Activity of antimicrobial peptides on *Xanthomonas arboricola* pv. *pruni* populations and infection on *Prunus sp*.

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Abstract: *Xanthomonas arboricola* pv. *pruni* is the causal agent of bacterial spot disease of stone fruits (*Prunus* spp.), an economically important disease in the major stone-fruit-producing areas worldwide. Disease control is mainly achieved by preventive applications of copper-based plant protection products. However, due to the negative environmental impact, there is a strong demand to reduce copper use in crop protection. Antimicrobial peptides are an alternative to conventional compounds used in plant disease control. This work was aimed at evaluating the activity of antimicrobial peptides against *X. arboricola* pv. *pruni in vitro* and on host plants. Antimicrobial peptide BP100 and two of its derivatives were able to inhibit bacterial growth *in vitro*, with minimal inhibitory concentration from 2.5 to 10/50 µM. BP100 inhibited bacterial multiplication and significantly decreased *X. arboricola* pv. *pruni* population levels and disease severity on *Prunus* GF-677 leaves when applied in preventative and curative strategies. The persistence of BP100 activity on plant surface lasted up to five days, being optimal 24-48 h before pathogen inoculation. A 48 h post-infection effect of BP100 was also observed. These results demonstrate the ability of antimicrobial peptides to reduce pathogen epiphytic populations to levels below the minimum infective concentration, and thus to prevent infections.

Key words: *Xanthomonas*, *Prunus*, antimicrobial peptide, XapCAST, integrated disease management

Introduction

The Gram-negative bacterium *Xanthomonas arboricola* pv. *pruni* (Xap) (Vauterin et al. 1995) causes bacterial spot disease of stone fruits. The disease is widespread in all the major stone fruit-growing areas of the world, although in some countries only local and sporadic outbreaks have been reported (EPPO 2022).

X. arboricola pv. *pruni* infection on *Prunus sp*. produces necrotic angular spots on leaves, spots or sunken lesions on fruit, and stem canker. Severe infections may result in tree defoliation and fruit drop, with the consequent negative economic impact (Stefani 2010).

The pathogen overwinters in cankers, infected dormant buds, leaf scars, and on leaf debris on the orchard ground. These are important primary inoculum sources (Zaccardelli et al. 1998). Wind and rain contribute to bacterial dissemination. Bacteria multiply epiphytically on young leaves and penetrate through stomata or wounds, causing leaf lesions, which provide the secondary inoculum for infections on fruits, twigs and trunks. Warm temperatures and high relative humidity are required for bacterial multiplication (Morales et al. 2017). A disease forecaster (XapCAST) was developed to predict inoculum potential, risk of infection and onset of symptoms (Morales et al. 2017; Morales et al. 2018a; Morales et al. 2018b).

Disease management integrates measures to avoid pathogen introduction in new areas, the use of tolerant or resistant cultivars, cultural methods, and chemical control. Copper-based compounds are applied preventatively at late host dormant stage (phenological phases A and

B) and early in the growing season (Stefani 2010). However, the moderate efficacy, plant phytotoxicity (Lalancette and McFarland 2007), the risk of resistance development in the bacterium (Giovanardi et al. 2017; Vanneste et al. 2005), and the negative environmental impact, limit copper use.

Antimicrobial peptides have been considered as promising candidates for the development of novel antimicrobial compounds (Montesinos, 2007). BP100, a synthetic peptide based on a cecropin A-melittin hybrid (CECMEL11 library), has shown high activity against the plant pathogens *Erwinia amylovora*, *Pseudomonas syringae* and *Xanthomonas axonopodis*, with low minimal inhibitory concentration (MIC), low cytotoxicity and high stability to protease degradation (Badosa et al. 2007). BP100 derivatives designed for expression in plant systems are also effective in the inhibition of several plant pathogenic bacteria (Badosa et al. 2013).

This work was aimed at evaluating the activity of antimicrobial peptide BP100 and two of its derivatives against *X. arboricola* pv. *pruni in vitro*, on epiphytic populations on leaves, and on bacterial infections on *Prunus sp*. BP100 reduced pathogen epiphytic populations to levels below the minimum infective concentration, and thus prevented bacterial infections.

Material and methods

Peptides

Antimicrobial peptide BP100 (H-KKLFKKILKYL-NH2) was provided by LIPPSO (University of Girona, Spain) at >90% purity. Its derivatives BP178 and BP235, designed to be expressed in plants (Badosa et al. 2013) were purchased from CASLO Laboratory ApS (Lyngby, Denmark) (>90% purity).

Bacterial strains

*A*ctivity of peptides was determined against seven *X. arboricola* pv. *pruni* strains isolated from different host and geographic regions (Table 1). Bacterial suspensions were prepared in sterile distilled water from LB agar cultures grown at 27°C for 24-48 h.

In vitro antibacterial activity of BP100 and its derivatives

Lyophilized peptides were solubilized in sterile distilled water to final concentrations of 500, 250, 100, 50, 25 and 10 µM. Twenty microliters of each dilution were mixed in a microtiter plate well with 20 µl of the bacterial suspensions (10^8 cfu/mL; OD=0.2 at 600 nm) and 160 µl of LB broth to a total volume of 200 µl. Final peptide concentrations assayed were 50, 25, 10, 5, and 2.5 µM. Microplates were incubated for 48 h at 25°C in Bioscreen C incubator with 20 s shaking before hourly absorbance measurement. Distilled sterile water was added instead of peptides in positive controls. Similarly, negative controls with sterile distilled water instead of bacterial suspensions were included. Bacterial growth was determined by optical density measurement at 600 nm. Three replicates for each combination of strain, compound and concentration were used. The experiment was repeated twice. The minimum inhibitory concentration (MIC) was determined as the lowest peptide concentration with no growth at the end of the experiment. Bactericidal activity of peptides was assessed by adding resazurin (5µM), a cell health indicator, to each well at the end of experiment. Fluorescence (λex 520, λem 590) was measured after 24-hour incubation. The minimum bactericidal concentration (MBC) was determined as the lowest peptide concentration were no viable cells were detected.

In planta activity of BP100 and derivatives

The effect of BP100 on bacterial epiphytic populations and infection was analyzed on peach-almond hybrid GF-677 potted plants (Agromillora Group, Spain). For preventative experiments, plants were sprayed until runoff (5 mL/leaf) with a solution of BP100 100 µM in sterile distilled water, and maintained under greenhouse conditions for up to five days. Leaves were randomly collected daily from plants and surface-spray inoculated with bacterial suspensions of strains CITA 33 or CFBP5530 to final concentration of 10^4 cfu/leaf. Then, leaves were placed on moistened filter paper and sealed in a polyethylene bag to maintain high RH (> 98%). Leaves were incubated for 14 days at 25 °C and 12-h light photoperiod in a controlled environment chamber (model MLR-350; Sanyo, Gunma, Japan) for pathogen multiplication and infection (Morales et al., 2018a). In curative experiments, GF-677 detached leaves were inoculated with bacterial suspensions and incubated for 3, 24 or 48h at optimal conditions for infection. Then, leaves were treated with BP100 100 µM and incubated at the same conditions for 14 days. Water-treated control plants were included. Three replicates of ten leaves were used for each sampling time. Population densities of *X. arboricola* pv. *pruni* on GF-677 leaf surface were assessed on samples of five leaves per replicate 48 h after pathogen inoculation. Each sample was placed in a sterile plastic bag containing 50 ml of extraction buffer (7.10 g $Na HPO_4$, 2.72 g KH_2PO_4, and 1 g peptone per 1000 mL of distilled water), and ground in a lab blender (Stomacher, IUL Instruments, Germany) for 5 min. Aliquots and serial dilutions were plated on YPGA and incubated for 72 h at 27 °C. Disease severity was assessed 14 days after pathogen inoculation on the other set of leaves according to Morales et al. (2018b). A completely randomized experimental design was used. The experiment was performed twice. The effects of BP100 treatment on bacterial population and disease severity was determined using the GLM procedure of SAS software version 9.4 (SAS Institute Inc. Cary, NC, USA), after confirmation of the homogeneity of variance and normality.Tukey's HSD test was used for mean comparison.

Results and discussion

BP100 and its derivatives BP178 and BP235 were highly effective in inhibiting *in vitro* multiplication of *X. arboricola* pv. *pruni* strains (Table 1). MIC ranged from 2.5 to 10 µM against six out of seven strains and from 10 to 50 µM for strain CFBP 5563. The bactericidal activity was demonstrated by the resazurin-based method (Table 1). These results confirm that antimicrobial peptides BP100, BP178 and BP235 inhibit bacterial growth and have a direct bactericidal activity on the Gram-negative plant pathogenic bacterium *X. arboricola* pv. *pruni*.

BP100 treatment reduced *X. arboricola* pv. *pruni* populations on *Prunus* GF-677 leaves when applied in both preventative and curative strategies (prior to or after pathogen inoculation, respectively) (Figure 1 top panels). Bacterial populations dropped from 10^6 cfu/g fresh leaf to 10^2-10^4 cfu/g fresh leaf with BP100 treatment. The analysis of variance indicated a significant effect of treatment ($P < 0.0001$, $R^2 > 0.91$) on bacterial populations and disease severity in both strategies of peptide application. For the two strains of *X. arboricola* pv. *pruni*, peptide treatment maintained the pathogen populations below the minimum infective dose of 10^6 cfu/ml (equivalent to 10^6 cfu/g leaf fresh weight) (Morales et al. 2028a). Regarding the preventative strategy, the antibacterial activity of BP100 persisted on *Prunus* GF-677 leaves up to five days before pathogen inoculation, with a significant reduction in epiphytic bacterial populations compared to non-treated control (Figure 1A). The optimal activity was observed when BP100 was applied preventatively 24-48 h before pathogen inoculation. Accordingly, the disease

severity decreased significantly in BP100 treated leaves (Figure 1A bottom panel). A significant reduction in disease severity of up to 75-100 % compared to non-treated controls was observed. Interestingly, when BP100 was applied in a curative strategy, *X. arboricola* pv. *pruni* CITA33 and CFBP5530 populations decreased significantly compared to non-treated control (Figure 1B top panel). A decrease from ~6 log CFU/g (non-treated control plants) to ~2-3 log CFU/ mL in BP100 treatments was observed, demonstrating the post-infection activity of BP100 against *X. arboricola* pv. *pruni* when applied from 3 up to 48 h after inoculation. Moreover, a significant reduction in disease severity, ranging from 50 to 75% compared to non-treated control, was achieved in BP100 curatively treated leaves (Figure 1B bottom panel).

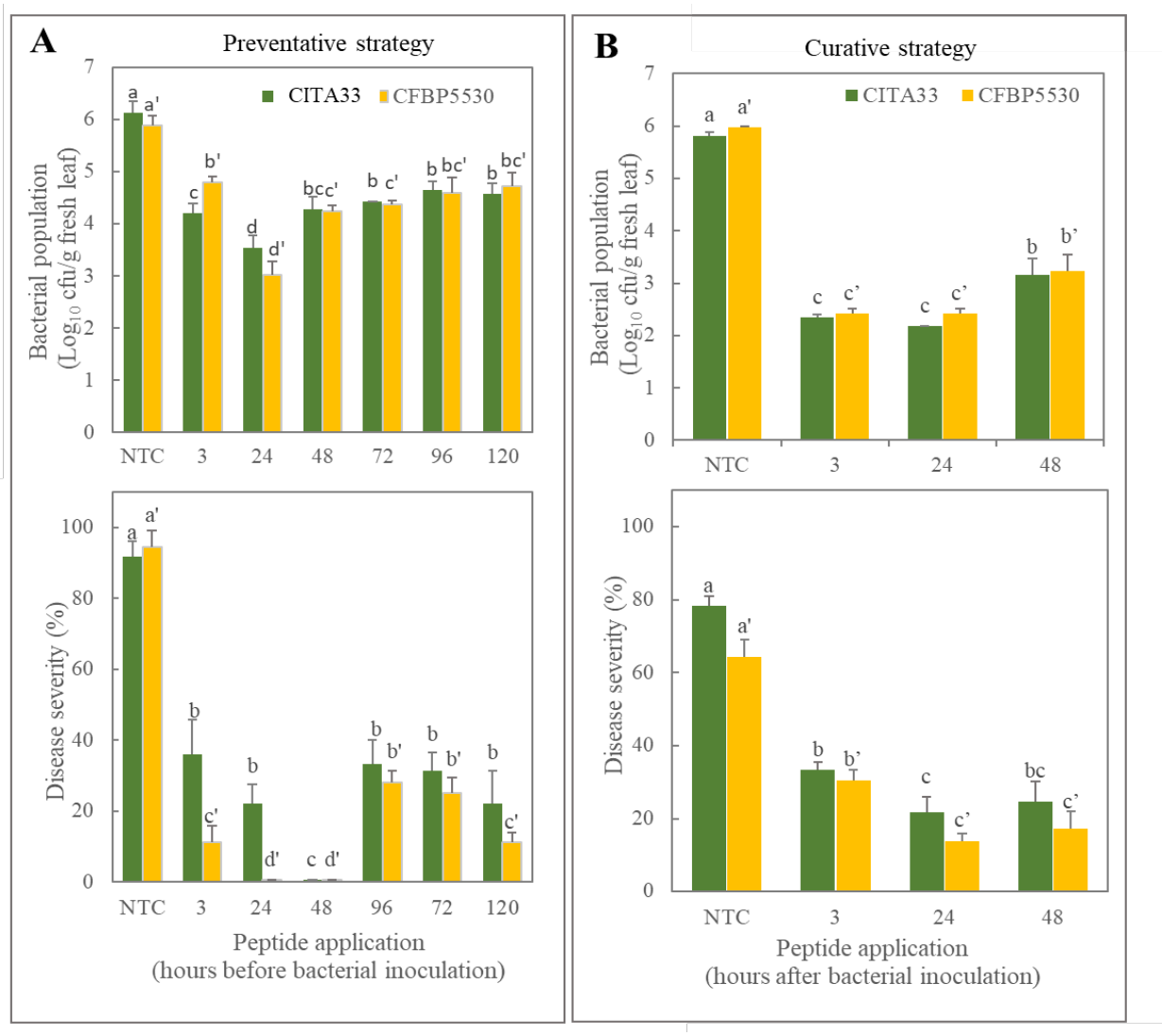

Figure 1. Bacterial populations (top panels) and disease severity (bottom panels) in GF-677 leaves treated with BP100 (100 µM) at different time intervals according to a preventative (A) or curative (B) strategy, compared to non-treated control plants (NTC). Two *X. arboricola* pv. *pruni* strains were used (CITA33 and CFBP5530). Values are the mean of two experiments and three replicates per experiment. Error bars are the standard error. Different letters indicate significant differences according to Tukey's mean separation test ($P = 0.05$).

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of peptides BP100, BP178 and BP235 against *X. arboricola* pv. *pruni*.

Strain [z]	Host	Geographic region	MIC /MBC (µM)
CFBP 3894	*Prunus salicina*	New Zealand	2.5
CFBP 3903	*P. domestica*	Italy	2.5
CFBP 5530	*P. persica*	Italy	2.5-10.0
CFBP 5563	*P. persica*	France	10.0-50.0
CFBP 5725	*P. persica*	EUA	5.0
CITA 33	*P. amygdalus*	Spain	2.5
IVIA 3162	*P. amygdalus*	Spain	5.0

[z] CITA, Centro de Investigación y Tecnología Agroalimentaria de Aragón, Zaragoza, Spain; CFBP, Collection Française de Bactéries Phytopathogénes, Angers, France; IVIA, Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain.

The experiments demonstrated that BP100 inhibits *X. arboricola* pv. *pruni* multiplication and decreases epiphytic bacterial populations to levels below the infective dose, so preventing infections and disease development. These results state the potential of BP100 to avoid *X. arboricola* pv. *pruni* infections on host plants. The new knowledge on the persistence of peptide activity on host leaves will be the basis for BP100 evaluation under field conditions.

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