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Effectiveness of tailocins produced by *Pseudomonas fluorescens* SF4c in controlling the bacterial-spot disease in tomatoes caused by *Xanthomonas vesicatoria*

Analía Príncipe, Maricruz Fernandez, Milenka Torasso, Agustina Godino, Sonia Fischer*

Departamento de Ciencias Naturales, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto-CONICET, Agencia Postal No. 3, X580BYA Río Cuarto, Córdoba, Argentina

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ABSTRACT

The development of alternatives for the use of chemical pesticides for plant disease control is the present-day and ongoing challenge for achieving sustainable agriculture. *Pseudomonas fluorescens* SF4c, native strain from wheat, produces tailocins (phage-tail-like bacteriocins) with antimicrobial activity against several phytopathogenic strains. We thus investigated the efficacy of foliar application of these bacteriocins to control the bacterial-spot disease in tomato caused by *Xanthomonas vesicatoria* Xcv Bv5-4a. The disease severity and incidence index were reduced by 44 and 36%, respectively; while the number of viable cells of *X. vesicatoria* Xcv Bv5-4a decreased after bacteriocin treatment. Furthermore, bacteriocin was effective in reducing bacterial-spot-disease symptoms on tomato fruits even when applied 12 h after infection. Tailocin activity was not affected by abiotic influences such as adjuvant, light and temperature and, biotic factors such as apoplastic-fluids. In contrast, no antibacterial activity of these tailocins was observed when the bacteriocin was exposed to extremely dry conditions. Finally, that no cytotoxic effects on mammalian cells were observed with this representative tailocins is highly significant and demonstrates the safety of such compounds in humans. All these findings indicate that the SF4c tailocins represent an attractive alternative to copper-containing bactericides for use in the control of bacterial spot.

1. Introduction

The tomato (*Lycopersicon esculentum*) is one of the most highly produced vegetables worldwide in terms of its direct consumption in the raw state, its inclusion in food recipes, and its marketing in industrial preparations. The global production of tomato has accordingly been increasing in recent years. Ten countries are responsible for 90% of the world output; with the greatest producer being China; followed by India, the United States, Turkey, and Egypt. The rest of the tomatoes produced are harvested in the southern hemisphere—*i. e.*, in Brazil, Chile, and Argentina (FAOSTAT; http://faostat.fao.org/).

Foliar pathogens are a major problem causing severe losses in tomato yields. The bacterial-spot disease, caused by different *Xanthomonas* species—such as *X. vesicatoria*, *X. euvesicatoria*, *X. gardneri*, and *X. perforans* (Constantin et al., 2016)—is a disease distributed worldwide that reduces the yield and quality of this crop. The bacterial spot is favored by warm temperatures and high relative humidity (Potnis et al., 2015). *Xanthomonas* spp. enters into the host through hydathodes, stomata, or wounds. Once inside, the bacteria migrate into the host tissues and multiply either locally in the intercellular space or colonize the xylem to spread systemically throughout the plant (Büttner and Bonas, 2010).

To date, copper-based products are applied to control *Xanthomonas* spp. The effectiveness of these chemicals on the control of bacterial spot, however, is variable. Itako et al. (2015) demonstrated that the severity of disease varied in tomato plants treated with copper-based bactericides, from 27% to 66% depending on the tomato cultivars. Moreover, the continuous use of those antibacterial agents has favored the spread of copper-resistance genes among soil bacteria, including the pathogenic strains (Voloudakis et al., 2005). The emergence of copper resistance in strains of *Xanthomonas* was found to be associated with mobile elements such as plasmids that thus represent a significant risk of rapid and widespread propagation within the bacterial populations (Richard et al., 2017). Another disadvantage of copper-based bactericides is that they have negative effects on both human and animal health. In recent years the demand for chemical-free products by consumers has increased and this has resulted in the restricted use of chemicals (Buttimer et al., 2017). In addition, the absence of chemical residues is also necessary requirement to export food.

For these reasons, alternative technologies to copper bactericides

* Corresponding author.
E-mail address: sfischer@exa.unrc.edu.ar (S. Fischer).

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are being investigated. For example, photocatalytic nanoscale titanium dioxide (TiO_2) was evaluated to control *X. perforans*. In those experiments, the use of TiO_2/Zn reduced the incidence of bacterial-spot disease on tomatoes (Paret et al., 2013); but the compound was unfortunately phytotoxic, thus severely limiting its commercialization (Potnis et al., 2015). At present, an increased interest had developed in natural biologic agents—*e. g.*, secondary metabolites, bacteriophages, antagonistic bacteria—to replace conventional agricultural practices for achieving more sustainable, and safer, tomato production (Byrne et al., 2005; Bae et al., 2012; Cawoy et al., 2015; Hert et al., 2009; Moss et al., 2007; Munhoz et al., 2017; Yim et al., 2014).

Bacteriocins are a biologic alternative to chemicals for use in pest control. These proteinaceous compounds are capable of killing bacteria phylogenetically close to the producer strain (Ahmad et al., 2017; Ghequire et al., 2015). Several studies have demonstrated that bacteriocins are effective against bacteria that cause disease in plants. The application of these antibacterials for the biocontrol of phytopathogens, however, has thus far been limited, mainly owing to the paucity of research on those compounds' mechanism of action, rather than their intrinsic potential (Grinter et al., 2012).

Tailocins are phage-tail-like bacteriocins containing no head structures and therefore no DNA. Consequently, these multiprotein complexes do not replicate inside target cells (Ghequire and De Mot, 2014). Recently, we reported that tailocins from *P. fluorescens* SF4c have *in-vitro* antimicrobial activity against *X. vesicatoria* Xcv Bv5-4a—synonym of *X. axonopodis* pv *vesicatoria* Xcv Bv5-4a (Fernandez et al., 2017). In the present work, we evaluated the potential use of the SF4c tailocins to reduce the bacterial-spot severity under greenhouse conditions. Previous to field application, a knowledge of the stability of the agent to different abiotic and biotic influences, and its toxicity is necessary. In view of these considerations, we analyzed the influence of temperature, adjuvants, light, desiccation, and apoplast fluids on bacteriocin activity. Moreover, the cytotoxicity of SF4c tailocins was studied by assays on cultured green-monkey-kidney Vero cells and a determination of hemolytic activity in sheep red blood cells.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Pseudomonas fluorescens SF4c and *X. vesicatoria* Xcv Bv5-4a were grown in Luria-Bertani (LB) medium at 28–30 °C. Strain Xcv Bv5-4a was provided by the National Institute of Agricultural Technology (INTA, Bella Vista, Argentina).

For plant experiments, overnight cultures of strain Xcv Bv5-4a were diluted in LB broth and grown to an optical density at 600 nm (OD_{600}) = 0.8. The bacterial cells were then pelleted by centrifugation (3500 × *g*, for 10 min), and diluted to appropriate cell densities in sterile physiologic saline (0.9% [w/v] NaCl).

2.2. Production and purification of tailocins from P. fluorescens SF4c

Overnight cultures of *P. fluorescens* SF4c were diluted 1:100 in liquid LB medium and incubated at 30 °C with shaking at 150 r.p.m. to an OD_{600} = 0.3. Mitomycin C (final concentration 3 μg mL^{-1}) was then added and the incubation continued until the bacteria lysed. The debris were removed by centrifugation at 17,000 × *g* for 1 h at 4 °C and the supernatants thereafter filtered through a membrane of pore size of 0.45 μm. Next, the supernatants were precipitated with 60% (w/v) ammonium sulfate and incubated overnight at 4 °C. The pellets were harvested by centrifugation at 17,000 × *g* for 1 h at 4 °C and resuspended in 5 ml of TN50 buffer [50 mM NaCl, 10 mM Tris-HCl (pH = 7)]. Finally, the tailocins were sedimented at 58,000 x *g* for 1 h at 4 °C, resuspended in 1.5 ml of TN50 buffer, and sterilized by filtration through a membrane of 0.2-μm pore size of (Scholl and Martin, 2008; Fischer et al., 2012; Hockett and Baltrus, 2017). The antibacterial activity of the purified SF4c tailocins was assayed by the spot method against the sensitive strain Xcv Bv5-4a. The tailocins concentration was expressed as arbitrary units per milliliters (AU mL^{-1}), corresponding to the reciprocal of the highest dilution producing a clear inhibition of that indicator strain in the assay plates (Williams et al., 2008).

2.3. Plant growth

Tomato (*Lycopersicon esculentum* cv. HM7883) seeds were surface-sterilized and germinated in trays containing vermiculite:peat (1:1) under greenhouse conditions (16 h light at 28 °C and 70% humidity and 8 h darkness, 18 °C and 80% humidity). After 2 weeks, the seedlings were transferred to plastic pots containing vermiculite: peat (1:1) and grown under the same conditions. Plants were irrigated twice a week, either with tap water alone or, every 10 days, with Hoagland nutrient solution (Hoagland and Arnon, 1938).

2.4. Biocontrol assay under greenhouse conditions

An inoculum of the phytopathogen was prepared as described above. Ten young (four-true-leaf stage) tomato plants were inoculated by spraying a suspension of the strain Xcv Bv5-4a (10^8 colony-forming units [CFU] mL^{-1}) containing the surfactant Silwet L*Ag (Rizobacter Argentina SA) at 0.025% (v/v). Suspensions containing SF4c tailocins (1000 AU mL^{-1}) were sprayed onto leaflets 1 h before the inoculation with the pathogen, and two additional doses were applied at 48 and 96 h postinfection. The plants were maintained under the greenhouse conditions stated above throughout the experiment. Some plants were left uninoculated, while others were infected with strain Xcv Bv5-4a but treated with TN50 buffer instead of bacteriocin to serve as controls. The severity of bacterial-spot disease was assessed by visual estimation of the percentage of leaf tissue with lesions at 15 days after inoculation according to the disease-index scale described by Yim et al. (2014) with certain modifications: healthy leaflets (0) or sick leaflets (1–5). The severity index (SI) was calculated from the disease rating by the formula described by Bora et al. (2004):

$$SI(\%) = \frac{\sum (\text{rating number} \times \text{no. of leaflets in the rating})}{\text{Total number no. of leaflets} \times \text{highest rating}} \times 100$$

The reduction in disease severity, compared to the water control, was determined according to the formula described by Moss et al. (2007): Disease reduction (%) = ([Disease $severity_{control}$- Disease $severity_{treatment}$]/Disease $severity_{control}$) × 100.

The incidence was determined as the percent of diseased leaflets among the total number evaluated. For each treatment, 10 plants were used. The experiment consisted in three biologic replicates.

2.5. Antagonism assay

Before the antagonism assays, the strain Xcv Bv5-4a was marked with rifampicin, and called Xcv Bv5-4a-Rif50, to monitor the survival of the phytopathogen on the tomato leaf. To compare the growth of the strain Xcv Bv5-4a-Rif50 with that of the parental strain, both were grown in liquid LB medium as described by Fischer et al. (2010). Moreover, the virulence of strain Xcv Bv5-4a-Rif50 was checked in tomato plants and compared to wild type strain.

Tomato plants were infected with strain Xcv Bv5-4a-Rif50 and treated or untreated with bacteriocin as described above in the biocontrol assays. The population of the phytopathogen on tomato leaves was quantified at different times (0, 3, and 10 days postinoculation). At these times, 1 cm^2 leaf disks were macerated in sterile physiologic saline. The samples were then serially diluted and plated on LB medium supplemented with Rif 50 mg mL^{-1}. After incubation at 28 °C for 4–5 days, the colonies of Xcv Bv5-4a-Rif50 were counted. Population data were log_{10}-transformed. The experiment was carried out three times