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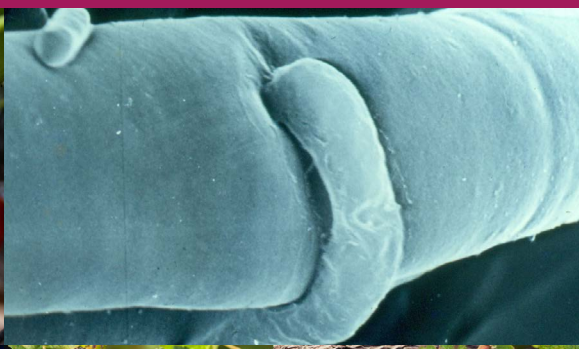
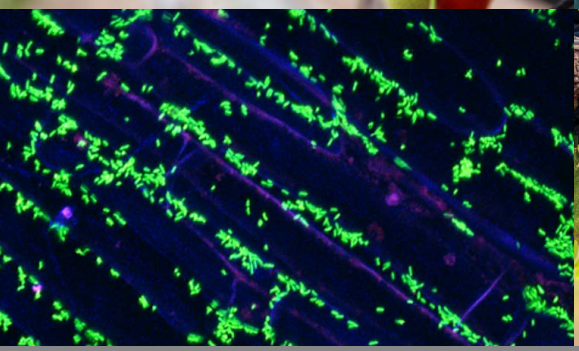


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Clonostachys rosea to control plant diseases

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1 Introduction

The ascomycete fungus *Clonostachys rosea* was reported as an aggressive mycoparasite in the late 1950s (Barnett and Lilly, 1962), and initial attempts to use it for biological control of plant diseases soon followed (Shigo, 1958). Since then, there has been a wealth of new knowledge emerging concerning the ecology, physiology and genetics of *C. rosea*, as well as concerning its applied use as a biological control agent (BCA) including formulation, application strategy, efficiency and safety. In this chapter, we use the definition of biological control as the use of living organisms for the control of plant pathogens/diseases in line with the recent update on the terminology, where biological control falls under the umbrella 'bioprotection', with the term BCAs being used only for living organisms, whereas products based on non-living, nature-based substances are another separate part of bioprotection (Stenberg et al., 2021). Due to the extensive literature available on *C. rosea*, this chapter does

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not represent a comprehensive review but rather aims to highlight selected aspects of *C. rosea* with respect to ecology, mechanisms of action, targeted crops and diseases and product development.

2 Taxonomy and sources

Based on morphology, *C. rosea* (Link) Schroers, Samuels, Seifert & W. Gams was identified as the anamorph of the teleomorph *Bionectria ochroleuca* (Schwein.) Schroers & Samuels (Schroers et al., 1999). This was later confirmed based on DNA sequence data, including internal transcribed spacer (ITS) ribosomal DNA and β-tubulin (*tub*) gene sequences (Schroers, 2001). Following the one-fungus, one-name principle, the use of *C. rosea* as the preferred species label was proposed due to its established use in the scientific literature (Rossman et al., 2013). Until 1999, strains of *C. rosea* were referred to as *Gliocladium roseum* Bainier, now considered a synonym that is sometimes still in use, especially in a more applied, biocontrol context. Two variants of *C. rosea* can be found in the literature, *C. rosea* forma (f.) *rosea* (*G. roseum*) and *C. rosea* f. *catenulata* (*G. catenulatum*), primarily distinguished by the colour of the conidia (white/yellow/salmon and green, respectively). However, a recent study using genealogical concordance phylogenetic species recognition indicates that the two variants constitute a single species (Moreira et al., 2016). Although the vast majority of reports of biological control of plant diseases involves the species *C. rosea*, there is evidence to suggest that certain strains from other, closely related species, also possess biocontrol properties (Table 1, Broberg et al., 2021; Sun et al., 2017; Krauss et al., 2006; García et al., 2003).

Strains of *C. rosea* have been isolated from all continents except Antarctica and from a wide range of habitats (Sun et al., 2020a; Sutton et al., 1997), indicating a cosmopolitan distribution. Strains are typically isolated from soil, fungi, plant debris and from plant parts including roots, leaves and flowers (Walker and Maude, 1975; Nobre et al., 2005; Mueller and Sinclair, 1986; García et al., 2003), but isolations from nematodes and insects are also reported (Verdejo-Lucas et al., 2002; Haarith et al., 2020). Strains of *C. rosea* are even present as endophytes in several halophyte plant species in coastal areas (You et al., 2017). This habitat distribution should be viewed in light of the ecological generalist lifestyle of *C. rosea*, which includes plant endophytism, rhizosphere competence, polyphagous ability and mycoparasitism (Shigo, 1958; Li et al., 2002; Chatterton and Punja, 2012; Saraiva et al., 2015; Maillard et al., 2020). The traits that form the basis of the nutritional versatility that characterises generalist behaviour in *C. rosea* is tightly connected with its ability to control plant diseases and its use as a BCA.

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Table 1 Species of *Clonostachys* with reported biocontrol properties.

Species	Authors	Mycobank ID	Mycobank link
Clonostachys byssicola	Schroers	#485119	http://www.mycobank.org/MB/485119
Clonostachys chloroleuca	G. M. Moreira, L. M. Abreu, L. H. Pfenning & H. J. Schroers	#816994	http://www.mycobank.org/MB/816994
Clonostachys rhizophaga	Schroers	#485120	http://www.mycobank.org/MB/485120
Clonostachys rosea	(Link) Schroers, Samuels, K. A. Seifert & W. Gams	#461067	http://www.mycobank.org/MB/461067
Clonostachys solani	(Harting) Schroers & W. Gams	#456098	http://www.mycobank.org/MB/456098
Clonostachys sp.	Not formally described		

3 Mechanisms of action

The mycoparasitic behaviour of *C. rosea* has attracted a lot of attention since its first description (Barnett and Lilly, 1962) and has been considered an important biocontrol trait for combatting plant pathogens (Karlsson et al., 2018). Biocontrol interactions leading to efficient biocontrol of plant diseases, however, can rely on a range of mechanisms of action beyond parasitism (Baker and Cook, 1974; Jensen et al., 2017; Köhl et al., 2019) and several of these may work in concert (Köhl et al., 2019). As mentioned above, the generalist lifestyle of *C. rosea* has equipped it with traits enabling competition for resources and space, and interference competition through antibiosis, in addition to its mycoparasitic ability (Sutton et al., 1997; Fatema et al., 2018). Its endophytic ability allows for establishment in plant organs close to potential pathogen entry points (Saraiva et al., 2015), and will in some cases result in activation of inducible plant defence responses (Kamou et al., 2020; Wang et al., 2019) leading to induced systemic resistance (ISR) (Lahoz et al., 2004). In the following sections, we will explore the contribution of these mechanisms to *C. rosea* biocontrol, and how it varies depending on the host plant and the pathogen which causes the disease.

3.1 Competition for space and nutrients

Competition for space and resources through priority colonisation ahead of the pathogen (Jensen et al., 2017) is reported to be important for the ability of *C. rosea* to control grey mould, caused by *Botrytis. cinerea*, in strawberry and raspberry flowers (Sutton et al., 1997). Reduced germination of *B. cinerea* conidia on raspberry and rose leaves, and subsequent control of grey mould,

was shown to depend on the competition for scarce nutrients (Yu and Sutton, 1997b; Morandi et al., 2000). It was also shown that competition with indigenous *Penicillium* and *Alternaria* spp. on rose leaves reduced control of *B. cinerea* by *C. rosea* (Morandi et al., 2000).

3.2 Mycoparasitism

The initial reports of mycoparasitic behaviour in *C. rosea* were based on agar plate interaction studies where *C. rosea* was able to overgrow and destroy established cultures of a range of fungi (Shigo, 1958; Barnett and Lilly, 1962). The attack was characterised by collapse of the surface mycelium of the fungal prey and the destruction of the dark pigment produced by some species. The attack involves attachment to the hyphae of the fungal prey and production of an appressorium, followed by penetration (Makkonen and Pohjakallio, 1960; Walker and Maude, 1975). Confocal fluorescence microscopy studies of the interaction between *C. rosea* expressing the green fluorescent protein and *F. oxysporum* forma specialis (f. sp.) *radicis lycopersici* expressing the red fluorescent protein confirmed production of an appressorium during the penetration (Karlsson et al., 2015). However, scanning electron microscopy analysis of the interaction between *C. rosea* and *B. cinerea* showed examples of direct penetration of *B. cinerea* conidia and germ tubes without the formation of appressoria, resulting in cytoplasmic disintegration (Li et al., 2002). Mycoparasitism of oomycete plant pathogens such as *Pythium aphanidermatum* and *P. ultimum* by *C. rosea* was also reported (Chatterton and Punja, 2009; Mamarabadi et al., 2009). Mycoparasitism was reported as an important mode of action for controlling *B. cinerea* on raspberry stems (Yu and Sutton, 1997b). Significant biocontrol of *Zymoseptoria tritici* causing septoria tritici blotch (STB) has been obtained in field experiments over several years using *C. rosea* (Jensen et al., 2019). As *C. rosea* was sprayed on the wheat crop after the initial pathogen infection of the leaves, mycoparasitism seems to contribute to biocontrol of STB.

3.3 Secretion of fungal cell wall-degrading enzymes

Secretion of fungal cell wall-degrading enzymes, such as chitinases, glucanases and proteases, is a component of the mycoparasitic attack (Pachenari and Dix, 1980). Chitinases and glucanases produced by *C. rosea* were confirmed to degrade the cell walls of taxonomically diverse plant pathogens from the oomycete genus *Pythium* and from the fungal genus *Fusarium* (Inglis and Kawchuk, 2002; Chatterton and Punja, 2009). However, the exact contribution of chitinases to biocontrol in *C. rosea* is difficult to assess; deletion of the *chiC2*, *ech37*, *ech42* and *ech58* chitinase genes resulted in mutants being impaired in their antagonistic ability towards other fungi but there was no

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reduction of their biocontrol ability (Table 2, Tzelepis et al., 2015; Mamarabadi et al., 2008b). Overexpression of the *ech37* ortholog (*chi67-1*) in the closely related species *C. chloroleuca* resulted in a mutant with higher chitinase activity in liquid cultures, higher rates of parasitism of *Sclerotinia sclerotiorum* sclerotia and higher efficiency to control *S. sclerotiorum* on soybean (Sun et al., 2017), thereby establishing a link between chitinase activity and biocontrol in *Clonostachys*. Nematodes are also a target for enzymes secreted from *C. rosea*; the extracellular serine protease PrC was shown to exhibit nematicidal activity against *Panagrellus redivivus* (Li et al., 2006). Nematode cuticle degradation products released by the proteolytic activity of PrC were also shown to protect *C. rosea* against oxidative stress by scavenging reactive oxygen species (Zou et al., 2010), a novel mechanism for alleviating environmental stress.

3.4 Secretion of antibiotic compounds

Production of secreted compounds with antifungal activity is another component of the mycoparasitic attack. Furthermore, competition for space and resources is intimately connected with the ability of *C. rosea* to defend occupied resources against other fungi through antibiosis. Antibiosis is therefore considered an important trait of *C. rosea* in biocontrol interactions, due to its ability to produce various secondary metabolites with antagonistic effects towards plant pathogens (Han et al., 2020; Saraiva et al., 2020). For example, the polyketide compounds Clonorosein A and B were shown to inhibit germ tube growth in both *B. cinerea* and *F. graminearum* (Fatema et al., 2018). Furthermore, deletion of the polyketide synthase gene *pks29* in *C. rosea* resulted in mutants with an impaired ability to control fusarium foot rot on barley (Table 2, Fatema et al., 2018). Non-ribosomal peptides are another important group of secondary metabolites in fungi, and *C. rosea* was shown to produce a mix of peptaibol compounds that inhibited growth of *S. sclerotiorum* (Rodriguez et al., 2011). Deletion of the non-ribosomal peptide synthetase genes *nps1*, *nps4* and *nps5* in *C. rosea* compromised the ability of the mutants to protect wheat seedlings against fusarium foot rot and nematode root disease (Table 2, Iqbal et al., 2019; Iqbal et al., 2020). *Clonostachys rosea* was also reported to produce glisoprenin compounds that specifically inhibited appressorium formation by the rice blast fungus *Magnaporthe oryzae*, but without any observable antifungal or antibacterial activities (Thines et al., 1998). Production of compounds with antibiotic effect towards bacteria (Zhai et al., 2016) and nematodes (Dong et al., 2005) by *C. rosea* illustrate the need to defend resources against other microorganisms inhabiting the same ecological niche and may be an important ability for establishment of *C. rosea* in the rhizosphere and phyllosphere. For example, germination of spores of the pathogen *Bipolaris solani* was inhibited on barley leaf surfaces by *C. rosea*, clearly indicating the involvement of antibiosis in competition (Jensen et al., 2016a).

Table 2 *Clonostachys* genes functionally validated for their role in antagonism, xenobiotic tolerance or biocontrol of plant diseases.

Family	Gene[1]	*Clonostachys* strain	Validated function	Reference
Chitinase	*ech58* *ech42* *ech37*	*C. rosea* IK726	Involved in antagonism against *Fusarium culmorum*	Mamarabadi et al., 2008b
	chi67-1	*C. chloroleuca* 67-1	Mycoparasitism of *Sclerotinia sclerotiorum* sclerotia Biocontrol of sclerotinia stem rot of soybean	Sun et al., 2017
	chiC2	*C. rosea* IK726	Antagonism against *Botrytis cinerea* and *Rhizoctonia solani*	Tzelepis et al., 2015
Transaldolase	*tal67*	*C. chloroleuca* 67-1	Antagonism against *B. cinerea* Mycoparasitism against *S. sclerotiorum* sclerotia Biocontrol of sclerotinia stem rot of soybean	Liu et al., 2016b
Phosphatase	*ssd1*	*C. chloroleuca* 67-1	Antagonism against *B. cinerea* Mycoparasitism against *S. sclerotiorum* sclerotia Biocontrol of sclerotinia stem rot of soybean	Lv et al., 2020
Hydrolase	*zhd101*	*C. rosea* IK726	Antagonism against *F. graminearum* Biocontrol of fusarium foot rot on wheat Tolerance to zearalenone mycotoxin	Kosawang et al., 2014b
Protein kinase	*mapk*	*C. chloroleuca* 67-1	Mycoparasitism of *S. sclerotiorum* sclerotia Biocontrol of sclerotinia stem rot of soybean	Sun et al., 2020b
Heat shock protein	*hsp*	*C. chloroleuca* 67-1	Mycoparasitism of *S. sclerotiorum* sclerotia	Sun et al., 2019
Perilipin	*per3*	*C. rosea HL-1-1*	Mycoparasitism of *S. sclerotiorum* sclerotia	Sun et al., 2015c
Hydrophobin	*hyd1* *hyd2* *hyd3*	*C. rosea IK726*	Antagonism against *B. cinerea*, *F. graminearum* and *R. solani* Plant root colonization	Dubey et al., 2014b
LysM protein	*lysm1* *lysm2*	*C. rosea IK726*	Antagonism against *B. cinerea* Biocontrol of grey mold on *Arabidopsis* and fusarium foot rot on wheat Root colonization	Dubey et al., 2020

ABC transporter	*abcG5*	*C. rosea IK726*	Antagonism against *F. graminarum* Biocontrol of fusarium foot rot on barley Tolerance to zearalenone mycotoxin and certain fungicides	Dubey et al., 2014a
	abcG29	*C. rosea IK726*	Biocontrol of grey mould on *Arabidopsis* and fusarium foot rot on barley	Dubey et al., 2016
	abcG6	*C. rosea IK726*	Tolerance to certain fungicides	Broberg et al., 2021
MFS transporter	*mfs464*	*C. rosea IK726*	Antagonism against *F. graminarum*	Nygren et al., 2018
Polyketide synthase	*pks22* *pks 29*	*C. rosea IK726*	Antagonism against *B. cinerea* and *F. graminearum* Biocontrol of fusarium foot rot on barley[2]	Fatema et al., 2018
Non-ribosomal peptide synthetase	*nps1* *nps4* *nps5*	*C. rosea IK726*	Antagonism against plant parasitic nematodes, *B. cinerea* and *F. graminearum* Biocontrol of nematode root disease[3] and fusarium foot rot on wheat	Iqbal et al., 2019, 2020

[1] There is currently no agreement on the use of gene name nomenclature in *Clonostachys*. We propose to use the gene nomenclature currently used for *Neurospora*, *Aspergillus* and *Trichoderma*, where a gene name consists of three small letters and a number (all italicised) while the corresponding protein is denoted by the same letters and number written in capital letters (non-italicized); e.g. *acl1* is the gene encoding the ATP citrate lyase protein ACL1. Abbreviations of species names should not be part of the gene name.
[2] Only deletion of *pks29*, not *pks22*, attenuated biocontrol of fusarium foot rot on barley.
[3] Measured as reduced numbers of plant pathogenic nematodes in wheat roots.

3.5 Tolerance towards antifungal compounds

The strong ability of *C. rosea* for interference competition through antibiosis as well as the production of toxic secondary metabolites from the fungal prey during mycoparasitism emphasises the need for toxin tolerance/detoxification mechanisms in *C. rosea*. *Clonostachys rosea* has indeed been shown to be highly tolerant towards the *Fusarium* mycotoxin zearalenone, with strong antifungal activity (Utermark and Karlovsky, 2007). This ability was shown to depend partly on direct detoxification of zearalenone to less toxic compounds by the ZHD101 lactone hydrolase (Takahashi-Ando et al., 2002; Kosawang et al., 2014b), and partly on active efflux from the cell with the ABCG5 ATP-binding cassette (ABC) transporter (Table 2, Dubey et al., 2014a). Both detoxification and efflux contribute to the ability of *C. rosea* to control fusarium foot rot disease on cereals (Dubey et al., 2014a; Kosawang et al., 2014b). Growth of *C. rosea* was not inhibited by high concentrations of the mycotoxin fumonisin B1, which suggests involvement of efflux transporters in the tolerance as the fumonisin B1 was not degraded (Chatterjee et al., 2016). In contrast, tolerance towards deoxynivalenol-type mycotoxins in *C. rosea* is likely to involve glycosylation followed by efflux (Demissie et al., 2020). Furthermore, *C. rosea* is also shown to be relatively tolerant towards phenazine produced by *Pseudomonas chlororaphis* (Karlsson et al., 2015) and certain xenobiotic substances including fungicides (Roberti et al., 2006; Dubey et al., 2014a).

3.6 Induction of plant disease resistance

There is clear evidence that many *Clonostachys* spp. strains, including *C. rosea*, can live as endophytes in plants (Maillard et al., 2020; Saraiva et al., 2015; Sutton et al., 2002; Chatterton and Punja, 2010; Mueller and Sinclair, 1986). This fact alone is significant for biocontrol as the BCA can be present at the sites of infection of plant pathogens. There is also accumulating evidence that this intimate interaction between *C. rosea* and plants can trigger defence gene expression in plants, as shown in tomato and wheat (Kamou et al., 2020; Mouekouba et al., 2014; Wang et al., 2019). This can be interpreted as the recognition of microbe-associated molecular patterns (MAMPs) from *C. rosea* by the plant and subsequent induction of pattern-triggered immunity (PTI) (Jones and Dangl, 2006; Köhl et al., 2019). However, to what extent this induction of plant defence gene expression by *C. rosea* translates into induced local or systemic resistance (ISR) is less clear. Colonisation of wheat seedlings by *C. rosea* resulted in induction of pathogenesis-related proteins that in turn resulted in significant growth inhibition of the pathogen *F. culmorum* (Roberti et al., 2008). Similarly, *C. rosea* inoculated on roots of tobacco plants triggered ISR in leaves against the biotrophic powdery mildew pathogen *Erysiphe*

orontii mediated by increased activity of 1,3-β-glucanases, 1,4-β-glycosidases, chitinases and peroxidases in the plant leaves (Lahoz et al., 2004). Recent data also show that *C. rosea* strains applied in soil resulted in reduced stem lesion length caused by the pitch canker pathogen *F. circinatum* in Monterey pine, indicating induced disease resistance in forest tree seedlings (Moraga-Suazo et al., 2016). Induced resistance triggered by *C. rosea* was also suggested as a possible biocontrol mechanism in canola against club root disease caused by *Plasmodiophora brassicae* (Lahlali and Peng, 2014) and in tomatoes against grey mould (Mouekouba et al., 2014; Wang et al., 2019), although it was difficult to clearly separate induced resistance from other mechanisms in these studies.

In addition to these mechanisms of biocontrol, *C. rosea* can also trigger an increased plant growth response (Fig. 1, Ravnskov et al., 2006; Johansen et al., 2005). Although plant growth promotion is not considered a biocontrol mechanism *per se*, it can for example result in avoidance of seedling damping-off caused by *Pythium* spp. if the plant seedlings establish faster in the field due to the microbial treatment.

4 Lessons from genomics and transcriptomics

Application of comparative genomics approaches can be very useful in research and application of biocontrol solutions. For example, it allows for accurate identification of species, populations and strains, it can be used for understanding modes of action and for identification of genetic markers associated with biocontrol traits. Genomic information from *Clonostachys* species has increased rapidly during the recent past. Genome sequence data are currently available for 56 different strains of *C. rosea* (Demissie et al., 2021; Wang et al., 2021; Broberg et al., 2018; Karlsson et al., 2015), 4 strains of *C. byssicola* (Broberg et al., 2021), 4 strains of *C. chloroleuca* (Broberg et al., 2021; Sun et al., 2015a), 1 strain of *C. solani* (Broberg et al., 2021), 3 strains of *C. rhizophaga* (Broberg et al., 2021; Liu et al., 2016a) and 1 strain representing an undescribed *Clonostachys* species (Broberg et al., 2021). The gene content in a species is partly shaped by selection and therefore reflects adaptations towards the ecological niche of the species. Hence, comparing the gene content in *Clonostachys* with other closely related species can provide important clues to the mechanistic basis of traits that are important for their use in biological control.

4.1 Genes encoding proteins involved in secondary metabolite biosynthesis and efflux

One feature that stands out when comparing gene content in *Clonostachys* with plant pathogenic *Fusarium* and mycoparasitic *Trichoderma* species, is the high number of genes involved in the biosynthesis of secondary metabolites,

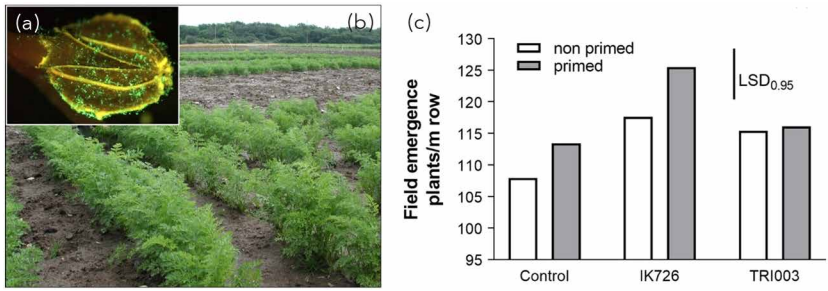

Figure 1 Seed coating and biopriming of carrot seed with *C. rosea* strain IK726 and *Trichoderma harzianum* strain TRI003 to improve field emergence of carrot. (a) Germination of an IK726 bioprimed seed showing hyphal regrowth of the *gfp*-marked IK726 mutant *d11*, (b) Establishment of the carrot plants in the field following seed coating and biopriming, and (c) Effect of IK726 and TRI003 seed coating and biopriming on plant establishment (Jensen, B., unpublished data).

including polyketide synthases, non-ribosomal peptide synthetases and cytochrome P450s (Karlsson et al., 2015; Broberg et al., 2021). In fungi, secondary metabolites perform a variety of functions including protection from biotic and abiotic stresses and interaction with other organisms (Keller et al., 2005; Osbourn, 2010). In *C. rosea*, 75% of the 32 predicted polyketide synthase genes are located in secondary metabolite biosynthetic clusters (Fatema et al., 2018), which is a higher proportion compared with the mentioned mycoparasitic *Trichoderma* species (50%). Gene expression analyses also show induced expression of 17 polyketide synthase genes in *C. rosea* during interactions with *B. cinerea* and *F. graminearum* (Fatema et al., 2018; Demissie et al., 2020; Nygren et al., 2018). Induced expression of 20 polyketide synthase genes was also correlated with pigmentation in *C. rosea* (Fatema et al., 2018). There are 17 predicted non-ribosomal peptide synthetase genes in the *C. rosea* genome (Karlsson et al., 2015; Broberg et al., 2021), from which *nps1*, *nps4*, *nps5* and *nps13* are shown to be induced during mycoparasitic interactions (Nygren et al., 2018; Iqbal et al., 2019; Iqbal et al., 2020). These data fit well with the idea of mycoparasitism and interference competition through antibiosis being an important component of the biocontrol ability of *C. rosea* (Karlsson et al., 2018).

The high numbers of genes associated with secondary metabolite biosynthesis are, not surprisingly, accompanied by equally high numbers of membrane transporter genes predicted to be involved in drug efflux (Karlsson et al., 2015; Nygren et al., 2018; Broberg et al., 2021). More specifically, this relates to the ABC transporter families (Kovalchuk and Driessen, 2010) ABC-B (multidrug resistance proteins), ABC-C (multidrug resistance-associated proteins) and ABC-G (pleiotropic drug resistance proteins), and the major facilitator superfamily (MFS) Drug:H+ Antiporter-2 family. Transcriptomic

analyses show that several members of these groups are induced in *C. rosea* and *C. chloroleuca* during interactions with other fungi and during exposure to fungal metabolites and mycotoxins (Kosawang et al., 2014a; Lysøe et al., 2017; Demissie et al., 2018; Nygren et al., 2018; Demissie et al., 2020; Sun et al., 2015b), but also during exposure to bacterial metabolites (Karlsson et al., 2015; Kamou et al., 2016). The ability to neutralise compounds with antifungal activity produced by other microorganisms or defence molecules produced by plants by efflux mechanisms may be an important trait contributing to the biocontrol property of *Clonostachys*. In addition, several sugar and small organic compound MFS transporter gene families contained high gene numbers in *C. rosea* (Nygren et al., 2018), perhaps involved in nutrient uptake.

4.2 Genes encoding fungal cell wall-degrading enzymes

As mentioned in the previous section, secretion of fungal cell wall-degrading enzymes such as chitinases and proteases is one of the suggested mechanisms involved in *C. rosea* biocontrol (Pachenari and Dix, 1980). However, this view is only partially corroborated from a genomics point of view. High numbers of serine protease genes in *C. rosea*, as well as in mycoparasitic *Trichoderma* species, suggests an involvement of these proteases in biotic interactions (Iqbal et al., 2018a). In contrast, *C. rosea* only possesses a moderate number of chitinases (14 genes) compared with certain *Trichoderma* species (Tzelepis et al., 2015). However, both protease and chitinase genes are induced during mycoparasitic interactions in *C. rosea* (Tzelepis et al., 2015; Iqbal et al., 2018a; Lysøe et al., 2017; Mamarabadi et al., 2008a) and *C. chloroleuca* (Sun et al., 2015b). High gene numbers of carbohydrate-active enzymes targeting components of plant cell walls, in particular xylan and rhamnose/pectin (Broberg et al., 2021; Karlsson et al., 2015; Atanasova et al., 2018), may provide the basis of the saprophytic capability of *Clonostachys* and be important for its establishment in soil and the rhizosphere.

4.3 Genes encoding small secreted proteins

Another notable difference between *Clonostachys* and *Trichoderma* species is the low numbers of hydrophobin and LysM protein genes in *Clonostachys*, compared with *Trichoderma*. Hydrophobins are small, cysteine-rich secreted proteins found only in fungi (Wösten, 2001). These proteins aggregate on the outer surface of fungal cell walls and develop amphipathic layers that perform a variety of biological functions in the life cycle of filamentous fungi, including a role during interactions between the fungus and the environment (Wösten, 2001). The *C. rosea* genome contains three class II hydrophobin (*hyd*) genes, which is in strong contrast with the *T. atroviride* and *T. virens* mycoparasites

that contain 10 and 9 hydrophobin-encoding genes, respectively (Dubey et al., 2014b). Gene deletion strains of *hyd1* and *hyd3* displayed more aggressive behaviour towards *B. cinerea*, *F. graminearum* and *Rhizoctonia solani*, which also translated into an increased ability to control *B. cinerea* infection of *Arabidopsis thaliana* leaves (Table 2, Dubey et al., 2014b). *Hyd1* and *hyd2* double deletion strains displayed enhanced root colonisation compared with the *C. rosea* wild type strain, while the Δ*hyd3* strain showed reduced root colonisation ability (Dubey et al., 2014b). Taken together, these data show that hydrophobins have an important role in mediating biotic interactions in *C. rosea*.

Lysin motif (LysM) domains are approximately 50 amino acids long carbohydrate-binding modules, reported in proteins from all kingdoms of life including fungi (Kombrink and Thomma, 2013). In fungi, LysM modules can be found with varying numbers of LysM modules either together with catalytic protein modules (referred to as LysM-containing proteins) or without any known catalytic module (referred to as LysM effectors) (de Jonge and Thomma, 2009). LysM effectors act as a virulence factor in plant pathogenic, entomopathogenic and mycoparasitic fungi, either by scavenging chitin oligomers, a well-known MAMP molecule, or by protecting the fungal cell wall against hydrolytic enzymes (Kombrink and Thomma, 2013; Cen et al., 2017; Romero-Contreras et al., 2019). *Clonostachys rosea* only contains three *lysm* genes, compared with 12 and 18 genes in *T. atroviride* and *T. virens*, respectively (Dubey et al., 2020). Gene deletion mutants of the two LysM effector genes *lysm1* and *lysm2* were reduced in their ability to control plant diseases caused by *F. graminearum* and *B. cinerea* (Fig. 2, Table 2, Dubey et al., 2020). Furthermore, a *lysm1* and *lysm2* double deletion strain displayed reduced ability to colonise wheat roots (Dubey et al., 2020).

5 Product development and commercialisation

5.1 Selecting the right strain

A strategy for developing a commercial BCA product embraces a whole range of criteria to be fulfilled such as selection, production and formulation of a microorganism into a storable product that can be easily applied for disease control in greenhouses and in fields (Köhl et al., 2011). A crucial part is the screening step for selection of strains efficient in plant disease control. An example of a screening procedure for selection of efficient biocontrol strains of *C. rosea* is from a Nordic research program 1990–1993. This screening system included the plant in question and simulated the natural conditions where the BCA is to be used (Knudsen et al., 1997; Teperi et al., 1998). This turned out to be a very successful strategy leading to selection of several *C. rosea* strains in Denmark and Finland. Among those the strain J1446 used in the commercial products LALSTOP G46 WG®, Prestop® and Gliomix® (Table 3) and the strain *C. rosea* IK726 (Table 4). Both strains have since been studied intensively in

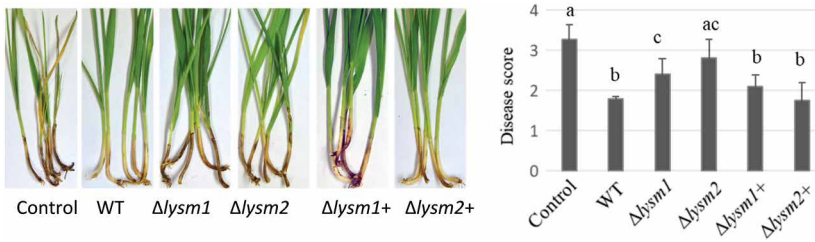

Figure 2 LysM effector proteins LYSM1 and LYSM2 contribute to the biocontrol of fusarium foot rot disease on wheat. Wheat seeds were coated with *C. rosea* conidia and planted in moist sand together with a *F. graminearum* agar plug. *Clonostachys* strains include wild type (WT), *lysm* gene deletion mutants (Δ*lysm1/2*) and deletion mutants complemented with a functional *lysm1/2* gene (Δ*lysm1/2*+). Seedlings were harvested 3 weeks post-inoculation and disease symptoms were scored on 0–4 scale. The figure is adapted from Dubey et al., 2020.

Denmark and Sweden for their efficacy and biocontrol traits (Jensen et al., 2007; Karlsson et al., 2015).

Reliance on *in vitro* screening procedures, such as dual agar plate tests, is not encouraged due to the complexity of the biocontrol trait, involving multiple and complex mechanisms depending on the BCA, pathogen, host plant and environment (Harman et al., 2004; Jensen et al., 2017; Köhl et al., 2019; Rojas et al., 2020). For example, the *Clonostachys* sp. strain CBS 192.96 efficiently controlled fusarium foot rot disease on wheat seedlings, caused by *F. graminearum*, despite its inability to suppress the growth of *F. graminearum in vitro* (Broberg et al., 2021). Another example is *C. rosea* strain IK726 that failed to suppress *in vitro* growth of *F. culmorum* but efficiently controlled foot rot disease on wheat seedlings caused by the same pathogen (Knudsen et al., 1997). We will not address all relevant steps outlined in Köhl et al. (2011) but in the following give some selected examples of aspects addressed for developing *C. rosea* strains into marketable BCAs.

5.2 Production and formulation: effects on viability and shelf life

Economical mass production of storable high-quality propagules, e.g. spores and chlamydospores, is a prerequisite for the successful development of fungal BCAs. The view that BCAs based on filamentous fungi should be produced by solid-state fermentation (i.e. (Köhl et al., 2011)) probably has its origin in the work with production of *Trichoderma* spp., where it was shown that solid fermentation resulted in the best spore quality and shelf life (Agosin et al., 1997). Solid media are also often applied for the production of *C. rosea* and a high spore production can be obtained, e.g. on wheat grains (James and Sutton, 1996; Sutton et al., 1997), a mixture of sphagnum peat and wheat bran (Fig. 3a and b, Jensen et al., 2000) or on mixtures of wheat bran and cornmeal (Zhang et al.,

2013; Zhang et al., 2015). Maximum spore concentration on a solid substrate is typically reached after 10–14 days (Jensen et al., 2002; Zhang et al., 2015). Nevertheless, the quality of the spores can in some cases be improved if the production period is extended from, e.g., 12 to 18 days (Jensen et al., 2002). This suggests that extending the production period a few days after maximum spore concentration is reached could be relevant in order to maintain viability and biocontrol efficacy for longer periods. High concentrations of *C. rosea* spores can also be produced during submerged liquid fermentation (de Andrade Carvalho et al., 2018). However, direct drying of propagules strongly decreases their viability (Jensen, 1999). Nevertheless, mixing *C. rosea* spores produced in liquid with, e.g. diatomic clay before drying can improve survival, shelf life and biocontrol efficacy significantly (Fig. 3c, Jensen, 1999; Jensen et al., 2002).

Likewise, the difficulties in stabilising spore viability are demonstrated for *T. harzianum* too (Muñoz et al., 1995; Agosin et al., 1997; Harman et al., 1991). The authors showed that spores from solid fermentation developed a thicker outer cell wall layer as compared to spores from liquid culture and suggested that this is important for the desiccation tolerance and enhanced shelf life of *Trichoderma* spores. This trait might also be relevant to study further for improving shelf life of *C. rosea* spores.

Another often overlooked trait of BCA spores is their ability to germinate fast as this should enhance their capability to control pathogens such as *Pythium* sp. that can infect a seed in less than 4 h after sowing (Taylor et al., 1991). Hence, *C. rosea* spores start germinating within 4–6 h (Fig. 4a, Jensen, 1999) which was considerably faster than for, e.g. *T. harzianum* spores (Fig. 4b). In fact, after 12 h >70% *C. rosea* spores had germinated as compared to <1% for *T. harzianum* (Fig. 4b). Similarly, Sutton and Peng (1993) showed faster germination of *C. rosea* than for *Trichoderma* and *Penicillium* on strawberry leaf disks incubated at 10–20°C. However, it should be noted that drying spores can strongly reduce both germinability and speed of germination (Fig. 4a and b). This should be taken into consideration when testing the efficacy of BCAs towards commercial application as tests only with dosages of freshly harvested spores might give misleading results (Jensen et al., 2000).

There is limited information available in the public domain concerning large-scale solid-state production of *C. rosea* at an industrial level. However, a two-step submerged/solid-state scale-up production process has been demonstrated to enhance *C. rosea* spore production (Krauss et al., 2002). Furthermore, a novel solid-state fermenter type based on enhanced growth area for *C. rosea* spores in the medium using optimised response surface methodology has been developed (Zhang et al., 2013; Zhang et al., 2015). Production of *Clonostachys* chlamydospores, i.e. thick-walled highly desiccation-tolerant resting structures, is an alternative approach that seems to work for the species *C. chloroleuca* (Sun et al., 2014). This method might also be relevant for *C. rosea* production.

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Table 3 Examples of commercial products based on *Clonostachys rosea*.

Product	Strain	Strain(s) registered in	Production company	Distributor(s)	Region
Baikekong 2®	Strain info not available	China	Harbin Baikekong Biotech. Ltd		China
Gliogen®	VKPM-F1324	Russia	Ecogen LLC	Ecogen LLC	Russia
Guangenbao®, Guanjunling®, Zhongbaofenzuan®	Strain info not available (Product mixed with *Bacillus subtilis*)	China	Chinese Acad. Agric. Sci., Beijing Qigao Biotechnol. Co., Ltd. and Beijing Green Agric. Sci. & Technol. Group Co. Ltd		China
Kamoi®	CPQBA 040-11 DRM 07	Brazil	Agrivalle Brasil Indústria		Brazil
Lalstop46^{WG}®	JI446	Canada, USA	Lallemand	Lallemand, Jetharvest	USA, Canada
Prestop®	J1446	EU, USA, Canada	[1]Danstar Ferment AG	[1]Verdera Danstar Ferment AG	EU (some countries), USA, Canada
Prestop 4B® (bee-vector delivery)	J1446	EU, USA, Canada,	[1]Danstar Ferment AG	Biobest (Flying doctors)	
Vectorite with CR-7®	CR-7	USA	Bee-Vectoring-Technology BVT	BVT	USA

[1]Danstar Ferment AG and Verdera are Lallemand Inc's subsidiary companies.

Table 4 Selected examples of biological control of diseases by application of *Clonostachys rosea*.

Plant/Pathogen	Disease	Isolate origin	Isolate ID	Experimental conditions	Application method	Effects on disease development	Other remarks	Reference
Strawberry								
B. cinerea	Grey mould	Soil, Finland	Verdana B[4] PreStop	Greenhouse	Bee delivery	Reduced berry infection and improve shelf-life of berries	*C. rosea* vectored by bumblebees	Van Delm et al., 2015
B. cinerea	Grey mould	Collection of microorganisms Embrapa Meio Ambiente, Brazil	LQC 62	Field trial	Spraying	Reduced grey mould incidence of fruits	UV-B tolerant *C. rosea* isolate. High UV-B radiation had no influence on efficacy	Nechet et al., 2017
B. cinerea	Grey mould	Strawberry fruit, Canada	PG-A-Fr-88-710	Field trial	Spraying and bee delivery	Reduced grey mould incidence of flowers and fruits	Weekly spraying treatment compared to bee delivery	Peng et al., 1992
B. cinerea	Grey mould	Brazilian ecosystems	Mixture of four isolates	Field trial	Spraying	Reduced flower and fruit infection. Increased yield	Integration with fungicide application	Cota et al., 2009
Raspberry								
B. cinerea	Grey mould	Strawberry fruit, Canada	PG-A-Fr-88-710	Field trial	Spraying and bee delivery	Reduced fruit rot	Comparing honeybee and bumblebee vectoring	Yu and Sutton, 1997a
Rose								
B. cinerea	Grey mould	Strawberry fruit, Canada	PG-A-Fr-88-710	Growth chamber	Drop inoculation	Reduction of *B. cinerea* sporulation	Interactions with indigenous fungi (*Alternaria*, *Aspergillus*, *Penicillium*)	Morandi et al., 2000

Tomato								
B. cinerea	Grey mould	Strawberry fruit, Canada	PG-A-Fr-88-710	Greenhouse, hydroponic	Spraying to deleafed stem wounds	Reduction of *B. cinerea* sporulation in wounds	*C. rosea* established endophytically in stems	Sutton et al., 2002
B. cinerea	Grey mould	Brazil	NCR19/F, NCR60/F, NCR61/F, NCR62/F	Growth chamber, 18°C	Spraying	Reduced incidence and severity of stem symptoms	Wounded stems of whole plants. Inoculation 1 day or at the same time as Bc	Borges et al., 2015
B. cinerea	Grey mould	China	Unknown	Growth chamber	Spraying detached tomato fruits	Reduced incidence and severity on fruits	*C. rosea* either 12 h before or 12 h after *B. cinerea*. Incubated at 25°C at, high humidity	Gong et al., 2017
B. cinerea	Grey mould	Turfy soil, China	Unknown	Growth chamber	Spraying detached leaves	Reduced severity	*B. cinerea* and *C. rosea* applied at the same time	Mouekouba et al., 2014
Alternaria solani, A. alternata	Early blight	Soil, Finland	Prestop, J1446	Greenhouse and field trial	Spraying	Lowered early blight disease severity	*C. rosea* was as effective as copper	Egel et al., 2019
Fusarium oxysporum f. sp. *radices lycopersici*	Root rot	Barley root, Denmark	IK726	Growth chamber	Seedling dip	Reduced disease severity	Microscopy showing *C. rosea* appresoria on *F. oxysporum* hyphae	Karlsson et al., 2015
F. oxysporum	Vascular wilt	Barley root, Denmark	IK726	Greenhouse	Root dip and drenching	Reduced wilt severity	Endophytic colonisation of root and stem	Højer, 2014
Cereals (wheat, barley)								
F. culmorum	Seedling blight, root rot	Barley root, Denmark	IK726	Field trials and growth chamber	Seed treatment	Reduced seedling blight, increased yield in field trial	Significant efficacy at soil temperatures ranging from 6-12°C	Jensen et al., 2000; Knudsen et al., 1995

(*Continued*)

Table 4 (*Continued*).

Plant/Pathogen	Disease	Isolate origin	Isolate ID	Experimental conditions	Application method	Effects on disease development	Other remarks	Reference
F. culmorum	Seedling blight	Wheat crown infected with *F. culmorum*	CR47	Growth chamber	Seedling treatment	Reduced seedling blight		Roberti et al., 2000
F. graminearum	Fusarium head blight	Pea plant, Canada	ACM941	Field trial	Spraying	Reduced FHB and DON content in grains, increased yield	Effects compared to the fungicide tebuconazole	Xue et al., 2009
F. graminearum	Fusarium head blight	Pea plant, Canada	CLO-1 (isolate ACM941)	Field trial	Spraying	Reduced FHB and DON content in grains, increased yield	3 cultivars, highest efficacy in resistant cultivar, dose-response relationship	Xue et al., 2014
Zymoseptoria tritici	Septoria tritici blotch	Barley root, Denmark	IK726	Field trial	Spraying	Reduced STB severity		Jensen et al., 2019
Puccinia triticina, Pu. hordei, Pu. coronata f. sp. *avenacea*	Rusts	Isolated from rust pustules on wheat and oat plants, Australia	H2	Growth chamber	Spraying	Reduced pustle number	Detached leaves on water agar	Wilson et al., 2020
Bipolaris sorokiniana	Leaf blotch	Barley roots, Denmark	IK726	Growth chamber	Spraying	Reduced severity and sporulation	Stored clay formulation. Timing and dosis decisive for control efficacy	Jensen et al., 2016a, b
Potato								
Rhizoctonia solani	Black scurf	*R. solani* hyphae	MpA	Greenhouse	Stem inoculation by agar plug	Reduced black scurf incidence and severity, increased yield of healthy tuber	*C. rosea* and *R. solani* was co-cultivated on stems	Salamone et al., 2018

F. avenaceum, F. coeruleum	Dry rot	Barley roots, Denmark	IK726	Growth chamber	Drop inoculation in wounds	Reduced dry rot severity		Samils et al., 2016
Helminthosporium solani	Silver scurf	Barley roots, Denmark	IK726	Growth chamber	Tuber treatment	Reduced silver scurf severity		Lysøe et al., 2017
Carrot								
A. dauci, A. radicina	Seedling blight	Barley root, Denmark	IK726	Growth chamber	Bio-priming	Eradication of seed-borne *Alternaria* spp., increased seedling stand	Colonisation visualized using *gfp*-tagged IIK726 mutant	Jensen et al., 2004
A. dauci, A. radicina	Seedling blight	Barley root, Denmark	IK726	Growth chamber and field trial	Seed treatment	Increased number of healthy seedlings		Koch et al., 2010
Pulses (pea, soy bean)								
Fusarium, Alternaria, Aphanomyces, Pythium	Pea root rot complex	Pea plant, Canada	ACM941 (ATTCC74447)	Greenhouse, field trial	Seed treatment	Enhanced seedling emergence and yield	*C. rosea* colonize seed, hypocotyl and root	Xue, 2003
Sclerotinia sclerotiorum	Stem rot	Vegetable crop, China	67-1 (*C. chloroleuca*)	Greenhouse	Leaf inoculation	Reduced stem rot severity		Sun et al., 2020b
S. sclerotiorum	Root rot	Suppressive soil, Argentina	BAFC3874	Greenhouse	Mixing into soil	Increased survival		Rodriguez et al., 2011
Pythium ultimum	Damping-off	Unknown	Unknown	Greenhouse	Bark inoculum mixed into the substrate	Reduced damping-off severity		Steinmetz and Schönbeck, 1994

(*Continued*)

Table 4 (*Continued*).

Plant/Pathogen	Disease	Isolate origin	Isolate ID	Experimental conditions	Application method	Effects on disease development	Other remarks	Reference
Oilseed rape								
Plasmodiophora brassicae	Clubroot	Barley roots, Denmark	IK726	Greenhouse	Seed treatment	Reduced disease severity and incidence, reduced *P. brassicae* biomass in roots	Highest control efficacy in resistant cultivar	Andersen et al., 2018
Pl. brassicae	Clubroot	Soil, Finland	Prestop (J1446)	Greenhouse	Soil drench at seeding	Reduced incidence and disease severity, reduced *P. brassicae* biomass in roots	The Prestop product more effective than a conidial suspension	Lahlali and Peng, 2014
Pl. brassicae	Clubroot	Soil, Finland	Prestop (J1446)	Greenhouse, field trials	Soil drench at seeding and seed treatment	Reduced club root severity	Soil drench is more efficient than seed treatment. In field only biocontrol effect in the resistant cultivar	Peng et al., 2011
Chinese cabbage								
Pl. brassicae	Clubroot	Soil, Finland	Prestop (J1446)	Field trial	Soil drench at seeding	Reduced clubroot disease severity	Biocontrol in susceptible cultivar	Peng et al., 2011

Py. tracheiphilum	Chinese cabbage	Leaf and head rot	IK726	Field trial	Spray application to soil surface under leaves	Reduced percentage of attacked plants and increased number of marketable heads	Sprayed to soil and leaf before head setting	Møller et al., 2003
Pine								
F. circinatum	Pitch canker	Pine tissues and soil	Cr7, Cr8	Growth chamber	Drenching	Reduced lesion length	Effective only with a resistant variety	Moraga-Suazo et al., 2016
Cocoa								
Moniliophthora roreri, *Phytophthora* spp., *Theobroma cacao*	Frosty pod rot, black pod	Panama	Several strains	Field trial	Spraying	Reduced disease severity, increased percentage healthy pods		Krauss et al., 2006
Rosellinia spp.	Root rot	Soil, Costa Rica	Several strains (*C. byssicola, C. rhizophaga*)	Greenhouse	Mixing into soil	Reduced disease severity		García et al., 2003

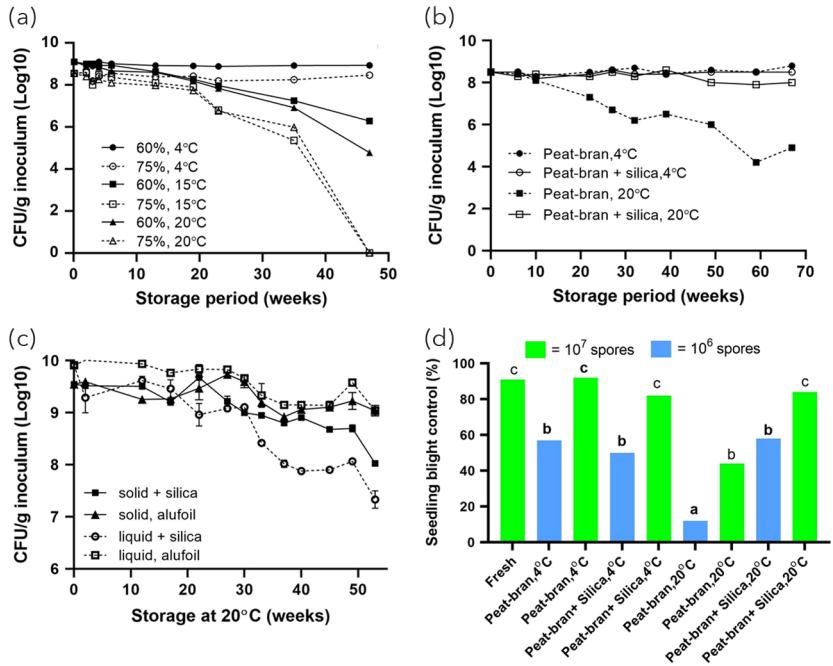

Figure 3 Influence of production, formulation and packaging of *C. rosea* isolate IK726 inoculum on shelf life (CFU/g inoculum) at 4°C and 20°C and on biocontrol efficacy. (a) Effect of water content in the solid peat-bran substrate, (b) Effect of packaging peat-bran inoculum with and without a silica desiccant, (c) Effect of formulating liquid or solid-state produced spores in a clay carrier and packaging the inoculum with silica or in sealed alufoil bags, and (d) Control of fusarium seedling blight using peat-bran inoculum packaged with and without a silica desiccant (Fig 2b) and stored for 29 weeks at 4°C and 20°C. Seeds infested with *F. culmorum* were coated with 10^6 or 10^7 spores/ml of *C. rosea* recovered from the stored formulations. The figures are modified from Jensen (1999). The *C. rosea* peat-bran inoculum was produced in a mixture of sphagnum peat, wheat bran and water (15:26:59, w/w/w) incubated for 2 weeks at 21°C. Subsequently, the inoculum was air-dried, milled and stored (See Jensen et al., 2000 and Jensen et al., 2002 for more details).

Shelf life and vitality of produced propagules can be optimised using various formulation and packing methods and by proper handling after production. Temperature and moisture content are key factors that influence the storage shelf life of *C. rosea* propagules produced both by solid and liquid fermentation (Fig. 3a–c). Significantly longer storage times of spores (months) with preserved biocontrol efficacy (Jensen et al., 2000; Jensen et al., 2002), or even several years at 4°C (Jensen, D. F., unpublished data), was obtained by drying the spores down and keeping them stored at low relative humidity. Raising the storage temperature above 15°C, however, can drastically reduce viability of the inoculum within a few months if the moisture content is not kept

low. By optimising the packaging of the inoculum, e.g. with the desiccant silica (Fig. 3b) or formulating the propagules in clay followed by airtight packaging (Fig. 3c) can increase shelf-life for more than half a year at 20°C and the biocontrol efficacy can be maintained (Fig. 3d, Jensen et al., 2002). Treatments of seed with *Clonostachys* spores several months before sowing is also a possibility but might give problems with shelf life depending on how dry the seed is and how the humidity and temperature are controlled during storage of treated seeds (Jensen et al., 2002).

5.3 Commercial products based on C. rosea

On a global scale, at least 10 different commercial products based on *C. rosea* strains are available (Table 3). Some products are available in several countries whereas others are only available in certain regions or countries. So, BCA products are registered in Brazil, China, the EU, Russia and the USA. According to product information, they can be used in a wide range of crops. These include grain crops, cabbage, legumes, vegetables, beans, tomato, cucumber, pepper, tomato, strawberry, raspberry, black current and other *Ribes* spp., blueberry, pome fruit (pear, apple, quince, various *Crataegus* spp.), stone fruit (apricot, peach, plums), herbs and aromatic plants, ornamental plants, potted plants, cut flowers, forest nursery tree seedlings and tree nuts (butternut, chestnut, macadamia, pecan, pistachio). Furthermore, these products are listed to target a wide range of disease types, including damping-off (caused by *Pythium* spp.

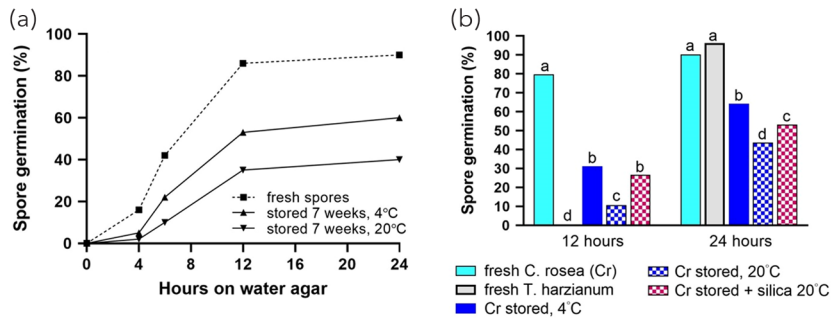

Figure 4 Time-dependent germination of *C. rosea* isolate IK726 on water agar. (a) Spores freshly harvested and undried or recovered from dried peat-bran inoculum stored at 4°C and 20°C, respectively and (b) Freshly harvested and undried spores of *C. rosea* and *T. harzianum* and spores of *C. rosea* recovered from dried peat-bran inoculum stored for 18 weeks with and without silica desiccant at 4°C and 20°C. The figures are modified from Jensen (1999). The *C. rosea* peat-bran inoculum was produced in a mixture of sphagnum peat, wheat bran and water (15:26:59, w/w/w) incubated for 2 weeks at 21°C. Subsequently, the inoculum was air-dried, milled and stored (See Jensen et al., 2000 for more details).

and *R. solani*), grey mould (*B. cinerea*), root- and stem base rot (*Phytophthora* spp. and *Sclerotinia* spp.), leaf spot diseases and fusarium head blight (*Fusarium* spp.).

6 Delivery and action of *C. rosea* as a biological control agent

Application of *C. rosea* has shown potential for biological control of diseases in many important crops. In Table 4, we have listed selected examples of experiments where significant biocontrol efficacy of various *C. rosea* isolates was demonstrated in field trials, greenhouse or in a growth chamber. The majority of examples involve the isolates J1446 (now commercialised in the products LALSTOP G46 WG®, Prestop® and GlioMix®) and IK726, both isolated in a Nordic research programme. Both soil-borne and seed-borne pathogens as well as pathogens attacking leaf, stem, flower and fruit can be controlled by *C. rosea*. It is important to note that the outcome of interactions between host plant genotype and the target pathogen is strongly influenced by the surrounding biotic and abiotic environment which decide the severity of the disease. Realising that an introduced BCA has to perform its action in this highly complex setting makes it difficult to predict if the BCA is going to be successful in a given niche in soil or on the plant. Therefore, successful biological disease control relies on finding the most efficient method for delivery of the BCA in an active state, at the right place, at the right time with the right dose.

6.1 Using pollinators for C. rosea *delivery*

The idea of using pollinators to deliver BCAs to flowers goes back to the work of Peng et al. (1992) and Sutton et al. (1997). They used honeybees or bumblebees to deliver spores of *C. rosea* to strawberry and raspberry flowers to control grey mould caused by *B. cinerea* (Table 4). Infection by the pathogen mainly occurs in newly opened flowers. In fact, honeybee vectoring (Peng et al., 1992) and bumblebee vectoring (Van Delm et al., 2015) during flowering resulted in a more efficient spore delivery and thereby better grey mould control in strawberry flowers and fruits as compared to weekly spraying with *C. rosea*. In raspberry, bumblebee delivery of spores controlled grey mould in flowers more efficiently than one *C. rosea* spray application at the beginning of flowering (Yu and Sutton, 1997a). The success of bee delivery is probably achieved because they visit the newly opened flowers delivering the spores with more precise timing, prior to the natural colonisation of flowers by *B. cinerea* (Peng et al., 1992). Using bumblebees instead of honeybees gave more stable results as honeybees were more prone to attraction to other crops flowering at the same time and therefore delivering the BCA to the wrong crop – a problem not seen with bumblebees (Sutton et al., 1997). This method with bumblebees and a

special hive construction to facilitate vectoring of *C. rosea* is described in more detail in Yu and Sutton (1997a) and is now used commercially both in Europe and in the USA (Table 3).

6.2 Seed coating for C. rosea *delivery*

Delivering the BCA with the seed is a common strategy used to control seed-borne, damping-off and seedling diseases (Table 4). Especially for seed-borne pathogens, it is an obvious approach (Knudsen et al., 1995; Jensen et al., 2000; Jensen et al., 2004; Bennett et al., 2009; Koch et al., 2010). For example, using *C. rosea* seed treatment for control of seedling diseases in cereals caused by *F. culmorum* and *B. sorokiniana* have consistently reduced the diseases in several field trials (Knudsen et al., 1995; Jensen et al., 2000). Root diseases can also be controlled by seed delivery as demonstrated for *P. brassicae*, the cause of clubroot in *Brassicae* species (Andersen et al., 2018; Peng et al., 2011), in pea against several soil-borne pathogens (Xue, 2003) and enhance field establishment of carrot plants (Fig. 1, Jensen, B. unpublished data). Efficient seed and root colonisation by *C. rosea* is probably required to obtain effects against soil-borne diseases (Fig. 1a, Xue, 2003; Jensen et al., 2004).

A special case of seed delivery is biopriming – a method first reported for *T. harzianum* (Harman et al., 1989) where the BCA was applied during the seed priming process. Priming is basically done by imbibition or controlled hydration of seed followed by a priming period at a reduced moisture content allowing seeds to go through the first reversible stage of germination but do not allow radical protrusion through the seed coat. The priming process can be completed after 12 to 14 days and after drying back the seeds, they can be stored until sowing. In general, priming results in more rapid germination and seedling emergence in the field, which is important to vegetables, like carrot, where seeds often are sown at low soil temperature and other unfavourable conditions for seedling establishment, e.g. pathogens causing seedling damping-off. Integration of *C. rosea* into the priming process has shown promising potential. Hence, biopriming of carrot seed with *C. rosea* resulted in a significant enhancement of the carrot plant stand in the field as compared to both primed and unprimed seed (Fig. 1, Jensen, B., unpublished data). Likewise, Bennett et al. (2009) showed that drum priming with and without different BCAs consistently improved the emergence of carrot seed in glasshouse trials and that *C. rosea* further shortened emergence time by two days as compared to unprimed seeds. However, in field experiments, no consistent effects on emergence and yield were seen for BCA primed seed (Bennett et al., 2009). The positive effects of biopriming on seedling establishment are probably related to the ability of *C. rosea* to colonise the seed during priming and to colonise root and rhizosphere after planting (Jensen et al., 2004; Bennett and

Whipps, 2008a,b). In some cases, the expected positive effects of seed priming can disappear or even result in drastically reduced seed quality if the seed lot harbours pathogens that are activated by the priming hydration (Jensen et al., 2004). However, the use of biopriming can minimise the risk of such adverse effects. For example, it was shown that the priming of carrot seeds naturally infected by *Alternaria* spp. lead to a lower healthy seedling stand than for nonprimed seed, mainly due to a high degree of post-emergence seedling death. In contrast, *C. rosea* biopriming resulted in a seedling stand that was significantly better than that of both nonprimed and seed primed without the BCA (Jensen et al., 2004).

6.3 Delivering C. rosea *to soil or plant growth substrates*

In Chinese cabbage, bottom rot caused by the soil-borne pathogen *P. tracheiphilum* can be a devastating problem. *Clonostachys rosea* spray application to the soil surface below the plants resulted in significant disease control and increased yield under commercial field production of Chinese cabbage (Table 4, Møller et al., 2003). Incorporation of the BCA into soil or growth substrate is another approach to control soil-borne pathogens in various crops. For practical use, the focus has mainly been on protecting crops in screen- or glasshouse production or in nurseries, but biocontrol effects have also been shown in field trials (Lahlali and Peng, 2014; Peng et al., 2011). Control of plant pathogenic nematodes by soil treatment with *C. rosea* has been demonstrated on small scale (Iqbal et al., 2018b) but controlling nematodes with *C. rosea* on larger field-scale need further testing. Summing up the available information from companies marketing *C. rosea* BCAs shows that different methods are in use for incorporation into the soil or plant growth substrates. Examples are watering or drip irrigation with the BCA that is used in protected high-value crops as well as dipping roots of small plants or cuttings in a spore suspension of *C. rosea* before planting. *C. rosea* could also have the potential for controlling several fungal plant diseases by watering or incorporating the BCA into golf greens.

6.4 Spray application of C. rosea

Spray application of *C. rosea* is relevant for controlling diseases in the phyllosphere such as the cereal diseases spot blotch in barley (Jensen et al., 2016a), FHB (Xue et al., 2009, 2014) and STB in wheat (Fig. 5, Jensen et al., 2019). Recently, Egel et al. (2019) demonstrated a significant reduction of early blight caused by *Alternaria solani* in tomatoes by spraying of Prestop® in field trials. This shows that spray application can be an important option for diseases in large agriculture field crops. Spray application is also used for the control of grey

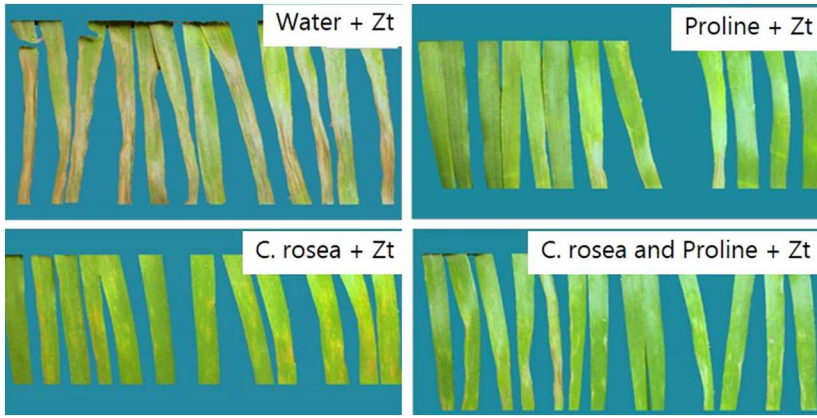

Figure 5 Control of septoria tritici blotch on wheat plants by spray application with *C. rosea* strain IK726 and the fungicide Proline® (a.i. Prothioconazole) in a growth chamber experiment. Leaves were sprayed with either *C. rosea*, Proline® or with both *C. rosea* and Proline® before inoculation with the pathogen *Z. tritici* (Zt) 24 h later. (Photos by Henrik Bendix Aas).

mould in strawberries and tomatoes (Sutton et al., 2002; Cota et al., 2008; Gong et al., 2017; Nechet et al., 2017). The Prestop® product is recommended for spray application on stems and wounds in vegetables against several diseases.

6.5 Delivery of C. rosea *in consortia*

Consortia, i.e. where two or more different BCA strains are combined, has often been suggested aiming at either additive or synergistic biocontrol effects against one disease or an approach for controlling different diseases by exploiting BCAs targeting different pathogens (e.g. Hoopen et al., 2010; Xu et al., 2011a,b; Krauss et al., 2013; Jensen et al., 2016b). Another strategy is to combine BCA(s) with other microorganisms for controlling plant diseases and at the same time alleviate other biotic or abiotic constraints to crop production. The complex plant microbiome can have an effect trait leading to healthy plants. On the other hand, microbiome function might also have an impact that makes it difficult to successfully establish BCAs or BCA consortia in the crop. However, research is at present mainly descriptive without much evidence for how to regulate the complex microbial communities and thereby facilitate their functions, e.g. biocontrol effect traits or how their functions can be compatible with implementing efficient BCA consortia. As reviewed by Xu et al. (2011a), it has generally been difficult to find published work demonstrating statistically significant improved consortia effects even if only two BCAs have been combined. There is also the issue that consortia members can be antagonistic towards each other leading to unsuccessful control, which is seen in several cases (Xu

et al., 2011a,b). Consortia formulations with *C. rosea* show, however, promising results in some cases. A combination of *C. rosea* and the arbuscular mycorrhiza *Glomus intraradices* was delivered to the rhizosphere of tomato plants. The two fungi showed mutual inhibition in the rhizosphere, but nevertheless resulted in synergistic plant growth promotion when combined (Ravnskov et al., 2006). Crops are often suffering from both insect pests and diseases, which necessitate multiple control measures. Therefore, a dual treatment approach involving BCAs targeting the different organisms without compromising the biocontrol traits of each other would be ideal. In a study of wheat, it was demonstrated that entomopathogenic fungi from the genus *Metarhizium* and *C. rosea* could be used in concert to control a root-feeding insect and a seed-borne disease in a single seed treatment (Keyser et al., 2016). Furthermore, an additive effect on biocontrol of tomato foot and root rot disease caused by *F. oxysporum* f. sp. *radicis lycopersici* was achieved by combining *C. rosea* with the phenazine-producing bacterium *P. chlororaphis* (Karlsson et al., 2015).

6.6 Role in integrated pest management (IPM)

Biological control should have a central role in IPM strategies aiming at reduced use of chemical pesticides as discussed elsewhere (Jensen et al., 2016b). *Clonostachys rosea* has shown tolerance to several chemical pesticides (Dubey et al., 2014a; Roberti et al., 2006), and therefore the BCA can be used in combination with various chemical pesticide treatments either in full recommended or in reduced doses of, e.g. fungicides, or in application schemes where BCAs and fungicides are alternated (Cota et al., 2009). Depending on the sensitivity to a pesticide, *C. rosea* might be applied together with the pesticide or with a time distance of a few days between the pesticide and the biocontrol treatments as outlined in the Prestop® info letter: https://verdera.fi/index.php/download_file/view/470/174/, from Lallemand/Verdera. The BCA could also be delivered at other time points in the cropping season to target other pathogens or even between seasons. Applying the BCA in the 'pre-harvest period' in which chemical control measures are not allowed is another option both in IPM and organic production (Jensen et al., 2016b). In addition to reducing the input of chemical pesticides, ongoing research also investigates if this strategy can prevent the build-up of pesticide resistance in pathogen populations when one or two pesticide applications are substituted with *C. rosea* treatments.

Plant disease resistance is a key factor in IPM strategies. Interestingly, it has been shown that plant cultivars harbouring resistance, or are less susceptible towards a disease, facilitate more efficient biocontrol traits as compared to the application of the BCA to a more susceptible cultivar (Yu and Sutton, 1997a; Andersen et al., 2018; Xue et al., 2014; Moraga-Suazo et al., 2016). Thus, when breeding for disease resistance the possibilities to exploit plant genotypes that also facilitate biocontrol effects in the crop should be in focus.

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6.7 Is C. rosea *pathogenic on plants?*

As mentioned in the previous section, there is an extensive amount of literature that reports on the biocontrol properties of *C. rosea* (Table 4, Sun et al., 2020a), without any negative effects on plant growth. However, over the years there

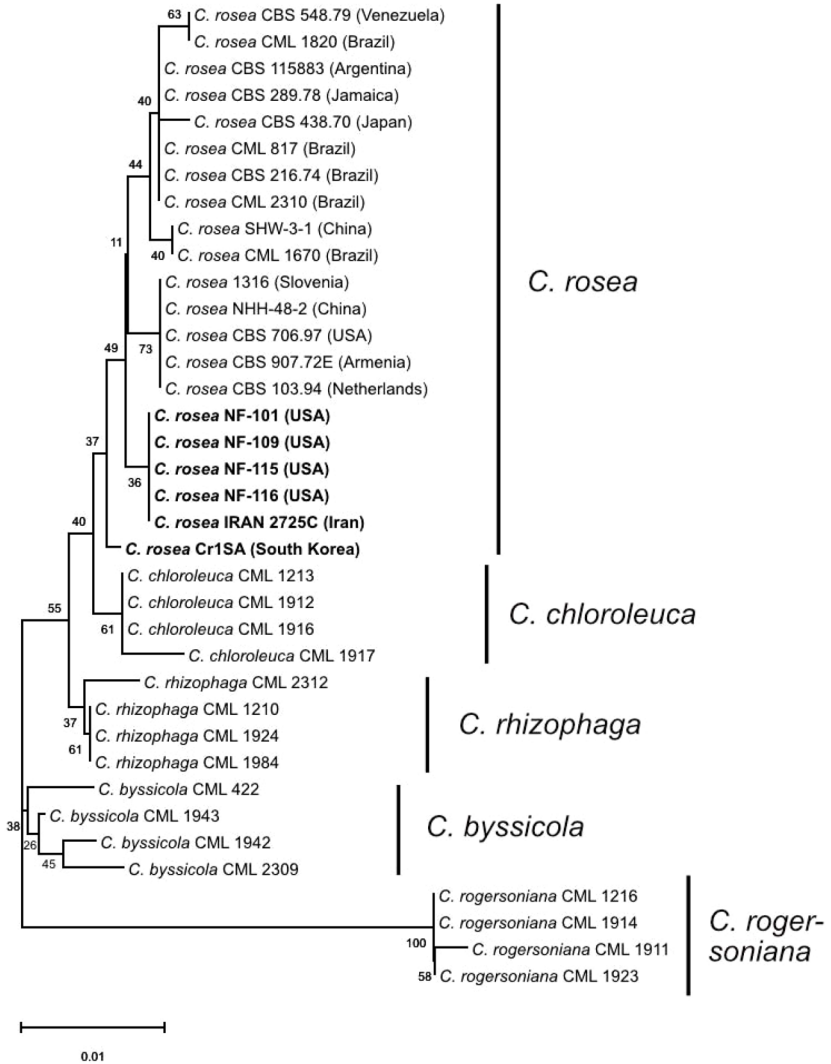

Figure 6 Phylogenetic analysis of *C.* strains. The tree is rooted with *C. rogersoniana*, and based on the internal transcribed spacer (ITS) and neighbour-joining analysis. Bootstrap support values are associated with branches. Sequence identifiers include species, strain ID and geographic origin. The bar marker indicates an average number of substitutions per site. Strains indicated in bold are reported to be pathogenic on plants.

have been a few reports on *C. rosea* being pathogenic to plants. For example, strains identified as *C. rosea* were reported to cause dry rot on potatoes (Theron and Holz, 1991), root rot on soybean (Bienapfl et al., 2012), wilt and crown rot on faba bean (Afshari and Hemmati, 2017) and root rot on orchids (Lee et al., 2020). This discrepancy between plant-beneficial and plant-detrimental properties of different strains of *C. rosea* is intriguing but not easily explained. The intimate association between *C. rosea* and plants, sometimes even involving systemic, asymptomatic colonisation (Saraiva et al., 2015; Mueller and Sinclair, 1986), indicate a delicate balance between the colonisation of *C. rosea* and the immune responses by the plant host. It is plausible that poor physiological status of the plant, a high inoculum of *C. rosea*, as well as certain genotype-by-genotype (*C. rosea* vs. plant) combinations may distort this balance and result in disease symptoms.

In some cases, sequencing of the ITS region was used together with morphology for species identification of plant pathogenic strains (Bienapfl et al., 2012; Afshari and Hemmati, 2017; Lee et al., 2020). A phylogenetic analysis of the ITS sequences of these strains, together with selected *C. rosea* strains representing a worldwide distribution (Broberg et al., 2018) and strains representing closely related *Clonostachys* species (Moreira et al., 2016) is presented in Fig. 6. First, this analysis confirms that the ITS region does not provide enough resolution for distinguishing between different *Clonostachys* species, as reported previously (Schroers, 2001; Abreu et al., 2014). However, it is interesting to note that all the *C. rosea* strains reported to be plant pathogenic clusters in a basal position within the *C. rosea* clade (albeit with low bootstrap support), distinct from the strains representing the worldwide collection. This may suggest that plant pathogenic *C. rosea* strains indeed form a genetically distinct group. Whether they form a separate, as yet undescribed, cryptic species remains to be investigated using other genetic markers such as ATP citrate lyase (*acl1*) and RNA polymerase II large subunit (*rpb1*) that are reported to be more suitable to resolve species boundaries in *Clonostachys* (Moreira et al., 2016).

7 Conclusion and future trends

A key to high efficacy and consistency in biocontrol is to identify and target vulnerable stages in the pathogen lifecycle and plant development. This can include the targeting of pathogen resting structures, temporarily protecting plant wounds and other pathogen entry points, or a more continuous interference with the plant tissue colonisation and dissemination of the pathogen. Basic studies of pathogen biology combined with advanced methods for tracing the presence and activity of the BCA is, therefore, an important aspect for successful implementation of biocontrol solutions. For *C. rosea*, the availability

of strains expressing the green fluorescent protein (Lübeck et al., 2002), and more recently, accurate and validated tools for DNA quantification (Gimeno et al., 2019) is therefore promising.

Different strains of *C. rosea* can display a considerable variation in biocontrol-related traits (Iqbal et al., 2020), which emphasises the importance of choosing the correct strain with a high biocontrol ability for the particular pathosystem in question. This is typically done in large screening experiments, involving the plant and the pathogen, in greenhouse or field settings resembling the conditions where the BCA will act (Köhl et al., 2011; Jensen et al., 2016b). The availability of high numbers of whole-genome sequenced *C. rosea* strains (Broberg et al., 2018) opens up new possibilities for including genetic markers coupled with specific traits as a decision-support in the screening procedure. Such genetic markers can also find applications in future attempts to breed for new *C. rosea* strains with specific traits, either through protoplast fusion approaches, which are used in *Trichoderma* (Stasz et al., 1988), or through sexual crosses followed by progeny selection. Given the intimate association between *C. rosea* and plants, genotype-by-genotype interaction effects are likely to have a considerable effect on biocontrol efficacy. This may, in fact, be exploited in plant breeding, where plant compatibility with beneficial microorganisms, including *C. rosea*, can be included as a breeding target alongside yield, quality and disease resistance.

Genetic improvement of BCA strains using CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats – CRISPR Associated protein 9) genome-editing technology is a promising approach for increasing the speed for developing biocontrol solutions, with increased genetic precision (Muñoz et al., 2019). In *Clonostachys*, there are already now several examples of genetic modifications that increase the biocontrol efficacy that may be a future target for CRISPR-Cas9 technology. For example, deletion of the *hyd1* and *hyd3* hydrophobin genes in *C. rosea* resulted in increased biocontrol ability towards *B. cinerea* on leaves (Dubey et al., 2014b). Furthermore, overexpression of the *chi67-1* endochitinase gene in *C. chloroleuca* increased chitinase production and subsequently the ability to control sclerotinia stem rot on soybean (Sun et al., 2017).

Microbiome research is leading to an increasing amount of detailed information not only on what microbial communities are to be found in plant/soil microbiomes but also on how microbiome function is emerging. How environment, plant cultivar and crop management affect microbiome functions will be important in forming strategies for sustainable healthy crop production in the future. Especially for the use of augmentative biocontrol, new detailed information on microbiome changes and their related functions will be important for establishing the correct timing and place to deliver the BCA(s) to the crop plant. In focus are also the pathobiomes where several

pathogen species are interacting in a way that modifies the outcome of infection. This is probably in most cases through direct interference with the plant defence responses, but can also relate to biocontrol interactions. An example of this is in FHB on wheat (Tan et al., 2021) where two *Fusarium* spp. interact, leading to a reduced effect of a bacterial biocontrol strain or chemical pesticide treatment in FHB control. Thus, having a focus on how successful *C. rosea* can be in controlling several pathogens found in complex natural pathobiomes should be important for future research. How the whole plant microbiome affects augmentative biocontrol effect traits is an important topic for research. Based on the information brought in this book chapter we believe that *C. rosea* will be an important model organism for such future studies. *Clonostachys rosea* is already today an important factor in sustainable plant protection strategies, and the recent developments in our understanding of its ecology, genetics and application promise an even more significant role in the future.

8 Where to look for further information

The following book chapter provides a good introduction to *C. rosea* and biological control:

- Jensen, D. F., Karlsson, M., Sarrocco, S. and Vannacci, G. (2016). Biological control using microorganisms as an alternative to disease resistance. In: Collinge, D. B. (Ed) *Plant Pathogen Resistance Biotechnology*. Wiley, New York and London, 341–363.

A key scientific conference involving *C. rosea* is the International Workshop on *Trichoderma* and *Gliocladium* (TG), held every second year. Other relevant conferences are the International Congress of Plant Pathology (ICPP) and the IOBC/WPRS Working Group meeting, Biological control of fungal and bacterial plant pathogens.

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