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Incidence of *'Candidatus* Liberibacter europaeus' and phytoplasmas in *Cacopsylla* species (Hemiptera: Psyllidae) and their host/shelter plants

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Abstract Psyllids, as vectors of phloem-restricted plant pathogens, are serious agricultural pests. Fruit tree phytoplasmas are transmitted by different *Cacopsylla* spp., while other psyllids are known vectors of liberibacters. Recently, the bacterium '*Candidatus* Liberibacter europaeus' was found in pear trees and in *Cacopsylla pyri* (Linnaeus), the vector of '*Ca*. Phytoplasma pyri'. This new species does not cause symptoms in plants and is probably a symbiont rather than a pathogen. Based on these findings and the assumption that '*Ca*. Liberibacter europaeus' is widespread, we studied its distribution in the genus *Cacopsylla* and in the respective host and shelter plants (where psyllids aestivate and overwinter), as well as its possible co-presence with '*Ca*. Phytoplasma' spp. We tested 14 *Cacopsylla* species and 11 plant species from north-western Italy, Hungary and Israel, characterized by warm oceanic, temperate continental and warm Mediterranean climatic conditions, respectively. '*Ca*. Liberibacter europaeus' was common within the *Cacopsylla* genus, being present in nine of the 14 species screened as well as in most host plants, whereas none of the shelter plants tested positive for this bacterium. Altogether, these findings indicate the presence of '*Ca*. Liberibacter europaeus' in continental zones, whereas it does not seem to be widespread in the Mediterranean region. Furthermore, lack of specific symptoms in all infected plants confirms an endophytic relationship with this bacterium, while its abundance in insects suggests a beneficial role for the host. Co-infections with phytoplasmas, observed in insects and plants, require further study to evaluate the possible interactions between them.

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Introduction

As vectors of phytopathogenic agents, many psyllids represent important pests of agricultural crops. The main plant pathogens associated with and transmitted by psyllids are viruses, such as the *Potato rugose stunting virus* (Tenorio *et al.* 2003), and bacteria such as '*Candidatus* Liberibacter' spp. and '*Candidatus* Phytoplasma' spp. (Hodkinson 2009). In the last few years, researchers have focused their interest on '*Ca.* Liberibacter' spp. because of the serious economic losses they cause worldwide. The genus '*Ca.* Liberibacter' includes phloem-limited Gram-negative bacteria belonging to the α-subdivision of the Proteobacteria (Jagoueix *et al.* 1994). To date, five species have been identified: Huanglongbing (HLB) disease, also known as citrus greening, is associated with '*Ca.* Liberibacter asiaticus', '*Ca.* Liberibacter americanus' and '*Ca.* Liberibacter africanus' and affects many citrus cultivars, as well as wild citrus. '*Candidatus* Liberibacter asiaticus' is present in Asia and America. '*Ca.* Liberibacter americanus' is common in the Americas, and '*Ca.* Liberibacter africanus' occurs in Africa (Gottwald 2010; Lin 1956; McLean and Oberholz 1965; Teixeira *et al.* 2005). '*Ca.* Liberibacter solanacearum', also known as '*Ca.* Liberibacter psyllaurous', is associated with newly emerging and economically important diseases of solanaceous crops. '*Ca.* Liberibacter solanacearum' is present in New Zealand (Liefting *et al.* 2008) and the Americas (Hansen *et al.* 2008; Munyaneza *et al.* 2009; Wen *et al.* 2009), and it was recently observed in Europe in carrots (Munyaneza *et al.* 2010). A new species described in Europe, '*Ca.* Liberibacter europaeus' (hereafter referred to as CLeu), infects *Cacopsylla pyri* (Linnaeus) and pear plants. However, even though the percentage of plants hosting CLeu was quite high, no specific symptoms were observed in infected pears, leading to the proposal that this bacterium behaves as an endophyte rather than a pathogen (Raddadi *et al.* 2011). Furthermore, studies of CLeu in *C. pyri* revealed high infection rates (Raddadi *et al.* 2011), suggesting that this bacterium has an endosymbiotic relationship with the insect, as suggested for other Liberibacter species (Duan *et al.* 2009).

To date, CLeu has been described only in pears and pear psyllids in northwestern Italy; however, there are no reported investigations into its occurrence in other European and Mediterranean regions, or in other plants or psyllid species. If the nature of the interaction of CLeu with both insects and plants is beneficial, it is likely to have a much wider distribution than that known today. Moreover, considering the fact that *C. pyri* and other species of the genus *Cacopsylla* are known vectors of phytoplasmas (Carraro *et al.* 1998a,b, 2004; Davies *et al.* 1992; Jarausch *et al.* 2003; Tedeschi and Alma 2004), possible interactions may have developed between these two microorganisms in both plants and insects.

The aim of this work was to investigate whether CLeu is capable of infecting different species of the genus *Cacopsylla* and their respective host and shelter plants, and to evaluate the spread of the bacterium in the species under investigation. Samples were collected in three different geographical areas: northwestern Italy (Piedmont and Aosta Valley), Central Hungary (Pest County) and Israel (the Galilee region), which are characterized by different climatic conditions. Indeed, according to the Köppen-Geiger classification, the Piedmont region falls into climate type Cfa (warm oceanic), Aosta Valley and Pest have climate type Dfb (temperate continental), and the Galilee is classified as climate type Csa (warm Mediterranean) (Peel *et al.* 2007). In addition, we investigated the possible copresence of CLeu and phytoplasmas.

Materials and methods

Insect sampling, species determination and DNA isolation In northwestern Italy, sampling was carried out in eight different locales in the Piedmont and Aosta Valley regions at altitudes between 500 and 1500 m above sea level (masl) from February until November in the years 2008–2010. Insects were collected from *Pyrus communis* L. (pear), *Malus domestica* (Borkh.) (apple), *Crataegus monogyna* (Jacquin) (hawthorn), *Prunus spinosa* (L.) (blackthorn), *Prunus domestica* (L.) (plum) and *Salix caprea* (L.) (goat willow) using the beat-tray method, and from *Abies alba* (Miller) (silver fir), *Picea abies* (L.) (Norway spruce), *Larix decidua* (Miller) (European larch) and *Pinus sylvestris* (L.) (Scots pine) by means of a sweep net (35 cm diam) with a 5-m telescopic handle

(Čermák and Lauterer 2008). For the beat-tray method, also called 'frappage', a white plastic tray (250× 350 mm) was held beneath the tree branches and the branches were struck several times to dislodge insects into the tray. The psyllids were then collected in glass tubes by means of a mouth aspirator and brought to the laboratory for further analyses (Horton 1994; Tedeschi *et al.* 2002).

The species *Cacopsylla pyrisuga* (Förster), *Cacopsylla pyricola* (Förster), *Cacopsylla peregrina* (Förster), *Cacopsylla crataegi* (Schrank), *Cacopsylla nigrita* (Zetterstedt), *Cacopsylla breviantennata* (Flor), *Cacopsylla pruni* (Scopoli) and *Cacopsylla ambigua* (Förster) were identified by examining the forewings and male and female terminalia (Ossiannilsson 1992). *Cacopsylla melanoneura* (Förster) and *Cacopsylla affinis* (Löw) were also collected. Due to the fact that these latter two species live together on *Crataegus* spp., migrate to conifers as shelter plants for aestivation and overwintering and have similar morphological characters, all *Cacopsylla* spp. specimens collected on hawthorn plants or conifers were identified by means of polymerase chain reaction (PCR) analyses using species-specific primers. All of the insects were stored in absolute ethanol at −20°C before analysis.

Cacopsylla melanoneura, *C. peregrina*, *C. affinis* and *C. crataegi* were collected on hawthorn in the Aosta Valley region; *C. melanoneura* was also sampled in apple orchards and from Norway spruce, Scots pine and European larch plants. In the same region, *C. nigrita* was found on Norway spruce, Scots pine, silver fir and European larch, while *C. ambigua* and *C. breviantennata* were collected from goat willow and Norway spruce, respectively. The species *C. pyricola* and *C. pyrisuga* were found in pear orchards in Piedmont.

In Hungary, the samplings were carried out in pear orchards from two sites in Pest County between May and July 2011 at altitudes between 110 and 149 masl. Specimens of *C. pyri, C. pyricola* and *C. pyrisuga* were collected using the beat-tray method and were identified by examining the forewings and male and female terminalia.

In Israel, the samplings were carried out in April 2010 in two different locales in the Upper Galilee region at altitudes between 700 and 1000 masl. The following species of the genus *Cacopsylla* were collected from different plants using the beat-tray method: *Cacopsylla bidens* (Sulc) from *Pyrus syriaca* (Boiss.) (wild pear), *Cacopsylla myrthi* (Puton) from *Rhamnus alaternus* (L.) (Mediterranean buckthorn) and *Cacopsylla pulchella* (Löw) from *Cercis siliquastrum* (L.) (Judas tree). The species were identified by examining the forewings and male and female terminalia.

The SDS-proteinase K-CTAB protocol (Sambrook *et al.* 1989), modified as reported previously (Raddadi *et al.* 2011), was used to extract DNA samples from single individuals of all psyllid species.

Plant sampling and DNA isolation The following plant species were sampled at the sites at which their respective psyllids were sampled: pear (41 samples), apple (20 samples), hawthorn (18 samples), plum (9 samples), blackthorn (10 samples), goat willow (5 samples), wild pear (3 samples), silver fir (8 samples), Norway spruce (8 samples), Scots pine (3 samples), European larch (3 samples). Because symptoms for CLeu have never been observed or described, secondary and tertiary branches from plants with or without symptoms of phytoplasma infection were randomly collected.

Total DNA was extracted from 100 mg (wet weight) plant phloem tissues that had been ground with liquid nitrogen in a sterile mortar, according to the DNeasy Plant Mini Kit protocol (Qiagen; Milan, Italy). The DNA was eluted in 100 μl of elution buffer and kept at −20°C before use.

PCR and restriction fragment length polymorphism (RFLP) analyses To discriminate between *C. melanoneura* and *C. affinis,* which cannot be distinguished based on morphological characteristics, the two primer pairs MEL_fw/MEL_rev and AFF_fw/AFF_rev were used in a duplex touch-down PCR according to Tedeschi and Nardi (2010). The reaction and cycling conditions were as described in the original paper. Each amplicon (5 μl) was separated by electrophoresis in a 1% agarose gel and the PCR products were visualized using a transilluminator after staining with ethidium bromide.

To verify the incidence of CLeu in psyllids and plants, heminested PCR of the 16S rRNA gene was performed using the specific primers Lib223F and Lib451R, as reported previously (Raddadi *et al.* 2011).

To assess the presence of phytoplasmas in psyllids that feed on fruit trees and in their host/shelter plants, PCR assays were carried out with universal phytoplasma

Table 1 Incidence of '*Candidatus* Liberibacter europaeus' (CLeu) and phytoplasmas in *Cacopsylla* spp. collected in northwestern Italy, Hungary and Israel, as determined by specific PCR assays

Cacopsylla species	Life stage	Region	Host/shelter plant	CLeu-infected insects	Phytoplasma-infected insects	CLeu + phytoplasma-infected insects
C. affinis	Overwintering adult	Aosta Valley[z]	European larch	0/1	0/1*	0/1
″	Overwintered adult	″	Hawthorn	4/28 (14.29%)	0/28*	0/28
″	Newly emerged adult	″	Hawthorn	0/1	0/1*	0/1
C. ambigua	Adult	Aosta Valley	Goat willow	1/14 (7.14%)	0/14**	0/14
C. bidens	Summer-form adult	Galilee[y]	Wild pear	0/10	0/10*	0/10
C. breviantennata	Overwintering adult	Piedmont[z]	Norway spruce	4/20 (20%)	0/20 **	0/20
C. crataegi	Adult	Aosta Valley	Hawthorn	0/6	0/6*	0/6
C. melanoneura	Newly emerged adult	Aosta Valley	Apple	3/6 (50%)	0/6*	0/6
″	Overwintered adult	″	Apple	22/115 (19.13%)	14/115 (12.17%)*	5/115 (4.35%)
″	Newly emerged adult	″	Hawthorn	0/2	0/2*	0/2
″	Overwintered adult	″	Hawthorn	7/39 (17.95%)	1/39 (2.56%)*	0/39
″	Aestivating adult	″	Norway spruce	0/16	0/13*	0/13
″	″	″	Scots pine	0/1	0/1*	0/1
″	″	″	European larch	0/6	0/6*	0/6
C. myrthi	Summer-form adult	Galilee	Mediterranean Buckthorn	0/6	0/6**	0/6
C. nigrita	Aestivating adult	Aosta Valley	Scots pine	1/2 (50%)	0/2**	0/2
″	″	″	Norway spuce	1/10 (10%)	0/10 **	0/10
″	″	″	Silver fir	0/1	0/1**	0/1
″	″	″	European larch	1/2 (50%)	0/2**	0/2
C. peregrina	Adult	Aosta Valley	Hawthorn	3/27 (11.11%)	0/27*	0/27
C. pruni	Overwintering adult	Piedmont	Plum	0/5	0/5*	0/5
″	″	″	Blackthorn	0/28	1/28 (3.57%)*	0/28
C. pulchella	Newly emerged adult	Galilee	Judas tree	0/13	0/13**	0/13
C. pyri	Summer-form adult	Pest County[x]	Pear	4/10 (40%)	2/10 (20%)*	1/10 (10%)
C. pyricola	Winter-form adult	Piedmont	Pear	2/13 (15.38%)	0/13*	0/13
″	Summer-form adult	Pest County	Pear	1/14 (7.14%)	2/14 (14.29%)*	0/14
C. pyrisuga	Newly emerged adult	Piedmont	Pear	18/21 (85.71%)	0/21*	0/21
″	Newly emerged adult	Pest County	Pear	0/4	0/4*	0/4

[z] Northwestern Italy

[y] Israel

[x] Hungary

* P1/P7+fO1/rO1+RFLP (*Ssp*I+*Rsa*I)

** P1/P7+F2n/R2

primers P1 and P7 (Schneider *et al.* 1995) followed by a nested PCR with specific primers for the 16SrX group phytoplasmas, fO1 and rO1 (Lorenz *et al.* 1995). The cycling and reaction conditions were as described in the original papers. Amplicons were then subjected to RFLP analyses with the restriction enzymes *Ssp*I (Fermentas; Vilnius, Lithuania) and *Rsa*I (Promega; Madison, WI, USA), following the suppliers' instructions, to determine the 16SrX subgroup (Seemüller and Schneider 2004). For all of the other psyllids and their respective host/shelter plants, PCR assays were carried out with the universal phytoplasma primers P1/P7 followed by a nested PCR with the other universal phytoplasma primer pair F2n/R2 (Gundersen and Lee 1996). Reaction and cycling conditions were the same as in the original paper.

Results

Incidence of CLeu in psyllids and plants A total of 421 specimens belonging to 14 different species of the Psylloidea superfamily were screened for the presence of CLeu (Table 1). Of these, 72 were positive for CLeu, corresponding to 17.1% of the individuals analyzed, whereas 20 out of 421 samples were found to be infected by '*Ca.* Phytoplasma' spp., corresponding to 4.75% of the individuals analyzed.

CLeu was detected in nine species of the genus *Cacopsylla* and in four species of host plants, including apple (the host plant of *C. melanoneura*), blackthorn (the host plant of *C. pruni*), hawthorn (the host plant of both *C. melanoneura* and *C. peregrina*), and pear (the host plant of *C. pyri*, *C. pyricola* and *C. pyrisuga*). With regard to geographical distribution, CLeu was found in 67 out of 364 (18.4%) specimens collected in Italy, in five out of 28 (17.9%) Hungarian individuals, and in none of the 46 Israeli psyllids.

CLeu was found in overwintered *C. melanoneura* adults collected from both apple and hawthorn plants and in newly emerged adults collected from apple plants; it was never found in specimens collected from conifers. Of the other psyllids collected from hawthorn plants, 11.1% of the *C. peregrina* and 14.3% of the *C. affinis* specimens analyzed were positive for CLeu, whereas *C. crataegi* was always negative for the

Table 2 Incidence of '*Candidatus* Liberibacter europaeus' (CLeu) and '*Candidatus* Phytoplasma' spp. in phloem tissues of host (top part) and shelter (bottom part) plants, as determined by specific PCR assays

Species	Region	CLeu-infected samples	Phytoplasma-infected samples	CLeu + phytoplasma-infected samples
Apple	Piedmont[z]	4/20 (20%)	7/20 (35%)*	3/20 (15%)
Blackthorn	Piedmont	3/10 (30%)	2/10 (20%)*	1/10 (10%)
Goat willow	Aosta Valley[z]	0/5	0/5**	0/5
Hawthorn	Aosta Valley	1/18 (5.55%)	3/18 (16.66%)*	1/18 (5.55%)
Pear	Piedmont	3/20 (15%)	9/20 (45%)*	2/20 (10%)
Pear	Pest County[y]	0/21	5/21 (23.81%)*	0/21
Plum	Piedmont	0/9	4/9 (44.44%)*	0/9
Wild pear	Galilee[x]	0/3	0/3*	0/3
European larch	Piedmont	0/3	0/3**	0/3
Norway spruce	Aosta Valley	0/4	0/4 **	0/4
Norway spruce	Piedmont	0/4	0/4 **	0/4
Scots pine	Aosta Valley	0/3	0/3**	0/3
Silver fir	Piedmont	0/4	0/4 **	0/4
Silver fir	Aosta Valley	0/4	0/4 **	0/4

[z] Northwestern Italy

[y] Hungary

[x] Israel

* P1/P7+fO1/rO1+RFLP (*Ssp*I+*Ssp*I)

** P1/P7+F2n/R2

presence of the bacterium. In *Cacopsylla* sp. adults aestivating and overwintering on conifers, CLeu was identified in three out of 15 *C. nigrita* and in four out of 20 *C. breviantennata* specimens.

The α-Proteobacterium was detected in three of the four pear-feeding species: *C. pyri*, *C. pyricola* and *C. pyrisuga*, with a high infection rate in the last (85.7%), and in one specimen of the willow feeder *C. ambigua*. Its presence was not detected in *C. crataegi*, *C. pruni*, *C. pulchella* or *C. myrthi*.

Analysis of the host and shelter plants revealed the presence of CLeu on apple and pear trees, but not on plum, and among the wild plants, on blackthorn and hawthorn. The bacterium was not found on goat willow or on coniferous plants (Table 2).

The PCR analyses using the 16SrX group-specific primers fO1/rO1 followed by RFLP with the restriction enzymes *Ssp*I and *Rsa*I detected '*Ca.* Phytoplasma mali' on apple and hawthorn plants from Italy and Hungary, '*Ca.* Phytoplasma pyri' on pear plants from Italy and Hungary, and '*Ca.* Phytoplasma prunorum' on plum and blackthorn from Italy.

Symptoms such as witches' broom, leaf yellowing/reddening and stunting were always matched to the presence of phytoplasmas, whereas no particular symptom was observed in the case of only CLeu infection. Mixed infections of liberibacter/phytoplasma were observed in apple, pear, hawthorn and blackthorn; however, we did not detect any difference in observable symptoms of doubly infected and phytoplasma-infected plants.

Goat willow and all of the coniferous plants tested were consistently free of phytoplasmas and CLeu.

Discussion

The results suggest that CLeu is widespread throughout European areas characterized by oceanic and continental climate types. This corroborates preliminary observations made in northwestern Italy, where the α-Proteobacterium was found in the oceanic and continental climates of the Piedmont and Aosta Valley regions, respectively (Raddadi *et al.* 2011). Moreover, this is the first report of CLeu in Hungary, confirming this bacterium's ability to spread in continental climates. On the other hand, we did not detect any CLeu infections in Israel. There are a variety of possible reasons for this, including the different climate, the presence of different host plants for many of the psyllid species tested (for instance, plants that do not belong to the Rosaceae family could be unsuitable for the development of CLeu), the possibility that the bacterium has simply not yet arrived, or the relatively low number of samples tested. Further studies are needed to exclude the presence of CLeu in this area, and clarify its spread throughout different Mediterranean regions.

CLeu was detected in all insect species analyzed in both Italy and Hungary except *C. crataegi* - although in this case very few individuals were tested - and *C. pruni*. This suggests that, at least in oceanic and continental areas within Europe, this bacterium is widespread within the *Cacopsylla* genus. Similarly, CLeu was present in all of the host plants of these species except for plum. However, we know that the principal host plant of *C. pruni* is blackthorn, whereas plum is only sporadically colonized by this insect (Jarausch *et al.* 2007). The occurrence of CLeu in blackthorn indicates that *C. pruni* can be exposed to CLeu, although the uptake rate is likely relatively low.

In contrast, CLeu was never found in conifers or goat willow. This could be due to the dissimilar compositions of these plants' phloem sap: whereas in most plants, conifers included, sucrose is the predominant component of phloem sap, in Rosaceae plants the sugar alcohol sorbitol is the main carbohydrate in the phloem (Zimmerman and Ziegler 1975). The presence of high concentrations of sorbitol may influence the presence and development of CLeu, creating a suitable environment. Very little information is available on the phloem composition of Rutaceae and Solanaceae plants, so it is difficult to compare the spread of CLeu and other '*Ca.* Liberibacter' spp. in relation to host plant characteristics. Further work is required to clarify this aspect. Note, also, that sampling limitations might obscure other factors with a potential influence on the failure to detect CLeu in conifers and goat willow: the bacterium could have been absent on the collected branches but present in other plant parts. Indeed, the uneven distribution of liberibacters in plants is well known (Tatineni *et al.* 2008; Teixeira *et al.* 2008a; Wen *et al.* 2009). In addition, the limited number of sampled trees leads to a smaller chance of encountering an infected host, especially when infection rates are low.

Similarly, phytoplasmas could not be detected in any of the conifer samples. As infections by a group

16SrXXI phytoplasma have been reported in *Pinus* spp. and other conifers (Kamińska *et al.* 2011; Schneider *et al.* 2005), further research might reveal the occurrence of phytoplasma-infected conifers in northwestern Italy.

In most of the insect species tested, we observed high infection rates of CLeu which, in the case of *C. pyrisuga*, were higher than the percentages observed in Florida, USA (8.7%) and Indonesia (45.2%) for *Diaphorina citri* with '*Ca*. Liberibacter asiaticus' (Manjunath *et al.* 2008; Subandiyah *et al.* 2000). Such a high infection rate supports a hypothesis of CLeu's role in the insect host as a beneficial symbiont, similar to '*Ca*. Liberibacter asiaticus' in *Diaphorina citri*, whose role as a beneficial symbiont rather than a simple ingested bacterium passing through the gut was suggested by analysis of the bacterium's genome content (Duan *et al.* 2009). However, for the most part, the interaction between liberibacters and their hosts remains unclear. In *Bactericera cockerelli* (Sulc), for instance, '*Ca*. L. solanacearum' has been reported to have a negative effect on its vector's population growth rate on tomato (Nachappa *et al.* 2012).

CLeu was found in aestivating, overwintering and newly emerged adults of monovoltine species, and in winter- and summer-form adults of polyvoltine species. It would be interesting to determine whether psyllids on shelter plants retain the bacterium during aestivation and overwintering. No CLeu could be detected in *C. melanoneura* specimens collected from conifers, but in *C. nigrita* and *C. breviantennata*, with *Salix* spp. and *Sorbus* spp. as their respective host plants, some CLeu-positive individuals were recorded on conifers. Considering the fact that the conifer plants themselves were always negative for these species, it is possible that these psyllids retained a level of infectivity from previous feeding activity on host plants, although further studies should be carried out to specify the origin of infection in these *Cacopsylla* species and the presence of this bacterium on different host plant species.

Preliminary transmission trials have demonstrated *C. pyri*'s ability to transmit CLeu to healthy pear plants (Raddadi *et al.* 2011). The finding of this bacterium in a high number of *Cacopsylla* species and on their respective host plants calls for verification of all of these species' transmission abilities. Their possible role as vectors will have a great influence on the spread of this bacterium.

Considering the fact that so far, symptoms related to CLeu have never been observed on infected plants, we cannot consider this liberibacter a plant pathogen, in agreement with previous observations by Raddadi *et al*. (2011).

In our study we found mixed infections of CLeu and 16SrX group phytoplasmas in psyllids and plants. The consequences of this co-presence on the insect host's fitness remain to be determined. On the other hand, mixed infections were found in both symptomatic and asymptomatic plants, but we were not able to find any correlation between the co-infection and phytoplasma symptom expression (data not shown). The simultaneous presence of '*Ca*. Liberibacter' spp. and group 16SrIX and 16SrI phytoplasmas has also been observed on citrus plants, but not in the insect vectors (Chen *et al.* 2009; Teixeira *et al.* 2008b), and there was no particular influence on symptom severity. The interactions between these bacteria in insects and plants thus warrant further investigation.

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