plant pathogens, while others are hemibiotrophic (having a distinct biotrophic phase where no symptoms are visible; De Silva et al., 2017). Thus, while the actual mechanisms of the endophyte-rust interactions are unknown at present, it is not surprising that they occur. These interactions are likely to be widespread across the invasive range and to have important implications in the biological control of this weed. Najberek et al. (2018) isolated several fungi from seeds in Italy and Switzerland (though their seed sterilisation process was very brief, at 5 s in 1% sodium hypochlorite) and found that occurrence of both *A. alternata* and *Cladosporium cladosporioides* (Fre.) G.A. de Vries was negatively correlated with various pathogens in the genus *Fusarium*. Thus, it is clear that a better understanding of endophyte occurrence in the field is required to enable rust establishment to be more predictable and successful.

It has proven difficult to measure the impact of the rust on Himalayan balsam at the population level in the field, since there is so much natural variation between plants within populations. The field results reported here indicate that the rust did not significantly reduce plant size, while a reduction was seen in both glasshouse experiments. However, the replication was low in the field experiments, and impact on seed set was not measured in either case. Nevertheless, we suggest that this discrepancy may be due to enhanced presence of endophytes in the field, compared with the more controlled environment. When *I. glandulifera* was grown simultaneously in a glasshouse and nearby field site, a total of two endophyte species were recorded in the glasshouse plants, but six in field plants (Ab Razak, 2019). However, in experiment 3, infection by rust in the field significantly increased the size inequality of plants, in which a few individuals appeared to be immune to infection and grew large. All of these individuals yielded *A. alternata* but it was also noticeable that rust-treated plants tended to contain more endophyte species, suggesting that addition of the pathogen may have stimulated growth of latent endophytes, making them more likely to be cultured (de Souza et al., 2017). By their very nature, tests of potential weed biological control agents must be performed in strictly controlled conditions, but the fact that these are likely to use plants with different endophyte communities to those in the field may explain why some pathogen introductions fail (Schwarzländer et al., 2018).
A final and most important feature of UK field populations of *I. glandulifera* is that they are known to differ genetically (Nagy & Korpelainen, 2015) and that populations with different origins are differentially susceptible to strains of the rust from India and Pakistan (Varia et al., 2016). This is likely to be the primary reason for variation in rust efficacy across sites. However, genetic differences in plant populations could account for the inter-site differences in endophyte communities and an important next step is to understand whether the different biotypes of *I. glandulifera* harbour different endophytes. It is also critical to understand whether rust strains of different origins have different interactions with the endophytes. Taken together, elucidation of these cryptic, but critical interactions, may better explain why rust impact on the host can be variable within and between weed populations. While challenging, this may enable the barriers to biological control of this invasive weed to be overcome.

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**Conflict of interest**

The authors declare no conflict of interest.

**Author contributions**

All authors contributed to the design of these experiments, which were executed by ACG, N AR, AFC and SVW. ACG conducted data analyses and wrote the paper, with substantial contributions from all authors.

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