Biological control of plant-parasitic nematodes by the fungus *Clonostachys rosea*

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Cover: Antagonism and biocontrol of fungus *Clonostachys rosea* against plant-parasitic nematodes. wheat plants in pot trial (upper panel), killed nematodes in culture filtrates of *C. rosea* (left) and fungal colonization in eggs of soybean cyst nematodes (right).

(photos: Upper and lower right by Mudassir Iqbal, lower left by Mukesh Dubey)

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Abstract

Plant diseases caused by plant-parasitic nematodes are serious constraints to sustainable crop production due to high yield losses, the persistent nature of these nematodes and a lack of efficient control methods. Biological control is a promising approach to reduce plant diseases caused by nematodes.

This study investigated the effect of the fungus *Clonostachys rosea* strain IK726 on nematode populations in a naturally nematode-infested soil planted with wheat in a climate chamber under controlled conditions. Populations of plant-parasitic nematodes extracted from soil and roots were 40 to 73% lower in soils when *C. rosea* was applied than in untreated soils, whereas non-parasitic nematodes were unaffected. Soil inoculation with *C. rosea* increased the shoot weight and shoot length of wheat plants by 20 and 24%, respectively. Light microscopy of *in vitro C. rosea*–nematode interactions did not reveal evidence of direct parasitism; however, culture filtrates of *C. rosea* grown in potato dextrose broth (PDB) exhibited toxicity towards nematodes and immobilized nematodes. A genome-wide analysis of protease genes showed that *C. rosea* contains more protease genes than other studied biocontrol fungi. A computational analysis of gene family evolution revealed a high gene copy number of serine protease subfamilies S8A, S9X and S33 in *C. rosea*, suggesting the involvement of these proteases in biotic interactions. A genome-wide association analysis of 53 strains of *C. rosea* further identified 279 single-nucleotide polymorphism markers that were significantly associated with the *in vitro* antagonism trait against plant-parasitic nematodes. Two non-ribosomal peptide synthetase (NRPS) genes (*nps4* and *nps5*) were identified in genomic regions associated with nematicidal activity whereas *nps1* was included based on previous published reports of functional studies. Gene deletion strains of *nps1*, *nps4* and *nps5* were generated and showed increased growth and conidiation rates. Culture filtrates from *C. rosea* Δ*nps1*, Δ*nps4* and Δ*nps5* strains exhibited reduced nematicidal activity and immobilized lower numbers of nematodes compared with the wild type after 24 h of incubation. However, NRPS deletion strains still possessed some nematicidal activity compared with the PDB control treatment, which may be due to the presence of additional nematicidal compounds or enzymes (e.g., serine proteases). Furthermore, Δ*nps1*, Δ*nps4* and Δ*nps5* strains showed reduced biocontrol efficacy in a naturally nematode-infested soil in a pot experiment and failed to reduce populations of nematodes in soil or in roots of wheat as efficiently as the wild type strain.

This study demonstrates that *C. rosea* can control plant-parasitic nematodes and improve the growth of plants at the same time. The antagonistic potential of *C. rosea* could be used to control plant-parasitic nematodes, which may contribute to reduced applications of chemicals as part of an integrated pest management programme.

Keywords: antagonism, antibiosis, biocontrol, fungi, nematodes, NRPS, proteases

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Biologisk bekämpning av växtparaitiska nematoder med svampen *Clonostachys rosea*

Abstract

Sjukdomar på växter som orsakas av växtparasitiska nematoder är ett allvarligt hot mot en hållbar växtproduktion genom höga skördeförluster och brist på effektiva växtskyddsmetoder. Biologisk bekämpning är en lovande metod för att minska skördeförluster orsakade av växtparasitiska nematoder. I den här studien undersöktes hur svampen *Clonostachys rosea* isolat IK726 påverkar nematod-populationer i en naturligt infekterad jord i ett försök med vete i klimatkammare med kontrollerad miljö. Populationerna av växtparasitiska nematoder i jorden minskade med 40 till 73% när *C. rosea* applicerades jämfört med jord utan *C. rosea*. Icke-växtparasitiska nematoder påverkades inte. Applicering av *C. rosea* i jorden ökade vikten och längden på veteplantorna med 20 respektive 24%. Undersökningar av interaktionen mellan *C. rosea* och nematoder med mikroskop resulterade inte i några bevis för direkt parasitism. Filtrat av potatis dextros vätskekulturer från *C. rosea* uppvisade dock toxicitet mot nematoder. En jämförande analys av proteas-gener i svampars arvsmassa visade att *C. rosea* innehåller fler proteas-gener än andra svampar som används för biologisk bekämpning. En evolutionär analys av genfamiljer av proteaser visade att underfamiljerna S8A, S9X och S33 innehåller höga antal gener i *C. rosea*, vilket indikerar att dessa proteaser har en viktig funktion i biotiska interaktioner. En storskalig associationsanalys av 53 isolat av *C. rosea* identifierade vidare 279 mutationer som segregerade med *in vitro* antagonism mot växtparasitiska nematoder. Två gener (*nps4* och *nps5*) för icke-ribosomala peptidsyntaser (NRPS) identifierades i de genomiska regioner som segregerade med antagonism, och ytterligare en NRPS gen (*nps1*) valdes ut för fortsatta funktionella studier baserat på befintlig litteratur. Mutanter av *C. rosea* där *nps1*, *nps4* respektive *nps5* inaktiverats med genetisk transformering uppvisade högre tillväxt och högre konidiering, jämfört med vildtypen. Filtrat från vätskekulturer av *C. rosea* mutanterna hade minskad toxicitet mot nematoder jämfört med vildtypen efter 24 timmars inkubation. Kulturfiltraten uppvisade dock fortfarande en viss toxicitet mot nematoder jämfört med kontrollbehandlingen, vilket indikerade att andra toxiska ämnen eller enzymer (t.ex. proteaser) fortfarande producerades av mutanterna. Inaktiveringen av *nps1*, *nps4* och *nps5* resulterade också i minskad förmåga till biologisk bekämpning av rotsjukdom hos vete orsakad av växtparasitiska nematoder hos mutanterna, jämfört med vildtypen.

Den här studien visar att *C. rosea* kan bekämpa sjukdomar orsakade av växtparasitiska nematoder och förbättra tillväxten hos växter. Dessa egenskaper kan potentiellt användas inom integrerat växtskydd och därmed bidra till minskad användning av kemiska växtskyddsmedel.

Keywords: biologisk bekämpning, svampen, nematoder, NRPS, proteaser

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Dedication

To my **beloved parents**

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Iqbal, M***., Dubey, M., McEwan, K., Menzel, U., Franko, M. A., Viketoft, M., Jensen, D. F. and Karlsson, M. (2018). Evaluation of *Clonostachys rosea* for control of plant-parasitic nematodes in soil and in roots of carrot and wheat. *Phytopathology*, 108(1), pp. 52-59.

II **Iqbal, M***., Dubey, M., Gudmundsson, M., Viketoft, M., Jensen, D. F. and Karlsson, M. (2018). Comparative evolutionary histories of fungal proteases reveal gene gains in the mycoparasitic and nematode-parasitic fungus *Clonostachys rosea*. *BMC Evolutionary Biology*, 18, 171.

III **Iqbal, M***., Dubey, M., Broberg, A., Viketoft. M., Jensen, D. F. and Karlsson, M. (2019). Deletion of the non-ribosomal peptide synthetase gene *nps1* in the fungus *Clonostachys rosea* attenuates antagonism and biocontrol of plant pathogenic *Fusarium* and nematodes. *Phytopathology*, 109(10), pp. 1698-1709.

IV **Iqbal, M***., Broberg, M., Haarith, D., Broberg, A., Bushley, K., Durling, M. B., Viketoft, M., Jensen, D. F., Dubey, M. and Karlsson, M. A genome-wide association study for *in vitro* antagonism of *Pratylenchus penetrans* nematodes identifies non-ribosomal peptide synthetases as biocontrol factors in the fungus *Clonostachys rosea* (submitted).

Papers I-III are reproduced with the permission of the publishers.

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Additional publications and manuscripts

1. Hyder, S., Inam, U. H. M., Fatima, N., Hannan, A., Alam, M. and **Iqbal, M**. (2019). First report of root rot of tomato caused by *Phytophthora syringae* in Pakistan. *Plant Disease*, 103(3), pp. 590.

2. Nygren, K., Dubey, M., Zapparata, A., **Iqbal, M**., Tzelepis, G. D., Durling, M. B., Jensen, D. F. and Karlsson, M. (2018). The mycoparasitic fungus *Clonostachys rosea* responds with both common and specific gene expression during interspecific interactions with fungal prey. *Evolutionary Applications*, 11(6), pp. 931-949.

3. Hyder, S., Inam, U. H. M., Ashfaq, M., Ahmad, A., Gondal, A. S and **Iqbal, M**. (2018). First report of *Pythium myriotylum* D., causing damping off and root rot in chili pepper (*Capsicum annum* L.) from Punjab, Pakistan. *Plant Disease*, 102(3), pp. 687.

4. **Iqbal**, M., Ehlers, R. U. and Waeyenberge, L. (2016). Molecular characterisation of novel isolates of entomopathogenic nematodes. *Nematology*, 18(3), pp. 277-291.

5. Khan, A. A., Dubey, M., Iqbal, Z., Atiq, M., Ahmed, W., **Iqbal, M**., Jensen, D. F. Karlsson, M. First report of *Penicillium citrinum* C., causing postharvest fruit rot of Kinnow (*Citrus reticulatae* B.) from Punjab, Pakistan (submitted).

6. Broberg, M., Dubey, M., **Iqbal, M**., Gudmundsson, M., Ihrmark, K., Schroers, H. J., Jensen, D. F., Durling, M. B. and Karlsson, M. Comparative genomics of the fungal genus *Clonostachys*, subgenus *Bionectria*, provides insights into the genetic background of mycoparasitism and biological control (manuscript).

The contribution of Mudassir Iqbal to the papers included in this thesis was as follows:

I Performed the experiments, generated the data and contributed to the analysis of data, wrote the paper with comments and suggestions from co-authors, and corresponded with the journal throughout the publication process.

II Performed the experiments, contributed to the analysis of the data and wrote the paper with comments and suggestions from co-authors, and corresponded with the journal throughout the publication process.

III Performed the experiments, generated the *nps1* deletion strains, carried out all phenotypic analyses of deletion strains, analysed the data and wrote the paper with comments and suggestions from co-authors, and corresponded with the journal throughout the publication process.

IV Performed the experiments, contributed to the analysis of the data and wrote the paper with comments and suggestions from co-authors, and corresponded with the journal throughout the publication process.

Abbreviations

BSA	Bovine Serum Albumin
CAFE	Computational Analysis of Gene Family Evolution
CDD	Conserved Domain Database
CFU	Colony Forming Unit
DPI	Days Post-Inoculation
E-flask	Erlenmeyer flask
ETP	Epipolythiodiketopiperazine
GWAS	Genome-Wide Association Study
hyg	Hygromycin
lfsr	Local False Sign Rate
MEGA	Molecular Evolutionary Genetics Analysis
NCBI	National Center for Biotechnology Information
NRPS	Non-Ribosomal Peptide Synthetase
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
SNA	Synthetic Nutrient Medium
SNP	Single Nucleotide Polymorphism
TCDB	Transporter Classification Database
UHPLC-MS	Ultra-High Performance Liquid Chromatography-Mass Spectrometry
WT	Wild Type

1 Introduction

Nematodes are multicellular, pseudocoelomate, unsegmented worm-like animals, and commonly described as filiform or threadlike, a feature reflected by the taxon name *nema* (Greek, *nema* = thread) and its nominative plural *nemata*. The most recent classification by De Ley & Blaxter (2002) classified nematodes into a separate phylum, Nematoda (Kingdom Animalia); however, based on the most recent molecular phylogenetic analyses, twelve clades are now recognized within the Nematoda (Megen *et al.*, 2009; Holterman *et al.*, 2006).

Nematodes are the most numerous Metazoa on earth, comprising > 25,000 known species, and display a wide range of feeding habits, including animal parasitism, plant parasitism, bacterivory, fungivory and omnivory. They occur in almost every habitat, although they are essentially aquatic animals (Fig. 1; Decraemer & Hunt, 2013; Blaxter 2003). A range of different factors such as soil moisture, relative humidity and other environmental factors greatly influence nematode locomotion and survival. Soil structure is very important because pore size affects the ease with which nematodes can move through the soil interstices. In general, sandy soils provide the best environment for terrestrial nematodes; however, certain specialized nematodes can also colonize saturated clay soils. The soil pH influences nematodes to a certain degree, although temperature is considered to have a greater influence on nematode survival (Decraemer & Hunt, 2013).

1.1 Plant-parasitic nematodes and their divisions

To date, more than 4100 known species of plant-parasitic nematodes have been described, displaying a wide range of interactions with their hosts (Decraemer & Hunt, 2013). Plant-parasitic nematodes are obligate biotrophs. Based on their parasitic behaviour, they have been classified into different groups, such as sedentary endoparasites, migratory endoparasites, semi-endoparasites and ectoparasites (Jones *et al.*, 2013).

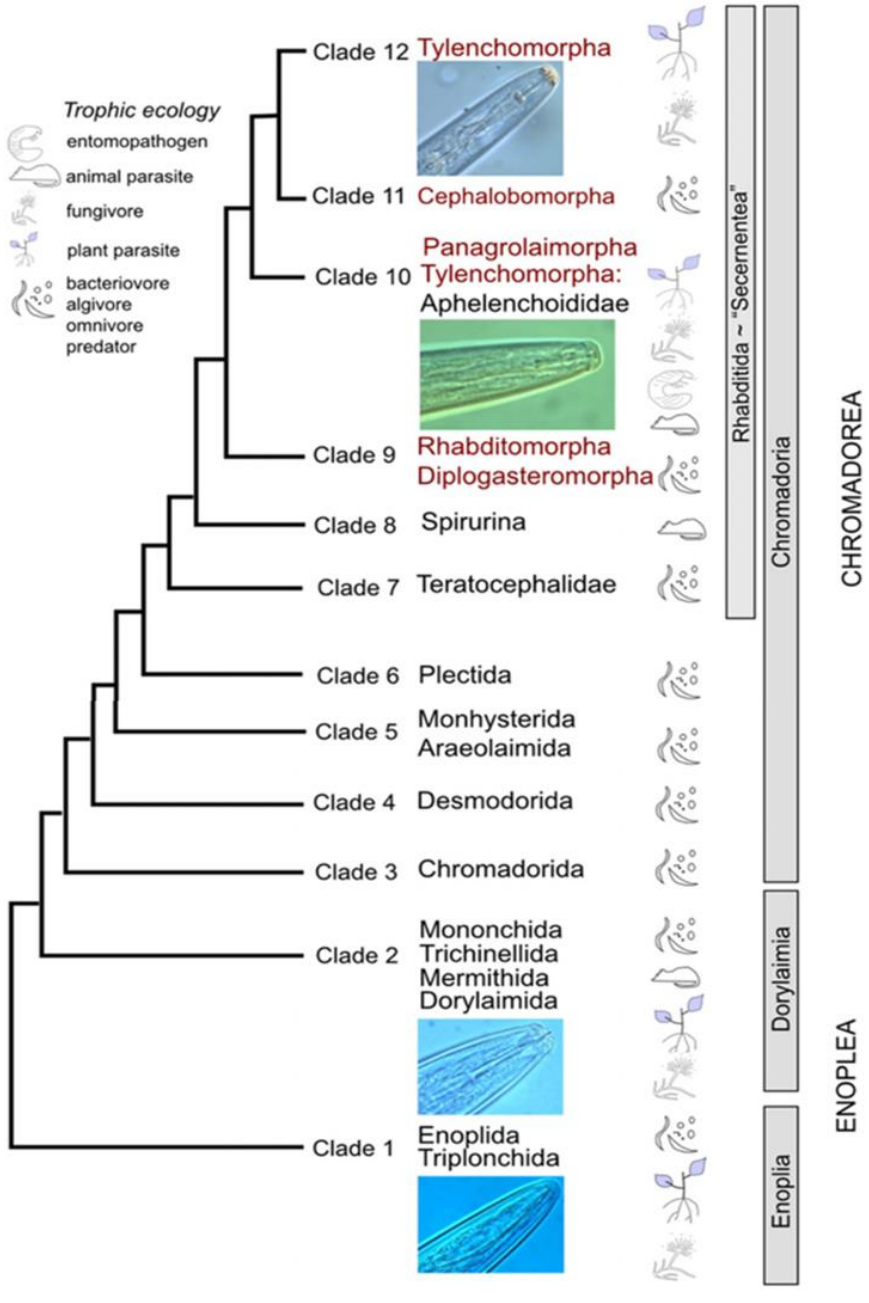

Figure 1. Interrelationship of nematode clades Megen *et al.* (2009). Different feeding groups of nematodes are shown alongside their phylogenetic positions. Infraorders (which are below suborders) are indicated in red. Figure from Jones *et al.* (2013) and reproduced with permission of the publisher.

1.1.1 Sedentary endoparasitic nematodes

Endoparasitism involves the entire nematode penetrating the root tissue and developing a permanent feeding site. This type of parasitism is shown by root-knot nematodes (*Meloidogyne* spp.) and cyst nematodes (*Heterodera* and *Globodera* spp.).

Root-knot nematodes

Root-knot nematodes are distributed globally and the genus *Meloidogyne* contains at least 98 known species, which parasitize almost every species of vascular plant (Jones *et al.*, 2013). Four species that are economically very important are *Meloidogyne arenaria*, *M. incognita*, *M. javanica* and *M. hapla* (Moens *et al.*, 2009).

Root-knot nematode eggs are confined within gelatinous egg sacs and are commonly found on the surface of galled roots and, occasionally, occur within galls. Following embryogenesis, the first moult occurs within the egg, giving rise to the infective, second-stage juvenile (J2). The hatching of *Meloidogyne* J2 from eggs is usually temperature dependent and occurs without a plant-root stimulus; however, root diffusates in some instances stimulate hatching (Curtis *et al.*, 2009). When J2 leave the egg masses, they start infecting galled roots in the vicinity or enter into new roots. Penetration generally occurs directly behind the root cap, but it can occur at any site. J2 penetrate the rigid root cell walls via a combination of physical damage, caused by thrusting their stylet, and enzymatic breakdown due to the secretion of cellulolytic and pectinolytic enzymes (Karssen *et al.*, 2013). After penetrating the plant, they start moving upwards in the vascular cylinder towards the zone of differentiation. The J2 starts to establish a permanent feeding site, which comprises numerous giant cells. These cells function as specialized sinks, providing nutrients to the now sedentary J2. The head of the J2 is implanted in the peripheral vascular tissue. After feeding, the J2 swells and undergoes three further moults to reach the reproductive adult stage. J3 and J4 stages lack a functional stylet and do not feed. Males are generally vermiform and leave the root. Adult females continue to feed and expand to become pear-shaped.

Root-knot nematodes have unbalanced sex ratios. They show an extraordinary range of reproductive strategies, ranging from amphimixis to obligatory mitotic parthenogenesis (Chitwood & Perry, 2009). Most species are parthenogenetic, with males only developing under hostile conditions. The response of a plant to parasitism by root-knot nematodes depends on the host

plant and cultivar. Different factors such as crop rotation, nematode population density, season and soil type influence the intensity of the damage. Typical symptoms include wilting, stunted growth, leaf discoloration, deformation and heavily galled roots (Jones *et al.*, 2013; Hofmann & Grundler, 2007).

Cyst nematodes

Cyst nematodes are also endoparasitic nematodes, as mentioned above, and have great economic importance across the world. The most devastating species include potato cyst nematodes (*Globodera rostochiensis* and *G. pallida*), the soybean cyst nematode (*Heterodera glycines*) and cereal cyst nematodes (*H. filipjevi* and *H. avenae*). Economic losses are hard to define; however, soybean cyst nematodes are responsible for annual losses of 1.5 billion US dollars in the USA alone (Chen *et al.*, 2011). Potato cyst nematodes have been estimated to cause the loss of approximately 9% of total potato production globally (Turner & Rowe, 2006). Losses caused by cereal cyst nematodes depend to a large extent on environmental factors but occasionally can cause the loss of up to 90% of the crop (Nicol *et al.*, 2011).

Cyst nematodes moult to form the J2 during the egg dormant stage of the life cycle, which depends on the species and environmental factors. Hatching is influenced by host-originated chemical cues present in root diffusates and this stage is considered the end of dormancy (Perry, 2002). The J2 then finds its host, attacks, migrates devastatingly and intracellularly through the root until it reaches the inner cortex where it finds a suitable cell for developing as a feeding site, which becomes the initial syncytial cell (Golinowski *et al.*, 1997). A syncytium is a large multinucleate feeding structure that is formed by the disintegration of the plant cell wall, which begins at the plasmodesmata and leads to the fusion of protoplasts of adjacent cells. The nematode stays at this feeding site for some weeks and undergoes three further moults to the J3, J4 and finally the adult stage. Females keep growing until their spherical bodies burst through the root surface, whereas males return to a vermiform body shape, exit the plant via a root and follow sex pheromone gradients to find females. After fertilization, the female dies and her body wall transforms into a cyst, which encloses the next generation of eggs.

1.1.2 Migratory endoparasitic nematodes

All life stages of migratory endoparasitic nematodes can be found within plant tissues. Unlike sedentary endoparasites, nematode species that belong to this group do not induce permanent feeding sites, but rather feed and reproduce while migrating between or through plant cells. Three nematode families are adapted

to a migratory endoparasitic lifestyle. Of these, nematode species belonging to the family Pratylenchidae primarily inhabit cortical cells in roots and other belowground plant parts. Species in the families Anguinidae and Aphelenchoididae have an unusual lifestyle because they parasitize aboveground parts of plants, such as stems, buds, leaves and seeds. Three genera in the Pratylenchidae family, i.e., *Pratylenchus* (lesion nematodes), *Hirschmanniella* (rice root nematodes) and *Radopholus* (burrowing nematodes), are economically very important (Duncan & Moens, 2013).

More than 60 known species of root-lesion nematode are found worldwide. Root-knot and cyst nematodes have the greatest negative impact on crops globally, with root-lesion nematodes ranked third (Castillo & Vovlas, 2007). Root-lesion nematodes are economically very important and are often underrated pests of various crops, such as cereals, causing wheat yield losses of up to 30% in Australia (Vanstone *et al.*, 2008). They also infect a range of other crops, including banana, coffee, sugarcane, legumes, maize and numerous vegetables and fruit trees (Castillo & Vovlas, 2007). Some root-lesion nematode species are known for their enzymatic degradation of host tissues or for inducing hormonal imbalances that cause galling, swelling and other tissue distortions, and others are known for causing visible lesions when phenolic compounds are released in and around wounded cells (Duncan & Moens, 2013).

The life cycle of root-lesion nematodes lasts 3–8 weeks, depending on the species and environmental factors. After developing within the egg to the J1 stage, the nematode moults to form the J2, which hatches from the egg. All succeeding juvenile and adult stages are worm-like and mobile and can have the ability to infect host plant roots. Adults and juveniles can enter and leave roots and females lay eggs either inside the roots or in soil in the vicinity of roots. Although males are common in most species, some species do not produce males; however, reproduction often happens by parthenogenesis (Duncan & Moens, 2013; Jones *et al.*, 2013).

1.1.3 Semi-endoparasitic nematodes

Only the anterior part of the body of semi-endoparasitic nematodes penetrates the root while the posterior part remains in the soil. This lifestyle is represented by the *Tylenchulus* and *Rotylenchulus* genera.

In economic terms, the reniform nematode (*Rotylenchulus reniformis*) is a very important species and attacks a large number of annual and perennial species. Crop injury caused by *R. reniformis* is prevalent in tropical and subtropical regions and it is an important pest in the southern USA. The broad host range includes more than 350 plant species, including many economically

important vegetables, fruits, ornamentals and fibre crops, as well as weeds that support nematode populations in the absence of host crops (Chen *et al.*, 2011; Robinson *et al.*, 1997). The aboveground symptoms caused by *R. reniformis* infection look like those caused by moisture and nutrient deficiencies. However, root necrosis increases and root growth diminishes with limited secondary root development. Infected plants are also severely stunted and chlorotic. The degree of crop loss depends on nematode population density, crop growth conditions and temperature.

R. reniformis has a peculiar life cycle. The J2 hatch from 8–10-day-old eggs and undergo three successive moults without feeding, and then grow into vermiform adult males or females within 7–9 days. The young females are the infective stage. They enter into the roots of plants, inserting about one-third of their anterior body, and establish feeding sites on endodermal and pericycle cells (Megen *et al.*, 2009; Gaur & Perry, 1991). Males are present in large numbers in most populations. Generally, males do not feed and remain in the soil; however, they are attracted to females and several may become entrapped in the gelatinous matrix produced by the female. In some populations, males are rare or absent, and these populations may reproduce by parthenogenesis.

1.1.4 Ectoparasitic nematodes

Ectoparasitic nematodes live and feed on the external surface of a host and do not enter the host tissue. Instead, they use their stylet to perforate the host tissue and feed on the cytoplasm. The ectoparasitic nematode group includes sting nematodes (*Belonolaimus* spp.), stubby root (*Trichodorus* spp.), dagger (*Xiphinema* spp.) and needle (*Longidorus* spp.) nematodes. (Decraemer & Hunt, 2013; Jones *et al.*, 2013; Moens *et al.*, 2009). Ectoparasitic nematodes can be grouped into three categories based on their parasitic behaviour. i) Migratory ectoparasites, such as species belonging to the genera *Belonolaimus*, *Dolichodorus* and *Trichodorus*, which generally stay vermiform throughout their entire life cycle and feed for short periods of time along the root system. ii). Sedentary ectoparasites, which may feed for several days on the same cell, either a cortical or an epidermal cell (Gheysen *et al.*, 2000). iii). Semi- or facultative ecto-endoparasites, such as species belonging to the *Helicotylenchus* and *Hoplolaimus* genera, which sometimes may act as ectoparasites (Decraemer & Geraert, 2013).

Ectoparasitic nematodes normally lay eggs in the soil, where they develop up to the J1 stage. Nematodes moult for the first time while still within the egg. Second stage juveniles leave the egg and move into the soil, find a host root, and penetrate the epidermal cells of the root surface with their stylet. After feeding

for a period, the nematode removes its stylet and moves to a new site on the root to feed. After a period of time, the nematode undergoes three further moults and develops into an adult male or female. All stages of ectoparasitic nematodes from J2 to adult are motile and vermiform and vigorously feed on host roots (Decraemer & Geraert, 2013).

1.2 Importance of plant-parasitic nematodes in field crops

Plant diseases caused by plant-parasitic nematodes remain a problem in all field crops. As mentioned earlier in section 1, nematode survival is favoured by sandy soils, moisture and high temperature. In Sweden, these conditions are common on farms growing root and horticultural crops and, consequently, sugar beets, tomato, potatoes and carrots are among the crops most affected by diseases caused by nematodes.

Over the past decades, organic farming has increased rapidly throughout the world and is likely to expand further in the future given that it is being supported by legislation and subsidies in many countries. Features of organic crop production, for instance intercropping, compost use, perennial lays, mulching and the frequent cultivation of legume species, favour the development of nematode disease. In a survey carried out in Germany on organic farms, growers reported increased nematode problems after shifting to organic production (Hallmann *et al.*, 2007). Increases in mean temperature and higher precipitation due to the ongoing process of climate change will also favour the development of crop diseases caused by nematodes, especially under organic settings. It is also likely that nematode problems are under-reported by farmers due to a lack of knowledge about the causal organism of these diseases and through confusing nematode disease symptoms with those caused by other organisms. The plant-parasitic nematode genera that have the greatest impact on Swedish agriculture are root-knot nematodes (*Meloidogyne* spp.), cyst nematodes (*Heterodera* spp.), root-lesion nematodes (*Pratylenchus* spp.), stubby root nematodes (*Trichodorus* spp. and *Paratrichodorus* spp.) and needle nematodes (*Longidorus* spp.) (Nicol *et al.*, 2007).

1.3 Crop losses caused by plant-parasitic nematodes

Crop losses due to plant-parasitic nematodes not only have an economic impact on agricultural crop production but also can affect food security. Plant-parasitic nematodes constitute 15% of the total population of nematodes and on average are estimated to cause annual crop losses worldwide of up to 14.6%. In economic

terms, this is equivalent to losses of 157 billion US dollars annually (Abad *et al.*, 2008); however, occasionally, parasitism of a crop by nematodes can lead to the complete loss of a marketable yield (Nicol *et al.*, 2011; Barker & Koenning, 1998).

Cereal cyst nematodes (*Heterodera* spp.) can significantly decrease wheat yields and negatively affect other important cereals, including barley and oats. *H. avenae* in wheat has been estimated to cause yield losses of 30 to 100%, causing economic losses of 3.4 million US dollars every year in Idaho, Oregon and Washington (Nicol *et al.*, 2007; Bonfil *et al.*, 2004). In addition, root-lesion nematodes (*Pratylenchus* spp.) can cause further wheat losses (Lasserre *et al.*, 1994). Cyst nematodes (*G. pallida* and *G. rostochiensis*) are major pathogens of potato and are estimated to cause losses of 50 million GBP in the UK alone (Jones *et al.*, 2017). *M. chitwoodi* is also considered an important pest of potato (Brown *et al.*, 2006). Almost 100 species of plant-parasitic nematodes from various genera have been reported to affect rice production and can cause yield losses of 10–25% (Trudgill & Blok, 2001). Among them, the most important species is *M. graminicola*, which is able to reduce yields by up to 80% (Soriano *et al.*, 2000). Similarly, 50 species of plant-parasitic nematodes are known to parasitize maize. Among these, the needle nematode (*Longidorus breviannulatus*), which is associated with stunting in maize, is able to cause economic losses of up to 60% (Norton, 1983). Root-knot nematodes and *Ditylenchus destructor* parasitize sweet potato and have caused 100% yield losses in major sweet potato-producing countries, including in China, which is the top producer (Xu *et al.*, 2015; Decraemer & Hunt, 2013; Zhang *et al.*, 2006). More than 90 species of plant-parasitic nematodes from several genera parasitize umbelliferous crops, including carrot. Root-lesion nematodes (*Pratylenchus* spp.) and root-knot nematodes species, such as *M. hapla* and *M. javanica*, are important species affecting carrot production and are able to cause yield losses ranging from 20% up to 77% (Kim *et al.*, 2016; Hay *et al.*, 2004; Koenning *et al.*, 1999).

1.4 Disease management of plant-parasitic nematodes

Chemical control methods can effectively control plant-parasitic nematodes in many cases (Barker & Koenning, 1998). However, because of their high cost, their toxicity to livestock and plants, their negative impact on biodiversity (Beketov *et al.*, 2013; Abad *et al.*, 2008), the development of resistance in nematode pathogen populations (Yamashita & Viglierchio, 1987; Yamashita *et al.*, 1986) and the banning of chemical soil disinfestation for nematode control in many countries, the use of chemicals to control nematode diseases is now

limited. Furthermore, the use of synthetic chemicals to control pests and diseases is prohibited in organic farming systems. These factors underscore the need to find effective, novel and environment-friendly alternatives that can be used instead of the legislatively withdrawn nematicides.

Certain soil-borne microorganisms, such as nematode-parasitic fungi, could potentially be used to control plant-parasitic nematodes. Basic research on nematophagous fungi belonging to the order Orbiliales (family Orbiliaceae) that develop specialized nematode-trapping structures has been conducted to understand their interaction biology (Nordbring-Hertz *et al.*, 2006). Certain fungal species from genera such as *Purpureocillium*, *Penicillium* and *Trichoderma* also have potential as biological control agents (Martinez-Beringola *et al.*, 2013; Atkins *et al.*, 2005; Zhao *et al.*, 2005; Sharon *et al.*, 2001). *Trichoderma koningii* and *T.* confer (cf.) *harzianum* are reported to control the root-knot nematode *M. arenaria* (Sharon *et al.*, 2001; Windham *et al.*, 1989) and *T.* cf. *harzianum* can control the citrus nematode *Tylenchulus semipenetrans* (Reddy *et al.*, 1996a). The egg pathogenic fungus *Purpureocillium lilacinum* (previously *Paecilomyces lilacinus*) has been extensively tested against plant-parasitic nematodes (Atkins *et al.*, 2005) and successfully controls *M. javanica* and the potato cyst nematode *G. rostochiensis* (Cannayane & Sivakumar, 2001). However, its ability to control these nematodes under field conditions is inconsistent (Kerry & Evans, 1996). *P. lilacinum* and the nematode-trapping fungus *Monacrosporium lysipagum*, either alone or in combination, are able to reduce the number of *M. javanica* galls and juveniles by 62 and 94%, respectively, and to reduce the number of cysts caused by *H. avenae* by 65% (Khan *et al.*, 2006).

1.5 The fungal biocontrol agent *Clonostachys rosea*

The ascomycete *Clonostachys rosea* (Link: Fr.) Schroers, Samuels, Seifert & W. Gams, comb. nov. (Schroers *et al.*, 1999) is a soil-borne mycoparasitic fungus and an effective biocontrol agent against fungal crop pathogens such as *Alternaria* spp. (Jensen *et al.*, 2004), *Bipolaris sorokiniana* (Jensen *et al.*, 2002), *Botrytis cinerea* (Sutton *et al.*, 1997), *Fusarium culmorum* (Jensen *et al.*, 2000), *F. graminearum* (Hue *et al.*, 2009) and *Sclerotinia sclerotiorum* (Rodríguez *et al.*, 2011).

However, recently it has been shown that certain *Clonostachys* strains can potentially affect a range of different plant-parasitic nematodes (Fig. 2) through direct parasitism on several nematode life stages, including eggs and cysts (Zou *et al.*, 2010a; Zhang *et al.*, 2008; Li *et al.*, 2006; Sutton *et al.*, 1997). *C. rosea* is also reported to produce nematicidal compounds, such as leptosins, chetracin A,

chaetocin and gliocladines A, B, C, D and E (Dong *et al.*, 2005), enzymes, such as extracellular serine proteases (Zou *et al.*, 2010a) and chitinases (Tzelepis *et al.*, 2015; Gan *et al.*, 2007), which may be involved in the biocontrol effect against plant-parasitic nematodes. Some reports suggest that certain strains of *C. rosea* can interact with plant roots and trigger local and systemic resistance responses that suppress fungal diseases (Mouekouba *et al.*, 2014; Roberti *et al.*, 2008). The effect of induced resistance against nematodes needs to be studied further. Previous studies have also reported that *C. rosea* is involved in plant growth promotion (Roberti *et al.*, 2008; Ravnskov *et al.*, 2006).

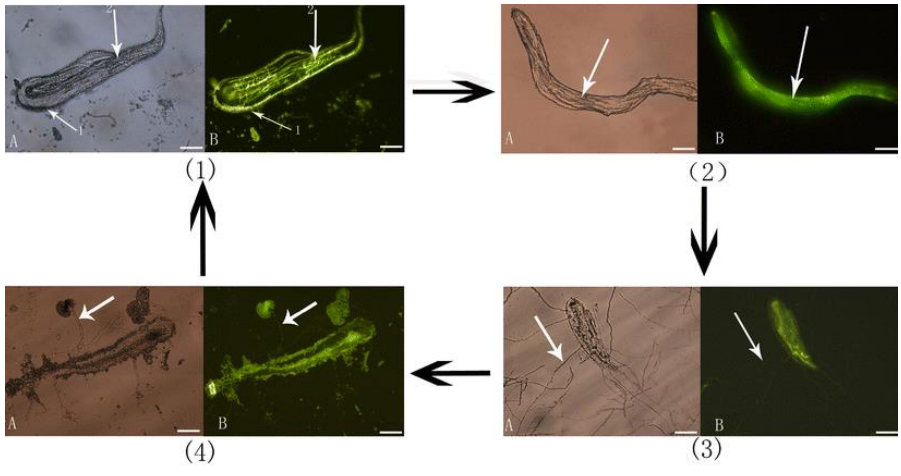

Figure 2. Light microscopy (A) and fluorescence microscopy (B) images of *Clonostachys rosea* strain 611 infecting the nematode *Panagrellus redivivus*. (1) Conidia attached to the nematode cuticle (arrow 1), proliferating and mycelia penetrating the nematode body (arrow 2). (2) Mycelia growing inside the nematode body. (3) Mycelia growing out from the nematode body. (4) The fungus producing abundant conidia. Scale bar = 20 µm. Figure from Zhang *et al*. (2008) and reproduced with the permission of the publisher.

1.6 The role of proteases in fungal biocontrol agents

Proteases are considered to play an important role in fungal adaptations to new environments and ecological niches, especially in biotic interactions (Prasad *et al.*, 2015). Fungal serine proteases have been suggested to play an important role in both pathogenic and mutualistic associations (Kamou *et al.*, 2016; Donatti *et al.*, 2008; Reddy *et al.*, 1996b).

Expanded gene sets of subtilisin-like serine proteases have been reported for entomopathogenic fungi, such as *Cordyceps militaris* (Zheng *et al.*, 2012), *Metarhizium* spp. (Gao *et al.*, 2011) *Beauveria bassiana* (Xiao *et al.*, 2012), and for nematode-parasitizing fungi, such as *Hirsutella minnesotensis* (Lai *et al.*,

2014), *Drechslerella stenobrocha* (Liu *et al.*, 2014), *Arthrobotrys oligospora* (Yang *et al.*, 2011), *Pochonia chlamydosporia* (Larriba *et al.*, 2014), *P. lilacinum* (Prasad *et al.*, 2015) and *M. haptotylum* (Meerupati *et al.*, 2013). The situation is more complex in mycoparasitic fungi because *T. virens* and *T. atroviride* have been reported to have expanded gene sets of serine proteases (Kubicek *et al.*, 2011), whereas *T. reesei* and *T. longibrachiatum* have contracted gene sets (Xie *et al.*, 2014). Overexpression of serine proteases Tvsp1 in *T. virens* (Pozo *et al.*, 2004) and Prb1 in *T.* cf. *harzianum* (Flores *et al.*, 1997) enhanced the ability of these mycoparasites to protect cotton seedlings against the pathogen *Rhizoctonia solani*. Furthermore, serine proteases PRA1 from *T.* cf. *harzianum* and SprT from *T. longibrachiatum* (Chen *et al.*, 2009) reduced egg hatching of *M. incognita* (Suarez *et al.*, 2004), and ThSS45 from *T.* cf. *harzianum* inhibited growth of *A. alternata* (Fan *et al.*, 2014). Serine proteases from several different nematode-parasitizing fungi, including *Dactylella shizishanna*, *Lecanicillium psalliotae*, *H. rhossiliensis*, *A. oligospora* and *P. lilacinum*, have been purified and shown to be involved in the degradation of the cuticle and the progression of the nematode infection (Yang *et al.*, 2011; Li *et al.*, 2010; Wang *et al.*, 2009; Wang *et al.*, 2006; Yang *et al.*, 2005; Minglian *et al.*, 2004).

Numerous serine protease genes in *C. rosea* strain IK726 are upregulated during parasitism of the silver scurf pathogen of potato (*Helminthosporium solani*) (Lysøe *et al.*, 2017). Furthermore, the *C. rosea* serine protease PrC is involved in the infection of nematodes (Zou *et al.*, 2010a), emphasizing the importance of proteases produced by this biological control agent.

1.7 The role of non-ribosomal peptide synthetases in fungal biocontrol agents

Secondary metabolites produced by biological control agents may influence the outcome of their interactions with plant pathogenic microorganisms and plants. Non-ribosomal peptide synthetases (NRPSs) are multifunctional, modular, megasynthases that play a role in the biosynthesis of small bioactive non-ribosomal peptides, utilizing a thiotemplate mechanism that is independent of the ribosomal protein synthesis machinery (Zou *et al.*, 2010a; Grünewald & Marahiel, 2006; Reiber *et al.*, 2005; Finking & Marahiel, 2004; Sieber & Marahiel, 2003; Mootz *et al.*, 2002). NRPSs synthesize peptides using modules, with each module responsible for incorporating a specific amino acid into the final peptide product. Each module typically contains at least three core domains: an adenylation domain, a thiolation or peptidyl carrier protein domain and a condensation domain. The adenylation domain recognizes and activates

the substrate, using ATP as an energy source (May *et al.*, 2002; Stachelhaus *et al.*, 1999; Conti *et al.*, 1997). The thiolation or peptidyl carrier protein domain accepts the activated substrate, which is bound to a 4′-phosphopantetheine cofactor as a thioester bond (Weber *et al.*, 2000), and transfers the substrate to a condensation domain, which is responsible for peptide bond formation between adjacent substrates on the megasynthase complex (Finking & Marahiel, 2004; Keating *et al.*, 2002; Keating *et al.*, 2000; Weber *et al.*, 2000).

The genome of *C. rosea* strain IK726 contains seventeen *nps* genes, including the *nps1* gene, which was predicted to encode an epipolythiodiketopiperazine (ETP) synthetase (Karlsson *et al.*, 2015), which is possibly involved in the biosynthesis of nematicidal epipolysulfanyldioxopiperazine compounds (Dong *et al.*, 2005). ETPs are derivatives of cyclic peptides and constitute a poorly studied class of secondary metabolites (Gardiner & Howlett, 2005). Dong *et al.* (2005) isolated nine verticillin-type epipolysulfanyldioxopiperazine compounds (i.e., gliocladin A–E, verticillin A, 11-deoxyverticillin A, Sch52900 and Sch52901) compounds from *C. rosea* strain 1A and all compounds showed toxicity towards *Caenorhabditis elegans* and *P. redivivus* nematodes, with more than 50% mortality after 24 h of incubation.

2 Objective of the study

The overall goal of this study was to investigate whether the fungus *C. rosea* strain IK726 can be used as a biological control agent against plant-parasitic nematodes in different crops and to investigate the possible mechanisms involved in nematode control by *C. rosea*.

This thesis is divided into four projects and the specific objectives were to:

- Investigate the effect of *C. rosea* on nematode communities, plant health and possible mechanisms involved in nematode control (paper I).

- Investigate the evolutionary history of the protease gene family in *C. rosea* and other hypocrealean fungi with an emphasis on their role in mycoparasitic and nematode parasitic lifestyles (paper II).

- Study the role of the *C. rosea* non-ribosomal peptide synthetase NPS1 gene product in antagonism and biocontrol of nematodes (paper III).

- Study genetic variations associated with nematode antagonism and biocontrol among *C. rosea* strains (paper IV).

3 Materials and Methods

3.1 Fungal strains and maintenance conditions

C. rosea strains (Broberg *et al.*, 2018), mutants derived from the *C. rosea* strain IK726 wild type (WT), *B. cinerea* strain B05.10 and *F. graminearum* strain PH-1 were maintained on potato dextrose agar (PDA) medium (Oxoid, Basingstoke, Hampshire, England) at 25°C in darkness. Petri plates of PDA were inoculated from stock cultures preserved in 20% (w/v) glycerol at –80°C.

3.2 Biomining of fungal proteases

Whole-genome nucleotide and protein sequences of ten hypocrealean fungi (*H. minnesotensis* (Lai *et al.*, 2014), *H. sinensis*, *H. thompsonii*, *Metarhizium robertsii* (Agrawal *et al.*, 2015; Hu *et al.*, 2014; Hu *et al.*, 2013), *T. virens*, *T. reesei*, *T. atroviride* (Kubicek *et al.*, 2011; Martinez *et al.*, 2008), *F. solani*, *F. graminearum* (Coleman *et al.*, 2009; Cuomo *et al.*, 2007) and *C. rosea* (Karlsson *et al.*, 2015)) and *Neurospora crassa* (Galagan *et al.*, 2003) were retrieved from the National Center for Biotechnology Information (NCBI). Protease information for *M. robertsii* was retrieved from Hu *et al.* (2014). Proteases were identified and classified into families by BLASTp (Altschul *et al.*, 1997) against the MEROPS peptidase database (Rawlings *et al.*, 2016).

3.3 Protease gene family evolution

Previous phylogenetic placements of *C. rosea* within the Hypocreales order were retrieved from the literature (Karlsson *et al.*, 2015; Sung *et al.*, 2007; James *et al.*, 2006) and used for the analysis of protease gene family evolution. Branch lengths were calculated based on a four-gene alignment, including actin, glyceraldehyde 3-phosphate dehydrogenase, DNA-directed RNA polymerase II subunit B and translation elongation factor 1 alpha. Coding gene sequences were retrieved from the respective genome sequences. Each gene was aligned

individually using Clustal W (Larkin *et al.*, 2007) in MEGA ver. 6 (Tamura *et al.*, 2013), concatenated and used to calculate branch lengths in MEGA ver. 6. The species phylogeny obtained was calibrated to the fossil record by setting the split between *H. minnesotensis* and *H. sinensis* to 29 million years, as described by Lai *et al.* (2014).

Gene family evolution analysis was performed on protease families that contained ≥ 2 genes in at least one species and were present in ≥ 2 species. The program CAFE (Computational Analysis of Gene Family Evolution) ver. 3 (Han *et al.*, 2013) was used to test whether gene family sizes were compatible with a stochastic birth and death model, to estimate gene family size in extinct species and to identify lineages with accelerated rates of gene gain or loss. A mutation rate (λ) of 0.017 was estimated based on the data.

3.4 *In vitro* nematode antagonism assays

Migratory endoparasitic nematodes (*Pratylenchus penetrans*) were purchased from the Laboratory of Nematology, Department of Plant Sciences, Wageningen University, Netherlands, and mixed nematode communities were extracted from naturally infested soil for use during *in vitro* antagonism assays. The level of nematicidal activity in liquid cultures of 53 *C. rosea* strains, including IK726, was measured by analysing culture filtrates. Culture filtrates were produced by inoculating 50 ml of potato dextrose broth (PDB) or synthetic nutrient medium (SNA) (Nirenberg, 1976) in E-flasks with 1×10^6 conidia/flask, and then incubating at 25°C on a rotating shaker (120 rpm) for five days under dark conditions. The fungal biomass was separated from the broth by filtering it through three layers of filter paper (Ahlstrom-Munksjö, Helsinki, Finland). The culture filtrate was further sterile filtrated through a 0.45 µm cellulose membrane (Sarstedt, Nümbrecht, Germany). The *C. rosea* IK726 culture filtrate that was produced in SNA medium was subjected to heat treatment (80°C) in order to denature secreted proteins and heat labile metabolites (Dubey *et al.*, 2014).

Eight hundred µl of culture filtrate or liquid media (control) were mixed with 200 µl of water containing approximately 100 nematodes, either *P. penetrans* or a mixed community of nematodes, and placed in a well of a 24-well plastic plate, followed by incubation at 25°C in the dark for 24 h. The number of dead nematodes was determined by washing the nematodes with cold water and by performing a touch assay under a light microscope (Wild M5A Heerbrugg, Heerbrugg, Switzerland) at 50× magnification (Gill *et al.*, 2003).

3.5 Genome-wide association study

The genome assemblies of 53 *C. rosea* strains, including IK726, were retrieved from previously published work (Broberg *et al.*, 2018). A genome-wide association study (GWAS) was executed using PLINK ver. 1.90 (Purcell *et al.*, 2007), using the parameters—maf 0.1—hwe 1e-5 for SNP filtering. The single-nucleotide polymorphism (SNPs) were annotated using the ANNOVAR software (Wang *et al.*, 2010). The resulting beta and beta standard deviation from the PLINK analysis were used as input to the R package ashr for empirical Bayesian multiple hypothesis testing and for estimating the local false sign rate (lfsr) for significance approximation (Stephens, 2016). We used an lfsr of $\leq 1 \times 10^{-10}$ as a cut-off for significance. Identification of conserved protein modules and features were made using the conserved domain database (CDD, Marchler-Bauer *et al*., 2017) and the transporter classification database (TCDB, Saier *et al*., 2016).

3.6 Evaluation of *C. rosea* IK726 to control nematodes

Soil containing a naturally diverse community of nematodes, including plant-parasitic genera, was collected from a wheat field during fallow conditions in Säby, close to Uppsala, Sweden. The soil was cleaned to remove clumps and mixed with sand (particle size, 0.50 mm) (Rådasand, Lidköping, Sweden) to a final content of 20% vol/vol. The formulation of *C. rosea* strain IK726 was prepared by following the procedure described by Jensen *et al.* (2000). Spore viability and the concentration of *C. rosea* strains in the formulations were determined using serial dilutions followed by mixing with soil to reach a final concentration of 2.5×10^5 viable colony forming units (CFU)/g of soil. Plastic pots (9 × 9 × 10 cm) were filled with 500 g of *C. rosea*-inoculated soil or uninoculated soil (control). Eight wheat seeds (*Triticum aestivum*, winter wheat variety "Stava") were planted in each pot and placed in a growth cabinet at 15°C with a 12 h/12 h light/dark photoperiod and a relative humidity of 70 ± 5%. All pots were subjected to the same watering regime throughout the seven-week experimental period.

When the experiment was harvested, plants were cut at the root–shoot junction at the surface of the soil; plant fresh shoot weight (g), dry shoot weight (g) and shoot length (cm) were recorded. Living nematodes were extracted from 15 g of soil and 1 g of harvested roots using a modified Baermann funnel method (Viketoft *et al.*, 2005; Baermann, 1917). Nematodes were counted under a light microscope (Wild M5A Heerbrugg, Heerbrugg, Switzerland). The classification of nematodes to genus level was based on an analysis of morphological characters.

3.7 Gene expression analysis and nucleic acid manipulation

Gene expression of S8A serine protease genes (*prs1* to *prs18*) and three NRPS genes (*nps1*, *nps4* and *nps5*) was determined during fungal–fungal interactions when *C. rosea* was challenged in a dual plate assay with *B. cinerea*, *F. graminearum* or with itself on 9-cm diameter Petri plates containing PDA. Cultures were incubated at 25°C in darkness. In order to compensate for differences in mycelial growth, *C. rosea* was inoculated five days before inoculating *B. cinerea* or *F. graminearum*. The edge of the growing front (7 to 10 mm) of *C. rosea* mycelium was harvested at contact with *B. cinerea* or *F. graminearum* mycelia. Mycelium harvested at the same stage when *C. rosea* confronted itself served as the control treatment.

The expression pattern of *C. rosea* protease genes was also investigated using different protein compounds. E-flasks containing PDB were inoculated with *C. rosea* conidia (2.5×10^5) and incubated at 25°C on a rotating shaker (100 rpm) for 4 days in darkness. Fungal biomass was separated from the broth by filtration using sterilized Miracloth (EMD Millipore, Billerica, MA) and mycelia were washed twice with 20 ml sterile water to completely remove the PDB. The washed mycelia were then transferred into new E-flasks containing 25 ml of sterile water (control) or a sterile water solution containing either 1% bovine serum albumin (BSA, Sigma Aldrich, Steinheim, Germany), 1% collagen (Sigma-Aldrich, Steinheim, Germany) or 75 g/l of milk powder (Semper). The BSA and collagen solutions were sterile filtrated through a 0.45 µm cellulose membrane (Sarstedt, Nümbrecht, Germany). The milk powder solution was sterilized by autoclaving. E-flasks were incubated at 25°C on a rotating shaker (100 rpm) for 6 h under dark conditions to induce protease gene expression before harvesting mycelia.

Total RNA extraction was carried out using a Qiagen RNeasy kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). RNA was treated with RNase-free DNase I (Fermentas, St. Leon-Rot, Germany) and concentration was determined spectrophotometrically using a NanoDrop-1000 (Thermo Scientific, Wilmington, DE). One microgram of total RNA was reverse transcribed in a total volume of 20 µl using a Maxima first strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany) followed by 10-fold dilution. Transcript levels were quantified using an iQ5 qPCR system (Bio-Rad, Hercules, CA) using the SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA), as described previous (Kamou *et al.*, 2016). Relative expression levels of the investigated NRPS and serine protease genes were calculated in relation to the

reference gene β-tubulin (*tub*) (Mamarabadi *et al.*, 2008) using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

3.8 Phylogenetic analysis of NRPS families in *C. rosea*

A reference set of fungal NRPS protein sequences was retrieved from Bushley and Turgeon (2010) for phylogenetic analyses. Adenylation domains of NPS1 were predicted using SMART, a simple modular architecture research tool (Letunic & Bork, 2018). Adenylation domain sequences were extracted and aligned using Clustal W, and a phylogenetic tree was constructed using MEGA ver. 6 (Tamura *et al.*, 2013). The evolutionary history of NRPS families based on adenylation domains was inferred using a maximum likelihood method. The LG amino acid substitution model (Le & Gascuel, 2008) was identified as optimal for modelling gamma distributed rates among sites and partial deletion of gaps was performed for phylogenetic tree analysis. Statistical support was determined by 1000-iteration bootstrap resampling.

The adenylation domains of NPS4 and NPS5 proteins were extracted using HMMER ver. 3.1b2 and a custom HMMER model created for fungal adenylation domains (Bushley & Turgeon, 2010). The adenylation domains were aligned with mafft (Katoh *et al.*, 2002), along with a reference set of adenylation domains from fungal NRPS with known chemical products (Bushley & Turgeon, 2010) as outgroups. A maximum likelihood phylogenetic tree was created using RAxML (Stamatakis *et al.*, 2008) with the best-fitting protein model (RTREVF) estimated by ProtTest (Abascal *et al.*, 2005).

3.9 Construction of NRPS gene deletion cassettes and transformation

A three-fragment multisite gateway cloning system (Invitrogen, Carlsbad, CA) was used to delete NRPS genes (*nps1*, *nps4* and *nps5*) in *C. rosea*. Gateway entry clones of the PCR fragments were generated according to the manufacturer's instructions (Invitrogen, Carlsbad, CA); entry clones for the hygromycin-resistance cassette (hygB) were constructed during previous studies (Dubey *et al.*, 2013, 2012). The gateway LR recombination reaction was performed using the entry plasmid of the respective fragments and the destination vector pPm43GW (Karimi *et al.*, 2005) to generate a deletion cassette following the conditions described by the manufacturer (Invitrogen, Carlsbad, CA). Each deletion cassette contained upstream and downstream flanking sequences of approximately 1200 to 2000 bp together with the marker gene. Transformation

of *C. rosea* IK726 was performed using an *Agrobacterium tumefaciens* protocol, as described previously (Dubey *et al.*, 2016; Utermark & Karlovsky, 2008).

3.10 Phenotypic analyses of the NRPS deletion strains of *C. rosea*

3.10.1 Growth and conidiation rate

In order to measure the growth rate of *C. rosea* WT and NRPS deletion strains (*nps1*, *nps4* and *nps5*), a 5-mm diameter agar plug was excised from the growing mycelia and transferred to PDA plates. Cultures were incubated at 25°C under dark conditions and colony diameter was measured daily for up to seven days post-inoculation (DPI). Conidiation rates of *C. rosea* NRPS deletion strains and WT were determined as follows: conidia were harvested from 2-week-old cultures maintained on PDA plates at 25°C under dark conditions by adding 7-ml of sterile water to the culture and then scraping the surface of the mycelium with a glass rod to release the conidia. Conidia were counted using a haemocytometer (Hausser Scientific, Horsham, PA).

3.10.2 *In vitro* antagonism assay

The nematicidal inhibition level was measured in PDB cultures of *C. rosea* WT and NRPS deletion strains (*nps1*, *nps4* and *nps5*). The same procedure was used to produce culture filtrate and to assess nematode mortality as described earlier in section 3.4.

3.10.3 Secondary metabolite analysis

To examine the involvement of NRPS in secondary metabolite production, culture filtrates of *C. rosea* WT and NRPS deletion strains (*nps1*, *nps4* and *nps5*) were further analysed by performing ultra-high-performance liquid chromatography–mass spectrometry (UHPLC-MS). UHPLC-MS analysis was performed using an Agilent 1290 Infinity II UHPLC (Agilent, Palo Alto, CA, USA) attached to a Bruker maXis Impact QTOF MS (Bruker Daltonic GmbH., Bremen, Germany). Samples (1 µl) were analysed on a reversed phase UHPLC column (2.1 × 50 mm, 1.5 µm, Accucore Vanquish, Thermo Scientific, Waltham, MA), eluted using acetonitrile (MeCN) gradients in water and 0.2% formic acid (i.e., 10% to 95% MeCN for 3 min and 95% MeCN for 1.2 min at 0.9 ml/min). The MS was used in positive mode in the range m/z 50–1500.

Compass Data Analysis 4.3 software (Bruker Daltonics) was used to calibrate mass spectra against sodium formate clusters to create extracted-ion chromatograms corresponding to expected substances, and to convert data to mzXML format. Ion-chromatogram peak picking was conducted within the R software environment using the program XCMS (Smith *et al.*, 2006) and the centWave method (Tautenhahn *et al.*, 2008).

3.10.4 *In planta* fusarium foot rot assay

A fusarium foot rot assay on wheat (*T. aestivum*, winter wheat variety "Stava") seedlings was performed to determine the efficacy of *C. rosea* WT and NRPS deletion strains (*nps1*, *nps4* and *nps5*) as biocontrol agents against *F. graminearum*, following the procedure described in previous studies (Dubey *et al.*, 2016; Dubey *et al.*, 2014; Knudsen *et al.*, 1995). The experiment was conducted in a growth cabinet under controlled conditions: i.e., a light/dark photoperiod of 12 h/12 h with 150 μmol m^{-2} s^{-1} light intensity, a temperature of 15°C ± 1°C and a relative humidity of 80 ± 5%. Seedlings were harvested three weeks post-inoculation and disease symptoms were scored based on a 0 to 4 scale, as described previously (Dubey *et al.*, 2016; Dubey *et al.*, 2014; Knudsen *et al.*, 1995).

3.10.5 *In planta* nematode root disease assay

A nematode root disease assay on wheat (*T. aestivum*, winter wheat variety "Stava") was carried out to evaluate the biocontrol efficacy of *C. rosea* WT and NRPS deletion strains using the same method as that described earlier in section 3.5. When the experiment was harvested, the same parameters regarding nematodes and plant growth were taken into account as described in section 3.6.

4 Results and Discussion

4.1 Analysis of *in vitro* nematode antagonism

Observations of interaction bioassays using light microscopy did not reveal any signs of *C. rosea* strain IK726 attaching to or penetrating nematodes, which indicates that *C. rosea* does not directly parasitize nematodes (paper I). However, culture filtrates of 53 *C. rosea* strains grown in PDB medium exhibited varying levels of toxicity against *P. penetrans* nematodes compared with the control treatment after 24 h of incubation (paper IV, Fig. 3). An assessment of the antagonistic ability of *C. rosea* strain IK726 against a mixed community of nematodes revealed that plant-parasitic nematode genera showed higher mortality in culture filtrates than non-parasitic nematode groups (paper I). Varying levels of toxicity in culture filtrates indicated the production and secretion of nematicidal metabolites and/or enzymes by *C. rosea* strains. *C. rosea* strain 1A grown on wheat kernels for 20 days has previously been reported to produce nematicidal ETPs (Dong *et al.*, 2005), and it is possible that similar compounds were produced by *C. rosea* strains in the liquid culture conditions used in the present study. *C. rosea* strain 611 has also been shown to produce the extracellular serine protease PrC during growth on PDB, which killed up to 80% of *P. redivivus* nematodes after 48 h of incubation (Li *et al.*, 2006).

SNA medium was used to produce the culture filtrate of *C. rosea* IK726 to evaluate whether the nematicidal activity was heat stable (paper I). The heat treatment reduced but did not eliminate the toxic effects of culture filtrates on nematodes (mixed community) after 12 h of incubation. After prolonged incubation, nematode mortality levels in heat-treated culture filtrates were not significantly different from those in sterile SNA medium (controls) (paper I, Fig. 4). A previous study reported that boiling crude extracts of *C. rosea* strain 611 reduced nematicidal activity by up to 80% (Li *et al.*, 2006), which is very similar to the results obtained for *C. rosea* strain IK726 in the current work. This shows that the major nematicidal activity in culture filtrates of *C. rosea* stems from heat-labile compounds or enzymes, but that some activity still remains after heat treatment.

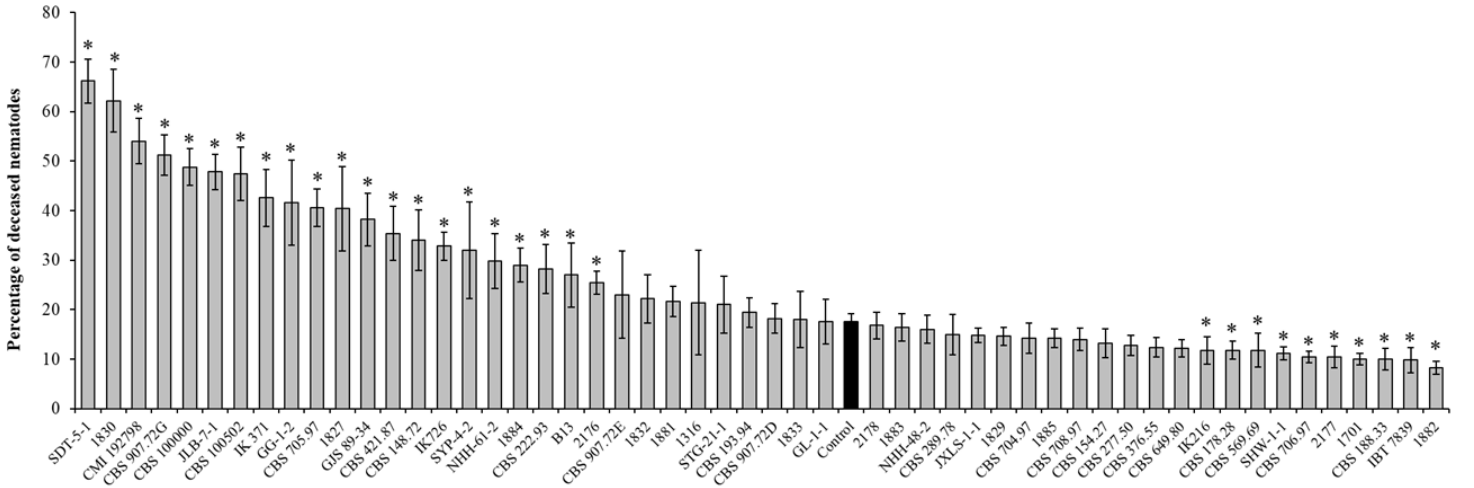

Figure 3. Mortality of migratory endoparasitic nematodes (*Pratylenchus penetrans*) in culture filtrates of 53 *Clonostachys rosea* strains in potato dextrose broth. Nematode mortality was assessed in culture filtrates from *C. rosea* strains and in sterile liquid medium (control) after 24 h of incubation at 25°C. Error bars represent the standard deviation based on five biological replicates. Asterisks indicate a significant difference ($P \leq 0.05$) between a *C. rosea* culture filtrate and the control, as determined by Fisher's least significant difference (paper IV).

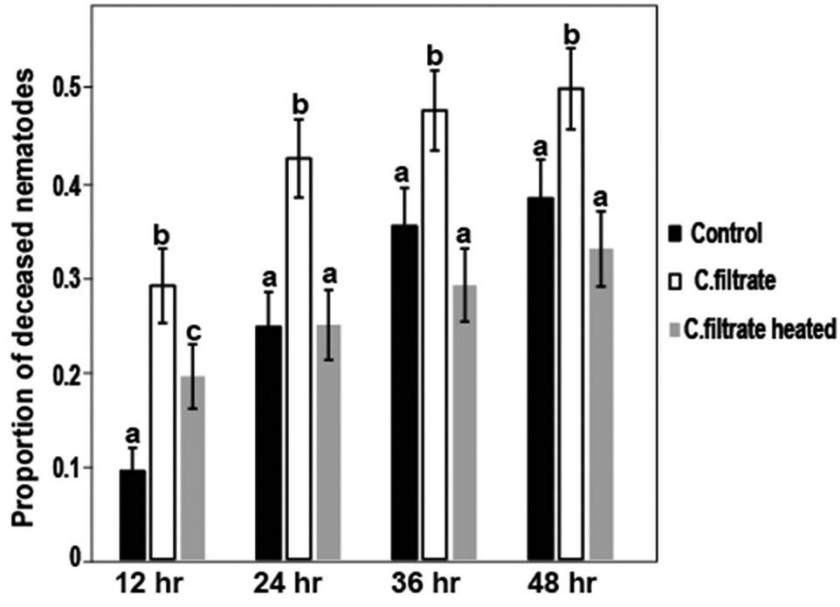

Figure 4. Nematode mortality in *Clonostachys rosea* IK726 culture filtrates in synthetic nutrient medium (SNA) over time. Nematode mortality was assessed in SNA culture filtrates (C. filtrate), heat-treated culture filtrates (C. filtrate heated) and in sterile SNA medium (control) after 12, 24, 36 and 48 h of incubation. Error bars represent the standard deviation based on five biological replicates. Treatments marked with different letters within each time point are significantly different ($P \leq 0.05$) as determined by a chi-squared test. Figure from Iqbal *et al*. (2018a) and reproduced with the permission of the publisher (paper I).

4.2 Genome-wide association study analysis

There was significant variation among *C. rosea* strains regarding their antagonistic ability against *P. penetrans* nematodes (Fig. 3), which suggests that a GWA analysis of the trait is possible. The empirical multiple hypothesis correction identified a total of 279 SNP markers that were associated with *in vitro* antagonism against *P. penetrans* at $\text{lfsr} \leq 1 \times 10^{-10}$ (paper IV).

Two NRPSs genes (*nps4* and *nps5*) were identified in the genomic regions associated with the trait and, therefore, these genes were studied in more detail (paper IV). The *nps1* gene was selected for functional studies (paper III) based on previous suggestions that NPS1 is involved in producing nematicidal compounds in *C. rosea* (Karlsson *et al.*, 2015; Dong *et al.*, 2005). Thus, functional studies were performed on *nps1*, *nps4* and *nps5* genes to determine their role in the biocontrol of nematodes.

4.3 Effect of *C. rosea* on nematode communities and plant health

Screens of the naturally infested soil sample revealed that it contained a number of plant-parasitic nematode genera (i.e., *Heterodera*, *Helicotylenchus*, *Pratylenchus*, *Paratylenchus*, *Tylenchorhynchus*, *Trichodorus* and *Rotylenchus*) and other trophic groups, i.e., bacterivorous, fungivorous and omnivorous nematodes. The application of *C. rosea* to soil in pots of wheat plants decreased the number of plant-parasitic nematodes recovered from the pot soil, with the exception of *Trichodorus* (paper I, Fig. 5). Similarly, the number of nematodes extracted from roots (*Pratylenchus* spp. and *Heterodera* J2) was also reduced compared with the control treatment without *C. rosea*. This reduction in plant-parasitic nematode numbers also correlated with a better growth performance of wheat plants and an absence of disease symptoms usually associated with nematode damage of roots, such as yellowing of leaves and chlorosis (Jones *et al.*, 2013). The *C. rosea* treatment also reduced the populations of *Heterodera* spp., *Helicotylenchus* spp., *Trichodorus* spp. and *Pratylenchus* spp. when applied to soil in pots without plants. This indicates that *C. rosea* has a direct effect on nematode survival that this effect is not dependent on the presence of plants (paper I).

Interestingly, non-parasitic nematode groups (i.e., bacterivorous, fungivorous and omnivorous) populations did not appear to be negatively affected by the *C. rosea* treatment (paper I, Fig. 5), which suggests that the activity of *C. rosea* has a certain level of specificity. This is further supported by the *in vitro* study, in which plant-parasitic nematodes showed higher levels of mortality in culture filtrates of *C. rosea* than other nematode groups (paper I). Previously, Hallmann and Sikora (1996) showed that culture filtrates of *F. oxysporum* inactivated 60–100% of plant-parasitic nematodes while bacterivorous and fungivorous nematodes were unaffected. Similarly, culture filtrates of *P. lilacinum* killed up to 100% of plant-parasitic nematodes while on average only 25% of bacterivorous, fungivorous and entomopathogenic nematodes were killed (Cayrol *et al.*, 1989). *C. rosea* IK726 can effectively colonize the root surface of tomato (Karlsson *et al.*, 2015) and, therefore, it may have a greater impact on plant-parasitic nematodes that live in the same ecological niche (Hallmann & Sikora, 1996). Similar effects on nematode populations and plant growth were observed when *C. rosea* IK726 was grown in soil with carrot plants (paper I).

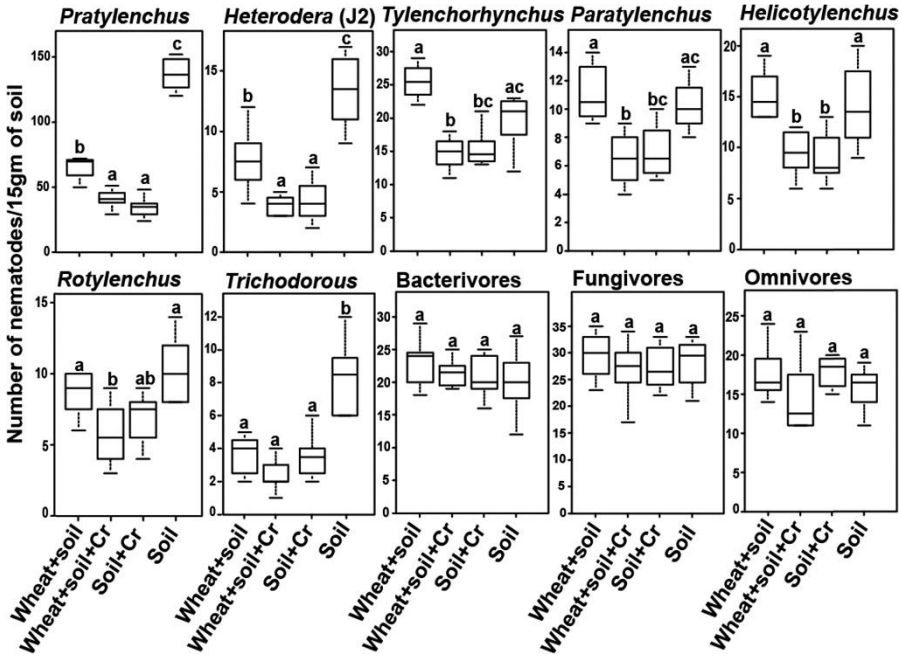

Figure 5. Effect of *Clonostachys rosea* IK726 (Cr) on plant-parasitic nematode genera and trophic groups recovered from wheat plant soil. The horizontal axis represents the different treatment combinations while the vertical axis shows the number of nematodes extracted per 15 g of soil. Treatments marked with different letters in each nematode group are significantly different as determined by the Tukey method ($P \leq 0.05$). Eight biological replicates were included per treatment. Bars show medians and boxes indicate the 25th and 75th percentiles. Figure from Iqbal *et al*. (2018a) and reproduced with the permission of the publisher (paper I).

4.4 Analysis of protease gene family evolution

Based on the reported involvement of the PrC serine protease in nematode antagonism, the evolution of protease genes in *C. rosea* was studied. The highest number of protease genes was found in *C. rosea* and a comprehensive analysis using CAFE revealed five serine protease families, S1A, S8A, S9X, S12 and S33, which were recognized as evolving non-randomly. Among them four serine protease families, S1A, S8A, S9X and S33, were found to be expanded in *C. rosea* (paper II); however, only the S8A family was studied in detail (Fig. 6). The analysis also revealed an expansion of serine protease gene copy numbers in *T. virens* and *T. atroviride* (mycoparasites) and in *M. robertsii* (entomopathogenic), suggesting that serine proteases play an important role in ecological niche adaptation (paper II). The expansion of serine protease gene families in *C. rosea* and in the other mycoparasites (*T. virens* and *T. atroviride*),

but not in the nematode-parasitizing fungus *H. minnesotensis*, indicates that these protease gene families are coupled with a mycoparasitic lifestyle. However, it is worth mentioning that the opportunistic lifestyle of *C. rosea* and of some *Trichoderma* species (*T. longibrachiatum*, *T. atroviride* and *T.* cf. *harzianum*) includes antagonism towards nematodes (Chen *et al.*, 2009; Sharon *et al.*, 2001). Gene deletion of serine protease S8A PrC in *C. rosea* has been reported to attenuate antagonism against nematodes (Zou *et al.*, 2010a; Zou *et al.*, 2010b). This suggests a connection between the expanded gene copy number of serine proteases in *C. rosea* and nematode virulence in *C. rosea*. Previously it has been shown that the serine protease SprT from *T. longibrachiatum* and PRAI (a trypsin-like protease) from *T.* cf. *harzianum* decreased the number of *M. incognita* eggs that hatched (Chitwood & Perry, 2009; Suarez *et al.*, 2004).

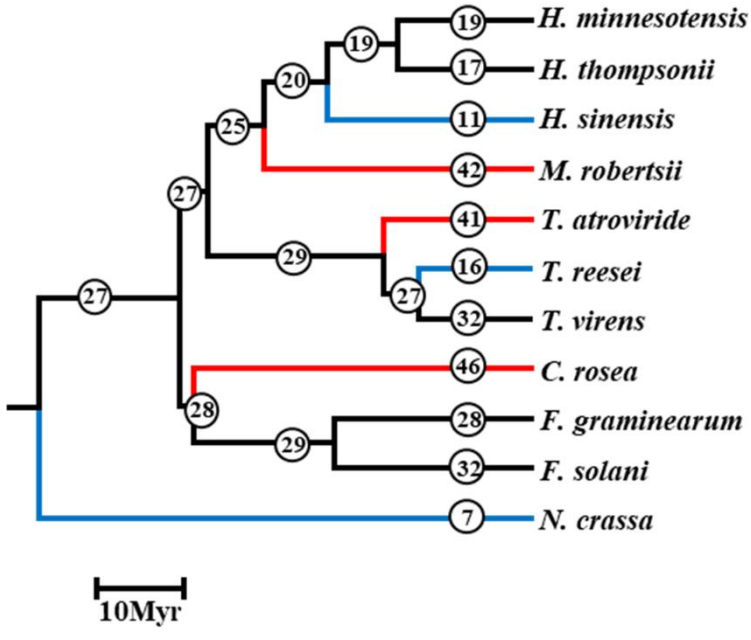

Figure 6. Distribution of serine protease gene gain and loss in the S8A family. Circled numbers indicate the number of serine protease genes in the S8A family in extant species and estimates of the total number of serine protease genes for ancestral species. Red lineages show a significant ($P \leq 0.05$) expansion of serine protease genes and blue lineages represent a significant ($P \leq 0.05$) contraction of serine protease genes. Figure from Iqbal *et al.* (2018b) and reproduced with the permission of the publisher (paper II).

4.5 Gene expression analysis

Only one (*prs6*) out of eighteen S8A serine protease genes was induced during confrontation with *F. graminearum* compared with the control treatment (paper II, Fig. 7A). A previous study reported that numerous *C. rosea* IK726 serine protease genes were induced during parasitism of the potato pathogen *H. solani* (Lysøe et al., 2017). It is possible that several proteases take part in the deterioration and nutrient discharge from dead fungal prey (Sun *et al.*, 2015; Steindorff *et al.*, 2014; Vieira *et al.*, 2013); however, certain proteases also have roles in detecting fungal prey. The *prb1* protease gene in *T. atroviride* is upregulated just prior to hyphal contact with *R. solani*, and overexpression in mutant strains leads to better protection of cotton seedlings compared with that conferred by the WT *T. atroviride* strain (Flores *et al.*, 1997). Furthermore, the upregulation of the *prs6* gene during physical contact between *F. graminearum* and *C. rosea* in this study is compatible with the PRS6 protein having such a sensing function. A low level of *C. rosea* PrC serine protease is essential for the induction of the *prC* gene by the presence of nematode cuticle (Zou *et al.*, 2010a), providing further support for this idea.

Complex transcriptional response patterns were observed for *C. rosea* S8A serine protease genes when *C. rosea* was grown on different protein sources. During growth on milk powder, eight S8A serine protease genes were induced at different levels (3- to 34-fold change) compared with the control treatment (water). Six *C. rosea* genes were induced during growth on BSA at varying levels (12- to 22-fold change) compared with the control treatment. Collagen is a major constituent of the nematode cuticle and, therefore, it was included in this assay; however, interestingly, none of the investigated S8A serine protease genes were induced during growth on collagen (paper II). This contradicts previous findings that a *prc* gene in *C. rosea* strain 611 (which is likely to be an orthologue of *prs14* in *C. rosea* strain IK726) was induced by adding nematode cuticle material to the growth medium (Zou *et al.*, 2010a). Nematode cuticle material also induced subtilisin-like serine proteases in the nematophagous fungus *M. haptotylum* (Ahren *et al.*, 2005). It is likely that additional factors other than the presence of collagen in nematode cuticle are necessary for the induction of S8A serine protease genes. However, other intrinsic differences between the *C. rosea* strain IK726 (from Denmark) and strain 611 (from China) might also explain the difference in transcriptional responses.

The *nps1* gene was induced during confrontations with *B. cinerea* (paper III, Fig. 7B), and *nps4* and *nps5* genes were similarly induced (paper IV), which suggests that NRPSs play important roles during interspecific fungal interactions. None of these genes were induced during confrontations with *F. graminearum*. Previously, it has been shown that the *nps13* gene in *C. rosea*

IK726 was induced when confronted with *B. cinerea* but not induced during interactions with *F. graminearum* (Nygren *et al.*, 2018). Similarly, in the truffle-parasitizing fungus *Tolypocladium ophioglossoides*, the NRPS-like gene TOPH 03459 was highly upregulated during growth in yeast malt broth compared with broths that contained material from the truffle host, i.e., *Elaphomyces muricatus* (Quandt *et al.*, 2016).

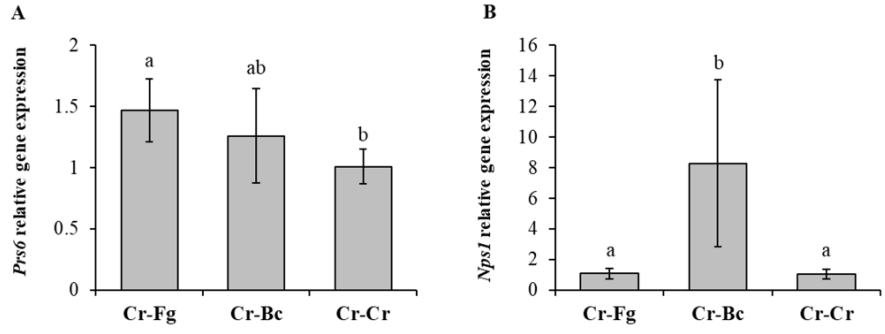

Figure 7. Expression analyses of the S8A serine protease gene *prs6* (A) and the non-ribosomal peptide synthetase gene *nps1* (B) determined by performing RT-qPCR during interactions between *Clonostachys rosea* and its fungal prey. The interactions were *C. rosea* with *Fusarium graminearum* (Cr-Fg), *C. rosea* with *Botrytis cinerea* (Cr-Bc) and *C. rosea* with itself (Cr-Cr), which acted as the control. Relative expression was calculated as the ratio between the target genes and tubulin using the $2^{-\Delta\Delta CT}$ method. *Prs6* gene expression was significantly higher ($P \leq 0.05$) during interactions with Cr-Fg than during Cr-Bc or Cr-Cr interactions (A), while *nps1* gene expression during the Cr-Bc interaction was significantly higher ($P \leq 0.05$) than during the Cr-Fg or Cr-Cr interactions (B), as determined by the Tukey-Kramer test. Error bars represent the standard deviation based on five biological replicates. Figure adapted from Iqbal *et al*. (2019, 2018b,) and reproduced with the permission of publishers (paper II & III).

4.6 Phylogenetic analysis of NRPS families in *C. rosea*

Phylogenetic analysis of the NPS1 protein sequence based on adenylation domains resolved nine major NRPS subfamilies, as shown previously (Bushley & Turgeon, 2010). The predicted NPS1 protein sequence was contained in two adenylation domains. The first predicted NPS1 adenylation domain clustered in the ChNPS11/ETP module 1 synthetase subfamily of toxins (e.g., gliotoxin and sirodesmin) with 99% bootstrap support, while the other adenylation domain clustered in the ChNPS12/ETP module 2 subfamily (paper III).

Phylogenetic analysis of NPS4 and NPS5 showed that both of these NRPSs belong to the euascomycete synthase clade, which has been shown to be expanded in euascomycete fungi and to contain primarily multimodular NRPSs, many of which may have niche-specialized functions (Bushley & Turgeon,

2010). The *C. rosea* NRPS4 belonged to the large clade containing peptaibol synthetases, including TEX1 from *T. virens*, and encodes an eleven modular amino acid peptaibol synthase. Three of the adenylation domains from *C. rosea* NRPS5 were grouped in a clade containing NRPS8 from the corn pathogen *Cochliobolus heterostrophus* and peramine synthase from *Epichloë*/*Neotyphodium* endophytes of grasses. However, the remaining two adenylation domains of NRPS5 grouped closely with the second module of PS1 and PS4, two NRPSs involved in the synthesis of ergot alkaloids in *Claviceps purpurea* (paper IV).

4.7 Phenotypic analyses of NRPS deletion strains

4.7.1 Growth and conidiation

Deletion of the *nps1* gene in *C. rosea* led to an increased growth and conidiation rate on PDA plates compared with WT (paper III, Fig. 8A and B). Similar results were obtained using the *nps4* and *nps5* deletion strains (paper IV). These results indicate that metabolites produced by NRPS might have a toxic effect on the fungus itself and exert an inhibitory effect on *C. rosea* growth. Alternatively, deletion of NRPS genes could allow the distribution of resources for growth and conidiation that would otherwise be used for secondary metabolite biosynthesis (Calvo *et al.*, 2002), i.e., there is likely to be a trade-off between using resources for growth versus secondary metabolite production. Mukherjee *et al*. (2018, 2012) also reported similar results in studies involving the mycoparasite *T. virens* in which deletion strains of NRPS genes (i.e., *tex7*, *tex8*, *tex10* and *tex13*) showed increased growth rates compared with the WT. The results of the present study are also in line with previous findings that deletion of the polyketide synthetase genes *pks22* and *pks29* in *C. rosea* strain IK726 resulted in increased conidiation, showing that the production of secondary metabolites may have an important implication on growth and development in *C. rosea* (Fatema *et al.*, 2018).

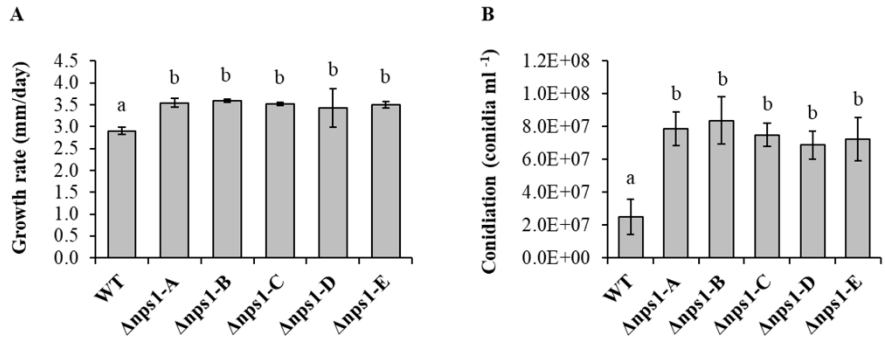

Figure 8. Growth and conidiation rate of *Clonostachys rosea* wild type and *nps1* deletion strains on potato dextrose agar (PDA) Petri plates. (A) Growth rate was measured daily up to seven days post-inoculation (DPI). (B) Conidiation was measured at 14-DPI. The growth rate experiment was performed using three biological replicates whereas the conidiation experiment was performed using four biological replicates. Error bars represent the standard deviation and different letters show statistically significant differences between treatments within an experiment based on the Tukey-Kramer test. Figure from Iqbal *et al.* (2019) and reproduced with the permission of the publisher (paper III).

4.7.2 Deletion of *nps1*, *nps4* and *nps5* genes attenuates *in vitro* antagonism against nematodes

The antagonistic ability of Δ*nps1* strains was evaluated in culture filtrates against a mixed community of nematodes, whereas *P. penetrans* nematodes were used to assess the antagonistic potential of Δ*nps4* and Δ*nps5* strains. The results showed that the antagonistic impact of Δ*nps1* strains was significantly lower than that of *C. rosea* WT (paper III, Fig. 9), which suggests that NPS1 is involved in biosynthesizing a metabolite with nematicidal activity. Δ*nps4* and Δ*nps5* strains also displayed lower antagonism against *P. penetrans* than WT in a similar manner to that observed for the *C. rosea* Δ*nps1* strains (paper IV). Previously, nine verticillin-type ETP compounds (i.e., gliocladin A–E, verticillin A, 11-deoxyverticillin A, Sch52900 and Sch52901) were isolated from *C. rosea* strain 1A and all these compounds had shown toxicity against *C. elegans* and *P. redivivus* nematodes, with more than 50% nematode mortality following 24 h of incubation (Dong *et al.*, 2005).

However, deletion of *nps1*, *nps4* or *nps5* genes did not abolish 100% of the *C. rosea* antagonistic ability; culture filtrates of these strains still possessed a certain degree of nematicidal activity compared with the control treatment (papers III and IV, Fig. 9), which suggests that other associated compounds or enzymes, such as serine proteases, are involved in nematode antagonism, as discussed in section 4.5.

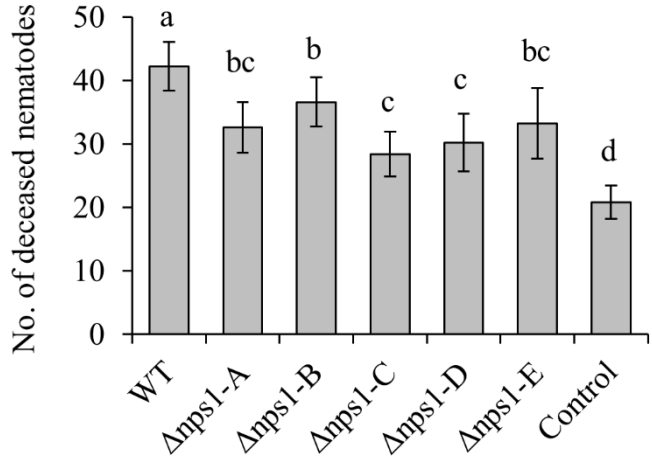

Figure 9. Nematode mortality in culture filtrates of *Clonostachys rosea* wild type (WT) and Δ*nps1* strains in potato dextrose broth (PDB). Nematode mortality was measured after 24 h of incubation in culture filtrates of *C. rosea* WT, Δ*nps1* strains and in sterile PDB medium (control). Error bars represent the standard deviation based on five biological replicates. Different letters indicate a statistically significant difference ($P \leq 0.05$) between treatments as determined by Fisher's least significant difference test. Figure from Iqbal *et al.* (2019) and reproduced with the permission of the publisher (paper III).

4.7.3 Secondary metabolite analysis of *C. rosea* wild type and NRPS deletion strains

UHPLC-MS analysis of 2-week-old culture filtrates indicated that the *C. rosea* WT strain contained the compound 10-hydroxy-8-decenoic acid, which has previously been reported to have nematicidal activity (Soman *et al.*, 2001; Anke *et al.*, 1996). However, nuclear magnetic resonance spectroscopy failed to confirm the structure.

UHPLC-MS analysis of culture filtrates from *C. rosea* WT and NRPS deletion strains did not detect the production of any known nematicidal dioxopiperazines (i.e., gliocladin A–E, verticillin A, 11-deoxyverticillin A, Sch52900 or Sch52901) (Dong *et al.*, 2005) (papers III and IV). The failure to detect these molecules or metabolites was perhaps because of differences in growth conditions (e.g., PDB versus wheat kernels in the Dong et al. (2005) study) or differences between strains. Another possible explanation might be that the concentration of the metabolite was too low for detection in the studied cultures. Moreover, PDB might not be an ideal medium for *C. rosea* cultures to produce the NRPS metabolite.

4.7.4 Deletion of *nps1*, *nps4* and *nps5* genes reduces *C. rosea* biocontrol efficacy against fusarium foot rot disease

C. rosea nps1 deletion strains showed a diminished ability to inhibit fusarium foot rot disease caused by *F. graminearum* on wheat seedlings compared with WT (paper III, Fig. 10). Moreover, *nps4* and *nps5* deletion strains also showed significantly reduced biocontrol efficacy compared with WT (paper IV). Previously, the reduced growth rate of *C. rosea* deletion strains of the *zhd101* (*Zearalenone lactonohydrolase*) gene contributed to a reduction in biocontrol potential (Kosawang *et al.*, 2014). However, the NRPS deletion strains in the current work showed a higher growth rate on PDA plates compared with the WT; it is therefore likely that the reduced biocontrol efficacy is because of a failure to produce metabolites that carry the fungal-growth-inhibiting effect. Fatema *et al.* (2018) reported similar findings that deletion strains of the polyketide synthase gene *pks29* in *C. rosea* strain IK726 lessened the biocontrol efficiency of *C. rosea* against *F. graminearum*, which resulted in enhanced disease symptoms in barley.

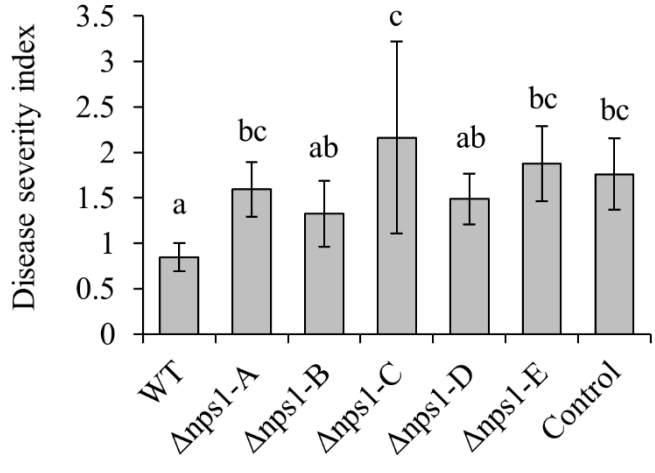

Figure 10. In planta biocontrol efficacy of *Clonostachys rosea* wild type (WT) and Δ*nps1* strains against *Fusarium graminearum* foot rot disease on wheat. Wheat seeds were treated with *C. rosea* conidia (either WT or Δ*nps1* strains) and grown in moist sand together with an *F. graminearum* agar plug. Error bars denote the standard deviation based on five biological replicates. Each replicate contained the mean of 12 to 15 plants. Different letters indicate a statistically significant difference ($P \leq 0.05$) as determined by Fisher's least significant difference test. Figure from Iqbal *et al.* (2019) and reproduced with the permission of the publisher (paper III).

4.7.5 Deletion of *nps1*, *nps4* and *nps5* genes reduces *C. rosea* biocontrol efficacy against nematode root disease

Significantly higher numbers of nematodes were recovered from soil and wheat roots after eight weeks when soil was inoculated with Δ*nps1* strains of *C. rosea* compared with WT. This indicates that *nps1* deletion significantly reduced the biocontrol potential of *C. rosea* to control plant-parasitic nematodes in soil as well as in roots of wheat, which suggests that NPS1 is a biocontrol factor against nematodes (paper III, Fig. 11A and B). Likewise, *C. rosea* Δ*nps4* and Δ*nps5* strains also showed reduced biocontrol ability against plant-parasitic nematodes compared with WT (paper IV). Previous studies have reported that *C. rosea* produces numerous epipolysulfanyldioxopiperazine compounds with nematicidal activity under *in vitro* conditions (Zou *et al.*, 2010a; Zou *et al.*, 2010b; Li *et al.*, 2006; Dong *et al.*, 2005) and it is likely that *C. rosea* produces similar compounds when inoculated in soil.

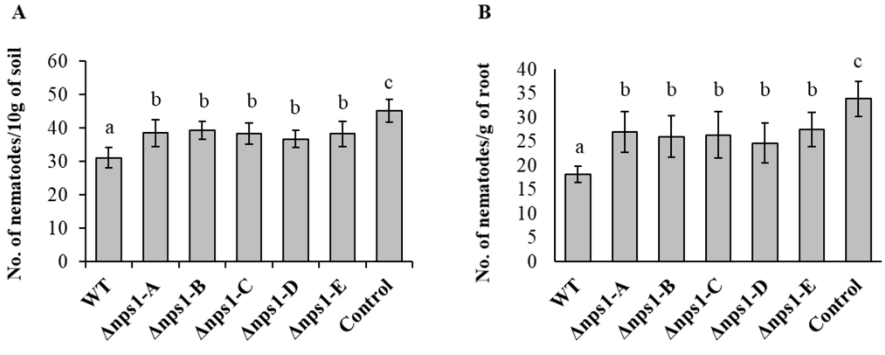

Figure 11. Effect of *Clonostachys rosea* wild type (WT) and Δ*nps1* strains on nematode populations extracted from soil (A) and wheat roots (B) in pots planted with wheat. (A) Impact of inoculating soil with *C. rosea* WT or Δ*nps1* strains on nematode populations, including plant-parasitic nematode genera (i.e., *Boleodorus*, *Helicotylenchus*, *Heterodera* J2, *Merlinus*, *Pratylenchus*, *Paratylenchus* and *Rotylenchus*) and other trophic groups (i.e., bacterivorous, fungivorous and omnivorous nematodes). (B) Two genera of plant-parasitic nematodes, i.e., *Pratylenchus* spp. and *Heterodera* J2, were recovered from roots. Soil that was not inoculated with *C. rosea* acted as the control. Error bars represent the standard deviation based on eight biological replicates. Different letters indicate statistically significant differences ($P \leq 0.05$) between treatments based on the Tukey-Kramer test. Figure from Iqbal *et al.* (2019) and reproduced with permission of the publisher (paper III).

5 Summary

This study aimed to investigate whether the fungus *C. rosea* strain IK726 is able to control plant-parasitic nematodes and, if so, to determine which of the possible mechanisms are used by this fungus to control plant-parasitic nematodes.

The main conclusion from this study are as follows:

1. The application of naturally infested soil with *C. rosea* decreases the population of plant-parasitic nematodes and is positively correlated with improved growth of carrots and wheat (paper I).
2. The action of *C. rosea* shows a certain level of specificity towards plant-parasitic nematodes compared with non-parasitic groups given that *in vitro* and *in planta* assays revealed a higher mortality rate for plant-parasitic nematodes (paper I).
3. Antibiosis through secreted metabolites or enzymes contributes to the antagonistic effect against nematodes (papers I, III and IV).
4. Serine protease subfamilies S1A, S8A, S9X and S33 evolved non-randomly in *C. rosea* (paper II).
5. The increased gene copy number of serine protease subfamilies S8A, S9X and S33 in *C. rosea* and other related mycoparasitic fungi from the same order indicates the involvement of serine proteases in biotic interactions (paper II).
6. Induction of the serine protease gene *prs6* during the interaction of *C. rosea* with *F. graminearum* suggests the involvement of PRS6 in mycoparasitism, perhaps by releasing oligopeptide fragments as part of a sensing mechanism (paper II).
7. Intrinsic variation exists among *C. rosea* strains in terms of antagonism and biocontrol of plant-parasitic nematodes (paper IV).

8. Non-ribosomal peptide synthetase *nps1*, *nps4* and *nps5* gene products influence growth and conidiation in *C. rosea* (papers III and IV).
9. Deletion strains of *nps1*, *nps4* and *nps5* displayed attenuated antagonism against nematodes, which indicates that NRPS-encoding genes play an important role in antagonism against nematodes (papers III and IV).
10. *In planta* nematode root disease assays revealed that *nps1*, *nps4* and *nps5* genes in *C. rosea* are required for efficient biocontrol of plant-parasitic nematodes in soil as well as in roots to protect plants and thereby improve plant growth (papers III and IV).
11. *In planta* fusarium foot rot assays on wheat showed that *nps1*, *nps4* and *nps5* genes are important in *C. rosea* for efficient biocontrol of fusarium foot rot disease on wheat caused by *F. graminearum* (papers III and IV).

6 Future perspectives

Biological control of plant-parasitic nematodes by the fungus *C. rosea* and possible interaction mechanisms were investigated in this study. Previous knowledge about the biocontrol of plant-parasitic nematodes using *C. rosea* was limited although *in vitro* antagonism against nematodes had been reported. This study showed that *C. rosea* can control plant-parasitic nematodes and elucidated possible mechanisms used by this fungus. However, the following aspects need further investigation.

In planta assays and soil inoculation of *C. rosea* have negative impacts on plant-parasitic nematode genera and reduced these populations considerably more than populations of other trophic groups (i.e., bacterivorous, fungivorous and omnivorous nematodes). This is further supported by observations of the effect of *C. rosea* culture filtrates on nematode populations given that plant-parasitic nematodes were more negatively affected by secreted metabolites or enzymes than non-parasitic nematodes. Thus, it would be valuable to investigate this area further by studying the cuticle composition of different groups of nematodes or the biology of nematode groups in more depth to further understand this interesting finding.

The application of *C. rosea* to soil significantly reduced the number of plant-parasitic nematodes and positively increased the shoot length or shoot weight of carrots and wheat. It would be interesting to investigate whether plant growth improved because *C. rosea* induced resistance or if this effect was directly related to a lower number of plant-parasitic nematodes.

The most likely mechanism involved in the control of plant-parasitic nematodes by *C. rosea* is antibiosis through the secretion of metabolites or enzymes. However, the current work did not succeed in identifying compounds secreted by *C. rosea*. Identifying secreted compounds or enzymes with nematicidal properties that antagonize nematodes would be an exciting aspect to explore further.

In the current study, the induction of eighteen serine protease genes was tested during growth on protein compounds, including collagen, because collagen is a major constituent of nematode cuticle. However, unexpectedly none of the investigated serine protease genes were induced during growth on

collagen, indicating that additional factors other than the presence of collagen in the nematode cuticle contribute to the induction of serine protease expression. Further investigations are needed to uncover these other factors and to investigate all the genes in the serine protease gene family to identify candidate genes that contribute to the antagonism of plant-parasitic nematodes.

The *C. rosea* genome contains seventeen non-ribosomal peptide synthetase genes (*nps*). The generation of gene deletion strains of three NRPS genes (*nps1*, *nps4* and *nps5*) revealed that NRPS genes play an important role during *in vitro* antagonism and biocontrol of fusarium foot rot and root diseases caused by nematodes. However, to obtain a more comprehensive view of the role of NRPSs, the other NRPS genes in *C. rosea* need to be explored further by performing functional studies.

The results obtained from the current study showed that *C. rosea* is able to control plant-parasitic nematodes during *in vitro* and *in planta* assays under controlled conditions. However, *C. rosea* biocontrol efficacy against nematodes now needs to be evaluated under field conditions.

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Popular science summary

Nematodes are present everywhere where life exists and are essentially water loving-animals. There are probably more than one million species in total, making them the most abundant animals on Earth. More than 25,000 nematode species are known, including plant-parasitic, bacterivorous, fungivorous and omnivorous nematodes. To date, 4100 species of plant-parasitic nematodes have been described. Diseases caused by these nematodes are estimated to result in the annual loss of 14.6% of crops produced globally, causing economic losses of approximately 157 billion US dollars a year.

To prevent these huge losses, chemical-based nematicides have been used to control these tiny animals. However, the indiscriminate use of these chemicals is now known to be associated with a range of issues, such as the accumulation of pesticide residues in soil and water, toxicity to livestock, potential negative impacts on biodiversity, the environment and human health, and the risk that the nematode population may develop resistance to nematicides. Consequently, chemical-based nematicides are banned in many countries and are not allowed in organic farming. To obtain high levels of agricultural productivity in the future, biological control of plant-parasitic nematodes using naturally occurring microbial antagonists represents a promising alternative to chemical-based nematicides. One such antagonist is the biocontrol fungus *Clonostachys rosea*, which is mainly mycoparasitic (i.e., it attacks and parasitizes other plant pathogenic fungi); however, *C. rosea* has also been reported to have negative impacts on plant-parasitic nematodes.

The main focus of the present study was to investigate the impact of applying *Clonostachys rosea* strain IK726 to an agricultural soil that was naturally infested with nematodes. The soil was mixed with *C. rosea* and afterwards planted with wheat and carrot under controlled conditions. At the end of the experiment, plants were harvested and nematode communities were extracted, counted, identified and grouped based on morphological characters. Nematode communities in treated and untreated soil with and without crops were compared to evaluate the impact of *C. rosea*. Plant-parasitic nematode populations extracted from soil and roots were 40–73% lower in soils treated with *C. rosea*

compared with untreated soil, and plant growth was correspondingly enhanced. Non-parasitic nematodes were unaffected by *C. rosea*-treated soil.

Another focus of this work was to try to determine how *C. rosea* antagonizes plant-parasitic nematodes. Direct parasitism of nematodes was not observed. However, nematodes were unable to grow in a medium in which the fungus had been grown, suggesting that *C. rosea* can produce substances that are harmful to nematodes, such as nematicidal compounds and enzymes such as proteases (which help to breakdown proteins), thereby creating hostile conditions for nematode survival. A genome-wide analysis of protease genes revealed that *C. rosea* contains more proteases than other studied fungi. Furthermore, a computational analysis of gene family evolution revealed that *C. rosea* has increased gene copy numbers of the serine protease family, suggesting that serine proteases may play an important role in biotic interactions.

A genome-wide association study of 53 strains of the biocontrol fungus *C. rosea* was performed against the root-lesion nematode *Pratylenchus penetrans* under *in vitro* conditions. An empirical Bayesian multiple hypothesis testing approach detected 279 single nucleotide polymorphism markers that were significantly associated with a nematode antagonism trait. The putative non-ribosomal peptide synthetase genes *nps4* and *nps5* were located in the identified genomic regions while *nps1* was selected for use in further investigations based on previously published reports. Deletion of the *nps1*, *nps4* and *nps5* genes revealed that NPS1, NPS4 and NPS5 play an important role in fungal growth, conidiation and antagonism against plant-parasitic nematodes. Furthermore, NPS1, NPS4 and NPS5 are important for biocontrol of fusarium root rot and nematode root diseases in wheat.

In summary, the results presented in this thesis show that *C. rosea* strain IK726 can decrease populations of plant-parasitic nematodes in naturally infested soil and, thus, improve the growth of plants. The main mechanism responsible for antagonizing plant-parasitic nematodes is likely to be antibiosis, i.e., the secretion metabolites or enzymes that are harmful to plant-parasitic nematodes. This antagonistic potential can be used to control plant-parasitic nematodes, which may help to reduce applications of chemicals to soils as part of an integrated pest management programme.

Populärvetenskaplig sammanfattning

Nematoder finns överallt där liv existerar, men behöver vatten för sin överlevnad. Det finns antagligen mer än en miljon olika arter, vilket gör nematoder till det vanligaste djuret på jorden. Fler än 25000 arter är beskrivna, och inkluderar olika livsstilar såsom växtparasiter, bakterieätare, svampätare och allätare. Till dags dato har 4100 arter av växtparasitiska nematoder beskrivits. Växtsjukdomar orsakade av nematoder har beräknats att orsaka en global skördeförlust på 14.6% vilket motsvarar en ekonomisk förlust på 157 miljarder amerikanska dollar.

Kemiska växtskyddsmedel har använts för att minska skadorna från dessa djur. Storskalig användning av dessa kemikalier kan dock resultera i flera problem, såsom ansamling av dessa medel i mark och vatten, toxiska effekter på tamdjur, negativa effekter på biodiversitet, miljön och hälsoeffekter på människor, samt utveckling av resistens hos växtparasitiska nematoder. Därför har användningen av kemiska växtskyddsmedel mot nematoder förbjudits i många länder och användningen är inte heller tillåten inom ekologisk odling. Biologisk bekämpning av växtparasitiska nematoder med naturliga mikrobiella antagonister kan potentiellt användas som ett alternativ till kemiska växtskyddsmedel för att bibehålla en framtida hög produktion inom jordbruket. En sådan antagonist är svampen *Clonostachys rosea* som kan användas för biologisk bekämpning genom sin förmåga att parasitera andra svampar och växtparasitiska nematoder.

Ett mål med detta projekt var att undersöka hur *C. rosea* isolat IK726 påverkar nematodsamhället i jordbruksmark. Naturligt infekterad jord blandades med *C. rosea* och effekten på tillväxten av vete undersöktes under kontrollerade miljöbetingelser i en klimatkammare. Veteplantorna skördades i slutet av experimentet och nematodsamhället undersöktes, räknades och identifierades baserat på morfologi. Sammansättningen på nematodsamhället jämfördes mellan behandlingar med och utan *C. rosea*, samt med och utan växter. Genom att blanda in *C. rosea* i jorden så minskade antalet växtparasitiska nematoder med 40-73% jämfört med kontrollbehandligen utan *C. rosea*, med ökad tillväxt hos plantorna som följd. Nematoder som inte är växtparasitiska påverkades inte av behandlingen.

Ett annat mål inom projektet var att undersöka vilka mekanismer som *C. rosea* använder för att minska antalet växtparasitiska nematoder. Direkt parasitism kunde inte observeras. Däremot så kunde inte nematoder överleva i vätskekulturer där *C. rosea* tidigare hade växt, vilket indikerar att *C. rosea* utsöndrar ämnen eller enzymer som påverkar nematoderna negativt. En jämförande studie av antalet proteas-gener i arvsmassan hos *C. rosea* visade att *C. rosea* har fler gener jämfört med andra liknande svampar. En evolutionär studie visade vidare att *C. rosea* har speciellt många gener för serin-proteaser, vilket tyder på att dessa har en viktig funktion för *C. rosea*.

Förmågan att producera ämnen som påverkar rotsårsnematoder negativt undersöktes hos 53 isolat av *C. rosea*. En statistisk analys identifierade vidare 279 mutationer som kunde kopplas till antagonismen mot växtparasitiska nematoder. Två gener (*nps4* och *nps5*) för icke-ribosomala peptidsyntaser (NRPS) identifierades i de genomiska regioner som segregerade med antagonism, och ytterligare en NRPS gen (*nps1*) valdes ut för fortsatta funktionella studier baserat på befintlig litteratur. Funktionella studier av mutanter där generna hade inaktiverats visade att NPS1, NPS4 och NPS5 fyller en viktig funktion för svampens tillväxt, produktion av sporer och för antagonism mot nematoder. Mutanterna kunde inte heller minska mängden sjukdom på vete orsakad av nematoder lika bra som vildtypen där generna fortfarande var intakta.

Sammanfattningsvis så visar resultaten i denna avhandling att *C. rosea* IK726 kan minska mängden växtparasitiska nematoder i naturligt infekterad jord och därmed förbättra tillväxten hos grödor. Den främsta mekanismen är troligen produktion av ämnen och enzymer som har en negativ påverkan på nematoderna. Svampen *C. rosea* kan potentiellt användas inom integrerat växtskydd och därmed bidra till minskad användning av kemiska växtskyddsmedel.

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