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SHORT NOTES

Pollen as a possible pathway for the dissemination of *Pseudomonas syringae* pv. *actinidiae* and bacterial canker of kiwifruit

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Summary. Pollen collected in a kiwifruit orchard with symptoms of bacterial canker and naturally contaminated by *Pseudomonas syringae* pv. *actinidiae* (Psa), was used to pollinate an experimental orchard, in order to confirm its role, under commercial orchard conditions, in disseminating the pathogen and, possibly, contributing to disease spread. A pollen lot, certified free from Psa, was used with the same methods as a control. Two pollination techniques were used: dusting (dry pollen) and spraying (pollen suspension in water). The orchard was monitored during 2 years from experimental pollination, with regular sampling of flowers, fruits, leaves, and vines, to check for Psa as an epiphyte or endophyte, and for bacterial canker symptoms. Psa was recovered from flowers, fruitlets and leaves during the first season, mainly in plots where contaminated pollen had been sprayed in water suspension. From early August until harvesting time (mid-October), Psa detection was possible only on leaves. No symptoms developed during the first season after pollination. No endophytic Psa was detected in pruned vines in the following winter. During the second season, detection and isolation of Psa was erratic, but direct isolation was achieved from four plots. During the second season after pollination, typical leaf symptoms were observed on a few vines, and Psa was isolated and identified. Our results suggest that Psa could be disseminated via contaminated kiwifruit pollen as a pathway for spread of bacterial canker. However, further pollination experiments are needed to establish, beyond any doubt, whether contaminated pollen may contribute to possible disease outbreaks.

Key words: *Actinidia deliciosa*, artificial pollination, pathogen survival, disease spread.

Introduction

In good agricultural practices, artificial pollination of kiwifruit is extensively used by orchardists, to increase flower fertility, enhance fruit size and increase yields (Hopping and Hacking, 1983; Gonzales *et al*., 1998). The mechanical application of pollen in kiwifruit orchards is usually achieved using two techniques: either dusting with dry pollen applicators or application in water suspensions with a pumping/spraying device. In both cases, usually, 400–500 g of pollen (250 to 750 g) are applied per ha, through dusting or spraying under the crop canopy (Galliano *et al*., 2008).

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Pollen of *Actinidia* spp. has been shown to be a good substrate for *Pseudomonas syringae* pv. *actinidiae* (Psa) colonisation and survival (Gallelli *et al*., 2011b; Vanneste *et al*., 2011) and as a pathway for its spread (Stefani and Giovanardi, 2011). Pollen may therefore serve as vehicle for dispersal of Psa in kiwifruit orchards or, in other cases, transfer the bacteria from infested to non-infested areas (Biosecurity New Zealand, 2010; MAF, 2011). An investigation in New Zealand of 250 pollen samples during MAF surveillance activities, aiming to understand initial outbreaks of Psa in local kiwifruit orchards, confirmed that Psa was widely present on pollen, but undetected since 2007 (Richardson *et al*., 2012). Although Card *et al*., (2007) concluded that there are no pollen transmitted bacteria, other authors have observed that pollen dispersal of bacterial pathogen and disease outbreaks might be connected in some pathosystems.

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For example, pollen dispersal has been suggested for *Erwinia amylovora* and fireblight of pome fruits (van der Zwet and Bell, 1992; Johnson and Stockwell, 1998), and for *Xanthomonas arboricola* pv. *juglandis* and bacterial canker of walnut (Garcin *et al*., 2001; Giovanardi *et al*., 2010). A Pest Risk Analysis for Psa was prepared and published by EPPO (2012): pollen was recognized as a possible pathway for Psa, although further studies were suggested to confirm if pollen could spread the pathogen. In December 2012 the European Commission published a Decision regarding measures to prevent the introduction and spread of Psa within the Union. Article 2 of this Decision specified that pollen lots may be introduced into the Union only if they are each accompanied by a phytosanitary certificate (European Commission, 2012). This Decision officially recognizes pollen as a possible pathway for the introduction and spread of Psa. Several companies are providing *Actinidia* pollen for kiwifruit pollination, and worldwide trade of pollen may be as much as several tons per year. This trade has been from one hemisphere to another one, thus increasing the risk of movement of Psa.

To date, there has been no indication that survival and dispersal of Psa through pollen by pollination in commercial kiwifruit orchards may be related to the establishment of local Psa populations and with outbreaks of bacterial canker. Therefore, a kiwifruit orchard was planted in isolation, in a field more than 100 km away from any area where commercial kiwifruit orchards were present. Two different pollination techniques were applied in the orchard, with Psa-free or naturally contaminated pollen, to assess the likelihood of Psa survival and dissemination, and development of bacterial canker.

Materials and methods

Plants and pollen

Two-year-old female plants of *Actinidia deliciosa* cv. Hayward were used to plant an experimental orchard south of Reggio Emilia, Emilia Romagna region, Italy, in December 2011. Male plants were also planted, one for each five female plants. Orchard planting and management followed the usual commercial kiwifruit production practices in the Emilia Romagna region (www.ermesagricoltura.it).

Pollen was collected in May 2011 from a kiwifruit orchard located in the Province of Ravenna, Romagna, Italy. The orchard showed bacterial canker symptoms at the time of pollen collection. A pollen lot (approx. 150 g) was analysed for the presence of Psa and found to be contaminated with the pathogen: assessment was carried out using the detection methods of Rees-George *et al*. (2010). Pollen analysis was further carried out in triplicate on three subsamples obtained from the contaminated lot following the methods described by Vanneste *et al*. (2011) and Stefani and Giovanardi (2011). Contamination by Psa was assessed to be 6×10^4 cfu g^{-1}. Commercial, non-contaminated pollen was used as a control, after analysis carried out in parallel with the contaminated pollen. Pollen samples were stored at -20°C until used for pollination.

Experimental treatments

Four treatments of four plots each, randomly distributed, were applied to the orchard plants. These were: 1) dry and 2) wet pollination with naturally contaminated pollen; 3) dry and 4) wet pollination with non-contaminated pollen. The pollination treatments were applied to the plants on 21 May 2012. Dry pollination was carried out using a plastic dust sprayer. Wet pollination used a suspension of 4 g of pollen in 1 L of sterile distilled water. Ten g of contaminated pollen or 10 g of non-contaminated pollen were used in total during the pollination of each treatment , applied to four adult plants in four replicates (total of 16 plants per treatment). This applied approx. 440 g of pollen per ha, slightly less than recommended for adult kiwifruit orchards (approx. 500 g ha^{-1}; Galliano *et al*., 2008). Each replicate was separated from the neighbouring plots by an additional untreated adult plant. Pollination was carried out when approx. 90% of flowers were opened.

Monitoring and identification of Psa populations

Monitoring and sampling started the day following pollination and were then carried out bi-weekly thereafter, until mid October (20–21 weeks after pollination) during the two following growing seasons (2012 and 2013). Sampling was of flowers, fruitlets, fruits and leaves, and took five of each of these per plot (replicate). For each sampling time, samples were either five leaves, flowers or fruitlets / fruits per plant. Each sample was washed in an appropriate quantity of 0.5% Tween in sterile saline in an Erlenmeyer flask on a rotary shaker for 1 h at room temperature (24

± 3°C). Washing fluids were filtered into centrifuge tubes through sterile gauze and centrifuged al 10,000 × g for 20 min at 4°C. Pellets were then suspended in 1–2 mL of sterile, distilled water. The suspended pellets and their 1:10, 1:100, 1:1000 dilutions were used to confirm the presence of Psa by PCR (Rees-George *et al*., 2010) and plating onto modified NSA (Crosse, 1959), supplemented with 80 ppm cephalexin and 200 ppm cycloheximide. Putative Psa colonies, isolated and purified on modified NSA, were subject to hypersensitive reaction (HR) on tobacco mesophyll (Klement, 1963); HR positive colonies were further identified by PCR (Rees-George *et al*., 2010; Gallelli *et al*., 2011a) and confirmed by rep-PCR using the BOX primer (Versalović *et al*., 1991; Scortichini *et al*., 2002; Vanneste *et al*., 2010). As positive controls, three pure cultures were used: Psa NCPPB 3739 (Japanese strain, low virulence), Psa ISF ACT1 (Italian strain, high virulence) and *Pseudomonas syringae* pv. *syringae*, strain DLS 427, isolated from necrotic leaf spots on *Actinidia deliciosa* and present in the bacteria collection of the Department of Life Sciences, Reggio Emilia, Italy.

Monitoring symptom development

During each sampling day, both in 2012 and in 2013, kiwifruit vines were carefully inspected by one operator per side for typical symptoms of bacterial canker, especially searching for spots and lesions on leaves. Any organ showing symptoms resembling those by Psa was sampled and analysed within 1 h, to allow maximum recovery of Psa. Direct isolation, HR screening on tobacco mesophyll of suspected colonies and PCR (described above) were used to identify bacteria from possible disease samples.

Results and discussion

Results of Psa analyses and detection for both years of the experiment are shown in Tables 1 and 2. Table 1 shows data of detection of Psa within 2 weeks after pollination. Isolation of Psa confirmed the establishment of the pathogen on leaves and fruitlets. Psa was easily detected in washing fluids with the PCR protocol of Rees-George *et al*. (2010), and confirmed using the PCR protocol of Gallelli *et al*. (2011a). The application of both protocols gave the expected amplicons, thus confirming the presence of Psa associated with the field samples collected after pollination with contaminated pollen. Direct isolation from flowers and leaves on all those plots was always positive for Psa, confirming the viability of the pathogen in the orchard 1 day after pollination. On plants pollinated with Psa contaminated dry pollen (dusting), direct isolation on modified NSA showed a number of colonies about 100 times less than for plants pollinated with con-

Table 1. Establishment of detectable *Pseudomonas syringae* pv. *actinidiae* (Psa) populations on kiwifruit plants after pollination with contaminated pollen (wet and dry pollination). On flowers and leaves (1st), sampling was carried out 24 h after pollination (first and second column). On fruitlets and leaves (2nd), sampling was 2 weeks after pollination (third and fourth column). From the third sampling, Psa populations on leaves and fruits were not calculated, but detection was done by PCR and direct isolation (DI).

Treatment	Sampling[a] (cfu mL^{-1}/ cfu mL^{-1}) 2012–2013			
	Flowers[b]	Leaves 1st sampling	Fruitlets	Leaves 2nd sampling
Wet pollination (clean pollen)	-/-[c]	-/-	-/-	-/-
Wet pollination (contaminated pollen)	2×10^4/ -	5.75×10^2/ -	3.50×10^3/ -	PCR+ and DI+ / PCR+ and DI-[d]
Dust pollination (clean pollen)	-/-	-/-	-/-	-/-
Dust pollination (contaminated pollen)	3.75×10^2/ -	4.25×10^2/ -	5.0×10^2/ -	-/-

[a] cfu mL^{-1}, Average number of colony forming units obtained from four replicates of four plants.
[b] 2012/2013 = Psa detection in 2012/Psa detection in 2013.
[c] Psa was not detected in either year.
[d] PCR and/or direct isolation (DI) were positive in at least one replicate.

Table 2. Time of sampling and detection of *Pseudomonas syringae* pv. *actinidiae* (Psa) in field experiments after pollination (wet and dry) with naturally contaminated pollen in 2012. In 2013 pollination was natural and first sampling was taken at full bloom. Results shown summarise detection of Psa in two following growing seasons, 2012 and 2013. The epiphytic presence of Psa in 2013 was erratic and on leaves only. An asterisk indicates that symptoms associated with presence of Psa were detected on plants during the 2013 season.

Method of pollination	Time of sampling after pollination[a] (2012/2013)[b]							
	24 h	2 wks	4 wks	6 wks	8 wks	12 wks	16 wks	21 wks
Wet (Flowers)	+/-							
Wet (Fruits)		+/-	+/-	+/-	+/-	-/-	-/-	-/-
Wet (Leaves)	+/+	+/+	+/+	+/+*	-/-	+/+*	+/-	+/-
Dust (Flowers)	+/-							
Dust (Fruits)		+/ -	+/ -	-/-	-/-	-/-	-/-	-/-
Dust (Leaves)	+/-	+/-	-/-	+/+	+/+*	-/-	-/-	-/-

[a] Hours/weeks after pollination (artificial in 2012 and natural in 2013). First sampling was carried out 24 h after artificial pollination on flowers and leaves in 2012 and at full bloom in 2013. Other samplings were bi-weekly in both growing seasons (2012–2013), from the end of bloom to harvesting (mid October).

[b] 2012/2013 = Psa detection in 2012/Psa detection in 2013. Positive or negative Psa detection in 2012 and 2013 in the plots.

* Foliar symptoms, typical for Psa infection, were seen and sampled, and from those symptoms the pathogen was isolated in pure culture and identified as Psa.

taminated wet pollen. More precisely, direct isolation gave recovery of approx. 4×10^2 cfu mL^{-1} from wet pollination and approx. 2×10^4 cfu mL^{-1} for dry pollination, from the first sampling. This was probably due to the more favourable surface conditions produced by wetting, where bacteria could take advantage of a thin water layer for survival. Flowers always gave greater numbers of bacteria than leaves, where samples were collected from the same plot: the final concentrate obtained from five flowers contained an average of 2×10^4 cfu mL^{-1} (4×10^3 cfu per flower), whereas the final concentrate from leaf washings contained approx. 4–6 × 10^2 cfu mL^{-1} (0.8–1.2 × 10^2 cfu g^{-1} leaf fresh weight). In similar experiments carried out under controlled conditions in a containment glasshouse (Stefani and Giovanardi, 2011), contaminated pollen transferred viable Psa cells to kiwifruit flowers and leaves. Flowers represented a good location for survival and, possibly, initial multiplication of small Psa populations, especially in case of wet pollination.

Analysis of washings from fruitlets and fruits allowed detection of epiphytic Psa populations until early August 2012 from plants pollinated with wet contaminated pollen, whereas fruitlets or fruits collected from dust-pollinated plants were positive for Psa, mainly only with PCR, until early July. On leaves, Psa was regularly detected during May to early August on wet-pollinated plants. Thereafter, detection of the pathogen was erratic, but sometimes also positive in October. Psa was not detected on leaves of dust-pollinated plants from early July onwards (Table 2).

During wintertime, samples of pruning residues were analysed for endophytic presence of Psa for each treatment and replicate in the experiment. Psa was not detected on that plant material, either with PCR or direct isolation.

During the growing season of 2013 (May to October) Psa detection and isolation were erratic. Three samples from wet pollinated plots and two samples from a dust pollinated plot were positive for Psa in May, June and July (Table. 2). These results suggest that small Psa populations may have survived during the winter and then multiply in spring of the following year. Detection of Psa during the second growing season after artificial pollination was successful in scattered sites inside the orchard. Psa was also detected on a few control plants, which were pollinated with non-contaminated pollen the previ-

ous year. Detection and isolation of Psa in 2013 indicated establishment and movement of the pathogen inside the orchard, possibly from wind driven rain or pollinating insects.

During the spring and summer of 2013, necrotic spots resembling Psa lesions were seen on leaves of three wet-pollinated plants out of 16 (disease incidence = 19%), that had been pollinated the previous year, and one dust-pollinated plant out of 16 (incidence = 6%). From those lesions Psa was isolated. A comparison of their BOX fingerprints with those obtained from Psa characterized the previous year confirmed their identity. Therefore, we assume that the bacteria were from the contaminated pollen used the previous year for pollination. To our knowledge, this is the first report of the possibility of Psa spread inside a kiwifruit orchard from contaminated pollen, and its connection with typical foliar lesions caused by the pathogen. The disease was only observed as leaf spots. No cankers were observed on any vines until autumn 2013. No symptoms were detected on any of the plants pollinated with non-contaminated pollen, throughout the season.

These results suggest that Psa overwinters on host plants. Since no symptoms were observed during 2012, and all samples collected to detect the pathogen internally in vines were negative for Psa, our data suggest that overwintering of the pathogen could be in dormant buds, as described for other *Pseudomonas syringae* pathovars (Kennelly *et al*., 2007) or *Erwinia amylovora* (Bonn, 1978; Paulin, 1981). One symptom described for Psa in early season is the browning and necrosis of buds (Balestra and Varvaro, 2008), thus confirming the importance of buds for pathogen overwintering. In our orchard no infection started from leaf scars, thus leading to possible formation of cankers along the vines. Cankers were never observed during the 2013 growing season. Furthermore, no endophytic Psa was detected in vines during the 2012–13 winter. The detection of first symptoms as foliar necrotic spots is in agreement with results from other studies, documenting that Psa can infect plants through stomata and hydatodes (Serizawa *et al*., 1989). The pathogen may also enter host plants through wounds (e.g. hail damage or pruning cuts).

Molecular detection of Psa and its isolation on semiselective medium showed that establishment of epiphytic Psa populations in the orchard from contaminated pollen is possible, although establishment was much less when pollen was applied as a dust. Detection and isolation of Psa from wet-pollinated plants was more consistent, probably because prolonged leaf/flower wetness supported the early stages of Psa colonisation, as previously observed (Stefani and Giovanardi, 2011). Leaf/flower wetness may have supported Psa as a plant epiphyte, especially because the pollination treatments were applied shortly before sunset. The wet pollination treatment may have solubilised metabolites on leaf and flower surfaces assisting bacterial survival and growth.

The detection of typical disease symptoms the following year after pollination only on a few plants (three out of 16 for the wet pollination treatment) may indicate that naturally contaminated pollen was not carrying enough inoculum to cause an outbreak within the season of pollen application. Nevertheless, the results presented here indicate that pollen transfer has potential to start initial disease foci and disease establishment, as is frequently the case for phytopathogenic bacteria with epiphytic life cycle phases (Sigee, 1993). If contaminated pollen originating in areas where bacterial canker is present is not checked/certified and is applied for two or more seasons in a kiwifruit orchard, then the potential of establishment of small Psa populations in a disease free area may be high.

Our results indicate how Psa may have been spread from continent to continent. The international trade of kiwifruit pollen has been very difficult to monitor, as no traceability has been requested during past years. A rough calculation of possible pollen lots traded yearly on world markets indicates a quantity of approx. 35–40 tons (Stefani, unpublished). Inconsistencies and uncertainties in information related to the viability of Psa on pollen, the role of pollen in spreading the pathogen and the disease, have been expressed several times in the recent past (MAF, 2011; EPPO, 2012). The role of pollen in initiating bacterial canker in kiwifruit orchards after pollination has been questioned. Nonetheless, Biosecurity Australia (2011) rated the probability of pathogen spread as high, once introduced through contaminated pollen. Our preliminary experimental data suggest that a naturally contaminated pollen lot with a bacterial load of 6×10^4 cfu g^{-1}, might be sufficient to contaminate an orchard, to build up small, scattered population of Psa on the canopy and to possibly cause a disease outbreak 2 years after artificial pollination. Since our experimental kiwifruit

orchard was planted in isolation conditions (at least 100 km from the nearest kiwifruit orchards) it is unlikely that the Psa we isolated originated from other infected orchards present in the eastern part of the Emilia Romagna region. However, further pollination experiments are needed to establish, beyond any doubt, whether contaminated pollen may contribute to possible disease outbreaks. This final demonstration will greatly help assessors involved in pest risk analysis (PRA), National Plant Protection Organisations and stakeholders to carefully evaluate the need to establish the phytosanitary status of pollen and its possible certification as pathogen-free.

Phytopathogenic bacteria, and among them Psa, may spread over long distances through trade of plant propagation material, such as cuttings, plantlets and *in vitro* plants. For Psa, this has been consistently highlighted in the published PRAs (i.e. Biosecurity Australia, 2011; EPPO, 2012). Therefore, the introduction and spread of Psa in several kiwifruit orchards in Europe, New Zealand and Chile may be linked with the trade of such multiplication material. Mazzaglia *et al*. (2012) reported that all isolates of the virulent type of Psa obtained from the recent European disease outbreak belong to one genetic lineage, suggesting a possible Chinese origin of the European and New Zealand epidemics. More recently, Butler *et al*. (2013), analysing the presence of shared SNPs in the core genomes of Psa isolates from New Zealand, Italy and Chile, suggested that the close similarities of these genome sequences indicates a very recent common ancestor of Chinese origin. Therefore, the recent disease outbreaks in Italy and New Zealand are unlikely to have derived one from another, but they have China as a common source. The usual worldwide non-traceability of pollen lots during the last decades, particularly relating to origin and possible mislabelling (as reported in at least one case in New Zealand by the New Zealand Commerce Commission), suggests that much more pollen quantities of possible Chinese origin have been marketed before and during the first years of disease outbreaks. On this point very few data are available (European Commission, 2013). Nonetheless, reports of Chinese pollen possibly contaminated with Psa are available (MAF, 2011).

Our experiments have highlighted the biological possibility of pollen-associated spread of Psa in kiwifruit orchards after artificial pollination, and later development of bacterial disease. Further studies using tagged strains of Psa would confirm if pollen transfer occurs for this pathogen, and would add an important component to international and local PRAs for this key pathogen threatening international kiwifruit production (Biosecurity Australia, 2011; EPPO, 2012; MPI, 2013).

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