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Short Communication

A Phage Therapy Model for the Prevention of *Pseudomonas syringae* pv. *actinidiae* Infection of Kiwifruit Plants

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Abstract

Great efforts have been made with chemicals and pesticides to contain the spread of *Pseudomonas syringae* pv. *actinidiae* (*Psa*) responsible for kiwifruit canker. Unfortunately, only partial results were obtained for this bacterial pandemic, and alternative remedies were proposed to avoid soil pollution and the onset of antibiotic resistance. Among these, phage therapy represents a possible tool with low environmental impact and high specificity. Several phages have been isolated and tested for the capacity to kill *Psa* in vitro, but experiments to verify their efficacy in vivo are still lacking. In the present study, we demonstrated that the phage φPSA2 (previously characterized) contains the spread of *Psa* inside plant tissue and reduces the symptoms of the disease. Our data are a strong indication for the efficiency of this phage and open the possibility of developing a phage therapy based on φPSA2 to counteract the bacterial canker of kiwifruit.

Keywords: bacterial canker, kiwifruit, phage therapy, *Pseudomonas syringae* pv. *actinidiae*

Bacterial canker, caused by *Pseudomonas syringae* pv. *actinidiae* (*Psa*) infection, is one of the main factors affecting kiwifruit production worldwide (Scortichini et al. 2012). Over the last decade, major kiwifruit producing countries, such as China, New Zealand (NZ), and Italy have been strongly affected by this pathogen, leading to substantial economic loss (Donati et al. 2020; Guroo et al. 2017; Qin et al. 2013; Young 2012). The pathogen is extremely virulent, spreading rapidly between plants (Donati et al. 2020; Vanneste 2017). The main symptoms of the disease include brown spots on the leaves with extensive chlorotic regions, canker on trunks and canes, plant wilting, and bloom necrosis (Kim et al. 2017).

Many strategies to control the spread of the infection have been developed, such as the removal of the infected parts of the plants or whole orchards (Vanneste 2017), and the use of chemicals (e.g., antibiotics, copper, and pesticides) (Cameron and Sarojini 2014; Corrado et al. 2018; Marcelletti et al. 2011). However, these strategies turned out to be quite ineffective; as a systemic pathology, the removal of infected canes does not arrest the progression of the disease, and the grubbing up of whole orchards results in severe economic loss. Moreover, the application of chemical agents may induce resistance in pathogens (Colombi et al. 2017) and cause phytotoxic symptoms to kiwifruit as well as environmental pollution. Indeed, more than 80% of *Psa* isolates from kiwifruit orchards in New Zealand showed copper resistance or tolerance (Pereira et al. 2021; Sundin et al. 2016). Additionally, antibiotic use in food production has been banned in Europe due to rising antimicrobial resistance, and there is a mandate for a 50% reduction in copper use by 2030. One approach that has proved profitable is the breeding of *Psa*-tolerant or resistant kiwifruit cultivars. In 2012, the new cultivar 'Zesy002', commonly known as 'G3', was licensed by Zespri to growers as a replacement for the highly susceptible kiwifruit cultivar 'Hort16A'. The *Psa*-tolerant cultivar 'G3' has allowed the New Zealand kiwifruit industry to survive and even expand, but exploring alternative ways to manage the disease in the future is still essential, especially as *Psa* could adapt to the new cultivar. In this scenario, phage therapy, which uses lytic bacteriophages to treat bacterial infections, represents an eco-friendly and sustainable biocontrol method relative to current control strategies (Holtappels et al. 2021). The main advantage of phage therapy is the specificity of bacteriophages for their targets (Yu et al. 2016), which allows killing pathogens with no effects on the normal microbiota (Almeida et al. 2009; Liu et al. 2021; Pereira et al. 2011; Rios et al. 2016; Silva et al. 2014; Vieira et al. 2012).

The effectiveness of phage therapy has been widely demonstrated in vitro (Balogh et al. 2010; Caflisch et al. 2019; Di Lallo et al. 2014; Jamal et al. 2019), and in the last two decades, positive results from the "phage therapy" application in plant pathology have been obtained (Adriaenssens et al. 2012; Fujiwara et al. 2011; Gašić et al. 2018; Ravensdale et al. 2007). Many commercial preparations have been approved for "phage therapy" in agriculture, e.g., AgriPhage from OmniLytics (Salt Lake City, UT) which is already used to deal with *Pseudomonas syringae* pv. *tomato* infections of tomatoes (Balogh et al. 2003). Despite that, no effective system for the control of kiwifruit bacterial canker has been developed so far, and few studies have evaluated the effectiveness of phage treatment on kiwifruit plants in vivo (Flores et al. 2020; Pinheiro et al. 2020; Song et al. 2021).

Recently, 258 phages representative of Myoviridae, Podoviridae, and Siphoviridae families, active against *Psa*, were identified (Frampton et al. 2014). A member of Podoviridae family, φPSA2 phage, isolated by Di Lallo et al. (2014) from raw sewage, was characterized for its ability to infect *Psa* in vitro. φPSA2 showed a lytic cycle, a broad host range, a stable activity at pH ranging from 5.0 to 9.0, and up to a temperature of 50°C. With these features, φPSA2 represents a good candidate as a control agent for kiwifruit canker. To develop a phage therapy based on φPSA2, it was essential to verify the reliability of the phage preparation and treatment as well as the phage survival on the plant leaves. In this framework, we investigated the use of φPSA2 prophylaxis in reducing damage induced by *Psa* infection. For this purpose, we pretreated kiwifruit plants with φPSA2 before *Psa* inoculation with the high virulent biovar 3 strain CRA-FRU 8.43 (Scortichini et al. 2012).

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First, we verified the persistence and viability of φPSA2 suspension on the leaf surface, since the stability on the leaf is an important property in using a phage as a biocontrol for *Psa*-induced kiwifruit canker. To this end, in vitro grown kiwifruit seedlings were used. *Actinidia deliciosa* cv. 'Hayward' seeds were sterilized with 70% (v/v) ethanol for 1 min and a 1% (v/v) sodium hypochlorite solution containing 0.05% (v/v) Tween-20 for 10 min, washed with sterile water five times for a total of 10 min, and sowed on half-strength Murashige and Skoog (MS, Duchefa Biochemie, Haarlem, the Netherlands) medium, supplemented with 3% (w/v) sucrose and 0.7% (w/v) agar at a pH of 5.8. Seeds were stratified for 3 days at 4°C in the dark and moved to the growth chamber at 22°C, 80% humidity, under a 16/8 h light/dark cycle. After 3 weeks from germination, kiwifruit seedlings were spray inoculated with the φPSA2 suspension (2 ml/plant), prepared as described by Di Lallo et al. (2014) at 5×10^8 plaque-forming units per ml (PFU/ml), and the titer of the phage was determined after 1, 3, and 8 days using the strain *Psa* CRA-FRU 8.43 as the indicator. For this purpose, leaf discs of 1 cm diameter from three different leaves of each plant (both cotyledons and true leaves were used to collect leaf discs) were placed in 1.5-ml tubes, weighed, and homogenized with a pestle in 300 µl of sterile 10 mM $MgSO_4$. The phage suspension was serially diluted and titrated by double layer plaque assay. The phage starting titer was $1.8 \pm 0.6 \times 10^6$ PFU/mg of fresh weight (F.W.) and, as shown in Fig. 1A, the titer remained at $1.0 \pm 0.8 \times 10^6$ PFU/mg F.W. after 8 days, indicating that φPSA2 is very stable on the leaf surfaces.

Once the persistence and viability of φPSA2 was verified, its ability to prevent *Psa* infection was then determined by quantifying the bacterial growth inside plant tissue in kiwifruit plantlets with or without phage pretreatment. For this purpose, in vitro grown kiwifruit seedlings were used to avoid possible microbial contamination which could interfere with *Psa* quantification. Kiwifruit plantlets germinated in MS medium and grown for 3 weeks (20 for each experiment) were divided in two groups. One group was treated with φPSA2 by filling the plant containers with a phage suspension at 5×10^8 PFU/ml in 100 ml of SM buffer (10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10 mM $MgSO_4$) and incubating for 2 min. The other group was treated with the same volume of SM alone as the control. After 24 h, both groups were inoculated with *Psa* CRA-FRU 8.43 suspension at 5×10^8 colony-forming units per ml (CFU/ml), prepared as described in Di Lallo et al. (2014). Briefly, containers with kiwifruit plantlets were filled with 100 ml of the bacterial suspension, containing 0.0025% (v/v) Silwet L-77 (Lehle Seeds, Round Rock, TX) to facilitate leaf wetting, and incubated for 2 min (McAtee et al. 2018). The bacterial growth inside plant tissue was then compared at different times postinfection. For this purpose, leaf discs of 1 cm diameter obtained from infected plants, were placed in 1.5-ml

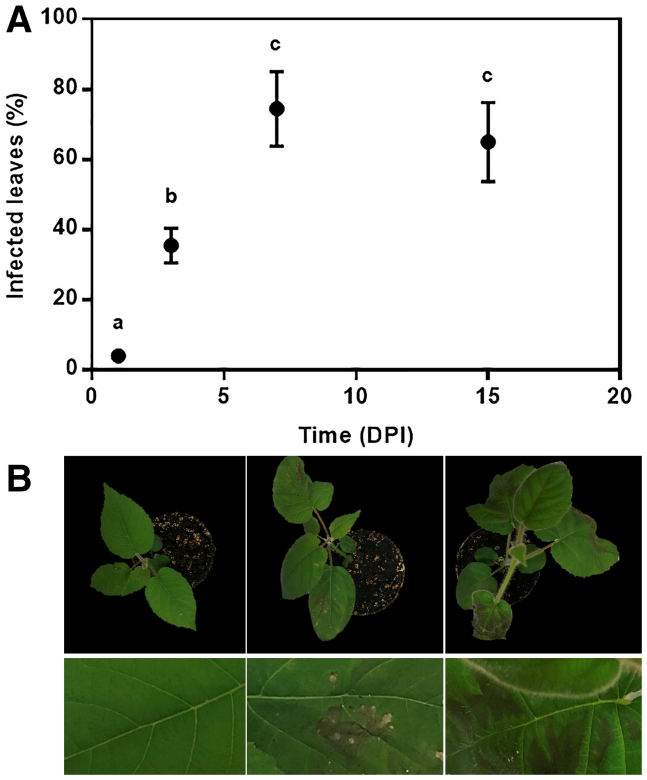

Fig. 2. *Psa* infection of kiwifruit plants grown in greenhouse conditions. **A,** Kiwifruit plants were spray inoculated with 5×10^8 CFU/ml *Psa*, and the progression of disease was determined at 1, 3, 7, and 15 days postinoculation (DPI) as the percentage of leaves showing at least a visible lesion on their surface. The experiment was performed three times with 20 plants for each experiment. Data are the means ± SD. Lowercase letters indicate that values are significantly different (statistical analysis was performed using one-way analysis of variance by Tukey's multiple comparison test; $P < 0.05$). **B,** A representative infected plant and a magnification of a leaf are shown. Pictures were taken at 0 (left panels), 7 (middle panels), and 15 (right panels) DPI.

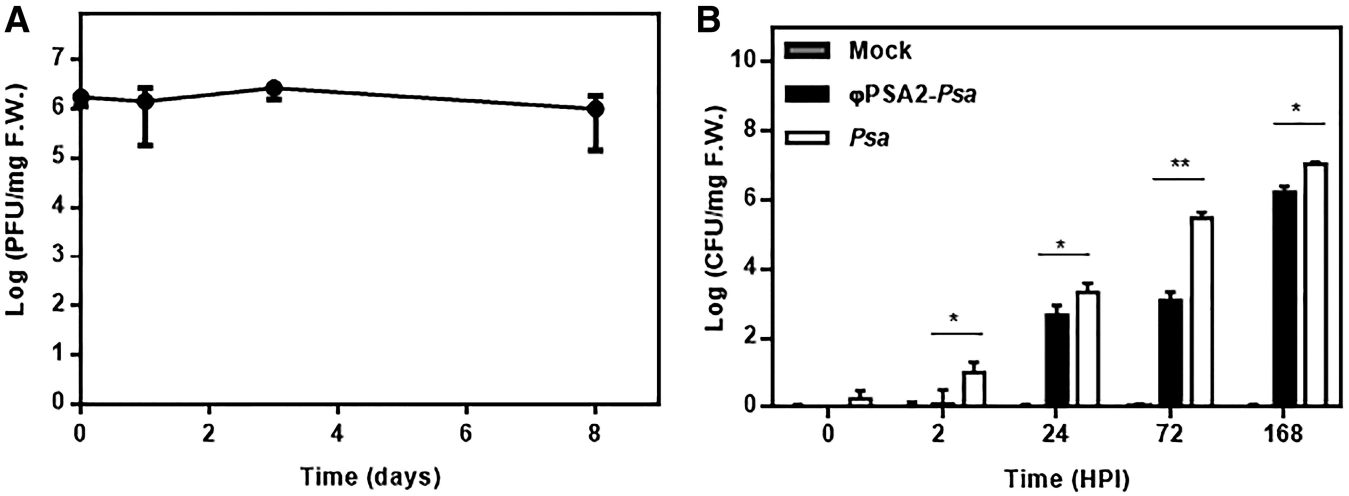

Fig. 1. Stability of φPSA2 on leaf surface and its effect on in planta bacterial growth. **A,** Kiwifruit (10 seedlings) were spray inoculated with 5×10^8 PFU/ml φPSA2 suspension, and the titer of the phage was determined immediately after phage application and then after 1, 3, and 8 days. Phage PFU were normalized to mg of leaf. Data are the means ± SD. **B,** Kiwifruit plantlets 3 weeks after germination were treated with φPSA2 24 h prior to *Psa* inoculation (φPSA2-*Psa*); the control group of kiwifruit plants was treated with phage buffer without the addition of φPSA2 (*Psa*). Bacterial quantification was determined at 0, 2, 24, 72, and 168 h postinoculation. The experiment was performed three times using leaf discs derived from 20 plantlets that were considered as individual replicates for statistical analysis. Water-treated (mock) seedlings were included as a control. Data are the means ± SD. Statistical significance was assessed by the unpaired Student's *t* test, $*P < 0.05$; $**P < 0.01$.

tubes, surface sterilized in 70% (v/v) ethanol to remove bacteria outside the leaf, washed with sterile water, and homogenized with a pestle in 300 µl of sterile 10 mM $MgSO_4$. Serial dilutions were prepared in 10 mM $MgSO_4$ and 100 µl of solution was inoculated on Nutrient agar medium to allow bacterial growth. After 72 h of incubation at 26°C, the CFU/mg of F.W. was calculated. As shown in Fig. 1B, *Psa* replication inside plant tissue was significantly reduced by φPSA2 pretreatment. The effect was particularly significant at 72 h postinfection (HPI), with a difference in bacterial growth of more than 100-fold (from 3.0×10^5 to 1.3×10^3 CFU/mg of leaf) ($P = 0.0081$). After 1 week (168 HPI), the difference in bacterial enumeration was still evident, although to a lower extent. It is also interesting to note that already at 2 HPI, leaves of φPSA2 pretreated plants displayed a lower amount of bacteria, suggesting that φPSA2 immediately kills bacteria on the leaf surface, thus reducing their entry into the plant tissue.

The ability of φPSA2 to prevent the disease symptoms of *Psa* infection was then investigated on kiwifruit plants grown in greenhouse conditions. Kiwifruit seeds were germinated on MS medium and grown for 2 weeks before being transplanted to soil. One-month-old plants were inoculated with *Psa* by extensively spraying both sides of fully expanded leaves with 20 ml of a 5×10^8 CFU/ml bacterial suspension, as described by Mauri et al. (2016). *Psa* infection of plants induced clear disease symptoms as necrotic spots and chlorotic regions on the leaves, as can be observed in the panels of Fig. 2B, where a representative infected plant is shown. The progression of the disease was determined by evaluating the percentage of leaves that displayed symptoms at 1, 3, 7, and 15 DPI. As shown in the graph reported in Fig. 2A, the percentage of infected leaves increased up to 7 DPI. The slight decrease observed at 15 DPI compared with 7 DPI was not statistically significant ($P = 0.47$);

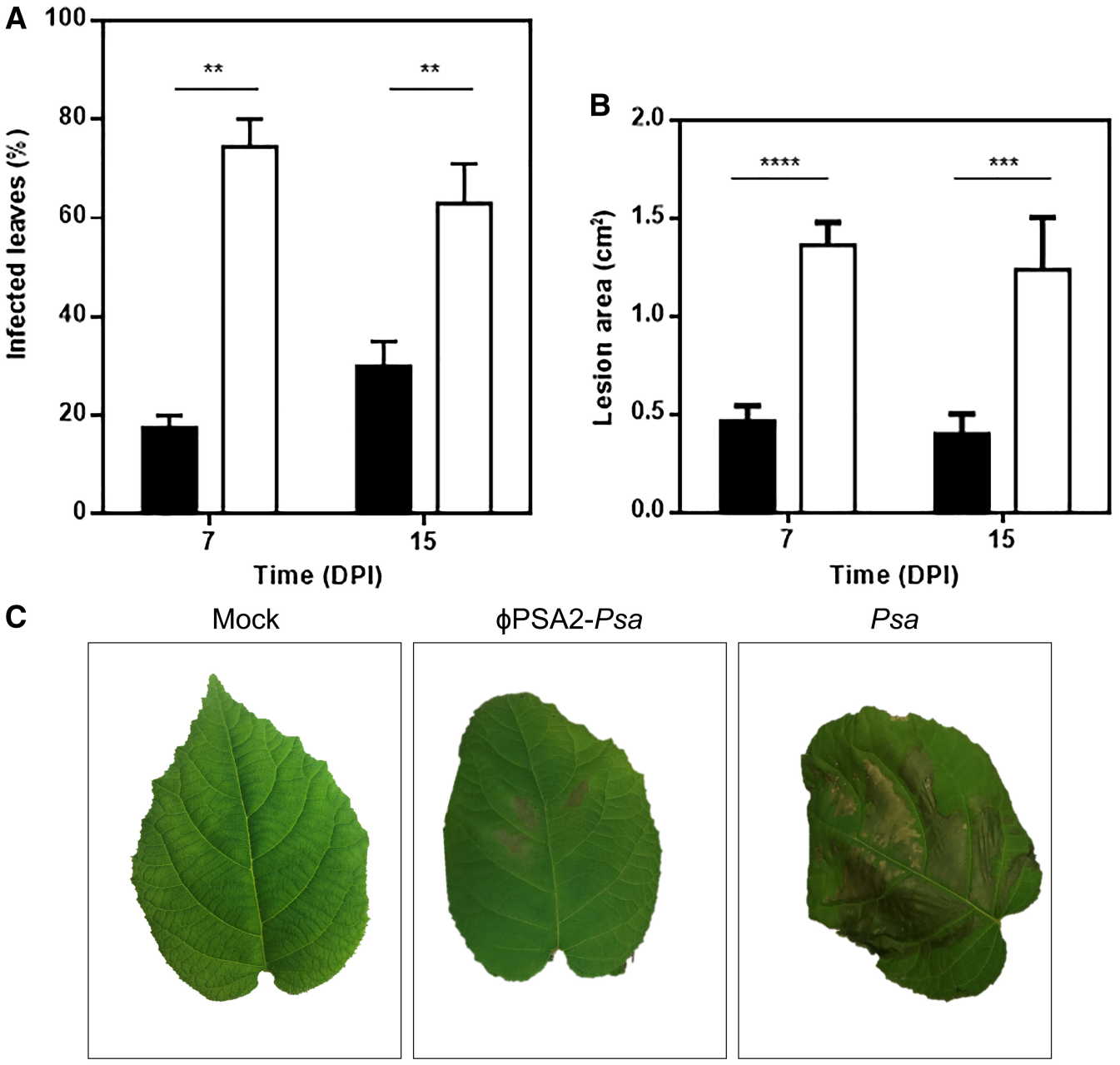

Fig. 3. φPSA2 treatment reduced the symptoms induced by *Psa* infection. Kiwifruit plants, sprayed (black bars) or not (white bars) with 5×10^8 PFU/ml φPSA2, were inoculated with 5×10^8 CFU/ml *Psa*. The experiment was repeated three times using 20 plants for each experiment. **A,** Percentage of leaves showing at least a visible lesion evaluated at 7 and 15 days postinoculation (DPI). Data are the means ± SD. Statistical significance was assessed by the unpaired Student's *t* test, $^{**}P < 0.01$. **B,** Area of lesions evaluated at 7 and 15 DPI. The area was measured by analyzing with ImageJ software (Schneider et al. 2012) digital pictures of infected leaves showing disease symptoms. Values are the means ± SD. Statistical significance was assessed by the unpaired Student's *t* test, $^{***}P < 0.001$; $^{****}P < 0.0001$. **C,** Representative leaves showing necrotic lesions. The pictures were taken at 15 DPI.

however, it must be considered that the percentage of infected leaves at longer times after inoculation is affected by the development of not infected new young leaves.

To evaluate the ability of φPSA2 to prevent *Psa* infection, kiwifruit plants were sprayed with 20 ml of a suspension of 5×10^8 PFU/ml φPSA2 the day before *Psa* inoculation, and the percentage of infected leaves was assessed at 7 and 15 DPI. As shown in Fig. 3A, 17.5% of the leaves pretreated with φPSA2 at 7 DPI displayed lesions compared with 74.5% of the untreated plants, indicating that φPSA2 treatment reduced the symptoms of the disease by 77%; the reduction of symptoms by φPSA2 treatment at 15 DPI was 50%, which was slightly lower but still significant. In addition, to better evaluate the effect of the phage administration on preventing symptoms of *Psa* infection, the area of necrotic regions of leaves, pretreated or not with φPSA2, was measured according to de Jong et al. (2019). As reported in Fig. 3B, phage treatment reduced the size of the lesions at both 7 and 15 DPI by about 70%. The effect of φPSA2 is also evident in Fig. 3C, where representative leaves, pretreated or not with the phage, are shown, indicating that, besides decreasing the number of infected leaves, φPSA2 sensibly reduced the severity of symptoms.

In conclusion, the results here reported that the application of φPSA2 to kiwifruit seedlings is effective in preventing *Psa* replication inside plant tissues and in reducing the number and size of pathological lesions induced by *Psa*. The φPSA2 application by spray treatments turned out to be an efficient strategy that was already reported to function in the control of diseases that mainly manifest at the level of phyllosphere (Flores et al. 2020; Holtappels et al. 2021; Pinheiro et al. 2020). Presumably, the action of the phage is accomplished by killing the bacteria present on the leaves' surface, thus limiting their entry into the plants.

The reduction of symptoms observed after φPSA2 treatment was noticeable and comparable to that reported in the literature for a mixture of four phages closely related to φPSA2 that were isolated using *Psa* biovar 3 strains obtained from Chilean kiwifruit orchards (Flores et al. 2020).

Furthermore, our results demonstrated that the phage remains viable and stable on the leaves under the adopted experimental conditions. This result is of particular relevance since phages degrade very rapidly on plant surfaces; hence, the stability of phages on the phyllosphere is a major limiting factor for phage employment as a biocontrol strategy (Jones et al. 2007).

Nowadays, the fight against *Psa* can be carried out mainly through preventive measures. These measures may not be sufficient in the case of emergence of particularly virulent *Psa* strains, as we have had the opportunity to experience over the years at the beginning of the last decade. The initial shock, which led to the eradication of thousands of hectares around the world, was overcome thanks to breeding and selection in New Zealand of new kiwifruit cultivars that are less sensitive to *Psa*. However, these new cultivars do not guarantee protection against future outbreaks with new *Psa* strains. Therefore, it is necessary to continue to develop new approaches to manage *Psa* outbreaks. Bacteriophages represent a concrete possibility, and several companies have already released phage-based products for agricultural crops (Pereira et al. 2021). However, there are still no phages approved for *Psa*-induced kiwifruit canker biocontrol due to a limited number of in vivo studies demonstrating their efficacy.

Our study supports the idea of using phages for *Psa* control and contributes to the knowledge of the in vivo effects of a bacteriophage previously only characterized in vitro. Even though more experiments are necessary to verify the possible employment of φPSA2 to prevent *Psa* infection in field conditions, this work represents an important step forward in the development of a phage therapy for the bacterial canker of kiwifruit.

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