

Intraspecific variation of virulence and host-specificity in the mycoparasitic fungus *Clonostachys rosea*

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Abstract

Parasite-host interactions can display a continuum from specialization to generalism. *Clonostachys rosea* is an ascomycete fungus known for its ability to parasitize other fungi, a phenomenon known as mycoparasitism, and for its use as a biological control agent. *Clonostachys rosea* is reported to parasitize range of different hosts, yet our understanding of the evolutionary dynamics shaping its virulence and host range remains limited. In this study, up to 63 *C. rosea* strains were assessed for virulence towards host fungi and an oomycete, under the hypothesis that strains differ in virulence and host specialization. Results revealed significant ($P \leq 0.05$) variation in virulence between *C. rosea* strains towards the host fungi *Botrytis cinerea*, *Fusarium graminearum* and *Rhizoctonia solani* and an overall lack of correlation between hosts, suggesting a certain level of host specificity. There was no significant correlation between *in vitro* antagonism of *F. graminearum* and biocontrol of fusarium root rot disease on wheat, emphasizing the complexity of the biocontrol trait. Differences in virulence was further hypothesized to partially depend on different interaction mechanisms towards the intrinsically different host fungi. Genome-wide association analysis identified seven single nucleotide polymorphisms significantly ($P \leq 1.6 \times 10^{-6}$) segregating with virulence or biocontrol. Different genomic regions segregated with virulence towards different hosts, suggest different underlying virulence determinants. These results are important for our increased understanding of the evolution and ecology of mycoparasitic fungi, with implications for their exploitation in agricultural applications.

Introduction

Microbial parasites of plants and animals can have devastating effects on ecosystem stability, agricultural production and food security. However, parasites can themselves be parasitized by other microorganisms, a phenomenon referred to as hyperparasitism (Gleason et al., 2014). The term hyperparasitism is used irrespective of the taxonomic origin of the hyperparasite and the parasite host and encompasses both obligate and facultative relationships (Gleason et al., 2014). Ecologically, hyperparasites can influence the population size and stability of the parasites, which in turn affects host populations through top-down cascades (Hairston et al., 1960). The ability of microbial hyperparasites to decrease plant pathogen populations through top-down cascades is the basis for their use as biological control agents (BCAs) in agriculture. Certain hyperparasites have therefore been studied to some extent, including species from the fungal genera *Trichoderma* (Druzhinina et al., 2011) and *Clonostachys* (Jensen et al., 2022), the CHV1 virus (Milgroom and Cortesi, 2004) and the oomycete *Pythium oligandrum* (Benhamou et al., 2012). Our understanding of the population dynamics and evolution of microbial hyperparasites can at best be described as superficial, likely due to the inconspicuous nature of these microorganisms, and the inherent difficulties in studying them. Additionally, available research is in many aspects driven within an applied anthropogenic context with the aim to reduce yield losses in agriculture, which have contributed to the lack of empirical data regarding the evolutionary dynamics affecting hyperparasite populations.

The specific case where one fungus is parasitizing another fungus is referred to as mycoparasitism (Druzhinina et al., 2011). Mycoparasitism can involve a hyperparasitic interaction if the host fungus constitutes a pathogen, causing disease on another host. Well-known examples are species of filamentous hypocrealean fungi from the *Trichoderma* and *Clonostachys* genera. Most of these species are necrotrophic mycoparasites with broad host range, evidenced by the fact that single species, and sometimes single strains, are used commercially as efficient BCAs against a range of different plant pathogenic fungi including *Fusarium*, *Botrytis* and *Rhizoctonia* (Jensen et al., 2022; Woo et al., 2023). Both *Trichoderma* and *Clonostachys* spp. can directly parasitize other fungi by sensing the presence and growing towards the fungal host, penetrating cells using cell wall-degrading enzymes and by producing toxic specialized metabolites that poison the host (Karlsson et al., 2017).

Parasite-host interactions can display a continuum from specialization to generalism (Barrett et al., 2009). Parasite specialization may involve a complete inability to infect many hosts or a lower performance on certain hosts. At the other end of the spectrum, generalists can parasitize a multitude of host species. Host heterogeneity is hypothesized to be an important ecological driver for the evolution of parasite host range (Thrall et al., 2007). The parasite specialization-to-generalism continuum is also predicted to shape the infection mechanisms and virulence factors of parasites (Barrett et al., 2009). Although *Trichoderma* and *Clonostachys* spp. mycoparasites are assumed to have broad host ranges with little specificity, accumulating evidence predicts that a certain level of host specificity may exist. *Trichoderma* and *Clonostachys* strains from the same species can exhibit significant differences in *in vitro* antagonism against a host species and in biocontrol of a specific disease (Mamani et al., 2026; Iqbal et al., 2020, Anees et al., 2010b), suggesting specialization. Transcriptomic and small RNA sequencing data shows that *C. rosea* reacts with both common and specific gene expression during interactions with *Botrytis cinerea* and *Fusarium graminearum*, suggesting that *C. rosea* can distinguish between host species and modulate its responses accordingly (Nygren, 2018; Piombo et al., 2021, 2022). Anees et al. (2010a) showed that *Trichoderma* strains isolated from disease patches caused by the pathogen *Rhizoctonia solani* on sugar beet were more antagonistic against *R. solani* than strains isolated from field soil outside the patches, which also resulted in higher biocontrol ability. Taken together, this shows the potential for very fast and local adaptation of mycoparasite populations, by selection of highly virulent strains driven by interactions with host fungi.

The identification of strains displaying host-specific virulence could further improve the exploitation of these interactions for biocontrol applications. Biocontrol refers to the use of living agents, directly or indirectly, to suppress the growth, survival, or impact of target pests or pathogens, representing a promising alternative to conventional control strategies (Stenberg et al, 2021). Although promising, a number of challenges still remain for biocontrol to reach its full potential, including variation in efficacy, formulation, shelf-life, and poor establishment in soils. High strain-to-strain variation in biocontrol ability is a long-standing axiom in biocontrol research (Hermosa et al. 2000, Jensen et al. 2016, Knudsen et al. 1997). The relatively limited number of strains currently exploited in biocontrol suggest the identification and selection of additional strains to improve efficacy and specificity of this application. Evaluating potential BCAs under *in vitro* conditions may however not necessarily translate into effective biocontrol

performance. The reason being that the efficacy of BCAs is influenced by the complexity of the environment in which they are introduced, competition for space, plant effects and abiotic factors that may influence their establishment and efficacy (Scott and Punja, 2025). In this context, understanding the genetic basis underlying strain variation in virulence and host association could provide a complementary strategy for identifying traits associated with biocontrol efficacy.

Genome-wide association (GWA) analysis constitutes a powerful tool to study the genetic architecture of phenotypic traits. It is based on the identification of single nucleotide polymorphisms (SNPs) in different individuals that is used to establish relationships between genetic and phenotypic variation (Dutta et al., 2023, Bush & Moore, 2012). A population genomic dataset of *C. rosea* is available (Broberg et al., 2018; Manyara et al., 2026) and has been used to identify SNPs segregating with growth rate at cold temperature (Broberg et al., 2018) and with the ability of *C. rosea* to produce nematocidal compounds (Iqbal et al., 2020). Importantly, two separate lineages of *C. rosea* with different reproductive strategies are identified: one homothallic lineage capable of selfing and one presumably outcrossing heterothallic lineage (Manyara et al., 2026). As GWA analyses depend on enough recombination between individuals, it is recommended to exclude homothallic *C. rosea* strains from such investigations (Manyara et al., 2026).

In this study, we evaluated the virulence of 63 *C. rosea* strains using an *in vitro* interaction assay with a range of taxonomically different host species, including Ascomycota, Basidiomycota and Oomycota. We hypothesize that (i) *C. rosea* strains differ in virulence towards specific hosts, (ii) *C. rosea* strains differ in their level of host specialization towards different hosts, (iii) *in vitro* antagonism is not correlating with biocontrol efficacy, and (iv) variation in virulence towards different hosts is associated with specific genetic determinants. Our results show that *C. rosea* strains indeed differ significantly in their virulence and host specificity, while there is no significant correlation between *in vitro* antagonism and biocontrol. Furthermore, a GWA analysis showed that different genomic regions are segregating with virulence towards different host species, indicating mechanistic differences of virulence. This study offers empirical evidence providing novel insights into the knowledge-gap on the evolution of parasitism in fungal-fungal interactions. These results are important for our increased understanding of the fundamental ecology of mycoparasites, with clear implications for their exploitation as BCAs in agricultural production.

Materials and methods

Fungal strains and culture conditions

Sixty-three strains of *C. rosea* (Table S1), *B. cinerea* strain B05.10, *F. graminearum* strain PH-1, *R. solani* strain AG2-2IIIB and *Aphanomyces euteiches* strain SE51 were maintained on potato dextrose agar (PDA; Oxoid, Cambridge, UK) medium at 20°C. The *C. rosea* collection included eleven homothallic and 52 heterothallic strains (Table S1, Manyara et al. 2026).

In vitro confrontation assays

The ability of *C. rosea* strains to antagonize and overgrow taxonomically distinct hosts was assessed using an *in vitro* agar plate (nine cm diameter) confrontation assay on PDA medium as described before (Dubey et al. 2014). The growth rate of *B. cinerea*, *F. graminearum*, *R. solani* and *A. euteiches* was measured daily until their mycelial front touched the *C. rosea* mycelial front. After mycelial contact between *C. rosea* strains and the hosts, the growth rate of *C. rosea* strains was measured until *C. rosea* reached the other side of the plate and completely colonized the host mycelia. The intrinsic growth rate of *C. rosea*, *B. cinerea*, *F. graminearum*, *R. solani* and *A. euteiches* was measured on PDA medium, without confrontation. The plate confrontation assay was performed in at least three biological replicates.

Biocontrol assay

The biocontrol ability of *C. rosea* strains was evaluated for fusarium foot rot caused by *F. graminearum* using a sand seedling test (Knudsen et al., 1995, Dubey et al., 2014). Briefly, surface-sterilized wheat seeds were treated with *C. rosea* conidia (1×10^6 conidia/ml) in sterile water for 30 minutes on a rotary shaker at 100 rpm. Water-treated seeds were used as a control. Seeds were planted in a 2 cm hole in moist sand within individual 5x5x5 cm plastic pots, three seeds per hole. Pathogen inoculation was carried out by placing a 5 mm PDA plug with *F. graminearum* strain PH1 mycelia near the seeds. The holes were covered with moist sand, and the pots were incubated in a growth chamber with a 12-hour light ($150 \mu\text{mol m}^{-2} \text{s}^{-1}$) and 12-hour dark cycle, $70\% \pm 5\%$ humidity, and $15^\circ\text{C} \pm 1$ temperature. Seedlings were harvested two weeks after inoculation and disease severity was scored on a 0–4 scale (Knudsen et al. 1995): 0 = healthy, no symptoms; 1 = minor browning of roots/coleoptiles; 2 = moderate browning; 3 = severe browning; 4 = dead plants.

Genome-wide association analysis

A set of SNPs generated from the 63 *C. rosea* strains used in this study was retrieved from Manyara et al. (2026). Genome-wide association analysis was conducted on a subset of 52 heterothallic, potentially outcrossing *C. rosea* strains (Table S1) using the Genome Associated Prediction Integrated Tool (GAPIT) v3 package (Wang & Zhang 2021) in R v4.4.2 (R Core Team 2025). To reduce linkage disequilibrium (LD), SNPs were pruned with PLINK v1.90b7 (Purcell et al., 2007) using a window size of 50 SNPs, step size of 5 SNPs, and an LD threshold of 0.5. Additional filtering retained only SNPs with depth > 20, no missing data, and minor allele frequency > 10%. After filtering, 30166 SNPs remained for analysis. Genome-wide association was performed using a Multiple Loci Mixed Linear Model (MLMM) (Segura et al., 2012), incorporating kinship matrices and population assignment probabilities as covariates to control for population structure and relatedness. Phenotypic data included host growth rate inhibition, *C. rosea* overgrowth rate of hosts, and *C. rosea* biocontrol of fusarium foot and root rot disease on wheat, measured across the 52 strains. Statistical significance was determined using a Bonferroni threshold ($P \leq 1.6 \times 10^{-6}$; based on $0.05/30166$ SNPs) as well as a false discovery rate (FDR, $P \leq 0.05$). For each significant SNP, genomic features within ± 10 kb and ± 100 kb were extracted from the *C. rosea* IK726 v2 reference annotation (Broberg et al. 2018) for evaluation.

Statistical analysis

Differences between *C. rosea* strains regarding host growth rate inhibition, *C. rosea* overgrowth rate of hosts from the *in vitro* confrontation assay, and *C. rosea* biocontrol were determined using one-way analysis of variance (ANOVA) in Minitab 20 at the significance level $P \leq 0.05$. Post-hoc pairwise comparisons were made using the Tukey's and Fisher's test at the $P \leq 0.05$ significance level. Correlations between phenotypes were analyzed using Minitab 20.

Results

Antagonism of *Clonostachys rosea* strains against *Rhizoctonia solani*

The intrinsic growth rate of *R. solani* on PDA was 12.67 ± 0.27 mm/day. The growth rate of *R. solani* was significantly ($P \leq 0.05$) reduced when confronted with 27 out of 63 *C. rosea* strains (Fig. 1). In addition, there were significant ($P \leq 0.001$) differences between *C. rosea* strains in their ability to reduce *R. solani* growth rate (Fig. 1a; Table S2). The largest reduction in *R. solani* growth rate was observed in interactions with *C. rosea* strains CBS 907.72E, CBS 154.27 and CBS 705.97, where growth rate was reduced by 32.3, 28.3 and 27 percent, respectively.

After mycelial contact between *R. solani* and *C. rosea*, the growth rate for *C. rosea* strains when overgrowing the *R. solani* mycelia was measured. There were significant ($P \leq 0.001$) differences in growth rate between different *C. rosea* strains (Fig. 2a). The highest growth rate during *R. solani* overgrowth was observed for *C. rosea* strains IK216, CBS 548.79 and CBS 222.93, with 2.18, 2.15 and 2.08 mm/day, respectively. In contrast, the lowest growth rates were observed for *C. rosea* strains 1830, CBS 569.69 and CBS 100000, with growth rates of 0.13, 0.20 and 0.28 mm/day, respectively.

The intrinsic growth rates of *C. rosea* strains on PDA, without being confronted with another fungus, varied from 0.66 mm/day to 2.66 mm/day, and some of these differences were significant ($P \leq 0.05$) (Table S10). However, the correlation between *C. rosea* intrinsic growth rates and the growth rates during overgrowth on *R. solani* was not significant ($P = 0.073$) (Fig. S3). More specifically, growth rates of 54 *C. rosea* strains were lower during *R. solani* colonization compared with their intrinsic growth rate, while growth rates for *C. rosea* strains IBT7839, IK216, IK371, 1701, 1832, 1881, CBS 548.79, CBS 649.80, CBS 708.97 were higher during overgrowth of *R. solani* compared with their intrinsic growth rate.

Antagonism of *Clonostachys rosea* strains against *Botrytis cinerea*

The intrinsic growth rate of *B. cinerea* on PDA was 10.88 ± 0.87 mm/day. A significant ($P \leq 0.05$) reduction in growth rate of *B. cinerea* was observed upon confrontation with only one out of 63 *C. rosea* strains (Fig. 1b). This strain, 2176, reduced *B. cinerea* growth rate by 20.1 percent. Interestingly, eleven strains significantly ($P \leq 0.05$) increased *B. cinerea* growth rate up to 25.6 percent as compared with *B. cinerea* control. Furthermore, the effect on *B. cinerea* growth rate varied significantly ($P \leq 0.001$) between *C. rosea* strains (Fig. 1b; Table S3).

Significant ($P \leq 0.001$) differences between *C. rosea* strains in their ability to overgrow *B. cinerea* were observed (Fig. 2b; Table S7). The highest growth rates were recorded for strains IK216, B14 and CBS 103.94, reaching 1.87, 1.82 and 1.80 mm/day, respectively. The lowest growth rates were detected for strains GJS89-34, CBS 289.78 and SHW-3-1 with mean values of 0.52, 0.57 and 0.67 mm/day, respectively. There was no correlation ($P = 0.522$) between the intrinsic growth rate of *C. rosea* strains and their ability to overgrow *B. cinerea* (Fig. S5). For instance, 52 *C. rosea* strains displayed lower growth rates during *B. cinerea* colonization, whereas strains IK216, IK371, B14, 1827, 1829, 2177, CBS 115884, NHH-61-2, CBS 100502, CBS 708.97 and JLB-7-1 exhibited increased growth rates during overgrowth of *B. cinerea* compared with their intrinsic growth rate.

Antagonism of *Clonostachys rosea* strains against *Fusarium graminearum*

The intrinsic growth rate of *F. graminearum* on PDA was 7.9 ± 0.95 mm/day. A significant ($P \leq 0.001$) reduction in growth rate was observed upon confrontation with four out of 53 *C. rosea* strains (Fig. 1c). Differences in the ability to inhibit *F. graminearum* growth were statistically significant ($P \leq 0.05$) across *C. rosea* strains (Fig. 1; Table S4). Strains GG-1-2 and SHW-1-1 showed the most prominent inhibitory effects, decreasing *F. graminearum* growth rate by 35.1 percent, while strains CBS 907.72G and CBS 222.93 decreased *F. graminearum* growth rate by 33.5 percent.

In direct interaction with *F. graminearum*, the overgrowth ability of *C. rosea* strains varied significantly ($P \leq 0.001$, Fig. 2c; Table S8). The fastest overgrowth was observed in strains IK371, IK216 and 2178 with 2.27, 2.20 and 2.19 mm/day, respectively, while strains CBS 706.97, GJS89-34, CMI192798, CBS 178.28, CBS 188.33, CBS 222.93, CBS 421.87, CBS 277.50 and CBS 569.69 could not overgrow *F. graminearum* at all. There was no significant ($P = 0.477$) correlation between the *C. rosea* intrinsic growth rates and the growth rates during overgrowth of *F. graminearum* (Fig. S3).

Antagonism of *Clonostachys rosea* strains against *Aphanomyces euteiches*

Aphanomyces euteiches exhibited a mean intrinsic growth rate of 7.86 ± 0.25 mm/day. No significant reduction in growth rate was observed in *A. euteiches* when confronted with 52 *C. rosea* strains (Fig. 1d). During overgrowth of *A. euteiches* by *C. rosea*, growth rate measurements revealed no significant ($P = 0.053$) differences between strains (Fig. 2). Strains CBS 708.97, NHH-48-2 and 2176 displayed the highest growth rates, with values of 3.58, 3.42 and 2.60 mm/day. Strains CBS 569.69, SHW-1-1 and GJS89-34 exhibited the lowest growth rate, with a mean of 1.41, 1.60 and 1.62 mm/day, respectively. Intrinsic growth rates of *C. rosea* strains were not significantly ($P = 0.352$) correlated with overgrowth rates (Fig. S3).

Ability of *Clonostachys rosea* strains to control fusarium root rot caused by *F. graminearum*

Using an *in planta* bioassay on wheat, the *F. graminearum* infection treatment resulted in an average disease index of 3.27. In total, 19 out of 54 *C. rosea* strains significantly ($P < 0.05$) reduced the disease index (Fig. 3). There were significant ($P < 0.05$) differences between *C. rosea* strains in their ability to

control the disease. *C. rosea* strains IK726, SDT-5-1 and CBS 178.28 were recorded to be the most effective strains in reducing disease with disease index score of 1.84, 1.88 and 1.91 respectively.

Comparison of *Clonostachys rosea* antagonism towards different hosts and biocontrol activity

The antagonistic behavior of *C. rosea* strains towards different hosts (host specificity) were compared. There were no significant ($P \geq 0.07$) correlations between any host growth rate inhibition comparisons, with the exception for a positive correlation ($P = 0.029$, $R = 0.30$) between the growth rate inhibition of *B. cinerea* and *A. euteiches*. In the case of *C. rosea* overgrowth, there were no significant ($P \geq 0.05$) correlations between any host overgrowth rate comparisons, with the exception for a positive correlation ($P < 0.001$, $R = 0.54$) between the ability of *C. rosea* strains to overgrow *R. solani* and *B. cinerea*.

In vitro antagonism data towards *F. graminearum* were further compared with *C. rosea* biocontrol of fusarium foot and root rot disease caused by *Fusarium graminearum*. There were no significant correlations between neither *F. graminearum in vitro* growth rate reduction ($P \geq 0.273$, $R = -0.155$), nor *C. rosea* overgrowth on *F. graminearum* ($P \geq 0.155$, $R = -0.200$) when compared with biocontrol data.

Genome-wide genetic marker – trait associations

In the GWA analysis, 30166 SNPs across the *C. rosea* genome were tested for association with multiple virulence traits using a Bonferroni-corrected threshold of $P < 1.6 \times 10^{-6}$. Virulence data for *A. euteiches* interactions were not analyzed due to the lack of significant differences between *C. rosea* strains. Seven SNPs distributed across seven different contigs were significantly associated with *F. graminearum* biocontrol, *F. graminearum* growth rate inhibition, and *C. rosea* overgrowth on *B. cinerea*, *F. graminearum* and *R. solani* (Fig. 4; Table 1). Four nonsynonymous SNPs were in coding regions, while three were intergenic. For the *F. graminearum* growth rate inhibition trait, SNP marker CADEHS020000005.2_4130362 on contig CADEHS020000005.2 was in a predicted membrane Na^+/H^+ antiporter protein (Table 1). For the *C. rosea* host overgrowth rate trait, SNP markers were localized in genes predicted to encode malate synthase, a fungal transcription factor, and a F-box domain superfamily protein, respectively (Table 1). Three SNPs were in intergenic regions close to genes with predicted functions related to growth regulation and signal transduction.

Discussion

In this study, up to 63 strains of the mycoparasitic fungus *C. rosea* were assessed for virulence towards taxonomically different hosts. The selected *C. rosea* strains are genetically diverse (Manyara et al., 2026) and have previously been evaluated in terms of their growth rate at cold temperatures (Broberg et al., 2018) and their production of nematocidal compounds (Iqbal et al., 2020). The selected host microorganisms represent the phyla Ascomycota, Basidiomycota and Oomycota in order to maximize the differences in the virulence mechanisms employed by *C. rosea*. The selected hosts differ in cell wall composition (Mélida et al., 2013) and production of specialized metabolites (Chamoun et al., 2015, Serrano et al., 2017). In the current work, virulence was assessed by growth inhibition of the host species

and by *C. rosea* overgrowth of the hosts. The lack of correlation between the intrinsic growth rates of *C. rosea* strains without confrontation and their growth rates during host interaction shows that the presence of host species influences the behaviour of the mycoparasite. Therefore, *C. rosea* virulence assessments were not normalized by the intrinsic growth rates.

Different host species responded differently to the presence of *C. rosea* strains. The reduction of growth rate recorded for the hosts *B. cinerea*, *F. graminearum* and *R. solani*, suggests secretion of growth inhibitory specialized metabolites or enzymes from *C. rosea*. Several polyketide and non-ribosomal peptide compounds (Piombo et al., 2021; Han et al., 2020; Fatema et al., 2018; Rodrigues et al., 2011) are reported to be produced by *C. rosea*, and several of these display an inhibitory effect on other fungi (Piombo et al., 2021; Fatema et al., 2018; Iqbal et al., 2019; Rodrigues et al., 2011). Additionally, *C. rosea* is reported to produce glucanases, chitinases and cellulases (Chatterton and Punja, 2009; Mamarabadi et al., 2009; Inglis and Kawchuk, 2002), some that influence the interaction with hosts, such as the endochitinase-encoding genes *chiC2* and *chi67-1*, reported to contribute to the inhibitory activity of *C. rosea* against *R. solani*, *B. cinerea* and *Sclerotinia sclerotiorum* (Tzelepis et al., 2015; Sun et al., 2017). The lack of inhibitory activity of *C. rosea* on *A. euteiches* is similar to data reported in Xue et al. (2003), where *C. rosea* showed no inhibition of *A. euteiches* in dual culture before hyphal contact. The observation that eleven *C. rosea* strains increased the growth rate of *B. cinerea* during the initial stage of their interaction resembles data from Zapparata et al. (2021) where *F. graminearum* growth rate was stimulated by the presence of *T. gamsii* during confrontation. While a change in medium pH was excluded as a mechanism for this phenomenon, it was speculated that chemical compounds secreted by the mycoparasite caused metabolic changes in *F. graminearum* (Zapparata et al., 2021).

After hyphal contact, *C. rosea* strains differed in their ability to overgrow their hosts. Furthermore, the overall lack of correlation between virulence towards different hosts suggests a certain level of host specialization of individual *C. rosea* strains. It is likely that cell wall degrading enzymes produced by *C. rosea*, such as chitinases and glucanases (Mamarabadi et al., 2009), contribute to this ability, as been shown for the β -N-acetylhexosaminidase gene *nag1* and the endochitinase genes *ech42* and *ech37* (Mamarabadi et al., 2008). The particularly low rates of overgrowth of *F. graminearum* emphasize the necessity for *C. rosea* to adopt specific strategies depending on the host. It is reported that *F. graminearum* produces specialized metabolites that may be toxic for other fungi, including zearalenone (Utermark and Karlovsky, 2007), and nivalenol (Muller et al., 2012). Deoxynivalenol is reported to inhibit chitinase gene expression in the mycoparasite *Trichoderma atroviride* (Lutz et al., 2003), emphasizing the need for mycoparasites to adapt to their hosts. Taken together, the important observation that different strains of *C. rosea* exhibited different virulence levels towards *B. cinerea*, *F. graminearum* and *R. solani* suggests genetic variation in production of virulence determinants, which may constitute specialized metabolites, secreted enzymes, efflux transporter proteins or other factors (Jensen et al., 2022). Data on phenotypic intraspecific variation in *C. rosea* are rare, but variation in growth rate at different temperatures (Broberg et al. 2018), secretion of nematocidal compounds (Iqbal et al., 2020) and growth inhibition of *Fusarium graminearum* (Bahadoor et al., 2023) are reported. Intraspecific variation in *in vitro*

antagonism against *R. solani*, and biocontrol of damping off disease on carrot seedlings, is reported for *Trichoderma* spp. (Anees et al., 2010b).

The lack of correlation between antagonism and biocontrol of *C. rosea* against *F. graminearum* may appear surprising. However, previous studies have reported limited or absent correlation between *in vitro* antagonism and biocontrol efficacy, highlighting the difficulty of using *in vitro* assay alone to identify effective BCA strains (Knudsen et al., 1997). The reason behind this is likely that biocontrol is a complex trait involving several mechanisms (competition, antibiosis, parasitism and induction of plant defence), while the *in vitro* tests completely ignore the indirect, plant-related biocontrol effects. For example, *C. rosea* strain SYP-4-2 was recorded to have very high *in vitro* virulence on *F. graminearum*, while it recorded no significant reduction ($P \geq 0.194$) of disease index in the pot bioassay. In contrast, *C. rosea* strain CBS 178.28 was not able to overgrow *F. graminearum* *in vitro*, although the same strain significantly reduced ($P \leq 0.05$) the disease index in pot bioassay. Overall, the data highlights how *in vitro* responses alone do not reliably translate to biocontrol efficacy. Therefore, *in vitro* assays should not be used when screening for efficient BCAs.

The variation in virulence towards different hosts varied along the specialist-to-generalist continuum for different *C. rosea* strains. The observed variation across different fungal hosts can be associated with secreted compound of *C. rosea*, as previous studies on *lysM1* effector and *dcl2* dicer gene deletion strains demonstrated a reduced antagonistic ability of the mutants towards *B. cinerea*, whereas no reduction was observed towards *F. graminearum* (Dubey et al., 2020; Piombo et al., 2021). Similarly in this study, *C. rosea* strain CBS 421.87 displayed high virulence towards a single host, *R. solani*, and low virulence towards *B. cinerea*. We can hypothesize that certain *C. rosea* populations have evolved in environments dominated by few, abundant host species, while other have evolved in environments where host species are rare, providing ecological drivers for evolution of specialists and generalists, respectively (Jaenike, 1990). Increased abundance of *R. solani* in soil from sugar beet disease foci correlated with increased abundance of *Trichoderma* mycoparasites, as well as increased virulence in *Trichoderma* strains towards *R. solani* (Anees et al., 2010a), providing evidence that host species can drive specialization in mycoparasite populations. In the current work, we acknowledge the limited range of tested host species and the need for additional empirical data from sampling natural populations of *C. rosea*.

The observed continuous range of *C. rosea* virulence against host species suggests a polygenic inheritance of virulence with many alleles contributing small effects. This is expected given the complexity of the mycoparasitic infection process (Druzhinina et al., 2011; Karlsson et al., 2017). In the current work, the segregation of different genomic regions located on different scaffolds in *C. rosea* during interaction with different hosts provides further evidence for host-specific virulence mechanisms. The low number of segregating sites is expected given the low number of *C. rosea* strains available for the GWA analyses. Previously, transcriptomic data from *C. rosea* provides evidence of specific gene sets uniquely induced during interactions with *B. cinerea* or *F. graminearum*, respectively (Nygren et al., 2018; Piombo et al., 2021), as well as uniquely regulated small RNA sets (Piombo et al., 2022).

Out of the six SNPs identified to segregate with *F. graminearum* growth rate inhibition and *C. rosea* overgrowth on the three hosts, four SNPs were located in coding regions. SNP marker CADEHS020000005.2_4130362 associated with *F. graminearum* growth rate inhibition was located in a predicted membrane Na⁺/H⁺ antiporter protein. Similar transporters are reported to be involved in antifungal drug resistance and virulence in the human fungal pathogen *Cryptococcus neoformans* (Jung et al., 2012). For the *C. rosea* overgrowth rate in *B. cinerea* trait, SNP marker CADEHS020000004.2_2283774 was localized in the predicted malate synthase protein, which is part of the glyoxylate cycle. Inactivation of the glyoxylate cycle by deletion of the isocitrate lyase gene *icl* in *T. atroviride* resulted in mutants with a reduced ability to antagonise *B. cinerea* in plate confrontation assays (Dubey et al., 2013). SNP marker CADEHS020000596.2_157879 segregating with overgrowth of *F. graminearum* was located in a predicted Zn(II)₂Cys₆ transcription factor. A similar Zn(II)₂Cys₆ transcription factor was shown to regulate virulence and stress responses in *Penicillium expansum* (Zhu et al., 2025). Finally, SNP marker CADEHS020000009.2_1030681 segregating with overgrowth on *R. solani* was located in a predicted F-box domain-containing protein, possible involved in ubiquitin-dependent protein degradation (Nguyen et al., 2020; Kipreos et al., 2000). Establishment of causality between these genes and virulence requires further functional validation.

In this study, we show that strains of the mycoparasite *C. rosea* differ in virulence towards specific hosts, as well as in their level of host specialization. Furthermore, we show that different genomic regions are segregating with virulence towards different host species, indicating underlying mechanistic differences of virulence. This study offers empirical evidence on the evolution of parasitism in fungal–fungal interactions, emphasizing the need to further address the population dynamics and ecology of mycoparasites, with clear implications for their exploitation as BCAs in agricultural production.

Declarations

Competing interest

The authors declare that they have no competing interests. They have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

Alessandra Ruffino conducted the experiment, prepared the material, collected and analyzed the data and wrote the original draft of the manuscript. David Manyara and Edoardo Piombo performed the

bioinformatic data analysis. Esther Kuper performed part of the test and contributed to data analysis. Georgios Tzelepis, Hanna Friberg and Mukesh Dubey contributed to the study conception and design, supervised the research, and participated in the review and editing of the manuscript. Magnus Karlsson contributed to the study conception and methodology, supervised the overall project, and reviewed and edited the final manuscript. All authors read and approved the final manuscript.

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<https://doi.org/https://doi.org/10.1016/j.postharvbio.2025.113816>

Table

Tables 1 is available in the supplementary files section.

Figures

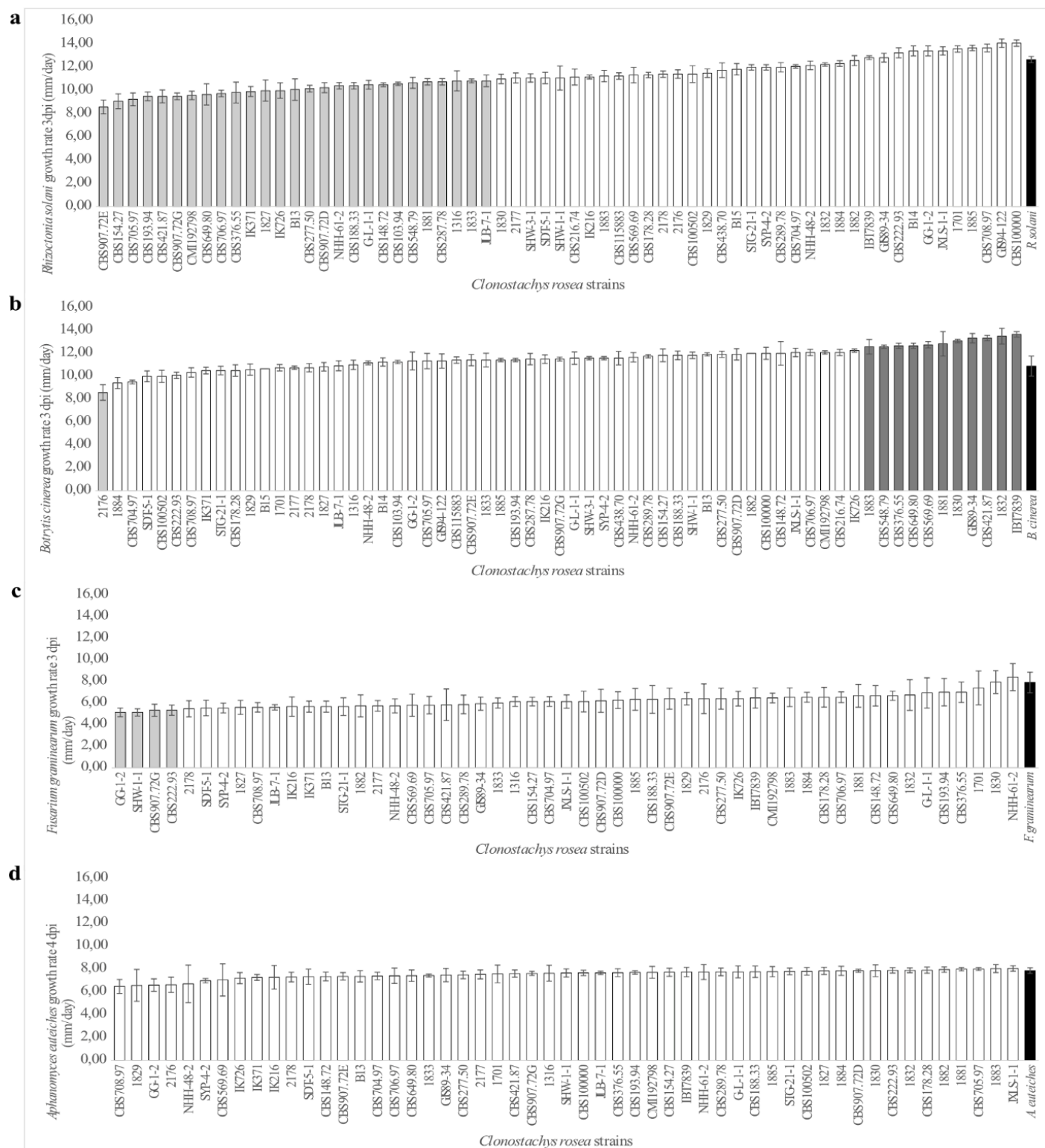


Figure 1

Growth rate inhibition of the tested hosts in interaction with *C. rosea* strains. In vitro assay conducted on potato dextrose agar and data expressed in mm/day. Each assay was compared with its tested host intrinsic growth rate as a control (black bars in the charts). Light grey bars indicate statistically significant growth inhibition ($P \leq 0.05$) of tested hosts within media based on Tukey's test. Dark grey bars indicate statistically significant growth promotion ($P \leq 0.05$) of tested hosts within media based on Tukey

a

Clonostachys rosea overgrowth on *Rhizoctonia solani* 10 dpi (mm/day)

b

Clonostachys rosea overgrowth on *Borytis cinerea* 10 dpi (mm/day)

c

Clonostachys rosea overgrowth on *Fusarium graminearum* 10 dpi (mm/day)

d

Clonostachys rosea overgrowth on *Aphanomyces etchiae* 10 dpi (mm/day)

Clonostachys rosea overgrowth rate on hosts. Data recorded 10 days after hyphal contact. In vitro assay conducted on potato dextrose agar and data expressed in mm/day. Error bars represent standard deviation based on at least 3 biological replicates. Rhizoctonia solanidata set was taken as a strain order reference. Strains marked in black represent missing data. (A) C. rosea overgrowth on Rhizoctonia solani. (B) C. rosea overgrowth on Botrytis cinerea. (C) C. rosea overgrowth on Fusarium graminearum. (D) C. rosea overgrowth on Aphanomyces euteiches

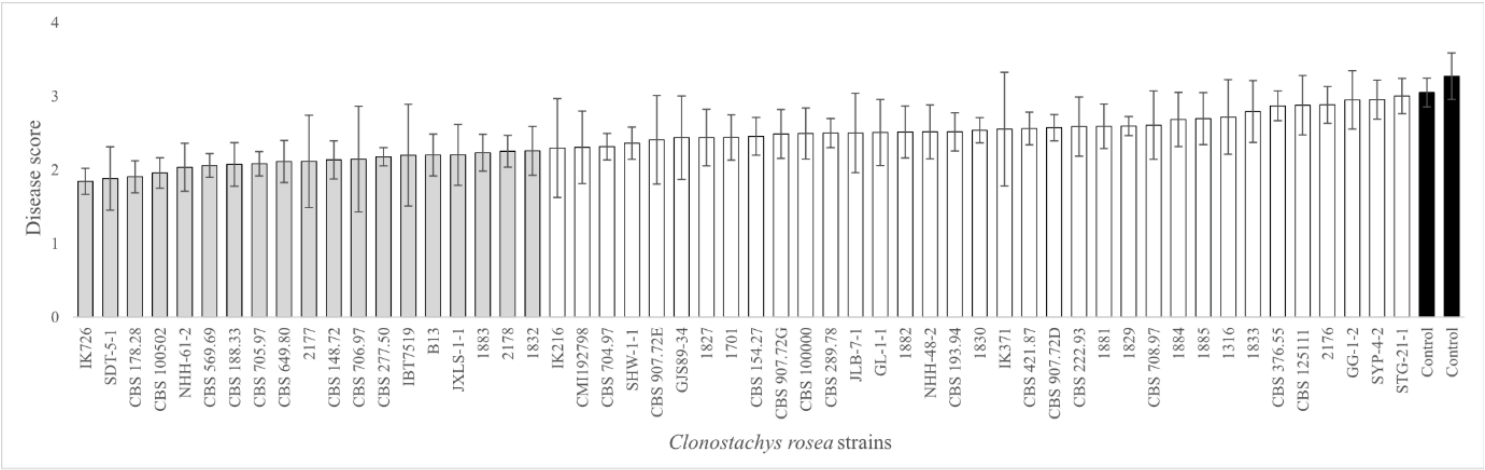


Figure 3

Screening of *Clonostachys rosea* strains biological control of fusarium foot rot disease on wheat caused by *F. graminearum*. Biocontrol assay conducted on pot and data expressed in disease severity score bases on a scale from 0 (healthy, no symptoms) to 5 (dead plant). *F. graminearum* was included as a control (black bars in the charts). Light grey bars indicate statistically significant disease index reduction ($P \leq 0.05$) within media based on Tukey’s test. Error bars represent standard deviation based on at least 4 biological replicates.

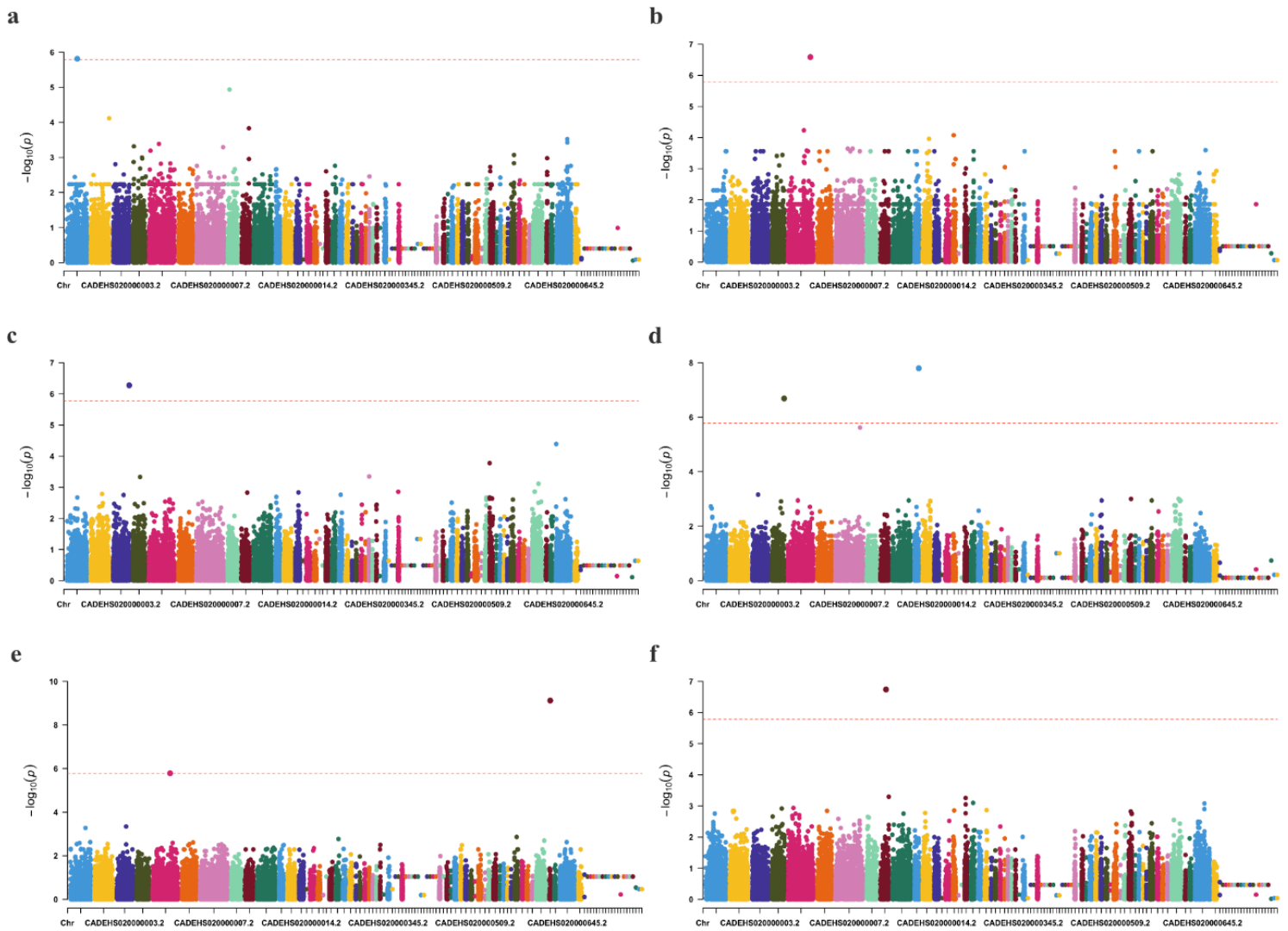


Figure 4

Genome-wide Manhattan plots of significant trait-associated SNPs in *Clonostachys rosea*. Panels A–E show Manhattan plots for five phenotypic traits measured across 52 *C. rosea* strains. (A) *C. rosea* biocontrol of *F. graminearum*. (B) *F. graminearum* growth inhibition. (C) *C. rosea* overgrowth on *A. euteiches*. (D) *C. rosea* overgrowth on *B. cinerea*. (E) *C. rosea* overgrowth on *F. graminearum*. (F) *C. rosea* overgrowth on *R. solani*. Each point represents a single SNP mapped onto the *C. rosea* IK726 reference genome, coloured by contig. The red dashed horizontal line marks the Bonferroni significance threshold ($P < 1.6 \times 10^{-6}$), above which associations were considered statistically significant.

Supplementary Files

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