Dispersal of *Aureobasidium pullulans* by pollinating insects to control *Botrytis* infection in strawberries

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Abstract

Infections with Botrytis cinerea *cause serious damage in strawberry production. The infection starts already at blossom. For* Botrytis *control several treatments are necessary and the spray applications increase the humidity in tunnels and favour* Botrytis *development.*

If an active ingredient could be dispersed by pollinating insects the substance will reach the flowers directly without using water. Within a research project Bio-Protect GmbH and Koppert BV developed a method combining pollinating with plant protection. The usefulness of the dispersal of Aureobasidium pullulans *through bumble bees and the control of* Botrytis *infection in a protected strawberry production was shown over a two years period. The trials were carried out in the Ortenau region, Baden-Württemberg, on the early variety Clery. The dispersal was measured by a strain specific qPCR and the disease control by evaluation*Botrytis *infection in comparison with untreated control tunnels.*

*It was shown that the bumble bees dispersed between 10^3 and 10^5*A. pullulans cells per *blossom and the efficiency in* Botrytis *control was between 60 and 80%.*

Keywords: *Aureobasidium pullulans*, antagonist, *Botrytis cinerea*, bumble bees, strawberries

Introduction

Fruitrotsare among the most serious infections in strawberries and most strategies to control the disease are just more or less successful, especially in organic production.The main infections in strawberries are caused by the grey mold pathogen (*Botrytis cinerea*) through blossoms. The occurrence of an infection mainly depends on the weather conditions during harvest.Therefore any kind of treatment hasto be protective and as an ideal case every blossom has to be treated. This optimal protection is under practical use with conventional treatment methods hardly to conduct.

Bio Protect GmbH developed biocontrol agents, which contain antagonistic strains of *Aureobasidium pullulans,* against several bacterial and fungal pathogens (Kunz 2004; Weiss, Mögel, and Kunz 2006). One of the *A. pullulans* formulations controls *Botrytis cinerea* in grapes (Achleitner 2010) and the application of *A. pullulans* to strawberry blossoms reduced infections by *Botrytis cinerea* (Adikaram, Joyce, and Terry 2002). Therefore first trials with spray applications of Boni Protect forte were done in organic strawberries, revealing increase in harvest and longer shelf life of the fruit (Mayr and Späth 2008).

Aim of the following study was to develop a method to treat as many blossoms as possible without enhancing treatment dates. If pollinating insects dispersed aplant protection agent during pollination, it should be possible to protect a maximum number of blossoms.

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Koppert BV was working on beneficial organisms for a long period of time, distributes bumble bees and developed several dispensing systems (Fig. 1) for the use with bumble bees.*Aureobasidium pullulans* in an appropriate formulation is predestinated to be dispersedin such a system. With this new innovative technique, it would be possible to improve the efficacy of the product and also reduce the application rate by bringing the product directly to the blossom.

Figure 1 Bumble bee hive with dispenser filled with a powdery formulation of *A. pullulans* in a strawberry tunnel.

Material and Methods

The trials were carried out in protected production of a Bioland farm in Sinzheim/Müllhofen, Ortenau region Baden-Württemberg on the early variety Clery. Trials were done over a two years period. In 2010 trials were realized in ten tunnels (length between 100 and 200m, total acreage 1.5 ha) and in 2011 in twelve tunnels (length 180-200m, total acreage 2.2 ha).

Trials were started at the beginning of bloom. Bumble bee hives were placed in the middle of each tunnel and the dispensers were filled with a modified formulation of Boni Protect forte containing the two strains of *A. pullulans* DSM14940 and DSM14941.

In vertical spacing from the bumble bee hives at different distances (in 2010 up to eight distances in 2011 fourdistances) at a time ten blossoms were collected to measure the dispersal of *Aureobasidium pullulans* by the bumble bees.The determination of DSM14940 was measured by a quantitative real time PCR (qPCR).

qPCR analyses were performed using a CFX96 Real-Time PCR Detection System (BioRad, Munich). Manual threshold was set to 180. As qPCR chemistry the QuantiTect® SYBR® Green PCR Master Mix (QIAGEN, Hilden) together with primers CF10-RAPD-F4 and CF10-RAPD-R6 in a reaction volume of 25 µl was used(Sickinger 2008). Maximum sample volume to be tested was 10 µl. The PCR protocol consisted of an initial denaturation step of 5 min at 95°C, followed by 40 cycles of denaturation at 94°C for 20 sec, annealing at 54.5°C for 20 sec and extension at 72°C for 20 sec. A melt analysis followed each PCR run to verify identity and homogeneity of the amplicon.

Samples consisted of washing fluids containing intact blastospores. No DNA extraction was performed prior to PCR analysis. For the analyses strawberry blossoms (in general 10) were collected in Whirl-Pak bags (Carl Roth GmbH, Karlsruhe), and incubated with 20 mlH_2O for 15 min. A 1 ml aliquot was removed and centrifuged for 1 min at 15,000 g. The supernatant was discarded and pellets resuspended in an equal volume of H_2O. Samples were either analyzed directly or stored at -20°C.

Absolute quantification of blastospores in samples was done by standardization with respect to serial dilutions of washed pure cultures of strain DSM14940.

Statistical analysis of log transformed data was done using one-way analysis of variance, and mean separation was accomplished using Tuckey's Multiple Comparison test ($p \leq 0.05$). Arithmetic means were retransformed to be described in the text.

Disease incidence was evaluated by counting *Botrytis* infection sites on flowers and fruits of 200 plants in each plot. At least four plots (different rows) were counted in each treatment (untreated, treated with new dispenser, treated with old dispenser). Symptomatic flowers and fruits were removed.In 2010 primary and also secondary *Botrytis*infections were distinguished.

Efficiencies of treatments against primary *Botrytis* were calculated according to Abbott (Abbott 1925). Statistical analyses of the data were done using one-way analysis of variance, and meanseparation was accomplished using either Student's t-test (($p \leq 0.05$) or Tuckey's Multiple Comparison Test ($p \leq 0.05$).

Results

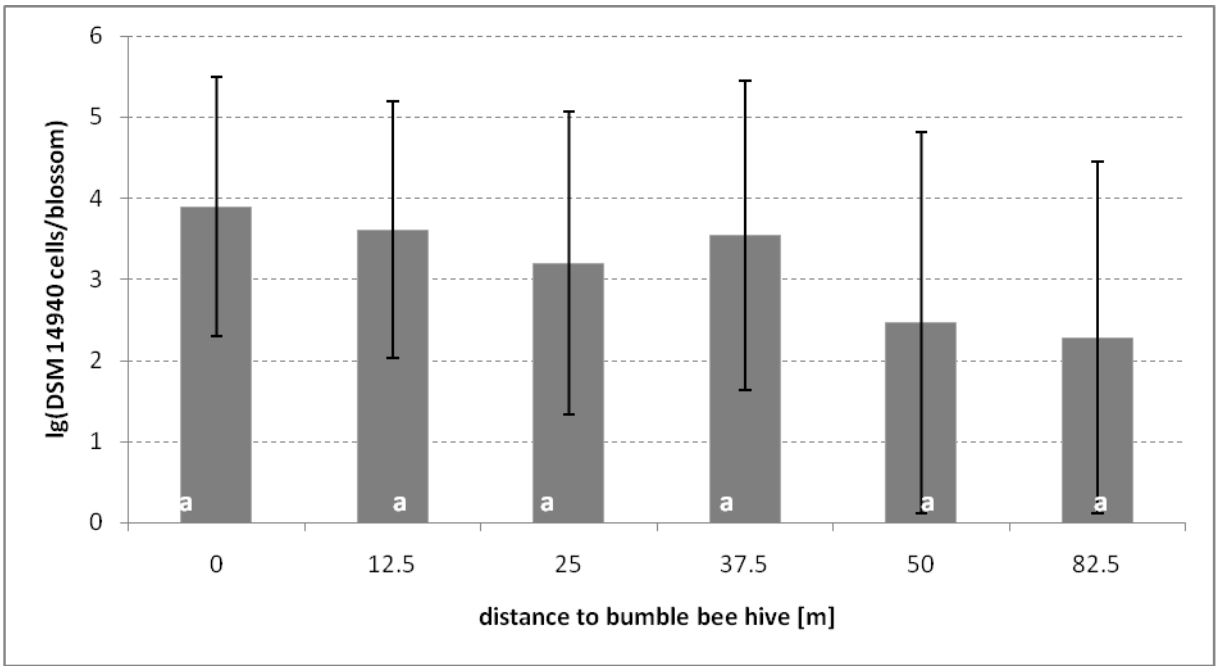

Figure 2: Average abundance of DSM14940 on blossoms of all treated tunnels in relation to the distance of the flowers to the bumble bee hives. The same letters in columns indicate no significant difference in Tuckey'sMultiple Comparison test ($p \leq 0.05$). n = 9-23

In 2010 dispersal of *A. pullulans* was evaluated in ten tunnels. Bumble bees reached all parts of the tunnels and a laminar distribution of *Aureobasidium pullulans*occurred. There was no significant influence of the distance to the bumble bee hive on DSM1940 cell numbers present on the blossoms (Fig. 2).

In both years during the whole sampling time in more than 80% of all taken samples from tunnels with dispensers DSMZ14940 was detected. On some sampling days even 97% of the samples were positive. The abundance of strain DSM14940 was up to 10^5 per blossom. On average between 10^2 and 10^4cells per blossom were found.

In 2010 evaluation of infections by *Botrytis cinerea* was done on 12.05.2010in the control tunnels 25 and 27 as well as in the treated tunnels 26, 28 and 29. These five tunnelswere comparablein size, configuration and exposure. In addition to the primary *Botrytis* infections caused through infected blossoms, secondary fruit infections caused by *Botrytis cinerea* were evaluated.

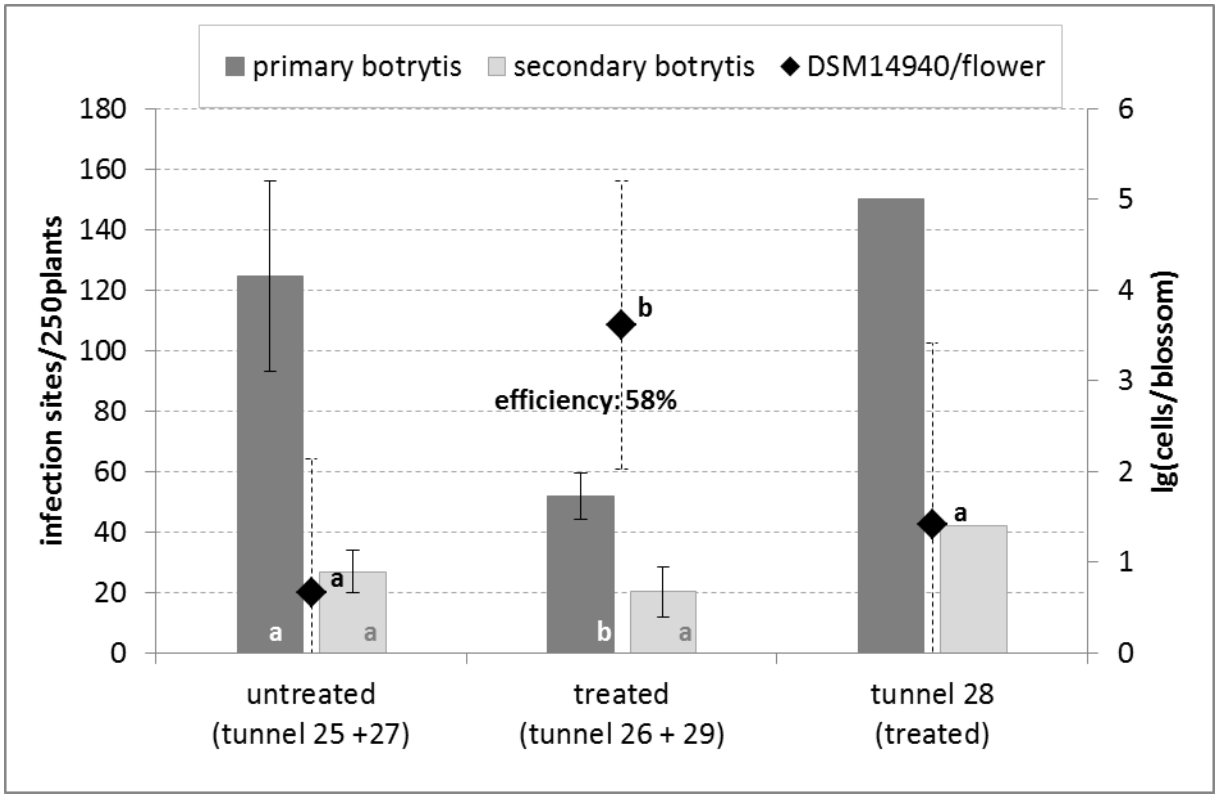

Figure 3: *Botrytis* infection sites and average abundance of DSM14940 on blossoms of treated and untreated tunnels in 2010. Tunnel 28 was not included in statistical analysis of disease incidence (n=2). Different letters in columns indicate a significant difference in Student's t-test ($p \leq 0.05$); n = 4. Different letters next to rhombs indicate a significant difference in Tuckey's Multiple Comparison test ($p \leq 0.05$); n = 18-40.

In tunnel 28 low activity of the bumble bee population was noticed during the trial. In consequence, the abundance of *A. pullulans* was significantly lower than in the other treated tunnels and the number of primary infections was higher. Therefore this tunnel was not included in calculations of means and in the statistical analysis (Fig. 3). In untreated tunnels in average less than 10 DSM14940 blastospores per blossom and in average 125 infection sites per 250 plants were found. In treated tunnels more than 1,000 DSM14940 blastospores per blossom and in average 52 infection sites per 250 plants were found (Fig.3). The dispersal of *Aureobasidium pullulans* was able to significantly reduce infections caused by *Botrytis cinerea* with an efficiency of 58%. The number of secondary infections was low and there was no difference between the treatments concerning secondary infections.

In 2010 function of the dispensers sometimes were restricted by high humidity. Therefore a second typeof new developed dispenser was included in the trials in 2011. Exposure, size and configuration of the tunnelswere morehomogenous than in 2010 but plant development in the first three tunnels (20-22) was lightly earlier than in the remaining tunnels. A first evaluation was carried out in tunnels 20-22 on the 19.04.2011(Fig.4).

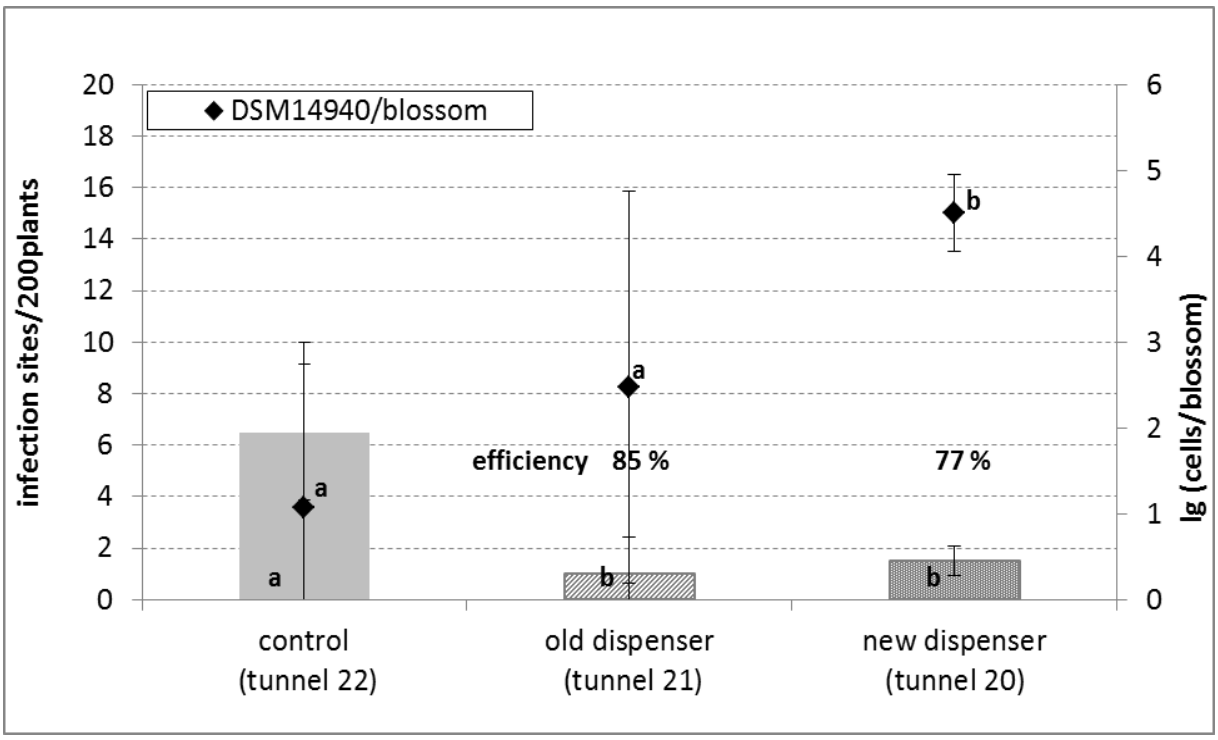

Fig.4: *Botrytis* infection sites and average abundance of DSM14940 on blossoms of treated and untreated tunnels in 2011 (1st evaluation). Different letters in columns (n=4) or in rhombs (n=16) indicate significant differences in Tuckey's Multiple Comparison test (p≤ 0.05).

In untreated tunnels in average 6 infection sites were found per 200 plants. Unless the DSM14940 cell number per blossom was significantly lower in tunnel with the old dispenser compared to tunnel with the new one, with both dispenser types a significant reduction of incidence was observed (Fig.4)showing efficiencies of 77% and 85%.

On 28.04.2011 a second evaluation in eight tunnels of similar shape (two control tunnels 22 and 25, three tunnels with old dispensers21,24 and 27 and three tunnels with the new dispenser type:20,23 and 26)was conducted (Fig.5).

Disease incidence increased to 18 infection sites per 200 plants in untreated tunnels.and was significantly reduced in the treated tunnels (Fig. 5) showing efficiencies of 55% and 65%. Again the cell count of DSM14940 was significantly higher in treated tunnels. In this case, cell counts in three tunnels with old dispenserswere compared to cell counts in three tunnels with new dispensers and no significant difference between the dispenser types were found. May be the difference between tunnel 21 and 20 (Fig. 4) was caused by differences in the activity of bumble bees.

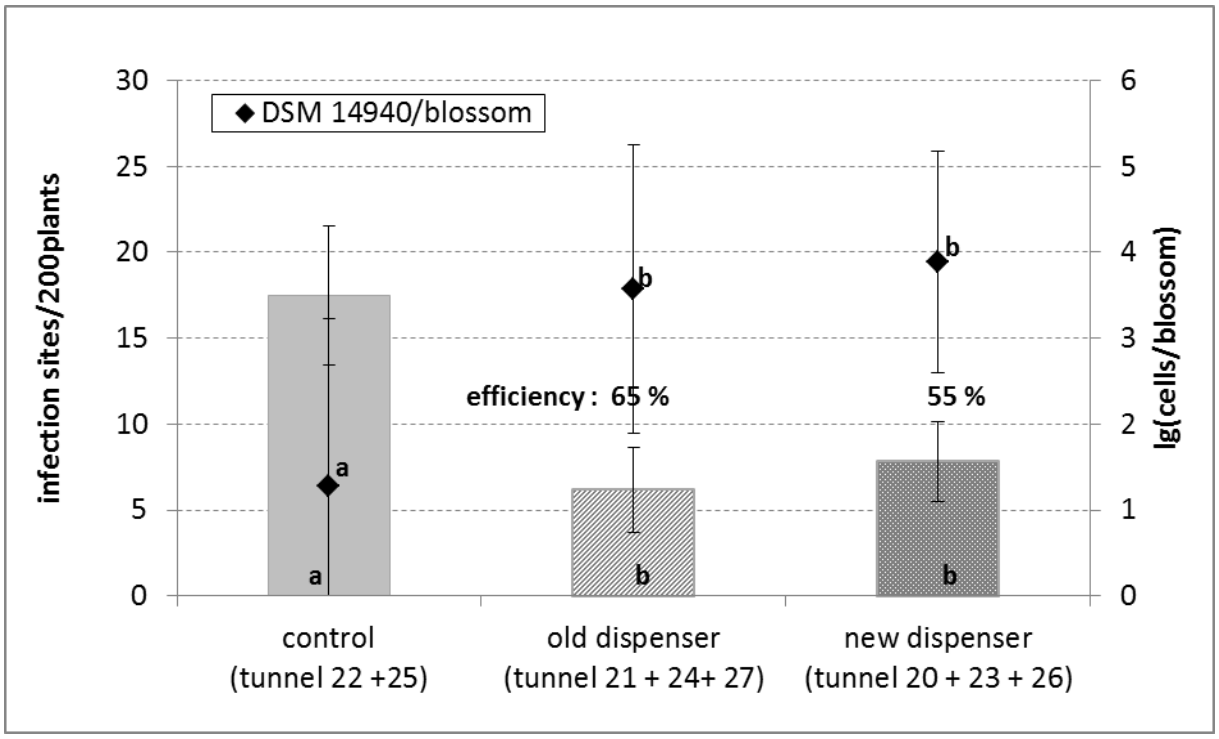

Fig.5: *Botrytis* infection sites and average abundance of DSM14940 on blossoms of treated and untreated tunnels in 2011 (2nd evaluation). Different letters in columns (n=6) or next to rhombs (n=32-48) indicate significant differencesin Tuckey's Multiple Comparison test (p≤ 0.05).

Discussion:

The results of thistwo year trials in protected strawberry production showed that it is possible to disperse *A. pullulans,* the active ingredient of the plant protection agentBoni Protect forte, to strawberry blossomsusing pollinating insects. As indicated in other studies (Adikaram, Joyce, and Terry 2002; Mayr and Späth 2008), inserting *A. pullulans* into strawberry blossoms reduced infections by *Botrytis cinerea.* Regarding the fact that in organic strawberry growing *Botrytis*control is difficult up to now, this innovative strategyshowing an efficiency in *Botrytis* control between 60-80% offers an alternative way in future plant protection.Using this method a reduction of amount of product compared to conventional treatment methods and also a reduction of labor for the grower can be achieved. Due to the fact that resistance of strainsof *Botrytis cinerea* against most common chemical plant protection agentsincreases (Weber 2011), this method will also obtain importance in integrated production.

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