

Control of *Botrytis cinerea* in strawberries with *Gliocladium catenulatum* vectored by bumblebees

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Abstract.

BACKGROUND: Grey mould *Botrytis cinerea* is the most common fruit rot disease in strawberries and shelf-life is often limited because of *Botrytis*. Generally the disease is controlled by spraying chemical products.

OBJECTIVE: To prevent resistance and to produce strawberries with fewer residues, it is required to find alternatives. In this study, bumblebees were used as vector to transport the antagonistic fungus *Gliocladium catenulatum* J1446 to the flowers in order to control *Botrytis*.

METHODS: A trial was conducted in strawberries grown in a plastic greenhouse. Bumblebees leaving the special hive become loaded with the microbial product. The following strategies were compared: untreated control, conventional chemical spray scheme, spray application with *G. catenulatum* or fenhexamid, *G. catenulatum* (vectored). The last strategy is also tested with an additional spraying with *G. catenulatum* (1x) or fenhexamid (1x). *Botrytis* infection and yield were evaluated.

RESULTS: Spray applications with *G. catenulatum* (3x) or Teldor (1x) did not reduce the *Botrytis* infection in this trial. *G. catenulatum* vectored by bumblebees reduced grey mould, and was comparable with the conventional chemical treatment scheme. Additional sprayings with *G. catenulatum* or fenhexamid did not generate an additional reduction.

CONCLUSIONS: This greenhouse trial demonstrates that *G. catenulatum* vectored by bumblebees can reduce *Botrytis* infection and improve shelf-life of strawberries at low to moderate disease pressure. This results in a more sustainable control with healthier strawberries with fewer residues.

Keywords: *Fragaria x ananassa* Duch., grey mould, residues, biological control

1. Introduction

Grey mould *Botrytis cinerea* is the most common fruit rot disease in strawberries and shelf-life is often limited because of *Botrytis* infections [1]. This problem can be reduced by cultivating strawberries under protection. However, under favourable conditions *Botrytis cinerea* can still cause serious losses. Infection by *Botrytis cinerea* occurs mainly during flowering [2], whereafter the pathogen can remain inactive for a long time and cause fruit rot pre- or post harvest [1, 3]. Since *Botrytis cinerea* is mainly infecting flowers, flower protection is considered as a key management strategy to control grey mould [4].

Generally in commercial strawberry greenhouses in Belgium, the disease is controlled by spraying chemical fungicides. However, chemical fungicides can result in the fungus developing resistance [5, 6] and for a sustainable disease control and producing strawberries with fewer residues, it is required to find non-chemical alternatives.

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In this study, the main aim is to test whether bumblebees can be used as vector to transport an antagonistic fungus to control fruit rot in a commercial strawberry greenhouse. Kovach *et al.* [7] found in Geneva, New York (USA) that honeybee- or bumblebee-delivered *Trichoderma harzianum* is a viable option for strawberry growers to control *Botrytis*. Mommaerts *et al.* [8] proved that bumblebees can vector a microbiological control agent to reduce *B. cinerea* incidence in greenhouse strawberries. Our study investigates if this control method was comparable with common chemical treatment schemes currently used in strawberry farms in Belgium. In addition, some extra spraying treatments were included to assess whether there was an added value and which strategy resulted into the best control of *Botrytis* fruit rot.

2. Material and methods

2.1. Field experiment

A field trial was conducted with strawberries grown in a peat substrate under a plastic greenhouse (not heated). Tray plants of cultivar ‘Elsanta’ were used for an autumn crop, and planted on 10 July 2013 at 9,6 plants per square meter.

To control *Botrytis*, bumblebees (*Bombus terrestris*) were used as vector to transport the antagonistic fungus *Gliocladium catenulatum* J1446 (Verdera B⁴) to the flowers using the Flying Doctors® system (Biobest, Westerlo, Belgium). This system consists of a bumblebee hive with an integrated product dispenser. Bumblebees leaving the hive walk through the dispenser filled with Verdera B⁴ and become loaded with the microbial product (FD: Flying Doctors® with Verdera B⁴). The trial need to be split up, since the bumblebees may not be mixed over the different control strategies. Therefore, the strategies including the FD were placed in one compartment, strategies without FD in another compartment (Fig.1). The total area of the trial was 1200 m²: a compartment of 800 m² was treated with the FD by using four bumblebee hives, another compartment of 400 m² was pollinated with bumblebees coming from two bumblebee hives with empty dispensers.

Within these compartments, the control strategies were placed by using four replicates of each control strategy. Each replicate consists of 60 plants (6,25 m²), randomized within the greenhouse correct compartment. In the two compartments, with and without the Flying Doctors system with Verdera B⁴, fruit yield and grading were registered each picking in four plots of 60 plants (6,25 m²). Fruits are graded as follows: large (diameter ≥ 35 mm), small (<35 mm), misshapen and rotten fruits.

2.2. Control strategies for Botrytis cinerea

The following control strategies were compared with *G. catenulatum* vectored by bumblebees: untreated control, conventional chemical spray scheme, a spray application with *G. catenulatum* (Prestop) (3 application) or with fenhexamid (Teldor) (1 application). In addition, it was investigated whether the efficacy of *G. catenulatum* (vectored) could be improved by combined spraying with *G. catenulatum* (1 application) or fenhexamid (1 application).

Control strategies without FD	Control strategies that include FD
Strategy 1: 4 replicates	Strategy 4: 4 replicates
Strategy 2: 4 replicates	Strategy 5: 4 replicates
Strategy 3: 4 replicates	Strategy 6: 4 replicates
Strategy 7: 4 replicates	
Compartment 1 (400m ²)	Compartment 2 (800m ²)

Fig. 1. Schematic overview of the trial setup. In each compartment the control strategies are placed depending whether they contain the Flying Doctors treatment or not. Each control strategy consist out of 4 replicates, randomly divided over the greenhouse compartment.

Table 1
Different control strategies to control *Botrytis cinerea* in a greenhouse strawberry trial

Control Strategy	31/07/13	7/08/13	12/08/13	30/08/13	9/09/13
1. Untreated control	/	/	/	/	/
2. Prestop (3x)	Prestop 6 kg/ha	Prestop 6 kg/ha	Prestop 6 kg/ha	/	/
3. Teldor (1x)	Teldor 1.5 kg/ha	/	/	/	/
4. FD Verdera B ⁴	8 g/3–4 days	8 g/3–4 days	8 g/3–4 days	8 g/3–4 days	8 g/3–4 days
5. FD Verdera B ⁴ + Prestop	8 g/3–4 days Prestop 6 kg/ha	8 g/3–4 days /	8 g/3–4 days /	8 g/3–4 days /	8 g/3–4 days /
6. FD Verdera B ⁴ + Teldor	8 g/3–4 days Teldor 1.5 kg/ha	8 g/3–4 days /	8 g/3–4 days /	8 g/3–4 days /	8 g/3–4 days /
7. Chemical control	Frupica 0.6 kg/ha	Frupica 0.6 kg/ha	Teldor 1.5 kg/ha	Teldor 1.5 kg/ha	Signum 1 kg/ha

The treatments were applied as shown in Table 1. Spraying with *G. catenulatum* was performed on 31 July, 7 August and 12 August at 6 kg/ha. Fenhexamid was sprayed once on 31 July at 1.5 kg/ha. The dispensers in the hives of FD-compartment were filled with 8 g Verdera B⁴ every three to four days (twice a week). There were four bumblebee hives filled with VerderaB⁴ in the compartment of 800 m². Inside this compartment, four plots were treated by spraying *G. catenulatum* (6 kg/ha) and four plots with fenhexamid (1.5 kg/ha), both on 31 July 2013. The chemical control object was treated with mepanipyrim (Frupica; 0.6 kg/ha) on 31 July and 7 August, with fenhexamid (Teldor; 1.5 kg/ha) on 12 and 30 August and with boscalid+pyraclostrobin (Signum; 1 kg/ha) on 9 September 2013.

For controlling other pests and diseases, it is important to pay attention to the use of crop protection products. These products should be compatible with *G. catenulatum* and with bumblebees. It is possible to lock bumblebees during and after treatment, but if this occurs no Verdera B⁴ can be distributed by the bumblebees. Products were selected to minimize the need for locking bumblebees as much as possible.

2.3. *Botrytis* inoculation and natural infection

Artificial inoculation with the pathogen *Botrytis cinerea* (isolate PCF232) was performed during flowering to test efficacy under higher (artificial) disease pressure. Spore suspension was used for inoculation at a concentration of 2×10^5 spores/mL with ¼ PDB (Potato Dextrose Broth). For each replicate, 50 open flowers were labelled and inoculated with 20 µL *B. cinerea* suspension per flower of the at 31 July and 7 August. On 13 August 25 flowers were labelled and inoculated. After inoculation the plants were covered with plastic for 24 h to ensure an elevated relative humidity.

Pre- and post harvest *Botrytis* infection were evaluated. At each harvest date, all ripe labelled fruits were picked. At five different dates 25 unlabeled fruits per plot were picked. Infested fruits were scored and discarded immediately while healthy fruits were stored at 4°C in separate closed plastic boxes with a moist filter paper. The presence of *B. cinerea* was scored 14 days after harvest. The strawberries with mycelium growth, were then selected and placed at 20°C for 2 days. This stimulated spore production and facilitated identification.

3. Results

3.1. Yield and grading

Harvest was between 12 August and 9 October 2013, divided over 15 pickings, with midharvest (50% of total production harvested) between 30 August and 1 September 2013. The yield in the FD compartment was 3,05 kg/m² (320 g/plant). There was no statistically significant difference with the control treatment (3,28 kg/m² or 340 g/plant). The grading the grading was also not significant and similar for the two treatments: approximately 56–60% large fruits, 38–42% small fruits and 1–2% misshapen fruits. Also here the small differences are negligible.

3.2. *Botrytis* assessments

Natural disease pressure. Of the five different picking dates, the occurrence of grey mould after cold storage did not differ statistically on 2/09 and 5/09 (Table 2). On 21/08, 26/08 and 16/09 there were statistically significant differences with the untreated control: the chemical control resulted in less fruits rotten on 26/08 and 16/09, FD showed a lower *Botrytis* infection on 26/08, and on 21/08 FD in combination with Teldor resulted in less fruits rotten. All data averaged showed for the untreated control 53.5% of fruit rotten after cold storage. For the sprayed objects with Prestop (strategy 2) and Teldor (strategy 3), the infection percentage was similar: 52.0 and 47.8, respectively. The chemical control showed a statistical lower infection with 40.3% fruits rotten. The FD treatment (strategy 4) resulted in 36.6% of rotten fruit, which is similar to the chemical strategy reference result, representing a statistically significant reduction in *Botrytis* infection compared to the untreated control. The strategies with FD in combination with Prestop or Teldor gave infection percentages of 41.5% and 39.2%, respectively, but were not significantly different to the other treatments.

Inoculated fruits. At seven different dates all ripe labelled fruits were picked. In total eight fruits were rotten at harvest. The first and second inoculation (on 31/07 and 7/08) showed significant differences between treatments, and with FD showing the lowest infection rate. The third inoculation did not result in statistical differences.

When all data of the three inoculations were averaged, the untreated control showed 63.1% of fruit rotten after the cold storage. The Prestop spray treatment (strategy 2) showed 66.0% of fruit rotten, with Teldor (strategy 3) showing a similar result with 63.3% of fruit rotten. All strategies with FD (strategy 4-5-6) gave lower percentages of rotten fruit, from 45.9 to 53.4%. The chemical control strategy (strategy 7) resulted in 55.7% of fruit rotten, while only strategy 6 (FD with 1 spray of Teldor) was significantly better compared to the untreated control, with 45.9% of fruit rotten (Table 3).

Table 2
Infection rates (%) on strawberry fruit after 14 days in cold storage at 4°C under natural disease pressure

Control strategy	21/08/13		26/08/13		2/09/13		5/09/13		16/09/13		All data	
1. Untreated control	58.0	a	54.0	a	62.0	ns	40.0	ns	58.7	ab	53.5	a
2. Prestop (3x)	50.0	ab	47.0	ab	76.0	ns	35.0	ns	54.0	ab	52.0	ab
3. Teldor (1x)	44.0	ab	54.0	a	64.0	ns	29.0	ns	56.0	ab	47.8	ab
4. FD Verdera B ⁴	37.0	ab	27.0	b	53.0	ns	28.0	ns	45.0	ab	36.3	b
5. FD Verdera B ⁴ + Prestop	41.0	ab	32.0	ab	55.0	ns	38.0	ns	49.0	ab	41.5	ab
6. FD Verdera B ⁴ + Teldor	27.0	b	36.0	ab	55.0	ns	38.7	ns	66.0	a	39.2	ab
7. Chemical control	35.0	ab	26.0	b	66.0	Ns	34.0	ns	38.0	b	40.3	b

Significance $p < 0.05$ according to Duncan.

Table 3
Infection rates (%) on strawberry fruit after inoculation with *B. cinerea* followed by 14 days in cold storage at 4°C.

Control strategy	Inoculation 31/07/13		Inoculation 7/08/13		Inoculation 13/08/13		All data	
1. Untreated control	65.6	ab	68.9	ab	54.9	ns	63.1	a
2. Prestop (3x)	73.8	a	75.4	ab	48.8	ns	66.0	a
3. Teldor (1x)	47.6	ab	76.9	a	65.4	ns	63.3	a
4. FD Verdera B ⁴	38.4	b	57.1	b	51.0	ns	48.9	ab
5. FD Verdera B ⁴ + Prestop	45.2	ab	57.3	b	57.8	ns	53.4	ab
6. FD Verdera B ⁴ + Teldor	33.7	b	64.9	ab	39.0	ns	45.9	b
7. Chemical control	44.0	ab	69.0	ab	54.2	ns	55.7	ab

Significance $p < 0.05$ according to Duncan.

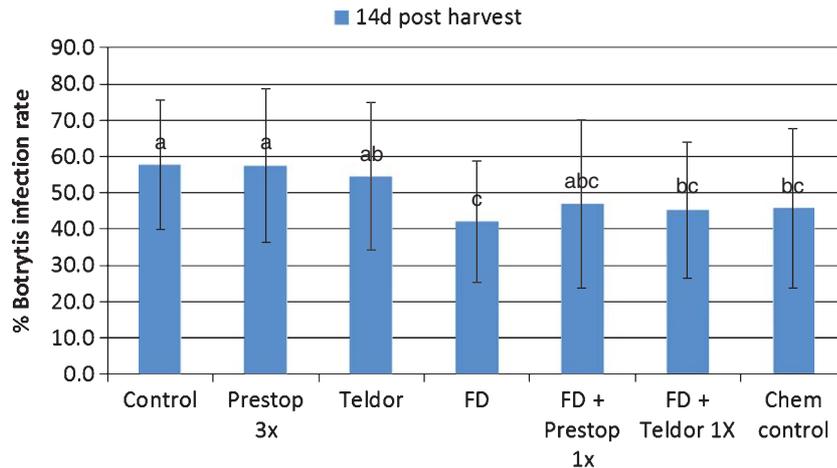


Fig. 2. Botrytis-infection rates (%) for all fruits assessed 14 days post harvest (significance $p < 0.05$ Duncan).

3.3. General efficacy

To obtain a general efficacy of the trial, all data of inoculated and non-inoculated fruits, were averaged (Fig. 2). The untreated control showed 57.7% of fruit rotten after cold storage. Three spray applications with *G. catenulatum* (57.5%) or 1 spray application with Teldor (54.6%) did not reduce the *Botrytis* infection. The conventional chemical treatment scheme caused a significant reduction of grey mould and resulted in 45.8% infection after cold storage. Also the FD strategy (strategy 4) showed a significant lower infection rate of 42.1%, which was comparable to the conventional chemical control strategy (strategy 7). The additional sprayings with Prestop or Teldor did not generate an additional reduction in infection compared to the FD strategy with 46.9 and 45.2% infection rates, respectively.

4. Discussion

The dispenser with Verdera B⁴ used in the Flying Doctors® System resulted in good fruit set and yield. Grading of fruit was similar for treated and untreated flowers. So there was no negative effect on the bumblebees nor on pollination. This is in accordance with the findings of Kovach *et al.* [7], who found no negative effects on bees loaded with *T. harzianum*, and those of Mommaerts *et al.* [8] who showed no negative effects on bumblebee foraging activity or working bumblebees survival.

*** Under natural disease pressure and after inoculation of flowers, lower percentages of rotten fruit due to *Botrytis* infection were found in most of the FD strategies and the conventional chemical control strategy compared to the untreated control, although not all these results were statistically significant. In general, disease pressure was moderately low in this trial, with the untreated control resulting in 53.5% rotten fruit after two weeks of storage. This is probably due to weather conditions during flowering, the main period for pre-harvest infections of *B. cinerea* [2]. During this period there were very high temperatures (during daytime $> 25^{\circ}\text{C}$) with low relative humidity (during daytime $< 50\text{--}60\%$) in the greenhouse (data not shown). Maas [1] described favourable temperatures for *Botrytis* spore production between $15\text{--}22^{\circ}\text{C}$ at high relative humidity or leaf wetness. Artificial inoculation could increase the infection rate to 63.1%, but it is still quite low for inoculated flowers. These relatively low percentages of rotten fruits after 14 days of cold storage made it difficult to obtain consistent significant differences between different control strategies. The variation between the replicates within one control strategy is relatively high, so under these conditions with low to moderate *Botrytis*-pressure, probably more fruits should have been assessed to find statistically more significant differences.

Nevertheless, when all assessed fruits – inoculated and non-inoculated – were evaluated, the number of fruits per plot increased. This trial showed that use of *G. catenulatum* vectored by bumblebees was able to reduce the

Botrytis infection in a commercial strawberry greenhouse. At low/moderate disease pressure as in this trial, the infection rate was comparable to a conventional chemical control strategy. This is in agreement with other trials conducted in different locations with several microbiological control agents. Most of these also showed a reduced infection of *Botrytis*. Weiß *et al.* [9] tested the yeast *Aureobasidium pullulans* dispersed by bumblebees in protected strawberry cultivation, and also obtained reduced *Botrytis cinerea* infection. Kovach *et al.* [7] also found that vectoring a microbiological control (*Trichoderma harzianum*) reduced the number of *Botrytis cinerea* infected strawberries to the same level as the control with spraying chemical fungicides. Also Shafir *et al.* [10] showed in Israel that it was possible to suppress *Botrytis cinerea* – at a low to medium disease pressure – in strawberry fields with *Trichoderma harzianum* T39 vectored by bumblebees, and this control was better than in the chemical treatment. In this Israeli trial, non of the treatments controlled *Botrytis* well at a high disease pressure. Mommaerts *et al.* [8] investigated the effect of *G. catenulatum* vectored by bumblebees, and showed that under a high disease pressure, the system could significantly increase the percentage of pre- and postharvest non-infected fruits. Due to weather conditions in our trial, we could not test the efficacy at high disease pressure. That might have been a reason that the additional sprays with Prestop or Teldor to the FD could not improve the results. At higher disease pressure, these additional spray treatments might have improved the results. This should be further investigated to find a complete strategy to make it possible at high disease pressure to control *Botrytis cinerea* with *G. catenulatum* vectored by bumblebees in strawberry greenhouses. This could extend shelf-life of greenhouse grown strawberries and a more sustainable control with healthier strawberries due to fewer residues can be achieved.

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