

## Frost promotes the pathogenicity of *Pseudomonas syringae* pv. *actinidiae* in *Actinidia chinensis* and *A. deliciosa* plants

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Frost occurs in all major areas of cultivation, presenting a threat for the production of kiwifruit crops worldwide. A series of experiments were performed on 1-year-old, potted plants or excised twigs of *Actinidia chinensis* and *A. deliciosa* to verify whether strict relationships exist between bacterial canker outbreaks from *Pseudomonas syringae* pv. *actinidiae* (Psa) attacks and the occurrence of autumn and winter frost events. The association between the occurrence of autumn frost and the sudden outbreak of bacterial canker in *A. chinensis* in central Italy has been confirmed. Both autumn and winter frosts promote Psa multiplication in the inoculated twigs of both species. The day after the frost, reddish exudates oozing from the inoculation sites were consistently observed in both species, and Psa was re-isolated in some cases. During the thawing of both *A. deliciosa* and *A. chinensis* twigs, the 2-cm upward and downward migration of Psa from the inoculation site was observed within 3 min, and the leaves were consistently colonized with the pathogen. A consistent brown discoloration, accompanied with a sour-sap odour, was observed throughout the length of the excised twigs of both *Actinidia* species after Psa inoculation and winter frost. Psa inoculation induced a remarkably higher necrosis in excised twigs that were not frozen compared with *P. s. pv. syringae* inoculation. Antifreeze protection using irrigation sprinklers did not influence the short-term period of Psa and *P. s. pv. syringae* multiplication in both *A. deliciosa* and *A. chinensis* twigs. Thus, the damage from frost, freeze thawing and the accumulation of Psa in *Actinidia* twigs promotes the migration of the pathogen within and between the orchards. Taken together, the results obtained in this study confirmed that *A. deliciosa* is more frost tolerant than *A. chinensis*, autumn frosts are more dangerous to these crops than winter frosts, and in the absence of Psa, young kiwifruit plants remain sensitive to frost.

**Keywords:** autumn frost, epidemiology, kiwifruit bacterial canker, systemic migration, winter frost

### Introduction

*Pseudomonas syringae* pv. *actinidiae* (Psa), the causal agent of bacterial canker in kiwifruit, severely damages and causes economic losses in all major areas of kiwifruit production worldwide (Marcelletti *et al.*, 2011; Mazzaglia *et al.*, 2012), with a pandemic population of the pathogen infecting both green (*Actinidia deliciosa*) and yellow-fleshed (*Actinidia chinensis*) cultivars and the pollinators of these crops (Chapman *et al.*, 2012; Scortichini *et al.*, 2012). The newly evolved Psa population is different from that of past outbreaks observed in Japan and Italy from 1980 to 1990 (Ferrante & Scortichini, 2009, 2010; Marcelletti *et al.*, 2011; Mazzaglia *et al.*, 2012).

The sudden, destructive epidemic of bacterial canker observed in central Italy on *A. chinensis* cultivars in 2008 has been associated with frost events recorded in the provinces of Latina and Rome in autumn–winter 2007–2008 and an unusually high amount of precipitation recorded in 2008 (Ferrante *et al.*, 2012). Recently, a relationship between the occurrence of bacterial canker

in both yellow- and green-fleshed kiwifruits and winter frost has also been postulated for the Shaanxi province in China (Zhao *et al.*, 2013). Frost events alone or in combination with strains of *P. syringae* pathovars (i.e. primarily the pathovar *syringae*) are considered as a major predisposing factor(s) for the initial colonization of resident bacterial pathogens and/or the subsequent penetration of phytopathogens (De Kam, 1982; Vigoroux, 1989; Sobiczewski & Jones, 1992; Tomihama *et al.*, 2009). Spring seasons with frequent rains and/or high humidity have also contributed to rapid Psa multiplication in Japan (Serizawa & Ichikawa, 1993a). In addition, the damage resulting from frost, bacterial colonization and the penetration of the leaf through the stomata represent important facets of the pathogen life cycle, as accumulated Psa systematically migrates from these sites through the petiole to reach young twigs and initiates the endophytic phase of the disease (Serizawa & Ichikawa, 1993b; Spinelli *et al.*, 2011; Ferrante *et al.*, 2012; Petriccione *et al.*, 2012).

Both *A. deliciosa* and *A. chinensis* are endemic to eastern Asia (i.e. China), with *A. deliciosa* located inland in the colder regions, and *A. chinensis* located in the eastern, warmer regions of the country. The two species occur together in mountainous areas and are separated vertically, with *A. deliciosa* located at higher altitudes

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(Warrington & Weston, 1990). In regions outside of its origin, the freezing tolerance of green-fleshed kiwifruit vines in the field under winter conditions varies from  $-10$  to  $-18^{\circ}\text{C}$  (Monet & Bastard, 1985; Kamota *et al.*, 1989; Dozier *et al.*, 1992). However, *A. deliciosa* cv. Hayward and the pollinator cv. Matua resisted deep winter frosts, as observed in 1985 in northern Italy when the minimum temperatures reached  $-23^{\circ}\text{C}$ . The plants resprouted in the following spring from the adventitious buds of the leader and the trunk, and the main damage consisted of a relevant decrease in the proportion of fruit-bearing shoots (Testolin & Messina, 1987). In France, serious injuries to *A. deliciosa* cv. Hayward were also observed on young shoots and flowers after spring frost and on fruits and leaves after autumn frost (Blanchet, 1985). Until 1981, kiwifruit plantations located in the Bay of Plenty on the northern coast of New Zealand occasionally sustained apparent freeze damage in the spring, autumn and winter (Hewitt & Young, 1981). In contrast, spring frosts have more recently (i.e. 2002–2009) been a threat to both green- and yellow-fleshed kiwifruits in this area, and consequently, many growers have installed frost protection systems (i.e. fans or water sprinklers) in their orchards ([http://www.nzherald.co.nz/business/news/article.cfm?c\\_id=3&objectid=3559952](http://www.nzherald.co.nz/business/news/article.cfm?c_id=3&objectid=3559952); [http://www.freshplaza.com/news\\_detail.asp?id=50230](http://www.freshplaza.com/news_detail.asp?id=50230)). Protection from winter and spring frosts using water sprinklers is also a rather common practice in major kiwifruit production areas in Italy.

Because winter frost events occur in all major areas of kiwifruit cultivation, such as China, Italy and New Zealand, where *Psa* was recently identified, this study was initiated to determine whether the occurrence of frost events in the winter influences the multiplication and pathogenicity of this bacterium on both *A. deliciosa* and *A. chinensis* plants and organs and examine the effects of antifreeze protection through water sprinkling on infected, but apparently healthy, plants and freezing–thawing in the twigs after frost. In addition, to verify the relationship between frost occurrence and the sudden outbreak of kiwifruit bacterial canker observed in central Italy in 2008, the influence of frost events occurring in autumn during those same days in central Italy in November 2007 on *Psa* multiplication and plant colonization was also characterized. Because other pathogenic bacteria, such as *P. syringae* pv. *syringae*, might be present in kiwifruit orchards sustaining damage through bacterial canker via *Psa* (Ferrante & Scortichini, 2010), the effect of winter frost on the multiplication of this bacterium was also considered.

## Materials and methods

### Bacterial strains

*Pseudomonas syringae* pv. *actinidiae* (*Psa*) CRA-FRU 8.43 and *P. syringae* pv. *syringae* (*Pss*) CRA-FRU 10.31, both isolated in the province of Latina from *A. chinensis* cv. Hort16A in 2008 and 2009, respectively, were used for the inoculations. Both iso-

lates have been previously characterized (Ferrante & Scortichini, 2009, 2010; Marcelletti *et al.*, 2011). The bacteria were routinely maintained on nutrient agar (Oxoid) supplemented with 3% sucrose (NSA), at  $25$ – $27^{\circ}\text{C}$ . For the inoculations, colonies grown on NSA for 48 h were suspended in sterile saline (0.85% NaCl in distilled water) to a concentration of  $1$ – $2 \times 10^6$  colony-forming units (CFU)  $\text{mL}^{-1}$  or  $1$ – $2 \times 10^9$  CFU  $\text{mL}^{-1}$ .

### Autumn frost

One-year-old *A. deliciosa* cv. Hayward and *A. chinensis* cv. Soreli self-rooted, potted plants were used. During the growing season, the plants received normal fertilization and regular watering. One month before the frost treatment, 20 plants from each species were inoculated by gently puncturing the shoot with a sterile scalpel at three different sites. Subsequently,  $10 \mu\text{L}$  *Psa* suspension, at a concentration of  $1$ – $2 \times 10^6$  CFU  $\text{mL}^{-1}$ , were applied to the wound. Five plants from each species, which were not inoculated, served as controls. After inoculation, the plants were maintained in open-air conditions. In 2007, an autumn frost was recorded on 4 November (i.e. a 6-h duration with a minimum temperature of  $-8^{\circ}\text{C}$ ) and 5 November (i.e. a 2-h duration with a minimum temperature of  $-2^{\circ}\text{C}$ ) (Ferrante *et al.*, 2012); therefore, during two consecutive days of the first week of November 2012, 10 inoculated and five control plants from both species were placed in a climatic chamber programmed to gradually (over 2 h) reach and maintain the minimum temperatures recorded in 2007 on 4 and 5 November. The duration of the frost event included the 2 h required to reach the minimum temperature. The remaining 10 inoculated plants from each species were maintained in the open-air temperatures recorded for those days (i.e. varying from  $8$  to  $19^{\circ}\text{C}$ ). After the frost, the treated plants were maintained in open-air conditions. Two days after the frost, reisolations from both the frost-treated and non-frost-treated plants were obtained to count the *Psa* colonies from each inoculated site (i.e. 1–3 mm of tissue) according to well-established techniques (Ferrante & Scortichini, 2009). The putative *Psa* colonies from each isolation were streaked in purity onto NSA plates, incubated at  $25$ – $27^{\circ}\text{C}$  and identified through repetitive-PCR using BOX and ERIC primers (Ferrante & Scortichini, 2009, 2010). In addition, to verify whether the sudden autumn frost promoted rapid pathogen colonization of the leaves during thawing, the lower surface of 20 leaves from both the frosted and non-frosted plants (i.e. control plants) were gently sprayed (Sule & Seemuller, 1987) with  $1$ – $2 \times 10^6$  CFU  $\text{mL}^{-1}$  *Psa* at 30 min after the frost treatment. Twenty additional leaves were sprayed using the same procedure 2 h later. Subsequently, all plants were maintained in open-air. Reisolations from the exudates, water-soaked leaf spots and control leaves were performed the next day using previously established procedures (Ferrante & Scortichini, 2009).

### Winter frost

The interaction between winter frost and kiwifruit bacterial canker was assessed in two ways. The first experiment was conducted using healthy, 1-year-old *A. deliciosa* cv. Hayward and *A. chinensis* cv. Soreli self-rooted, potted plants, treated as described above. One day before the frost (i.e. 16 February 2012), 10 plants from each species were inoculated by gently wounding the shoot with a sterile scalpel at three different sites. Subsequently,  $10 \mu\text{L}$  *Psa* CRA-FRU 8.43 or *Pss* CRA-FRU 10.31 suspension, at a concentration of  $1$ – $2 \times 10^6$  CFU

mL<sup>-1</sup>, was placed onto the wound. Five plants from each species were not inoculated and served as controls. These plants were maintained in open-air conditions at a minimum temperature of 5°C. The inoculated plants were placed in a climatic chamber programmed to gradually reach and maintain a minimum temperature of -8°C for a total of 7 h (i.e. including the 2 h required to achieve the minimum temperature programmed), similar to the frost event recorded in the province of Latina and Rome on 17 February 2008 (Ferrante *et al.*, 2012). Two days after the frost, reisolation from each inoculated site from both frost-treated and non-frost-treated plants were performed to count the Psa and Pss colonies, as reported above. The putative Psa and Pss colonies from each isolation were streaked in purity onto NSA plates, incubated at 25–27°C and identified through repetitive-PCR using BOX and ERIC primers (Ferrante & Scortichini, 2009, 2010). A second experiment was conducted using healthy, excised 1-year-old twigs in accordance with Vigoroux (1974) and Weaver (1978). Briefly, 40 1-year-old *A. deliciosa* cv. Hayward and *A. chinensis* cv. Soreli twigs of 15–20 cm in length were excised on 8 March 2012 from the adult trees. All twigs were surface-disinfected according to Vigoroux (1974) and Weaver (1978). Subsequently, the twigs were placed on water-saturated cotton in the bottom of sterilized glass test tubes of 25 cm in length and 2.5 cm in diameter. The apical end of the twig was cut even with the upper edge of the tube and immediately inoculated with 10 µL 1–2 × 10<sup>9</sup> CFU mL<sup>-1</sup> Psa CRA-FRU 8.43 or Pss CRA-FRU 10.31 suspension, as indicated in similar, previous studies (Vigoroux, 1974; Weaver, 1978). This high bacterial concentration mimics the dispersal of bacterial inoculum in winter through the exudates. The 10 tubes of each species were sealed with aluminium foil and placed in an incubator at 15°C for 7 days. Ten control tubes from each species were treated in the same manner using only sterile saline. The other 20 tubes (i.e. 10 per species) were inoculated in the same manner and received, in addition, a frost exposure of -10°C for 36 h. After the frost, the tubes were placed in an incubator at 15°C for 7 days. Ten control twigs from each *Actinidia* species received frost treatment without inoculation. Subsequently, all twigs were examined for injuries, and the necrosis length was measured and recorded.

### Freezing–thawing after winter frost and kiwifruit shoot susceptibility

To verify whether freezing–thawing influences the systemic migration of Psa and Pss within kiwifruit twigs, 10 healthy, 1-year-old *A. deliciosa* cv. Hayward and *A. chinensis* cv. Soreli self-rooted, potted plants were placed in winter, on 19 December 2011, in a climatic chamber at -10°C for 6 h. Psa experiments were separated from the Pss experiments. The duration of the frost event included the 2 h required to reach the minimum temperature. This temperature and duration were observed in the provinces of Latina and Rome on 17 December 2010 (Ferrante *et al.*, 2012). Ten other plants were maintained in open-air conditions with no frost. After the frost treatment, the plants were transported into the laboratory for inoculation. Bacterial suspensions of 1–2 × 10<sup>9</sup> CFU mL<sup>-1</sup> were prepared as described above for Psa CRA-FRU 8.43 and Pss CRA-FRU 10.31. As soon as the twig tissues appeared water-soaked as a result of thawing, the twigs were punctured with a sterile syringe and 5-µL drops of the bacterial suspension were placed onto the wound, in accordance with Vigoroux (1989, 1999) and Cao *et al.* (1999). Each shoot was pricked in two places on the

internode, and the bacterial suspension was absorbed within 4–6 s. The plants that did not receive frost treatment were inoculated in the same manner. A total of 15 shoots (i.e. 30 inoculation sites) per treatment from each species were inoculated. Three minutes after the inoculation, at intervals of 0.5 cm upward and downward from the inoculation site, 5–8 mg of tissue was harvested and processed for reisolations according to the procedures described above (Ferrante & Scortichini, 2009). Ten fold serial dilutions were performed on NSA plates. In parallel, the presence of Psa in the inoculated tissue was also directly detected using duplex PCR according to the methods of Gallelli *et al.* (2011), thus avoiding reisolation. The presence of Pss CRA-FRU 10.31 in the inoculated tissues was verified through repetitive-PCR using BOX and ERIC primers on putative Pss colonies obtained from each reisolation (i.e. five single colonies from representative plates), and the identification was considered valid when there was total identity of the fingerprint pattern with that of the original isolate (Scortichini *et al.*, 2003).

### Antifreeze protection using water sprinklers

To verify whether antifreeze protection using water sprinklers influences the multiplication of Psa in infected but apparently healthy kiwifruit plants, 1-year-old *A. deliciosa* cv. Hayward and *A. chinensis* cv. Soreli self-rooted, potted plants trained in open-field conditions were inoculated with 1–2 × 10<sup>6</sup> CFU mL<sup>-1</sup> Psa CRA-FRU 8.43 at four different sites along the shoot. The inoculations were performed on five plants from each species 48 h before antifreeze protection, as described above. Based on the weather forecast, the day before the frost was expected the plants were transported to a kiwifruit orchard of Latina province (central Italy) equipped with an antifreeze protection system consisting of water sprinklers. The plants were placed in between the rows of the orchard to receive the antifreeze treatment. The water sprinkler antifreezing system was initiated at midnight and terminated at c. 06:00 AM. Five other plants from each species were placed in the orchard but did not receive antifreeze treatment. The experiment was performed on 10 February 2012, and the minimum temperature recorded was -2°C for 6 h. Three days after the antifreeze treatment, the plants were transported to the laboratory to count the Psa colonies. The reisolations were performed in accordance with the procedures described above.

### Statistical analysis

The data concerning the mean population size of Psa and Pss recovered from *Actinidia* spp. twigs after the frost events and the thawing process compared with plants that were not subjected to frost are expressed in log CFU mL<sup>-1</sup> ± standard deviation. The significance of these values was calculated using Student's *t*-test for the comparison of log means using the SPSS v. 16.0 (SPSS Inc.). Differences of *P* < 0.05 were considered significant and are indicated with different letters.

## Results

### Autumn frost

The effect of a sudden autumn frost on the multiplication of Psa within the twigs of *A. deliciosa* cv. Hayward and *A. chinensis* cv. Soreli is shown in Figure 1, and rep-

representative images of both kiwifruit twigs and leaves the day after the autumn frost events and the control inoculation site are shown in Figure 2(a–f). The *Psa* population significantly increased from *c.*  $3\text{--}4 \times 10^6$  CFU mL<sup>-1</sup> to  $4\text{--}5 \times 10^7$  CFU mL<sup>-1</sup> after the frost treatment and 2 days of incubation in open-air conditions (i.e. 6–18°C) compared with the non-frosted plants (Fig. 1). Notably, the sudden appearance of reddish exudates oozing from the inoculation sites in 40% of the *A. chinensis* and 30% of *A. deliciosa* plants was observed (Fig. 2a–c). The reisolations performed from these exudates facilitated the recovery of the pathogen in 60% (*A. deliciosa*) and 70% (*A. chinensis*) of cases. Extensive reddish spots beneath the epidermis appeared after frost along the twigs of both inoculated and non-inoculated plants (Fig. 2e). In addition, extensive water soaking was observed in the leaves sprayed with *Psa* during thawing (Fig. 2d), resulting in subsequent penetration of the pathogen potentially through both the stomata and/or tiny wounds resulting from frost damage. Indeed, the recovery of the pathogen from the 40 water-soaked leaf spots was 100% in both *Actinidia* species compared with 32.5% (*A. chinensis*) or 25% (*A. deliciosa*) of the control leaves. The leaf inoculation performed at 2 h later exhibited the positive reisolation of *Psa* at 27.5% (*A. chinensis*) and 22.5% (*A. deliciosa*). One week after frost, 70 and 20% of the inoculated *A. chinensis* and *A. deliciosa* plants, respectively, died. The remaining plants, however, exhibited extensive necrosis indicative of severe damage. A similar response was observed for the control plants that received only frost treatment.

### Winter frost

The effect of winter frost on the multiplication of *Psa* and *Pss* in *A. deliciosa* and *A. chinensis* twigs is shown in Figure 3. The frost event promoted a significantly higher multiplication of both *Psa* and *Pss* on both *A. chinensis* and *A. deliciosa* twigs, with concentrations higher than  $10^7$  CFU mL<sup>-1</sup> per inoculation site. One week after the frost, 40% of the inoculated *A. chinensis* plants died. The *A. deliciosa* plants, although severely damaged, survived. The control plants showed similar responses. After the frost, a consistent brown discoloration, accompanied by a sour-sap odour, was observed along the lengths of the twigs excised from both *A. deliciosa* and *A. chinensis* plants. In addition, the tissues exhibited a softened consistency. In contrast, the control twigs showed lighter discoloration along the twig length. *Psa* was consistently reisolated from the frozen twigs. In twigs inoculated with bacteria but not frozen, *Psa* induced a significantly higher necrosis compared with *Pss*. Indeed, the average necrosis lengths induced by *Psa* in *A. deliciosa* and *A. chinensis* was  $10.3 \pm 0.7$  cm and  $9.9 \pm 0.5$  cm, respectively. In contrast, *Pss* induced twig necrosis lengths of  $2.7 \pm 0.3$  cm in *A. deliciosa* and of  $2.5 \pm 0.2$  cm in *A. chinensis*. *Psa* and *Pss* were consistently reisolated from all inoculated twigs. The control twigs inoculated with only sterile saline did not show

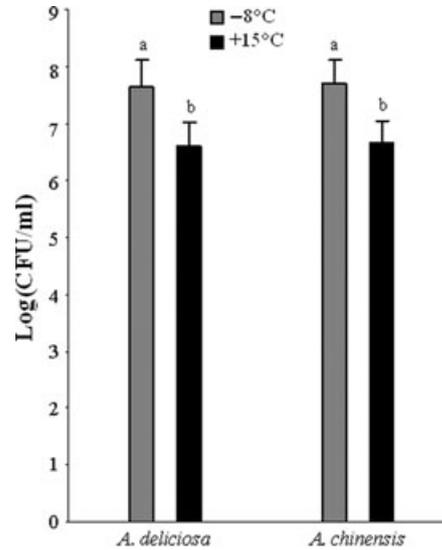


Figure 1 Mean population of *Pseudomonas syringae* pv. *actinidiae* in *Actinidia deliciosa* and *A. chinensis* twig inoculation sites after autumn frost events compared with unfrozen twigs. The vertical bars show the standard errors of the mean of log populations; different letters indicate the statistical significance at  $P < 0.05$  based on Student's *t* test for the comparison of log means.

any symptoms. The effect of *Psa* and *Pss* inoculation with and without frost treatment on excised *A. deliciosa* twigs is shown in Figure 4.

### Freezing thawing after winter frost

In *A. deliciosa* and *A. chinensis* twigs, both *P. syringae* pathovars migrated 2 cm upward and downward from the inoculation site within 3 min after the inoculation. In twigs inoculated but not frozen, both pathogens colonized solely within 0.5 cm upward and downward from the inoculation site. The migration of *Psa* and *Pss* in *A. deliciosa* twigs upon wounding during thawing compared with that in twigs that were not frozen is shown in Figure 5(a,b). The detection of *Psa* in the inoculated twigs using the duplex-PCR technique confirmed these findings (Fig. 6).

### Antifreeze protection

The mean *Psa* and *Pss* population in inoculated *A. deliciosa* and *A. chinensis* twigs obtained after antifreezing protection using water sprinkler irrigation is shown in Figure 7. In both *Actinidia* species, the concentration of the *P. syringae* pathovars did not significantly increase in the inoculation sites three days after receiving antifreeze protection during frost treatment at a minimum temperature of  $-2^{\circ}\text{C}$ . Indeed, the bacterial concentration remained at *c.*  $10^6$  CFU mL<sup>-1</sup>, which is consistent with that used for the inoculation and similar to those recorded for the frozen twigs.

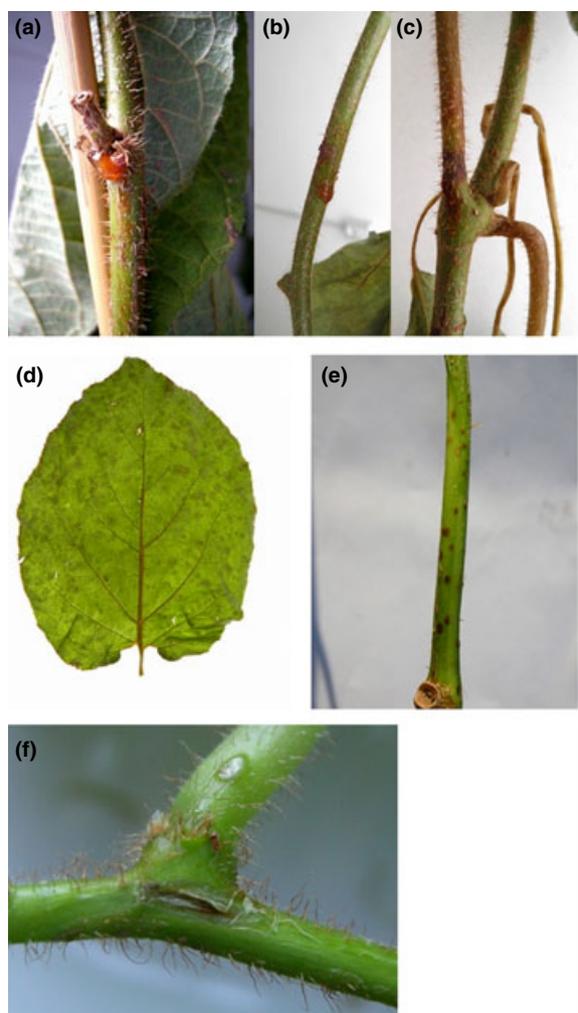


Figure 2 Effects of autumn frost on different *Actinidia deliciosa* and *A. chinensis* organs. (a–c) Reddish exudates obtained the day after the frost from a *Pseudomonas syringae* pv. *actinidiae* inoculation site (a and b: *A. deliciosa*; c: *A. chinensis*); (d) water-soaked spots on *A. deliciosa* leaf appearing 1 day after inoculation during thawing after autumn frost events; (e) reddish spots appeared along the lengths of *A. deliciosa* twigs the day after the frost events. The spots appeared on both inoculated and control plants; (f) inoculation site on the *A. deliciosa* unfrozen twig.

## Discussion

This study clearly showed that different frost events promote Psa multiplication within 1-year-old *A. deliciosa* and *A. chinensis* twigs and contribute to the spread of the pathogen and subsequent penetration into the host plants. In particular, this study confirmed the strict relationships between the autumn frost events of November 2007 in central Italy and the outbreak of bacterial canker of yellow-fleshed kiwifruit observed the following year (Ferrante *et al.*, 2012). Indeed, during the 2 days of incubation in the open-air conditions after frost, the Psa population in the frozen twigs increased 10 times more than the population in the twigs that were not subjected to

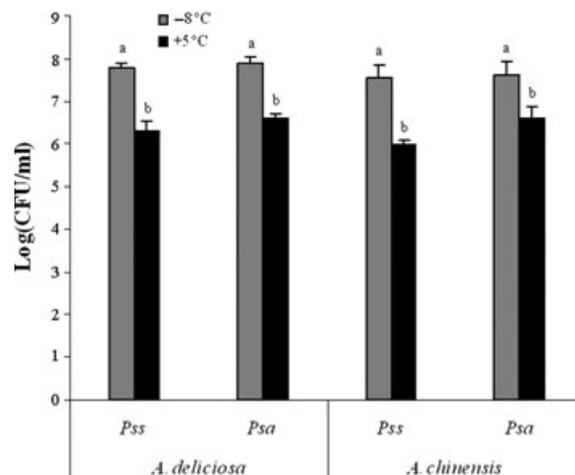


Figure 3 Mean population of *Pseudomonas syringae* pv. *actinidiae* (Psa) and *P. syringae* pv. *syringae* (Pss) in *Actinidia deliciosa* and *A. chinensis* twig inoculation sites after winter frost compared with the unfrozen twigs. The vertical bars show the standard errors of the mean of log populations; different letters indicate the statistical significance at  $P < 0.05$  based on Student's test for the comparison of log means.

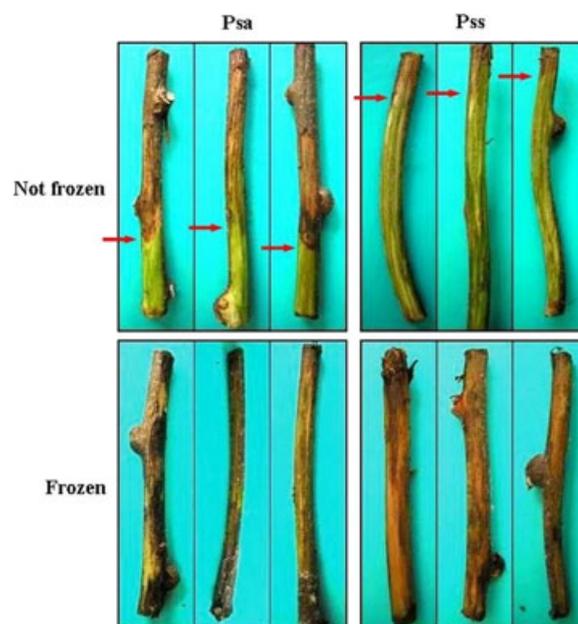


Figure 4 Effects of inoculation with *Pseudomonas syringae* pv. *actinidiae* (Psa) and *P. syringae* pv. *syringae* (Pss) on excised *Actinidia deliciosa* twigs after incubation at 15°C for 7 days. The twigs shown at the bottom of the panel also received frost treatment after bacterial inoculation and before incubation.

frost. This phenomenon occurred in both *Actinidia* species. Arguably, the outbreak of bacterial canker was promoted by pathogen previously established in the area. The release of water and nutrients from damaged plant cell walls might contribute to pathogen multiplication, as one of the major effects of freezing is induction of severe membrane damage (Stepenkus, 1984; Pearce, 2001; Beck

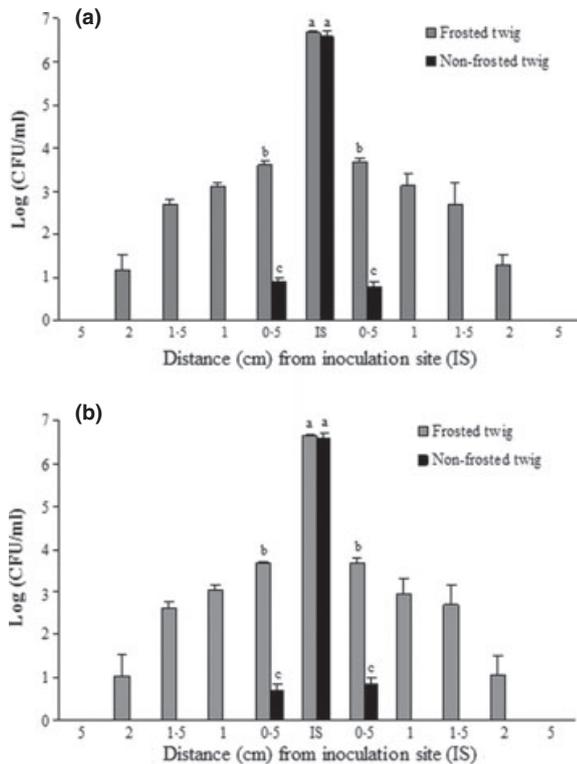


Figure 5 Mean population of *Pseudomonas syringae* pv. *actinidiae* (a) and *P. syringae* pv. *syringae* (b) in *A. deliciosa* twigs at 3 min after the inoculation performed during the thawing process compared with the unfrozen twigs. The figure represents the migration of the pathogen upward and downward from the inoculation site (IS). The vertical bars show the standard errors of the mean of log populations; different letters indicate the statistical significance at  $P < 0.05$  based on Student's test for the comparison of log means.

*et al.*, 2007), and sucrose and other simple sugars accumulate during cold (Mahajan & Tuteja, 2005). In contrast, plants inoculated 1 month before, which did not receive the frost treatment, did not show any symptom, and the pathogen concentration remained stable. Therefore, further studies are needed to identify the mechanism underlying the rapid *in planta* multiplication of the pathogen without the occurrence of frost events.

The autumn frost also promoted the sudden oozing of reddish exudates from the wounds, which, in some cases, contained the pathogen. This observation suggests a crucial step for the rapid dispersal of the bacterium within and between the orchard(s) through rain and wind. In general, reddish exudates are frequently associated with the typical symptoms of bacterial canker during winter events worldwide (Everett *et al.*, 2011; Vanneste *et al.*, 2011; Ferrante *et al.*, 2012; Zhao *et al.*, 2013). Indeed, the dispersal of *P. syringae* in the air is favoured by high humidity (i.e. 70–80%) and largely depends on the size of the drop of water. In addition, the organic matter, by increasing the vapour pressure of the droplets, can confer protection to the aerosolized bacterial cell (Marthi *et al.*, 1990). Therefore, the exudate could effectively confer protection for the spread of *Psa* within and between

kiwifruit orchards. Autumn frost also promoted an effective penetration of the pathogen into the leaf through stomata and/or tiny wounds, as revealed through the consistent reisolation (i.e. 100%) of *Psa* from the water-soaked spots appearing after frost and the inoculation during thawing. Thus, high humidity in the kiwifruit orchard after the autumn frost can potentially favour the effective penetration of the pathogen into the plant leaves. This finding was previously verified in sour cherry leaves infected with *Pss*, and in this case, only a low population of the pathogen (i.e.  $1.2 \times 10^3$  CFU mL<sup>-1</sup>) in the presence of high air humidity was required to initiate the infection (Sule & Seemuller, 1987).

Winter frost promoted the increased multiplication of *Psa* and *Pss* in both *A. deliciosa* and *A. chinensis* twigs compared with the twigs obtained from plants that were not frozen. Similar to the autumn frost, the multiplication of both *Psa* and *Pss* was increased 10 times more than that observed in the unfrozen twigs. Interestingly, the examination of the excised twigs revealed that *Psa* induced longer necrotic lesions than *Pss* in both *Actinidia* species. Although *Psa* is not an ice-nucleation active pathogen (Scortichini *et al.*, 2002), this bacterium causes more extensive damage to twigs during winter frost events than the *Pss* strain isolated from an infected kiwifruit orchard. Moreover, this study confirmed previous findings that *Pss* causes lesions of 2–4 cm in length in *A. deliciosa* cv. Hayward twigs over a 1 month period, with minimum temperatures reaching  $-4^{\circ}\text{C}$  during several days (Scortichini & Rossi, 1991). Whether the occurrence of mixed infection (i.e. *Psa* and *Pss*) promotes more extensive damage remains unknown.

Similar to other fruit tree species, such as peach and apricot, which incur damage through *P. syringae* pathogens (Vigoroux, 1989; Cao *et al.*, 2011), this study demonstrated that during thawing, *Psa* systemically migrates throughout the host vascular system within a few minutes after penetration. During thawing, the ice crystals formed upon the frost, which occupy the intercellular spaces, begin to melt, and the bacteria suspended in the water drops are absorbed into the intercellular spaces (Sule & Seemuller, 1987). Therefore, it is reasonable to conceive that this process occurs several times during winter, as observed for stone fruit crops (Vigoroux, 1999), and *Psa* could effectively colonize the relevant portions of 1-year-old kiwifruit twigs and subsequently migrate to the leader and main trunk during the following season, eventually producing exudates after a frost event. A similar phenomenon has been previously observed for *Agrobacterium tumefaciens* systemically invading grapevines, chrysanthemums and marguerite daisies, for which frost injury can induce a linear or confluent array of small tumours along the vascular system of infected plants (Kado, 2002).

During the antifreeze treatment, the minimum temperature in the orchard was  $-2^{\circ}\text{C}$  for c. 6 h. In contrast to the other frost events, the concentration of *Psa* and *Pss* in the twigs not receiving protection through irrigation was not significantly different from that in the twigs

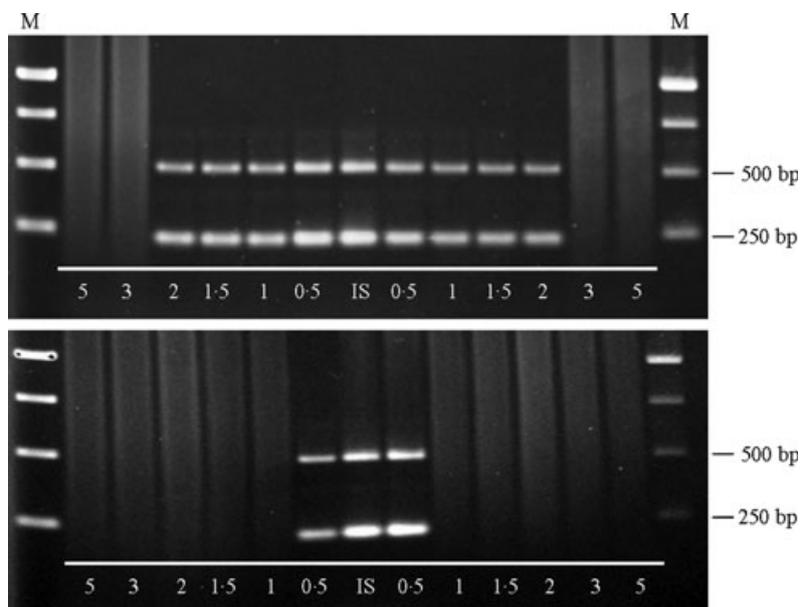


Figure 6 Agarose gel showing the PCR products obtained using the duplex-PCR methods of Gallelli *et al.* (2011) for detecting *Pseudomonas syringae* pv. *actinidiae* (Psa) colonies from the *Actinidia deliciosa* twigs inoculated during thawing. The technique was applied at each twig interval. Also see Figure 5. M: molecular size marker.

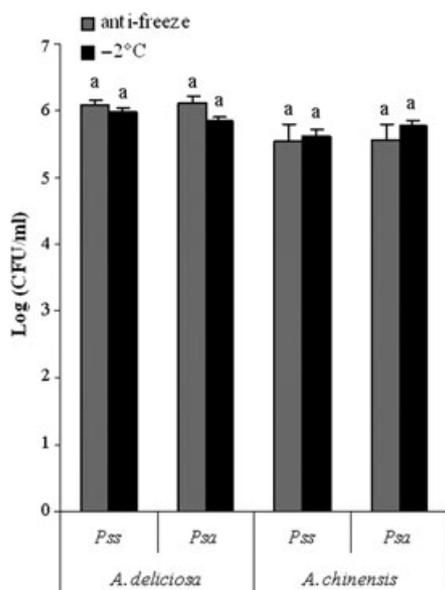


Figure 7 Mean population of *Pseudomonas syringae* pv. *actinidiae* (Psa) and *P. syringae* pv. *syringae* (Pss) in *Actinidia deliciosa* and *A. chinensis* twig inoculation sites after winter frost and the application of an antifreeze irrigation system compared with twigs that were not provided with antifreeze protection at a minimum temperature of  $-2^{\circ}\text{C}$ . The vertical bars show the standard errors of the mean of log populations; different letters indicate the statistical significance at  $P < 0.05$  based on Student's test for the comparison of log means.

receiving the antifreeze treatment. Thus, although the duration of the frost was as long as in the other frost events described above, it is probable that the temperature did not damage the plant cell wall, and consequently, the pathogens did not multiply. This result is consistent with the results of Levitt (1980), showing that

for frosts of short periods (i.e. 2–24 h), the duration of the frost is less important than the extent of the decrease in temperature. It can be argued that antifreeze protection using irrigation sprinklers did not influence the short-term period of Psa and Pss multiplication in both *A. deliciosa* and *A. chinensis* twigs. However, there is some concern that the extensive supply of water could favour the dispersal of the pathogen in the case of exudates.

Collectively, this study confirmed that *A. deliciosa* is more frost tolerant than *A. chinensis*, autumn frosts are more dangerous than winter frosts to these crops, and young kiwifruit plants are also sensitive to frost in the absence of Psa (Pyke *et al.*, 1986; Lu & Rieger, 1990; Warrington & Weston, 1990). Considering the growing importance of both kiwifruit species in global fruit production, breeding programmes to obtain Psa-resistant/tolerant cultivars with frost-resistant traits is desirable. Some *Actinidia* species, such as *A. arguta*, *A. kolomikta* and *A. polygama*, are more tolerant to frost than *A. deliciosa* and could be used for this purpose (Chat, 1995).

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