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SHORT COMMUNICATION

First sharpshooter species proven as vectors of *Xylella fastidiosa* subsp. *multiplex* in *Prunus salicina* trees in Brazil

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Abstract

Plum leaf scald (PLS), caused by the vector-borne bacterium *Xylella fastidiosa*, is a major obstacle to the expansion of plum crops in Brazil. In other affected crops, this pathogen is naturally transmitted mainly by sharpshooter leafhoppers (Hemiptera: Cicadellidae: Cicadellinae). Despite the importance of PLS, the vector species responsible for bacterial spread in plums remain unknown. This study, therefore, aimed to determine the *X. fastidiosa* transmission ability of three sharpshooter species commonly found in plum orchards in southern and southeastern Brazil: *Macugonalia cavifrons* (Stål), *Macugonalia leucomelas* (Walker), and *Sibovia sagata* (Signoret). After a 72-h acquisition access period to plum trees infected with *X. fastidiosa* subsp. *multiplex* (sequence type 67), the insects were transferred for an inoculation access period of 96 h to 10–20 healthy *Prunus salicina* plants for each sharpshooter species, using four insects per plant. Bacterial infection in the plants was verified 7 months after inoculation by polymerase chain reaction. Results showed that the three sharpshooter species were able to transmit *X. fastidiosa* to plums, with mean transmission efficiencies by single insects of 16%, 14%, and 18% for *M. cavifrons*, *M. leucomelas*, and *S. sagata*, respectively. To our knowledge, this study is the first to identify vector species and prove the transmission of a PLS strain of *X. fastidiosa* subsp. *multiplex* by sharpshooters in Brazil.

Keywords Plum leaf scald · Vector-borne bacterium · Transmission · Cicadellinae

Plum leaf scald (PLS), caused by the phytopathogenic bacterium *Xylella fastidiosa* subsp. *multiplex*, is the most destructive disease affecting plum trees in Brazil, resulting in severe yield losses and a drastic reduction in crop productivity. The disease symptoms become visible after a long incubation period and involve the appearance of leaf marginal chlorosis, which evolves to leaf necrosis, branch dieback, and eventually death (Hickel et al. 2001). PLS also influences fruit quality attributes, reducing fruit size, weight, and pulp firmness (Kleina et al. 2018). This disease has become a major factor limiting Japanese plum cultivation in the main producing states in Brazil, including Rio Grande do Sul, Santa Catarina, Paraná São Paulo, and Minas Gerais (Eidam and Pavanello 2012), making it necessary to import large quantities of the fruit annually to meet domestic consumption demand (Aliceweb 2018).

The bacterium is disseminated *via* infected propagation materials (He et al. 2000) and piercing-sucking insects specialized in xylem-sap feeding, such as sharpshooter leafhoppers (Hemiptera: Cicadellidae: Cicadellinae) and spittlebugs (Hemiptera: Cercopoidea) (Redak et al. 2004; Almeida et al. 2005). Transmission by insect vectors occurs in a non-circulative propagative manner, in which *X. fastidiosa* cells acquired from an infected plant attach to the anterior portion of the insect's digestive tract (foregut), where propagation and biofilm formation occur (Killiny and Almeida 2009). As the foregut has an ectodermal origin, the cuticular lining containing the bacterial biofilm is replaced during ecdysis, resulting

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in a loss of the nymphs' infectivity; in contrast, adults that acquire the bacterium remain infectious throughout their lifespan (Purcell and Finlay 1979).

The sharpshooter species reported as vectors in this study measure between 4.3 and 7.2 mm in length and belong to the Cicadellini tribe, which is cosmopolitan (Young 1968; Coletta-Filho et al. 2020). They have daytime habits, with peak of activities in the hottest hours of the day (Gravena et al. 1997). Although all sharpshooters are xylem-sap feeders, there is a species preference for specific parts of plants (Daugherty et al. 2010). *M. leucomelas* and *M. cavifrons* prefer to feed on the stems of young branches of the plants, while *S. sagata* prefers to feed on the leaf blade (M. B. Esteves, personal information). Adult longevity varies from 15 to 72 days, depending on the sharpshooter species, host plant, and environmental conditions (Browning et al. 1995; Paiva et al. 2001).

Although PLS is the most important plum disease in Brazil, the vector species of *X. fastidiosa* in this crop are unknown, and this knowledge is essential for the development of control tactics. Through sharpshooter and spittlebug surveys carried out in diseased orchards in the southern region of the country, some potential vector species have been identified (Hickel et al. 2001; Azevedo Filho et al. 2016). Hickel et al. (2001) detected the presence of *X. fastidiosa* in field-collected individuals of seven species of cicadellids and two species of cercopids. However, no experiments have been conducted to prove that these species can transmit *X. fastidiosa* to plums. This study was designed to verify the transmission of *X. fastidiosa* to plums by three sharpshooter species: *Macugonalia cavifrons* (Stål), *Macugonalia leucomelas* (Walker), and *Sibovia sagata* (Signoret), which have previously been reported in plum orchards in southern Brazil (Azevedo Filho et al. 2016).

Healthy plants of *Ocimum basilicum* L. (Lamiaceae) and *Vernonia condensata* Baker (Asteraceae) were cultivated for sharpshooter rearing. The basil was grown from seeds sown in styrofoam trays in the commercial substrate Rendimax® (Eucatex, Itapetininga, SP, Brazil). Seedlings approximately 10 cm in height were transplanted in 1.2-L plastic pots (11 × 14 cm [height × diameter]) containing the same substrate. Plants of *V. condensata* were obtained from cuttings (approximately 40 cm long) in 3-L plastic pots (15 × 20 cm [height × diameter]) with Rendimax®.

Plum trees for the transmission experiment were cultivated in screenhouses at the "Núcleo de Produção de Mudas de Itaberá" ("Coordenação de Assistência Técnica Integral do Governo de São Paulo" - CATI/SP) by grafting healthy scions of *Prunus salicina* Lindl. 'Reubennel' (Rosaceae) (Japanese plum) on *P. persica* (L.) Batsch 'Okinawa' rootstock. The grafted plants were grown in 2-L plastic bags with Rendimax®. The plum trees were regularly pruned at 10 cm above the grafting point so that young branches and leaves were available for bacterial inoculations. All plants were kept in a vector-proof screenhouse and fertilized *via* irrigation with macronutrients and micronutrients, as described by Esteves et al. (2019).

To obtain source plants of the pathogen, healthy plum trees were mechanically inoculated with the isolate 137amx of *X. fastidiosa* subsp. *multiplex*, which was isolated from plum trees with symptoms of PLS in Veranópolis, RS, Brazil, and classified as sequence type (ST) 67 (Coletta-Filho et al. 2017). Originally preserved at 80 °C in PW liquid with 30% glycerol, the isolate 137amx was recovered on periwinkle wilt gelrite (PWG) medium (Hill and Purcell 1995) at 28 °C for 15 days. After two transfers on PWG, the resulting colonies were scraped and suspended in phosphate-buffered saline (PBS) to obtain a turbid suspension. The cell suspension was inoculated using the pinprick method (Hopkins 1985) at three different points on the stems of young branches of 12 plum plants by applying 5 μL of the suspension per point. Five months after mechanical inoculation, samples of five randomly selected mature leaves from each plant were tested for *X. fastidiosa* infection by polymerase chain reaction (PCR).

The sharpshooters *M. cavifrons* and *M. leucomelas* were originally collected from plants of *Lagerstroemia indica* L. (Lythraceae) and *V. condensata*, while *S. sagata* was collected from *Peumus boldus* Molina (Monimiaceae), in Piracicaba, SP, Brazil. Approximately 50 adults of each species were placed in screened rearing cages (50 × 60 cm (base) by 70 cm [height]) containing plants of *V. condensata* for *M. cavifrons* and *O. basilicum* for *M. leucomelas* and *S. sagata*. Needle inoculations of *O. basilicum* and *V. condensata* with cell suspensions of the *X. fastidiosa* strain used in the present study yielded negative results by culturing (data not shown), indicating that these plant species do not support colonization by this strain and can serve as suitable hosts for producing healthy sharpshooters.

The insects were reared in a greenhouse equipped with a "pad-fan" type cooling system and a thermostat-driven heater for temperature control (25 ± 5 °C), as previously described (Marucci et al. 2003; Esteves et al. 2019). After an oviposition period of 2 weeks, adults were removed from the plants, leaving only eggs and nymphs. The adult progeny was used in the transmission experiments about 1–3 weeks after emergence.

Groups of 70 adult individuals of each sharpshooter species (*M. cavifrons*, *M. leucomelas*, and *S. sagata*) were placed inside screened rearing cages (50 × 60 cm [base] by 70 cm [height]) containing three plum plants diagnosed as positive for *X. fastidiosa* by PCR (source plants) with 5 months after mechanical inoculation (branches with young and mature leaves), for an acquisition access period (AAP) of 72 h. Subsequently, the insects were confined in groups of four individuals on the young shoots of healthy plum plants (test plants) for an inoculation access period (IAP) of 96 h, inside sleeve cages made of tulle fabric. Insect mortality was

recorded after the AAP and IAP. The experiment was repeated twice for *M. leucomelas* and *S. sagata*, but it was only performed once with *M. cavifrons*. Ten test plants were inoculated per sharpshooter species in each trial. Before the AAP, the individuals were confined for 48 h on five healthy plum plants, which served as negative controls to ensure that the insects were healthy before exposure to the source plants. After the IAP, all plants were sprayed with the insecticide imidacloprid (Provado® 200 SC) (0.24 mL/1.2 L) and maintained in a vector-proof screenhouse (anti-aphid screen), where they were fertilized with macronutrients and micronutrients, as described above. At 7 months after inoculation, the plants were rated for PLS symptoms and samples of five randomly selected mature leaves from each plant were subjected to PCR for the detection of *X. fastidiosa*.

Leaf samples comprising 0.1–0.12 g of sliced petioles were subjected to DNA extraction following the protocol of Minsavage et al. (1994), with modifications described by Marucci et al. (2008). The PCR was performed in a PTC-100 thermal cycler (MJ Research, Inc., Watertown, MA, USA) using the primer pair HL5/HL6 and the conditions described by Francis et al. (2006). The amplified DNA samples were subjected to 1% agarose gel electrophoresis; DNA fragments were visualized under UV light and documented in the Eagle Eye II system (Stratagene, La Jolla, CA, USA).

Since each test plant of the transmission experiment was inoculated by four insects, the probability of transmission of *X. fastidiosa* by a single insect (P) was estimated using the formula: $P = 1 - (1 - I)^{1/k}$, in which I refers to the proportion of infected plants and k is the number of insects used per test plant during the IAP (Swallow 1985). The mortality rates (%) of sharpshooters on plum were calculated based on the number of dead individuals on infected source plants, after the 72-h AAP and on healthy test plants, after the 96-h IAP. The mortality rates were transformed using the equation $[(n + 0.5)/100]^{0.5}$, where n is the sampled data. Data were subsequently subjected to analysis of variance and the Hartley test ($P < 0.05$) using the R software environment for statistical computing (R Core Team 2019).

The three species of sharpshooters tested (*M. cavifrons*, *M. leucomelas*, and *S. sagata*) were able to transmit the isolate 137amx (ST 67) of *X. fastidiosa* subsp. *multiplex* to healthy *P. salicina* 'Reubennel' (test plant) after a 72-h AAP on infected plants of this plum cultivar (Table 1). The transmission rate, calculated based on the proportion of infected test plants, ranged from 40% (*M. leucomelas*) (trial II) to 60% (*S. sagata*) (trial I), using four insects per test plant. The estimated transmission probability per insect in the two trials ranged from 11.9 to 16% for *M. leucomelas*, 15.9 to 20.4% for *S. sagata*, and 16% for *M. cavifrons* (Table 1).

Approximately 80% of PCR-positive test plants showed characteristic symptoms of PLS after inoculation by sharpshooters, which initially presented as irregular chlorosis at the edges of the leaves and evolved into the development of necrotic lesions on the entire leaf limb. None of the plum test plants exposed to the lab-reared sharpshooters before the AAP (negative controls) showed positive results for *X. fastidiosa* by PCR, showing that the vectors reared on *V. condensata* and *O. basilicum* were free of the pathogen and only became infected after exposure to source plants.

The mean mortality rates of sharpshooters after the 72-h AAP on *X. fastidiosa*–infected plum plants were low (<20%), without significant differences among the species (Table 2). Likewise, low mortality rates were observed for the three sharpshooter species after the IAP of 96 h in healthy plum plants (Table 2).

To our knowledge, this study is the first to show the transmission of a PLS strain of *X. fastidiosa* subsp. *multiplex* by sharpshooters in Brazil, confirming three species as vectors of the bacterium in plums. The transmission efficiencies of this *X. fastidiosa* strain by sharpshooters in plum (12–20%) were lower than those reported for *X. fastidiosa* subsp. *fastidiosa* in grapevines (20–100%) (Hill and Purcell 1995; Daugherty and Almeida 2009), but they were within the same range observed for strains of *X. fastidiosa* subsp. *pauca* in coffee, citrus (Marucci et al. 2008; Lopes and Krugner 2016), and *Catharanthus roseus* L. (Apocynaceae) (Esteves et al. 2019).

Among the sharpshooter species reported as vectors in the present study, *M. leucomelas* had previously been demonstrated as a vector of *X. fastidiosa* subsp. *pauca* in citrus (Lopes and Krugner 2016) and *C. roseus* (Esteves et al. 2019), with a single insect transmission rate of 16%, similar to that obtained in this study (12–16%). The other two species, *M. cavifrons* and *S. sagata*, were previously reported as vectors of a CVC strain (9a5c) of *X. fastidiosa* subsp. *pauca* in *C. roseus*, with transmission efficiencies of 3.3% and 7.1%, respectively, after bacterial acquisition from artificial diets (Esteves et al. 2019).

Sharpshooters are generally polyphagous, as they feed and develop on a wide range of host plants, including herbaceous and woody species (Paiva et al. 1996). Adult sharpshooters are very mobile and move seasonally between habitats (Lopes and Krugner 2016). For example, *M. leucomelas* is often found on herbaceous weeds in the ground cover of citrus orchards (Miranda et al. 2009) and, along with *M. cavifrons*, on weeds and shrubs in the edge of neighboring woods (Coelho et al. 2008; Giustolin et al. 2009). These characteristics increase the potential for *X. fastidiosa* dispersal among plants, and consequently, the probability of spreading diseases in susceptible crops.

Faunistic analyses carried out in orchards of Serra Gaúcha, a plum-producing region in the state of Rio Grande do Sul, Brazil, showed that *M. cavifrons* and *S. sagata* are abundantly and frequently present (Azevedo Filho et al. 2016), being classified as the dominant sharpshooters in the region. Together with *M. leucomelas*, these species are widely distributed in Brazil, being recorded in citrus groves of São Paulo

Table 1 Transmission rates of *Xylella fastidiosa* subsp. *multiplex* in *Prunus salicina* cv. Reubennel by sharpshooters in two trials

Trial	Sharpshooter species	No. of infected plants/no. of inoculated plants[a]	Transmission probability per insect[b]
I	*Macugonalia cavifrons*	5/10	0.160
	Macugonalia leucomelas	5/10	0.160
	Sibovia sagata	6/10	0.204
II	*Macugonalia leucomelas*	4/10	0.119
	Sibovia sagata	5/10	0.159

[a] Proportion of plum plants positive for *X. fastidiosa* by polymerase chain reaction (PCR) at 7 months after an inoculation access period (IAP) of 96 h with four insects per plant. Before the IAP, the insects were allowed an acquisition access period of 72 on infected plum trees

[b] Estimated probability of transmission by a single insect, as described by Swallow (1985), since each test plant was inoculated by four insects

State (Yamamoto et al. 2000) and in vineyards of Rio Grande do Sul (Ringenberg et al. 2010). The sharpshooters *M. cavifrons* and *M. leucomelas* were also collected from citrus groves in the states of Paraná (Nunes et al. 2008), as well as in coffee plantations in the state of São Paulo (Giustolin et al. 2009).

The low mortality rate for *M. cavifrons*, *M. leucomelas*, and *S. sagata* observed in this study during the 3–4 days of confinement in infected and healthy plants suggests that these sharpshooter species have an affinity for feeding on *P. salicina*, which increases their importance as vectors of *X. fastidiosa* in plum orchards. In a previous study, *S. sagata* showed a relatively high excretion rate (average of 1.91 mL per individual), an indirect measure of xylem-sap ingestion, when confined for 72 h on a susceptible plum cultivar (Kleina et al. 2020). The ability to transmit *X. fastidiosa* subsp. *multiplex*, the obvious affinity for feeding on plum trees, and the prevalence in plum orchards all support the hypothesis that these sharpshooter species play a significant role in PLS epidemiology.

Table 2 Sharpshooter mortality after the acquisition access period (AAP) and inoculation access period (IAP) of *Xylella fastidiosa* on *Prunus salicina* cv. Reubennel

Sharpshooter species	Mean mortality rate (%)	
	72-h AAP	96-h IAP
Macugonalia cavifrons	10.8±1.9 (70)[ns]	22.0±2.3(40)[ns]
Macugonalia leucomelas	17.7±2.6 (140)	14.0±2.2 (80)
Sibovia sagata	19.2±2.7 (140)	14–0±2.3 (80)

[ns] Means (±SEM) within the columns are not statistically different by the Hartley test ($P < 0.05$). The number of individuals evaluated is shown in parenthesis. The mortality rates (%) were obtained from dead individuals after the AAP of 72 h on infected plants and the IAP of 96 h on healthy plants. For the species *M. leucomelas* and *S. sagata*, the average was calculated from the individuals used in the two replications of the transmission experiment, totaling 140 insects for AAP and 80 for the IAP. For *M. cavifrons*, which was used in only one repetition of the experiment, the calculation of the average mortality was based on a total of 70 individuals for AAP and 40 for IAP

A previous study has shown that other sharpshooters, *e.g.*, *Bucephalogonia xanthophis* (Berg) and *Plesiommata corniculata* Young, collected in plum orchards in the state of Santa Catarina, Brazil, can carry *X. fastidiosa* (Hickel et al. 2001). In the state of Florida, USA, similar studies detected *X. fastidiosa* in the sharpshooters *Homalodisca coagulata* (Say), *Oncometopia orbona* (Fabricius), and *Paralaucizes irrorata* (F.), indicating these species as potential vectors of the bacterium in plum (Hopkins 1977; Younce and Chang 1987). The diversity of sharpshooter species collected in plum orchards (Azevedo Filho et al. 2016), combined with the fact that other species show natural infectivity by *X. fastidiosa* (Hickel et al. 2001), suggest that PLS has a wide range of vector species, as demonstrated by the CVC (Redak et al. 2004; Lopes and Krugner 2016) and coffee leaf scorch (Marucci et al. 2008). However, transmission experiments are required to prove a particular sharpshooter species as a vector. Although *X. fastidiosa* has low vector specificity among xylem-sap feeding insects (Almeida et al. 2005), the insect's ability to transmit the pathogen is dependent on its interactions with the bacterial strain and host plant (Lopes et al. 2009). Studies of *X. fastidiosa* strains from almonds and citrus have shown that not all sharpshooter species can transmit the bacterium (Lopes et al. 2009; Lopes and Krugner 2016).

In conclusion, the confirmation of three sharpshooter species as vectors of *X. fastidiosa* subsp. *multiplex* in plum shown in this work contributes to a greater understanding of PLS epidemiology and could help in the development of more efficient control strategies to reduce losses caused by this disease in Brazil. Future transmission studies involving other species of sharpshooters and PLS-causing *X. fastidiosa* strains should be carried out since there is a diversity of potential vectors (Azevedo Filho et al. 2016) and bacterial genotypes (Coletta-Filho et al. 2017) distributed in plum orchards in the southern and southeastern regions of the country.

Author contribution All authors contributed to the study conception and design. The first draft of the manuscript was written by the first author and all coauthors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

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