SHORT COMMUNICATION

Laboratory efficacy and fungicide compatibility of *Clonostachys rosea* against *Botrytis* blight on lowbush blueberry

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Reeh, K. W. and Cutler, G. C. 2013. **Laboratory efficacy and fungicide compatibility of *Clonostachys rosea* against *Botrytis* blight on lowbush blueberry**. Can. J. Plant Sci. **93**: 639–642. Lowbush blueberry (*Vaccinium angustifolium*) is an economically important crop. *Clonostachys rosea* is an endophytic fungus that can provide protection of plants from several pathogens, including *Botrytis cinerea*. It is unknown if *C. rosea* is able to colonize and protect *V. angustifolium*, and whether it is tolerant of fungicides commonly used for *B. cinerea* management in blueberry production. In a greenhouse experiment, pre-treatment of blueberry blossoms with *C. rosea* significantly reduced establishment of *B. cinerea,* but treatments after establishment of the pathogen were not effective. *Clonostachys rosea* demonstrated in vitro tolerance to the fungicide Switch®, but little or no tolerance to Pristine® and Maestro®. Our results are encouraging for additional field research examining the use of *C. rosea* as part of an integrated pest management program for *B. cinerea* control on lowbush blueberries.

Key words: *Vaccinium angustifolium*, *Botrytis cinerea*, biological control, fungicide compatibility, microbial antagonist, wild blueberry

Reeh, K. W. et Cutler, G. C. 2013. **Efficacité en laboratoire et compatibilité de *Clonostachys rosea* avec les fongicides employés contre la brûlure causée par *Botrytis* chez le bleuet nain**. Can. J. Plant Sci. **93**: 639–642. Le bleuet nain (*Vaccinium angustifolium*) est une culture d'importance économique. *Clonostachys rosea* est un champignon endophyte capable de protéger les végétaux contre plusieurs pathogènes, dont *Botrytis cinerea*. Néanmoins, on ignore si *C. rosea* peut coloniser et protéger *V. angustifolium*, et si le champignon tolère les fongicides couramment utilisés pour combattre *B. cinerea* dans les bleuetières. Lors d'une expérience en serre, le pré-traitement des inflorescences de bleuet avec *C. rosea* a significativement réduit l'établissement de *B. cinerea,* mais le traitement manque d'efficacité lorsqu'il survient après l'implantation du pathogène. *C. rosea* tolère le fongicide Switch® in vitro, mais présente peu de tolérance, voire aucune, à Pristine® et à Maestro®. Ces résultats incitent à entreprendre d'autres recherches sur le terrain en vue de déterminer si *C. rosea* pourrait s'insérer dans un programme de lutte intégrée contre *B. cinerea* dans les cultures de bleuet nain.

Mots clés: *Vaccinium angustifolium*, *Botrytis cinerea*, lutte biologique, compatibilité avec les fongicides, antagoniste microbien, bleuet sauvage

Botrytis cinerea Persoon:Fries (Sclerotiniaceae), the causative agent of grey mould and blossom blight, is a commonly encountered pathogen in lowbush blueberry (*Vaccinium angustifolium* Aiton) (Ericaceae) production. Blossom infections can cause high reductions in yield (Hildebrand et al. 2001). Botrytis blight is often prevented using an early spray of Pristine® (25.2% boscalid + 12.8% pyraclostrobin, BASF, Mississauga, ON) or Switch® (37.5% cyprodinil, 25.0% fludioxonil, Syngenta Canada Inc., Guelph, ON), or occasionally Maestro® (80% captan, Tomen Canada Inc., Toronto, ON). However, non-chemical methods for blueberry disease management are desirable.

The microbial antagonist *Clonostachys rosea* f. *rosea* (Link) Schroers (syn. *Gliocladium roseum* Bainier) is a beneficial endophytic fungus that has shown good efficacy against *B. cinerea* in several fruiting crops and ornamentals (Peng and Sutton 1991; Yu and Sutton 1997; Morandi et al. 2000; Kapongo et al. 2008). It is unknown whether *C. rosea* is able to colonize and protect *V. angustifolium*. *Clonostachys rosea* has shown tolerance to some fungicides (Harrison and Stewart 1988; Roberti et al. 2006), but its compatibility with the fungicides commonly used for *B. cinerea* management in lowbush blueberry is unknown. Here we describe a greenhouse experiment that evaluated the ability of *C. rosea* to

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Abbreviations: **BCS**, *Botrytis cinerea* suspensions; **CFU**, colony-forming units; **CRS**, *Clonostachys rosea* suspensions

control *B. cinerea* in blossoms of *V. angustifolium*. We also examined the in vitro susceptibility of *C. rosea* to three fungicides commonly used in *Botrytis* management.

METHODS AND MATERIALS

Plants and Cultures

Potted clones of *V. angustifolium* and a local wild blueberry isolate of *B. cinerea* (B94.a1) were provided by Dr. P. D. Hildebrand (Atlantic Food and Horticulture Research Centre, Agriculture and Agri-Food Canada, Kentville, NS). Plants were placed in a greenhouse (25°C ± 2°C, 16:8 L:D) with treatments randomly assigned to a triplicate of plants, which were watered daily. Every 4–6 d another triplicate of plants was established in order to block the experiment over time, for a total of five blocks.

Working cultures of *B. cinerea* were derived from stock cultures grown and maintained on Kings B – Glucose Media mixed in distilled water (Hildebrand et al. 2001). Cultures were kept under fluorescent lighting at room temperature (22°C), and were used in experiments when 8–11 d old. A powder formulation of *C. rosea* was provided by Dr. Peter Kevan (University of Guelph, Guelph, ON) and refrigerated (4°C) between uses.

The *B. cinerea* spore suspension was prepared by adding 1–3 mL sterile distilled water to the working culture, and then lightly brushing the conidia/mycelia with a sterile stainless steel spreader. Solution concentrations were standardized by performing counts using a haemocytometer (average of four replicate samples).

Botrytis cinerea suppression by *Clonostachys rosea*

Treatments utilized *B. cinerea* suspensions (BCS) of 10^5 spores mL^{-1} and *C. rosea* suspensions (CRS) of 1 g L^{-1}, and were as follows: (1) control: 10 μL BCS applied to each flower followed 24 h later with 10 μL per flower of sterile distilled water; (2) 10 μL CRS applied to each flower followed 24 h later with 10 μL of BCS to each flower; (3) 10 μL BCS applied to each flower, followed 24 h later with 10 μL of CRS to each flower.

All attached open flowers were inoculated with CRS or BCS by injecting suspensions into the open end of flowers using a micropipette. Unopened blossoms were marked and excluded from analysis. Plants were placed in plastic sleeves to prevent contact and moved to a "fogging box" (61 cm × 32 cm × 42 cm) inside of a growth chamber (Conviron Inc., Model E15, Winnipeg, MB). The fogging exposure, temperature and spore concentration used were ideal for obtaining *B. cinerea* infection in blossoms at stage F7-F8 (Hildebrand et al. 2001). Plants were subjected to 8 h of cool fog (sterile distilled water; Zoo med Repti Fogger™, San Luis Obispo, CA) and kept in the chamber at 20°C in complete darkness (Hildebrand et al. 2001). Plants were removed 24 h after the initial exposure, given the second inoculation, and then returned to the chamber. Forty-eight hours after the second exposure, treated blossoms from each plant were removed and placed on Whatman #1 filter paper in 10-cm petri dishes moistened with sterile distilled water. Blossoms from different plants were kept in separate dishes during culturing. Plates were kept in a growth chamber at 20°C and complete darkness, and analyzed for growth of *B. cinerea* after 3 d. Blossoms were scored positive for *B. cinerea* when conidiophores were present, as determined under a microscope. The numbers of blossoms with and without conidiophores was recorded for each plant. The data had normally distributed residuals and were analyzed using a standard least squares linear model at $\alpha = 0.05$. Means were separated using a Tukey test (SAS Institute, Inc. 2012).

Fungicide Tolerance

Bottles (1 L) containing 19.5 g of Difco Potato Dextrose Agar, topped to 488 g with distilled water, were mixed and autoclaved, and then cooled to 60°C in a water bath. Prior to pouring, 1.0 mL each of 100 ppm streptomycin sulphate and 2000 ppm Triton X-100 were added to each bottle (Peng et al. 1992). Bottles were amended with one of four fungicide treatments that reflected manufacturer-recommended maximum application rates. Formulated fungicides were dissolved in sterile distilled water in a beaker and added to the media. Residue in the beaker was rinsed into the bottle with sterile distilled water, resulting in a total solution weight of 500 g per bottle. Treatments were: (1) 10 g autoclaved distilled water (control); (2) Maestro 80 DF at 2250 g ha^{-1} per 1000 L ha^{-1} = 2.25 g L^{-1} = 1.125 g active ingredient (a.i.) topped to 10 g with distilled water; (2) Switch 62.5 WG at 975 g ha^{-1} per 200 L ha^{-1} = 4.875 g L^{-1} = 2.435 g a.i. topped to 10 g with distilled water; and (4) Pristine WDG at 1600 g ha^{-1} per 208 L ha^{-1} = 7.69 g L^{-1} = 3.845 g a.i. topped to 10 g with distilled water.

Clonostachys rosea dilutions of 1000, 100, 10 and 1 mg L^{-1} were prepared, with four blocks replicated over time. Aliquots (100 μL) of each dilution were plated on the amended-media using a sterile stainless steel spreader. Plates were stored at 22°C in complete darkness. The numbers of colony-forming units (CFU) were counted on day 4 after plating. Colony-forming units count data met assumptions of normality and equal variance, and were analyzed using a standard least squares linear model at $\alpha = 0.05$. Means were separated using a Tukey test (SAS Institute, Inc. 2012).

RESULTS

The percentage of blossoms per stem with *B. cinerea* conidiophores was significantly affected by treatment ($F_{2,2} = 55.5$, $P < 0.0001$). Less than 40% of blossoms had conidiophores when they were treated with *C. rosea* before exposure to *B. cinerea*, reducing disease incidence

by over 50% compared with the control and treatment where *C. rosea* was applied to blossoms after exposure to *B. cinerea* (Fig. 1). The overall percentage of bloom with conidiophore establishment varied among the experimental blocks ($F_{1,\ 1} = 7.4$, $P = 0.023$), but there was no treatment-block interaction ($F_{2,\ 2} = 0.95$, $P = 0.42$).

For the fungicide-amended media experiments, the number of CFU was significantly affected by treatment ($F_{3,\ 3} = 225.9$, $P < 0.0001$) (Fig. 2). Significantly more CFU were found in the control than in plates amended with fungicide ($P < 0.05$), but high numbers of CFU were found in the Switch treatment. Very few CFU were found in the Pristine treatment, and none in the Maestro treatments (Fig. 2). The mean number of CFU differed among the experimental blocks ($F_{1,\ 1} = 151.5$, $P < 0.0001$), decreasing with each successive block (over time) in the control and Switch treatments. Colony-forming unit counts did not differ across blocks for the Pristine and Maestro treatments, resulting in a significant interaction effect ($F_{3,\ 3} = 50.9$, $P < 0.0001$).

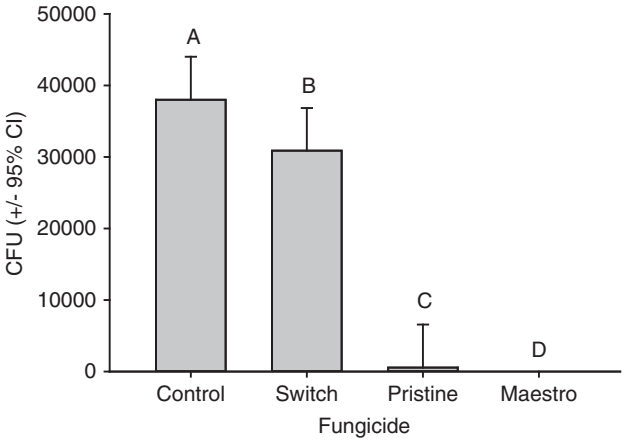

Fig. 2. Number of colony-forming units (CFU) of *Clonostachys rosea* on media containing the fungicides Switch (cyprodinil + fludioxonil), Pristine (pyraclostrobin + boscalid), or Maestro (captan). Bars with different letters are significantly different ($\alpha = 0.05$; Tukey test).

DISCUSSION

Consistent with previous findings (Peng and Sutton 1991; Yu and Sutton 1997; Morandi et al. 2000; Kapongo et al. 2008), our greenhouse experiment demonstrated that *C. rosea* can provide protection from *B. cinerea* infection, but that protection is only prophylactic. Although *C. rosea* was unable to reduce disease incidence when applied 24 h after *B. cinerea* treatment, synthetic fungicides currently registered for *B. cinerea* in lowbush blueberry also need to be applied before disease development if they are to be effective (Rosslenbroich and Stuebler 2000). Moreover, the ~50% disease reduction we observed in the greenhouse under conditions ideal for *B. cinerea* infection is comparable with the reduction provided by *C. rosea* in greenhouse-grown sweet pepper (Kapongo et al. 2008), and to that seen in highbush blueberry at peak bloom using sprays of Pristine and Switch (Elmhirst and Smith 2010). Our greenhouse experiments are a useful first step demonstrating the potential of *C. rosea* to suppress *Botrytis* blight on lowbush blueberry. Further evaluations should compare field efficacy with sprays of *C. rosea* to that achieved with synthetic fungicides.

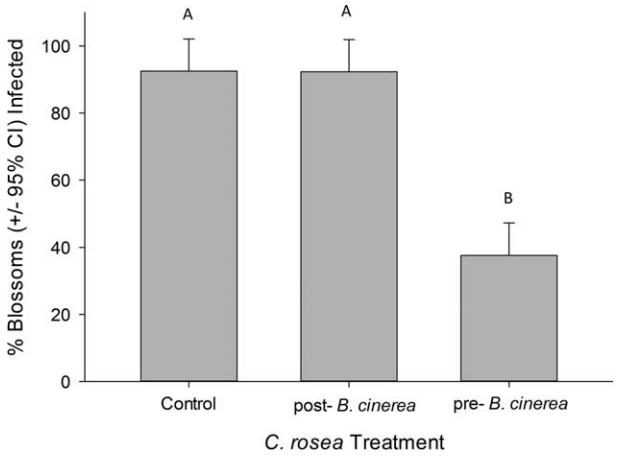

Fig. 1. Percent of lowbush blueberry blossoms infected with *Botrytis cinerea* following pre- or post-treatment with *Clonostachys rosea*. Bars with different letters are significantly different ($\alpha = 0.05$; Tukey test).

If they are to have optimal utility in integrated pest management, biological control agents should be compatible with pesticides also used in a particular agricultural system. We found that *C. rosea* activity was significantly inhibited by all fungicide treatments in vitro. Nonetheless, a large number of *C. rosea* CFU developed in media containing Switch. Despite the strong effect of Pristine on the germination of *C. rosea*, we did find some CFU, suggesting the possibility of isolating *C. rosea* strains that are more tolerant to Pristine. We did not see any growth of *C. rosea* with Maestro, but in vitro tolerance of *C. rosea* (*G. roseum*) to captan has been demonstrated (Harrison and Stewart 1988). Our exposures of *C. rosea* to fungicides in this experiment likely represented a worst case for the endophyte, since it was in continuous direct contact with nutrient media containing a fungicide. Antagonistic effects of Switch on *C. rosea* would probably be less severe in the field, suggesting integrated pest management programs for Botrytis blight using spray alternations or mixtures of *C. rosea* and Switch may be possible. Using microbial controls would reduce selection pressure for *B. cinerea* resistance to fungicides, while also reducing chemical inputs into the environment. In addition to spray applications, commercial pollinators can vector *C. rosea* for disease and insect management (Peng et al. 1992; Yu and Sutton 1997; Kapongo et al. 2008; Reeh 2012). Field experiments should be done next to confirm control of *Botrytis*

blight, and potentially other lowbush blueberry diseases, under realistic conditions.

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