A semi-natural evaluation of the potential of the rust fungus *Puccinia komarovii* var. *glanduliferae* as a biocontrol agent of *Impatiens glandulifera*

K.M. Pollardab\*, A.C. Gangeb, M.K. Seiera, C.A. Ellisona†

*a CABI Europe-UK, Bakeham Lane, Egham, Surrey TW20 9TY, UK*

*b Department of Biological Sciences, Royal Holloway, University of London, Egham, Surrey, TW20 0EX, UK*

†Deceased 19 April 2020

\*Corresponding author: email: Telephone: +44 (0) 1491 829080; Email: [k.pollard@cabi.org](mailto:k.pollard@cabi.org)

ORCID ID

Kathryn M. Pollard 0000-0002-0913-6743

Alan C. Gange 0000-0002-9918-1934

Marion K. Seier 0000-0002-6842-0808

Carol A. Ellison 0000-0002-8840-1639

ABSTRACT

Himalayan balsam (*Impatiens glandulifera*) is one of the most prolific non-native species in Europe. Since 2014, the highly-specific rust fungus, *Puccinia komarovii* var. *glanduliferae,* has been released into Great Britain as a classical biological control agent for this invasive weed. Prior to its release, research focused on ensuring the safety of the pathogen; elucidating its life cycle and verifying its host-specificity. However, limited studies were conducted to determine the likely impact of the rust on its host. Due to difficulties in assessing field populations, unforeseen complexities in the plant-pathogen relationship and the requirement for long-term monitoring, inoculation experiments were conducted to evaluate the effect of the rust on both seedlings and mature plants of Himalayan balsam under semi-natural conditions. The impact of the rust was determined through measuring plant-growth parameters in addition to assessing reproductive output. The rust significantly increased seedling mortality, with up to 80% of seedlings dying as a result of rust infection, or indirectly through the colonisation of secondary pathogens. Mature plants infected with the rust produced fewer leaves with a decrease in total plant biomass. Reproductive output was negatively affected through a reduction in both flower and seed production, but with no observed effects on seed viability. This study demonstrates the high potential of the rust for controlling fully susceptible field populations of Himalayan balsam.

*Key words:*

Biological control, fungus, Himalayan balsam, invasive species, rust pathogen

**1. Introduction**

*Impatiens glandulifera* Royle (Balsaminaceae), more commonly known as Himalayan balsam (Hb), is an annual plant introduced as a garden ornamental into Great Britain (GB) and much of Europe from its Himalayan native range in 1839, due to its eye-catching and attractive flowers (Coombe, 1959). Since then, the species has naturalised and spread rapidly along watercourses and, more recently, has started to colonise disturbed sites and woodland habitats (Andrews et al., 2009; Seeney et al., 2019). Hb is now regarded as one of the most widespread non-native invasive plant species in Europe (Tanner and Gange, 2020) and its negative impact on native biodiversity (invertebrates, plants and microbial communities), and even whole ecosystems, has been widely documented (Coakley and Petti, 2021). Small, isolated patches of Hb can be readily controlled using mechanical and chemical means. However, given the plant’s vast distribution along watercourses and the large number of seeds produced, which are frequently transported downstream to non-invaded areas, these techniques are not just labour intensive and environmentally damaging but also impractical on such large scales. Thus, Hb is an ideal candidate for classical biological control (CBC).

Weed CBC – the introduction of natural enemies from the native range of a plant species for the control of this species in its introduced invasive range – is a technique that has been practised for well over a century and the development of regulatory protocols and international standards has transformed the way it is performed today (Morin, 2020). Safety is the main focus of such programmes, ensuring that any imported biocontrol agents do not cause any unpredicted damage to non-target species, and the global safety record of weed CBC, particularly when utilising fungal natural enemies has been excellent (Barton, 2004, 2012; Morin, 2020). Nevertheless, in Europe, the implementation of weed CBC has lagged behind that of the rest of the world and, as a result, has a more conservative approach (Shaw et al., 2018; Sundh and Eilenberg, 2020). The CBC programme against Hb in GB commenced in 2006 with native range surveys in the foothills of the Himalayas, across western India and Pakistan. Although numerous arthropod and fungal natural enemies were found to be associated with Hb, based on their apparent field host-range and limited host-range testing under greenhouse conditions, the majority were found not to be sufficiently specific and was thus ruled out as potential biocontrol agents (Tanner, 2011; Varia et al., 2016). The rust fungus, *Puccinia komarovii* var. *glanduliferae* Tanner, C.A. Ellison, L. Kiss & H.C. Evans (Puccinales) (Pkg) causing high levels of damage to Hb plants in the field, however, was prioritised for further testing (Tanner et al., 2015). Rust pathogens are the most frequently utilised group of fungal biocontrol agents, due to their high levels of specificity, the detrimental effect they can have on their hosts and their ability to readily disperse over long distances with the wind (Hanley and Groves, 2002; Morin et al., 2002; Tomley and Evans, 2004; Evans et al., 2011; Giordano and Anderson, 2021). Comprehensive host-range testing of Pkg, conducted under quarantine conditions, found the rust to be highly host specific; only one non-target, *Impatiens balsamina* L., a minor ornamental species, was fully susceptible to Pkg, successfully supporting sporulation of the rust (Tanner et al., 2015). Based on a pest-risk assessment, a strain of the rust from India was approved for release in GB in 2014; subsequent approval was given for a second strain of Pkg from Pakistan, which was released in 2017 to tackle Hb stands not susceptible to the Indian strain (Shaw et al., 2018; Ellison et al., 2020).

Studies to determine the impact of Pkg on individual Hb plants or populations were not formally conducted prior to release. Unless undertaken experimentally in the native range, such evaluations are inherently difficult to conduct when restricted to working with an annual plant species under quarantine conditions. However, the potential impact of the rust can be predicted when taking into consideration the pathogen’s life cycle (macrocyclic and autoecious) and evidence from field observations in the native Himalayan range.The pathogen causes damage to its host, during two main life-cycle stages; as stem infection on seedlings in the spring, and as leaf infection on mature plants during the summer. Evidence of the likely impact the rust on Hb can also be drawn from a comparable pathosystem in mainland Europe where another distinct pathotype of the rust, *P. komarovii* ex *Impatiens parviflora* DC*,* is present; this pathotype exclusively infects *I. parviflora,* a small herbaceous plant, native to the mountains of central Asia. Studies of this pathosystem in Poland and Slovakia documented that the rust plays a significant role in regulating the population density of *I. parviflora* by not only increasing seedling mortality but also by decreasing flower, leaf and seed production (Eliáš, 1995; Piskorz and Klimko, 2006).

For Hb, initial field releases of Pkg have not been without constraints; a suboptimal release strategy, the presence of rust resistant biotypes and the complex nature of plant-pathogen compatibility are all considered to be contributing factors which have prevented high levels of disease on Hb and the persistence of the rust at many release sites (Pollard et al., 2019; 2021; Ellison et al., 2020). However, pre-release susceptibility testing of Hb populations to be targeted is now routinely conducted and has greatly improved infection with Pkg at selected field sites (Pollard et al., 2021). However, in order to accurately measure the impact of a biocontrol agent on the target weed in the field, long-term studies are required. Nonetheless, due to the costs and time associated with monitoring, such assessments are not always conducted. Therefore, in order to ascertain the impact of Pkg on Hb, a limited number of post-release semi-natural and greenhouse impact studies have been undertaken. A study by Currie et al. (2020) found that under greenhouse conditions, infection by the rust resulted in a significant decrease in plant biomass, but this change was not replicated under field conditions. The authors however, acknowledge the high variability of the plant in the field and the low number of replicates as possible reasons for why a change in biomass could not be detected (Currie et al., 2020). In order to prevent the growth and spread of this plant species in the wild, as regulated by specific UK legislation (Defra, 2021), this study did not measure the impact of the rust on seed set. Nevertheless, seed production is an important factor which will determine the effectiveness of the CBC programme against this annual species, as one individual Hb plant can produce between 500-5000 seeds (Love et al., 2013). More recently, a study by Pollard et al. (2021) assessing disease symptoms on Hb seedlings following basidiospore infection under semi-natural conditions, recorded disease incidence levels of up to 80% of seedlings in rust treatments. Nevertheless, progress of infection over time and seedling mortality was not accessed in this study.

The paper presented here reports on the results of a semi-natural field study to determine the potential impact that Pkg may have on both seedlings and mature plants of Hb and, consequently, its potential as a CBC agent.

**2. Materials and methods**

*2.1 Plant material*

Seeds of *I. glandulifera* were sourced from field collections at Harmondsworth Moor, Middlesex, UK (GPS: 51.49300, -0.48321) in 2017 and 2018. After collection, seeds were air dried at room temperature and stored in a refrigerator at 4 ºC for a minimum of four months before use. Following this, two procedures were used to establish Hb plants from stored seeds. Seeds were either: i) directly sown into pots for the basidiospore inoculation experiments as described under *2.3.1*; or ii) pre-germinated in moist Petri dishes at 4 ºC for two months (Tanner et al., 2015) ­for use in the urediniospore inoculation experiments, as described under *2.3.2.* Once germinated, the seeds for the urediniospore inoculation experiments were potted approximately 1 cm below the surface into seed trays containing 200 g multipurpose compost (Longacres, Bagshot, UK). Trays were maintained in a greenhouse chamber with a minimum temperature of 15 ºC for eight weeks. Lighting in the chamber was supplemented by high intensity discharge (HID) lamps, using a mix of high-pressure sodium and metal halide bulbs with a 12:12 (day/night cycle). Having reached the second to third whorl stage, plants were potted into 15 cm diameter, 1.5 L pots containing multipurpose compost, watered and placed outside in a protected area to prevent damage from wind. After a further two weeks, plants had developed four whorls of leaves and were at a suitable stage for inoculation.

*2.2 Pathogen material*

Two strains of Pkgwere used in this study; one collected from India (Rohtang Pass, Kullu Valley, Himachal Pradesh, IMI 398718) and one from Pakistan, (Lalazar, Kaghan Valley, Khyber Pakhtunkhwa Province, IMI 505791). For both rust strains, urediniospores used in the impact assessments were produced under controlled conditions on plants of Hbfrom Harmondsworth Moor as outlined in Pollard et al. (2021). For this, the abaxial leaf surface of Hb plants was inoculated using a urediniospore-talc mix (1:50 v/v), and sprayed lightly with sterile distilled water (SDW) before placing in a dew chamber at 15 ℃ for 24 hours. Inoculated plants were kept in a designated greenhouse chamber and urediniospores were regularly harvested into a Petri dish 2-4 weeks after inoculation and stored in a freezer at -20 ℃ until required. As the viability of urediniospores of Pkg can decrease over time, to ensure consistency, urediniospores harvested no more than one month prior to experimental use were used for all experiments (Pollard et al., 2021). After a further two weeks, as inoculated plants mature and infection progresses, teliospores develop. Infected leaves bearing telia were removed from inoculated plants, pressed and dried between newspaper at 22 ℃ for one week, before storing in a freezer until required. Infected leaf material produced during the previous six months was used for the study; no difference in teliospore germination has been observed for leaf pieces produced up to two years previously (Pollard, unpublished).

*2.3 Experimental work*

In order to determine the impact of Pkg on Hb, two separate inoculation experiments were set up under semi-natural conditions, each using one of the two main infecting spore stages: the urediniospores (the repeating, asexual spore stage) and the basidiospores (the haploid, gamete stage).

*2.3.1 Impact of* Puccinia komarovii *var.* glanduliferae *infection on seedlings of* Impatiens glandulifera

To investigate the impact of the rust on seedlings of Hb during spring, an experiment was conducted under semi-natural conditions in the CABI grounds, Egham, UK. The experiment was set up in January 2018, using the methodology from Pollard et al. (2021) adapted as follows; in order to mimic natural seedling densities in the field in GB, 25 Hb seeds were evenly distributed across 10 L pots, containing multipurpose compost. Pressed and dried leaves bearing telia of each respective rust strain were cut into approximately 1 cm2 pieces and 2 g of these fragments were placed into individual Petri dishes. Negative control pots were treated with healthy, pressed and dried Hb leaf pieces prepared in the same way. To simulate how developing seedlings are exposed to the rust in the field, 2 g of either telia-bearing leaf pieces or healthy, dried leaf pieces were scattered over the surface of each individual pot on top of the seeds and 300 g of sieved multipurpose compost was sprinkled evenly over the surface of each pot. Each treatment i.e. with the rust strain ex India, ex Pakistan and the healthy leaf pieces, was repeated six times to give a total of 18 individual pots. Each pot was watered lightly before being placed inside a fine mesh sleeve supported by a 50 cm bamboo cane with a metal hoop at the top. This served to prevent seedlings from touching the side of the mesh, provide protection from frost and enabled sufficient air flow without mixing leaf pieces and thus, rust strains. Pots were randomly positioned 0.5 m apart in separate blocks for each rust treatment, in an open grassy area. The control plants were positioned between each rust treatment to determine whether cross-infection between the two rust strains had occurred and to monitor for the potential dispersal of the rust. On average, temperatures in the experimental plot ranged from 3.8 ℃ to 9.7 ℃ in January, increasing to 9.8 ℃ to 20.8 ℃ in May, with an average of 58 mm of rainfall per month. Thirteen weeks after the initial set up, when the seedlings had reached approximately 5 cm in height and the risk of frost had passed, the mesh sleeves were removed from individual pots and the total number of germinating seedlings was counted. A wire cage was erected around the experimental plot to prevent feeding damage from deer and plants were watered daily. To measure the impact of the rust on the seedlings over time, plant parameters were measured three times; two, five and eight weeks after the first symptoms of infection were observed. During each assessment, the total number of seedlings per pot and the number which had died back were counted. The height of each individual seedling, measured from the top of the soil to the tip of the highest leaf and the diameter of the stem at the first node were also recorded. The level of disease severity caused by the rust was categorised for each individual seedling, into one of four groups (Pollard et al., 2021);

0: No infection;

1: Low (area of infection covering <10% of stem surface, absence of, or very few aecial cups);

2: Medium (area of infection covering 10-50% of stem surface, aecial cups present);

3: High (area of infection covering 50-100% stem surface, aecial cups prevalent).

*2.3.2 Impact of* Puccinia komarovii *var*. glanduliferae *infection on mature plants of* Impatiens glandulifera

Inoculation experiments were set up to measure the impact of the rust on individual Hb plants, in the absence of any competing vegetation. The experiment was established in an open exposed plot in the CABI grounds at the end of May 2019. The experiment comprised two rust treatments (inoculation with the Indian and Pakistani rust strains respectively) and a negative control, where no rust was applied. The treatments were positioned 20 m apart from each other with the negative control treatment in between at equal distance to each of the treatments, to monitor for any potential cross-infection of rust strains. Each treatment consisted of 12 Hb plants, planted across six Tomorite growbags (Evergreen Garden Care Ltd, Surrey, UK) with two plants per bag. Each grow bag was positioned 0.5 m apart to form a rectangle on top of weed matting to prevent competition from other vegetation and allow for natural water drainage. To provide extra support, a 1.5 m bamboo cane was staked into the ground through the growbags and plants were loosely tied to the cane. A wire cage with shade netting was erected around each individual subplot to prevent feeding from deer and damage caused by wind at the exposed site. A sprinkler system was set up in the centre of each cage and plants were watered for 15 minutes twice a day. On average, temperatures ranged from 8.4 ℃ to 18.6 ℃ in May, increasing to 11.8 ℃ to 21.2 ℃ in September, with an average of 46 mm of rainfall per month.

Hb plants were inoculated three times during the growing season (June, July and August) with a period of five weeks between each inoculation. Plants were inoculated in the evening, when daytime temperatures had dropped to a maximum of 17˚C and, where possible, overnight rain had been forecasted. Inoculations were performed separately with urediniospores of each of the two selected rust strains. Urediniospores were suspended in 0.05% Tween-80 (Sigma-Aldrich) in SDW and adjusted to approximately 5 x 105 spores ml-1 using a haemocytometer. Using a small, hand held spray bottle, a total of 5 ml of the spore suspension was sprayed onto the abaxial leaf surface of all leaves on each of the plants. The negative control consisted of plants to which 5 ml of 0.05% Tween-80 in SDW was applied. Plants were monitored closely for development of disease symptoms. Four weeks after each inoculation date, a number of plant growth parameters were recorded. These included: plant height (measured from the top of the soil to the tip of the tallest leaf); the diameter of the stem at the first node; the total number of leaves, whorls and side shoots produced per plant. As flowers frequently fall from plants during the growing season, the total number of flower stalks was counted as a proxy. During each assessment the uredinial incidence was calculated by placing an acetate grid with 1 cm2 squares over the lower leaf surface of five randomly selected leaves per plant and counting the total number of uredinia in three randomly selected squares per leaf. Randomisation was achieved by using a random number generator to provide the leaf number (starting from the first leaves at the bottom) and square to be assessed. The incidence of uredinia was then calculated by averaging the number of uredinia produced for the five leaves assessed across the total experimental period of 15 weeks. For each individual plant the uredinial incidence per leaf was defined as below:

0 = No infection on any leaves

1 = Low – uredinia sparse, an average of ≤ 5 uredinia per cm2

2 = Medium – an average of 5-8 uredinia per cm2

3 = High – uredinia abundant, ≥ 8 uredinia per cm2

During the experimental period, fine mesh bags 0.25 x 0.25 cm were tied over any seed pods to capture and prevent the spread of seed. Individual bags containing seeds were removed from plants and the total number of seeds counted. At the end of the experiment, following the assessment of plant vegetative parameters and the rust infection levels, all mesh bags were removed and individual seed pods pressed lightly to collect any additional seeds. The total number of seeds produced per plant was counted. The average weight of one individual seed per plant was calculated by allowing seeds to air dry at room temperature for 14 days before determining the total combined weight of all seeds for each plant and dividing this by the number of individual seeds counted. Individual plants were cut back at the soil level and, due to the large volume of plant material, placed onto trays to pre-dry in an air-conditioned room at 21 ℃ for two weeks. Plants were then placed into large paper envelopes and dried in a drying oven at 70 ℃ until the weight was stable. The dry weight of each individual plants was then measured.

In order to assess seed viability, seeds were stored in a 4 ℃ incubator in the dark for four months before being placed onto dampened filter paper in a Petri dish as described in Tanner et al. (2015). Due to the large number of seeds produced per plant, a subset of 150 seeds per treatment was randomly selected and distributed across six replicate Petri dishes, each containing 25 seeds. Petri dishes were randomly positioned onto trays and returned to the incubator where they were regularly monitored for seed germination. The filter paper in the plates was rehydrated with SDW as required and, to account for any temperature differences within the incubator, the position of each tray and the petri dishes it contained were altered every week. The total number of germinating seeds was assessed after 12 weeks.

*2.4 Statistical Analysis*

Statistical analyses were performed using R Studio version 3.6.1 (R Core Team, 2019) and the packages lme4 (Bates et al., 2015), MASS (Venables and Ripley, 2002), plyr (Wickham, 2011) and Multcomp (Hothorn et al., 2008). Generalised linear models (GLMs) and generalised linear mixed models (GLMMS) with the appropriate families (Gaussian, Poisson, Binomial or negative binomial) were used for all analyses. For models with a Gaussian error structure, assumptions for normality and homogenous residuals were confirmed using qq-plots and the residuals plotted against fitted values. For count data, a Poisson distribution structure was used and data were tested and corrected for overdispersion by adding in an observation level effect or using a negative binomial structure where necessary. *P-*values were obtained using likelihood ratio tests (*χ2*), comparing full models with reduced ones, omitting predictor variables one at a time. Treatment and time, and the interaction between the two, were included in all models as independent variables. The significance of treatment and time individually were only tested if the interaction term was found to be non-significant. However, if significant a subset of the data was analysed, using results of each individual time period separately. Plant susceptibility and level of infection were also included as independent variables in the analysis of mature plants and seedlings, respectively. Individual plant and pot were also included as random effects in the impact assessment of mature plants and seedlings, respectively. Post-hoc analysis of any significant variables was performed pair-wise, using the Tukey test with Benjamini-Hochberg correction.

**3. Results**

*3.1 Impact* of Puccinia komarovii *var.* glanduliferae *infection on seedlings of* Impatiens glandulifera

*3.1.1 Disease development*

The first symptoms of seedling infection were observed 14 weeks after setting up the experiment, at the end of April, as red lesions developing on stems of infected seedlings (Fig. 1A). Aecial cups were first recorded 14 days later, and, after a further 10 days, aecia were producing a mass of orange aeciospores (Fig. 1B). Over time the stems of heavily infected plants became etiolated and warped, often losing their turgidity (Fig. 1C). These heavily infected stems frequently became colonised by a number of unidentified secondary pathogens and were more prone to physical damage (Fig. 1D). Localised infection on the stems and cotyledons of seedlings, where lesions were restricted in size, were also observed. Plants in the control trays, where non-infected Hb leaf material had been added, remained healthy and free from infection.

Chlorotic spots resulting from infection by aeciospores were first visible on the upper leaf surface of the lowest leaves of plants in rust treatments at the beginning of June. This occurred approximately seven weeks after the first signs of fungal infection on the stems (Fig. 1E). On the lower leaf surface, uredinia developed within these chlorotic lesions seven days later. Initial uredinial infection remained localised, visible on plants in close proximity to each other. However, after a further two weeks a few chlorotic spots indicative of uredinial infection appeared on plants within the control pots, indicating that the rust had spread.

*3.1.2 Seedling mortality*

Treatment with the rust resulted in a significantly greater proportion of seedlings dying during the experiment than naturally occurred in the control pots, where seedlings were not exposed to the rust (*χ²* = 177.20, df = 2, *P* < 0.001; control vs Indian or Pakistani strain *P*< 0.001). Mortality in individual rust treated pots ranged from 33-80% compared with 0.67% for the control (Fig. 2A). The total mortality of Hb seedlings was greatest in pots containing the Indian rust strain with an average of 65.31% ± 3.37 of seedlings dying, compared to an average of 48.64% ± 5.42 of seedling mortality in the Pakistani rust treatment (Indian strain v Pakistani strain, *P* < 0.01)*.* Although it was not possible to monitor the survival of individual seedlings over time, from observations seedlings more heavily infected with the rust, in particular those with ≥50% stem infection, died during the first few weeks of the experiment. Plants with the lowest levels of infection (<10%) were more likely to survive, as lesions remained localised, and seedlings outgrew infection: the average level of stem infection across the two rust treatments at the end of the experiment was 8%. In addition, at this time point, fewer than 25% of the surviving seedlings in rust treatments were infected by the rust.

*3.1.3 Seedling height*

During the experimental period, the height of Hb seedlings within pots containing either of the two rust treatments was found to be significantly taller than those without exposure to the rust in the control treatment (*χ²* = 7.82, df = 2, *P* = 0.02, Fig. 2B); by week 8, plants in the India and Pakistan rust treatments measured an average of 123.39 ± 5.44 cm and 105.81 ± 3.11 cm, respectively, compared to an average of 92.19 ± 2.48 cm for the control. The height difference at this point was only significant between plants in pots containing the Indian strain of the rust and the controls where no rust was applied (*P*= 0.01). However, at this time point, the majority of rust infected plants in both treatments had died (as outlined in section *3.1.2 Seedling mortality*), and those that remained had low, infection category 1, or no infection. In addition, the level of stem infection was found to have a significant impact on overall plant height, with the average height of a seedling decreasing with increasing levels of infection (*χ²* = 43.40, df = 3, *P* < 0.001, Fig, 2C).

*3.1.4 Stem diameter*

The interaction between treatment and monitoring time was found to have a significant effect on stem diameter (*χ²* = 9.52, df = 2, *P* < 0.01). Therefore, in order to determine the effect of treatment with the rust, a subset of the data was created for each time period. There was no difference in the diameter of the stem, measured at the first node, between rust and non-rust treatments during the first two monitoring periods. However, a marked difference in stem diameter of each individual plant was observed by week 8 (*χ²* = 11.75, df = 2, *P* < 0.01). At this time point, the average diameter of an individual plant in pots containing the rust fungus was significantly larger than those in the control pots, but similar in size between the two rust treatments (control v Indian and Pakistani strains, *P* < 0.01; Fig. 2D). At this time point, the remaining plants were either free from infection or had very low levels of infection (category 1). The level of infection on the stem was also found to significantly affect stem diameter, with more heavily infected plants, those in infection category 3, having a thinner stem at the first node than those not infected by the rust (*χ²* =136.04, df = 3, *P* < 0.001; Fig. 2E).

*3.2 Impact of* Puccinia komarovii *var.* glanduliferae *infection on mature plants of* Impatiens glandulifera

*3.2. 1 Disease development*

Throughout the experiment, chlorotic spots were consistently observed on the leaves of mature plants 10 days after inoculation with the rust. After a further five days, uredinia producing light powdery brown urediniospores were seen developing on the lower leaf surface. During the course of the experiment the initial inoculated leaves, in particular those on the first whorl, turned yellow and abscised from the plant. At this point, infection on these leaves had progressed to the development of telia bearing teliospores, the over-wintering spore stage. Natural spread of the rust, through urediniospore infection, was observed on newly developed leaves of the side shoots, not previously inoculated. However, infection remained localised and plants within the control treatment remained healthy and free from infection by the rust. During the course of the experiment three plants inoculated with the Pakistani rust strain and three from the control treatment, where no rust was applied, died. The mortality of these plants was not related to the rust, rather strong winds causing the hollow stems to snap. These samples were removed from the analysis.

*3.2.2 Plant growth parameters*

Treatment with the rust had no effect on plant height (Fig. 3A), stem diameter or the total number of whorls or side shoots each plant produced. However, plants infected by the rust fungus produced significantly fewer leaves than those in the control treatment and a difference between the two rust strains applied was also observed. This difference was first noticed at week 10, when plants treated with the Pakistani strain of the rust bore significantly fewer leaves than those treated with the Indian strain or those not having received rust treatment (*χ²*=10.00, df = 2, *P* < 0.01; Pakistan v control, *P* < 0.01; Pakistan v Indian, *P <* 0.05). By week 15, the difference was much more noticeable (Fig. 3B), with control plants producing nearly twice as many leaves as those treated with the Indian rust strain and nearly four times as many as those treated with the Pakistani rust strain (*χ²*=25.70, df = 2, *P* < 0.001; Indian v control, *P <* 0.01; Pakistani v control and Pakistani v Indian, *P* < 0.001). Treatment with the rust also resulted in a decrease in plant biomass, measured as dry weight of individual plants (*χ²* = 2.49, df = 1, *P*< 0.001). The average weight of an individual plant was 193.35 g ± 27.18 for the control, 144.24 g ± 12.91 for the Indian strain and 81.83 g ± 13.76 for the Pakistani strain treatment (Fig. 3C). However, only those treated with the strain of the rust from Pakistan were significantly different from the other treatments (control v Pakistan strain, *P* < 0.001*,* Pakistan strain v Indian strain, *P* < 0.01).

*3.2.3 Reproductive output*

The total number of flower stalks, measured as a proxy for the total number of flowers, remained similar throughout the experiment between all treatments until week 15. At this time fewer flower stalks were produced on plants treated with the Pakistani strain of rust than those treated with the Indian strain or with no rust at all (Fig. 3D) (*χ²* =17.64, df = 2, *P* < 0.001; Pakistani v control and Pakistani v Indian, *P* < 0.001). Critically, reproductive output was reduced by the rust *(χ²* = 12.06, df = 2, *P* < 0.01), with an average of 1,989.22 ± 296.56 seeds produced per plant for the non-rust, control treatment, 1,635 ± 222.18 seeds for treatment with the Indian rust strain and 872.33 ± 139.06 seeds for the Pakistani strain (Fig. 3E). The difference, however, was only significant between the Pakistani strain and the control, non-rust treatment and between the Pakistani strain and the Indian rust strain. No impact of the rust on the average weight of an individual seed, or on seed viability, assessed as germination on most filter paper in a Petri dish, was observed.

**4. Discussion**

The safety of weed CBC using fungal agents has been widely demonstrated with no unexpected impacts on non-target species recorded to date (Barton, 2012; Morin, 2020), and the same has been confirmed for Pkg, with infection only being recorded on Hb since its release in GB. However, not all releases have resulted in the expected level of weed control and many agents, arthropods as well as fungal pathogens, have failed to establish or to significantly reduce the population levels of the target plant (Morin et al., 2006). Coupled with this the impact of a biocontrol agent in the field is often variable, with efficacy being context-specific and thus dependent upon conditions at a particular site (Seastedt, 2015). Therefore, a balance between the time and costs associated with testing a large number of non-target species, many of which are often very distantly related and unlikely to be part of the agent’s fundamental host-range (Gilbert and Webb, 2007; Gilbert et al., 2015), and pre-release evaluation of the agent’s potential efficacy and impact on the target weed needs to be carefully struck (McClay and Balciunas, 2005).

Being the first fungal pathogen considered for weed CBC in the European region (Tanner et al., 2015), the evaluation of Pkg prior to release in GB focused predominantly on its safety. The assessment of a total of 74 non-target species demonstrated the high specifity of Pkg to Hb: 67 species were regarded as immune to infection showing no symptoms in response to the rust; seven species, all belong to the genus *Impatien*s, exhibited minor leaf necrosis and/or chlorosis; and only one, *I. balsamina* was classsed as fully susceptible (Tanner et al., 2015). Furthermore, ue to the nature of working with an annual plant species under quarantine conditions, only limited studies on the impact of the rust could be conducted at the time. The approval for release of a strain of Pkg from India in GB, however, also opened up the opportunity to undertake evaluations of the impact of Pkg on Hb under more natural conditions. Since the first releases undertaken in 2014, the establishment of the rust has been slow (Ellison et al., 2020). Reasons for this include, an initial suboptimal release strategy in which greenhouse-infected plants were planted out at field sites and releases were partly undertaken at shaded sites, now known to be less favourable for rust infection; as well as the previously unknown presence of rust-resistant populations of the weed (Pollard et al., 2019; Ellison et al., 2020). Whilst a second strain of Pkg from Pakistan was subsequently approved for release, not all Hb populations were found to be susceptible to these two strains. Moreover, at a large number of sites the rust failed to infect Hb seedlings the following year, despite high levels of leaf infection being observed the previous summer (Ellison et al., 2020). Since then, a study by Pollard et al (2021) has revealed that the plant-pathogen relationship between Pkg and Hb is more complex than originally thought, identifying populations susceptible to urediniospores but not basidiospores of the rust, and *vice versa*. In order to ensure a fully compatible plant-pathogen relationship prior to release of the rust at a site, the susceptibility of the targeted Hb populations is now determined through urediniospore and basidiospore inoculations. This technique, although only recently employed, has greatly improved establishment of the rust in field; high levels of infection and premature abscission of infected leaves are regularly observed during the summer and the rust is able to survive the winter and establish a population in stands of Hb the following year (personal observation). In order to monitor the effect of the rust on Hb populations in the field, long-term studies at these fully susceptible sites will be required.

The results of the study conducted here confirm that Pkghas a deleterious effect on both seedlings and mature plants of Hb under semi-natural field conditions; thus, the rust is considered to have great potential as a biological control agent at fully susceptible sites of the weed in GB. In the seedling infection experiment, the rust caused plant mortality levels between 33-80%; a significantly higher level than the 0.67 % seen to occur through thinning in the control, non-rust treatment. Although variable, the level of seedling infection reported here is comparable to that observed on plants from the same locality (Harmondsworth Moor) used as the positive control treatment in an experiment by Pollard et al. (2021), who compared both disease incidence and severity of the rust on seedlings from three rust-release sites in Wales, as well as those documented for the strain of the rust *P. komarovii* on *I. parviflora* in the field in continental Europe (Eliáš, 1995; Bacigálová et al., 1998; Piskorz and Klimko, 2006). The death of Hb seedlings is caused by the systemic infection with monokaryotic mycelium of the rust fungus resulting from basidiospore infection of the very young seedlings and leading to the production of spermogonia and subsequently aecia. The monokaryotic phase of the fungus causes swelling of the seedling stem, resulting from hypertrophy and/or hyperplasia of plant tissues within the vascular system (Larous and Lösel, 1993a, 1993b). Both plant height and stem diameter of individual Hb seedlings were found to be negatively affected by increasing levels of rust infection; more heavily infected seedlings grew smaller in height with thinner stems, being prone to physical damage and often dying prematurely. Similar findings were observed at a field site in Indiana, US, where seedlings of *Impatiens capensis* Meerb. were naturally infected by the rust *Puccinia recondita* Roberge ex Desm; at high densities of the plant, rust-infected seedlingsgrew slower and were smaller than those not infected (Lively et al., 1995). As observed in our study, stem infection also makes Hb plants more susceptible to colonisation by secondary pathogens. This is thought to be facilitated by the physiological and biochemical changes brought about as the rust sporulates on its host and thus, aids invasion by secondary, often necrotrophic, pathogens (De Nooij and Paul, 1992). For other plant-pathogen systems, infection of sporulating fruiting bodies by secondary pathogens has been frequently recorded, many of which resulted in either a synergistic or additive effect on disease symptoms and plant health, frequently increasing mortality (Hallett et al., 1990; De Nooij and Paul, 1992; Hallett and Ayres, 1992; Morin et al., 1993).

Systemic seedling infection is the expression of an intricate relationship between the plant and the pathogen which is only established when basidiospores infect the meristematic tissue at the growing point; thus, the timing of infection of the upcoming seedling is critical. If basidiospores fail to infect the meristem, the infection remains localised to either the stem or the cotyledons and hypertrophy/hyperplasia does not occur (Larous and Lösel, 1993a, 1993b). As a result, plants are able to outgrow or avoid infection completely with negligible impact on overall plant health and fitness (personal observation). This is demonstrated by the fact that the majority of the plants surviving the experimental period in both rust treatments were either free or had very low levels of infection (<10%). Despite being part of the rust treatments, these remaining plants had both a larger stem diameter and increased height, being up to 35 cm taller than those not exposed to the rust in the control treatment. The likely reason for this is the decrease in intraspecific competition in each pot, as those seedlings heavily infected by the rust had died. In the field, interspecific competition from other vegetation will likely influence the size of the non-infected Hb plants (as discussed below).

Results of the urediniospore inoculations reported here show that Pkg also brings about a negative impact on mature Hb plants. Infected plants produced fewer leaves and flowers and thus, less biomass than those not infected by the rust. A reduction in the size of infected Hb plants will certainly reduce the competitiveness of the weed in the field, enabling other components of the vegetation to become established at the invaded sites (Clewley et al., 2012). A decrease in biomass was also reported in greenhouse studies using the Indian strain of Pkg by Currie et al. (2020) and occurs as infection by Pkg progresses. Sporulation of the rust, as recorded for many foliar pathogens, results in modifications to chlorophyll and photosynthetic activity, acting as a carbon sink by drawing key nutrients and water away from the plant and/or altering plant-gene expression (Voegele et al., 2009); as a consequent reproductive output is also compromised (Gilbert, 2002). Here, we also found Hb infected by Pkg to produce fewer flowers and seeds. However, no impact on the weight of individual seeds or their subsequent viability was detected. Nevertheless, in a field situation, a reduction in seed production should, in the long-term, contribute to slowing down the prolific spread of Hb. The impact of the localised infection of urediniospore stage of Pkg on Hb was also comparable to that of the strain of this rust species attacking *I. parviflora* in continental Europe, i.e. infected *I. parviflora* populations also produce fewer flowers, leaves, and seeds and also present an overall decrease in biomass (Piskorz and Klimko, 2006). It is therefore probable that an equivalent impact would result from the broad introduction of Pkg on Hb populations in GB.

The evaluation of the impact of Pkgon both seedling and mature Hb under semi-natural conditions of our experiments did not account for the complex interactions between Hb and the biotic and abiotic factors of a natural ecosystem. One key aspect omitted from this study was competition of Hb with components of the local vegetation, as suggested by Čuda *et al.* (2015). As for many annual plant species, density plays the most significant role at the seedling emergence stage (Goldberg et al., 2001) affecting the availability of both space and nutrients required for growth, through both intra- and interspecific competition. In a field situation this would likely play a major role at preventing the development of larger Hb plants, which escape infection, as observed towards the end of our study. In addition, the premature death of seedlings at the start of the growing season may increase both the available space and nutrients for other plant species to take hold; species such as *Urtica dioica* L. and *Filipendula ulmaria* (L.) Maxim.are limited by Hb (Bieberich et al., 2020, 2021). Yet, for a significant impact to occur on a plant population, it is critical that the mortality of seedlings is not offset by an increase in the growth and reproduction of the adult plants (Gilbert, 2002). Nonetheless for the pathosystem Hb-Pkg in the field, a reduction in plant density through increased seedling mortality is likely to not only be counteracted by interspecific competition but, as the growing season progresses, by uredinial infection of the leaves decreasing both plant biomass and seed production. As Hb is a prolific invader and soil modifier, the impact of the rust will likely also depend upon the plant’s competitive environment at a particular site. Factors such as how long the plant has been present at a site and how this has impacted upon the microbial communities in the soil (Ruckli et al., 2014; Pattison et al., 2016; Gaggini et al., 2019) and the plants (Evans, 2009; Gange et al., 2019; Currie et al., 2020), the density of a Hb stand and the presence of native vegetation will all play an important role in determining not only the efficacy of the rust, but also the potential for re-colonisation by native vegetation.

Studies have shown that removal of Hb can lead to an increase in undesirable non-native or ruderal species (Hulme and Bremner, 2006; Wood et al., 2021) and that ecosystem restoration using native species may be required following its removal (Tanner and Gange, 2013). However, changes brought about through biological control are more gradual than alternative control methods such as mechanical or chemical means, and the progressive impact of Pkg is likely to prevent the sudden opening of vacant niches which would leave space for invasion of other undesirable exotic weeds. The results of the study presented here are promising, and corroborate with initial observations of the rust in the field; premature leaf abscission and high levels of seedling infection are likely to have deleterious effects on Hb populations. The CBC of Hb using the rust Pkg therefore offers a long-term, self-sustaining and environmentally benign method of control for this weed in GB.

**5. Conclusion**

This study demonstrates that the rust, Pkg can have a significant impact on fully susceptible Hb populations under semi-natural conditions and thus, holds great potential for controlling the invasive weed at fully susceptible sites in the field in GB. During experimental work, the rust was shown to exhibit a two-pronged attack, significantly increasing seedling mortality during the spring and substantially decreasing the total number of leaves, plant biomass and reproductive output of mature plants. These results are comparable to those reported for the *I. parviflora/P. komarovii* pathosystem. Whilst high levels of seedling infection and premature leaf abscission have been observed at a number of Pkg rust release sites, long-term monitoring of such sites and comparable sites without presence of the rust will be required in order confirm the experimental data collated under greenhouse and semi-natural conditions to date. Nevertheless, for control of Hb to be successful throughout GB, additional rust strains from the native range are required, to tackle the presence of Hb populations exhibiting resistance to the two strains currently released (Ellison et al., 2020; Kurose et al., 2020; Pollard et al., 2021).

**Acknowledgements**

The authors would like to thank Jacob Horner, Harri Hudson and Rik Peters for their assistance with data collection and to Janet Hannon and Nikolai Thom for assistance with setting up the experiments. Thanks also go and Sonal Varia for her invaluable advice and support throughout the project. The authors would also like to thank the two anonymous reviewers whose comments have greatly improved the manuscript. This work was financially supported by the UK Department for Environment Food and Rural Affairs (Defra) Reference 24151 and the Natural Environment Research Council (grant NE/N00244X/1 to ACG). CABI is an international intergovernmental organisation and we gratefully acknowledge the core financial support from our Member Countries (and lead agencies) including the United Kingdom (Department for International Development), China (Chinese Ministry of Agriculture), Australia (Australian Centre for International Agricultural Research), Canada (Agriculture and Agri-Food Canada), Netherlands (Directorate-General for International Cooperation), and Switzerland (Swiss Agency for Development and Cooperation). See <https://www.cabi.org/about-cabi/who-we-work-with/key-donors/> for full details.

**Conflict of interests**

The authors declare no conflict of interest.

**Author contributions**

KMP, ACG and CAE conceived and planned the experiments. The experiment was set up and analysed by KMP. The manuscript was written by KMP with contributions from ACG and MKS.

**Data availability statement**

The data that support the findings of this study are available from the corresponding author, KMP, upon request.

**References**

Andrews, M., Mauleb, H.G., Hodgec, S., Cherrill, A., Raven, J.A., 2009. Seed dormancy, nitrogen nutrition and shade acclimation of *Impatiens glandulifera*: Implications for successful invasion of deciduous woodland. Plant Ecol. Divers. 2, 145–153. <https://doi.org/10.1080/17550870903186256>

Bacigálová, K., Eliáš, P., Šrobárová, A., 1998. *Puccinia komarovii* - A rust fungus on *Impatiens parviflora* in Slovakia. Biologia (Bratisl). 53, 7–13.

Barton, J., 2012. Predictability of pathogen host range in classical biological control of weeds: An update. BioControl 57, 289–305. <https://doi.org/10.1007/s10526-011-9401-7>

Barton (née Frőhlich), J., 2004. How good are we at predicting the field host-range of fungal pathogens used for classical biological control of weeds? Biol. Control 31, 99–122. <https://doi.org/10.1016/j.biocontrol.2004.04.008>

Bates, D., Maechler, M., Bolker, B., Walker, S., 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw. 67, 1–48. <https://doi.org/doi:10.18637/jss.v067.i01>.

Bieberich, J., Feldhaar, H., Lauerer, M., 2020. Micro-habitat and season dependent impact of the invasive *Impatiens glandulifera* on native vegetation. NeoBiota 57, 109–131. <https://doi.org/10.3897/neobiota.57.51131>

Bieberich, J., Müller, S., Feldhaar, H., Lauerer, M., 2021. Invasive *Impatiens glandulifera*: A driver of changes in native vegetation? Ecol. Evol. 11, 1320–1333. <https://doi.org/10.1002/ece3.7135>

Clewley, G.D., Eschen, R., Shaw, R.H., Wright, D.J., 2012. The effectiveness of classical biological control of invasive plants. J. Appl. Ecol. 49, 1287–1295. <https://doi.org/10.1111/j.1365-2664.2012.02209.x>

Coakley, S., Petti, C., 2021. Impacts of the invasive *Impatiens glandulifera*: Lessons learned from one of Europe’s top invasive species 10, 619. <https://doi.org/https://doi.org/10.3390/biology10070619>

Coombe, D.E., 1959. Notes on some British plants seen in Austria. Veröffentlichungen des Geobot. Institutes 35, 128–137.

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, 1–12. <https://doi.org/10.1093/aobpla/plv033>

Currie, A.F., Gange, A.C., Ab Razak, N., Ellison, C.A., Maczey, N., Wood, S.V., 2020. Endophytic fungi in the invasive weed *Impatiens glandulifera*: a barrier to classical biological control? Weed Res. 60, 50–59. <https://doi.org/10.1111/wre.12396>

De Nooij, M.P., Paul, N.D., 1992. Invasion of rust (*Puccinia poarum*) pycnia and aecia on coltsfoot (*Tussilago farfara*) by secondary pathogens: Death of host leaves. Mycol. Res. 96, 309–312. <https://doi.org/10.1016/S0953-7562(09)80943-X>

Defra, 2021. Wildlife and Countryside Act 1981. Defra, UK. 297pp.

Eliáš, P., 1995. Stem fungi disease (*Puccinia komarovii*) on *Impatiens parviflora* in Slovakia: effects on population dynamics and its role in regulation of plant populations. Carinthia II 53, 14–16.

Ellison, C.A., Pollard, K.M., Varia, S., 2020. Potential of a coevolved rust fungus for the management of Himalayan balsam in the British Isles: First field releases. Weed Res. 44, 37–49. <https://doi.org/10.1111/wre.12403>

Evans, H.C., 2009. The endophyte-enemy release hypothesis: implications for classical biological control and plant invasions., in: Proceedings of the XII International Symposium on Biological Control of Weeds, La Grande Motte, France, 22-27 April, 2007. pp. 20–25. <https://doi.org/10.1079/9781845935061.0020>

Evans, K.J., Gomez, D.R., Jones, M.K., Oakey, H., Roush, R.T., 2011. Pathogenicity of *Phragmidium violaceum* isolates on European blackberry clones and on leaves of different ages. Plant Pathol. 60, 532–544. <https://doi.org/10.1111/j.1365-3059.2010.02396.x>

Gaggini, L., Rusterholz, H.P., Baur, B., 2019. The annual invasive plant *Impatiens glandulifera* reduces hyphal biomass of soil fungi in deciduous forests. Fungal Ecol. 39, 242–249. <https://doi.org/10.1016/j.funeco.2018.12.004>

Gange, A.C., Koricheva, J., Currie, A.F., Jaber, L.R., Vidal, S., 2019. Meta-analysis of the role of entomopathogenic and unspecialized fungal endophytes as plant bodyguards. New Phytol. 223, 2002–2010. <https://doi.org/10.1111/nph.15859>

Gilbert, G.S., 2002. Evolutionary ecology of plant diseases in natural ecosystems. Annu. Rev. Phytopathol. 40, 13–43. <https://doi.org/10.1146/annurev.phyto.40.021202.110417>

Gilbert, G.S., Briggs, H.M., Magarey, R., 2015. The impact of plant enemies shows a phylogenetic signal. PLoS One 10, 1–11. <https://doi.org/10.1371/journal.pone.0123758>

Gilbert, G.S., Webb, C.O., 2007. Phylogenetic signal in plant pathogen-host range. Proc. Natl. Acad. Sci. U. S. A. 104, 4979–4983. <https://doi.org/10.1073/pnas.0607968104>

Giordano, L., Anderson, F.E., 2021. Detrimental effect of the rust *Uromyces pencanus* on the invasive species *Nassella neesiana* (Chilean needle grass). Australas. Plant Pathol. 50, 299–307. <https://doi.org/10.1007/s13313-020-00773-x>

Goldberg, D.E., Turkington, R., Olsvig-Whittaker, L., Dyer, A.R., 2001. Density dependence in an annual plant community: Variation among life history stages. Ecol. Monogr. 71, 423–446. [https://doi.org/10.1890/0012-9615(2001)071[0423:DDIAAP]2.0.CO;2](https://doi.org/10.1890/0012-9615(2001)071%5B0423:DDIAAP%5D2.0.CO;2)

Hallett, S.G., Ayres, P.G., 1992. Invasion of rust (*Puccinia lagenophorae*) aecia on groundsel (*Senecio vulgaris*) by secondary pathogens: Death of the host. Mycol. Res. 96, 142–144. <https://doi.org/10.1016/S0953-7562(09)80929-5>

Hallett, S.G., Paul, P.N., Ayres, P.G., 1990. *Botrytis cinerea* kills groundsel (*Senecio vulgaris*) infected by rust (*Puccinia lagenophorae*). New Phytol. 114, 105–109. <https://doi.org/10.1111/j.1469-8137.1990.tb00380.x>

Hanley, M.E., Groves, R.H., 2002. Effect of the rust fungus *Puccinia chondrillina* TU 788 on plant size and plant size variability in *Chondrilla juncea*. Weed Res. 42, 370–376. <https://doi.org/10.1046/j.1365-3180.2002.00297.x>

Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric models. Biometrical J. 50, 346–363.

Hulme, P.E., Bremner, E.T., 2006. Assessing the impact of *Impatiens glandulifera* on riparian habitats: Partitioning diversity components following species removal. J. Appl. Ecol. 43, 43–50. <https://doi.org/10.1111/j.1365-2664.2005.01102.x>

Kurose, D., Pollard, K.M., Ellison, C.A., 2020. Chloroplast DNA analysis of the invasive weed, Himalayan balsam (*Impatiens glandulifera*), in the British Isles. Sci. Rep. 1–12. <https://doi.org/10.1038/s41598-020-67871-0>

Larous, L., Lösel, D.M., 1993a. Vascular infection by *Puccinia menthae* and other rust fungi. Mycol. Res. 97, 409–414. <https://doi.org/10.1016/S0953-7562(09)80127-5>

Larous, L., Lösel, D.M., 1993b. Strategies of pathogenicity in monokaryotic and dikaryotic phases of rust fungi, with special reference to vascular infection. Mycol. Res. 97, 415–420. <https://doi.org/10.1016/S0953-7562(09)80128-7>

Lively, C.M., Johnson, S.G., Delph, L.F., Clay, K., 1995. Thinning reduces the effect of rust infection on jewelweed (*Impatiens capensis*). Ecology 76, 1859–1862. <https://doi.org/10.2307/1940718>

Love, H.M., Maggs, C.A., Murray, T.E., Provan, J., 2013. Genetic evidence for predominantly hydrochoric gene flow in the invasive riparian plant *Impatiens glandulifera* (Himalayan balsam). Ann. Bot. 112, 1743–1750. <https://doi.org/10.1093/aob/mct227>

McClay, A.S., Balciunas, J.K., 2005. The role of pre-release efficacy assessment in selecting classical biological control agents for weeds - Applying the Anna Karenina principle. Biol. Control 35, 197–207. <https://doi.org/10.1016/j.biocontrol.2005.05.018>

Morin, L., 2020. Progress in biological control of weeds with plant pathogens. Annu. Rev. Phytopathol. 58, 1–23.

Morin, L., Auld, B.A., Brown, J.F., 1993. Interaction between *Puccinia xanthii* and facultative parasitic fungi on *Xanthum occidentale*. Biol. Control 3, 288–295.

Morin, L., Evans, K.J., Sheppard, A.W., 2006. Selection of pathogen agents in weed biological control: Critical issues and peculiarities in relation to arthropod agents. Aust. J. Entomol. 45, 349–365. <https://doi.org/10.1111/j.1440-6055.2006.00562.x>

Morin, L., Willis, A.J., Armstrong, J., Kriticos, D., 2002. Spread, epidemic development and impact of the bridal creeper rust in Australia: Summary of results, in: Proceedings of the 13th Australian Weeds Conference. Plant Protection Society of WA Inc., Perth, Australia, pp. 385–388.

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 Res. 56, 198–207. <https://doi.org/10.1111/wre.12200>

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 Soc. Bot. Pol. 75, 51–59. <https://doi.org/10.5586/asbp.2006.008>

Pollard, K.M., Varia, S., Ellison, C.A., 2019. Field releases of the rust fungus for the biological control of Himalayan balsam in the UK: Constraints to success. In: Hinz, H., Bon, M., Bourdôt, G., Critofaro, M., Desurmont, G., Kurose, D., Müller-Schärer, H., Rafter, M., Schaffner, U., Seier, M., Sforza, R., Smith, L., Stutz, S., Thomas, S., Weyl, P., Winston, R. (Eds.), XV International Symposium on Biological Control of Weeds. Organising Committee, XV International Symposium on Biological Control of Weeds, 26th-31st August, 2018, Engelberg, Switzerland, pp. 231–233.

Pollard, K.M., Varia, S., Seier, M.K., Ellison, C.A., 2021. Battling the biotypes of balsam: The biological control of *Impatiens glandulifera* using the rust fungus *Puccinia komarovii* var. *glanduliferae* in GB. Fungal Biol. 125, 637–645. <https://doi.org/10.1016/j.funbio.2021.03.005>

R Core Team, 2019. R: A language and environment for statistical computing. R Foundation for Statistical Computing.

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*. J. Chem. Ecol. 40, 371–378. <https://doi.org/10.1007/s10886-014-0421-5>

Seastedt, T.R., 2015. Biological control of invasive plant species: A reassessment for the Anthropocene. New Phytol. 205, 490–502. <https://doi.org/10.1111/nph.13065>

Seeney, A., Eastwood, S., Pattison, Z., Willby, N.J., Bull, C.D., 2019. All change at the water’s edge: Invasion by non-native riparian plants negatively impacts terrestrial invertebrates. Biol. Invasions 21, 1933–1946. <https://doi.org/10.1007/s10530-019-01947-5>

Shaw, R.H., Ellison, C.A., Marchante, H., Pratt, C.F., Schaffner, U., Sforza, R.F.H., Deltoro, V., 2018. Weed biological control in the European Union: From serendipity to strategy. BioControl 63, 333–347. <https://doi.org/10.1007/s10526-017-9844-6>

Sundh, I., Eilenberg, J., 2020. Why is the authorization of microbial biological control agents slower in the EU than in comparable jurisdictions? Pest Manag. Sci. <https://doi.org/10.1002/ps.6177>. <https://doi.org/10.1002/ps.6177>

Tanner, R.A., Gange, A.C., 2020. Himalayan balsam, *Impatiens glandulifera*: its ecology, invasion and management. Weed Res. 60, 4–7. <https://doi.org/10.1111/wre.12401>

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 Ecol. 214, 423–432. <https://doi.org/10.1007/s11258-013-0179-9>

Tanner, R.A., Pollard, K.M., Varia, S., Evans, H.C., Ellison, C.A., 2015. First release of a fungal classical biocontrol agent against an invasive alien weed in Europe: Biology of the rust, *Puccinia komarovii* var. *glanduliferae*. Plant Pathol. 64, 1130–1139. <https://doi.org/10.1111/ppa.12352>

Tanner. R.A., 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, UK. 236 pp.

Tomley, A.J., Evans, H.C., 2004. Establishment of, and preliminary impact studies on, the rust, *Maravalia cryptostegiae*, of the invasive alien weed, *Cryptostegia grandiflora* in Queensland, Australia. Plant Pathol. 53, 475–484. <https://doi.org/10.1046/j.0032-0862.2004.01054.x>

Varia, S., Pollard, K.M., Ellison, C.A., 2016. Implementing a novel weed management approach for Himalayan balsam: Progress on biological control in the UK. Outlooks Pest Manag. 27, 198–203. <https://doi.org/10.1564/v27>

Venables, W.N., Ripley, B.D., 2002. Modern applied statistics with S, 4th Ed. ed. Springer-Verlag, New York, USA.

Voegele, R.T., Hahn, M., Mendgen, K., 2009. The Uredinales: Cytology, biochemistry, and molecular biology. The Mycota 69–98. <https://doi.org/10.1007/978-3-540-87407-2_4>

Wickham, H., 2011. The split-apply-combine strategy for data analysis. J. Stat. Softw. 40, 1–29.

Wood, S. V., Maczey, N., Currie, A.F., Lowry, A.J., Rabiey, M., Ellison, C.A., Jackson, R.W., Gange, A.C., 2021. Rapid impact of *Impatiens glandulifera* control on above- and belowground invertebrate communities. Weed Res. 64, 35–44. <https://doi.org/10.1111/wre.12454>

**Fig. 1.** *Impatiens glandulifera* infected with the rust *Puccinia komarovii* var. *glanduliferae*: A) early stages of infection (note red lesions on stems); B) production of aecial cups and aeciospores on infected stems; C) heavily infected stems becoming warped and etiolated; D) infected seedlings colonised by secondary pathogens; E) surviving plants becoming infected by aeciospores of the rust forming chlorotic spots on leaves; F) production of uredinia and urediniospores abaxially on infected leaf.

**Fig. 2**. Impact of inoculation basidiospores of the rust fungus *Puccinia komarovii* var *glanduliferae* on seedlings of *Impatiens glandulifera* over time: A) survival of seedlings; B) total plant height, measured from soil level to tip of top leaf; C) impact of the level of stem infection on plant height; D) stem diameter, measured at the first node and; E) impact of level of rust infection on stem diameter at the first node. Infection categories: low - <10%, medium - 10-50%, high - >50%. All points and bars represent means ± SE, n = 6 for each treatment, 25 seedlings, Different letters above bar charts indicate significant pairwise differences between means at *p* < 0.05.

**Fig. 3.** Impact of inoculation with urediniospores of the rust fungus *Puccinia komarovii* var *glanduliferae* on mature plants of *Impatiens glandulifera* over time:A) plant height; B) total number of leaves produced per plant; C) biomass, measured as dry weight at week 15; D) total number of flower stalks produced per plant; E) total number of seeds produced per plant by week 15. All points and bars represent means ± SE, *n* = 9, 9 & 12 plants for the Control, India and Pakistan treatments respectively. Different letters above bar charts indicate significant pairwise differences between means at *p* < 0.01.



Fig 1.



Fig. 2.



Fig. 3