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Potential biological agents isolated from apple fail to control Glomerella leaf spot in the field

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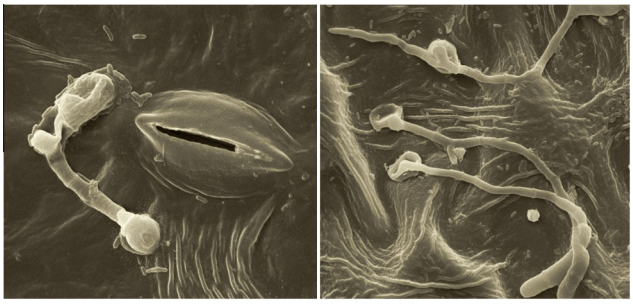
HIGHLIGHTS

- Serenade® showed no control of GLS in leaves or fruits.
- Bacterial isolates from apple showed no control of GLS in leaves.
- Leaf cover with the antagonists revealed flaws in the biofilm.

GRAPHICAL ABSTRACT



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ABSTRACT

Glomerella leaf spot (GLS) has great impact on apple crops in Paraná/Brazil because the majority of the orchards in the state are planted with the cultivar 'Gala', which is very susceptible to the disease. The objectives of this study were to evaluate several potential biological control agents as well as a commercial biocontrol product were applied to apple plants and compared with mancozeb for control of GLS on leaves and fruit. Test plots included an experiment station orchard as well as a commercial orchard. In the experimental orchard, treatments showed different effects between the three crop seasons. In the commercial orchard, the effects of the biological controls were not significantly different from control in the two areas evaluated. The commercial biocontrol product showed no effect on GLS in leaves and fruits. Leaf cover with cells of the antagonists visualized by scanning electron microscopy revealed flaws in the biofilm, enabling germination and appressoria formation by *Colletotrichum*.

1. Introduction

In the state of Paraná in Brazil, Glomerella leaf spot (GLS) is of great importance because the majority of orchards are planted with the cultivar 'Gala', which is very susceptible to disease. Moreover, the climate favors the development and dissemination of this pathogen. According to Katsurayama and Boneti (2009), GLS has increased in severity in Brazil, causing 75% defoliation by harvest under favorable conditions. The GLS is caused by *Colletotrichum* spp. The *Colletotrichum* genus was reclassified (Damm et al., 2012; Weir et al., 2012), and based on this new classification were described species causing the disease belonging to two complex, *Colletotrichum acutatum* complex and *Colletotrichum gloeosporioides* complex (Bragança, 2013). The disease has been controlled by applying dithiocarbamate fungicides in particular (Mancozeb, Metiram and Propineb), which show a high index of control (IC) at the beginning of the vegetative cycle of the apple tree (IC > 80%) and that is median at the end (IC > 70%) of each season, when the pressure inoculum increases. Control based on the application of protective fungicides results in large number of sprays that may reach up to 15 sprays per season (Katsurayama and Boneti, 2012).

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The standards of the Integrated Production of Apple in Brazil (IN SDC n.01 of 14/09/2006)[1] limit the use of fungicides per crop and often invalidates this control system. Thus, in view of the fact that the period prior to harvest is the most critical for disease develop, it may be stated that the treatments used today are ineffective due to restrictions on the grace period of fungicides (Valdebenito-Sanhueza et al., 2002; Katsurayama and Boneti, 2009, 2012).

Therefore, biological control is needed when fruit appear most vulnerable. However, the selection of antagonistic microorganisms must be made judiciously by determining the chances of success of the biological control program (Bettiol, 1997). Andrews (1992) and Bettiol (1997) drew attention to the fact that in many cases, due to their low cost and speed, the selection of antagonists is based solely on *in vitro* assays. However, they cautioned that there is a low correlation between results obtained under laboratory conditions and those obtained in the field.

Thus, antagonistic microorganisms with proven efficiency *in vitro* should show increased plant surface targeting and biocontrol of the disease target population and their survival should be examined for effectiveness under field conditions. Of the various biotic agents that may be used for biological control, the phylloplane bacteria are an unexplored alternative, particularly when compared with rhizobacteria (Lindow and Leveau, 2002). The phylloplane bacteria show potential because they use two strategies to survive in stressful environments: tolerance and escape. The former requires the ability to tolerate inhospitable conditions, such as ultraviolet radiation and low humidity, whereas the latter examines the ability of the bacteria to explore sites that offer less environmental stress (Andrews and Hirano, 1991; Beattie and Lindow, 1995, 1999; Wilson et al., 1999).

Given the above, the objectives of this study were (i) to evaluate the potential of bacterial isolated from the phylloplane of the apple tree to control GLS in an artificially inoculated orchard, that bacterial isolated had been proven efficacious using *in vitro* tests in a previous study (Moreira et al., 2014) where the bacterial isolated inhibited germination of the *C. acutatum* group by more than 60% and produced fixed and volatile compounds that inhibit the mycelial growth of *C. acutatum* group isolates from apple; (ii) to test the bacterial isolates and the commercial biofungicide Serenade® applied in addition to the fungicides used by the producers to control GLS; (iii) to observe biofilm formation on apple leaves treated with the antagonists and the effects of the antagonists on the germination of the pathogen by surface analyses with scanning electron microscopy.

2. Materials and methods

2.1. Biocontrol of Glomerella leaf spot in an experimental orchard following pathogen inoculation

The study was conducted in Curitiba/PR over three seasons (2008/09, 2009/10 and 2010/11). The experimental apple orchard of 'Gala', with rootstock Maruba and interstock M9 was established in 2007 and is situated at latitude 25°24′42.57″S and longitude 4 9°14′53.22″W. Using the Köppen classification, the climate is subtropical with cool summers and no set dry season. Meteorological data on precipitation and temperature were obtained from the Instituto Tecnológico Simepar (Station 25264916).

The experimental design was a randomized block with five treatments and five blocks, totaling 25 plots, each of which comprised four trees at a spacing of 0.50 m between trees and 1.50 m between rows. Two trees from each plot were considered sample tree. During seasons 2008/09 and 2009/10, all leaves of each working trees were evaluated, and during season 2010/11, a segment labeled branch containing 10 leaves was evaluated from each working plant.

The five treatments evaluated included three bacterial isolates (*Bacillus* sp., *Pseudomonas putida* and *Bacillus alcalophilus*), a fungicide control (Mancozeb, 2 g L^{-1}) and an untreated control. The bacterial isolates used in this study were obtained from Paraná by Rollemberg (2008).

The spraying of all treatments took place preventively every week from early December to May and totaled 20 sprays (mean) using a 5 L atomizer (Guarany, São Paulo, Brazil). The spray volume was calibrated to spray to run-off and was approximately 1 L per plot.

To prepare bacterial suspensions, isolates were streaked onto Nutrient Agar medium with sodium chloride (NaCl) 28 g L^{-1} (AES Laboratoire, Combourg, France) and maintained at 25 °C at a photoperiod of 12 h. After 24 h, 40 μL of the bacteria were transferred, using a platinum loop, to Erlenmeyer flasks containing Nutrient Broth liquid medium (13 g L^{-1}; Himedia, Curitiba, Brazil), and the flasks were maintained under laboratory conditions for 48 h under constant agitation (120 rpm) in a orbital shaker (Nova Ética, São Paulo, Brazil). Following this incubation, the optical distance (O.D.) for each bacterial isolate suspension was standardized using a spectrophotometer at O.D. (540 nm) = 0.2 (2×10^5 CFU/mL), and 8.5 g NaCl and 10 μL of Tween 80 was added to each liter of bacterial suspension.

Inoculation was performed following four applications of the bacterial and fungicide treatments. The inoculum was applied to the whole tree using a 700 mL manual atomizer (Guarany, São Paulo, Brazil). To prepare the inoculum, the isolate was streaked onto oatmeal-agar medium (40 g L^{-1} of oatmeal, 16 g L^{-1} agar, and 1000 mL distilled water), and plates were maintained at 25 ± 2 °C for 6 days. All trees were inoculated by spraying with an adjusted suspension of 1×10^4 conidia/mL using a hemacytometer.

Incidence was determined by the number of leaves with symptoms compared with the total number of leaves measured, and the severity was evaluated using the diagrammatic scale of Kowata et al. (2010). The evaluations were conducted from February to May during seasons 2008/09 and 2010/11 and from January to May during the season 2009/10. In 2008/09, the evaluations began when the incidence was almost 10%, and in 2009/10, evaluations began when incidence was almost 7%. During the 2010/11 season, the evaluations were initiated prior to the beginning of the epidemic.

2.2. Biocontrol of Glomerella leaf spot under commercial orchard conditions with natural inoculum

2.2.1. Experimental area and treatments

Research plots were located in two areas of a commercial apple orchard of 'Gala' with rootstock Maruba and interstock M9 with a spacing of 4 m between rows and 0.75 m between plants following 12 years of planting in the Campo Largo/PR. Area 1 was located at latitude −25°42′25.63″ and longitude −49°54′21.50″, and area 2 was at latitude −25°42′13.98″ and longitude −49°54′61.43″. Using Köppen classification, the climate is subtropical with cool summers and no set dry season (Cfb). Meteorological data on rainfall, relative humidity and temperature were obtained from the Instituto Tecnológico Simepar (station 25264916).

The number of favorable days (FD) to the occurrence of GLS was calculated as proposed by Katsurayama et al. (2000) who consider FD as days where the leaf wetness duration (LWD) is over 10 h (LWD > 10 h) and the temperature is above 15 °C ($T > 15$ °C). The

[1] Normative instruction SDC n.1 September 14, 2006, approving the specific technical standards for integrated production of apple, apple-NTEPI. Published in the Official Gazette on September 21, 2006.