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 rosea 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 10^8 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 the pathogen” (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 (Paulet and Belanger 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 Bacillus 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) (Écogén 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. rosea has been reclassified as Clonostachys rosea (Schroers et al. 1999)]. These antagonists have been the subject of numerous reviews (e.g. Papavias 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, Algaras, Sweden) (Engstedt 2007), Trichodex (Makhteshim Chemical Works, Beer...
Sheva, Israel) (Elad 2000), PlantShield™ (BioWorks Inc., Fairport, New York, USA) (Haran 2000), and Sentinel (Agrimtech 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 *Clonostachys rosea* have also been reported to suppress *B. cinerea* in strawberry under greenhouse and field conditions (Table 1).

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Application method, conc.</th>
<th>Temp. (°C)</th>
<th>Trial</th>
<th>Disease reduction</th>
<th>Location</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trich.</em></td>
<td>spray: 10⁶</td>
<td>n.r.</td>
<td>F</td>
<td>yes (fruit)</td>
<td>Victoria, Australia</td>
<td>Washington et al. 1999</td>
</tr>
<tr>
<td><em>Trich.</em></td>
<td>spray: 10⁷</td>
<td>n.r.</td>
<td>F</td>
<td>yes (fruit)</td>
<td>England</td>
<td>Tronsmo and Dennis 1977</td>
</tr>
<tr>
<td><em>Trich.</em></td>
<td>spray: 10⁸</td>
<td>10-25</td>
<td>G</td>
<td>yes (flower)</td>
<td>Israel</td>
<td>Freeman et al. 2004</td>
</tr>
<tr>
<td><em>Trich.</em></td>
<td>spray: 10⁷</td>
<td>n.r.</td>
<td>G</td>
<td>variable (fruit)</td>
<td>Italy</td>
<td>Gullino et al. 1989</td>
</tr>
<tr>
<td><em>Trich.</em></td>
<td>n.r.</td>
<td>F</td>
<td>yes (fruit)</td>
<td>Romania</td>
<td>Susan and Teodorescu 1993</td>
<td></td>
</tr>
<tr>
<td><em>Trich.</em></td>
<td>spray: 10⁻⁸</td>
<td>12</td>
<td>G</td>
<td>no (fruit)</td>
<td>Norway</td>
<td>Hjeljord et al. 2000</td>
</tr>
<tr>
<td><em>Trich.</em></td>
<td>bees: 10⁸</td>
<td>8-30</td>
<td>F</td>
<td>yes (fruit)</td>
<td>Israel</td>
<td>Shafir et al. 2006</td>
</tr>
<tr>
<td><em>Trich.</em></td>
<td>spray: 10⁷</td>
<td>n.r.</td>
<td>F</td>
<td>no (fruit)</td>
<td>Finland</td>
<td>Prokkolma et al. 2003</td>
</tr>
<tr>
<td><em>Trich.</em></td>
<td>spray: 10⁷</td>
<td>17, 21</td>
<td>C</td>
<td>yes (leaf)</td>
<td>UK</td>
<td>Robinson-Boyer et al. 2009</td>
</tr>
<tr>
<td><em>Trich.</em></td>
<td>spray: 10⁷</td>
<td>20</td>
<td>C</td>
<td>yes (leaf)</td>
<td>UK</td>
<td>Xu et al. 2010</td>
</tr>
<tr>
<td><em>Trich.</em></td>
<td>spray: 10⁷</td>
<td>n.r.</td>
<td>F</td>
<td>variable (fruit)</td>
<td>Norway</td>
<td>Stewart and von 1998</td>
</tr>
</tbody>
</table>

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 above-mentioned *Trichoderma*-based products claim activity down to 10°C (Table 1). In numerous reports, sprays containing ≤10⁷ colony forming units (CFU) ml⁻¹ of commercial or unformulated *Trichoderma* strains and *Clonostachys* rosea have been reported sufficient to reduce infection by *B. cinerea* (Table 1).

### MATERIALS AND METHODS

**Fungal strains and inoculum preparation**

Six unformulated fungal strains were used in bioassays and field trials: *B. cinerea* Be 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 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-
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 10^4 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 10^7 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 10^5 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 coinnoculated. 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 10^5 conidia ml^-1 of *B. cinerea*, alone or mixed with 10^5, 10^6 or 10^7 conidia ml^-1 of *C. rosea* Gr336, *T. atroviride* P1, *T. harzianum* T22™, *T. harzianum* TB8, or *T. polysporum* Tps35. Control flowers were treated with sterile water instead of conidial suspensions. Six replicates of three flowers per treatment were randomized in larger trays. 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.

<table>
<thead>
<tr>
<th>Trial type</th>
<th>Site</th>
<th>Year</th>
<th>Cultivar</th>
<th>Bed design/irrigation</th>
<th>Plot length (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Mamadal, 58°14´N</td>
<td>2000</td>
<td>Korona</td>
<td>MR/DI</td>
<td>6</td>
</tr>
<tr>
<td>I</td>
<td>Idse, 58°58´N</td>
<td>2000</td>
<td>Korona</td>
<td>MR/OS</td>
<td>6</td>
</tr>
<tr>
<td>I</td>
<td>Kvelde, 59°10´N</td>
<td>2000</td>
<td>Korona</td>
<td>DR/OS</td>
<td>6</td>
</tr>
<tr>
<td>I</td>
<td>Eina, 60°37´N</td>
<td>2000</td>
<td>Korona</td>
<td>DR/DI</td>
<td>6</td>
</tr>
<tr>
<td>I</td>
<td>Validal, 62°19´N</td>
<td>2000</td>
<td>Polka</td>
<td>MR/OS</td>
<td>6</td>
</tr>
<tr>
<td>II</td>
<td>Kise, 60°47´N</td>
<td>2000</td>
<td>Korona</td>
<td>DR/DI</td>
<td>6</td>
</tr>
<tr>
<td>II</td>
<td>Kolbu, 60°37´N</td>
<td>2001-2002</td>
<td>Korona</td>
<td>DR/OS</td>
<td>6</td>
</tr>
<tr>
<td>II</td>
<td>Frogn, 59°41´N</td>
<td>2002-2003</td>
<td>Korona</td>
<td>DR/OS</td>
<td>3</td>
</tr>
</tbody>
</table>

| Field Trial Type I | Sprays during flowering with T-22™ PlanterBox, or standard fungicides: The chemical treatments were 0.3% Euparen® M (50% tollylfluanid, Bayer AG Leverkusen, Germany) and 0.05% Switch® (37.5% cyprodinil + 25% fludioxonil), 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 concentration 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 during flowering with T-22™ PlanterBox, or standard fungicides: The chemical treatments were 0.3% Euparen® M (50% tollylfluanid, Bayer AG Leverkusen, Germany) and 0.05% Switch® (37.5% cyprodinil + 25% fludioxonil), 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 concentration 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).

**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 Mini-tab 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.

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.

<table>
<thead>
<tr>
<th>Site and year</th>
<th>Mean temperature</th>
<th>Accumulated precipitation</th>
<th>Untreated control plots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flower</td>
<td>Treatm.</td>
<td>Harv.</td>
<td>Flower</td>
</tr>
<tr>
<td>Marnardal 2000</td>
<td>11.8</td>
<td>11.5 (8.9-16.6)</td>
<td>15.0</td>
</tr>
<tr>
<td>Idse 2000</td>
<td>10.3</td>
<td>n.r.</td>
<td>13.4</td>
</tr>
<tr>
<td>Kvele 2000</td>
<td>10.8</td>
<td>10.9 (8.9-14.0)</td>
<td>14.3</td>
</tr>
<tr>
<td>Eina 2000</td>
<td>11.7</td>
<td>n.r.</td>
<td>16.2</td>
</tr>
<tr>
<td>Valldal 2000</td>
<td>11.9</td>
<td>11.3 (8.3-14.2)</td>
<td>16.4</td>
</tr>
<tr>
<td>Valldal 2001</td>
<td>13.0</td>
<td>12.9 (9.7-18.2)</td>
<td>16.4</td>
</tr>
<tr>
<td>Kise 2002</td>
<td>15.3</td>
<td>15.1 (12.5-17.6)</td>
<td>14.7</td>
</tr>
<tr>
<td>Kise 2003</td>
<td>14.6</td>
<td>14.6 (13.4-15.5)</td>
<td>18.4</td>
</tr>
<tr>
<td>Kolbu 2001</td>
<td>15.5</td>
<td>15.2 (11.2-20.3)</td>
<td>14.3</td>
</tr>
<tr>
<td>Kolbu 2002</td>
<td>15.7</td>
<td>16.0 (14.2-20.3)</td>
<td>17.1</td>
</tr>
<tr>
<td>Frog 2002</td>
<td>14.7</td>
<td>n.r.</td>
<td>13.4</td>
</tr>
<tr>
<td>Frog 2003</td>
<td>15.1</td>
<td>15.3 (12.1-18.7)</td>
<td>18.5</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Site</th>
<th>Control</th>
<th>Fungicides(^a)</th>
<th>Treatment</th>
<th>Clonostachys(^a) sprays per week</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Treatm.(^b)</td>
<td>Once</td>
<td>Twice</td>
</tr>
<tr>
<td>Marnardal</td>
<td>53.7 ± 3.6 a(^c)</td>
<td>10.4 ± 2.0 b</td>
<td>64.3 ± 7.3 a</td>
<td>44.1 ± 5.7 a</td>
</tr>
<tr>
<td>Idse</td>
<td>56.7 ± 3.9 a</td>
<td>3.4 ± 1.1 b</td>
<td>14.7 ± 2.8 a</td>
<td>17.3 ± 5.5 a</td>
</tr>
<tr>
<td>Kvele</td>
<td>25.5 ± 3.9 a</td>
<td>12.9 ± 1.0 b</td>
<td>25.3 ± 3.5 a</td>
<td>24.5 ± 1.2 a</td>
</tr>
<tr>
<td>Eina</td>
<td>71.0 ± 7.1 a</td>
<td>72.8 ± 7.4 a</td>
<td>71.5 ± 6.4 a</td>
<td>64.3 ± 5.9 a</td>
</tr>
<tr>
<td>Valldal</td>
<td>4.7 ± 1.3 a</td>
<td>1.9 ± 1.5 b</td>
<td>4.5 ± 1.6 a</td>
<td>5.7 ± 0.8 a</td>
</tr>
</tbody>
</table>

\(^a\) 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
\(^d\) Healthy fruits at harvest
\(^e\) Extensive fruit damage by insects; trial aborted after 3 harvests; data not included in regression analysis of grey mould and precipitation
\(^f\) Includes disease in wounds caused by bees in enclosures; data not included in regression analysis of grey mould and precipitation

0001X2, 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 IIB. 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-
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).

**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
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 10^7 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 (10^7 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 sites 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 (Table 3). 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 propagation 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 co-inoculated 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 10^5 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-

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**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.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Trial 1</th>
<th>Trial 2</th>
<th>Trial 1</th>
<th>Trial 2</th>
<th>Trial 1</th>
<th>Trial 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No antagonist</td>
<td>2.9 ± 0.9 a</td>
<td>2.7 ± 1.6 a</td>
<td>2.9 ± 0.9 a</td>
<td>2.7 ± 1.6 a</td>
<td>2.9 ± 0.9 a</td>
<td>2.7 ± 1.6 a</td>
</tr>
<tr>
<td><em>T. harzianum</em> T22</td>
<td>3.8 ± 0.4 a</td>
<td>3.9 ± 0.2 a</td>
<td>3.3 ± 0.4 a</td>
<td>3.7 ± 0.2 a</td>
<td>1.5 ± 0.6 b</td>
<td>1.8 ± 0.8 a</td>
</tr>
<tr>
<td><em>T. atroviride</em> P1</td>
<td>3.5 ± 0.4 a</td>
<td>2.4 ± 1.4 a</td>
<td>3.6 ± 0.5 a</td>
<td>3.6 ± 0.3 a</td>
<td>0.0 ± 0.1 b</td>
<td>0.0 ± 0.1 b</td>
</tr>
<tr>
<td><em>T. harzianum</em> TB8</td>
<td>3.4 ± 0.7 a</td>
<td>3.0 ± 1.5 a</td>
<td>2.8 ± 1.1 a</td>
<td>4.0 ± 0.2 a</td>
<td>1.1 ± 0.2 b</td>
<td>1.6 ± 0.5 a</td>
</tr>
<tr>
<td><em>T. polysporum</em> Tp53</td>
<td>3.5 ± 0.2 a</td>
<td>3.8 ± 0.3 a</td>
<td>3.2 ± 0.5 a</td>
<td>3.8 ± 0.5 a</td>
<td>0.4 ± 0.3 b</td>
<td>0.6 ± 0.5 b</td>
</tr>
<tr>
<td><em>C. rosea</em> Gr336</td>
<td>3.7 ± 0.2 a</td>
<td>3.8 ± 0.4 a</td>
<td>3.5 ± 0.3 a</td>
<td>1.7 ± 1.0 a</td>
<td>0.5 ± 0.6 b</td>
<td>0.9 ± 1.0 b</td>
</tr>
</tbody>
</table>

* 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 ≤ 0.05)
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 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 cooinculated Trichoderma conidia at 15°C suggests that the antagonistic mechanism involved occurs too quickly to be based on the accepted antagonistic mechanisms of mycoparasitism, bioassays, 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 Tricho- derma 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-stimu- lating 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 straw- berry 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.

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