The European Journal of Plant Science and Biotechnology ©2011 Global Science Books

Attempts to Reduce Strawberry Grey Mould (*Botrytis cinerea*) in Norway Using Fungal Antagonists

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ABSTRACT

Grey mould (causal agent *Botrytis cinerea*) is the most serious disease of field-grown strawberries in Norway. As this disease has become increasingly difficult to control with chemical fungicides, alternative control measures based on application of commercially available or laboratory strains of antagonistic fungi were investigated in field trials at eight locations in Norway. Formulated or unformulated strains of *Trichoderma* spp. and *Clonostachys roseum* were applied during flowering using sprays (10^3-10^6 conidia ml^{-1}) or bumblebees (*Bombus terrestris*). At harvest, the incidence of grey mould on untreated plants varied from 4 to 70% at different trial sites and was positively correlated with the amount of precipitation during harvesting ($P < 0.001$). The biocontrol treatments did not reduce disease at any location. A bioassay was used to assess the ability of the tested antagonists to prevent flower infection by *B. cinerea* under controlled conditions (high humidity, low or high temperatures, various antagonist concentrations). All antagonist strains prevented infection at 25°C at a spray concentration of 10^6 conidia ml^{-1}. However, at 15°C, which was the mean temperature during field trials, at least 10^8 conidia ml^{-1} of the antagonists were required to provide significant disease control. These results imply that the recommended concentrations of these antagonists are insufficient to prevent flower infection by *B. cinerea* under disease-conducive field conditions of high humidity and cool temperatures.

Keywords: bioassay, biological control, *Clonostachys*, *Fragaria* × *ananassa*, *Gliocladium roseum*, *Trichoderma*

INTRODUCTION

Strawberries (*Fragaria* × *ananassa*) are a valued commodity in Norway, as in many other countries. In the United States, the world's largest producer of strawberries, these are the fifth highest consumed fresh fruit (Economic Research Service 2007). Unfortunately, strawberries are highly susceptible to pre- and postharvest grey mould. The disease results from infection of the flowers during bloom by *Botrytis cinerea*, an ubiquitous and versatile plant pathogenic fungus which causes serious economic losses in fruit, vegetable and ornamental crops throughout the world (Elad *et al*. 2004). The pathogen is the target of most fungicide applications to strawberries in Norway (Sæthre *et al.* 1999; Stensvand and Christiansen 2000), as in other parts of the world, and has developed resistance to many of the most commonly-used fungicides (Elad *et al.* 1992; Leroux 2004; Myresiotis *et al.* 2007; Jacometti *et al*. 2010). In recent years, concerns about pesticide residues in food crops and fungicide-resistant pathogens have led to a demand for alternative methods to control plant pathogens such as *B. cinerea*.

One such alternative method is biological control, broadly defined as "the reduction of the amount of inoculum or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man" (Cook and Baker 1983). To date, most successful applications of biocontrol microorganisms have been in greenhouse crops and preceding postharvest storage, where temperature and humidity can be adjusted to the pathogen's disadvantage (Paulitz and Bélanger 2001; Spadaro and Gullino 2004; Sharma *et al.* 2009). Results of field trials have been more variable, and it is commonly agreed that additional information is needed on interactions between pathogen, host plant and antagonists under varying climatic conditions (Fravel 1999; Ojiambo and Scherm 2006; Vinale 2008).

Biological control of *B. cinerea* has been considered a promising alternative to fungicides since the 1950s, when a number of antagonistic bacteria and fungi were found capable of inhibiting this pathogen (Newhook 1951; Wood 1951). There are now numerous reports of inhibition of *B. cinerea* following deliberate introduction of microbial antagonists on various crops. Reduction of postharvest grey mould of apples, grapes, strawberries, pears, tomatoes and cherries has been achieved by preharvest or postharvest applications of various bacteria (e.g. *Pseudomonas* spp. and *Bacilllus* spp.), yeasts (e.g. *Candida* spp. and *Metschnikowia fructicola*), and filamentous fungi (e.g. *Trichoderma* spp.) (recently reviewed by Sharma *et al.* 2009; Jacometti *et al.* 2010). These successful biocontrol trials have resulted in several commercial products for postharvest protection against grey mould: e.g. Aspire® (*Candida oleophila* 1-182) (Ecogen Inc., USA), Biosave® (*Pseudomonas syringae* 10LP-110) (Eco Science Corp., USA), and Shemer® (*Metschnikowia fructicola* Y-27328) (AgroGreen Co., Israel).

Among the most studied fungal biocontrol agents of *B. cinerea* are species of *Trichoderma* and *Gliocladium/Clonostachys* [the mycoparasite *G. roseum* has been reclassified as *Clonostachys rosea* (Schroers *et al.* 1999)]. These antagonists have been the subject of numerous reviews (e.g. Papavisas 1985; Sutton *et al.* 1997; Hjeljord and Tronsmo 1998; Kubicek *et al.* 2001; Vinale *et al.* 2007) as well as a 2-volume book (Harman and Kubicek 1998; Kubicek and Harman 1998). Several commercial biopesticides based on *Trichoderma* strains have been registered or recommended for use as foliar sprays to control *B. cinerea*, e.g. Binab® T WP (Binab Bio-Innovation AB, Älgarås, Sweden) (Engstedt 2007), Trichodex (Makhteshim Chemical Works, Beer

Received: 6 October, 2010. Accepted: 18 December, 2010.

Original Research Paper

Table 1 Reports on biological control of *Botrytis cinerea* infection of strawberry leaves, flowers or preharvest fruit by *Clonostachys rosea* (*Clon.*) or *Trichoderma strains* (*Trich.*), unformulated (U) or as commercial products (P). Trials were conducted in the field (F), greenhouse (G), or under controlled conditions (C).

Antagonist	Application method, conc.[a]	Temp. (°C)[b]	Trial	Disease reduction	Location	Reference
Trich. U	spray: 10^6	n.r.	F	yes (fruit)	Victoria, Australia	Washington *et al.* 1999
Trich. U	spray: 10^7	n.r.	F	yes (fruit)	England	Tronsmo and Dennis 1977
Trich. U	spray: 10^6	10-25	G	yes (flower)	Israel	Freeman *et al.* 2004
Trich. U	spray: 10^7	n.r.	G	variable (fruit)	Italy	Gullino *et al.* 1989
Trich. U	n.r.	n.r.	F	yes (fruit)	Romania	Sesan and Teodorescu 1993
Trich. U	spray: 10^4, bees: 10^8	n.r.	F	yes (fruit)	New York, USA	Kovach *et al.* 2000
Trich. U	spray: 10^4, bees: 10^8	n.r.	F	yes (fruit)	New York, USA	Harman 2000
Trich. P	spray: 10^{3-6}	12	G	no (fruit)	Norway	Hjeljord *et al.* 2000
Trich. P	bees: 10^9	8-30	F	yes (fruit)	Israel	Shafir *et al.* 2006
Trich. P	spray: 10^3	n.r.	F	no (fruit)	Finland	Prokkoloa *et al.* 2003
Trich. P	spray: 10^6	17, 21	C	yes (leaf)	UK	Robinson-Boyer *et al.* 2009
Trich. P	spray: 10^6	20	C	yes (leaf)	UK	Xu *et al.* 2010
Trich. P	spray: 10^3	n.r.	F	variable (fruit)	Norway	Stensvand 1998
Clon. U	spray: 10^6	10-25	F	yes (leaf)	Ontario, Canada	Peng and Sutton 1991
Clon. U	spray: 10^6	10-25	F,G	yes (flower)	Ontario, Canada	Sutton *et al.* 1997
Clon. U	spray: 10^7	10-25	F,G	yes (leaf)	Ontario, Canada	Sutton and Peng 1993
Trich. U						
Clon. U	spray: 10^7, bees: 10^8	20-30	F,G	yes (fruit)	Ontario, Canada	Peng *et al.* 1992
Clon. U	spray: 10^6	16,18	F	yes (leaf, fruit)	Brazil	Cota *et al.* 2008, 2009
Clon. U	spray: 10^6	22	C	yes (leaf)	Denmark	Mamarabadi *et al.* 2008

[a] Spray concentration: CFU ml^{-1}, formulation concentration for bee vectoring: CFU g^{-1}. If not reported, concentration of product spray was calculated from label declaration and recommended dosage (RD): Binab® T WP: $\geq 10^5$ CFU g^{-1}, RD 2 g $liter^{-1}$; PlantShield®: $\geq 10^7$ CFU g^{-1}, RD 2 g $liter^{-1}$

[b] Reported temperature range or mean during experiments, n.r. = not reported

Sheva, Israel) (Elad 2000), PlantShield® (BioWorks Inc., Fairport, New York, USA) (Harman 2000), and Sentinel® (Agrimm Technologies Ltd., Lincoln, New Zealand). One of the first reports of successful biocontrol of *B. cinerea* on strawberry was based on the use of *Trichoderma* species (Tronsmo and Dennis 1977), and products specifically claiming to reduce grey mould in strawberry (e.g. Binab® T WP) are currently available in Scandinavia. Several unformulated isolates of *Trichoderma* spp. and *C. rosea* have also been reported to suppress *B. cinerea* in strawberry under greenhouse and field conditions (**Table 1**).

Strawberry grey mould often develops following infection during flowering, after which *B. cinerea* usually remains quiescent in the developing fruit until high humidity or ripening induces renewed mycelial growth (Jarvis 1964; Bulger *et al.* 1987; Prusky and Lichter 2008). Inhibition of flower infection necessitates antagonist activity at relatively cool temperatures. Manufacturers of the abovementioned *Trichoderma*-based products claim activity down to 10°C, according to information on product labels, and biocontrol of *B. cinerea* at 10°C by *T. harzianum* and *C. rosea* has been reported (O'Neill *et al*. 1996; **Table 1**). The concentration of the antagonist inoculum is another factor known to affect biocontrol of *B. cinerea* (Dubos 1987; Gullino *et al.* 1989). In numerous reports, sprays containing $\leq 10^6$ colony forming units (CFU) ml^{-1} of commercial or unformulated *Trichoderma* strains and *C. rosea* have been reported sufficient to reduce infection by *B. cinerea* (**Table 1**).

Attempts at biocontrol of strawberry grey mould in Norway have given variable results. The *Trichoderma* product Binab® T WP performed better than a chemical fungicide in one trial, but had no effect in others (Stensvand 1997, 1998; Hjeljord *et al*. 2000). In response to published reports and commercial advertisements, several organic and conventional farmers in Norway wished to test the ability of fungal biocontrol agents to control grey mould in field-grown strawberries. Two commercial *Trichoderma* products (PlanterBox® and Binab® T WP), as well as two well-characterized antagonists, *T. atroviride* P1 (formerly known as *T. harzianum* P1, ATCC 74508) (Tronsmo 1991) and *C. rosea* Pg88-710 (Peng and Sutton 1991), have been reported to reduce grey mould in strawberry in other locations (**Table 1**) and were included in our field trials and laboratory experiments.

The objective of the trials reported here was to assess the field performance of these products and strains compared to that of chemical fungicides commonly used against grey mould in Norway (Stensvand 1997, 1998). A second objective was to investigate the effects of temperature and antagonist inoculum concentration on the ability of the relevant *Trichoderma* and *Clonostachys* species to prevent flower infection by *B. cinerea* under controlled conditions.

MATERIALS AND METHODS

Fungal strains and inoculum preparation

Six unformulated fungal strains were used in bioassays and field trials: *B. cinerea* Bc 101 (isolated from an infected strawberry at Grimstad, Norway); *T. atroviride* P1 (ATCC 74508) (Tronsmo 1991); *T. harzianum* T22, isolated from the commercial product T-22™ PlanterBox (BioWorks, Inc., Fairport, New York, USA); *T. harzianum* TB8, isolated from the commercial product Binab® T WP (Binab Bio-Innovation AB, Sweden); *T. polysporum* Tp53, isolated from the commercial product Binab® T WP; and *C. rosea* Gr336 (subcultured from strain Pg88-710, received from John Sutton, University of Guelph, Ontario, Canada). These strains were stored in 20% glycerol at –80°C as stock cultures and routinely cultivated at room temperature (20 to 23°C) on potato dextrose agar (PDA).

Before inoculum preparation, conidia from actively-growing cultures were transferred to new PDA plates and incubated at room temperature for 2 weeks, at which time the cultures were actively sporulating. Inocula for use in bioassays were made by transferring a small amount of sporulating mycelium to a bottle containing 5 ml sterile tap water, shaking by hand for 1 min to disperse conidia, and filtering through sterile cotton to remove mycelial fragments. The concentration of the resulting suspension was determined using a hemacytometer and diluted to the desired concentration. Inocula for field trials were made in the same way, except that larger amounts of conidia were harvested by rubbing sterile water over colonized PDA plates, using a sterile glass rod. Inocula for bioassays were made shortly before use, while inocula for field trials were prepared as concentrated suspensions and stored at 4°C for up to 2 weeks before use. Preliminary tests showed that concentrated suspensions of fresh or nutrient-activated conidia (see below) could be stored at 4°C for at least 2 months with no loss of germinability (Hjeljord, unpublished data).

Nutrient-activated *T. atroviride* P1 conidia were prepared as previously described (Hjeljord *et al.* 2001); briefly, conidia were washed from 2 to 3-week-old PDA cultures, suspended in 500 ml potato dextrose broth (PDB) in 1-liter bottles at a final concentra-

Table 2 Description of the field trial sites in southern Norway.

Trial type[a]	Site	Year	Cultivar	Bed design/irrigation[b]	Plot length (m)
I	Marnardal, 58°14´N	2000	Korona	MR/DI	6
I	Idse, 58°58´N	2000	Korona	MR/OS	6
I	Kvelde, 59°10´N	2000	Korona	DR/OS	6
I	Eina, 60°37´N	2000	Korona	DR/DI	6
I	Valldal, 62°19´N	2000	Polka	MR/OS	6
II	Kise, 60°47´N	2001-2003	Korona	DR/DI	6
II	Kolbu, 60°37´N	2001-2002	Korona	DR/OS	6
III	Frogn, 59°41´N	2002-2003	Korona	DR/OS	3

[a] Trial types and treatments: I. Sprays during flowering with T-22™ PlanterBox (active ingredient (a.i.) *Trichoderma harzianum*), *Clonostachys rosea* Gr336, or fungicides (0.3% Euparen® M, a.i. tolylfluanid, and 0.05% Switch®, a.i. cyprodinil + fludioxonil). II. Sprays during flowering with *T. atroviride* P1 or *C. rosea* Gr336. III. Sprays during flowering with Binab® T WP (a.i. *T. harzianum* ATCC 20476 + *T. polysporum* ATCC 20475) or fungicides (0.05% Switch®, alternating with 0.15% Teldor®, a.i. fenhexamid), or use of bee-vectored *Trichoderma* (Binab® T Vector in bumblebee hives)

[b] DR: double row with plastic mulch; MR: matted row; planting density: 3 to 4 plants m^{-1}; DI: drip irrigation; OS: overhead sprinkler

tion of approximately 1×10^7 conidia ml^{-1} and incubated for 6 h at 22°C on a reciprocal shaker at 150 rpm. The nutrient-activated but still ungerminated conidia were then removed from the solution by vacuum filtration over Whatman GF/C filters, washed three times in sterile water and resuspended as a concentrated suspension in 50 ml sterile water. The activated conidia were stored at 4°C for up to 2 weeks and were diluted in tap water to a final concentration of 10^6 conidia ml^{-1} shortly before use.

The commercial products, T-22™ PlanterBox (BioWorks, Inc., Fairport, New York, USA) and Binab® T WP (Binab Bio-Innovation AB, Älgarås, Sweden), were stored in their original packaging at 4°C and prepared on the day of application according to label instructions, i.e. 4 g $liter^{-1}$ T-22™ PlanterBox or 2 g $liter^{-1}$ Binab® T WP, the latter supplemented with 10 g $liter^{-1}$ sucrose. Hemacytometer (Bürker, Brand, Wertheim, Germany) counts of the prepared sprays showed that they contained approximately 10^6 and 10^3 conidia ml^{-1}, respectively.

Bioassays

The bioassay conditions were designed to be highly conducive to infection by *B. cinerea*. Humidity was maintained at ≥ 90%, and since preliminary experiments indicated that 10^6 conidia ml^{-1} of *B. cinerea* gave rapid and reproducible flower infection, this concentration was used for the experiments. Conidia of the pathogen and putative antagonist were mixed and coinoculated. In both greenhouse and field-grown flowers, signs of natural infection during the bioassay were close to zero in flowers collected just before opening. In the present study, newly-opened greenhouse and field-grown strawberry (‘Korona’) flowers were placed in perforated plastic stands (empty pipette tip racks) with their stems in water. Each flower was inoculated at 3 points at the base of the receptacle with 10 µl drops of a spore suspension containing 10^6 conidia ml^{-1} of *B. cinerea*, alone or mixed with 10^6, 10^7 or 10^8 conidia ml^{-1} of *C. rosea* Gr336, *T. atroviride* P1, *T. harzianum* T22™, *T. harzianum* TB8, or *T. polysporum* Tp53. Control flowers were treated with sterile water instead of conidial suspensions. Six replicates of three flowers per treatment were randomized in larger trays. Water to a depth of two cm was added to the bottom of the trays, and these were then covered with aluminum foil and incubated at 15 ± 1 or 25 ± 1°C. The flowers were inspected daily for necroses on the abaxial surface of the sepals, under the inoculation points, and the number of days until each inoculation point became visibly necrotic was recorded. The experiments were repeated on different dates.

Field trials

From 2000 to 2003, field trials were carried out in commercial farms growing strawberries in open fields or plastic tunnels at eight locations in Norway (**Table 2**). Each trial was arranged as a randomized complete block design with three replicates. Treatments were applied during flowering using motorized back pack-sprayers delivering approximately 70 ml spray suspension per plant when sprayed to runoff. The fields were harvested five or six times, and the weight and number of healthy and diseased berries were recorded. Weather data were recorded at weather stations located 10–60 km from the farms.

Field Trial Type I (sprays of *C. rosea* Gr336, T-22™ PlanterBox, or standard fungicides): The chemical treatments were 0.3% Euparen® M (50% tolylfluanid, Bayer AG, Leverkusen, Germany) and 0.05% Switch® (37.5% cyprodinil + 25% fludioxinil, Syngenta Crop Protection, Basel, Switzerland), applied alternately for a total of five weekly applications during flowering. Spray suspensions of T-22™ PlanterBox or *C. rosea* Gr336 (final concentrations 10^6 conidia ml^{-1}) were applied once or twice weekly during flowering (i.e., two different treatment schedules, a total of five or nine times, respectively).

Field Trial Type II (sprays of *C. rosea* Gr336, nutrient-activated or non-activated *T. atroviride* P1): *C. rosea*, *T. atroviride* or a mixture of the two antagonists were sprayed once 2 weeks before flowering and once again 4 to 6 weeks after the field was harvested. During flowering, sprays of nutrient-activated *T. atroviride* were applied to plots treated the previous autumn and the same spring with *T. atroviride* or mixture, while *C. rosea* was applied to plots treated with *C. rosea* in autumn and spring, i.e. a total of three different treatments. Antagonists were applied six to seven times during flowering at a spray concentration of 10^6 conidia ml^{-1}. Controls were not treated. After two of the harvests, 50 apparently healthy berries from each treatment replicate were incubated in high humidity at 20°C and inspected daily for grey mould symptoms.

Field Trial Type III (Bumblebee-vectored Binab T, Binab T sprays or standard fungicide sprays). Bumblebee hives were equipped with dispensers (Binab T Dos, Binab Bio-Innovation AB, Älgarås, Sweden) containing Binab® T Vector, 3×10^7 CFU g^{-1}. In Trial Type IIIA (2002), bumblebee hives with or without Binab® T Vec-tor dispensers were set up in two separate heated large plastic tunnels (Haygrove Ltd., Herefordshire, UK) or in ten six meter long enclosures of insect-proof netting in the field (five enclosures with hives containing Binab® T Vector, five enclosures with hives without Binab). In Trial Type IIIB (2003), plants grown in two heated tunnels and in the field were given the same treatments: four replicates per treatment were sprayed with water (control), with Binab® T WP, or with 0.05% Switch®, alternating with 0.15% Teldor® (50% fenhexamid, Bayer AG, Leverkusen, Germany). Fungicides were applied four times during flowering, and Binab sprays were applied nine times during flowering. Hives containing Binab® T Vector were placed in the Binab® T WP-sprayed plots in the tunnel and field during the flowering period, according to the recommendations of the manufacturer (Binab Bio-Innovation AB 2006). The bumblebees in the field had access to all of the experimental plots. In order to monitor delivery of *Trichoderma* conidia to flowers, 25 flowers with dehiscent anthers were collected from each treatment replicate, including controls, on three dates. Each flower was pressed onto a plate of *Trichoderma* selective medium (Elad *et al.* 1981), which was then incubated in the dark at 20°C for 2 weeks and scored for presence of *Trichoderma* colonies.

Data analysis

The percentages by weight of grey mould in the field trials were arcsine square root transformed before analysis; non-transformed data are presented. The relationship between incidence of grey mould in fruit harvested from treated and untreated plots and cumulative precipitation during the harvest period was subject to

regression analysis. Areas under the disease progress curves (AUDPC), derived from cumulative daily infection during the bioassays, were compared by analysis of variance and, when appropriate, means were separated using Tukey's test or compared with the *B. cinerea* control using Dunnett's method (P = 0.05). All calculations were performed using Microsoft Excel v.X and Minitab v.15.

RESULTS

Field trials

Disease pressure varied considerably during the various field trials, as indicated by the incidence of grey mould in the untreated controls (**Table 3**). Regardless of the level of disease pressure, there was no statistically significant effect on disease incidence of any of the biocontrol treatments during field trial types I and II (**Table 4**, and data not shown). Fungicide application during flowering (field trial type I) significantly reduced the incidence of grey mould relative to untreated controls in all but the trial with the greatest disease pressure (**Table 4**). Antagonist-treated berries often remained symptomless longer than the untreated controls in postharvest storage trials, but the difference was usually not significant ($P > 0.05$) (data not shown).

Regression analysis showed that there was a significant linear relationship between cumulative precipitation during the harvesting period and amount of grey mould in the harvested fruit from the untreated control plots, as well as from all antagonist treatments, during the field trials (**Fig. 1**). The relationship between grey mould in fungicide-treated fruit and precipitation during harvesting was not linear, and reflected the ability of fungicides to protect fruit during all but the greatest amount of precipitation (equation of the line of best fit for fungicides: $y = 0.070 - 0.007X + 0.0001X^2$, $P = 0.033$; X = mm precipitation). Precipitation and temperature during flowering were not significantly related to incidence of grey mould in the harvest; in fact, the trial with the greatest amount of precipitation during flowering had the least amount of disease in the harvested fruit (**Table 3**).

The effect of bumblebee-vectored *Trichoderma* on grey mould in harvested strawberries was investigated in field trial types IIIA and IIIB. To determine the vectoring efficacy of the bumblebees, flower samples taken from experimental plots in large plastic tunnels, in enclosures in the field, and in the open field were tested for the presence of *Trichoderma*. In trial IIIA, *Trichoderma* was found in ap-

Table 5 Field trial types IIIA and IIIB. Incidence of grey mould in harvested strawberries pollinated during flowering by bumblebees from hives with or without dispensers containing formulated *Trichoderma* conidia (Binab® T Vector). In each trial, 3 replicate plots in plastic tunnels or in the open field were given the same treatments.

	Disease incidence (weight %)	
	Tunnels	Field
Trial IIIA: (Hives in separate plastic tunnels or field enclosures)		
Bumblebees	1.9 ± 0.0 a[w]	61.2 ± 4.5 b
Bumblebees vectoring *Trichoderma*	1.5 ± 0.0 a	62.8 ± 4.7 b
Trial IIIB: (Bumblebees vectoring *Trichoderma* had access to all plots)		
Water control	0.3 ± 0.0 c[x]	4.0 ± 0.8 de
Fungicide spray[y]	0.4 ± 0.2 c	1.0 ± 0.5 d
Trichoderma spray[z]	0.4 ± 0.2 c	4.2 ± 1.2 e

[w] Trial IIIA: values (± standard deviation) within columns having the same letter do not differ significantly (Tukey's test, $P \leq 0.05$)
[x] Trial IIIB: values (± standard deviation) within columns having the same letter do not differ significantly (Tukey´s test, $P \leq 0.05$)
[y] Fungicide sprays: alternately 0.05% Switch® (37.5% cyprodinil + 25% fludioxonil) and 1.5% Teldor® (50% fenhexamid)
[z] Binab® T WP spray (*T. harzianum* ATCC 20476 + *T. polysporum* ATCC 20475)

Table 3 Temperature (°C) and precipitation (mm) during the flowering and harvest periods, and grey mould and yield in untreated strawberries (controls) at the trial sites.

Site and year	Mean temperature			Accumulated precipitation		Untreated control plots[a]	
	Flower.[b]	Treatm.[c]	Harv.[d]	Flower.[b]	Harv.[d]	Rot (wt.%)	Yield (kg)[e]
Marnardal 2000	11.8	11.5 (8.9-16.6)	15.0	94.6	88.6	50.8	7.9
Idse 2000	10.3	n.r.	13.4	98.6	27.6	16.5	34.1
Kvelde 2000	10.8	10.9 (8.9-14.0)	14.3	90.2	66.6	25.4	34.2
Eina 2000	11.7	n.r.	16.2	98.5	120.5	70.2	15.1
Valldal 2000	11.4	11.3 (8.3-14.2)	16.4	196.8	0.2	4.9	32.4
Valldal 2001	13.0	12.9 (9.7-18.2)	16.4	44.3	25.8	8.9	9.4
Kise 2002	15.3	15.1 (12.5-17.6)	14.7	21.7	40.2	21.3	28.2
Kise 2003	14.6	14.6 (13.4-15.5)	18.4	63.4	22.8	16.9	27.5
Kolbu 2001	15.5	15.2 (11.2-20.3)	14.5	32.4	110.4[f]	16.5[f]	23.2
Kolbu 2002	15.7	16.0 (14.2-20.3)	17.1	32.9	59.6[g]	70.2[g]	1.8
Frogn 2002	14.7	n.r.	13.4	74.0	32.8[h]	61.2[h]	17.5
Frogn 2003	15.1	15.3 (12.1-18.7)	18.5	80.2	30.4	4.0	20.5

[a] Data are averages of 3 replicates at each trial site
[b] Mean temperature or accumulated precipitation for the entire flowering period
[c] Mean temperature for all spraying dates during flowering, with the lowest and highest daily mean temperature in parentheses; n.r. = temperature not recorded
[d] Mean temperature or accumulated precipitation for the entire harvesting period
[e] Healthy fruits at harvest
[f] Precipitation occurred after most of the yield was harvested; data not included in regression analysis of grey mould and precipitation
[g] Extensive fruit damage by insects; trial aborted after 3 harvests; data not included in regression analysis of grey mould and precipitation
[h] Includes disease in wounds caused by bees in enclosures; data not included in regression analysis of grey mould and precipitation

Table 4 Field trial type I. Percentage by weight of strawberries showing grey mould at harvest, following fungicide or antagonist spray applications during flowering. Data are means of three replicate plots per treatment.

Site	Treatment					
	Control	Fungicides[y]	*Trichoderma*[w] sprays per week		*Clonostachys*[x] sprays per week	
			Once	Twice	Once	Twice
Marnardal	53.7 ± 3.6 a[z]	10.4 ± 2.0 b	54.3 ± 7.3 a	50.9 ± 10.3 a	44.1 ± 5.7 a	37.2 ± 5.9 a
Idse	16.7 ± 3.9 a	3.4 ± 1.1 b	13.7 ± 2.8 a	14.7 ± 3.3 a	17.3 ± 5.5 a	15.5 ± 2.8 a
Kvelde	25.5 ± 3.9 a	12.9 ± 1.0 b	28.7 ± 3.7 a	25.3 ± 3.5 a	24.5 ± 1.2 a	24.4 ± 1.7 a
Eina	71.0 ± 7.1 a	72.8 ± 7.4 a	71.6 ± 3.6 a	71.5 ± 6.4 a	64.3 ± 5.9 a	69.1 ± 12.2 a
Valldal	4.7 ± 1.3 a	0.9 ± 1.5 b	5.0 ± 0.8 a	5.5 ± 1.6 a	5.7 ± 0.8 a	5.1 ± 1.2 a

[w] Sprays prepared from the commercial product T-22™ PlanterBox
[x] Sprays prepared from unformulated *C. rosea* Gr336
[y] Fungicide sprays: alternately 0.05% Switch® (37.5% cyprodinil + 25% fludioxonil) and 1.5% Teldor® (50% fenhexamid)
[z] Values (± standard deviation) in the same row with different letters differ significantly, according to Tukey´s test, $P \leq 0.05$

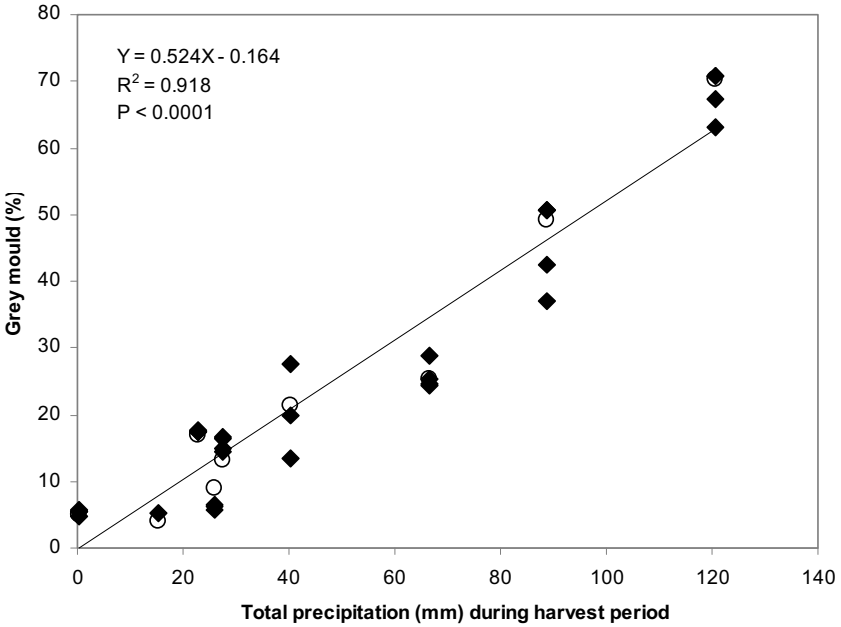

Fig. 1 Relationship between precipitation during the harvesting period and incidence of grey mould (% by weight) in harvested strawberries, as recorded in field trials at various locations in Norway. Data points are means of three replicates of antagonist treatments (solid diamonds) or non-treated controls (open circles) in each of nine field trials. Treatments varied among locations and comprise spray applications of antagonists in field trials types I, II and III (see text for details). There was no significant difference between controls and treatments at any trial.

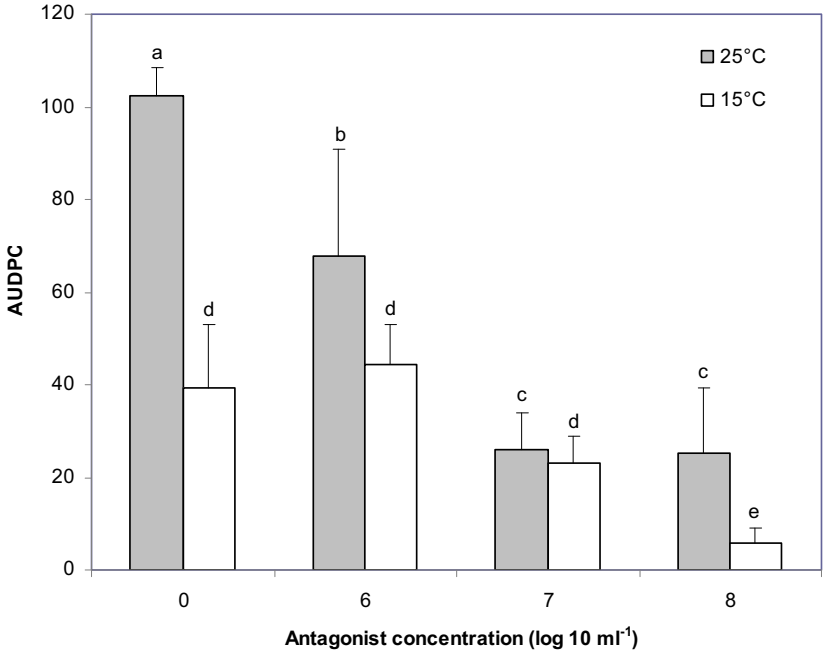

Fig. 2 Area under the disease progress curve (AUDPC) based on cumulative infection of detached strawberry flowers when *Botrytis cinerea* (10^6 conidia ml^{-1}) was inoculated alone or together with various concentrations of *Trichoderma harzianum* T22 (10^6, 10^7, or 10^8 conidia ml^{-1}). Inoculated flowers (6 replicates of 3 flowers, each flower with three inoculation points) were incubated under high humidity for 8 days at 15 or 25°C. For each temperature, values (bars indicate standard deviation of the mean) having different letters differ significantly (Tukey's test, $P < 0.01$).

proximately half of the sampled flowers from the tunnel with the hive containing *Trichoderma* spores, i.e. 57 ± 5, 65 ± 32 and 55 ± 34% of the flowers, at distances of 1, 7 and 15 m from the hive, respectively (the standard deviation following ± reflects the variation between three sampling dates). No *Trichoderma* was found in flowers from the tunnel containing the hive without *Trichoderma* spores. In the open field, all sampled flowers inside the enclosures with *Trichoderma* hives contained *Trichoderma* conidia, while no *Trichoderma* was found in flowers from enclosures with control hives. In trial IIIB, a hive with *Trichoderma* conidia was placed in the open field during flowering, and plots were sprayed with water, fungicides or *Trichoderma* conidia. *Trichoderma* was detected in all flowers sampled shortly after spray application of the antagonist, but not in flowers treated with water or fungicides, indicating that little *Trichoderma* was spread by bumblebees in this trial (data not shown). *Trichoderma* application by bees or sprays did not significantly reduce incidence of grey mould in the harvested fruit in either trial IIIA or trial IIIB (**Table 5**).

Bioassay

Detached strawberry flowers were inoculated with different concentrations of *T. harzianum* T22™ conidia mixed with *B. cinerea* conidia and incubated at 15 or 25°C. Analysis of the areas under the disease progress curve (AUDPC, in which disease severity is indicated by the area) showed that at

Table 6 Area under the disease progress curve (AUDPC) values for incidence of grey mould on detached strawberry flowers when *Botrytis cinerea* (10^6 conidia ml^{-1}) was co-inoculated with various concentrations of antagonists (10^6, 10^7, 10^8 conidia ml^{-1}) in bioassays. Each treatment in each trial had 5 replicates of 3 flowers, each with 3 inoculation points. AUDPC values (mean ± standard deviation) were calculated from disease incidence data collected daily for 8 days at 15°C.

Antagonist	10^6		10^7		10^8	
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
No antagonist	2.9 ± 0.9 a	2.7 ± 1.6 a	2.9 ± 0.9 a	2.7 ± 1.6 a	2.9 ± 0.9 a	2.7 ± 1.6 a
T. harzianum T22	3.8 ± 0.4 a	3.9 ± 0.2 a	3.3 ± 0.4 a	3.7 ± 0.2 a	1.5 ± 0.6 b	1.8 ± 0.8 a
T. atroviride P1	3.5 ± 0.4 a	2.4 ± 1.4 a	3.6 ± 0.5 a	3.6 ± 0.3 a	0.0 ± 0.1 b	0.0 ± 0.1 b
T. harzianum TB8	3.4 ± 0.7 a	3.0 ± 1.5 a	2.8 ± 1.1 a	4.0 ± 0.1 a	1.1 ± 0.2 b	1.6 ± 0.5 a
T. polysporum Tp53	3.5 ± 0.2 a	3.8 ± 0.3 a	3.2 ± 0.5 a	3.8 ± 0.5 a	0.4 ± 0.3 b	0.6 ± 0.5 b
C. rosea Gr336	3.7 ± 0.2 a	3.8 ± 0.4 a	3.5 ± 0.3 a	1.7 ± 1.0 a	0.5 ± 0.6 b	0.9 ± 1.0 b

[x] Values within the same column followed by the same letter are not significantly different from the *B. cinerea* control (without antagonist), according to Dunnett´s method ($P \leq 0.05$)

25°C, an inoculum consisting of 10^6 *Trichoderma* conidia ml^{-1} was sufficient to significantly inhibit flower infection by *B. cinerea.* At 15°C, however, the *Trichoderma* concentration had to be increased to 10^8 conidia ml^{-1} to significantly inhibit infection (**Fig. 2**). Flowers were inoculated with conidial suspensions of each of the antagonist strains used in the field trials, in concentrations of 10^6, 10^7 or 10^8 CFU ml^{-1} and mixed with *B. cinerea* conidia (10^6 CFU ml^{-1}). All showed the same result: at 15°C, significant inhibition of flower infection was only achieved by an antagonist inoculum concentration of 10^8 CFU ml^{-1} (**Table 6**).

DISCUSSION

Biocontrol of grey mould

We attempted to suppress *B.* cinerea infection of field-grown strawberry using antagonists and application methods recommended in the literature and by the manufacturers of *Trichoderma* products currently used to control *B. cinerea* in the greenhouse and field. None of the antagonist applications resulted in control of grey mould in the harvested fruit, nor was the postharvest shelf life of treated strawberries improved.

Precipitation during harvesting period

Incidence of grey mould in antagonist-treated and untreated berries was highly correlated with precipitation during the harvesting period, in accordance with Jarvis (1962) and Sutton (1998). The importance of humidity specifically during the harvesting period was suggested by weather data from the trial locations showing that precipitation during flowering was not correlated with disease incidence in the harvested fruit. It would be expected that precipitation during flowering could negatively impact biocontrol both by washing off the introduced antagonist and by favoring infection by the pathogen. In fact, the trial site with the most precipitation during flowering showed very little disease in the harvest, apparently due to the low amount of precipitation during the harvesting period (**Table 3**).

In contrast to the biocontrol treatments, application of chemical fungicides during flowering resulted in a significant reduction in grey mould in all trials, regardless of precipitation during the harvesting period, except at the trial location with the largest amount of precipitation during harvesting (**Tables 3, 4**). Disease reduction in harvested fruit treated during flowering with fungicides might be due not only to prevention of flower infection, but also to systemic and protective effects of fungicide residues remaining in or on developing fruit and other tissues that may otherwise serve as alternative infection sources. A systemic (Switch) and a contact product (Euparen M) were applied in our trials. Antagonistic fungi may not have such residual effects, and thus, inhibition of flower infection by antagonists as a control strategy may not be sufficient under disease-conducive weather during harvesting.

Effect of antagonist inoculum concentration

According to the literature, an antagonist concentration of 10^6 conidia ml^{-1} should be sufficient for inhibition of *B. cinerea* (**Table 1**), and this was confirmed by our bioassays at 25°C. Many of the previously reported trials were performed at 20-25°C (**Table 1**). However, at 15°C, which is a more realistic temperature for the flowering period in Norway (**Table 3**), this antagonist concentration had no effect on flower infection in the bioassays (**Fig. 2**). Significant control at 15°C necessitated a 100-fold increase in antagonist inoculum concentration, to 10^8 conidia ml^{-1}; this requirement was found for all of the antagonist strains tested (**Table 6**). These results are consistent with a recently-published theoretical model for biological control of foliar plant diseases using *Trichoderma/Botrytis* as an exemplar system (Jeger *et al.* 2009). This model showed that the concentration and activity of the antagonist are among the most important factors determining the outcome of the biocontrol program. In this model, reduction in activity (e.g. growth, antibiotic production) of the antagonist necessitates compensation by a higher propagule concentration to achieve the same competitive advantage. Although the model focused on colonization of leaf surfaces, it appears that the principles are relevant to competition in general, and that factors affecting activity (such as non-optimal temperature) also affect the effective concentration of the antagonist.

Our bioassay was deliberately disease-conducive, including pathogen conidia coinoculated with antagonist conidia, cool temperatures, and high humidity. As even small changes in temperature and humidity can affect the ability of *Trichoderma* and *Clonostachys* to inhibit growth of *B. cinerea* (Hannusch and Boland 1996), this bioassay may have underestimated the biocontrol ability of the antagonists under less stringent conditions, e.g. lower disease pressure or lower humidity. Nonetheless, the field trials demonstrated that regardless of disease pressure, an antagonist inoculum of 10^6 conidia ml^{-1} was unable to reduce flower infection at the cool temperatures that occur under field conditions in Norway.

Bee vectoring of antagonists

Bee vectoring of *Clonostachys* and *Trichoderma* conidia has been reported to be an efficient method of delivering antagonists to the infection court (Peng *et al.* 1992; Yu and Sutton 1997; Kovach *et al.* 2000; Shafir *et al.* 2006; Mommaerts *et al.* 2008). Although our experiments were not designed to quantify the number of *Trichoderma* conidia delivered to flowers by bumblebees, other studies have reported 10^4 antagonist CFU per bumblebee or honeybee-visited flower, with good control of grey mould as a result (Peng *et al.* 1992; Yu and Sutton 1997; Shafir *et al.* 2006). The disadvantage of bee delivery is the potentially large variation in proportion of flowers receiving detectable amounts of antagonist, probably related to decreased bee foraging when more desirable flowers are available, or during cool weather, wind and rain. Under such weather conditions, bumblebees were reported to forage more ac-

tively than honeybees (Yu and Sutton 1997), which is why bumblebees were used in the present study. The lack of disease control by bumblebee-vectored *Trichoderma* seen in the present study may have been due to variation in delivery of antagonist conidia to flowers in amounts sufficient to inhibit infection by *B. cinerea*, especially under disease-conducive temperatures.

Antagonistic mechanisms

The importance for biocontrol of antagonist concentration and temperatures favoring antagonist activity has been pointed out previously (Dubos 1987; Eden *et al.* 1996; Hannusch and Boland 1996). The present study showed that even at a temperature more conducive to germination and growth of *B. cinerea* than to that of the antagonists, sufficiently concentrated antagonist inocula were capable of inhibiting the pathogen. At 15°C, *B. cinerea* conidia can produce infective germ tubes within 6 hours, long before antagonists such as *Trichoderma* spp. germinate (Hjeljord *et al.* 2001). Inhibition of *B. cinerea* infection by coinoculated *Trichoderma* conidia at 15°C suggests that the antagonistic mechanism involved occurs too quickly to be based on the accepted antagonistic mechanisms of mycoparasitism, antibiosis, or induced plant defenses. A fourth mechanism, competition for nutrients or space, is considered to be the most important antagonistic mechanism by which microbial antagonists control *B. cinerea* infection of flowers (Blakeman and Fokkema 1982). The question remains whether slowly-germinating conidia of antagonists such as *Trichoderma* are capable of sequestering nutrients in a nutrient-rich microhabitat, such as water films enriched with nectar or pollen on newly-opened flowers, at a rate that can inhibit germination of *B. cinerea*. Other respiration-related antagonism by conidia initiating germination, such as competition for dissolved oxygen, would be more rapid. Dissolved oxygen availability in water films is known to affect growth of fungi (Deacon 2006). Respiration-related antagonism by germinating conidia would be reduced at low temperatures, and effective competition would necessitate a greater number of respiring cells. Competition for germination-stimulating factors such as glucose or oxygen (Hjeljord *et al.* 2001; Hjeljord and Tronsmo 2003) would be consistent with the observed antagonist concentration effect as well as with the previously-mentioned biological control model (Jeger *et al.* 2009).

CONCLUSION

Although the field trials reported here were carried out according to recommendations in the scientific literature and by producers of commercial products, antagonist treatments did not affect incidence of strawberry grey mould under field conditions in Norway, regardless of disease pressure (i.e., disease incidence in untreated controls). Bioassays under controlled conditions showed that the recommended inoculum concentrations of *C. rosea* and four *Trichoderma* antagonists were insufficient to inhibit flower infection by *B. cinerea* at temperatures typically recorded during strawberry flowering, while a 100-fold increase in antagonist concentration produced significant biocontrol activity under the same conditions. Although production of such highly concentrated inocula of *Trichoderma* or *Clonostachys* may not be economically or practically feasible, these results give insight into antagonistic mechanisms worth pursuing in a search for more effective biocontrol products or agents.

ACKNOWLEDGEMENTS

The field trials were performed with the skillful assistance of G. Bergaust, A. Dobson, B. Hageberg, S. Haraldstad, J. K. Henriksen, T. Idsø, N. E. Linge, A. Nes, J. Skaug, K. Sola, H. B. Stensrud, E. Stople, and R. Sunde. Financial support was provided by Innovation Norway and the Norwegian Research Council.

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