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

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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

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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

with an n = 9.

### 2.6. Inoculation of tomato fruits

The immature tomato fruits were surface-sterilized with 70% (v/v) aqueous ethanol. An aliquot (20 µl) of inoculum of strain Xcv Bv5-4a ($10^5$ CFU $mL^{-1}$) was injected onto the fruits. For each fruit, a total of 9 infection sites were inoculated. After 30 min of infection, the bacteriocin (500 AU $mL^{-1}$) was inoculated at each site. For the controls, *cf*. the section on the biocontrol assay above. The fruits were incubated in a humid chamber at room temperature. The infection was monitored daily for 15 days. For comparison, fruits were treated with bacteriocin (500 AU $mL^{-1}$) at 12, 24, and 48 h after the infection with strain Xcv Bv5-4a ($10^5$ CFU $mL^{-1}$) and likewise monitored daily. For each treatment, two tomato fruits were used. Each experiment was performed in triplicate.

### 2.7. Assays by scanning electron microscopy (SEM)

Samples of tomato-leaflet segments (0.5 × 1 cm) of the biocontrol assays (*cf*. the section above; in this instance, the surfactant Silwet L*Ag was not used) were collected for ultrastructural evaluation throughout the SEM analysis. These samples were fixed with ethanol 96 °C/distilled water/formaldehyde/acetic acid (5/3.5/1/0.5), pH 5.5, for 24 h at room temperature before dehydration in an ethanol series (70, 80, 90, 96, and 100%) for 12 h at each concentration. The samples were then desiccated in a critical-point drier CPD 030 (Leica EM, Leica Microsystem GmbH, Vienna, Austria), mounted on holders, and metallized with gold. The samples were observed with a scanning electron microscope (LEO Electron Microscope Model 1450VP, Carl Zeiss SMT AG Com., Germany). Electron micrographs were taken at various magnifications *in situ* by the SOFTWARE (LEO-32) with images of the phylloplane region being generated at random for each sample at several magnifications and then digitally recorded.

### 2.8. Effects of tomato apoplast fluids, temperature, adjuvant, desiccation, and light on bacteriocin activity

Apoplast fluids were extracted by vacuum infiltration according to O'Leary et al. (2014) with some modifications. The procedure stated in brief: Six-week-old tomato leaves were surface-disinfected, washed with double-distilled water, dried with absorbent paper, and infiltrated with sterile double-distilled water before transfer to a 10-mL syringe that was then placed in a 50 mL Falcon centrifuge tube. The apoplast fluids were collected by centrifugation at 2200 × *g* for 20 min at 4 °C and finally stored at −20 °C. The protein content was determined according to Bradford, (1976).

The effect of apoplast fluids on bacteriocin activity was evaluated according to the protocol of Zeitler et al. (2013) with some modifications. Strain Xcv Bv5-4a ($10^5$ CFU $mL^{-1}$) was treated with 0 or 500 AU $mL^{-1}$ of bacteriocin in presence or absence of different concentrations (10 mg $mL^{-1}$, 20 mg $mL^{-1}$, or 40 mg $mL^{-1}$) of apoplast fluids. After an incubation of the cultures for 18 h, the cell density was measured as the $OD_{600}$. The experiment consisted in three technical replicates and three biologic replicates.

Serial dilutions of bacteriocin were exposed to different external conditions with respect to the temperature, the presence or absence of adjuvants, exposure or nonexposure to fluorescent light, or desiccation. To test the effect of temperature on bacteriocin activity, the samples were incubated at 30 °C or at 37 °C. To determine the influence of fluorescent light, the samples were maintained under the same greenhouse conditions as described for the plant experiments (200 µmol photons $m^{-2} s^{-1}$). In parallel, to assess the effect of adjuvants, the bacteriocins were serially diluted in TN50 buffer plus Silwet L*Ag at 0.025% (v/v) and then incubated at 30 °C. In all three trials the samples were tested at 0, 3, 7, and 15 days for antimicrobial activity against strain Xcv Bv5-4a.

For experiments on the effect of desiccation, a microtiter plate with the serial dilutions of bacteriocin was placed inside a desiccation jar containing silica gel. The jar was then stored under greenhouse conditions for 72 h.

At 12, 24, and 72 h after the initiation of desiccation 50 µl of distilled water was added to each well (three wells per treatment) and the bacteriocin activity analyzed.

### 2.9. Cytotoxicity assays on Vero cells and on hemolytic activity in sheep erythrocytes

Vero C-16 cells were grown in 96-well microplates containing 0.2 ml of Eagle's Minimal Essential Medium (MEM) (Gibco) and incubated at 37 °C for 48 h, in a 5% (v/v) $CO_2$-in-air atmosphere at a density of 3 × $10^4$ cells/well. At confluence the cells were washed with phosphate-buffered-saline (8 g $L^{-1}$ NaCl, 0.2 g $L^{-1}$ KCl, 0.2 g $L^{-1}$ $KH_2PO_4$, 1.44 g $L^{-1}$ $NaH_2PO_4 \cdot 2H_2O$; pH 7.2 [PBS]) to eliminate detached cells.

Serial dilutions of bacteriocin were performed (from 100 to 10,000 AU $mL^{-1}$) in the MEM added to each well. The plates were incubated for 72 h under the same conditions described above. After removal of the medium, the cells were washed with PBS (pH 7.2), 200 µl/well of neutral-red solution (30 µg $mL^{-1}$ in MEM) were added, and the plates were incubated at 37 °C for 2 h for promoting the incorporation of the dye into the cells. The cells were then washed 3 times with PBS and the incorporated dye extracted with acetic acid/ethanol/water (1/50/49). The plates were maintained with agitation for 20 min before $OD_{540}$ measurement in an ELISA reader (Labsystems Multiskan MS). The cells were monitored daily through an inverted optical microscope (Carl Zeiss, Jena). Wells containing cells with MEM medium alone were used as growth and sterility controls. In addition, wells containing cells in MEM medium plus TN50 buffer were used as a negative control, and wells with cells in MEM medium plus $H_2O_2$ (0.1% [v/v]) or $CuSO_4$ (4 g $L^{-1}$) were used as positive controls. The cytotoxic concentration of bacteriocin, expressed as the concentration (AU $mL^{-1}$) necessary to reduce cell viability by 50% was calculated by regression analysis. Six biologic replicates were done for each treatment.

The cytotoxicity of the bacteriocin was also assessed by the hemoglobin-release assay as described by Zeitler et al. (2013) with certain modifications. The procedure stated in brief: Sheep erythrocytes were centrifuged at 1500 × *g* for 2 min and washed 3 times with physiologic saline. To one mL of cells (1 × $10^9$ cells $mL^{-1}$) was added a given concentration of bacteriocin (0, 100, 250, 500, 1000, 1500, or 2000 AU $mL^{-1}$) followed by a 45-min incubation at 37 °C. The cells were then centrifuged at 1500 × *g* for 5 min at room temperature and the supernatants used to determine the hemoglobin released as assessed by measurements at $OD_{405}$ nm. As controls, TN50 buffer or physiologic saline were used. In addition, SDS (0.01% [w/v]) was also used to produce 100% hemolysis. Those concentrations of bacteriocin that effected a hemolysis greater than 25% were considered toxic. Three biologic technical replicates were performed for treatment, and the experiment was conducted three times.

### 2.10. Statistical analysis

The results were expressed as the mean values ± the standard errors (SE). The data were analyzed by the Student *t*-test or the one-way analysis of variance (ANOVA). The means were compared by Tukey's *post-hoc* test (ANOVA) ($p \leq 0.05$).

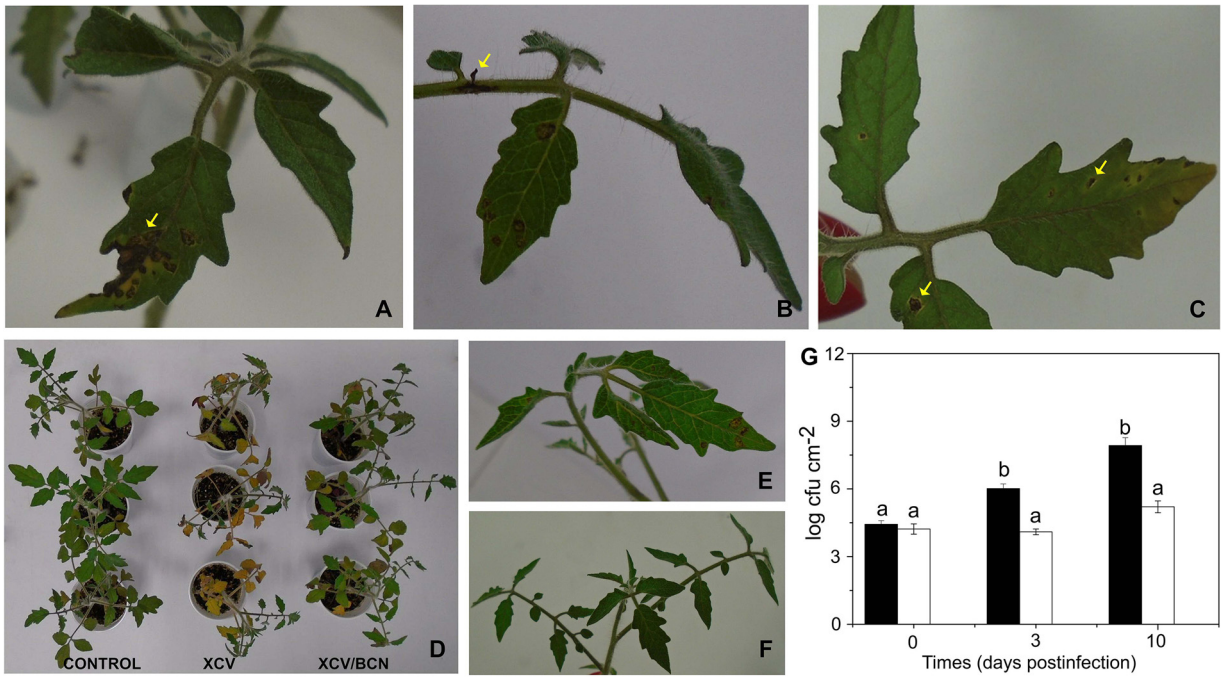


**Fig. 1.** Efficacy of bacteriocin application in reducing the severity of bacterial spot caused by *X. vesicatoria* (Xcv Bv5-4a) on tomato plants. Symptoms of disease in the leaves (panels A–C), stem (Panel B), or apical leaves (panels E–F) from plants untreated (panels A, B, and E) or treated with bacteriocin (panels C and F). In panels A, B, and C, the arrows mark characteristic bacterial spots. Panel D: Plants uninfected (CONTROL), infected with pathogen and untreated with bacteriocin (XCV), or infected and treated with bacteriocin (XCV/BCN) at 15 days postinfection. Panel G: The population size of strain Xcv Bv5-4a expressed as the logarithm of number of CFU recovered from these tomato leaves per $cm^2$ is plotted on the *ordinate* at 0, 3, and 10 days after inoculation, as indicated on the *abscissa*. The plants were infected with the pathogen and treated with bacteriocin (white bars) or left untreated (black bars). The experiment was repeated three times (n = 9). Different letters (above each bar) indicate significant differences among the treatments according to Tukey's *post-hoc* tests ($P \leq 0.05$). (For interpretation of this figure, the reader is referred to the web version of this article).

## 3. Results

### 3.1. Reduction of the incidence and severity of bacterial-spot disease by phage tail-like bacteriocins

We previously demonstrated that tailocins produced by *P. fluorescens* SF4c inhibited the growth *in vitro* of the phytopatogenic strain *X. vesicatoria* Xcv Bv5-4a (Fernandez et al., 2017). In the present work, biocontrol assays were performed on tomato plants under greenhouse conditions. To simulate a natural infection, a suspension of the phytopathogenic bacteria was sprayed on the leaf surfaces of the plants; but before that inoculation with the pathogen, SF4c tailocins were also applied to the plant surface, likewise by spraying. In addition, two doses of the tailocins were administered at 48 and 96 h after infection, and the development of disease was monitored daily. The characteristic symptoms of bacterial spot—such as water-soaked lesions on the leaves that subsequently became necrotic and dark brown with yellow halos—were more evident in the plants infected with the phytopathogen and untreated with tailocins than in the treated plants (Fig. 1, Panel A). Moreover, lesions on the stem were also detected in the infected but untreated plants (Fig. 1, Panel B). After 15 days postinfection, the chlorosis area in those plants enlarged and was followed by the complete necrosis of entire leaflets (Fig. 1, Panel D, Group XCV). In contrast, treatment with bacteriocin significantly reduced the chlorosis and necrosis symptoms on the leaflets, with a healthier appearance remaining on those than on the leaflets of the infected plants (Fig. 1, panels C and D). Along with those improvements, the respective severity and incidence indices decreased by approximately 44% and 36% (determined as described in Materials and Methods) compared to the corresponding values with the infected but untreated plants (Table 1). The treatment with bacteriocin was also effective in reducing the development of the disease on the apical leaves, with the incidence in those leaves being decreased from about 80% in the infected and untreated leaflets to some 12% of leaflets in the infected and treated plants (Table 1; Fig. 1, panels E and F). Therefore, the foliar application of SF4c tailocins resulted in a significant reduction of the symptoms of bacterial spot on

**Table 1**
Biocontrol of bacterial–spot disease in tomato plants under greenhouse conditions.

| Treatments[a] | Severity (%)[c] | Incidence (%)[d] | Incidence (%) on apical leaf[e] |
|---|---|---|---|
| Control[b] | 0 | 0 | 0 |
| XCV-water | 77.63 ± 2.23 A | 93.41 ± 0.57 A | 78.74 ± 13.53 A |
| XCV-TN50 Buffer | 73.9 ± 6.12 A | 96.07 ± 1.25 A | 80.5 ± 9.38 A |
| XCV-BCN | 43.78 ± 2.13 B | 57.28 ± 0.17 B | 12.1 ± 2.4 B |

[a] Four–week–old–tomato–seedling leaves were sprayed with water, TN50 buffer, or bacteriocin (BCN, 1000 AU $mL^{-1}$) 1 h before inoculation with *X. vesicatoria* Xcv Bv5-4a ($10^8$ CFU $mL^{-1}$), and at 48 and 96 h postinfection.

[b] Control corresponds to uninfected tomato plants.

[c] Disease severity is assessed on a 1–5 rating scale in which 0 = asymptomatic and 5 = dead leaflets. The disease index was calculated according to the following formula described by Bora et al. (2004) with some modifications: $DI(\%) = \frac{\sum(\text{rating number} \times \text{no of leaflets in the rating})}{\text{Total number no of leaflets} \times \text{highest rating}} \times 100$. Disease-severity and incidence data are an average of three independent experiments (n = 10) ± the standard errors.

[d] The incidence of bacterial spot was determined as the percentage of leaflets with symptoms per plant.

[e] The incidence of bacterial spot was determined at 22 days postinfection. Disease-severity and incidence data are an average of three independent experiments (n = 10) ± the standard errors. Different letters indicate significant differences among the treatments according to Tukey's *post-hoc* tests ($P \leq 0.05$).

those tomato plants. As expected, the treatment with water or TN50 buffer had no effect on the symptoms of bacterial spot. The treatment with TN50 buffer was included as a control since occasionally small reductions in disease severity had previously been attributable to phosphate buffer (Moss et al., 2007).

In order to analyze the effect of the bacteriocin on the growth of the phytopathogen in tomato plants, survival assays were carried out under greenhouse conditions. The number of viable cells of strain Xcv Bv5-4a-Rif50 was determined from leaflet discs at 0, 3, and 10 days post-infection. In plants untreated with SF4c tailocins, the population of phytopathogen on the leaflets increased over time (6.01 ± 0.205 and 7.92 ± 0.34 $\log_{10}$ CFU $cm^{-2}$, at 3 and 10 days postinfection respectively, Fig. 1, Panel G). These results were consistent with the observed increase in the development of the bacterial-spot symptoms. In plants treated with bacteriocins, however, the population of phytopathogenic bacteria was maintained to a low number of viable cells (4.5 ± 0.26 $\log_{10}$ CFU $cm^{-2}$, Fig. 1, Panel G) throughout the experiment, and the symptoms observed on the leaflets were also markedly reduced.

### 3.2. Visualization of tomato leaves by SEM

SEM images from the phylloplane of tomato leaves revealed the adherence of strain Xcv Bv5-4a onto epidermal cells at 24 h postinfection (Fig. 2, panels A and B). The number of bacteria increased even more at 72 h, and the cells were located mainly within the stomatal space embedded in an exopolymeric matrix (Fig. 2, panels E and F).

The foliar treatment with bacteriocin significantly reduced the colonization of the pathogen on the leaf surfaces at 24 and 72 h post-inoculation. At 24 h, few phytopathogenic bacteria were located on epidermal cells (Fig. 2, panels C and D), and the number of cells was further reduced at 72 h (Fig. 2, panels G and H). At this later time point, no cellular aggregates were observed on the stomata, while the accumulation of cellular debris was more evident. Of interest to us was the observation that SEM images revealed, in addition to the normal bacilli, the presence of the shorter Xcv Bv5-4a cells indicated in Fig. 2, (panels B, D, and F), which variants were more abundant in the plants treated with bacteriocin.

In plants sprayed with only water or bacteriocin (the two negative controls), no alterations were observed in the structure of the epidermal cells (Fig. 2, panels I and J).

### 3.3. Tomato-fruit protection by bacteriocin

The SF4c tailocins could also be applied to fruits as a biopreservative. In order to demonstrate the protection of fruits by bacteriocin, we conducted a trial. Strain Xcv Bv5-4a was first injected onto immature tomato fruits. Bacteriocin (500 AU $mL^{-1}$) was applied 30 min post-infection at the same inoculation point as used with the pathogen. The addition of TN50 Buffer, instead tailocins, was the control. After 7 days postinfection, the bacterial spots were detected on each side of the fruits inoculated with the phytopathogen but untreated with tailocins. In addition, on the infected fruit fungal growth occurs 10–15 days postinoculation (Fig. 3, Panel A and B). The bacteriocin treatment dramatically reduced symptoms on tomato fruits, indicating that bacteriocin is also effective in preventing the progression of the bacterial spot (Fig. 3, Panel A). The application of bacteriocin at 12 h after inoculation with strain Xcv Bv5-4 prevented the infection in addition to the development of symptoms on the fruits (Fig. 3, Panel C). Nevertheless, tailocins did not reduce the bacterial-spot symptoms when applied at as long as 24 and 48 h postinoculation with the phytopathogen (Fig. 3, Panel C).

### 3.4. Effect of different external influences on bacteriocin activity

Apoplast and cytoplasmic fluids from tomato leaves (rich in amino acids, proteases, and ions, among other components) are released at a lesion and have been known to inactivate antimicrobial peptides used for control of tomato diseases (Zeitler et al., 2013). Therefore, the apoplast fluids could conceivably inhibit the bacteriocin activity on the plant surface. For this reason, we analyzed the SF4c tailocins activity against strain Xcv Bv5-4a in presence of those fluids. The results demonstrated that the apoplast fluids released from tomato leaves at a lesion had no effect on the antibacterial activity of the bacteriocin (Fig. 4).

The stability of bacteriocin to temperature, adjuvants, light, and desiccation was also analyzed. The SF4c tailocins were routinely stored at 4 °C during long periods at a bacteriocin titer of $10^5$ AU $mL^{-1}$. A slight loss of the bacteriocin activity was detected when the samples had been exposed to 30 °C for lengthy periods, whereas the activity was reduced two orders of magnitude after 15 days of incubation at 37 °C. In addition, exposure to the adjuvant Silwet L*Ag or fluorescent light had almost no effect on antibacterial capability, whereas no bacteriocin activity at all remained in the samples rehydrated after three days of incubation under those desiccation conditions (Table 2).

### 3.5. Cytotoxicity assays

Biosafety studies of SF4c tailocins were carried out on simian-cell cultures. VERO cells were incubated with different concentrations of bacteriocins and their viability subsequently determined as described in Materials and Methods. The number of viable mammalian cells remained constant (approximately 84%) for all concentrations of bacteriocin tested in the assay (Fig. 5, Panel A). In addition, normal Vero-cell morphology was maintained, as observed by light microscopy (data not shown). Therefore, the SF4c tailocins proved to be noncytotoxic for VERO cells at all the concentrations assayed. In contrast, hydrogen peroxide and $CuSO_4$ (positive toxicity controls) were highly toxic to Vero cells. Both reduced drastically the cell viability down to 5.84 ± 0.2 and 5.73 ± 0.11%, respectively, of the control levels.

In the hemolysis assay, sheep red blood cells incubated with different concentrations of SF4c tailocins evidenced no hemolytic activity greater than 25% at all concentrations of bacteriocin evaluated (Fig. 5, Panel B). Similar results were obtained with physiologic saline or TN50 buffer. In contrast, sodium dodecyl sulfate (SDS) at 0.01% (w/v), tested as a positive-hemolysis control, effected a total release of hemoglobin.

## 4. Discussion

Bacteriocins are emerging as a promising alternative for reducing the application of agrochemicals in agriculture. One advantage of these compounds is their high specificity since they kill bacteria phylogenetically related to the bacteriocin-producing strain without altering the remaining microflora (Mills et al., 2017). The antimicrobial activity of many bacteriocins has been investigated *in vitro* against several phytopathogens (Dorosky et al., 2017; Ghequire et al., 2015; Grinter et al., 2013, 2012; Hammami et al., 2009; Jabeen et al., 2009), though a direct application of these antibacterial compounds to plants as biocontrol agents has still been poorly explored. Lavermicocca et al. (2002) evaluated the efficacy of a bacteriocin from *P. syringae* pv. *ciccaronei* in inhibiting the formation of overgrowths in olive plants caused by *P. syringae* subsp. *savastanoi* strains PVBa229 and PVBa304. That bacteriocin was also effective in controlling the multiplication of the epiphyte populations of the pathogen.

In our laboratory, we also demonstrated that bacteriocins produced by *P. fluorescens* have antimicrobial activity against several phytopathogenic strains of the genera *Xanthomonas* and *Pseudomonas* (Fernandez et al., 2017; Fischer et al., 2012; Godino et al., 2016). One of those bacteriocins is the SF4c tailocin, which particles adhere to the cell envelope of phytopathogenic strain *X. vesicatoria* Xcv Bv5–4a thereby causing damage through the production of a rapid leakage of intracellular materials followed by the death of the bacterium (Fernandez et al., 2017). In the present work, we evaluated the efficacy

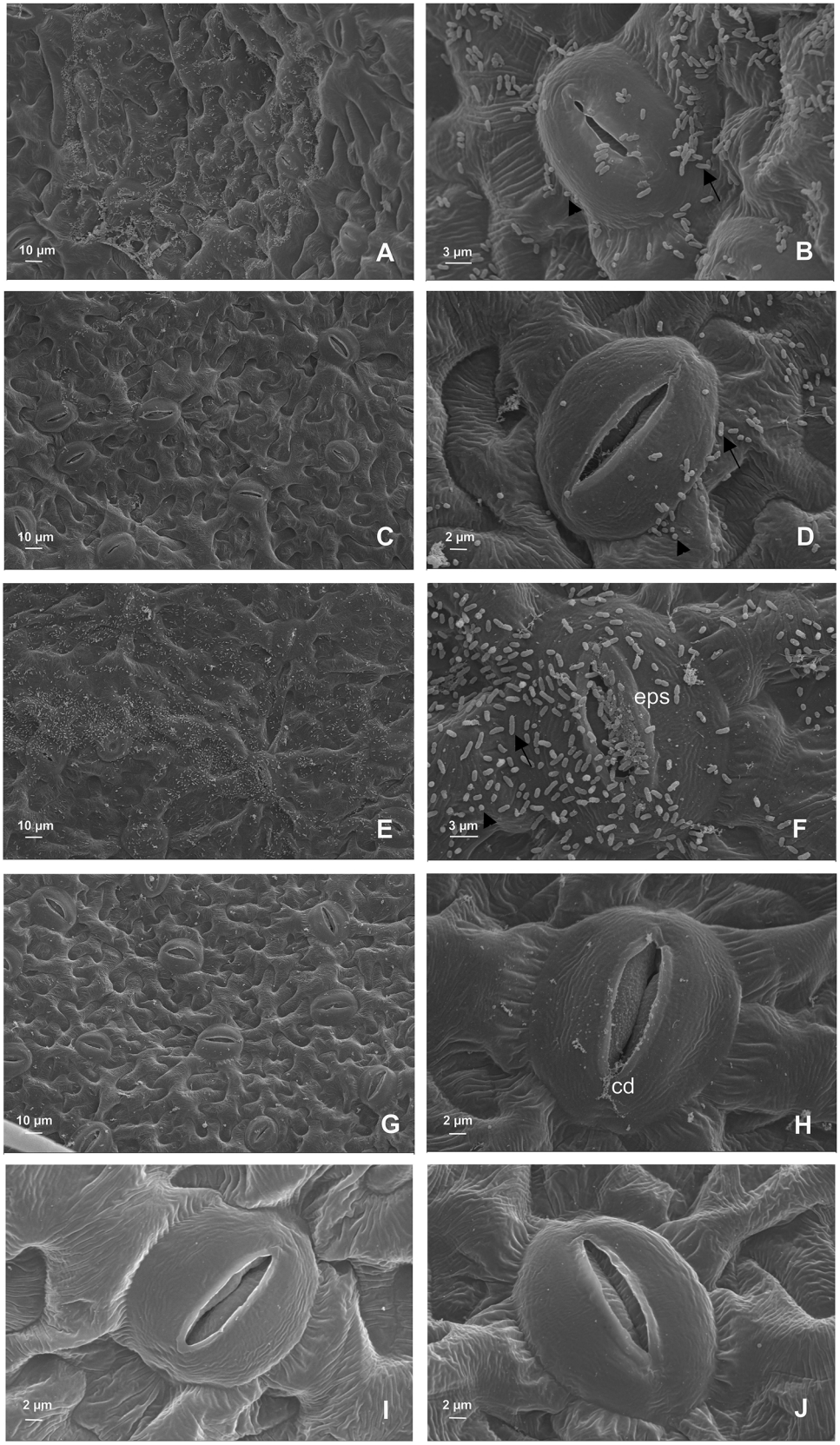


**Fig. 2.** Scanning electron micrographs of tomato leaves infected with strain Xcv Bv5-4a and untreated with bacteriocin (panels A, B, E, and F) or treated (panels C, D, G, and H). The images were recorded at 24 h (panels A–D) and 72 h (panels E–H) postinfection with the phytopathogen. Uninfected leaves sprayed with water (I) or bacteriocins (J) were used as controls. The scale bars represent 10 µm in panels A, C, E and G; 3 µm in B and F; and 2 µm in panels D and H–J. **Arrowhead in** B, D, and F, short Xcv Bv5-4a bacilli; **arrows in** B, D, and F, normal Xcv Bv5-4a cells; **eps**, exopolysaccharides; **cd**: cellular debris.

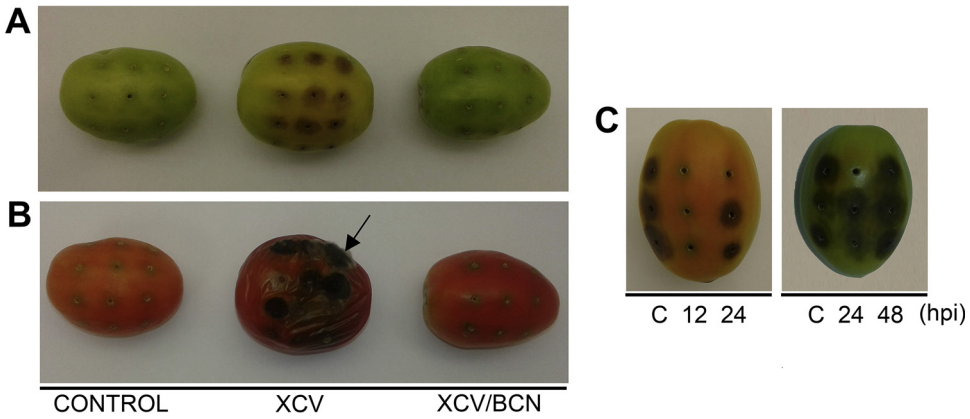


**Fig. 3.** Effect of SF4c tailocins on tomato fruits. *Xanthomonas vesicatoria* Xcv Bv5-4a ($1 \times 10^5$ cfu $mL^{-1}$) was injected into tomato fruits. After 30 min of infection, 500 AU $mL^{-1}$ of bacteriocin was injected at the same point on the fruits. The images correspond to 7 (Panel A) and 15 (Panel B) days postinfection. Fruits treated with bacteriocin at 12, 24 h (left), or at 24, 48 h (right) postinfection with the phytopathogen are shown in Panel C. The experiment was performed in triplicate (n = 2). XCV, Strain Xcv Bv5-4a; XCV/BCN, Strain Xcv Bv5-4a plus bacteriocin; hpi, h postinfection. The arrow points to the development of saprophytic fungi. (For interpretation of this figure, the reader is referred to the web version of this article).

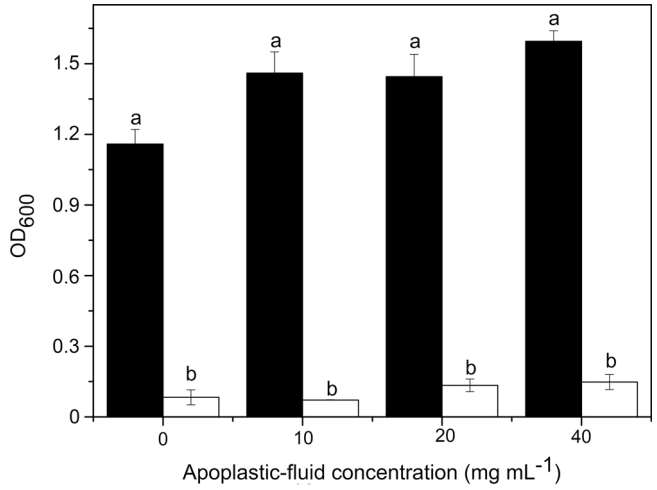


**Fig. 4.** Effect of apoplastic fluids on bacteriocin activity. Cultures of strain Xcv Bv5-4a were incubated with tomato apoplast fluids (0, 10, 20, or 40 mg $mL^{-1}$) in the absence (black bars) or presence (white bars) of bacteriocin as described in Materials and Methods. In the figure, the bacterial growth at 18 h, expressed as the $OD_{600}$, is plotted on the *ordinate* for each of the concentrations of apoplastic-fluid in mg $mL^{-1}$ indicated on the *abscissa*. The experiment consisted in three technical replicates and three biologic replicates. Different letters indicate significant differences in the values ($p < 0.05$) according to the Student *t*-test.

**Table 2**
Stability of phage tail-like bacteriocins from *P. fluorescens* SF4c under different abiotic conditions.

| | | SF4c Tailocins (AU $mL^{-1}$)[a] | | | |
|---|---|---|---|---|---|
| **Treatments** | **Days** | **0** | **3** | **7** | **15** |
| **4 °C** | | $10^5$ | $10^5$ | $10^5$ | $10^5$ |
| **30 °C** | | $10^5$ | $10^5$ | $10^5$ | $10^4$ |
| **37 °C** | | $10^5$ | $10^5$ | $10^4$ | $10^3$ |
| **Silwet L*Ag (0.025%)** | | $10^5$ | $10^5$ | $10^5$ | $10^4$ |
| **Light** | | $10^5$ | $10^5$ | $10^5$ | $10^4$ |
| | **Hours** | **0** | **12** | **24** | **72** |
| **Desiccation** | | $10^5$ | $10^2$ | $10^1$ | 0 |

[a] Concentration of SF4c tailocins determined as the reciprocal of the highest dilution that showed a clear inhibition of the indicator strain (Xcv Bv5-4a) in the plate (as described in Materials and Methods).

of the SF4c tailocins in controlling bacterial-spot disease under greenhouse conditions.

The symptoms of bacterial spot were detected in tomato plants after 5 days posinfection with *X. vesicatoria* Xcv Bv5–4a, and the disease advanced towards the newest leaves during all the time that the experiment lasted. The foliar application of the SF4c tailocins decreased the symptoms of bacterial spot and prevented the propagation of Xcv Bv5-4a cells to new leaves, thus reducing the incidence of the disease in the apical leaves. These results are in agreement with the findings of Yim et al. (2014), who observed a significant reduction of bacterial spot on apical tomato leaves when the plant was inoculated with *Methylobacterium* spp., strains producing 1-aminocyclopropane-1-carboxylate deaminase. The reduction in the disease, in that instance, was a result of a modulation of ethylene stress by that deaminase (Yim et al., 2014).

Survival trials carried out under greenhouse conditions demonstrated that the population of Xcv Bv5-4a cells exponentially increased in untreated plants while it remained in a low number in plants treated with the tailocins. Similar results had been found by Hert et al. (2009), who used an avirulent strain of *X. perforans* (a bacteriocin producer) against *X. euvesicatoria* (a bacterial-spot pathogen) on tomato leaves. In that instance, the strategy used was effective in controlling the disease by reducing the population of the pathogen in the phyllosphere below the threshold necessary for development of the lesion. In addition, Stromberg et al. (1999) demonstrated that the relationship between early population sizes of *Xanthomonas translucens* pv. *translucens* on wheat leaves and later disease severity is logistic rather than linear. This means that it is not necessary to completely eliminate the pathogen from surfaces of wheat leaves to greatly limit disease development. Therefore, the biocontrol is effective if phytopathogen populations are maintained below the threshold of population size (Stromberg et al., 2004).

In our experiments, the decrease in the phytopathogen population found in leaflets treated with SF4c bacteriocins was corroborated by SEM. A significant reduction in the pathogen cells were observed mainly at specific sites such as the stomata from the plants infected with strain Xcv Bv5-4a and treated with bacteriocin. Stomata are one of the most common targets for the phytopathogenic bacteria to use to establish themselves inside the plant and trigger disease; this suggests that SF4c tailocins could be reducing the number cells at that specific site, thus avoiding the subsequent colonization of the internal plant tissue by the phytopathogen along with the ensuing development of the disease. The use of a bioactive organocopper compound produced by *Pseudomonas aeruginosa* LV had produced a similar reduction in the populations of *Xanthomonas citri* subsp. *citri* strain 306 and a resulting lower bacterial colonization of the stomata in orange leaves. The application of this organocopper compound was more effective in reducing the symptoms of citrus canker when applied to leaf surfaces one hour before infecting with the pathogen. In addition, changes in the cell shape of *X. citri* subsp. *citri* strain 306 were also reported after such applications (de Oliveira et al., 2016).

Bacteriocins have been widely studied for use in the conservation of foods against the growth of spoilage organisms (fungi and bacteria) on tomatoes (Chopra et al., 2015; Temitope and Eberechukwu Oluchi, 2015) and other foods (Rai et al., 2016). In the present work, the effectiveness of bacteriocin was observed when was applied to fruit even

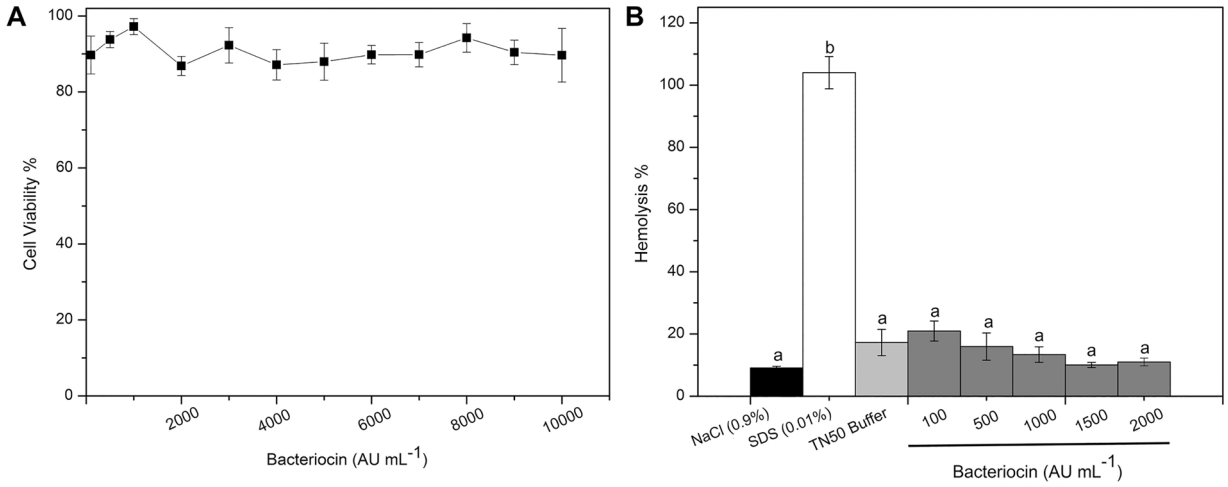


**Fig. 5.** Panel A: Effect of SF4c tailocins on the VERO cell line. In the figure, the percent cell viability is plotted on the *ordinate* as a function of the SF4c tailocins concentration, expressed as AU $mL^{-1}$ on the *abscissa*. Panel B: Hemolytic activity of SF4c tailocins towards sheep red blood cells. In the figure, the percent hemolysis is plotted on the *ordinate* for erythrocytes exposed to either progressive concentrations of bacteriocin in AU $mL^{-1}$ or the other additives indicated on the *abscissa*. SDS, sodium dodecyl sulfate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

12 h after infection with the phytopathogen. In this situation, the reduction of symptoms was significant compared to those of the untreated infected fruits. All these results indicate that the SF4c tailocins could be sprayed both on the plant for preventing the disease and on the fruit subsequent to harvest as a food preservative.

The effectiveness of a therapy against plant infection based on biological control agents depends not only on the susceptibility of the target bacterium but also on the environmental conditions that could affect the survival or stability of those agents, with a poor stability representing a major disadvantage for application in the field (Abhilash et al., 2016; Iriarte et al., 2007). For this reason, we analyzed the antibacterial activity of the SF4c tailocins in presence of different abiotic conditions—namely, temperature, fluorescent light, and desiccation—in addition to the effect of the plant's own apoplast fluids. Zeitler et al. (2013) had demonstrated that the synthetic peptides SP1-1, SP10-2, and SP10-5 had antibacterial activity against phytopathogens of tomato; but when those antimicrobials were exposed to tomato-apoplast fluids, the activity of the agents was reduced, indicating that the activity of those peptides would also be diminished in the plant. In the present work, we demonstrated that apoplast fluids did not affect the antimicrobial activity of the SF4c tailocins. In addition, the coadjuvant Silwet L*Ag, fluorescent light, and a 30 °C temperature had almost no effect on the activity of that bacteriocin, while exposure to 37 °C only partially reduced its potency. In contrast, desiccation was the most aggressive exogenous condition since the tailocin's activity became reduced below detectable levels in plates after a drying period of three days. This effect was presumably owing to the proteinaceous nature of the bacteriocin, whose structure could be denatured under an extreme absence of water of hydratration, as was accordingly observed here. Therefore, so as to prevent such denaturation, the application of this type of compound should be performed in presence of adjuvants that favor its rapid absorption into the tissues of the plant. Likewise, Iriarte et al. (2007) observed that the efficacy of bacteriophage formulations, against *Xanthomonas campestris* pv *vesicatoria* was reduced by desiccation, fluorescent and/or ultraviolet light, temperature, and the presence of copper compounds (Iriarte et al., 2007). Therefore, new phage formulations to improve disease-control efficacy were subsequently developed (Balogh et al., 2003; Iriarte et al., 2007), in which the presence of powdered skim milk plus sucrose eliminated the reduction of phage survival caused by external influences such as desiccation and temperature, among others (Iriarte et al., 2007).

The application of bacteriocins as biological tools to phytopathogen control, like that of other compounds, requires exhaustive studies to demonstrate the safety involved. For this reason, we performed cytotoxicity assays on sheep red blood cells and Vero simian-kidney cells in culture. All concentrations of the SF4c tailocins tested indicated the absence of any toxicity on either cell type whatsoever. Similar results had been described by Fagundes et al. (2016), who demonstrated that the bacteriocin aureocin A53, a preservative used in tomato and meat, was neither toxic to the cell lines assayed nor hemolytic against sheep erythrocytes. Moreover, a cytotoxicity assay had also demonstrated the bacteriocin sorensin to be noncytotoxic (Chopra et al., 2015). We wish to emphasize here that after treatment with the SF4c tailocins the plant likewise remained as healthy as the uninfected controls, thus indicating nonphytotoxicy to tomato plants as well. A comparable nontoxicity had also been the finding in rice, when plants were treated with melittin, a synthetic peptide, to control bacterial-leaf blight (Shi et al., 2016).

In conclusion, to the best of our knowledge, this report documents the first description of the application of tailocins for the biological control of bacterial-spot disease in the tomato. New protocols are being developed in our laboratory (formulations that include a mixture of bacteriocins, application time of the tailocins, etc) in order to achieve an improved biological control. So that bacteriocins can be considered as an alternative to the chemical bactericides currently used for the treatment of bacterial spot disease.

## Conflict of interest

None declared

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