



# Investigating hyperparasites as potential biological control agents of rust pathogens on cereal crops

A. Wilson<sup>1</sup> · W. S. Cuddy<sup>1,2</sup> · R. F. Park<sup>1</sup> · G. F. S. Harm<sup>3</sup> · M. J. Priest<sup>4</sup> · J. Bailey<sup>4</sup> · M. C. Moffitt<sup>3</sup>

Received: 21 August 2019 / Accepted: 28 February 2020 / Published online: 18 March 2020  
© Australasian Plant Pathology Society Inc. 2020

## Abstract

Rust pathogens cause damage to cereal crops around the world, leading to reduced yield and profit. Current methods of rust control include fungicides, resistant cultivars, and preventative agronomic practices. Some hyperparasites are antagonists of plant pathogens and may provide a potential method of biocontrol against increasingly virulent strains of rust. Very little is known about the mechanism of inhibition of rust growth by hyperparasites, however, isolation of new strains and subsequent characterisation may reveal new treatment strategies for the control of rust. Here we report the isolation of six new fungal hyperparasites and their effects on the development of three *Puccinia* rust pathogens were examined in vitro, to determine the potential of each as biocontrol agents of rust. Cut-leaf sections of rust-susceptible wheat, barley and oat cultivars were treated with fungal hyperparasite conidia prior to infection with the rust species; *Puccinia triticina*, *P. hordei*, and *P. coronata* f. sp. *avenae*, respectively. Inhibition of rust spore germination tests were also performed on water agar plates co-inoculated with the isolates. In leaf sections, rust pustule number was significantly ( $P < 0.01$ ) lower for all six isolates tested: *Penicillium brevicompactum*, *Clonostachys rosea*, *Simplicillium aogashimaense*, *Neosascochyta* sp., *Lecanicillium psalliotae* and *Epicoccum nigrum*. The results of these experiments suggest that the mechanisms underlying the reduction in pustule number could include one or more of the processes of direct parasitism, antagonism by antibiosis, competition, and/or induction of host plant resistance.

**Keywords** *Puccinia* · Hyperparasites · Phytopathogen · Rust · Biocontrol

## Introduction

Many fungal hyperparasites are from the phylum Ascomycota and occur naturally as plant mycoflora. Hyperparasites display a number of mechanisms that prevent plant pathogens from infecting their hosts, including; antibiosis from enzyme production; physical blockage of stomata; competitive exclusion of pathogens; induced resistance in the plant; and direct

parasitism (Moricca & Ragazzi 2008). These antagonistic interactions between the plant pathogen and hyperparasite can inhibit the growth and/or reproductive ability of the pathogenic organism, thus enabling a reduction in severity of disease caused in the plant (Pal & Gardener 2006).

Biotrophic rust pathogens of cereals cause some of the most damaging plant diseases in agriculture. These pathogens grow and reproduce in living host tissues of the foliage, stems and seed heads, impairing photosynthesis by reducing green leaf area and increasing the transpiration rate of the plant (Robert et al. 2005). This results in reduced grain yield and even plant death in severe cases. The control of rust in wheat is especially important for food security (Chaves et al. 2013), and there is interest in fungal hyperparasites as biological control agents as they grow quickly, are derived naturally, and potentially have less impact on the environment than chemical fungicides (Pal & Gardener 2006). Rust fungi are pathogenic organisms of the division Basidiomycota, order Pucciniales, and more than half of the known species are from the genus *Puccinia* (Margulis 2009). The purpose of this study was to

✉ M. C. Moffitt  
m.moffitt@westernsydney.edu.au

<sup>1</sup> Plant Breeding Institute, University of Sydney, Cobbitty, Australia  
<sup>2</sup> NSW Department of Primary Industries, Woodbridge Road, Menangle, NSW, Australia  
<sup>3</sup> Western Sydney University, Campbelltown Campus, Campbelltown, Australia  
<sup>4</sup> NSW Department of Primary Industries, Plant Pathology & Mycology Herbarium, Forest Lodge Road, Orange, NSW, Australia

focus on rust pathogens that have cereal crops as hosts, specifically: *P. triticina* (causal agent of wheat leaf rust), *P. hordei* (barley leaf rust), and *P. coronata* f. sp. *avenae* (*Pca*) (oat crown rust).

Relatively few hyperparasites of biotrophic foliar pathogens have been identified, and little is known about the molecular mechanisms of hyperparasitism, or the effectiveness on pathogen inhibition in the field. Fungal hyperparasites found growing in pustules of *P. graminis* and *P. striiformis* have previously been studied and were identified as species of *Verticillium*, *Alternaria* and *Cladosporium* (Mendgen 1981). Several *Cladosporium* species have been reported to parasitize rust fungi and appear to impact spore viability (Zhao et al. 2016; Zhan et al. 2014), and Zheng et al. (2017) found that a strain of *Alternaria alternata* had the ability to hyperparasitize *P. striiformis* f. sp. *tritici* (Pst). The use of hyperparasites in controlling diseases such as the rusts may be an environmentally friendly alternative, or an additional tool for the integrated control of these damaging pathogens. Furthermore, understanding their mechanism for inhibiting rust growth could be applied to the development of novel treatments of rust diseases.

## Materials and methods

**Hyperparasite isolation** All fungal hyperparasite strains were originally isolated from rust pustules on wheat and oat plants grown in the greenhouse at the University of Sydney Plant Breeding Institute (PBI), Cobbitty Campus, NSW, while isolates from rust pustules on willow and poplar were collected from the field (Table 1). Pure isolates of each strain were obtained by the selection of a single spore after a dilution series.

**Hyperparasite DNA extraction, amplification, purification and sequencing** Genomic DNA (gDNA) was extracted from each strain using Isolate II plant DNA kit (Bioline). Polymerase chain reaction (PCR) was used to amplify the internal transcribed spacer (ITS) for the purpose of identifying sampled fungal strains [White et al. 1990]; 16S primers were used to check for any bacterial contamination or symbionts [Clifford et al. 2012]. Amplified DNA products were verified on a 1.5% agarose gel and DNA concentration and quality were checked on a Nanodrop 2000 (Thermo Scientific). The PCR products were purified using a QIAquick PCR purification kit (QIAGEN) as per the manufacturer's instructions. For the sequencing of the amplified PCR products, an optimized PCR thermocycling method designed by Platt et al. [2007] was performed on an Eppendorf Master-cycler (Eppendorf), and the final products were purified by ethanol precipitation. The samples were prepared for amplicon sequencing using BigDye® Terminator v3.1 chemistry (Applied Biosystems, USA)

and analysed on an ABI 3730 Capillary Sequencer (Applied Biosystems, USA) at the Ramaciotti Centre at the University of New South Wales. BioEdit software (Ibis Biosciences) was used to analyse sequence data and correct reads. Sequences were registered in GenBank (Table 1).

**Hyperparasite identification** The complementary (dual read) ITS sequence for each strain was obtained from the alignment of the forward and reverse ITS sequences in BioEdit. The complementary sequence was entered into the BLASTn suite and the resultant top hit was determined by sequence identity, coverage, E-value and BLAST scores. The sequence match was used as the preliminary identification for each strain. To confirm sequence-based identifications, pure cultures were sent to The NSW Plant Pathology and Mycology Herbarium (Orange, NSW, Australia) for morphological based identification.

**Plants and rust isolates** The rust susceptible cultivars 'Morocco' (wheat), 'Gus' (barley), and 'Swan' (oat) were used in all experiments. Single isolates of *P. triticina*, *P. hordei*, and *P. coronata* f. sp. *avenae* (*Pca*) maintained as dried urediniospores in liquid nitrogen at the Plant Breeding Institute were used (Table 2).

**Hyperparasite culture preparation and growth** All hyperparasite isolates were cultured on 90 mm plates of PDA agar (39 g L<sup>-1</sup>) at Western Sydney University, either from spore stock (-80 °C) or culture plugs. The cultures were grown in an aging room with diurnal light cycle at 25 °C until sporulation (approx. 5–14 d).

**Preparation of plants** Uniform seed of each cultivar was sown at a depth of 15 mm in 10 cm pots filled with a commercial seedling potting mix treated by heat pasteurization overnight at 70 °C and, upon planting, were fertilized with 100 mL Aquasol (Yates) at 1.6 g L<sup>-1</sup> water. The seedlings were grown for 10 d in a greenhouse at PBI, where they were maintained at 20–24 °C and grown in trays to enable watering from below, every second day. Seedlings were thinned out to five evenly spaced plants per pot.

**Hyperparasite conidial inoculum and rust urediniospore suspensions** Hyperparasite conidial inocula were created by flooding 90 mm plates of each pure culture with a 10 mL solution of 0.02% Tween 20 (vol/vol), using a disposable plate spreader to gently dislodge conidia. The spore suspension was syringed off and transferred to a 15 mL Falcon tube. Conidial concentration of all isolates was corrected to approximately 1 × 10<sup>6</sup> spores mL<sup>-1</sup> as determined by haemocytometer. Rust urediniospore suspensions were created by measuring 30 µg rust spores per 5 mL 0.02% Tween 20 (vol/vol) and mixing by vortex. The experimental design generator EDGAR II

**Table 1** Fungal isolates used in experiments, identified by internal transcribed spacer (ITS) sequence information

Sample identifier	Plant host	Rust species	Location	Identification of fungal isolate	Percentage identity <sup>d</sup>	GenBank Accession no.
HOCR17	Oat	<i>Puccinia coronata</i> f. sp. <i>avenae</i>	Cobbitty, NSW Australia <sup>a</sup>	<i>Penicillium brevicompactum</i>	100%	MK070106
HWLR12	Wheat	<i>Puccinia triticina</i>	Cobbitty, NSW Australia <sup>a</sup>	<i>Clonostachys rosea</i>	99%	MK070105
HWYR21	Wheat	<i>Puccinia striiformis</i> f. sp. <i>tritici</i>	Cobbitty, NSW Australia <sup>a</sup>	<i>Simplicillium aogashimaense</i>	100%	MK070107
HWLR27	Willow	<i>Melampsora epitea</i>	Camden, NSW Australia <sup>b</sup>	<i>Neosascochyta</i> sp.	100%	MK070108
HPOP30	Poplar	<i>Melampsora larici-populina</i>	Camden, NSW Australia <sup>c</sup>	<i>Epicoccum nigrum</i>	99%	MK070109
HWLR35	Wheat	<i>Puccinia triticina</i>	Cobbitty, NSW Australia <sup>a</sup>	<i>Lecanicillium psalliotae</i>	100%	MK070110

<sup>a</sup> Host plants grown in a greenhouse at the University of Sydney Plant Breeding Institute (PBI), Cobbitty Campus, NSW coordinates (−34.056048, 150.692804)

<sup>b</sup> Field isolates from coordinates (−34.055186, 150.700535)

<sup>c</sup> Field isolates from coordinates (−34.057331, 150.691394)

<sup>d</sup> Percentage identity is based on BLAST analysis of ITS sequence

(version 2.0) [Brown 2005] was used to produce a completely randomized design with equal replication for plate placement in light shelves. Temperature, humidity and dew point were measured and recorded by a data logger (EL-USB-2-LCD+, Instrument Choice). The experiments consisted of rust controls, hyperparasite controls and untreated leaf sections as well as rust/hyperparasite treatments.

**Agar plate co-inoculations** A rust urediniospore solution (200  $\mu$ L) was transferred to each rust control plate. Co-inoculation plates consisted of 100  $\mu$ L of conidial inoculum and 100  $\mu$ L of rust urediniospore solution, dispensed onto each plate and spread over the surface with a disposable plate spreader. Conidial inoculum (200  $\mu$ L) was dispensed onto each hyperparasite control plate, shaking tubes to homogenize solution between each application. All plates were incubated at room temperature (25 °C) with a 12 h diurnal light cycle (with a measured intensity of 20  $\mu$ M photons  $m^{-2} s^{-1}$ ) for 24–48 h at PBI Cobbitty Campus. Three replicates were conducted on 60 mm plates containing water agar (1%), PDA (39 g  $L^{-1}$ ) or half concentration of PDA (19.5 g  $L^{-1}$ ). Milli-Q water was sterilized prior to use by autoclave. Rust germination scores were taken after 24 h to determine if germination was impacted by co-inoculation with isolates, compared to control plates. Scores were calculated by counting the number of germinated urediniospores out of a total of 100 urediniospores, in 2–4 fields of view (FOV) on each replicate

plate and then averaged. In this study, germination was defined by the presence of a germ tube equal to, or larger than the diameter of the rust urediniospore.

**Cut-leaf assays** Seeds of each susceptible cultivar were grown as described until first leaves of seedlings appeared from coleoptile (6–7 d), when they were treated with maleic hydrazide (2 mL  $L^{-1}$ ) to retard plant growth. The maleic hydrazide was applied on dry soil at a rate of 50 mL per 4" pot using a Te Pari automatic dosing gun once the coleoptile was fully emerged. Leaf sections (approximately 2 cm in length) were cut and plated adaxial side up on water agar (0.6%) containing benzimidazole (Sigma) at a rate of 35 ppm to facilitate longevity of cut leaves. Hyperparasite inoculation of leaf sections by disposable spray packs was conducted at an approximate rate of 0.5 mL per plate prior to rust infection. Rust inoculation was conducted by atomising with an airbridge, 4 d after hyperparasite inoculation. Unless otherwise stated, each treatment consisted of three replicate plates containing three cut-leaf sections. Plates of cut leaves were kept in darkness for the initial 24 h to promote rust germination, and then kept at 24 °C under diurnal light in growth rooms at PBI. The controls were sprayed with 0.02% Tween 20 (vol/vol) at the same rate as treatments. The number of rust pustules in the middle 2 cm area of each leaf section was determined by microscopy 10 d later. Micrograph images of these results were taken at 16–40 x magnification using ProgRes Microscope Camera and software by Jenoptik.

**Table 2** Details of the cereal rust pathogen isolates and host plants and used in experiments

Rust collection identification number	Pathotype	Rust	Plant host used
539	76–1,3,5,10,12	<i>P. triticina</i>	'Morocco' wheat
570	200P+	<i>P. hordei</i>	'Gus' barley
636	1107–1,6,7,10,12	<i>P. coronata</i> f. sp. <i>avenae</i>	'Swan' oat

**Data analysis** In all experiments, data were analyzed using R [R core team 2013] and R Studio version 1.0.153 [RStudio Team 2017] for statistical analysis. Parametric assumptions were met and data were within normal requirements to determine analysis of variance (ANOVA) and significance ( $P$ -values). Multiple comparisons of means (Tukey's contrasts) were conducted to determine differences between treatment groups.

## Results

**Isolation and identification of new hyperparasites** Six strains of ascomycetes isolated from rust pustules on wheat, oat, willow and poplar were used in this study. The identification of each strain was initially determined by BLAST analysis of the 18S rRNA gene and internal transcribed spacer (ITS) sequences and subsequently confirmed by morphological characterization (Table 1). Each of the hyperparasite strains were then analysed for their ability to inhibit germination and reduce rust pustule formation on cereal plant leaves.

**Agar plate co-inoculations** To analyse the direct inhibition of rust urediniospore germination by hyperparasites, urediniospores and conidia were co-inoculated on agar plates and the average percentage of germinated rust urediniospores visualized after 24 h (Fig. 1). There were significant ( $P < 0.01$ ) reductions in urediniospore germination when treated with *P. brevicompactum* and *C. rosea* in all three rust species tested, and significant ( $P < 0.01$ ) reductions in germination of *P. tritricina* and *P. hordei* but not *Pca* when treated with *S. aogashimaense*. Significant ( $P < 0.01$ ) reductions in germination of *P. hordei* were recorded when treated with *Neosascochyta* sp. and *E. nigrum* and significant ( $P < 0.05$ ) reductions of *Pca* germination when treated with *Neosascochyta* sp. and *E. nigrum*. There were no significant reductions in rust urediniospore germination in any of the rust species when treated with *L. psalliotae*, and determination of direct hyperparasitic interaction was inconclusive by microscopy.

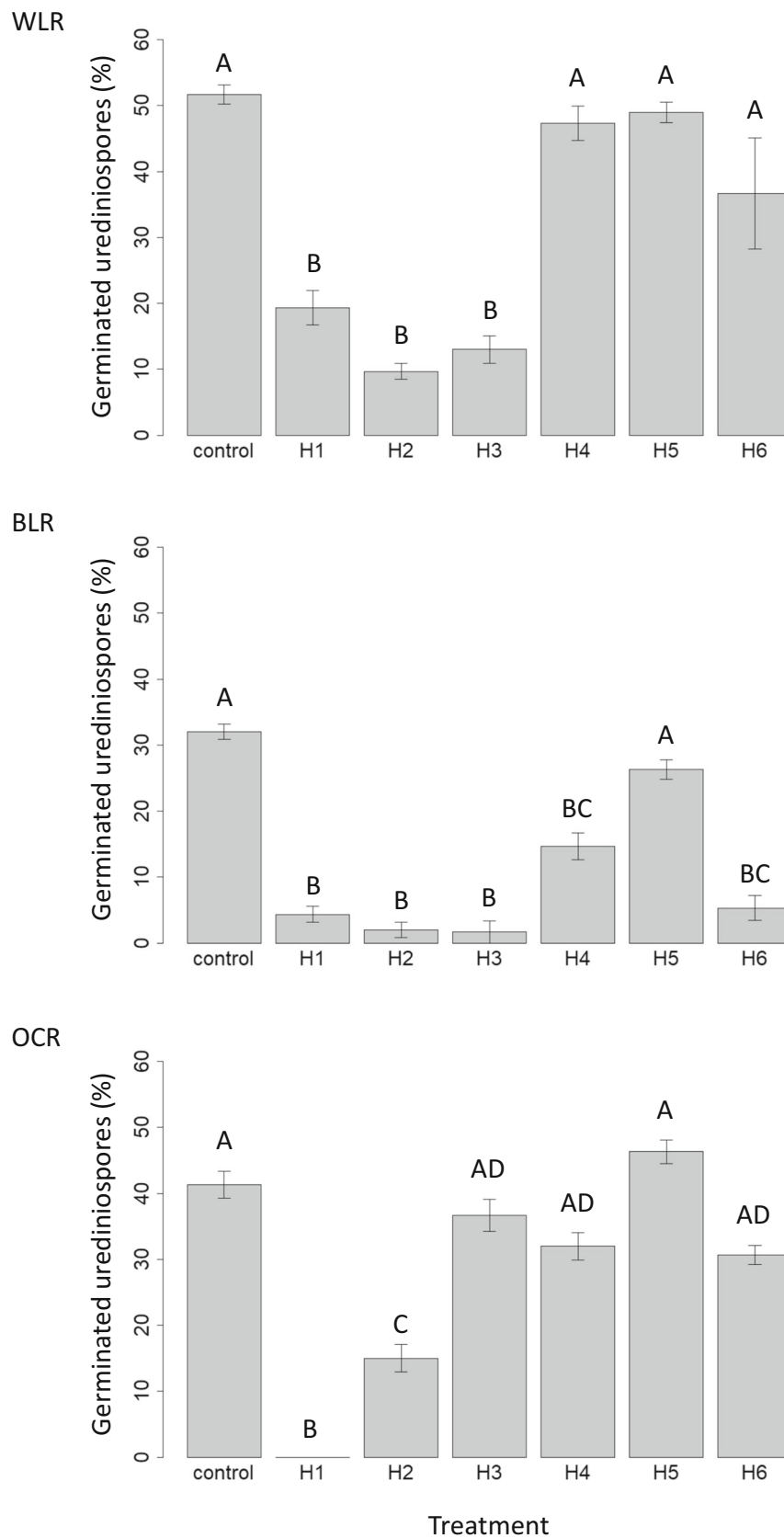
**Cut leaf assays** These assays were conducted to investigate the effects of hyperparasite inoculum on rust pathogen development during colonization of plant tissue. Using microscopy at 16–40 x magnifications, the number of rust pustules that developed were counted per 2 cm leaf section. All treatments (*P. brevicompactum*, *C. rosea*, *S. aogashimaense*, *Neosascochyta* sp., *L. psalliotae*, and *E. nigrum*) on all three rust species displayed a significant ( $P < 0.01$ ) reduction in the average number of rust pustules that developed compared to controls, except *P. brevicompactum* treatment of *Pca* on oat, which actually displayed increased pustule number compared to controls (Fig. 2). In tests with *P. tritricina* and *P. hordei*, all isolates

showed a decrease in pustule number that was also significantly more than the reduction shown by *P. brevicompactum*.

Microscopic observations showed fungal colonization of rust pustules on leaf sections, particularly in *C. rosea*, *S. aogashimaense*, *Neosascochyta* sp., and *L. psalliotae*. Other observations included abnormal pustule growth in most treatments, reductions in pustule size in treatments with *L. psalliotae* and *Neosascochyta* sp., aborted rust lesions in *P. brevicompactum*, and *E. nigrum* and low sporulation produced from pustules that were present in each treatment type. Necrotic patches in the leaf surface were present in treatments with *S. aogashimaense* and *E. nigrum*. In some cases, such as *S. aogashimaense* and *C. rosea*, heavy fungal colonization created complex networks of mycelium over pustules, physically containing urediniospores. Fungal growth appeared to be present on rust uredinia, and absent on healthy leaf surfaces.

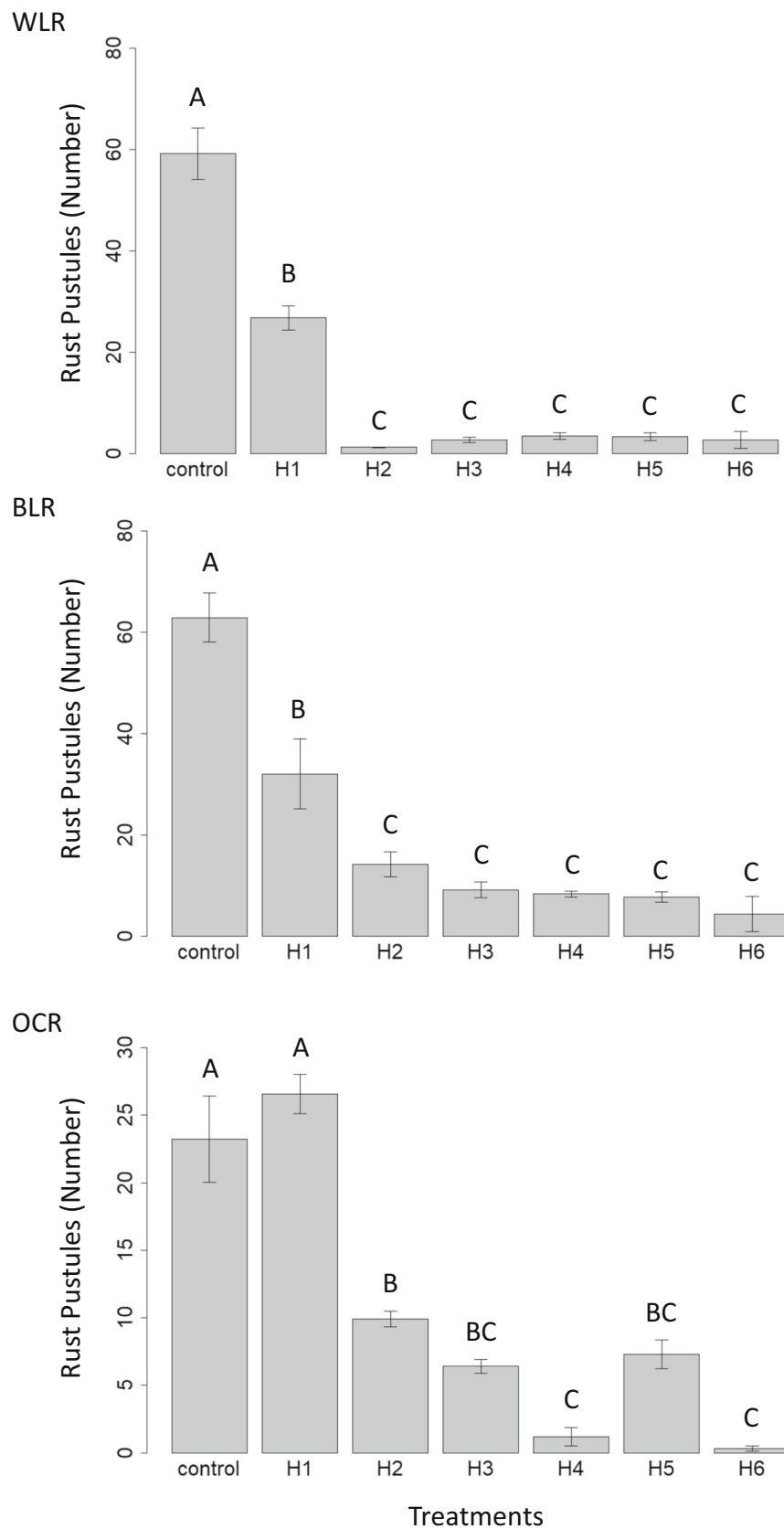
## Discussion

Although hyperparasite interactions are not well understood, different species appear to employ different modes of action against rust fungi. *Penicillium brevicompactum* and *Clonostachys rosea* isolates significantly reduced the germination rate of all three *Puccinia* spp. in co-inoculations. The inhibition of rust urediniospore germination on agar indicated that *P. brevicompactum* and *C. rosea* may act as antagonists by antibiosis that prevents rust spore germination. *P. brevicompactum* is known to produce mycophenolic acid, an antibacterial and antifungal compound, and a mycotoxin. It has been reported as the cause of spoilage in stored food (Pitt 2006) and other members of this family are known as saprobes with aggressive colonization strategies. Treatments with *P. brevicompactum* produced minor reductions in pustule size on cut leaves compared to other isolates, which although not quantified in this study, may be an indication of reduced sporulation. The suppression of *Botrytis cinerea* sporulation by foliar application of *Penicillium* spp. was reported by Fokkema (1993). Minimal parasitic colonization of pustules on detached leaf sections show that it is not likely to be acting by direct hyperparasitism, and aborted rust pustules indicated activation of plant defense responses (Ramachandran et al. 2017). Pustule development on cut leaves treated with *C. rosea* was reduced significantly, and although there were aborted rust colonies and necrotic patches, pustules present on leaf sections were heavily colonized with mycelial growth. These results indicate that *C. rosea* is able to inhibit rust spore germination, and that it perhaps has other antagonistic actions including competition and direct parasitism of rust urediniospores. The antifungal activity of *C. rosea* has previously been confirmed against *Sclerotinia sclerotiorum* (Rodriguez et al. 2015). *C. rosea* also significantly reduced



**Fig. 1** Percentage of germinated rust urediniospores on agar plates co-inoculated with hyperparasite isolates at 24 h ( $\pm$  s.e.). WLR: *P. triticina*, BLR: *P. hordei*, OCR: *P. coronata*. Treatments: H1: *P. brevicompactum*,

H2: *C. rosea*, H3: *S. aogashimaense*, H4: *Neoscochyta* sp., H5: *L. psalliotae*, H6: *E. nigrum*. Bars with different letters (A-D) differ significantly from each other ( $P < 0.05$ )



**Fig. 2** Average number of rust pustules per 2 cm cut-leaf section ( $\pm$  s.e.). WLR: *P. triticina*, BLR: *P. hordei*, OCR: *P. coronata*. Treatments: H1: *P. brevicompactum*, H2: *C. rosea*, H3: *S. aogashimaense*, H4:

*Neoscochyta* sp., H5: *L. psalliotae*, H6: *E. nigrum*. Bars with different letters (A-C) differ significantly from each other ( $P < 0.01$ )

disease caused by *Fusarium culmorum* in wheat (Roberti et al. 2008; Jensen et al. 2000) and foliar application of *C. rosea* reduced spot blotch in barley (Jensen et al. 2016) through activation of the host plant defense system in addition to production of toxic metabolites and direct hyperparasitism (Roberti et al. 2008).

There were significant reductions in urediniospore germination of *P. triticina* and *P. hordei* but not *Pca* when treated with *S. aogashimaense*, indicating the production of antifungal compounds that may have specific actions against different rust species. However, necrotic patches in the leaf surface also indicate the activation of a plant response. In other studies, the *Simplicillium* species *S. lanosoniveum* reduced pustule formation of *Phakopsora pachyrhizi*, the causal agent of soybean rust, and also decreased urediniospore germination rate by hyperparasitism (Ward et al. 2012), direct interactions have also been confirmed by TEM and SEM, illustrating the penetration of *P. pachyrhizi* urediniospores by *S. lanosoniveum* (Gauthier et al. 2014). A reduction in spore production demonstrates the potential of hyperparasites to break the re-infection cycle on crops (Szandala & Backhouse 2001), and hyperparasites have been studied for the ability to reduce the viability of spores produced. For example, *Alternaria alternata* reduced pustule density and impaired the production and germination rate of *Pst* urediniospores (Zheng et al. 2017).

Significant reductions in germination of *P. hordei* and *Pca* were recorded when treated with *Neosascochyta* sp. and *E. nigrum*. There was no significant difference in germination of *P. triticina*. Both these hyperparasites also significantly reduced numbers of rust pustules on cut leaves for all three rust pathogens. *E. nigrum* is an endophyte and a plant saprophyte (Schol-Schwarz 1959). It acts as an antagonist of rust and is likely to enable plant recognition of the pathogen. It is well known that some fungal endophytes found asymptotically within plant tissues can contribute to disease suppression and improve fitness (Lugtenberg et al. 2016). As an antagonist, *E. nigrum* has been shown to reduce sporulation of *B. cinerea* (Szandala & Backhouse 2001) and is known to produce antifungal compounds. There is little published information on the *Neosascochyta* sp. studied here. It may inhibit germination as shown by co-inoculation results, however, the presence of the hyperparasite inoculum on the plant leaves prior to rust infection may also initiate the pathogen recognition process, prevent rust infection by antagonism, or indicate production of antibiotic metabolites active against *P. hordei* and *Pca*, but not *P. triticina*.

*Lecanicillium psalliotae* was the only isolate that did not significantly inhibit the germination of any of the cereal rust pathogens in co-inoculations. However, when used as a treatment on cut leaf sections, the rust pustules that developed were heavily colonized, potentially indicating direct hyperparasitism. *L. psalliotae* was shown to act as a hyperparasite of soybean rust fungus urediniospores in culture (Saksirirat & Hoppe 1991) and another *Lecanicillium* species, *L. lecanii*,

was shown to be effective against white rust, by parasitism of spores in *P. horiana* of chrysanthemum (Whipps 1993). Reduced rust pustule numbers in cut-leaf assays and the presence of necrotic lesions indicates antagonism by competition or plant defense initiation may also play a role in the hyperparasitism of *L. psalliotae*.

## Conclusions

Early experimental success with hyperparasites of rust illustrates the potential of these isolates for control of foliar phytopathogens. This study also highlights that different strains of hyperparasite possess different mechanisms of action. Further investigation of each of these strains, particularly molecular and biochemical studies may help to provide an understanding for their action in rust inhibition.

**Acknowledgements** The funding for this project was provided by the Grains Research and Development Corporation (GRDC) (Number: 9174986) through the Undergraduate Honours Scholarship (UHS11004) to AW and the Western Sydney University Researcher Development Award to MCM.

## References

- Brown J (2005) EDGAR II experimental design generator and randomizer. Cereals Research Department John Innes Centre <http://www.edgarweborguk> Viewed 26 May 2017
- Chaves MS, Martinelli JA, Wesp-Guterres C, Graichen FAS, Brammer SP, Scagliusi SM, da Silva PR, Wiethölter P, Torres GAM, Lau EY, Consoli L, Chaves ALS (2013) The importance for food security of maintaining rust resistance in wheat. *Food Secur* 5:157–176
- Clifford RJ, Milillo M, Prestwood J, Quintero R, Zurawski DV, Kwak YI (2012) Detection of bacterial 16S rRNA and identification of four clinically important Bacteria by real-time PCR. *PLoS One* 7: e48558. <https://doi.org/10.1371/journal.pone.0048558>
- Fokkema NJ (1993) Opportunities and problems of control of foliar pathogens with micro-organisms. *Pestic Sci* 37:411–416
- Gauthier NW, Maruthachalam K, Subbarao KV, Brown M, Xiao Y, Robertson CL, Schneider RW (2014) Mycoparasitism of *Phakopsora pachyrhizi*, the soybean rust pathogen, by *Simplicillium lanosoniveum*. *Biol Control* 76:87–94
- Jensen B, Knudsen IMB, Jensen DF (2000) Biological seed treatment of cereals with fresh and long-term stored formulations of *Clonostachys rosea*: biocontrol efficacy against *Fusarium culmorum*. *Eur J Plant Pathol* 106:233–242
- Jensen B, Lübeck PS, Jorgensen HJL (2016) *Clonostachys rosea* reduces spot blotch in barley by inhibiting prepenetration growth and sporulation of *Bipolaris sorokiniana* without inducing resistance. *Pest Manag Sci* 72:2231–2239
- Lugtenberg BJJ, Caradus JR, Johnson LJ (2016) Fungal endophytes for sustainable crop production. *FEMS Microbiol Ecol* 92(12). <https://doi.org/10.1093/femsec/fiw194>
- Margulis L (2009) ‘Kingdom Fungi’ in kingdoms and domains - an illustrated guide to the phyla of life on earth (4th Edn.), Elsevier Inc.
- Mendgen K (1981) Growth of *Verticillium lecanii* in pustules of stripe rust (*Puccinia striiformis*). *J Phytopathol* 102:301–309

- Moricca S, Ragazzi A (2008) 'Biological and integrated means to control rust diseases' in Integrated Management of Diseases caused by Fungi, Phytoplasma and Bacteria. Ciancio, A & Mukerji (eds.). Springer science and business media. B. V
- Pal KK, Gardener BM (2006) Biological control of plant pathogens. The Plant Health Instructor APS net <https://doi.org/10.1094/PHI-A-2006-1117-02>
- Pitt JI (2006) *Penicillium* and related genera. 'Food spoilage Microorganisms' in food science technology and nutrition. Blackburn C (ed.). Woodhead publishing limited. Elsevier science direct
- Platt AR, Woodhall RW, George AL (2007) Improved DNA sequencing quality and efficiency using an optimized fast cycle sequencing protocol. *Biotechniques* 43:58–62
- Ramachandran SR, Yin C, Kud J, Tanaka K, Mahoney AK, Xiao F, Hulbert SH (2017) Effectors from wheat rust fungi suppress multiple plant defense responses. *Phytopathology* 107(1):75–83
- R Core Team (2013) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, <http://www.R-project.org/>
- Robert C, Bancal MO, Ney B, Lannou C (2005) Wheat leaf photosynthesis loss due to leaf rust, with respect to lesion development and leaf nitrogen status. *New Phytol* 165:227–241
- Roberti R, Veronesi AR, Cesari A, Cascone A, Di Berardino I, Bertini L, Caruso C (2008) Induction of PR proteins and resistance by the biocontrol agent *Clonostachys rosea* in wheat plants infected with *Fusarium culmorum*. *Plant Sci* 175:339–347
- Rodriguez MA, Rothen C, Lo TE, Cabrera GM, Godeas AM (2015) Suppressive soil against *Sclerotinia sclerotiorum* as a source of potential biocontrol agents: selection and evaluation of *Clonostachys rosea* BAFC1646. *Biocontrol Sci Tech* 25:1388–1409
- RStudio Team (2017) RStudio: integrated development for r. RStudio, Inc., Boston, MA. <http://www.rstudio.com/>
- Saksirat W, Hoppe HH (1991) Secretion of extracellular enzymes by *Verticillium psalliotae* Treschow and *Verticillium lecanii* (Zimm.) Viegas during growth on uredospores of the soybean rust fungus (*Phakopsora pachyrhizi* Syd.) in liquid cultures. *J Phytopathol* 131:161–173
- Schol-Schwarz MB (1959) The genus *Epicoccum* link. *British Mycological Society* 42:149–173
- Szandala ES, Backhouse D (2001) Suppression of sporulation of *Botrytis cinerea* by antagonists applied after infection. *Australas Plant Pathol* 30:165–170
- Ward NA, Robertson CL, Chanda AK, Schneider RW (2012) Effects of *Simplicillium lanosoniem* on *Phakopsora pachyrhizi*, the soybean rust pathogen, and its use as a biological control agent. *Phytopathology* 102:749–760
- Whipps JM (1993) A review of white rust (*Puccinia horiana* Henn.) disease on chrysanthemum and the potential for its biological control with *Verticillium lecanii* (Zimm.). *Ann Appl Biol* 122:173–187
- White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for Phylogenetics. *PCR Protocols*:315–322. <https://doi.org/10.1016/B978-0-12-372180-8.50042-1>
- Zhan G, Tian Y, Wang F, Chen X, Guo J, Jiao M, Huang L, Kang Z (2014) A novel fungal hyperparasite of *Puccinia striiformis* f. sp. *tritici*, the causal agent of wheat stripe rust. *PLoS One* 9:e111484
- Zhao J, Wang M, Chen X, Kang Z (2016) Role of alternate hosts in epidemiology and pathogen variation of cereal rusts. *Annu Rev Phytopathol* 54:207–228
- Zheng L, Zhao J, Liang X, Zhan G, Jiang S, Kang Z (2017) Identification of a novel *Alternaria alternata* strain able to hyperparasitize *Puccinia striiformis* f. sp. *tritici*, the causal agent of wheat stripe rust. *Front Microbiol* 8(71)