Biological Control 80 (2015) 40–49

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Understanding the mechanism of biological control of passionfruit bacterial blight promoted by autochthonous phylloplane bacteria

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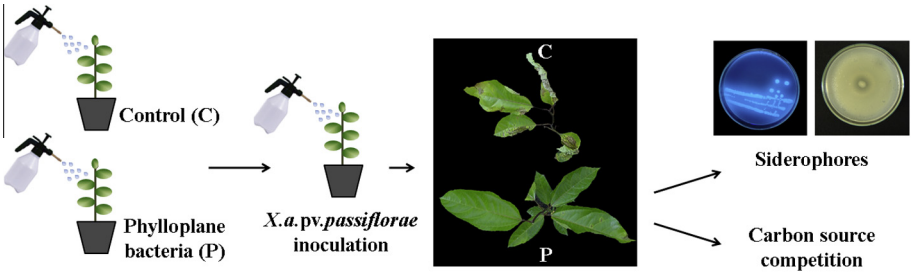
HIGHLIGHTS

- Phylloplane bacteria were effective in reducing the bacterial blight severity.
- Iron and nitrogenate compounds competition explains bacterial blight control.
- Disease control ability is not related to antibiosis nor to resistance induction.
- Siderophore-producing organisms may lead to a false-positive result for antibiosis.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 22 April 2014
Accepted 22 September 2014

Keywords:
Phyllosphere
Biocontrol
Competition
Iron
Nitrogen
Siderophores

ABSTRACT

The knowledge of biological control mechanism that provides a significant reduction in disease severity can guide the screening procedures and allow the discovery of new possibilities for disease control. In this study, a reduction of bacterial blight severity, caused by three *Xanthomonas axonopodis* pv. *passiflorae* (Xap) strains, was demonstrated by using nine indigenous passionfruit phylloplane bacteria regardless of the pathogen origin. Experiments were done to elucidate preemptive exclusion through carbon sources utilization profiles, siderophores production, iron competition and antibiosis. Furthermore, peroxidase activity and spatial separation assays were conducted to evaluate the ability to induce systemic resistance. Identification by 16S rRNA gene sequencing revealed that nine phylloplane strains show highest similarity to *Arthrobacter*, *Curtobacterium*, *Enterobacter*, *Microbacterium*, *Pseudomonas* or *Stenotrophomonas*. It is concluded that competition for iron and nitrogenate compounds on leaves explains the ability of bacterial phylloplane antagonists to control the disease. For siderophore-producing organisms that present antibiosis evidence, complementary iron supplementation assay is mandatory, to avoid misinterpretation. Results suggest that lack of these compounds could limit the optimal conditions for phylloplane colonization by Xap along the infection process.

1. Introduction

Passionfruit is a native tropical plant from Brazil, widely cultivated for medicinal, cosmetical and juice purposes, being the yellow passionfruit (*Passiflora edulis* f. *flavicarpa*) the most common species cultivated. Among the diseases that impact the crop productivity, bacterial blight is one of the most important for decreasing the period of commercial exploitation on vines. Besides being widespread at locations where this crop is cultivated, this disease has limited ways of control (Boro et al., 2011).

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http://dx.doi.org/10.1016/j.biocontrol.2014.09.011
1049-9644/

Bacterial blight is caused by *Xanthomonas axonopodis* pv. *passiflorae* (Xap) that infects leaves and fruits (Gonçalves and Rosato, 2000; Malavolta et al., 2001; Halfeld-Vieira and Nechet, 2006). Like other typical xanthomonads, this pathogen is a phylloplane inhabitant bacteria which survives associated with plants as residents, providing an inoculum source for disease development (Stall et al., 1993).

In spite of the lack of information on epidemiological role of this epiphytic population, it is assumed that pathogenic bacteria have a resident phase on healthy plants prior to infection, which is related to cell density (Hirano and Upper, 1983; Sharon et al., 1982; Stromberg et al., 1999). This relationship was demonstrated for common blight of bean where a base number of 5×10^6 CFU epiphytic cells of *Xanthomonas a.* pv. *phaseoli* per 20 cm^2 of leaf tissue was considered as necessary to trigger the disease development (Weller and Saettler, 1980). Likewise, for *Xanthomonas a.* pv. *citri*, the epiphytic survival and biofilm formation represent important events prior to development of canker disease (Rigano et al., 2007).

This information suggests that the maintenance of resident populations of the pathogen on plant surface at low levels, through direct antagonism mechanisms, can result in an efficient disease control strategy.

In the context of biological control, autochthonous phylloplane microorganisms could play this role using carbon and nitrogen compounds, the main limiting resources for bacterial growth on leaves (Wilson and Lindow, 1994ab; Mercier and Lindow, 2000; Parangan-Smith and Lindow, 2013), to establish and maintain their own population. This preemptive competitive exclusion capability is provided by nutritional similarity for carbon and organic nitrogen sources between the antagonist and the pathogen (Wilson and Lindow, 1994a,b; Dianese et al., 2003).

Preemptive exclusion of pathogen populations can also be provided through production of antimicrobial metabolites by antagonists. In this sense, antibiosis configures the biological control mechanism, as reported for broad range plant pathogens (Cavaglieri et al., 2004; Zhou et al., 2012; Suárez-Estrella et al., 2013 and Lin et al., 2014).

Besides the mechanisms that prevent the pathogen population establishment on leaves, plant indigenous bacteria, either isolated from rhizosphere or from phylloplane, can prime plants as elicitors prior to infection by a pathogen, triggering induced systemic resistance response (Halfeld-Vieira et al., 2006; Romeiro et al., 2010). In such case, even though the pathogen establishes the minimum necessary population to cause infection, the enhanced level of resistance against the pathogen results in reduced disease development (Conrath et al., 2002).

Therefore, the objective of this research was to test the hypothesis that preemptive exclusion mechanisms and/or induced resistance are important traits for antagonistic phylloplane bacteria on the biological control of passionfruit bacterial blight. In order to test this hypothesis, the common antagonistic features among nine isolates selected *in vivo* that demonstrated efficacy on the control of the disease were investigated.

2. Materials and methods

2.1. Bacterial strains

Healthy passionfruit leaves were randomly collected from non-commercial cultivation plants in 7 municipalities from São Paulo, Pará and Roraima states in Brazil, totalling about 56 samples. Leaves were cut in halves and each half was deposited in a 250-mL Erlenmeyer flask with 100 mL of sterile saline solution (0.85% NaCl) and 200 μL of Tween 80. It was then shaken for 20 min at 200 rpm. Serial dilutions were performed at the rate of 1:1000. Later on, 100 μL aliquot of each suspension were plated on 523 culture medium (Kado and Heskett, 1970) and spreaded with a Drigalski spatula. After incubation at 27 °C for 4 days, morphological different individual colonies were transferred to a test tube with the same medium (adapted from Halfeld-Vieira et al., 2004).

From 224 passionfruit phylloplane bacteria, nine were selected according to the reduction on disease severity compared to the other treatments, in trials conducted as thoroughly described below in item 2.2.1. On these trials, conducted at least three times, each group of isolates was tested against one single Xap strain obtained from its corresponding state, with 3 replications per treatment. Thus, the phylloplane bacteria from plants collected in São Paulo were tested against the isolate XapSP, from Pará against XapPA and from Roraima against XapRR. Only antagonists from São Paulo (11SP, 18SP, 22SP, 28SP and 48SP) and Roraima (29RR, 46RR, 98RR and 133RR) were able to reduce the disease severity and therefore were used to test the hypothesis.

2.2. Greenhouse experiments

2.2.1. Ability to control the bacterial blight caused by corresponding Xap strain

Two independent experiments were conducted. Antagonists 11SP, 18SP, 22SP, 28SP, 48SP were tested against the isolate XapSP, and 29RR, 46RR, 98RR, 133RR were tested against XapRR.

Yellow passionfruit plants obtained from seeds taken from one single fruit were cultivated in 1000-mL pots filled with potting media and maintained in greenhouse.

For each antagonist, a 72-hour-old bacterial culture grown on 523 culture medium (Kado and Heskett, 1970) was harvested with distilled water, its concentration adjusted to an optical density of 0.3 absorbance at a wavelength of 540 nm ($\sim 5.0 \times 10^8$ CFU per mL) in a spectrophotometer. Groups of 20 plants (one plant per pot) with five expanded leaves were sprayed with only one selected antagonist isolate, and randomized in the greenhouse. Plants sprayed with distilled water were used as a control. To represent the chemical control, streptomycin sulfate (0.1 g L^{-1}) was used for the assay with XapSP and copper oxychloride (3.0 g L^{-1}) for experiment with XapRR.

After 4 days, all plants were inoculated with a Xap suspension adjusted to 0.15 absorbance at a wavelength of 540 nm ($\sim 2.0 \times 10^8$ CFU per mL) and maintained in a humidity chamber for 24 h. Then, all plants returned to greenhouse conditions.

The experiment entailed a completely randomized design with 20 replications per treatment. Fourteen days after the symptoms of the disease are evident, severity was visually evaluated according to the estimate of leaf symptomatic area for entire plant and corrected with image analysis software Assess 2.0 (Lamari, 2008) when necessary.

Severity values were subject to analysis of variance (ANOVA) using the PROC GLM procedure of SAS 9.2 (SAS Institute Inc., Cary, NC). Fisher's protected LSD was used for separation of means ($P \leqslant 0.05$) and significative differences, specifically with the control, were confirmed by Dunnett test ($P \leqslant 0.05$).

2.2.2. Ability to control the bacterial blight caused by distinct Xap strains

To test the efficacy of selected phylloplane bacteria for disease control caused by different Xap strains, two experiments were conducted. Plant cultivation, bacterial growth, suspension concentration adjustment, colonization with antagonists, inoculation procedures and disease assessment were performed as described before. In one experiment, the antagonists 11SP, 18SP, 22SP, 28SP, 48SP were tested against XapPA, XapSP and XapRR. In the other, antagonists 29RR, 46RR, 98RR, 133RR were also tested against the three above-mentioned Xap strains. To