SHORT COMMUNICATION

THE IMPORTANCE OF THE MAIN COLONIZATION AND PENETRATION SITES OF *PSEUDOMONAS SYRINGAE* pv. *ACTINIDIAE* AND PREVAILING WEATHER CONDITIONS IN THE DEVELOPMENT OF EPIDEMICS IN YELLOW KIWIFRUIT, RECENTLY OBSERVED IN CENTRAL ITALY

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SUMMARY

During the years 2008-2011 repeated isolations from symptomatic organs and plant parts of *Actinidia chinensis* cvs Hort16A and Jintao, grown in the provinces of Latina and Rome (Latium, central Italy), were done with the aim of determining the main colonization and penetration sites exploited by *Pseudomonas syringae* pv. *actinidiae* (*Psa*). Isolations were also made from exudates, pruned twigs and from tissues surrounding elastic laces used to tie young shoots. Artificial inoculations were performed to identify the main avenues for pathogen colonization. Climatic data (maximum, minimum and average temperature as well as rainfall) were recorded by weather stations established in the areas of yellow-fleshed kiwifruit cultivation. The bacterium appeared to be capable of colonizing the host all year round and to systemically migrate from young leaves to twigs. Unprotected lenticels, fruit stalks and leaf scars, as well as pruning wounds and tissues surrounding the elastic laces were easily colonized by the pathogen. The whitish exudates yielded, in many cases, pure cultures of the bacterium. A clear-cut relationship was found between the occurrence of frost events during winter 2007-2008 and the first outbreaks of bacterial canker on *A. chinensis* in the following spring-autumn. Moreover, in 2008, in the infected area, the rainfall was 30-35% higher than the average, which may have promoted the massive spread of the pathogen on the very susceptible yellow-fleshed cultivars. The disease cycle of *Psa* on *A. chinensis* in central Italy is postulated and described.

Key words: bacterial canker, disease cycle, frost, pruning, yellow-fleshed kiwifruit.

Pseudomonas syringae pv. *actinidiae* (*Psa*) the causal agent of bacterial canker of kiwifruit, is currently causing severe economic losses to both yellow-fleshed (*Actinidia chinensis*) and green-fleshed (*A. deliciosa*) cultivars and pollinators world-wide. In Italy, the pathogen was isolated 20 years ago in Latium (central Italy) from *A. deliciosa* (Scortichini, 1994) but it never caused substantial damages to this crop. Suddenly, during spring-autumn 2008, symptoms resembling those incited by *Psa* were observed, again in Latium, for the first time on yellow kiwifruits of cvs Hort16A and Jintao and on their pollinators, namely CK2 and CK3, and the pathogen was always isolated from symptomatic plants and identified (Balestra *et al*., 2009; Ferrante and Scortichini, 2009). During 2009-2010, massive epidemics occurred in the whole Latium region as well as in other regions of northern and southern Italy on both *A. chinensis* and *A. deliciosa*. In-depth molecular studies revealed that the *Psa* population now causing severe damages to kiwifruits differed from that of the past outbreaks in Italy and Japan (Ferrante and Scortichini, 2010; Gallelli *et al*., 2011; Marcelletti and Scortichini, 2011), and did not evolve from the original bacterial population (Marcelletti *et al*., 2011). Currently, a novel virulent population of the pathogen, introduced in new areas most probably through latently infected plant material and/or pollen, is present in four continents causing severe damage everywhere (Scortichini *et al*., 2012).

Epidemiological studies concerning bacterial canker of kiwifruit were mainly conducted in Japan in 1980-1990's (Serizawa *et al*., 1989; Serizawa and Ichikawa, 1993a, 1993b). In Italy, since the first occurrence of the disease in 2008 till now, many isolations were made from symptomatic and symptomless kiwifruit plants in the attempt to determine the main *Psa* penetration sites. In parallel, *ad hoc* artificial inoculations were done to confirm the preferred pathways for pathogen colonization. Some agronomical practices, such as winter pruning and tying of young shoots that cause wounds and/or macroscopic lesions to the plants, were also considered as putatively responsible for enhancing the chances of plant colonization by the pathogen. Thus, *Psa* presence in the tissues surrounding the damaged plant organs was monitored. In addition, some climatic parameters (i.e. temperature, precipitations) were recorded during the last five years in the areas of kiwifruit production of the Latium provinces of Latina and Rome, and the relationship of climatic conditions with the sudden appear-

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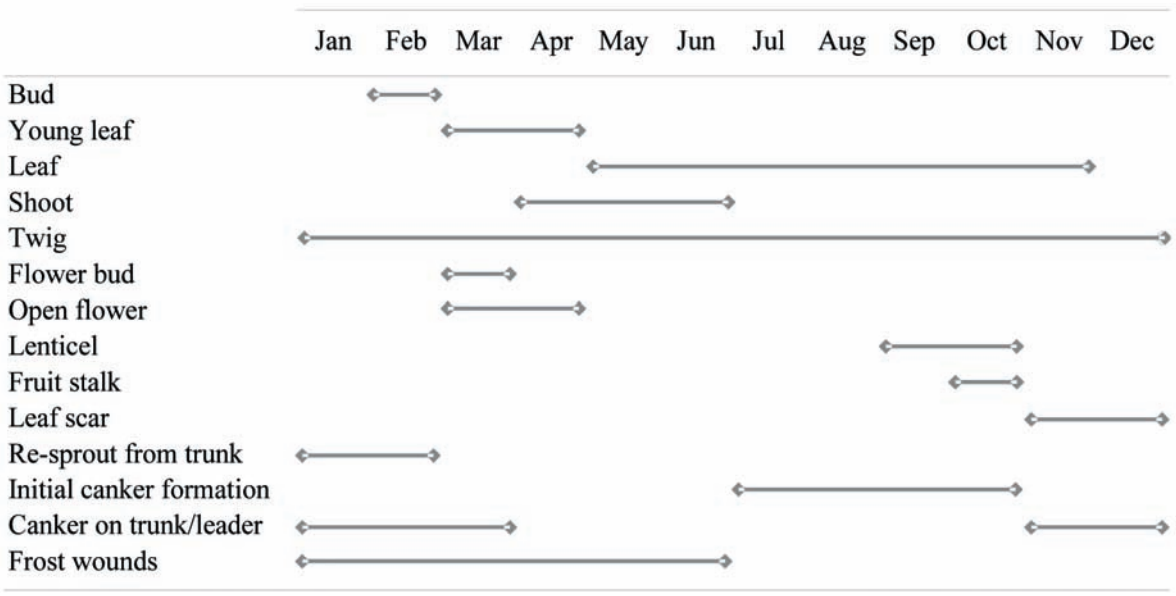

Fig. 1. Organs and plant parts of *Actinidia chinensis* from which *Pseudomonas syringae* pv. *actinidiae* was consistently isolated and the month when its presence was ascertained.

ance of bacterial canker was investigated. In this paper, we postulate that the frost events that took place in November 2007 and February 2008 in the provinces of Latina and Rome acted as the main initial stress factor inciting outbreaks of *Psa* in *A. chinensis* orchards of central Italy.

During 2008-2011, isolations were repeatedly made from the following symptomatic plant organs and parts of *A. chinensis*: leaf buds, young and old leaves, shoots, one-year-old twigs, flower buds, open flowers, symptomless lenticels along the twig, trunk and leader, fruit stalk, leaf scars and suckers. The plants parts indicated in Fig. 1 were collected twice a month from infected orchards and tested for the presence of *Psa,* performing 10 isolations per plant part, so as to define the preference of the bacterium according to the period of the year. In addition, samples were taken from the margin of cankers along the trunk and leader, including the exudates, and from frost injuries immediately after the frost event as well as from woody tissues of twigs two months after frost. Isolations were also done from plant tissue taken from the margin of pruning cuts and from twig lesions caused by the tying of shoots with elastic laces. From each sample, fragments 1-5 mm in size were crushed in sterile mortars in 3 ml sterile saline (0.85% NaCl in distilled water). An aliquot of 0.1 ml of a tenfold serial dilution of the extract was spread on nutrient agar (Oxoid, UK) with 5% sucrose (NSA) and incubated at 25±1°C for 72 h. Whitish exudates oozing from trunks or leaders were directly streaked onto NSA plates or from suspensions diluted (1:10 and 1:100) in sterile saline. Representative levan-positive, round colonies were selected for further identification. All putative *Psa* colonies were preliminary assayed by PCR (Koh and Nou, 2002) and further identified by repetitive-sequence (rep) PCR using BOX and ERIC primer sets (Ferrante and Scortichini, 2009, 2010).

During spring, representative *Psa* isolates (i.e. three isolates obtained during 2008-2011 from each kiwifruit organs and/or plant parts reported in Fig. 1) were inoculated in potted, two-year-old *A. chinensis* cv. Jintao plants. A suspension of *ca* 1-2×10^7 CFU/ml, of a 48 h NA culture was inoculated into the leaf parenchyma next to a vein with a sterile hypodermic syringe via the abaxial side of the leaf. For each isolate, two leaves were inoculated. After inoculation, plants were incubated at 100% relative humidity and natural lighting until the appearance of symptoms. Re-isolations were done after symptom appearance, about 10 days post inoculation (dpi). In all cases, re-isolations from symptomatic plants were successful. To promote penetration, bacterial suspensions were also sprayed on the lower surface of other young and mature leaves or placed directly onto the lenticels. In this case, the assessment of bacterial presence continued for up to one month post inoculation. Re-isolations were always done in NSA. Confirmation of bacterial identity was by PCR according to Koh and Nou (2000) and rep-PCR (i.e. BOX and ERIC primer sets). Moreover, the systemic migration of the pathogen to the shoots through young leaves, as described by Serizawa and Ichikawa (1993c) for green kiwifruit, was determined for yellow kiwifruit by injecting a 10 µl drop of a bacterial suspension into the veins of young leaves (four punctures per leaf, three leaves per plant) and by subsequent re-isolations from surface-disinfected leaf petioles and shoots up to 20 days post inoculation. The experiment was performed on five plants and was repeated for three times. In all cases, the bacterium was

re-isolated from leaf petioles and stems.

Psa was consistently isolated (i.e. recovery was successful from 40% of the buds and 90% of the lenticels) during 2008-2011 from the organs and/or *A. chinensis* plant parts as shown in Fig. 1. The whitish exudates yielded a pure culture of the bacterium in 80% of the cases. The pathogen was also frequently isolated from the margin of the lesions induced by winter pruning and tying of the shoots with elastic laces. Interestingly, *Psa* was isolated until June from woody tissues of symptomless twigs from plants exposed to late winter frosts. In these cases, tissues appeared water-soaked. In summer, during periods of heavy rainfall, it was also possible to observe the initial development of new cankers along the trunk and leader. Noteworthy, *Psa* was frequently isolated also in early spring from suckers that developed after cutting back heavily infected plants in the previous year (i.e. spring-summer). After harvest of 2009-2011, *Psa* was also isolated from all sites that were not naturally protected by suberin such as fruit stalks and leaf scars. The pathogen could be isolated in 70% of the cases from fruit stalks just three days after harvest and from symptomatic lenticels, especially in late summer, in 90% of the cases. Artificial inoculation tests revealed that *Psa* could survive in the tissues surrounding the lenticels.

One-year-old potted *A. chinensis* cv. Hort16A were inoculated by injecting a 1×10^7 CFU/ml bacterial suspension. To determine the presence and multiplication of the bacteria, 5 mm of tissue immediately next to the point of inoculation were sampled during a period of time ranging from 3 days to a month and ground in 1 ml of sterile saline. Then, serial dilutions were spotted onto NSA medium and colonies were counted two days after incubation at 27°C.

Psa is capable to move systemically from the leaves to young shoots of *A. chinensis* through the leaf veins and petioles. In fact, one week after leaf inoculation, the pathogen was recovered from symptomless shoots (i.e. up to 12-14 cm from the shoot tip) and from the longitudinal cankers that appeared along the shoot. Most probably, the *Psa* moves from the leaf veins to the shoot through the xylem vessels as observed by Spinelli *et al.* (2011). From the frequently repetead surveys carried out in 2008-2011 and the subsequent isolations and artificial inoculations, it appeared evident that, in the present epidemic phase, *Psa* could colonize different organs of the host throughout year. The *Psa* disease cycle on *A.*

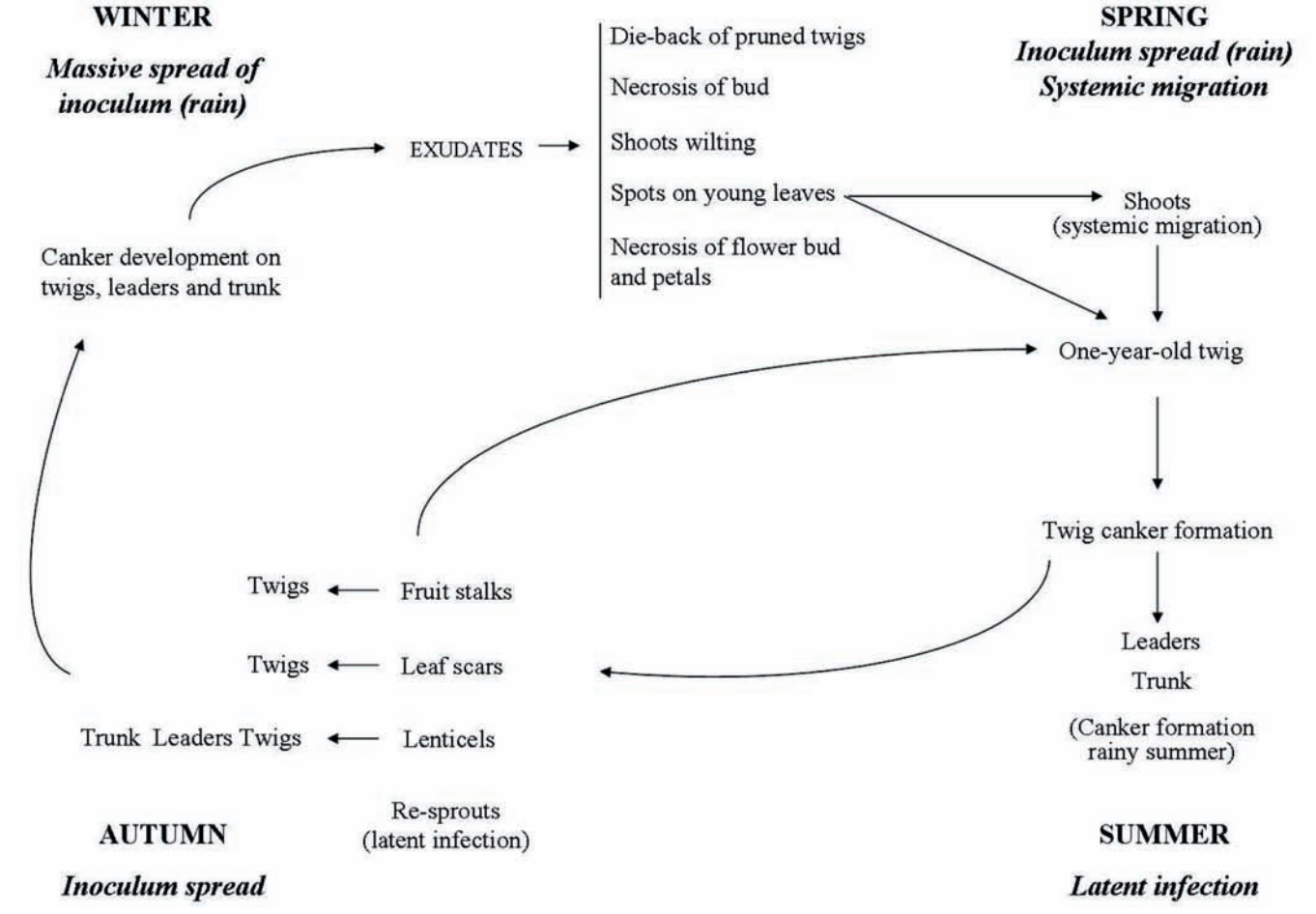

Fig. 2. Cycle of disease of *Pseudomonas syringae* pv. *actinidiae* on *Actinidia chinensis* as verified in Latium (central Italy).

chinensis in central Italy based on the results of this study is shown in Fig. 2.

The cycle starts with inoculum dispersal at the end winter-early spring through exudates oozing from old cankers. Field evidence suggests that, like in Japan (Serizawa *et al*., 1989; Serizawa and Ichikawa, 1993a), in central Italy rain and wind can effectively displace bacterial inoculum to the young shoots. Subsequently, flower buds are attacked and show symptoms (i.e. necrosis) and their infection, depending on the weather conditions, may lead to petal necrosis or massive drop of flowers during full bloom. Another critical part of the cycle, after leaf colonization, is the systemic movement of the pathogen inside the shoot through leaf veins and petioles that allows the bacterium to rapidly reach one-year-old twigs. Once inside, it can start canker formation. Penetration and subsequent colonization of the plant occurs through the lenticels, fruit stalk and leaf scars, as well as through wounds (provoked by early and late frosts, hail, pruning, tying of shoots, etc.). The practice to cut back the trunk of a heavily infected plant, growing in an infected area, and the subsequent training of the new sprouts appears risky since these are easily colonized by the pathogen. The wounds incited by frosts during winter, appear to be the preferred avenues for penetration and subsequent multiplication of the pathogen (Ferrante and Scortichini, unpublished data). In fact, once the wound is penetrated, *Psa* multiplies in

Table 1. Early and late frost events recorded in the areas of *Actinidia chinensis* cultivation of the provinces of Latina (LT) and Rome (RM) from 2007 to 2011 as recorded by thermometers placed in the farms by the Apofruit technical staff.

Date of frost event	Municipality/Province	Min T recorded (°C)	Event duration (h)
November 4 2007	Velletri (RM)	- 8	6
	Aprilia (LT)	- 9	6
	Latina (LT)	- 7	4
	Cisterna (LT)	- 5	4
November 5 2007	Velletri (RM)	- 2	4
	Aprilia (LT)	- 2	4
	Latina (LT)	- 1	4
	Cisterna (LT)	- 1	4
February 17 2008	Velletri (RM)	- 8	7
	Aprilia (LT)	- 9	7
	Latina (LT)	- 7	6
	Cisterna (LT)	- 5	6
February 1 2010	Velletri (RM)	- 5	3
	Aprilia (LT)	- 5	3
	Latina (LT)	- 3	3
	Cisterna (LT)	- 2	3
December 17 2010	Velletri (RM)	- 11	10
	Aprilia (LT)	- 12	10
	Latina (LT)	- 8	8
	Cisterna (LT)	- 7	8
March 8 2011	Velletri (RM)	- 8	6
	Aprilia (LT)	- 8	6
	Latina (LT)	- 7	5
	Cisterna (LT)	- 5	4

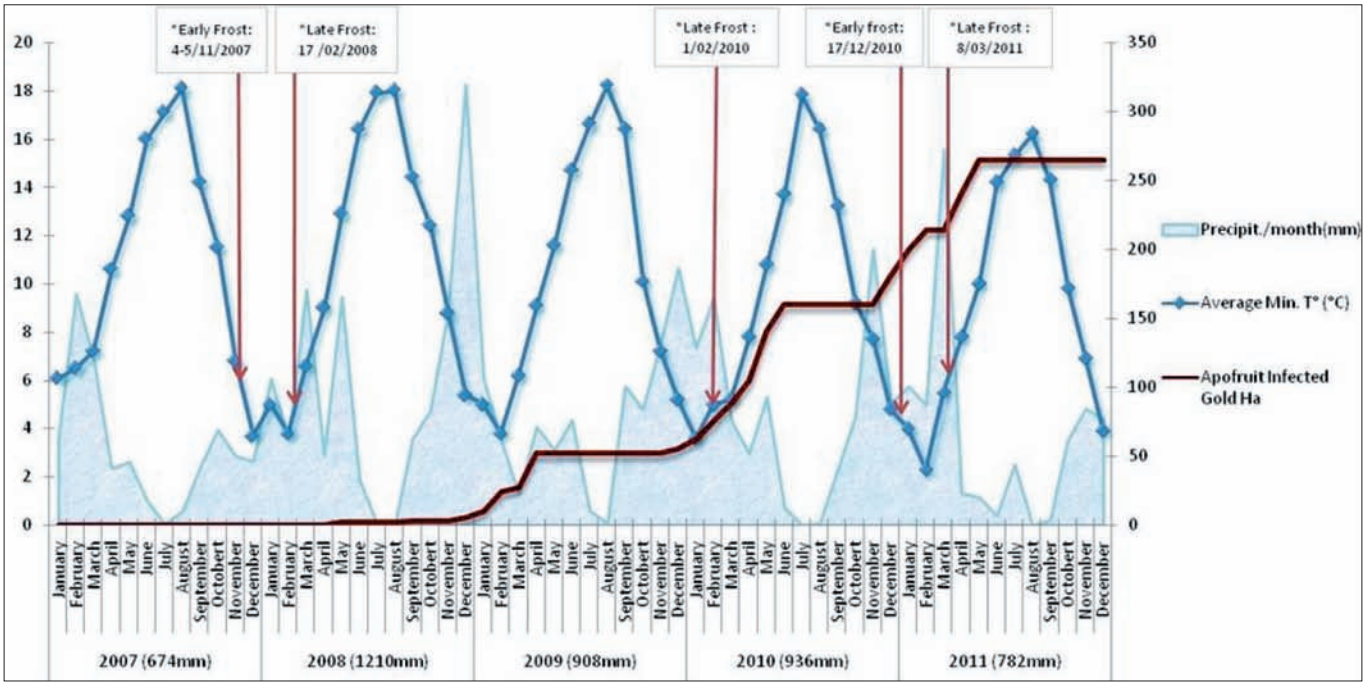

Fig. 3. Average temperatures and precipitations recorded from 2007 to 2011 in the kiwifruit production areas of Latina and Rome provinces. For each year, the total rainfall is reported in brackets. The purple curve shows the increase of bacterial canker incidence in the *Actinidia chinensis* surface area (Ha) tended by Apofruit-Aprilia in the same period. See also Table 1 for the frost events.

the surrounding tissues in spring and early summer.

The average temperature and precipitations of the last five years recorded in the areas of *A. chinensis* cultivation of the provinces of Latina and Rome are reported in Fig. 3, whereas Table 1 reports the frost events recorded in the same period. Fig. 3 also shows the cumulative hectares of yellow-fleshed kiwifruit cultivars, managed by Aprofruit Italy-Aprilia, affected by bacterial canker in the same period, in the same area. There is an obvious relationship between the frost events of 2007-2008 (see also Table 1) and the first outbreaks of bacterial canker. In the orchards, the first visible signs of the disease (i.e. leaf spotting) were observed in May-June 2008, as confirmed by isolations performed in that period (Ferrante and Scortichini, 2009), whereas the first signs of canker formation along the trunk and leader appeared in November-December 2008. It should be pointed out that the frost of November 4th and 5th 2007, damaged plants that were not yet in the dormant stage for, in central Italy, in that period a large number of leaves still persist. A clear-cut increase in bacterial canker spreading was observed after the severe late frost of February 1st 2010, as well as after the frost events of winter 2010-2011. In 2008 the total precipitation in the kiwi-growing area was 1,210 mm, i.e. 30-35% higher than the yearly average of 850-900 mm. In particular, October and November 2008 were very rainy, a condition that may have contributed either to the initial massive spread of *Psa* or to its rapid multiplication (i.e. canker formation) in the very susceptible *A. chinensis* cvs Hort16A and Jintao. To worsen the situation, during the same period very circumscribed measures for reducing *Psa* inoculum had been enforced, limitedly to a few orchards.

Frost alone or in combination with ice-nucleating strains of *P. syringae* is/are well known as major predisposing factor(s) for the subsequent colonization of micro-organisms, including pathogens belonging to the *Pseudomonas syringae* complex (Weaver, 1978; Sobicewski and Jones, 1992; Vigoroux, 1989; Cao *et al*., 1999; Kennelly *et al*., 2007; Tomihama *et al*., 2009; De Kam, 2010). However, in the case of bacterial canker of yellow-fleshed kiwifruit in central Italy, the role(s) played by the possible presence of ice-nucleating *P. syringae* pv. *syringae* (*Pss*) strains in *A. chinensis* tissues remains to be determined. Interestingly, it was previously ascertained that *Pss* was capable of inducing necrotic lesions to *A. deliciosa* tissues during the dormant period of the plant in presence of temperatures below 0°C (Scortichini and Rossi, 1991). In any case, it seems plausible to conclude that frost alone, by causing wounds that were subsequently colonized by *Psa,* would have largely contributed to the initial outbreaks of bacterial canker, as supported by the dramatic increase of the acreage damaged by *Psa* following the frost events of 2007-2008, 2010 and 2010-2011 (Fig. 2). Frost can also act as a stress factor, inciting the virulence of pathogens (Arnold *et al*., 2007). In fact, under stress conditions in

the host, the bacterial competence for DNA uptake is activated so that a pathogen can acquire foreign genetic material, possibly including pathogenicity and/or virulence factors.

Anecdotal observations refer to the observation of symptoms (i.e. leaf spotting) resembling those caused by *Psa,* on *A. chinensis* cultivated in the provinces of Latina and Rome also in 2007. If this were true, it would represent a further confirmation that the frosts of winter 2007-2008 massively enhanced the colonization of the wounds by the pathogen since its presence in the area greatly augmented during 2008.

Pruning wounds can enhance the penetration of phytopathogenic pseudomonads in fruit trees as reported for *P. s*. pv. *persicae, P. s.* pv. *syringae* and *P. s*. pv. *morsprunorum* in peach, sweet cherry and plum, respectively (Young, 1988; Vigoroux and Bussi, 1998; Hinrichs-Berger, 2004; Carroll *et al.,* 2010). *Psa* can infect pruning wounds, possibly, long after the cut. Furthermore, this bacterium has very frequently been found in the tissues surrounding the elastic laces used to tie the young shoots to the poles, possibly as a consequence of the lesions provoked by the laces to the growing shoots. Therefore, common agronomical practices such as pruning and tying can easily open avenues to *Psa* penetration and colonization of host tissues.

It can be concluded that a combination of factors seems to have played a role of paramount relevance in initiating the epidemics of bacterial canker on *A. chinensis* in central Italy consequent to: (i) presence of very susceptible cultivars such as Hort16A and Jintao extensively grown, in some cases, on a large acreage; (ii) presence of a very aggressive population of the specific pathogen; (iii) occurrence of predisposing stress factors like winter frosts and heavy rainfalls, that enable the massive colonization, multiplication and dispersal of bacterial inoculum in the area; (iv) underestimation of the risk potential during the early phases of the epidemics; (v) delayed implementation of measures aiming at lowering the inoculum potential in the whole area; (vi) unavailability of registered chemicals for control.

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