

UNIVERSIDADE FEDERAL DO PARANÁ

HELOISA THOMAZI KLEINA

CHALLENGES FOR PLUM LEAF SCALD MANAGEMENT:
APPROACHES ON FRUIT QUALITY, VECTOR INTERACTIONS, VOLATILE
COMPOUNDS AND TRANSMISSION

CURITIBA

2019

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COMPOUNDS AND TRANSMISSION

Tese apresentada ao Programa de Pós Graduação em Agronomia – Produção Vegetal, Departamento de Fitotecnia e Fitossanitarismo, Setor de Ciências Agrárias, Universidade Federal do Paraná, como parte das exigências para obtenção do título de Doutor em Ciências.

Comitê de orientação: Dr^a Louise Larissa May De Mio, Dr. Marco Antônio Dalbó e Dr. João Roberto Spotti Lopes.

CURITIBA

2019

K64c Kleina, Heloisa Thomazi
Challenges for plum leaf scald management: approaches on fruit quality, vector interactions, volatile compounds and transmission / Heloisa Thomazi Kleina. - Curitiba, 2019.
124 p.: il.,

Tese (Doutorado) - Universidade Federal do Paraná. Setor de Ciências Agrárias, Programa de Pós-Graduação em Agronomia - (Produção Vegetal).

Orientadora: Louise Larissa May De Mio

Coorientador: Marco Antônio Dalbó

Coorientador: João Roberto Spotti Lopes

1. Ameixa - Pós-colheita. 2. Frutas - Poda. 3. Ameixa - Doenças e pragas. 4. Frutas - Qualidade - Controle. 5. Folhas - Anatomia. I. De Mio, Louise Larissa May (Orientadora). II. Dalbó, Marco Antônio (Coorientador). III. Lopes, João Roberto Spotti (Coorientador). IV. Título. V. Universidade Federal do Paraná.

CDU 582.711.713



MINISTÉRIO DA EDUCAÇÃO
SETOR SETOR DE CIÊNCIAS AGRARIAS
UNIVERSIDADE FEDERAL DO PARANÁ
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO AGRONOMIA
(PRODUÇÃO VEGETAL) - 40001016031P6

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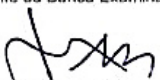
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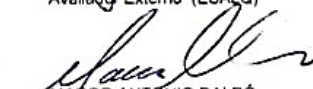


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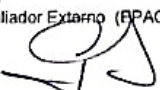
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Dedico este trabalho aos meus pais, Hilda e Luiz Carlos, que dignamente me apresentaram o caminho da honestidade e da persistência. E ao meu marido, Elias, por ter acreditado e me incentivado em todos os momentos.

AGRADECIMENTOS

À Deus, por me guiar e por me dar tranquilidade e sabedoria nessa trajetória árdua, porém gratificante. Agradeço por mais esta conquista e pela oportunidade por Ele concedida para que tudo isso se concretizasse.

Aos meus familiares, de maneira especial aos meus pais, Luiz Carlos e Hilda, por terem me proporcionado uma vida digna, onde pude crescer acreditando que tudo é possível, desde que sejamos honestos e íntegros de caráter.

Ao meu marido Elias, por todo apoio e compreensão ofertado nos momentos de ausência, por todo auxílio, esforço e paciência dedicados para que esse trabalho se consolidasse. Eu te amo.

À minha irmã Patrícia e ao meu cunhado Luis Henrique, por todo amor e carinho oferecidos e pelo apoio incondicional em todas as minhas decisões.

Aos meus queridos sogros, Jorge e Celina, e à minha cunhada Georgia, por me acolherem como uma verdadeira filha/irmã e por sempre estarem ao meu lado. Minha profunda gratidão.

À minha orientadora Dra. Louise Larissa May De Mio, por ter acreditado no meu potencial desde a primeira entrevista, por ter me estimulado a persistir mesmo nos momentos de dificuldade e principalmente, pelo respeito e amizade construídos neste período.

Aos meus co-orientadores Dr. João Lopes e Dr. Marco Dalbó, por todo suporte na execução desse trabalho e por sempre estarem dispostos à ajudar, mesmo que a distância.

Aos membros da banca examinadora de defesa, Dr. Francisco de Assis Marques, Dr. João Lopes e Dr. Marco Dalbó, por terem aceitado o convite e pelas valiosas contribuições realizadas nesse trabalho.

À Dra. Giselda Alves, Dra. Juliana Dogenhardt Goldbach e Dr. Cícero Deschamps pela participação e contribuição na banca examinadora de pré-defesa.

Ao professor Dr. Angelo Jacomino, por ter aceitado a parceria na condução desse trabalho e também por todas as considerações realizadas para que o nosso artigo fosse aceito.

Ao professor Thiago Oliveira, pela paciência, empenho, dedicação e atenção demandada às análises estatísticas do segundo capítulo.

Às minhas duas estagiárias, Eduarda Ramos e Karla Kudlawiec, por terem aceitado trabalhar com essa doença tão desafiadora e por terem sido dedicadas e responsáveis na condução dos experimentos.

A todos os estagiários e técnicos do laboratório de Pós Colheita da ESALQ/USP, em especial a doutoranda Thais Pádua, que passava horas me ajudando a avaliar os experimentos sempre com um sorriso no rosto.

A todos meus colegas do laboratório de Insetos Vetores da ESALQ/USP, por toda ajuda, receptividade e risadas inesquecíveis. Em especial, gostaria de agradecer minhas amigas Mariana Bossi Esteves, Nathalie Maluta e Taciana Azevedo Kuhn, por terem me acolhido tão bem e por sempre terem me auxiliado quando precisei, sem vocês eu não teria conseguido.

À minha amiga venezuelana Meira Ballesteros, por ter sido essa pessoa maravilhosa em todos os sentidos e por ter muita paciência em me ensinar a tão temida química, não medindo esforços para que esse trabalho fosse concluído com primor.

À Dra. Tamires Melo, por ter aceitado entrar nesse projeto aos 45 do segundo tempo e por ter me auxiliado, de maneira admirável, nas análises estatísticas e discussões do terceiro capítulo.

Ao Centro de Diagnóstico "Marcos Enrietti", especialmente à Neide Cristiane Cavalli, por ter me ensinado os primeiros passos na biologia molecular.

Aos produtores Waldomiro Gayer Neto, Paulo Cosmo e Luis Filipe Cosmo, pela cessão das áreas para a instalação dos experimentos. Agradeço também à seus funcionários de campo, em especial Sr. Raimundo e Sr. Ronaldo, pela ajuda e colaboração para que os ensaios fossem realizados.

Aos técnicos do LEMID, Tiago e Simone, por toda a ajuda nesses 4 anos de trabalho.

Aos meus colegas e amigos do LEMID, que conheci por ocasião do doutorado, mas que permanecerão por toda a vida. Obrigada por estarem ao meu lado nessa trajetória, por poder compartilhar minhas alegrias e sempre ser amparada nos momentos de dificuldade. Tenho orgulho do grupo que formamos. Com certeza, sem vocês tudo seria muito mais difícil.

A todos os professores do Programa de Pós Graduação em Produção Vegetal da Universidade Federal do Paraná, em especial aos professores Álvaro Figueredo dos Santos e Henrique da Silva Silveira Duarte.

À Universidade Federal do Paraná e ao Curso de Pós Graduação em Produção Vegetal, pela oportunidade de aprendizado e realização do doutorado.

À secretária do Programa de Pós Graduação da UFPR, Lucimara Antunes, sempre muito solícita em ajudar todos os alunos com a parte burocrática da pós-graduação.

À ESALQ/USP e EPAGRI, pelo suporte para que meus experimentos pudessem ser concluídos.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pela concessão da bolsa de estudos.

A todos os meus amigos, pelos momentos de descontração e por sempre torcerem pelo meu sucesso.

A todos, que por ventura não tenha citado, e que de alguma maneira contribuíram para que tudo isso se tornasse realidade.

“The difference between a successful person and others is not a lack of strength, not a lack of knowledge, but rather a lack in will”.

Vince Lombardi

RESUMO

A ameixeira é considerada uma cultura promissora no Brasil, tendo em vista que a atual oferta da fruta não supre a demanda interna do País. No entanto, pomares comerciais não tem alcançado o potencial produtivo esperado, devido a ocorrência da doença escaldadura das folhas da ameixeira (EFA), ocasionada pela bactéria *Xylella fastidiosa*. Este patógeno causa a oclusão dos feixes xilemáticos, bloqueando o fluxo de água e nutrientes em seus hospedeiros. Os sintomas de dessecamento ocorrem nas folhas e podem ser confundidos com sintomas de deficiência hídrica. No entanto, a interferência dessa doença nos atributos de qualidade pós-colheita de ameixas ainda não foi relatada. A transmissão desta doença ocorre pela disseminação de material propagativo infectado e por insetos vetores. A transmissão por tesouras de poda pode ser uma nova forma de dispersão da doença no campo, porém nenhum estudo sobre essa forma de disseminação foi realizado. A EFA é uma doença sem cura, por isso a identificação de genótipos de ameixeiras que não são contaminadas sob condições de campo, abre novas perspectivas para resolver definitivamente este problema no Brasil. Contudo, os mecanismos envolvidos nessa forma de resistência precisam ser investigados. Portanto, o presente estudo objetivou: i) determinar a influência da escaldadura das folhas nos atributos de qualidade pós-colheita; ii) determinar se a resistência dos novos genótipos ('SC7' e 'SC13') de ameixeira estaria relacionada com a interação entre inseto-planta (preferência de pouso e comportamento alimentar de vetores); iii) investigar o perfil volátil de genótipos de ameixeira com diferentes níveis de resistência à escaldadura das folhas; e iv) avaliar a disseminação de *X. fastidiosa* por tesouras de poda. Os resultados encontrados nessa pesquisa mostraram que: i) frutos retirados de plantas com escaldadura apresentaram menor tamanho, maior perda de peso, menor firmeza e maior incidência de podridão parda. Os atributos de cor diminuíram gradualmente durante o período pós-colheita em frutos removidos de plantas infectadas com *X. fastidiosa*. A atividade das enzimas PME e PAL, a respiração e a produção de etileno foi maior em ameixas provenientes de árvores contaminadas pela EFA. ii) 'SC7' foi menos preferido para pouso por ambas as espécies de vetores. 'SC13' reduziu as taxas de ingestão de seiva de *S. sagata* e afetou as atividades estiletares de *B. xanthophis*. 'SC7' e 'SC13' possuem maiores células epidérmicas e espessas camadas de cera epicuticular do que a cultivar suscetível 'Letícia'. iii) os principais constituintes do óleo essencial dos genótipos de ameixeira foram fitol (46,76%), hidroxitolueno butilado (11,26%), limoneno (2,48%), (*E,E*)- α farneseno (1,95%), β -pineno (1,54 %) e linalol (1,33%). 'Letícia' apresentou o maior teor de alcanos totais. 'Simka' apresentou a maior quantidade de álcools, enquanto 'Fortune' e 'SC13' exibiram a maior concentração de cetonas. Cedrol não foi detectado em genótipos suscetíveis, no entanto foi encontrado em 'SC7' e 'SC15'. (*E,E*)- α farneseno foi verificado em grande quantidade na cultivar 'Fortune'. De acordo com a análise dos componentes principais, os seis genótipos avaliados podem ser divididos em quatro grupos baseados na semelhança do perfil volátil; iv) Não foram observados sintomas de escaldadura nas plantas-teste aos 510 dias após a poda, porém uma amostra do genótipo 'SC7' foi detectada como positiva por *qPCR* aos 450 dias após a poda. Recomenda-se a sanitização das ferramentas de poda, a fim de evitar a disseminação da doença nos pomares.

Palavras-chave: *Xylella fastidiosa*. *Prunus salicina*. Pós-colheita. Insetos vetores. Compostos voláteis. Poda.

ABSTRACT

Plum is considered a promising crop for further development in Brazil, since current supply does not meet the national demand. However, commercial orchards have not reached the expected productive potential due to the occurrence of leaf scald disease (PLS), caused by the bacterium *Xylella fastidiosa*. This pathogen induce the occlusion of xylem bundles, blocking the water and nutrient flow in their hosts. Desiccation symptoms occur on leaves and may be confused with water deficiency symptoms. However, the interference of this disease on postharvest quality attributes of plums has not been reported. Disease transmission occurs through the dissemination of infected propagative material and by insect vectors. Transmission by pruning shears may be a new form of spread in the field, but no study about this mode of dissemination has been performed. PLS is a disease without a cure, so the identification of plum genotypes that are not contaminated under field conditions open new perspectives to definitively solve this problem in Brazil. However, the mechanisms involved in this form of resistance need to be investigated. Therefore, the current study aimed to: i) determine the influence of leaf scald on postharvest quality attributes; ii) determine if the resistance of the new plum genotypes (SC7 and SC13) could be related to the insect-plant interaction (particularly vector settling and feeding behavior); iii) investigate the volatile profile of plum genotypes with different resistance levels to leaf scald disease; and iv) evaluate the dissemination of *X. fastidiosa* by pruning shears. The results found in this research showed that: i) fruit removed from plants with leaf scald showed reduced size, greater weight loss, lower firmness and higher incidence of brown rot. Color attributes gradually decreased during the postharvest period in fruit removed from plants infected with *X. fastidiosa*. PME and PAL activity, respiration and ethylene production were higher in plums from trees with PLS; ii) 'SC7' was less preferred for settling by both vector species. 'SC13' reduced sap ingestion rates of *S. sagata* and affected stylet activities of *B. xanthophis*. 'SC7' and 'SC13' have larger epidermal cells and thicker layers of epicuticular wax than the susceptible cultivar 'Laetitia'. iii) major constituents in the essential oil from the plum genotypes were phytol (46.76%), butylated hydroxytoluene (11.26%), limonene (2.48%), (*E,E*)- α farnesene (1.95%), β -pinene (1.54%) and linalool (1.33%). 'Laetitia' presented the highest total alkane content. 'Simka' showed the highest alcohol content, while 'Fortune' and 'SC13' exhibited the highest concentration of ketones. Cedrol was not detected in susceptible genotypes, however was found in 'SC7' and 'SC15'. (*E,E*)- α farnesene was observed in high amount in 'Fortune'. According principal components analysis, the six evaluated genotypes can be divided in four groups based on the resemblance of the volatile profile; iv) No leaf scorch symptoms were observed on test plants at 510 days after pruning, however, one sample of the 'SC7' genotype was detected as positive by *q*PCR at 450 days post pruning. Sanitation of pruning tools is recommended, in order to avoid the disease spread in the orchards.

Key-words: *Xylella fastidiosa*. *Prunus salicina*. Postharvest. Insect vectors. Volatile compounds. Pruning.

LIST OF FIGURES

CHAPTER 1

- Fig. 1.** Meteorological data from the Curitiba (Parana) automatic station (Lat. 25.43°; Lon. 49.26°; Height above sea level 923.5 m) for the (a) 2015/2016 and (b) 2016/2017 growing seasons, provided by the Instituto Nacional de Meteorologia (INMET); and (c) data from the Paranapanema (São Paulo) automatic station (Lat. 25.23°; Lon. 48.43°; Height above sea level 610 m) for the 2015/2016 growing season, provided by the Centro Integrado de Informações Agrometeorológicas (CIIAGRO).32
- Fig. 2.** Fresh weight loss and pulp firmness of plum fruit collected from plants with and without symptoms of leaf scald, from the Parana (PR) and São Paulo (SP) growing regions. Growing season 2015/2016: A and B, ‘Gulfblaze’ cultivar (SP); C and D, ‘Reubennel’ cultivar (PR); E and F, ‘Reubennel’ cultivar (SP). Growing season 2016/2017: G and H, ‘Reubennel’ Cultivar (PR).34
- Fig. 3.** Chroma (C*) and hue angle (°hue) of plum fruit collected from plants with and without symptoms of leaf scald, from the Parana (PR) and São Paulo (SP) growing regions. Growing season 2015/2016: A and B, ‘Gulfblaze’ (SP) cultivar; C and D, ‘Reubennel’ (PR) cultivar; E and F, ‘Reubennel’ cultivar (SP).35
- Fig. 4.** Titratable acidity (% of malic acid) and total soluble solid (% TSS) content in plum fruit collected from plants with and without symptoms of leaf scald, from the Parana (PR) and São Paulo (SP) growing regions. Growing season 2015/2016: A and B, ‘Gulfblaze’ (SP) cultivar; C and D, ‘Reubennel’ (PR) cultivar; E and F, ‘Reubennel’ (SP) cultivar. Growing season 2016/2017: G and H, ‘Reubennel’ (PR) cultivar.37
- Fig. 5.** Activity of the enzymes pectin methylesterase (PME) and phenylalanine ammonia-lyase (PAL) in plum fruit collected from plants with and without leaf scald symptoms, from the Parana (PR) and São Paulo (SP) growing regions. Growing season 2015/2016: A and B, ‘Gulfblaze’ (SP) cultivar; C and D, ‘Reubennel’ (PR) cultivar; and E and F, ‘Reubennel’ (SP) cultivar.38
- Fig. 6.** CO₂ and ethylene production in plum fruit collected from plants with and without leaf scald symptoms, from the Parana (PR) and São Paulo (SP) growing regions. Growing season 2015/2016: A and B, ‘Gulfblaze’ (SP) cultivar; C and D, ‘Reubennel’ (SP) cultivar.39

CHAPTER 2

- Fig. 1.** Cumulative honeydew excretion (mL) for *Sibovia sagata* in three plum genotypes (Laetitia, SC7, SC13) after 24, 48 and 72 hours. The bars represent the standard error of the mean. The dots were shifted to the best view of the error bars.74
- Fig. 2.** Cumulative honeydew excretion (mL) for *Bucephalogonia xanthophis* in three plum genotypes (Laetitia, SC7, SC13) after 24, 48 and 72 hours. The bars represent the standard error of the mean. The dots were shifted to the best view of the error bars.....75
- Fig. 3.** Light photomicroscopy of plum leaf tissues. Transverse sections of plum leaves 30 days after sprouting in greenhouse. (1A – 1B) ‘SC7’ genotype. Increase 20x and 40x, respectively. (2A – 2B) ‘SC13’ genotype. Increase 20x and 40x, respectively. (3A – 3B) ‘Laetitia’ genotype. Increase 20x and 40x, respectively. Ep – epidermis; Pp – palisade parenchyma; PI – lacunar parenchyma; Xi-xylem.76
- Fig. 4.** Scanning photomicroscopy of plum leaf tissue. Top view of the adaxial face. (1A – 1B) Epidermis of ‘SC7’ genotype, with surface increase (1000x) and epicuticular layer detail, respectively. (2A – 2B) Epidermis of ‘SC13’ genotype, with surface increase (1000x) and epicuticular layer detail (3000x), respectively. (3A – 3B) Epidermis of ‘Laetitia’ genotype, with surface increase (1000x) and detail of the epicuticular layer (3000x), respectively.77
- Supplementary Fig. S1.** Half-normal plots with simulated envelopes for the deviance residuals from Poisson-Normal model fitted to the (a) *Bucephalogonia xanthophis* and (b) *Sibovia sagata* settling preference data.....78
- Supplementary Fig. S2.** Half-normal plots with simulated envelopes for the deviance residuals from Poisson-Normal model fitted to the (a) *Bucephalogonia xanthophis* and (b) *Sibovia sagata* honeydew excretion data.78
- Supplementary Fig. S3.** Descriptive analysis to settling preference for (a) *Bucephalogonia xanthophis* and (b) *Sibovia sagata* in plum genotypes (‘Laetitia’, ‘SC7’ and ‘SC13’). ‘Other’ represents the incidence of dead insects or insects perched on the walls and basis of the cages throughout the time (hour).79
- Supplementary Fig. S4.** Descriptive analysis to honeydew excretion for (a) *Bucephalogonia xanthophis* and (b) *Sibovia sagata* in plum genotypes (‘Laetitia’, ‘SC7’ and ‘SC13’) throughout the time (hour).80

CHAPTER 3

Figure 1 – Relative area (%) of the volatile compounds (cedrol^a, (*Z*)- β -damascenone^a, dendrolasin^a, (*E,E*)- α -farnesene, (*E,Z*)-geranyl linalool^a and *cis*-linalool oxide) emitted by six plum genotypes with different levels of resistance to leaf scald disease (*Xylella fastidiosa*).94

Figure 2 – A, C and E) Score plots of the samples. Principal components analysis (PCA) for six plum genotypes with different levels of resistance to plum leaf scald disease (*Xylella fastidiosa*). B, D and F) Loading plots of volatile compounds.....95

Figure 3 – Terpene pathway explain the occurrence of majority volatile compounds found in six plum genotypes with different levels of resistance to plum leaf scald disease (*Xylella fastidiosa*).....97

LIST OF TABLES

CHAPTER 1

- Table 1.** Diameter (mm) and fresh weight (g) of fruit collected from diseased and healthy plum cultivars in two growing seasons in different localities.31
- Table 2.** Cumulative incidence¹ (%) of brown rot (*Monilinia fructicola*) on plum fruit collected from plants with and without leaf scald symptoms in the 2015/2016 growing season.40

CHAPTER 2

- Table 1.** Overall mean number (\pm SE)^a of insects perched on treatments (plum genotypes) within experiments^b for *Bucephalagonia xanthophis* and *Sibovia sagata* settling preference72
- Table 2.** Mean number (\pm SE)^a of insects perched on treatments (plum genotypes) at successive periods after release in choice assays for *Sibovia sagata* settling preference.72
- Table 3.** Mean non-sequential EPG variable values for the probing behavior of *Bucephalagonia xanthophis* on three plum genotypes (treatment) during a 6-h recording.73
- Table 4.** Mean sequential EPG variable values for the probing behavior of *Bucephalagonia xanthophis* on three plum genotypes (treatments) during a 6-h recording.74
- Supplementary Table S1.** Parameter estimates by REML and their 95% simultaneous parametric bootstrap confidence intervals for settling preference of *Bucephalagonia xanthophis* and *Sibovia sagata*.80
- Supplementary Table S2.** Parameter estimates by REML and their 95% simultaneous parametric bootstrap confidence intervals for honeydew excretion of *Bucephalagonia xanthophis* and *Sibovia sagata*.81
- Supplementary Table S3.** Intraclass correlation coefficient between times by treatment.82

Supplementary Table S4. Backward procedure based on LRT to test experiment, cultivar, time, and interactions effects for settling preference of *Bucephalagonia xanthophis* and *Sibovia sagata*.82

Supplementary Table S5. Backward procedure based on LRT to test cultivar, time, and interaction effect for honeydew excretion of *Bucephalagonia xanthophis* and *Sibovia sagata*.83

Supplementary Table 6. Mean of cumulative honeydew excretion (mL) for *Bucephalagonia xanthophis* and *Sibovia sagata* and the respectively standard error of treatment (plum genotypes) within time (hours).83

CHAPTER 3

Table 1 - Plum genotypes, origin, parental of crosses and resistance level of plum leaf scald disease (PLS).88

Table 2 - Volatile compounds detected and quantified in plum leaves samples of six genotypes with different levels of resistance to leaf scald disease (*Xylella fastidiosa*) by GC-MS (Unit: relative area %).92

Table 3 - Unidentified volatile compounds quantified in plum leaves samples of six genotypes with different levels of resistance to leaf scald disease (*Xylella fastidiosa*) by GC-MS (Unit: relative area %).103

CHAPTER 4

Table 1. Ct values (cycle threshold) for plum leaves samples from seedlings pruned after cut in infected tree with plum leaf scald.111

LIST OF ACRONYMS

- AA – Ascorbic Acid
- AOAC – Official Methods of Analysis
- CA – California
- CAPES – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
- CIIAGRO – Centro Integrado de Informações Agrometeorológicas
- CLS – Coffee Leaf Scorch
- CNPQ – Conselho Nacional de Desenvolvimento Científico e Tecnológico
- CPD – Critical Point Drying
- CVC – Citrus Variegated Chlorosis
- DCFI – 2,6-Dichlorophenol Indolphenol-Sodium Indicator
- DNA – Deoxyribonucleic Acid
- EDTA – Ethylenediaminetetraacetic Acid
- EPAGRI – Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina
- EPG – Electrical Penetration Graphs
- ESALQ – Escola Superior de Agricultura “Luiz de Queiróz”
- FWL – Fresh Weight Loss
- FWS – Fresh Weight of the Sample
- GC – Gas Chromatograph
- GLMM – Generalized liner mixed effects model
- IAC – Instituto Agrônômico de Campinas
- IFW – Initial Fresh Weight
- INMET – Instituto Nacional de Meteorologia
- IPM – Integrated Pest Management
- LME – Linear Mixed Effects Model
- LRT – Likelihood Ratio Test
- LSD – Least Significant Difference
- MA – Massachusetts
- N – potencial drops during xylem phase
- np – non-probing
- NWEI – Number of waveform events per insect
- P – Probability
- PD – Pierce Disease

PAL – Phenylalanine Ammonia-Lyase
PCR – Polymerase Chain Reaction
PLS – Plum Leaf Scald
PME – Pectin Methylesterase
PPW – Proportion of individuals that produced a specific waveform type
PR – Parana
QSD – Quick Decline Syndrom
REML – Restricted Maximum Likelihood
SP – São Paulo
TA – Titratable Acidity
TBE – Tris/borate/EDTA
TSS – Total Soluble solids
UFPR – Universidade Federal do Paraná
USA – United States of America
USP – Universidade de São Paulo
UV – Ultra violet radiation
Xc – Contact/pre ingestion on xylem
Xi – Active ingestion on xylem
WDE – Waveform duration (min) per event
WDI – Total of waveform duration (min) per insect

LIST OF ABBREVIATIONS

B. xanthophis – *Bucephalagonia xanthophis*

Ltd. – Limited

S. sagata – *Sibovia sagata*

subsp. – subspecie

X. fastidiosa – *Xylella fastidiosa*

SUMMARY

1. GENERAL INTRODUCTION	22
CHAPTER I - POSTHARVEST QUALITY OF PLUMS IN RESPONSE TO THE OCCURRENCE OF LEAF SCALD DISEASE	25
ABSTRACT	25
1. INTRODUCTION	26
2. MATERIALS AND METHODS	27
2.1 Plant material and experimental design.....	27
2.2 Detection of <i>Xylella fastidiosa</i> in plum trees.....	28
2.3 Physical and chemical analyses	28
2.4 Biochemical analyses	29
2.5 Physiological analyses	29
2.6 Brown rot incidence.....	30
2.7 Statistical analysis	30
3. RESULTS	30
3.1 Effect of leaf scald on fresh weight loss and pulp firmness	33
3.2 Effect of leaf scald on fruit skin colour	34
3.3 Effect of leaf scald on pH, TA, TSS, and AA in fruit	36
3.4 Effect of leaf scald on PME activity.....	37
3.5 Effect of leaf scald on PAL activity	38
3.6 Effect of leaf scald on CO ₂ and ethylene production in fruit	39
4. DISCUSSION	40
5. CONCLUSIONS	43
ACKNOWLEDGEMENTS	43
REFERENCES	43

CHAPTER II - ASSOCIATION OF LEAF MORPHOLOGY AND VECTOR SETTLING AND FEEDING BEHAVIOR WITH RESISTANCE OF PLUM GENOTYPES TO LEAF SCALD DISEASE	48
ABSTRACT	48
1. INTRODUCTION	50
2. MATERIALS AND METHODS.....	52
2.1 Biological material: sharpshooters and plants.....	52
2.2 Settling preference assay	53
2.3 Statistical analysis of settling preference assay	54
2.4 Evaluation of sap ingestion (confinement assay)	55
2.5 Statistical analysis of sap ingestion assay	56
2.6 Probing behavior assay with <i>B. xanthophis</i>	57
2.7 Comparative leaf anatomy (preparation).....	59
2.8 Light Microscopy	59
2.9 Scanning Electron Microscopy.....	60
3. RESULTS.....	60
3.1 Settling preference.....	60
3.2 Evaluation of sap ingestion (confinement assay)	61
3.3 Probing behavior of <i>B. xanthophis</i>	61
3.4 Comparative leaf anatomy.....	62
4. DISCUSSION.....	62
ACKNOWLEDGEMENT	67
CHAPTER III. IDENTIFICATION OF VOLATILE COMPOUNDS FROM SIX PLUM GENOTYPES AS AN ATTEMPT TO UNDERSTAND THE RESISTANCE MECHANISM TO <i>Xylella fastidiosa</i> VECTORS.....	84
ABSTRACT	84
1. INTRODUCTION	85
2. MATERIAL AND METHODS	87

2.1	Plant Material and Sample Preparation	87
2.2	Volatile Extraction.....	88
2.3	Volatile Analysis.	88
2.4	Experimental Design and Statistical Analyses.....	89
3.	RESULTS.....	89
4.	DISCUSSION.....	95
	REFERENCES	98
	SUPPLEMENTARY MATERIAL.....	103
	CHAPTER IV. MECHANICAL TRANSMISSION OF <i>Xylella fastidiosa</i> TO <i>Prunus salicina</i> USING PRUNING SHEARS	105
	ABSTRACT	105
	RESUMO.....	106
	REFERENCES	112
	GENERAL CONCLUSIONS	116
	GENERAL REFERENCES	117
	APPENDICES.....	121

1. GENERAL INTRODUCTION

Plum is one of the most widespread temperate fruit in the world, and can be cultivated in several regions of the globe, due to the wide variety of species (CASTRO et al., 2008). Two of them stand out in the world production: *Prunus domestica* L., originated in the Caucasus region, but not commonly cultivated in Brazil (CASTRO et al., 2008); and *Prunus salicina* Lindl, originated in the Far East China, but cultivated for the first time in Japan for food purposes (NAKASU; CASTRO, 1990; OLIVEIRA, 2012).

The Japanese plum (family *Rosaceae*, subfamily *Prunoideae* and genus *Prunus*), is part of the group of stone fruits adapted to milder temperatures (KLABUNDE et al., 2014), predominantly in temperate climates, since they need a lower requirement of chilling hours when compared to European cultivars (CARVALHO; RASEIRA, 1989).

The world's largest producers of plum in order of relevance are China, Romania, United States, Serbia, Iran, Turkey, Chile, India, Italy and France (FAOSTAT, 2017). In Brazil, Santa Catarina state was considered the main producer of the fruit, with 18,513 tons of annual production (SÍNTESE ANUAL DA AGRICULTURA DE SANTA CATARINA, 2017), followed by Rio Grande do Sul (17,361 tons) (LEVANTAMENTO SISTEMÁTICO DA PRODUÇÃO AGRÍCOLA, 2017) and Paraná (10,288 tons) (ANDRADE, 2017).

This fruit has great national acceptance, but domestic production does not supply the Brazilian demand (MADAIL, 2003). From 2015 to 2017, imports grew by 27.5% (AGROSTAT/MAPA, 2018). In 2017 alone, approximately 12,600 tons of plums were imported (AGROSTAT/MAPA, 2018). On the other hand, exports did not reach 1,700 tons in that year (AGROSTAT/MAPA, 2018). Given these numbers, the Brazilian market of fresh plums presents great potential for expansion and good investment prospects.

Among the obstacles to the activity in Brazil, the lack of cultivars with good adaptability, the production of low-quality fruit and in particular the occurrence of plum leaf scald disease (PLS) should be highlighted (MADAIL, 2007). PLS was first reported in South America (Argentina) in 1930s (FERNANDEZ-VALIELA, BAKARCIC, 1954), and then in United States (FRENCH et al., 1977), Paraguay and Brazil (FRENCH, KITAJIMA, 1978) in 1970s. Currently, this disease is widespread in all fruit production centers in the country (Santa Catarina, Rio Grande do Sul, Paraná, São Paulo and Minas Gerais) (CASTRO; NAKASU; PEREIRA, 2008), causing a reduction up to 60 % in the useful life of orchards (MULLER, 2013).

Plum leaf scald is caused by *Xylella fastidiosa* (WELLS et al. 1987), a gram-negative bacterium that inhabits the xylem vessels of more than 350 plant species (EUROPEAN FOOD SAFETY AUTHORITY & EUROPEAN CENTER FOR DISEASE PREVENTION AND CONTROL, 2016) causing diseases in economic crops, such as grapevine, peach, plum, mulberry, citrus, coffee, macadamia (ALMEIDA and NUNNEY, 2015; BALDI and LA PORTA, 2017; GULDUR et al., 2005; HARTUNG et al. 1994; HOPKINS and PURCELL, 2002; JANSE and OBRADOVIC, 2010) and more recently in almond, oleander and olive trees in Europe (SAPONARI et al. 2013).

This bacterium presents phylogenetic diversity, with five subspecies already described: *fastidiosa* (alfalfa and grapevines strains), *morus* (mulberry strains), *multiplex* (plum, almond and peach strains), *pauca* (coffee, citrus and olive strains) and *sandyi* (oleander strains) (NUNNEY et al. 2014; SCHAAD et al. 2004; SCHUENZEL et al. 2005). In plums, a slightly irregular chlorosis on the leaf edges become visible after a long incubation period, when the pathogen titers achieve high densities (DAUGHERTY et al. 2011; RAJU et al. 1982), clogging the water conducting vessels (reduces the hydraulic conductance and sap flow) causing premature plants death (RAJU et al. 1982). However, these symptoms are not observed directly on the fruit. In citrus infected with citrus variegated chlorosis (*Xylella fastidiosa* subsp. *pauca*), physical and chemical changes have been reported in postharvest period (Agrios, 2005), which leads us to believe that plum fruit can also be affected by the presence of the bacterium. To date, no information has been found in the literature concerning how PLS interferes in the postharvest quality attributes of plums.

The disease is disseminated across long distances via infected but asymptomatic propagative material (JANSE and OBRADOVIC, 2010), grafting techniques (HE et al. 2000) and by sharpshooter leafhoppers (Hemiptera: Cicadellidae: Cicadellinae) (COLETTA-FILHO et al. 2016). The vector transmission occurs in three stages: a) the insect feeds on an infectious plant and acquires the bacterium; b) the bacterium remains attached in the insect foregut; c) the infected insect feeds on healthy host plants (BACKUS et al. 2015), characterizing the main spread form in the field. Another potential mode of dissemination was discovered for Pierce's disease (PD) in grapevines using contaminated pruning equipment (KRELL et al. 2007). In a study of PLS spatial distribution dynamics, Ferreira (2016) concluded that the onset of symptoms occurred within the same planting row, raising suspicions that cultural practices, such as pruning, could be able to transmit the bacterium. Nevertheless, no information is available about

this transmission type for PLS. If confirmed, the discovery of this new form of dissemination could imply in preventive control techniques, which would reduce potentially the secondary dissemination within the orchards.

In Brazil, the management strategy for PLS is to contain the pathogen dispersion, once, this pathosystem does not have an efficient applied control proposal (MULLER, 2013). The main measures used are the planting of healthy propagative material, eradication of infected trees, pruning of symptomatic branches and insecticides for the vectors (COLETTA-FILHO et al. 2016). Since 90s decade, breeding programs have been seeking by cultivars that prolong the useful life of the orchards (DALBÓ et al. 2018).

Recently, the Agriculture Research and Rural Extension of Santa Catarina (EPAGRI) identified genotypes that do not manifest plum leaf scald symptoms in the field and have favorable postharvest attributes (DALBÓ et al. 2018). However, the stability of this form of resistance and the mechanisms involved in this process have not been clarified and need to be investigated to ensure long-term management success. Thus, our hypothesis are that these new plum genotypes present morphological, structural and biochemical characteristics that could alter the spread of the disease and modify the epidemiology of this pathosystem. Some assumptions, such as the non-preference for settling and the reduction of xylem sap intake by the sharpshooters may explain this resistance, once these new genotypes are infected only by graft inoculation, supposing that the mechanism of resistance may be related to a vector transmission barrier. Another theory is that these plants may produce volatile compounds with repellent action, interfering with insect behavior.

Greater understanding about PLS is essential, once few information is available in the literature. Increased knowledge about the disease could facilitate the development of efficient management techniques and help small-scale producers and/or those with low levels of technology in the fruit scene. Therefore, the current study aimed to: i) determine the influence of leaf scald on postharvest quality attributes; ii) verify if the resistance of the new plum genotypes would be determined by the insect-plant relationship (particularly related with vector settling and feeding behavior linked with alterations in leaf morphology); iii) investigate the leaf volatile profile of plum genotypes with different resistance levels to leaf scald disease; and iv) evaluate the dissemination of the *Xylella fastidiosa* by pruning shears.

CHAPTER I - POSTHARVEST QUALITY OF PLUMS IN RESPONSE TO THE OCCURRENCE OF LEAF SCALD DISEASE¹

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ABSTRACT

Plum leaf scald (PLS) is considered the main barrier to expanding plum cultivation in Brazil. The disease causes water flow to be obstructed in the xylem of infected plants. Infected host plants produce fruit of lower quality; however, no information is available about the postharvest quality attributes in plum. Fruit from the ‘Gulfblaze’ and ‘Reubennel’ plant cultivars, both with and without PLS symptoms, were collected from commercial orchards. Bacterial presence was confirmed using a polymerase chain reaction, and the physicochemical, biochemical, and physiological properties were evaluated. Brown rot incidence was also determined during the ripening period. According to a molecular analysis, symptomatic plants used in the assay carried *Xylella fastidiosa* (which causes PLS), but healthy plants did not. Fruit collected from infected plants had a reduced diameter and weight in the 2015/2016 growing season. There was a greater reduction in pulp firmness and higher pectin methylesterase enzyme activity in fruit harvested from infected compared with healthy plum trees. The total soluble solids content was higher in fruit from ‘Gulfblaze’ and ‘Reubennel’ diseased compared with disease-free plants. A higher incidence of brown rot was observed in plants with leaf scald symptoms. The highest phenylalanine ammonia-lyase activity was observed in fruit from ‘Gulfblaze’ diseased plants. CO₂ and ethylene production was higher in the infected than healthy plants, indicating that the presence of *X. fastidiosa* can accelerate fruit ripening mechanisms.

Keywords: Fruit quality; *Xylella fastidiosa*; Xylem obstruction

¹ This manuscript is published on Postharvest Biology and Technology Journal.

1. Introduction

Plum is considered promising as a crop for further development in Brazil, since current supply does not meet national demand (Kist et al., 2012). However, commercial orchards have not achieved their expected productive potential due to the occurrence of plum leaf scald (PLS) disease, which is caused by the bacterium *Xylella fastidiosa*. This pathogen has a wide range of hosts and causes various diseases, including grapevine (Pierce's disease) (Hopkins, 1985), citrus (citrus variegated chlorosis; CVC) (Rossetti et al., 1990), and coffee (coffee leaf scorch; CLS) (Paradela-Filho et al., 1995). The disease is disseminated via infected propagative material and insect vectors, such as sharpshooter leafhoppers (Hemiptera: Cicadellidae, Cicadellinae) (Coletta-Filho et al., 2016).

The main symptoms of PLS occur in the leaves, only becoming visible after a long incubation period (approximately eight to nine months without hydric stress), after which the bacterial population is distributed systemically in the plum trees (Raju et al., 1982; Almeida and Nunney, 2015). Initially, the disease manifests as a slightly irregular chlorosis on the leaf edges. This chlorosis intensifies at the end of the vegetative cycle, resulting in marginal desiccation (Raju et al., 1982). However, these symptoms are not observed directly on the fruit.

Vascular diseases, such as PLS, block xylem transport and induce water stress in their hosts, increasing the resistance to water flow (Tyree and Sperry, 1989; Fletcher and Wayadande, 2002). Plum trees colonized by *X. fastidiosa* present similar symptoms to those of water deficiency, due to occlusion of the xylem bundles by bacterial cluster formation (Janissen et al., 2015). In general, tissue dehydration can lead to disturbances during the postharvest period, because the resultant increased respiration and ethylene production accelerates ripening processes. In addition, water scarcity causes a reduction in fruit diameter, weight loss, flavour changes, and decreased pulp firmness (González-Altozano and Castel, 1999; Girona et al., 2003). Physical and chemical changes have been reported during processing in citrus plants with CVC symptoms; this citrus disease affects not only the production, but also postharvest quality of these fruit (Agrios, 2005).

Plum is a highly perishable fruit (Margarim et al., 2007). Therefore, its shelf life is reduced by any physiological or metabolic alterations that interfere negatively with its maturation process, compromising its commercialization and consequently affecting the prices paid to producers.

To date, no information has been found in the literature concerning how PLS interferes in the postharvest quality attributes of plums. This study therefore aimed to

determine the influence of leaf scald disease on the physical, chemical, physiological, and biochemical characteristics of plum fruit.

2. Materials and methods

2.1 Plant material and experimental design

Two Japanese plum (*Prunus salicina* Lindl) cultivars ('Gulfblaze' and 'Reubennel') were selected from Sigma Agroambiental Ltd., which is located in the producing region of Paranapanema, São Paulo (SP), Brazil. 'Reubennel' was also collected from a commercial orchard located in the metropolitan region of Curitiba (Araucaria), Parana (PR), Brazil.

The treatments comprised: 1) fruit from plants with leaf scald symptoms; and 2) fruit from healthy plants. A total of 500 fruit per treatment per collection was used for each of the following cultivars: 'Gulfblaze' (SP); 'Reubennel' (PR) and 'Reubennel' (SP). Plum trees were standardized according to canopy size and fruit number, so that the relationship between fruit and leaves was the same among treatments. The environmental conditions and agricultural management were similar for each cultivar in each locality. Symptomatic trees were located at least 200 m away from healthy trees within the same field.

Plums were manually harvested in the morning, randomly selected from several plant heights at the commercial ripening stage. Fruit that presented with fungal or insect lesions or mechanical damage were discarded. Selected fruit were stored in a controlled temperature chamber at 22 °C and 85 % relative humidity in darkness.

The following fruit characteristics were evaluated in the 2015/2016 growing season: diameter, fresh weight, pulp firmness, fresh weight loss, colouration, pH, titratable acidity (TA), content of total soluble solids (TSS), ascorbic acid content, pectin methylesterase (PME) and phenylalanine ammonia-lyase (PAL) enzyme activity, CO₂ and ethylene production, and brown rot incidence. In the 2016/17 growing season, further analyses were conducted to measure diameter, fresh weight, fresh weight loss, pulp firmness, TA, and TSS content for 'Reubennel' (PR) fruit, which were stored under the same conditions as fruit analysed in the previous growing season.

2.2 *Detection of Xylella fastidiosa in plum trees*

In order to detect the bacterium (*X. fastidiosa*) in symptomatic and asymptomatic plants, DNA was extracted from the petioles using a Biopur Mini Spin Planta Extraction Kit (Mobius Life Sciences, Pinhais, PR, Brazil), according to the manufacturer's instructions. The extracted DNA samples were amplified using a pair of specific primers, RST31 and RST33 (Minsavage et al., 1994). The amplification was performed in a PTC-100 thermal cycler (Research Inc., Watertown, MA, USA), programmed with the following conditions: one initial cycle of 94 °C for 5 min; 32 denaturation cycles at 94 °C for 40 s; annealing at 50 °C for 40 s; an extension at 72 °C for 59 s; followed by one stabilization cycle at 72 °C, indefinitely. Polymerase chain reaction (PCR) products were separated by agarose gel electrophoresis (1.5 %) with a Tris/borate/EDTA (TBE) buffer (89 mM Tris, 89 mM boric acid, and 2 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0). The amplified fragments were visualized under ultraviolet light, and documented using the Eagle Eye II system (Stratagene, LaJolla, CA, USA).

2.3 *Physical and chemical analyses*

Transversal and longitudinal diameter (mm) was determined using a digital calliper (Vonder PPV 1506). Mean fresh fruit weight (g) was measured using an analytical balance (Balmak MP5) when fruit was harvested. Fresh weight loss (%) during ripening was estimated according to the formula: $FWL = \left[\frac{IFW - FWS}{IFW} \right] \times 100$, where: *FWL* = fresh weight loss during ripening (%), *IFW* = initial fresh weight of the sample, and *FWS* = fresh weight of the sample at the evaluated ripening periods.

A digital penetrometer (TR-Turoni) with a tip diameter of 8 mm was used to measure pulp firmness from two readings taken from opposite sides of each fruit's equatorial region. The results were expressed in Newton (N) as the average of the two readings.

Skin colour was determined using a colorimeter (Model CR-300; Minolta Co., Ltd, Osaka, Japan) that had previously been calibrated on a white surface. For this evaluation, two readings were taken from opposite sides of the equatorial region of the fruit, evaluating three colour parameters: *L** (luminosity), *a** (colouration in the red to green region), and *b** (colouration in the yellow to blue region). The results were expressed as hue angle (hue °) ($h^\circ = \tan^{-1}(b^*/a^*)$) and in chroma (*C**) ($C = (a^*^2 + b^*^2)^{1/2}$) (Minolta, 1994).

The pH of the pure juice was determined using a digital potentiometer (MS Tecnopeon mPA-210, Piracicaba, Brazil), standardized buffer solutions of pH 4.0 and 7.0. TA was determined using 0.1 N NaOH solution to titrate 10 mL of fruit juice diluted in 90 mL of distilled water, until a pH of 8.1 was achieved. Values were expressed as % of malic acid equivalent (Instituto Adolfo Lutz, 1985). The TSS content was determined using a digital benchtop refractometer (Palette PR-101; Atago Co Ltd., Tokyo, Japan), with results expressed as %. Ascorbic acid (AA) content was determined by reducing the 2,6-dichlorophenol indolfenol-sodium indicator (DCFI) with ascorbic acid, using titration (AOAC, 1997). All analyses were performed on the first, third, fifth, and seventh days after harvest.

2.4 *Biochemical analyses*

PME (EC 3.1.1.11) activity was determined from the carboxylic acid groups released by the action of this enzyme, using titration (Kertesz, 1951). The enzymatic activity measurement results were expressed in nmol of hydrolysed esters per gramme of pulp per unit of time ($\text{nmol g}^{-1} \text{min}^{-1}$).

PAL (EC 4.3.1.5) activity was determined using the conversion rate of L-phenylalanine to trans-Cinnamic acid at 290 nm, according to a methodology adapted by Peixoto et al. (1999). The result of this enzymatic activity was expressed in mmol min^{-1} . All analyses were performed on the first, third, fifth, and seventh days after harvest.

2.5 *Physiological analyses*

Freshly harvested plums from the ‘Gulfblaze’ (SP) and ‘Reubennel’ (SP) cultivars were placed in 0.59 L hermetically sealed glass jars, and maintained at 25 °C for 1 h. A headspace volume of 1 mL was removed from the glass jars with a syringe (Gastight; Hamilton, Nevada, USA) via a rubber and injected into a gas chromatograph (Thermo Finnigan Trace GC 2000, Thermo Fisher Scientific, Waltham, MA, USA) coupled to flame ionization detector and equipped with two Porapak N columns (1.8 m and 4 m in length for ethylene and CO₂ samples, respectively). The carrier gases used in these analyses were hydrogen (27 mL min⁻¹ constant flow) and nitrogen (30 mL min⁻¹ constant flow) for quantifying CO₂ concentration and determining ethylene, respectively. Temperatures were maintained in the apparatus at 140 °C (column), 120 °C (injector), 250 °C (detector), and 350 °C (methanator). Standard CO₂ and ethylene gases were used

to elaborate the standard curves. CO₂ and ethylene quantifications were determined from the difference between the initial gas concentration inside the containers at the time of closure and after 1 h, expressed as mg kg⁻¹ s⁻¹ for CO₂ and as ng kg⁻¹ s⁻¹ for ethylene (C₂H₄). Evaluations were performed on the first, third, fifth, sixth, and seventh days after harvest. Ten replicates were performed, with each replicate comprising three fruit.

2.6 *Brown rot incidence*

The cumulative incidence of brown rot disease (%) was obtained by counting fruit (180 fruit per treatment) showing visible symptoms of the pathogen (*Monilinia fructicola*) at seven days postharvest.

2.7 *Statistical analysis*

The trials followed a completely randomized design, comprising two treatments (fruit from diseased and fruit from healthy plants) and 20 replicates (with each replicate comprising one fruit). The data were submitted to an analysis of variance (ANOVA). A Tukey test (0.01 %) was used to separate averages for the variables of brown rot incidence, diameter, and fresh weight. For the other variables, the separation of averages was performed using a least significant difference (LSD) test (P = 0.01) over time. Data were represented as means and standard errors. The R statistical software package (R Development Core Team, 2012) was used for the analysis. All experiments were undertaken in duplicate and, when the treatments did not differ, the means of the two assays were used.

3 **Results**

Bacterial presence resulted in a significant difference in fruit diameter (Table 1) among different cultivars and localities (Fig. 1). Fruit collected from plants infected with *X. fastidiosa* had a smaller diameter, compared with healthy plants, for the ‘Gulfblaze’ (SP), ‘Reubennel’ (PR), and ‘Reubennel’ (SP) cultivars (P < 0.01). Pathogen presence also reduced fresh fruit weight in 2015/2016. In 2016/2017, however, no significant differences in diameter and fresh weight between fruit from diseased and healthy ‘Reubennel’ (PR) plants were observed (Table 1).

Table 1. Diameter (mm) and fresh weight (g) of fruit collected from diseased and healthy plum cultivars in two growing seasons in different localities.

Variable	Season	Cultivar/State	Healthy plants	Diseased plants
Diameter (mm)	2015/16	'Gulfblaze' (SP)	39.99 a	35.19 b
		'Reubennel' (SP)	43.64 a	38.93 b
		'Reubennel' (PR)	44.98 a	40.71 b
Fresh weight (g)	2016/17	'Reubennel' (PR)	50.242 a	48.621 a
	2015/16	'Gulfblaze' (SP)	53.15 a	29.49 b
		'Reubennel' (SP)	50.25 a	35.78 b
		'Reubennel' (PR)	59.41 a	46.74 b
	2016/17	'Reubennel' (PR)	77.875 a	74.541 a

Means followed by the same letter in the line do not differ according to a Tukey test ($P < 0.01$).

The results consist of the mean of two replicates. PR (Parana, Brazil); SP: São Paulo, Brazil).

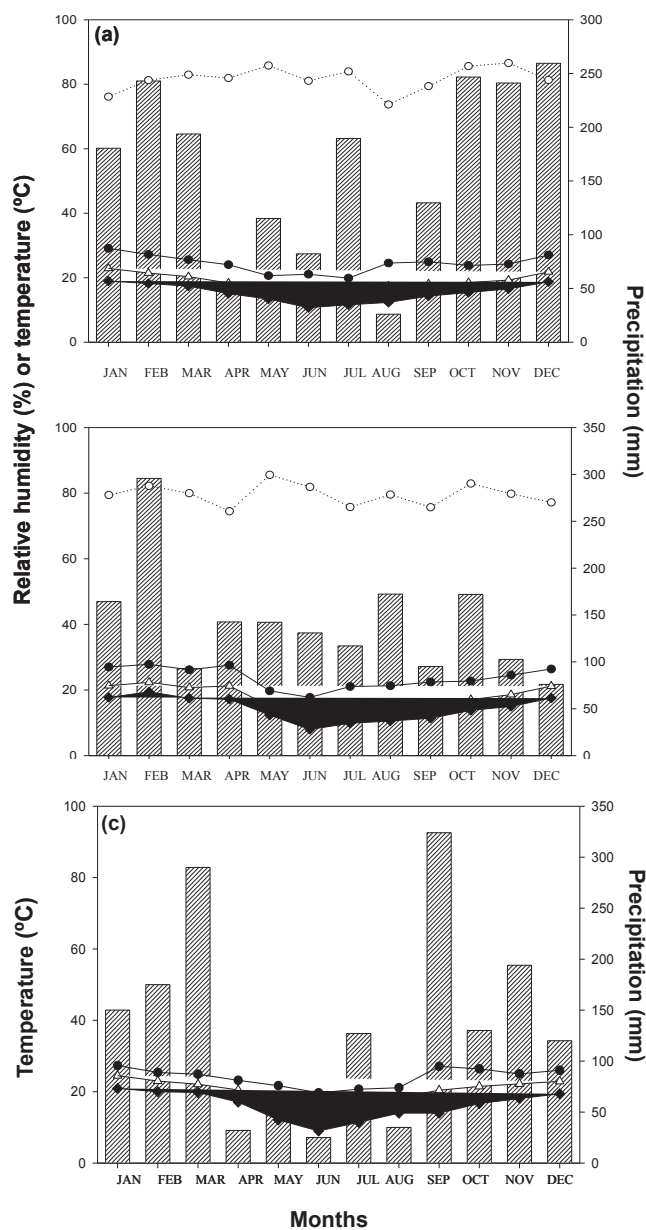
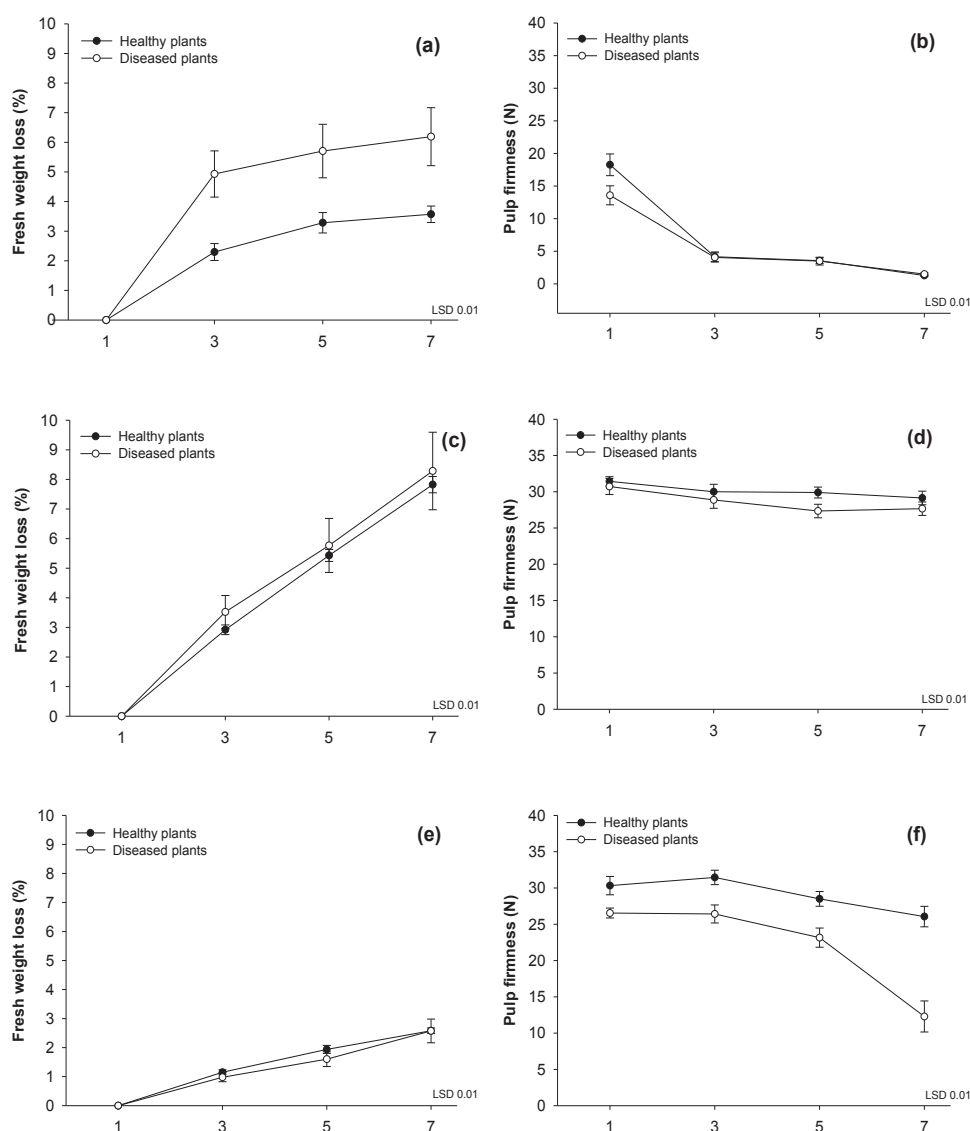


Fig. 1. Meteorological data from the Curitiba (Parana) automatic station (Lat. 25.43°; Lon. 49.26°; Height above sea level 923.5 m) for the (a) 2015/2016 and (b) 2016/2017 growing seasons, provided by the Instituto Nacional de Meteorologia (INMET); and (c) data from the Paranapanema (São Paulo) automatic station (Lat. 25.23°; Lon. 48.43°; Height above sea level 610 m) for the 2015/2016 growing season, provided by the Centro Integrado de Informações Agrometeorológicas (CIIAGRO). The dotted lines with empty circles represent average relative humidity, and vertical bars represent precipitation. Temperature is represented by a solid line: maximum temperature (—●—), medium temperature (—▲—), and minimum temperature (—◆—).

3.1 Effect of leaf scald on fresh weight loss and pulp firmness

‘Gulfblaze’ (SP) fruit collected from symptomatic plants showed a marked loss of fresh weight (5 %) in the initial evaluation (Fig. 2A). In contrast, fruit harvested from healthy trees of the same cultivar maintained their weight during the ripening period. No significant differences in weight loss were detected among treatments for the ‘Reubennel’ (PR and SP) cultivar (Fig. 2C, E, and G) in both growing seasons. The main difference was observed between localities in 2015/2016, with ‘Reubennel’ (PR) presenting a weight loss of 8 % and 2 % in ‘Reubennel’ (SP).



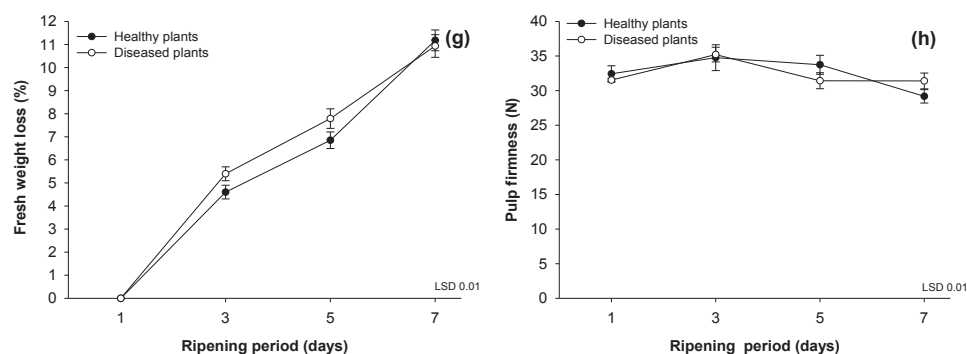


Fig. 2. Fresh weight loss and pulp firmness of plum fruit collected from plants with and without symptoms of leaf scald, from the Parana (PR) and São Paulo (SP) growing regions. Growing season 2015/2016: A and B, ‘Gulfblaze’ cultivar (SP); C and D, ‘Reubennel’ cultivar (PR); E and F, ‘Reubennel’ cultivar (SP). Growing season 2016/2017: G and H, ‘Reubennel’ Cultivar (PR). Each point corresponds to the average of 20 repetitions. The least significant difference (LSD) was used to calculate separation of means. Bars represent the standard error.

In all treatments, pulp firmness reduced throughout the ripening period, but with values that were lower in fruit harvested from plants with leaf scald symptoms (Fig. 2). In the first evaluation, fruit from ‘Gulfblaze’ (SP) healthy plants presented superior firmness (20 N) than fruit from trees with leaf scald symptoms (15 N) (Fig. 2B). Differences between treatments were observed on all evaluation dates for ‘Reubennel’ (SP) (Fig 2F). On the seventh day of evaluation, fruit harvested from ‘Reubennel’ (SP) plants carrying the bacteria showed a pulp firmness of 12.3 N, compared with 26 N in fruit harvested from healthy plants; this indicated that pulp firmness in fruit from healthy trees declines more slowly than in diseased plants. In both growing seasons, fruit firmness in ‘Reubennel’ (PR) remained close to 30 N in all treatments (Fig. 2D and H).

3.2 Effect of leaf scald on fruit skin colour

Chromaticity is a unit that expresses colour intensity. Chroma (C^*) values close to zero constitute neutral colours, while values close to 60 represent more vivid colours. Hue angles ($^{\circ}$ hue) closer to 90° and 0° characterize yellow and reddish fruit, respectively. Chroma and hue angle values gradually decreased during the postharvest period. The lowest chromaticity values were observed in fruit collected from diseased plants. Differences between treatments were observed for ‘Gulfblaze’ (SP) in all evaluations

(Fig. 3A), but only in the harvest period for ‘Reubennel’ (PR) (Fig. 3C). For ‘Reubennel’ (SP), the chroma values only differed on the seventh day of ripening, with chromaticity values of 12.4 and 18 for fruit from diseased and healthy plants, respectively (Fig. 3E).

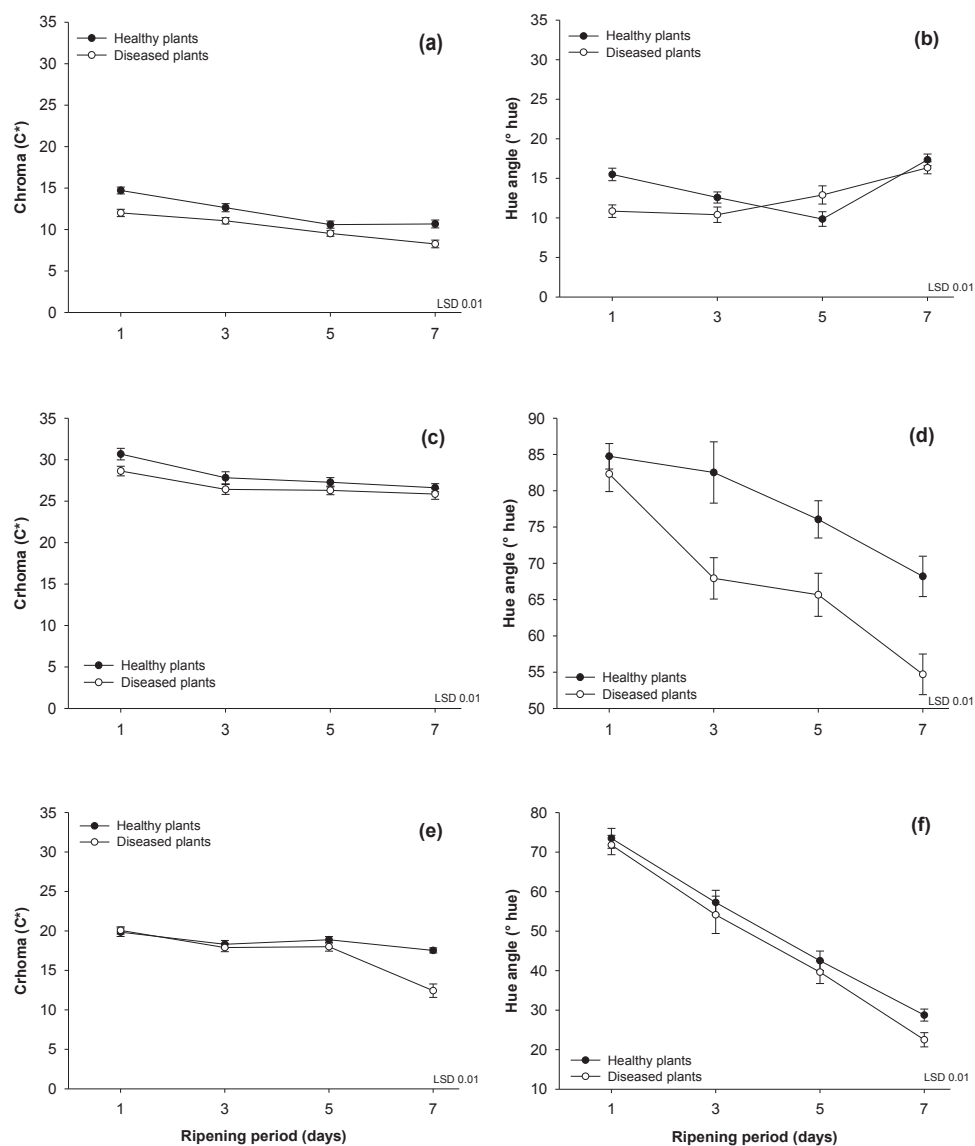


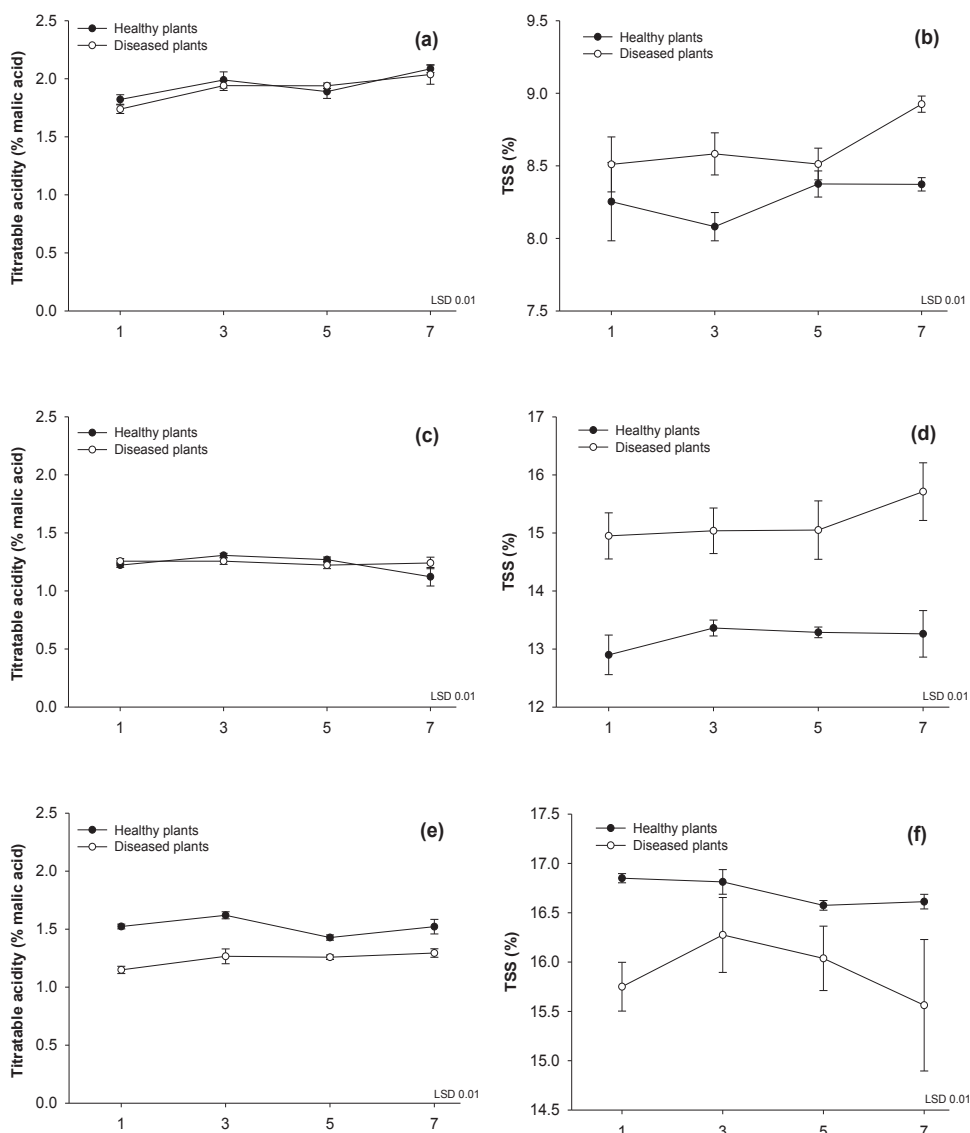
Fig. 3. Chroma (C*) and hue angle (°hue) of plum fruit collected from plants with and without symptoms of leaf scald, from the Parana (PR) and São Paulo (SP) growing regions. Growing season 2015/2016: A and B, ‘Gulfblaze’ (SP) cultivar; C and D, ‘Reubennel’ (PR) cultivar; E and F, ‘Reubennel’ cultivar (SP). Each point corresponds to the average of 20 repetitions. The least significant difference (LSD) was used to calculate the separation of means. Bars represent the standard error.

There were no significant differences in hue angle values among ‘Gulfblaze’ (SP) fruit ($P < 0.01$). ‘Reubennel’ (SP) showed differences only on the seventh day of ripening,

when fruit from diseased plants had a reddish colouration (23.3°) compared with the value (32.8°) for fruit removed from disease-free trees. The difference in colouration in ‘Reubennel’ (PR) had already been determined during the first evaluation, and was maintained until the seventh day of ripening (Fig. 3D).

3.3 Effect of leaf scald on pH, TA, TSS, and AA in fruit

No differences between treatments were observed in either pH or AA content. Acidity levels for ‘Gulfblaze’ (SP) and ‘Reubennel’ (PR) remained close to 1.5 % throughout the ripening period (Fig. 4). Fruit from healthy ‘Reubennel’ (SP) plants were more acidic than fruit harvested from diseased trees.



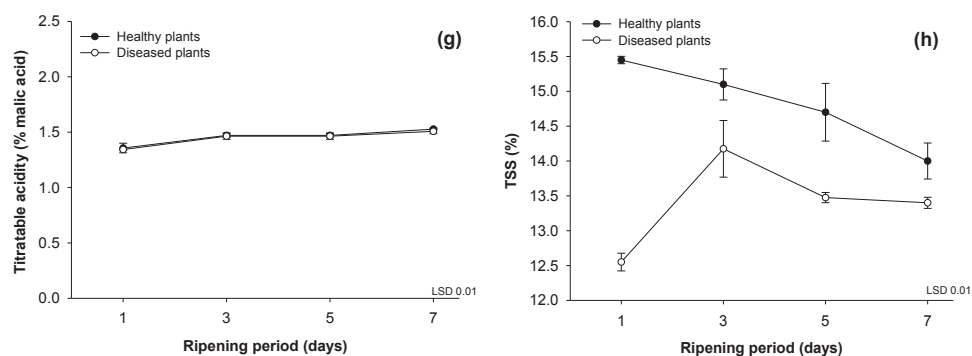


Fig. 4. Titratable acidity (% of malic acid) and total soluble solid (% TSS) content in plum fruit collected from plants with and without symptoms of leaf scald, from the Parana (PR) and São Paulo (SP) growing regions. Growing season 2015/2016: A and B, ‘Gulfblaze’ (SP) cultivar; C and D, ‘Reubennel’ (PR) cultivar; E and F, ‘Reubennel’ (SP) cultivar. Growing season 2016/2017: G and H, ‘Reubennel’ (PR) cultivar. Each point corresponds to the average of 20 repetitions. The least significant difference (LSD) was used to calculate separation of means. Bars represent the standard error.

TSS content increased during the 2015/16 fruit ripening period for the ‘Gulfblaze’ (SP) and ‘Reubennel’ (PR) cultivars. In addition, fruit from diseased trees had a sweeter flavour than fruit removed from healthy plants. However, leaf scald incidence did not result in increased sugar content in ‘Reubennel’ (SP), because the maximum amount of soluble solids (16.9 %) was observed in fruit from healthy plants. For ‘Reubennel’ (PR) in 2016/2017, no significant differences in acidity levels were observed. TSS content had an opposite behaviour to the 2015/16 growing season. Fruit from healthy plants showed a higher sugar concentration in the first evaluation, but no difference was observed between treatments in subsequent evaluations (Fig. 4H).

3.4 Effect of leaf scald on PME activity

PME enzyme activity was higher in fruit from ‘Gulfblaze’ (SP) and ‘Reubennel’ (SP) plants colonized by *X. fastidiosa*, compared to fruit from disease-free plants; the enzymatic peak for both cultivars was observed on the third day of ripening, with values of 1708 and 746.6 nmol g⁻¹ min⁻¹, respectively (Fig. 5A and E). No significant differences were observed among treatments for ‘Reubennel’ (PR) during the ripening period (Fig. 5C).

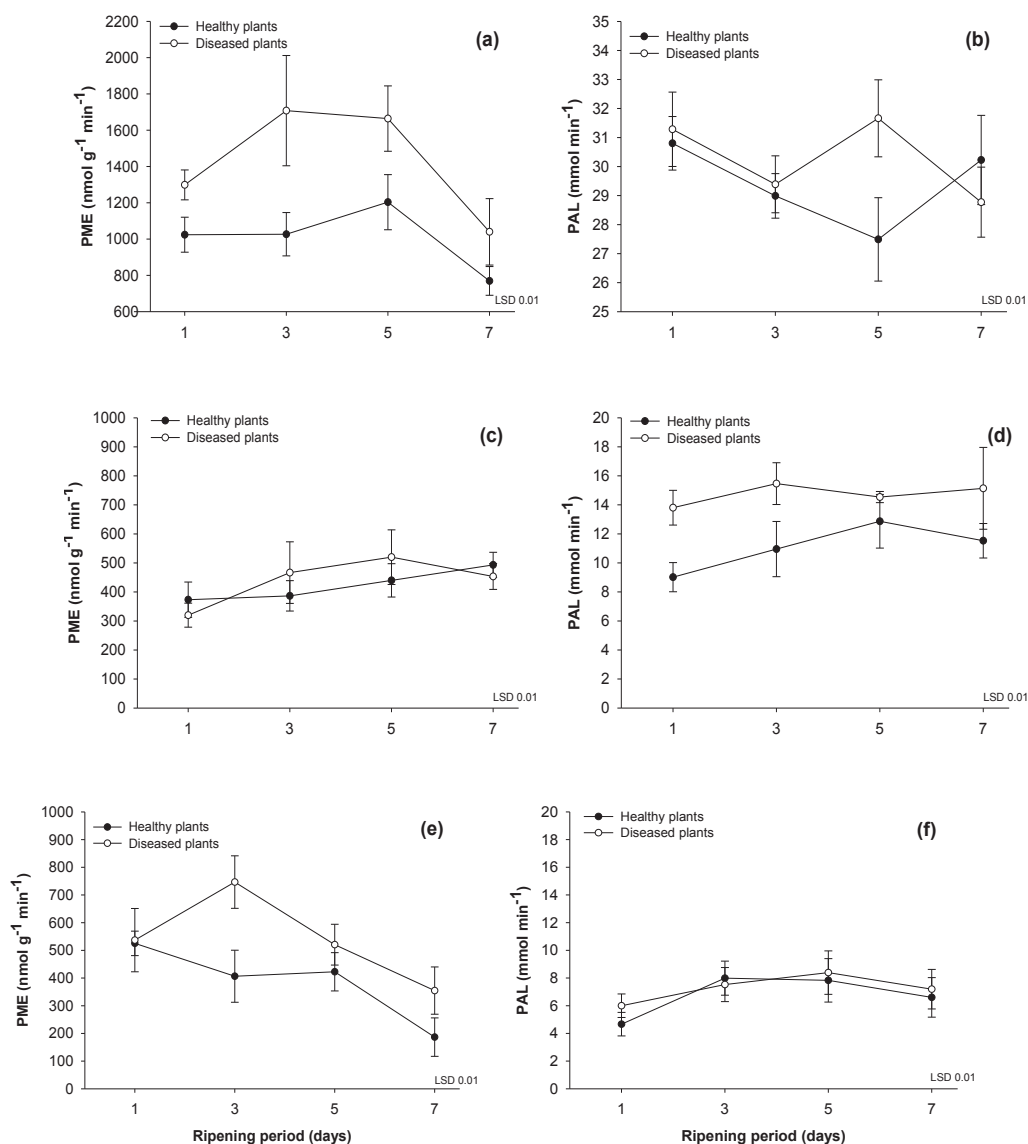


Fig. 5. Activity of the enzymes pectin methylesterase (PME) and phenylalanine ammonia-lyase (PAL) in plum fruit collected from plants with and without leaf scald symptoms, from the Parana (PR) and São Paulo (SP) growing regions. Growing season 2015/2016: A and B, 'Gulfblaze' (SP) cultivar; C and D, 'Reubennel' (PR) cultivar; and E and F, 'Reubennel' (SP) cultivar. Each point corresponds to the average of 20 repetitions. The least significant difference (LSD) was used to calculate separation of means. Bars represent the standard error.

3.5 Effect of leaf scald on PAL activity

The highest PAL activity was found in fruit from diseased 'Gulfblaze' (SP) plants. The enzymatic peak (32 mmol min⁻¹) for these fruit was observed on the fifth day of

ripening. However, fruit collected from healthy ‘Gulfblaze’ (SP) trees presented enzymatic activity of 27 mmol min^{-1} (Fig. 5B).

3.6 Effect of leaf scald on CO_2 and ethylene production in fruit

The climacteric ripening pattern in plum is represented by an increase in CO_2 and ethylene production during postharvest storage (Fig. 6). For both cultivars, fruit collected from symptomatic trees produced more CO_2 and ethylene than disease-free trees. The maximum CO_2 peak for ‘Reubennel’ (SP) occurred concomitantly with the maximum value of ethylene production on the sixth day of ripening. The rate of increase in CO_2 was determined on the third day of ripening for fruit from infected ‘Gulfblaze’ (SP) trees, while an increase in ethylene was observed on the sixth day of ripening (Fig. 6).

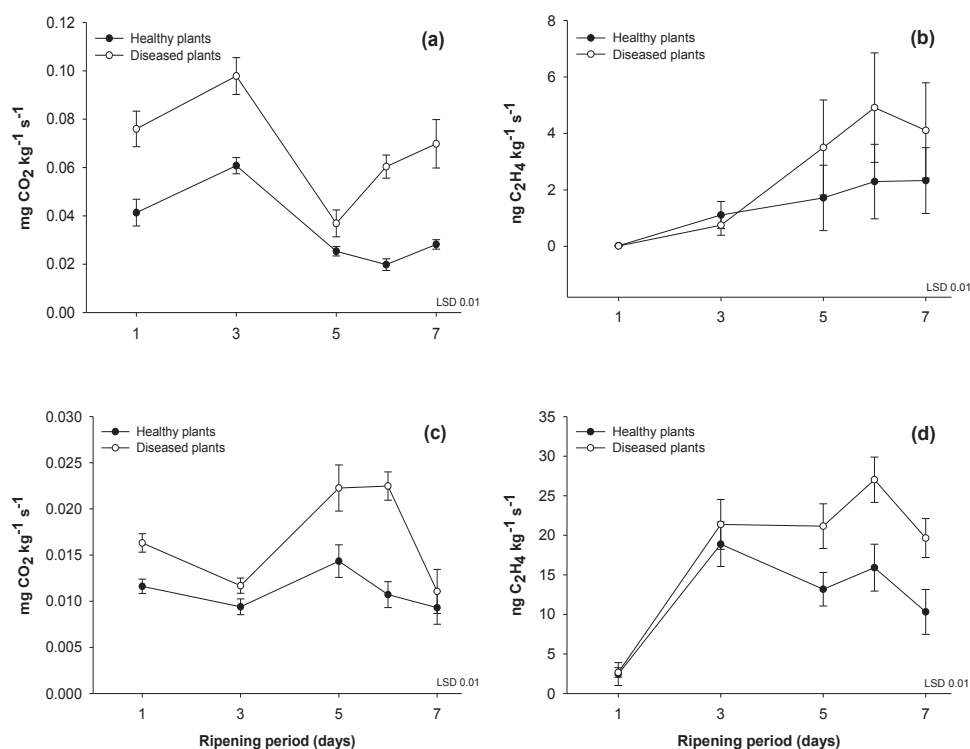


Fig. 6. CO_2 and ethylene production in plum fruit collected from plants with and without leaf scald symptoms, from the Parana (PR) and São Paulo (SP) growing regions. Growing season 2015/2016: A and B, ‘Gulfblaze’ (SP) cultivar; C and D, ‘Reubennel’ (SP) cultivar. Each point corresponds to the average of 20 repetitions. The least significant difference (LSD) was used to calculate separation of means. Bars represent the standard error.

3.7 Effect of leaf scald on fruit rot incidence

Fruit harvested from both cultivars with leaf scald symptoms showed a higher incidence of brown rot during the experiment in the 2015/2016 growing season, compared with fruit harvested from healthy plants (Table 2).

Table 2. Cumulative incidence¹ (%) of brown rot (*Monilinia fructicola*) on plum fruit collected from plants with and without leaf scald symptoms in the 2015/2016 growing season.

Cultivar	Healthy plants	Diseased plants
‘Gulfb blaze’ (SP)	20.00 b	42.20 a
‘Reubennel’ (SP)	17.77 b	29.44 a
‘Reubennel’ (PR)	23.88 b	32.77 a

Means followed by the same letter (lower case in columns) do not differ according to a Tukey's test ($P < 0.01$). ¹7 d postharvest. PR (Parana, Brazil); SP: São Paulo, Brazil).

4. Discussion

PLS interferes directly in the postharvest quality attributes of plum fruit. Compared with fruit from healthy trees, fruit from diseased trees in general presented with a lower weight, diameter, and firmness, higher brown rot incidence and sugar concentrations, as well as increased PME and PAL enzymatic activity, and higher levels of ethylene and CO₂ production. Very low availability of water and nutrients during the growth stages of *X. fastidiosa*-infected plants can trigger irreversible changes in the fruit, such as a drastic reduction in size, weight loss, and alterations in physicochemical characteristics (Johnson and Phene, 2008). Citrus fruit affected by *X. fastidiosa* are often one third smaller than healthy fruit (Agrios, 2005).

Water stress caused by PLS incidence has a huge impact on physiological and biochemical processes in infected plants. Some plum cultivars have a greater sensitivity to drought conditions than do others. In this study, fruit from infected ‘Gulfb blaze’ trees showed a reduced diameter and weight. In contrast, no significant differences in weight loss were detected among treatments for ‘Reubennel’ (PR and SP) cultivars in both growing seasons. However, in 2015/2016 it was observed that ‘Reubennel’ (PR) presented a higher increase in weight loss than ‘Reubennel’ (SP). In this context, climatic conditions may either intensify or mitigate the effects of the disease during the postharvest period. Conditions in years of higher average temperatures favour the development and

survival of the inoculum inside the plant. There were mild temperatures throughout the 2016/2017 growing season, suggesting the hypothesis that low temperatures could effectively eliminate or reduce the bacterial population, as already reported for *X. fastidiosa* subspecies *fastidiosa*, a causal agent of Pierce's disease in vine (Burbank and Stenger, 2016).

Firmness is one of the most significant variables in determining the postharvest quality and commercial value of fruit (Ozturk et al., 2012). The reduction of pulp firmness in fruit from infected plum trees may be related to decreased stiffness and to pulp softening. This marked loss of tissue water occurs because of decreases in turgescence pressure and the increased activity of pectin-degrading enzymes in the cell walls (Malgarim et al., 2007), as well as compromising the relationship between essential nutrients for the maintenance of membrane integrity, such as calcium and nitrogen.

Cuticular wax is developed during fruit ontogeny in a large range of fruit, including plum (Wisuthiphaet et al., 2014), and is relevant to fruit quality (Chu et al., 2018). Based on this, it is possible to propose another hypothesis concerning the high water loss in fruit harvested from *X. fastidiosa*-infected trees during the postharvest period. Diseased plants suffer in response to drought because of occlusion of the xylem vessels, which affects the entire tree, including the fruit. This stress could modify the dynamics of wax composition in the fruit epidermis, causing a reduced water retention capacity and rapid dehydration of this vegetal organ. According to some authors, however, postharvest water loss is not related to total wax content, but to the lipid composition of the cuticle (Parsons et al., 2013), and can vary between species, cultivars, and environmental conditions (Vichi et al., 2016).

In plums, PME is one of the main degradation enzymes (Manganaris et al., 2008). The activity of this enzyme is related to changes in characteristics of the middle lamella polysaccharides, which act to increase the depolymerization and solubilization of pectins present in the cell wall (Resende et al., 2004). PME activity is raised by increasing CO₂ production in climacteric fruit (Valero et al., 2013). Thus, the higher concentration of CO₂ present in fruit from diseased compared with healthy plants may be a cause of the increased PME activity observed in the present study, which promotes fruit softening (Tucker, 1993).

TSS, AA, pH and TA are important indicators during plum ripening (Liu et al., 2014). The acid content usually tends to decrease with maturation, because organic acids are consumed by respiration (Majeed and Jawandha, 2016). However, in the present

work, no significant decreases in acidity levels were observed for the treatments, since malic acid and pH remained practically constant throughout the evaluation period. Plum trees with leaf scald suffer from water deficiency, leading to low water availability for the fruit and therefore favouring sugar concentration and attenuation of the acidity content.

Another assumption is that plants infected by *X. fastidiosa* usually suffer from potassium deficiency (Vitti et al., 1989). Potassium plays an important role in carbohydrate metabolism and, compared with healthy plants, this deficiency would be expected to result in lower starch and higher sugar concentrations, followed by increased β -amylase and acid invertase enzyme activity (Lavon et al., 1995). A study of vine leaves infected by *X. fastidiosa* has demonstrated a relationship between potassium deficiency and low starch concentration (Goodwin et al., 1988). Thus, the atypical behaviour of the cultivar 'Reubennel' (SP) in relation to sugar distribution dynamics may result from the circumstances in which these plants were grown, in which they might have received only a low quantity of nutrients, including potassium. High temperatures are known to occur frequently in the Paranapanema (SP) region, mainly in the months preceding the harvest. For this reason, leaf scald becomes more intense in these areas, and may cause metabolic and enzymatic imbalances leading to starch accumulation in plant organs.

Colour attributes are an important factor in perceptions of plum quality (Liu et al., 2014). In the present study, the plum colour characteristics (chroma and hue angle) gradually decreased during postharvest storage, especially in the fruit from contaminated trees. Plants with PLS suffer from defoliation, resulting in increased solar radiation in the canopy. This may directly interfere in colour change and fruit ripening processes, promoting chlorophyll degradation and stimulating the synthesis of anthocyanins and carotenoids (Awad et al., 2001); this leads to the fruit skin having a darkened colour appearance.

PAL plays a crucial role in the secondary metabolism of plants, being one of the most studied enzymes with a role in protecting against biotic and abiotic stresses (Hyun et al., 2011). In the case of plum, PAL is one of the key enzymes involved in anthocyanin biosynthesis (Wu et al., 2011), in protection from UV radiation, and in the regulation of some molecules (Dixon and Paiva, 1995). The 'Gulfblaze' cultivar is considered highly resistant to *X. fastidiosa* infection (Rouse and Sherman, 1997), which suggests that these plants have the ability to signal and defend against this bacterium, unlike 'Reubennel',

which is highly susceptible to leaf scald. However, in the experimental conditions both cultivars showed behaviours of susceptibility to infection.

The respiratory rate in climacteric fruit rises with maturation processes (Liu et al., 2014). The highest CO₂ and ethylene production was correlated with the presence of *X. fastidiosa*. Increased respiration and ethylene production would act to intensify senescence processes, therefore increasing fruit exposure to pathogen attack during the postharvest period; this confirms the highest incidence of brown rot observed in the leaf scald symptomatic trees. The high amount of sugars observed in the fruit of contaminated plants, associated with the lower stiffness of the pulp compared with healthy fruit, may have facilitated the occurrence of brown rot.

5. Conclusions

In conclusion, in our study we have demonstrated the existence of great variations in plum postharvest attributes related to leaf scald disease. Fruit removed from plants with leaf scald showed reduced size, greater weight loss, lower firmness, and higher incidence of brown rot when compared with healthy plants. In addition, colour attributes gradually decreased during the postharvest period in fruit removed from plants infected with *X. fastidiosa*. PME and PAL enzyme activity, respiration, and ethylene production was higher in plums from trees contaminated by PLS than in fruit from disease-free plants.

Acknowledgements

The authors would like to thank the “College of Agriculture Luiz de Queiroz (ESALQ)” and “Federal University of Parana (UFPR)”, as well as the “Coordination of Improvement of Higher Education Personnel (CAPES)”, for their support and provision of resources necessary for our research.

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CHAPTER II - ASSOCIATION OF LEAF MORPHOLOGY AND VECTOR SETTLING AND FEEDING BEHAVIOR WITH RESISTANCE OF PLUM GENOTYPES TO LEAF SCALD DISEASE²

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ABSTRACT

Plum leaf scald (PLS) is a serious disease caused by *Xylella fastidiosa*, a pathogenic bacterium transmitted by sharpshooters (Hemiptera: Cicadellidae: Cicadellinae). The development of plum genotypes that apparently are not infected under field conditions has opened new prospects for disease control based on host plant resistance. Because

² Prepared in accordance with the standards of Phytopathology Journal.

‘SC13’ genotype can be infected by graft inoculation and ‘SC7’ is considered immune (since even grafting with infected plants the genotype showed no symptoms) we believe that the mechanism of resistance may be related to a vector transmission barrier. Here we investigated the settling preferences and xylem sap ingestion rates of the sharpshooters *Bucephalagonia xanthophis* and *Sibovia sagata*, as well as the stylet probing behavior of *B. xanthophis* using the Electrical Penetration Graph (EPG) technique, on three Japanese plum genotypes (‘SC7’, ‘SC13’ and ‘Laetitia’) with different levels of field resistance to PLS. In the settling experiments, the resistant genotype ‘SC7’ was less preferred by both vector species. Another resistant genotype (‘SC13’) did not affect settling, but reduced sap ingestion rates of *S. sagata*. ‘SC13’ also affected stylet activities of *B. xanthophis*, by increasing the duration of waveforms np (non-probing) and N (interruption of sustained ingestion during xylem phase), and reducing the duration of waveform Xi (active xylem sap ingestion). Comparative light and scanning microscopy indicated that ‘SC7’ and ‘SC13’ have larger epidermal cells and thick layers of epicuticular wax than ‘Laetitia’. The observed variations in leaf anatomy and settling/feeding behavior of the sharpshooters suggest that vector-plant interaction aspects may be involved in the field resistance of plum genotypes.

Key words: Disease resistance, gram-negative bacterium, vector behavior, *Prunus salicina*.

INTRODUCTION

Plum cultivation is a promising horticultural activity for Brazilian southern states (Paraná, Santa Catarina e Rio Grande do Sul), considering the high and currently unmet demand in the national market, with 33,575 tons of imported fruit, valued at US\$ 38 million in 2016 (Aliceweb 2018). Plum production in Brazil is based on Japanese cultivars (*Prunus salicina* Lindl), primarily due to their lower chilling requirement compared with European cultivars (*Prunus domestica* L.) (Ojima et al. 1983).

Nevertheless, in the 1970s plum leaf scald (PLS) almost decimated orchards in southern Brazil (Eidam et al. 2012). This disease is caused by the bacterium *Xylella fastidiosa* (Wells et al. 1987), which colonizes the foregut of xylem-sap feeding insects and the xylem of infected plants (Chatterjee et al. 2008), such as grapevine (Pierce's disease) (Hopkins 1985), citrus (citrus variegated chlorosis; CVC) (Rossetti et al. 1990), coffee (coffee leaf scorch; CLS) (Paradela-Filho et al. 1995), and more recently in olive trees (olive quick decline syndrome; QDS) (Saponari et al. 2014). In general, disease symptoms (leaf scorch) occur only after a long incubation period, when the pathogen titers achieve high densities (Daugherty et al. 2011), clogging the water conducting vessels, which reduces the hydraulic conductance and sap flow, weakening, and in more severe cases, causing premature plants death (Raju et al. 1982).

The dissemination of *X. fastidiosa* occurs through infected propagative material, such as in grafting techniques (He et al. 2000) and by piercing-sucking insects that are specialized xylem feeders, such as numerous sharpshooter species (Hemiptera: Cicadellidae: Cicadellinae) and spittlebugs (Hemiptera: Cercopoidea) (Redak et al. 2004). Among these, the sharpshooters *Bucephalogonia xanthophis* (Berg) is considered a key vector of *X. fastidiosa* in citrus in Brazil (Dellapé, et al., 2016; Lopes and Krugner, 2016), and *Sibovia sagata* (Signoret), which is a potential vector, are commonly found

in plum-producing regions in Brazil (Azevedo Filho et al. 2016; Hickel et al. 2001). Pathogen transmission by sharpshooters occurs in a non-circulative persistent manner, whereby *X. fastidiosa* multiplies in the functional foregut (cibarium and precibarium) of the insect, but there is no latency period between acquisition and inoculation, nor transovarial and transstadial transmission (Purcell and Finlay 1979).

Nowadays, PLS is once again a limiting factor for crop expansion, mainly due to the lack of research information and efficient management proposals. Control alternatives used are planting healthy nursery trees, roguing, insecticide applications and even fertilizations that promote vegetative development (Coletta-Filho et al. 2016).

The use of resistant varieties is an efficient method to control plant diseases (Sadasivan 1975). For Brazilian conditions, there are few plum cultivars with an acceptable level of resistance to PLS ('Carazinho', 'Sanguinea', 'Chatard' and 'Piamontesa'), but they typically produce low-quality fruit (Dalbó et al. 2018). In addition, the inheritance of resistance is polygenic and predominantly recessive (Dalbó et al. 2010). Recently, the breeding program of the Agriculture Research and Rural Extension of Santa Catarina (EPAGRI) identified genotypes ('SC7' and 'SC13') that do not manifest plum leaf scald symptoms in the field and have great fruit quality, such as appearance, flavor and lower acidity at maturity (Dalbó et al. 2018). Detection of *X. fastidiosa* in these field-maintained plants was regularly performed by PCR, and the results were always consistently negative for ten years, indicating that these genotypes were not infected under field conditions, despite being close to infected plants of susceptible varieties. Besides that, 'SC13' genotype was colonized by *X. fastidiosa* only when scions were grafted to infected plants (xylem vessels connected) (Dalbó et al. 2018). On the other hand, 'SC7' was considered immune, since, even grafting with infected plants the genotype showed no symptoms. These facts suggesting that these genotypes

might have a resistance mechanism that blocks the *X. fastidiosa* transmission by insect vectors. In view of this, the identification of plum genotypes that do not become infected under field conditions has opened new prospects to control PLS in Brazil. However, the stability of this form of resistance and the mechanisms involved in this process have not been clarified and need to be investigated to ensure long-term management success.

In the present study, we hypothesized that the field resistance of plum genotypes could be influenced by the insect-plant relationship, particularly vector settling and feeding behavior. Therefore, we investigated sharpshooter vector settling preferences, suitability for xylem-sap feeding and the differences in leaf morphology among plum genotypes that differ in field resistance to PLS. Two sharpshooter species commonly found in plum orchards of southern and southeastern Brazil and likely associated with PLS spread, *B. xanthophis* and *S. sagata* (Azevedo Filho et al. 2016), were selected for the study.

MATERIALS AND METHODS

Biological material: sharpshooters and plants. Adults of the sharpshooters *B. xanthophis* and *S. sagata* were obtained from bacterium-free colonies at the Department of Entomology and Acarology (USP/ESALQ), Piracicaba, SP, Brazil. The insects were reared in aphid-proof cages (55 x 55 x 32 cm) on *Vernonia condensata* Baker (Asteraceae) plants as previously described (Marucci et al. 2003), in a greenhouse equipped with a pad-fan type cooling system and thermostat-activated heater for temperature control. The plants were raised in plastic pots (Nutriplan® 15 cm x 20 cm x 14.5 cm) containing a substrate mix composed of shredded pine bark, peat, and expanded vermiculite at 60% w/w moisture content (Tropstrato HT Vida Verde, Mogi Mirim, SP, Brazil).

Two Japanese plum genotypes that showed field resistance ['SC7': crossing with ('Laetitia' x 'Piamontesa') x 'Fortune'; and 'SC13': crossing 'SC7' x Fortune] and the reference cultivar 'Laetitia', that is widely cultivated in southern Brazil and presented leaf scald symptoms in the field, were selected for the study. Healthy nursery trees of these genotypes were grafted in A9 rootstock and planted in plastic pots (Nutriplan® 12.5 cm x 17 cm x 12 cm; 2 L) containing the aforementioned substrate mix and fertilized by weekly fertigation [magnesium sulfate (0.83 g/L), potassium nitrate (0.36 g/L), monoammonium phosphate (0.14 g/L), zinc (0.02 g/L), copper (0.03 g/L), iron (0.09 g/L), ammonium nitrate (0.08g/L) and calcium nitrate (0.91g/L)]. The plants were kept in a greenhouse until they reached approximately 40 to 50 cm in height.

Settling preference assay. Independent settling preference assays were conducted for *B. xanthophis* and *S. sagata* on plum genotypes ('SC7', 'SC13' and 'Laetitia'), by releasing 40 non-sexed adults into each observation cage (base of 75 x 75cm, height of 115 cm) with a translucent plastic cover (Bugdorm type, model BD2400F, MegaView Science Co, Taichung Taiwan). Before releasing the insects, they were starved for 20 to 30 min in 50-ml conic plastic tubes (Falcon™) (17 mm O.D.; 120 mm length) closed with screw caps and covered with black tape, in groups of 10 individuals per tube (four tubes per replicate). The tubes were placed on a flight platform positioned in the top of the cage (20 to 30 cm above the top of the plants) and then were opened to allow the insects to exit. Inside the cage, six healthy nursery trees (two of each plum genotype) were arranged randomly in a circle, forming an experimental arena, according to the design described by Fereres et al. (1999). The total number of insects settled on each plant was assessed at 3, 6, 9, 24, 27, 30, 48, 51, and 54 h after the release, which was always done between 8:00 and 9:00 a.m. local time. Due to experimental restrictions that did not allow the counting of dead insects and those that were perched

either on the walls or at the base of the cage over time, a new level of treatment (named ‘others’) was added to represent this class of individuals.

Three independent experiments were conducted for each vector species over time. The experimental design was a randomized complete block with four replicates in experiment 1 and three replicates in experiments 2 and 3.

Statistical analysis of settling preference assay. The response variable in the analysis is the average number of insects settled per experimental unit (plant). Since plants were observed over time, the study design was longitudinal with successive counts not independent from each other. Although the number of insects per cage is fixed, the number of insects observed on each cultivar (treatment) is random, resulting in a Poisson process.

Let y_{ijkl} be a realization of a random variable that represents the number of insects evaluated in the i -th experiment, ($i = 1, 2, 3$), j -th cage (block), ($j = 1, 2, 3, 4$), on the l -th cultivar, ($l = 1, 2, 3$), at time θk , $k = 1, 2, \dots, 9$.

The number of insect counts per plant are assumed to be Poisson distributed, $y_{ijkl|b_{ij},d_{ijl}} \sim P(\mu_{ijkl})$, conditional on the above covariates. Thus, the logarithm of expected insect count is modeled using a generalized linear mixed effects model (GLMM) (Schall 1991). The fixed effects included in the linear predictor are experiment, cultivar and time, while the random effects are cage within experiment, and an observation-level random effect to account for overdispersion. The linear predictor of the complete model is given by:

$$\log(\mu_{ijkl}) = \mu + a_i + b_{ij} + \tau_l + (\alpha\tau)_{il} + \theta_k + (\tau\theta)_{lk} + (\alpha\tau\theta)_{ilk} + d_i$$

$$j|l \mathbf{b} \sim N(0, \mathbf{G}) \text{ and } d_{ijkl} \sim N(0, \sigma_d^2), \quad (1)$$

where μ is the overall mean; a_i is the fixed effect of the i -th experiment; b_{ij} is the random effect for the block within each experiment; τ_l is the fixed effect of l -th cultivar; $(\alpha\tau)_{il}$ is

the interaction effect between experiment and cultivar; θ_k is the fixed effect of time at the k -th repeated measurement; $(\tau \theta)_{lk}$ is the interaction effect between treatment and time; $(\alpha \theta)_{ilk}$ is the interaction effect among experiment, cultivar and time; and d_{ijk} is the random effect to account for overdispersion. Moreover, we assume that b_{ij} and d_{ijk} are independent, that they have the same correlation among times within experiments, and an unstructured matrix \mathbf{G} for the random effects of cage within experiment.

The restricted maximum likelihood (REML) estimation was used to obtain the estimates of variance components. Furthermore, 95% simultaneous confidence intervals were calculated for the parameters of the Poisson-Normal model using the parametric bootstrap approach with 5.000 pseudo-samples (Table S1).

The selection of fixed effects was made using the backward procedure based on a likelihood ratio test (LRT) between nested models to test interactions and main effects at the 5% significant level. In addition, the half-normal plot with simulated envelopes (Moral et al. 2017) was used to assess the goodness of fit of the Poisson-Normal model fitted to the data (Figure S1). Finally, we used simultaneous tests for multiple comparisons of means (Tukey contrasts) at the 5% significant level, with adjusting P-values using Hommel's correction (Hommel 1988).

Evaluation of sap ingestion (confinement assay). The ingestion rate was indirectly measured by collecting the liquid excreted by insects per unit of time (Marucci et al. 2005). For the evaluation of sap ingestion rates under laboratory conditions ($25^{\circ}\text{C} \pm 2$) by *B. xanthophis* and *S. sagata* on healthy plum genotypes ('SC7', 'SC13' and 'Laetitia'), three non-sexed adults of each sharpshooter species were confined on branch sections using a cage similar to that described by Andersen et al. (1992). The cage was composed of a 50 ml transparent conic plastic tube (Falcon™) (30 mm O.D.; 115 mm length) with sponge stopper, coupled in a 15 mL scaled conic plastic tube (Falcon™) (17

mm O.D.; 120 mm length). These cages were positioned on leaves at the middle third of the plant, attached at an angle to allow the collection tube to remain hung vertically, in order to favor the honeydew flow to the scaled recipient. Each plant contained a single cage.

The experimental design was completely randomized, with seven replicates (seven plants with three insects per cage) for *S. sagata* and eight replicates (eight plants with three insects per cage) for *B. xanthophis* per cultivar ('SC7', 'SC13' and 'Laetitia'). The volume of excreted honeydew (mL) in each replicate was recorded visually 24, 48 and 72 h after the sharpshooter were confined on the branches.

Statistical analysis of sap ingestion assay. Let y_{ijk} be a realization of the random variable that represents the honeydew volume in the i -th cage, ($i = 1, 2, \dots, I$), j -th cultivar, ($j = 1, 2, 3$) at time θ_k , ($k = 1, 2, 3$). The honeydew excretion data are assumed to be normally distributed, $y_{ijk|b_i} \sim N(\mu_{ijk}, \sigma_\epsilon^2)$ conditional on the above covariates. Thus, the expected value of honeydew excretion is modeled using a linear mixed effects model (LME) (Laird and Ware 1982). The linear predictor of the complete model is given by:

$$\mu_{ijk} = \mu + \tau_j + \theta_k + (\tau\theta)_{ik} + b_i \quad (2)$$

$$b_i \sim N(0, \sigma_{1j}^2),$$

where μ is the overall mean; τ_j is the fixed effect of j -th level of cultivar; θ_k is the fixed effect of time at the k -th repeated measurement; $(\tau\theta)_{ik}$ is the interaction effect between cultivar and time; and b_i is the random effect of cage. Because the random effect of cage may influence differently each level of cultivar, we assume that b_i has a specific variance by level of this one (σ_{1j}^2). The experimental error ϵ_{ijk} is assumed to be normally distributed with mean zero and variance σ_ϵ^2 and independent of random effect b_i . As the

variability of honeydew excretion increases over time differently by cultivar, a variance function given by:

$$Var(\epsilon_{ijk}) = \sigma_{\epsilon}^2 \exp\left(\frac{\delta_j \theta_k}{12}\right),$$

was included in the model. The parameter δ_j is unrestricted, enabling the variance to increase or decrease by cultivar over time θ_k (Pinheiro and Bates, 2000).

The restricted maximum likelihood (REML) approach was used to estimate fixed effects and variance components of the LME model. The parametric bootstrap approach was used to compute the 95% simultaneous confidence intervals for model's parameters based on 10.000 pseudo-samples (Table S2). This method resamples both random effects and residuals by simulating from an estimated distribution after model fitting in order to obtain 10.000 sets of estimates for all parameters. Thus, we compute the bootstrap-t intervals, with left- and right tail errors both equal to $\frac{\alpha}{2} = 0.025$, considering a bootstrap bias correction (Davison and Hinkley 1997).

The fixed effects selection, residual analysis (Figure S2), and simultaneous tests for multiple comparisons of means were done as described in the statistical analysis for settling preference. To assess the similarity between honeydew volumes measured by cultivar at different times, the intraclass correlation coefficient was used (Model S1 and Table S3).

Probing behavior assay with *B. xanthophis*. The Electrical Penetration Graph (EPG) technique was used to monitor the stylet activities of *B. xanthophis* on the three plum genotypes ('SC7', 'SC13' and 'Laetitia'). First, adult females were anesthetized with CO₂ for 5 to 8 s, and then immobilized under a dissecting microscope by using a vacuum device similar to that described by van Helden and Tjallingii (2000). The tip of a 37- μ m gold wire, 3 cm long was attached on the sharpshooter pronotum with a droplet of conductive silver paint (Ted Pella, Inc., Colloidal Silver Liquid, Redding, CA, USA).

The opposite end of the gold wire was attached with a droplet of silver paint to a thin copper wire (3 cm long x 1 mm diameter), which was connected to the EPG head stage amplifier. Another copper electrode (10 cm long, 2 mm in diameter) was inserted into the soil of the plant container. After a 1-h starvation period, the wired sharpshooters were placed individually on the stem of a plant, inside a Faraday cage (for electrical noise isolation) under laboratory conditions ($25\pm 1^\circ\text{C}$).

The EPG waveforms were recorded and observed in real time on a computer screen, using a Direct Current (DC) 8-channel EPG device, model Giga-8d, with Stylet+ for Windows software (EPG Systems, Wageningen, Netherlands). Each sharpshooter was monitored for 6 h and a total of 17, 15 and 18 individuals (replicates) were analyzed on the plum genotypes ‘Laetitia’ ‘SC7’ and ‘SC13’, respectively.

EPG waveform types previously described for *B. xanthophis* (Miranda et al., 2009) and *Philaenus spumarius* L. (Hemiptera: Aphrophoridae) (Cornara et al. 2018) were identified and associated with stylet activities, as follows: np, non-probing (stylets out of the plant); C, pathway through epidermis and parenchyma (including both stylet penetration and withdrawal); R, resting during xylem activity; N, interruption of sustained ingestion during xylem phase; Xc, xylem contact/pre-ingestion; and Xi, active intake of xylem sap.

Data were analyzed for EPG variables during the 6 h recording period. The output as given by the workbook of Sarria et al. (2009) for each sharpshooter was used to calculate the means for each EPG sequential and non-sequential variables. Selected non-sequential EPG variables (mean \pm SE) were calculated and compared among cultivars, as described by Backus et al. (2007): PPW, proportion of individuals that produced a specific waveform type; NWEI, (mean) number of waveform events per insect; WDI, (total)

waveform duration (min) per insect, and WDE, (mean) waveform duration (min) per event.

The experimental design was completely randomized, and prior to analysis, normality of residuals and homogeneity of variance were checked. All EPG parameters were analyzed by a Tukey's test ($P < 0.05$). If the data did not show homogeneity and normal distribution according to the Shapiro-Wilk normality test, a nonparametric Kruskal-Wallis H test ($P < 0.05$) was performed. All data were analyzed using IBM Statistics SPSS 19.0 software (IBM Corporation, Armonk, NY).

Comparative leaf anatomy (preparation). The assays of histological analysis of plum leaves were conducted at Department of Plant Pathology and Nematology (USP/ESALQ, Piracicaba, SP, Brazil). Ten fully expanded leaves (10.0 cm length x 5.0 cm width) were collected and cut in little fragments (leaf blade without veins) with a slide. The fragments were fixed in Karnovsky's solution (1992) (glutaraldehyde 2% and paraformaldehyde 2.5% in 0.05M cacodylate buffer pH 7.2) with vacuum pump (ca. 0.1 mm Hg) for 10 to 5 min, then cooled in microtubes. In the next step, the samples were dehydrated in an increasing series of ethanol concentration (30, 50, 70, 90 and 3 times in 100% for 30 min in each stage) (Marques et al. 2007).

Light Microscopy. Tissue samples were pre-infiltrated (8 to 12 h in a 1:1 Technovit acrylic resin mixture/absolute ethanol) and then infiltrated into the pure resin for 24 h. Blocks were immersed in historesin solution and hardener (15 mL:1 mL) deposited on a plastic mold and polymerized at room temperature. The hardened blocks were sectioned on a Leica RM2355 hand microtome (Leica Biosystems, Nussloch, Germany). Sections (7 μ m thick) were placed on glass slides and stained with blue toluidine (pH 4.5) for 2 min. Digital recording was done with the blades mounted in Entellan and examined in a Zeiss Axioimager D2 (Zeiss, 287 Oberkochen, Germany).

Axio Vision SE64 software was used to configure and analyze the images. Photographs were processed with Image - Pro Plus software (version 4.5.0.29; Media Cybernetics, Rockville, MD).

Scanning Electron Microscopy. Dehydrated leaf samples were placed inside porous cages in a drying chamber for Critical Point Drying (CPD). Blocks were fixed on stubs and placed in a chamber with 0.1 to 0.05 mbar pressure and the target was bombarded with gold ions and deposited on the sample (Dedavid et al. 2007). Digital images were acquired in a LEO (Zeiss) 435 VP Scanning Electron Microscope. The LEO SRV-32 IEW software was used for configuration and image analysis. Photographs were processed and adjusted in CorelDraw 12[®] software (Corel, Ontario, Canada).

RESULTS

Settling preference. For both sharpshooter species (*B. xanthophis* and *S. sagata*), significant interaction effects between experiment and cultivar were observed at the 5% level of significance (Table S4). Moreover, significant interactions between cultivar and time were verified for *S. sagata* (P value = 0.015) (Table S4).

By analyzing the interactions, it was possible to observe that 'SC7' was less preferred for settling by *B. xanthophis* within experiments 1 and 3, whereas both 'Laetitia' and 'SC7' were the less preferred for settling in experiment 2 (Table 1). 'SC13' was the most preferred genotype by *B. xanthophis*, in all experiments.

For *S. sagata*, 'SC7' was less preferred for settling in experiments 1 and 2. In experiment 3, no differences were observed among cultivars (Table 1), but 'Laetitia' was the genotype most preferred for settling in experiment 2.

The number of *S. sagata* individuals perched on 'Laetitia', 'SC7' and 'SC13' did not change over time (Table 2). 'SC7' was less preferred than 'SC13' until 24 hours,

and equal to 'Laetitia' from the 6 hours onwards (Table 2). From the evaluation of 27 hours onwards, there was no difference in settling preferences among the tested genotypes ('Laetitia', 'SC7' and 'SC13' treatments) (Table 2).

Evaluation of sap ingestion (confinement assay). For *S. sagata*, a significant interaction effect between cultivar and time was observed (<0.0001) (Table S5), whereas for *B. xanthophis* only time was significant (p value <0.0001) (Table S5).

Considering the amount of honeydew excreted over time by *S. sagata* on 'Laetitia', the mean excretion was lower at 24 h (2.27 mL) than at the other evaluation periods (Figure 1 and Table S6). For 'SC7' and 'SC13', there was no significant difference among mean excreted volumes over time of evaluation for this sharpshooter species (Figure 1 and Table S6). Comparing the plum genotypes, no significant difference was observed in the first evaluation period (24 h), but in further evaluations the highest volume of excreted honeydew was recorded when *S. sagata* fed on 'Laetitia', differing statistically from 'SC13'. For *B. xanthophis*, the amount of honeydew excreted at 24 h was lower than that observed in subsequent evaluations for all treatments, but there was no significant difference among the genotypes (Figure 2 and Table S4).

Probing behavior of *B. xanthophis*. The analysis of the 6-h EPG recordings of *B. xanthophis* on the three plum genotypes showed significant differences for the mean waveform duration per event (WDE) of np, Xi and N (Table 3). The WDE of np ($P = 0.03$) was significantly shorter on 'Laetitia' than on 'SC13', and waveform N (interruption of sustained ingestion during xylem phase) ($P < 0.01$) was significantly shorter on 'Laetitia' than on 'SC7' and 'SC13'. In addition, the WDE of Xi was longer on 'Laetitia' and 'SC7' than on 'SC13'; each Xi event (active xylem sap ingestion) was approximately three times longer on 'Laetitia' than on 'SC13' (Table 3). No significant

differences ($P > 0.05$) were found among the three plum genotypes for the other non-sequential (Table 3) and sequential (Table 4) variables analyzed.

Comparative leaf anatomy. The cuticle on the adaxial surface was thicker in ‘Laetitia’ and thinner on the abaxial side than in the other genotypes. The epidermal cells were oval in shape (in transverse section). ‘SC7’ and ‘SC13’ had larger epidermal cells (Figure 3).

‘Laetitia’ had major intercellular spaces between the lacunar parenchyma cells. ‘SC7’ and ‘SC13’, on the other hand, showed aggregate lacunar parenchyma cells, with small intercellular spaces. The vascular system was composed of a variable number of side vascular bundles. For xylem bundles, it was not observed differences among the genotypes (Figure 3).

Scanning microscopy showed that the epicuticular layer showed variations as to shape and wax distribution. ‘SC7’ and ‘SC13’ had depressions and a denser waxy layer than ‘Laetitia’ (Figure 4). However, ‘Laetitia’ showed no depressions (Figure 4).

DISCUSSION

Non-preference of insects to a particular host occurs when the plant becomes less interesting for feeding, oviposition or shelter when compared to the other host plant on equal terms (Smith 2005). The results of this current research revealed differences in settling preference and/or feeding behavior by the sharpshooters *B. xanthophis* and *S. sagata* among resistant genotypes and the reference cultivar. Both vector species showed non-preference for settling on the resistant plum genotype ‘SC7’ in at least two out of three repetitions of the choice experiments. Honeydew excretion assays showed reduced feeding (lower xylem sap ingestion rates) of *S. sagata* on ‘SC13’, another resistant genotype under field conditions (Dalbó et al. 2018). The analyses of EPG parameters

indicated that 'SC13' also affected stylet activities of *B. xanthophis* in the xylem, by reducing the mean duration of waveform Xi, which is associated with xylem sap ingestion. The comparative analysis of the leaf anatomy showed different characteristics between the plum genotypes, with possible implications on vector stylet penetration. Since *X. fastidiosa* is a xylem-limited pathogen, xylem sap feeding is considered essential for its transmission by insect vectors.

Piercing-sucking insects use a variety of chemical and visual cues during orientation, host selection and feeding acceptance (Bullas-Appleton et al. 2004; Fereres and Moreno 2009). The first orientation and host selection stimulus is based on olfactory and visual cues (Todd et al. 1990). Leaf coloration, for example, can modify insect flight orientation, since plants with chlorosis symptoms become more attractive to aphids for landing when compared to healthy ones (Ajayi and Dewar 1983; Baker 1960). Susceptible plums, the main source of inoculum in orchards, show yellowing leaves in the early stages of the leaf scald disease, which may alter the sharpshooter's orientation behavior, influencing the spread under natural conditions. On the other hand, cultivars with a certain degree of resistance can avoid or delay the symptom appearance, modifying the disease epidemiology (Rivière and Galiana 2007).

The initial stimulus for feeding is influenced by physical characteristics intrinsic to the host (Ayayee et al. 2014) added to the presence of phytochemical substances with phagostimulant and/or phagodeterrent function (Silva et al. 2014). Some plant-related factors may be responsible for greater or lower preference during vector feeding. Preformed structural resistance factors (passives, constitutives) are physical barriers intrinsic to plant species that include trichomes, cuticle waxes (Stevenson et al. 1993) and thickness of epidermis/parenchyma (Lonsdale 1983). Plant cell walls are composites of minute plant fibers interlaced with many different chains of simple sugars, or polymers,

that make the structure strong, like ‘building blocks’ (Carpita and McCann, 2000), which could effectively alter the feeding pattern of the sharpshooters. Another defense mechanisms utilized by the host is the postformed structural resistance factors (actives, inducibles), like crystals (Xing et al. 2017), cork layers, tyloses and lignification, which appear after the pathogen infection. The lowest values for sap ingestion, obtained by indirect measurement of the excretion, were observed for *S. sagata* on ‘SC13’ genotype, which shows field resistance to leaf scald disease (Dalbó et al. 2018). Interestingly, the genotypes that presented the lowest number of insects settled (‘SC7’) or lower ingestion rates and active ingestion in xylem (Xi) (‘SC13’), also showed thicker leaf epidermal cells and denser epicuticular layers than ‘Laetitia’ (reference cultivar), leading us to hypothesize that these morphological characteristics act as physical impediment that may pose some degree of difficulty for vector’s stylet penetration. For *Aphis fabae*, when epicuticular waxes were stripped from oats, aphids penetrated earlier than on oats with the intact wax layer (Powell et al. 1999). The presence of the wax layer on cuticle of young *Eucalyptus globulus* leaves influenced the feeding behavior of two psyllids species, since it makes the leaf more slippery, hindering the insertion of the stylet (Schoonhoven et al. 2005).

Although all the individuals tested were able to probe, perform waveform C and reach the xylem vessels (Xc) on the three plum genotypes, the mean duration of waveform np (non-probe) was lower on ‘Laetitia’, indicating that the insects initiated the probes faster on that genotype. However, the lack of significant differences in sequential variables (e.g. time from the beginning of EPG recording or from the first probe to the first Xc or Xi) suggest no physical barriers to reach xylem after the probe is initiated.

Plants have some complex strategies to defend against insects (Huot et al. 2013) and injuries caused by insects can change the plant physiology, which in turn may explain

the resistance or tolerance phenomena. Volatile compounds released from plants can be altered with the action of biotic stressors that include damage caused by herbivores (Turlings and Wackers 2004). Some studies with piercing-sucking-insect suggest a stepwise mechanism, which can be described in four sequential hypotheses: H1: activation of defense mechanisms; H2: defense affects food properties; H3: food properties include changes in odor; and H4: food properties affect taste. Stylet penetration usually causes minimum mechanical damage in plants, but it may induce the defense apparatus by the emission of volatile compounds (Agrawal et al. 2012), such as GLVs (Green Leaf Volatiles) and terpenoids (Gosset et al., 2009). For the glassy-winged sharpshooter, *Homalodisca vitripennis* (Germar), volatiles released from *Prostanthera ovalifolia* (commonly known as ‘mint bush’) are an effective deterrent altering attraction cues and preventing oviposition on this species (Rathé et al. 2014). The activation of specific defense sites can also influence the feeding performance (Fritz and Simms 1992, Agrawal et al. 1999) and in some cases alter the food quality, indicating the importance of gustatory signals for host plant selection and acceptance by leafhoppers (Backus and McLean 1985) and more specifically by vectors of *X. fastidiosa* (Brodbeck et al. 1990, Andersen et al. 2005).

Sharpshooter leafhoppers are insects that ingest exclusively in xylem bundles (Purcell and Finlay 1979). This conductive vessel consists of 98% water and organic solutes, predominantly amino acids and organic acids (Brodbeck et al. 1990). The concentration of the organic solutes found in xylem (Andersen 1989; Brodbeck et al. 1990), as well as the dimensions of the conductive vessels (Chatelet et al. 2011) can influence the vector feeding in the host plant. Comparative studies with grape varieties resistant to *X. fastidiosa* indicated that those with shorter and narrower xylem vessels and thicker parenchyma could limit bacterial movement within the conductors (Chatelet et al.

2011). For the glassy-winged sharpshooter, it was observed that host preference changed over time between *Vitis rupestris* × *Vitis arizonica/candicans* (D8909-17 grapevine hybrid) and *Vitis girdiana*. This fact could be explained by a disconnection between insect requirements and xylem sap composition, forcing the vector to find a new host that satisfied their nutritional demands (Fritschi et al. 2007). Bi et al. (2005) correlated the seasonal occurrence of glassy-winged sharpshooter population shifts among orange (*Citrus sinensis* L.) and lemon (*C. lemon* L.) trees with variations in xylem sap composition, suggesting that the concentration of amino acids may influence host selection by these insects. The leaf morphological analyses in our work did not allow a comparison of the xylem vessel structures among the plum genotypes.

In conclusion, we have demonstrated the existence of variations in settling and feeding behavior by the vectors in plum genotypes with different levels of field resistance to leaf scald disease. These findings on vector-plant relationships contribute to our understanding of the two mechanisms possibly involved in the field resistance of genotypes ‘SC7’ and ‘SC13’. Other studies involving the identification of volatile compounds produced by these plants and the quantification and identification of the wax components present in the leaves, associated with electrophysiology of gustatory sensillae should be performed, since the results of this research suggests non-preference of these two vector species to settle on the plum genotype ‘SC7’. The other resistant genotype (‘SC13’) does not affect settling, but reduces xylem feeding based on the combined results of honeydew excretion and EPG experiments. Because *X. fastidiosa* is a xylem-limited bacterium (Purcell and Finlay, 1979), xylem structure and sap composition in resistant and susceptible plants also may play an important role in sharpshooter settling and feeding behavior and need to be addressed in future investigation. Finally, transmission experiments using vectors and artificial inoculation approaches (e.g. needle

inoculation) should be carried out with susceptible and resistant cultivars to check if there is any level of resistance against bacterial infection and multiplication in the host plant. The use of resistant cultivars is an important strategy for integrated pest management (IPM), by reducing the amount of insecticides used in the orchards.

ACKNOWLEDGEMENT

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001. The last two authors received research fellowships from National Council for Scientific and Technological Development (CNPq)/Brazil. We thank the anonymous reviewers for many useful comments and suggestions, which helped to improve the manuscript.

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Table 1. Overall mean number (\pm SE)^a of insects perched on treatments (plum genotypes) within experiments^b for *Bucephalagonia xanthophis* and *Sibovia sagata* settling preference

Sharpshooter specie	Treatment	Exp. 1		Exp. 2		Exp. 3	
<i>Bucephalagonia xanthophis</i>	Laetitia	4.97 (\pm 0.3)	bA	5.63 (\pm 0.4)	aA	5.11 (\pm 0.4)	bA
	SC7	3.33 (\pm 0.3)	aA	5.70 (\pm 0.6)	aB	3.59 (\pm 0.3)	aA
	SC13	10.17 (\pm 0.5)	cB	7.19 (\pm 0.4)	bA	12.07 (\pm 1.2)	cB
	Other	21.53 (\pm 0.7)	dA	21.48 (\pm 1.0)	cA	19.22 (\pm 1.1)	dA
<i>Sibovia sagata</i>	Laetitia	8.86 (\pm 1.0)	bA	17.63 (\pm 0.8)	cB	8.37 (\pm 1.0)	aA
	SC7	5.42 (\pm 0.6)	aA	5.26 (\pm 0.6)	aA	8.44 (\pm 1.1)	aA
	SC13	11.83 (\pm 1.3)	bcA	10.26 (\pm 1.0)	bA	11.81 (\pm 1.0)	aA
	Other	13.89 (\pm 1.3)	cA	6.85 (\pm 0.7)	aB	11.37 (\pm 0.6)	aA

^a Means followed by the same letter (lower case in columns and upper case in lines) do not differ according to Tukey's pairwise comparison test with Hommel's correction, considering $\alpha = 0.05$.

^b Three experiments were set up over time in a complete randomized design with four blocks (cages) in the first one and three blocks in the following two experiments for each sharpshooter species.

Table 2. Mean number (\pm SE)^a of insects perched on treatments (plum genotypes) at successive periods after release in choice assays for *Sibovia sagata* settling preference.

Time (h)	Treatment							
	Laetitia		SC7		SC13		Other	
3	9.6 (\pm 1.5)	aB	4.3 (\pm 1.2)	aA	12.9 (\pm 2.6)	aB	13.2 (\pm 2.4)	aB
6	9.1 (\pm 1.7)	aAB	4.8 (\pm 1.1)	aA	14.5 (\pm 2.3)	aB	11.6 (\pm 2.5)	aB
9	9.3 (\pm 1.7)	aAB	4.4 (\pm 1.0)	aA	14.5 (\pm 2.3)	aB	11.8 (\pm 2.6)	aAB
24	9.9 (\pm 1.7)	aAB	6.0 (\pm 1.6)	aA	14.6 (\pm 2.1)	aB	9.5 (\pm 1.9)	aAB
27	12.1 (\pm 2.2)	aA	6.5 (\pm 1.3)	aA	10.0 (\pm 1.5)	aA	11.4 (\pm 1.9)	aA
30	13.1 (\pm 2.7)	aA	6.7 (\pm 1.5)	aA	10.7 (\pm 1.3)	aA	9.5 (\pm 2.0)	aA
48	13.0 (\pm 2.2)	aA	7.0 (\pm 1.5)	aA	9.0 (\pm 1.7)	aA	11.0 (\pm 1.3)	aA
51	13.3 (\pm 2.6)	aA	8.1 (\pm 1.5)	aA	8.8 (\pm 2.3)	aA	9.8 (\pm 2.0)	aA
54	12.7 (\pm 2.5)	aA	8.7 (\pm 1.6)	aA	7.2 (\pm 1.7)	aA	11.4 (\pm 1.9)	aA

^a Means followed by the same letter (lower case in columns and upper case in lines) do not differ according to Tukey's pairwise comparison test with Hommel's correction, considering $\alpha = 0.05$.

^b Other: represents the incidence of dead insects or insects perched on the walls and basis of the cages throughout the time (hour).

Table 3. Mean non-sequential EPG variable values for the probing behavior of *Bucephalogonia xanthophis* on three plum genotypes (treatment) during a 6-h recording.

Non-sequential variables ^a	Treatment	PPW ^b	NWEI ^c	p^d	WDI ^e	p	WDE ^f	p
Np	Laetitia	17/17	2.5 (\pm 0.5)		12.4 (\pm 4.5)		4.6 (\pm 1.7) a	
	SC13	18/18	3.5 (\pm 0.5)	0.11	35.0 (\pm 8.8)	0.33	8.9 (\pm 1.7) bc	<u>0.03</u>
	SC7	15/15	3.7 (\pm 1.0)		32.34 (\pm 9.6)		8.2 (\pm 1.7) ac	
C	Laetitia	17/17	10.1 (\pm 1.5)		72.9 (\pm 12.9)		23.2 (\pm 2.7) a	
	SC13	18/18	12.5 (\pm 1.5)	0.33	131.7 (\pm 45.7)	0.29	24.9 (\pm 2.8) a	0.39
	SC7	15/15	13.3 (\pm 1.7)		94.9 (\pm 13.7)		12.8 (\pm 1.6) a	
Probe	Laetitia	17/17	2.6 (\pm 0.5)		347.6 (\pm 4.5)		133.3 (\pm 21.0)	
	SC13	18/18	3.3 (\pm 0.5)	0.55	334.1 (\pm 13.0)	0.23	101.2 (\pm 15.5)	0.37
	SC7	15/15	3.7 (\pm 0.9)		327.6 (\pm 9.6)		84.7 (\pm 14.9)	
R	Laetitia	16/17	9.78 (\pm 1.7)		151.0 (\pm 20.8)		14.8 (\pm 1.4)	
	SC13	18/18	12.6 (\pm 1.9)	0.88	156.8 (\pm 21.9)	0.32	12.5 (\pm 1.4)	0.16
	SC7	15/15	9.3 (\pm 1.3)		152.4 (\pm 21.5)		16.3 (\pm 1.9)	
N	Laetitia	6/17	2.1 (\pm 1.2)		14.4 (\pm 7.1)		7.0 (\pm 0.7) a	
	SC13	11/18	3.8 (\pm 1.5)	0.16	33.31 (\pm 12.0)	0.16	8.6 (\pm 0.4) b	<u><0.01</u>
	SC7	10/15	2.1 (\pm 0.6)		19.7 (\pm 5.6)		9.5 (\pm 0.9) b	
Xc	Laetitia	17/17	6.2 (\pm 0.9)		28.6 (\pm 5.1)		5.3 (\pm 0.7)	
	SC13	18/18	6.4 (\pm 1.0)	0.90	24.2 (\pm 4.3)	0.75	3.8 (\pm 0.3)	0.11
	SC7	15/15	5.4 (\pm 0.7)		23.5 (\pm 4.6)		4.2 (\pm 0.6)	
Xi	Laetitia	14/17	5.7 (\pm 1.4)		92.9 (\pm 28.4)		16.9 (\pm 2.9) a	
	SC13	18/18	10.1 (\pm 2.0)	0.10	62.0 (\pm 16.7)	0.84	6.2 (\pm 0.9) b	<u><0.01</u>
	SC7	15/15	6.1 (\pm 0.9)		71.9 (\pm 17.9)		11.0 (\pm 1.9) a	
Xi > 10min	Laetitia	12/17	1.94 (\pm 0.6)		78.8 (\pm 26.8)		39.5 (\pm 6.3)	
	SC13	12/18	1.6 (\pm 0.4)	0.90	42.6 (\pm 16.6)	0.80	27.4 (\pm 3.5)	0.17
	SC7	13/15	1.9 (\pm 0.5)		55.4 (\pm 18.8)		28.6 (\pm 5.1)	

^a Waveforms: np, non-probing; C, pathway through epidermis/parenchyma including both stylet penetration and withdrawal; R, resting during xylem activity; N, interruption of sustained ingestion during xylem phase (duration in seconds); Xc, and xylem contact/pre-ingestion; and Xi, active xylem sap ingestion.

^b Non-sequential variables: PPW, proportion of individuals that produced the waveform type; NWEI, number of waveform events per insect; WDI, total waveform duration (min) per insect; WDE, waveform duration (min) per event (Backus et al. 2007).

^c Statistical comparisons were made by Tukey's test for Gaussian distribution variables or non-parametric Kruskal-Wallis test for non-Gaussian distribution variables. Underline-type indicates significant differences ($p < 0.05$).

Table 4. Mean sequential EPG variable values for the probing behavior of *Bucephalagonia xanthophis* on three plum genotypes (treatments) during a 6-h recording.

Sequential variables ^a	Treatment	Mean \pm (SE)	<i>p</i> -value ^b
N° of probes to the 1 st Xc	Laetitia	1.6 (\pm 0.3)	0.82
	SC13	1.2 (\pm 0.1)	
	SC7	1.7 (\pm 0.4)	
N° of probes until the 1 st Xi	Laetitia	1.5 (\pm 0.4)	0.62
	SC13	1.5 (\pm 0.2)	
	SC7	1.67 (\pm 0.4)	
Time from the beginning of EPG to the 1 st Xc	Laetitia	28.3 (\pm 6.7)	0.29
	SC13	28.1 (\pm 10.6)	
	SC7	20.3 (\pm 8.5)	
Time from the beginning of EPG to the 1 st Xi	Laetitia	50.8 (\pm 18.2)	0.67
	SC13	47.11 (\pm 4.0)	
	SC7	32.6 (\pm 10.5)	
Time from 1 st probe to 1 st Xc	Laetitia	26.2 (\pm 7.0)	0.53
	SC13	25.6 (\pm 10.6)	
	SC7	19.4 (\pm 8.5)	
Time from 1 st probe to 1 st Xi	Laetitia	48.4 (\pm 18.1)	0.83
	SC13	44.6 (\pm 13.9)	
	SC7	31.4 (\pm 10.6)	

^a Xc and Xi represent the xylem contact/pre-ingestion and active intake of xylem sap (active ingestion), respectively; ^b Statistical comparisons between 'Laetitia', 'SC7' and 'SC13' genotypes for each parameter were made by: Tukeys test for Gaussian distribution variables or non-parametric Kruskal-Wallis test for non-Gaussian distribution variables. Underline-type indicates significant differences ($p < 0.05$).

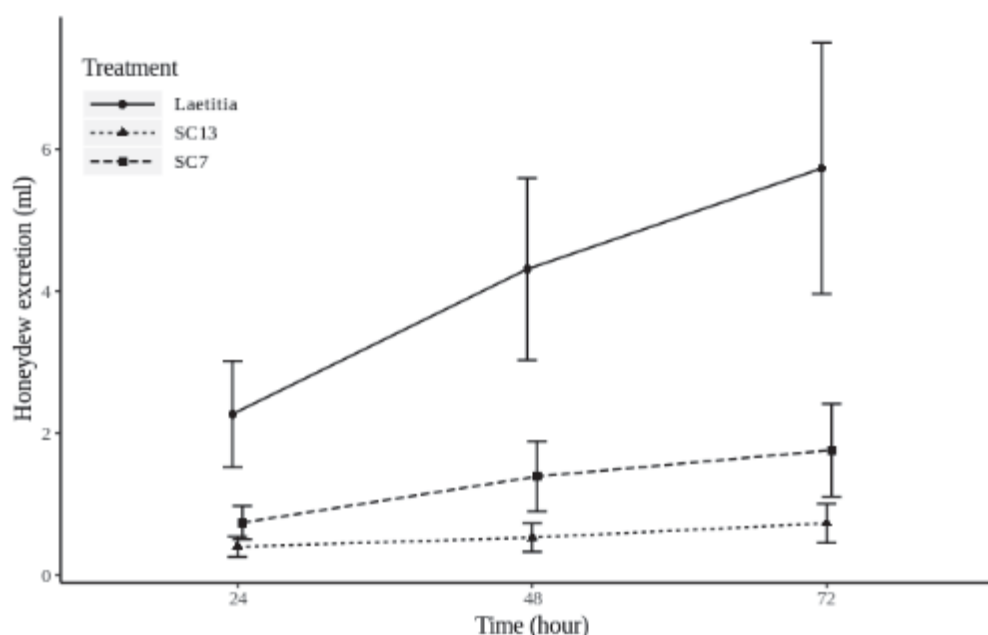


Fig. 1. Cumulative honeydew excretion (mL) for *Sibovia sagata* in three plum genotypes (Laetitia, SC7, SC13) after 24, 48 and 72 hours. The bars represent the standard error of the mean. The dots were shifted to the best view of the error bars.

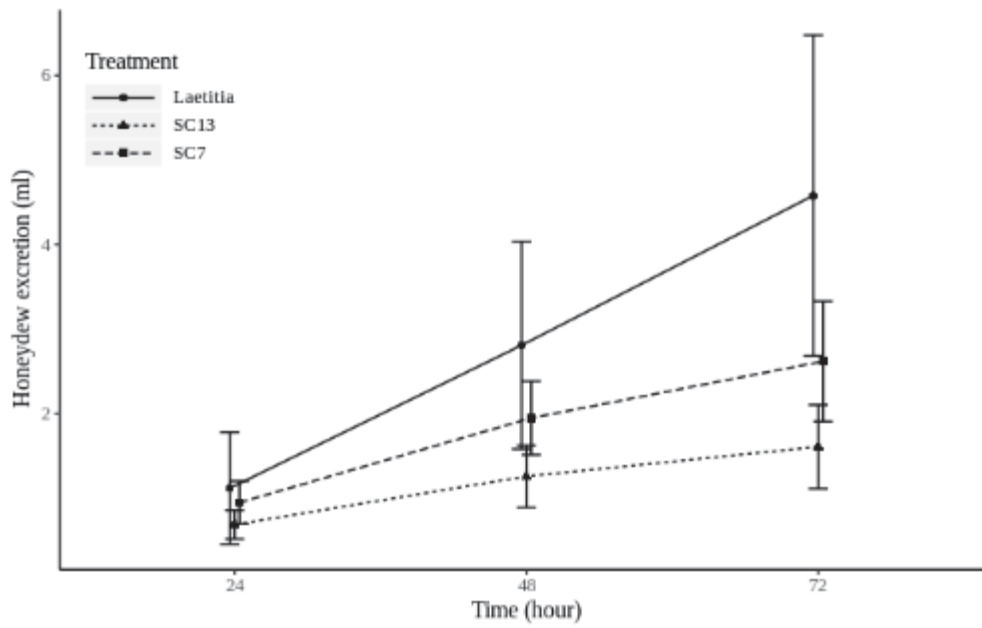


Fig. 2. Cumulative honeydew excretion (mL) for *Bucephalagonia xanthophis* in three plum genotypes (Laetitia, SC7, SC13) after 24, 48 and 72 hours. The bars represent the standard error of the mean. The dots were shifted to the best view of the error bars.

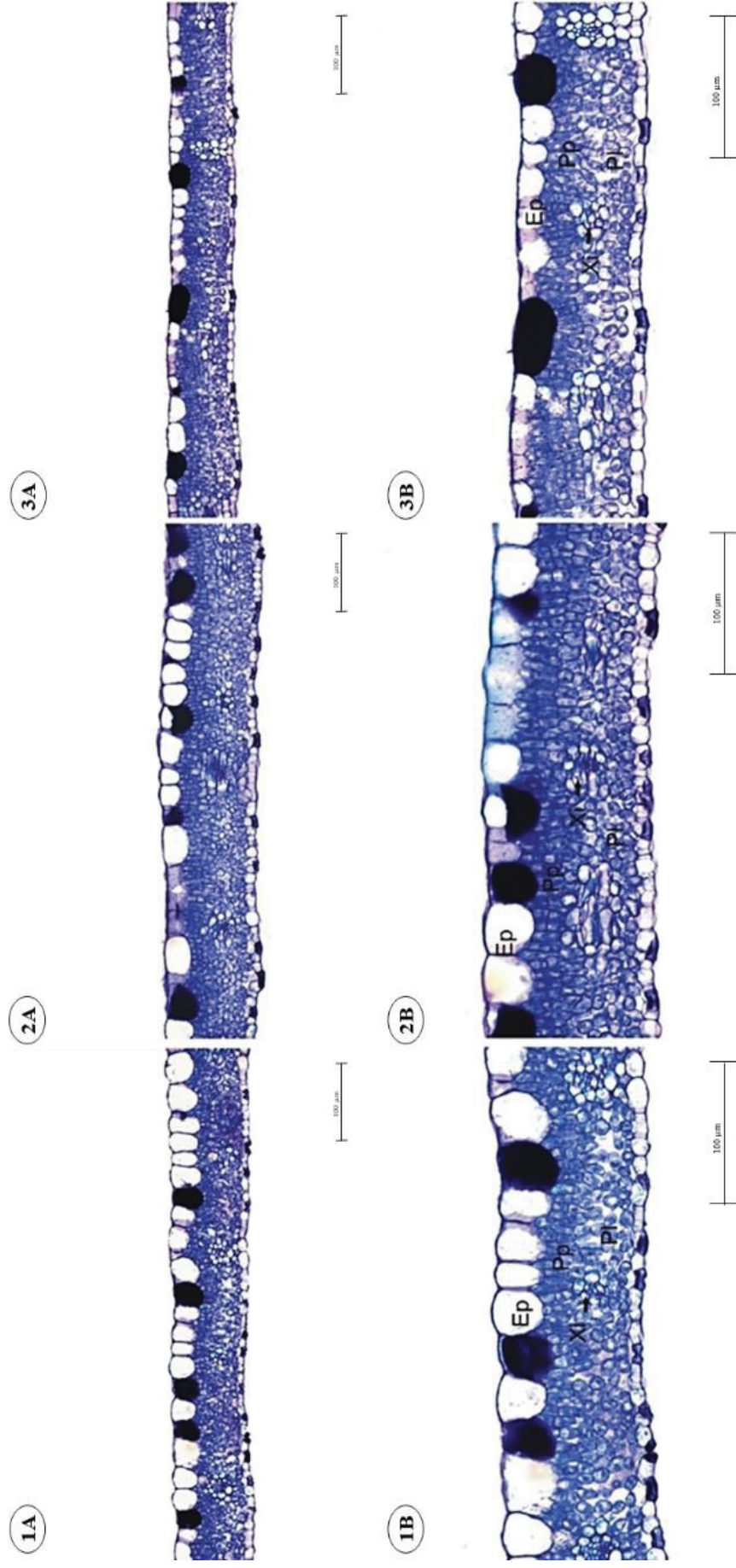


Fig. 3. Light photomicroscopy of plum leaf tissues. Transverse sections of plum leaves 30 days after sprouting in greenhouse. (1A – 1B) ‘SC7’ genotype. Increase 20x and 40x, respectively. (2A – 2B) ‘SC13’ genotype. Increase 20x and 40x, respectively. (3A – 3B) ‘Laetitia’ genotype. Increase 20x and 40x, respectively. Ep – epidermis; Pp – palisade parenchyma; Pi – lacunar parenchyma; Xi – xylem.

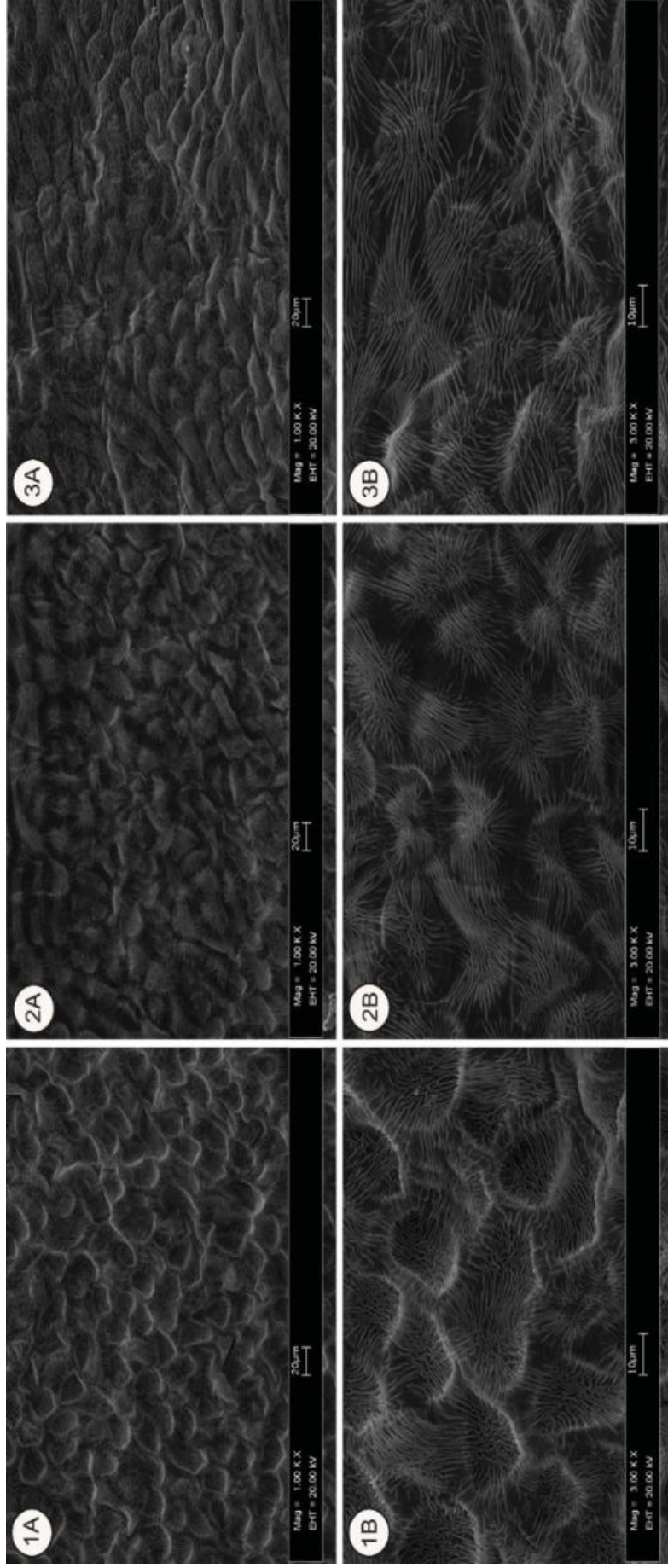
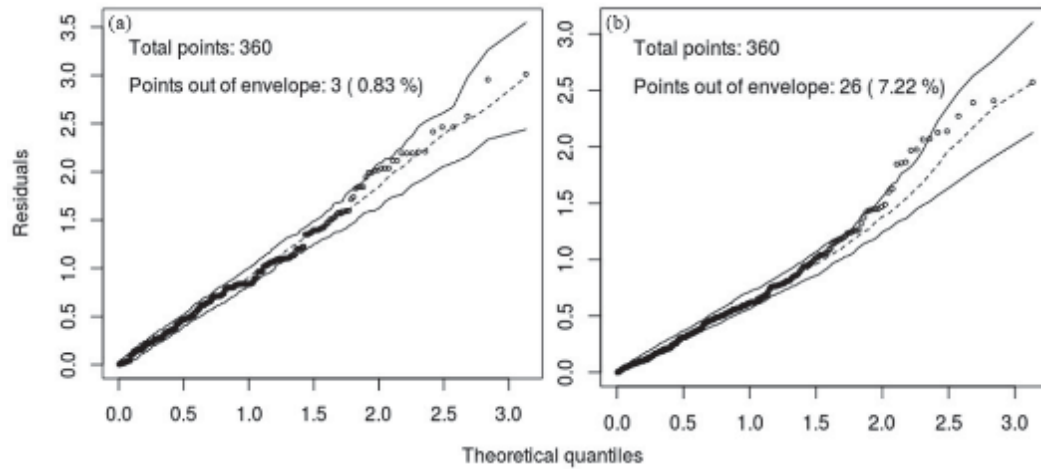
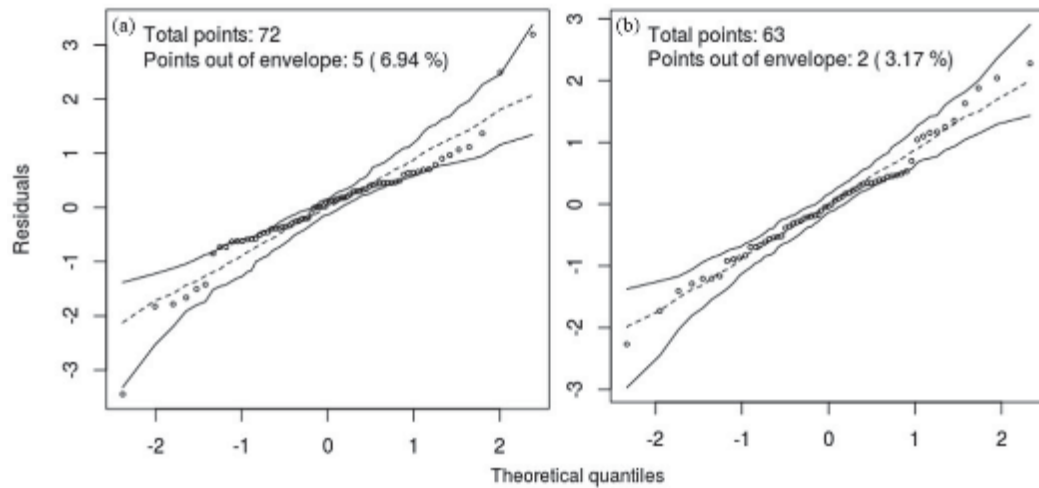


Fig. 4. Scanning photomicroscopy of plum leaf tissue. Top view of the adaxial face. (1A – 1B) Epidermis of ‘SC7’ genotype, with surface increase (1000x) and epicuticular layer detail, respectively. (2A – 2B) Epidermis of ‘SC13’ genotype, with surface increase (1000x) and epicuticular layer detail (3000x), respectively. (3A – 3B) Epidermis of ‘Laetitia’ genotype, with surface increase (1000x) and detail of the epicuticular layer (3000x), respectively.

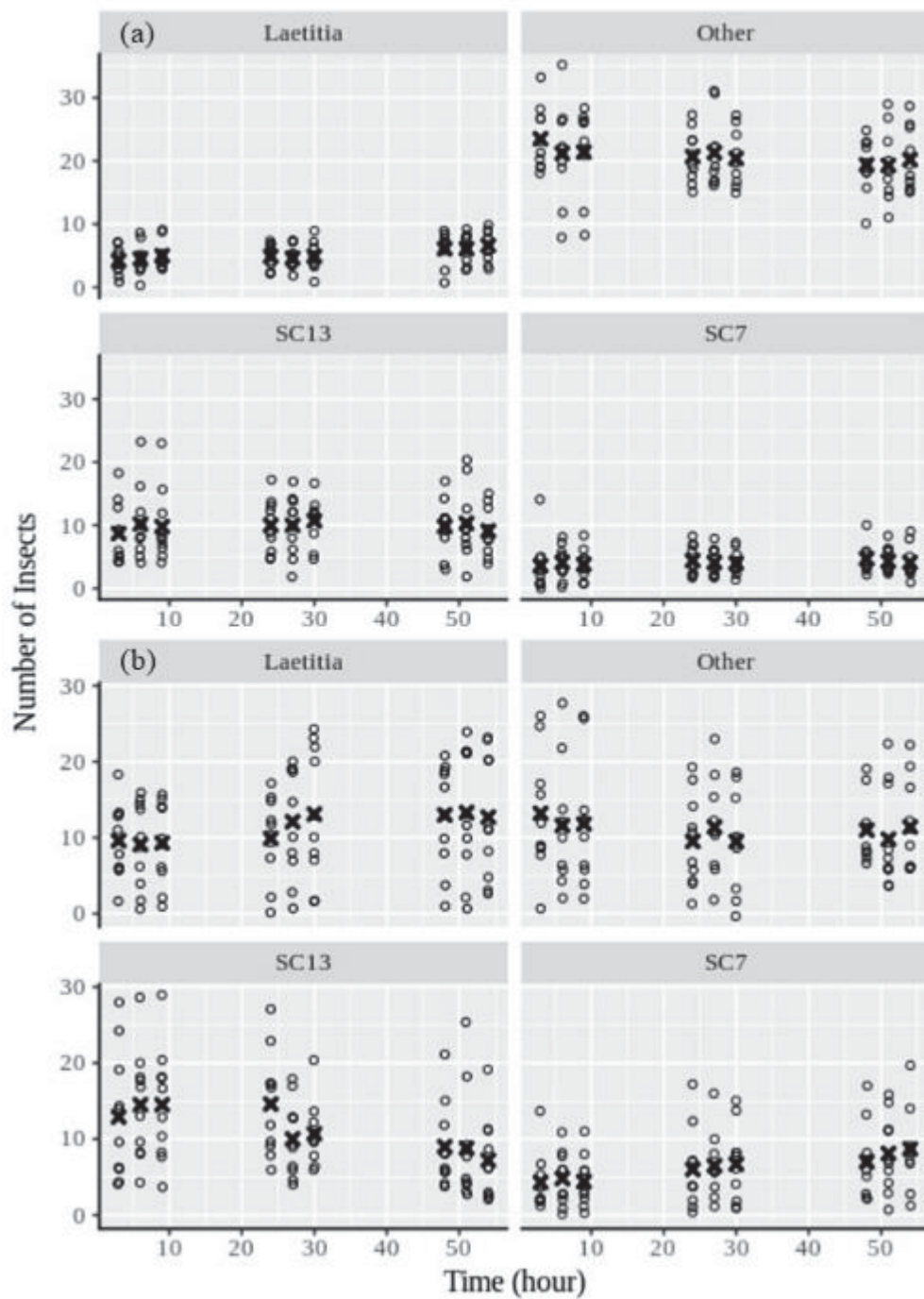
Supplementary Material



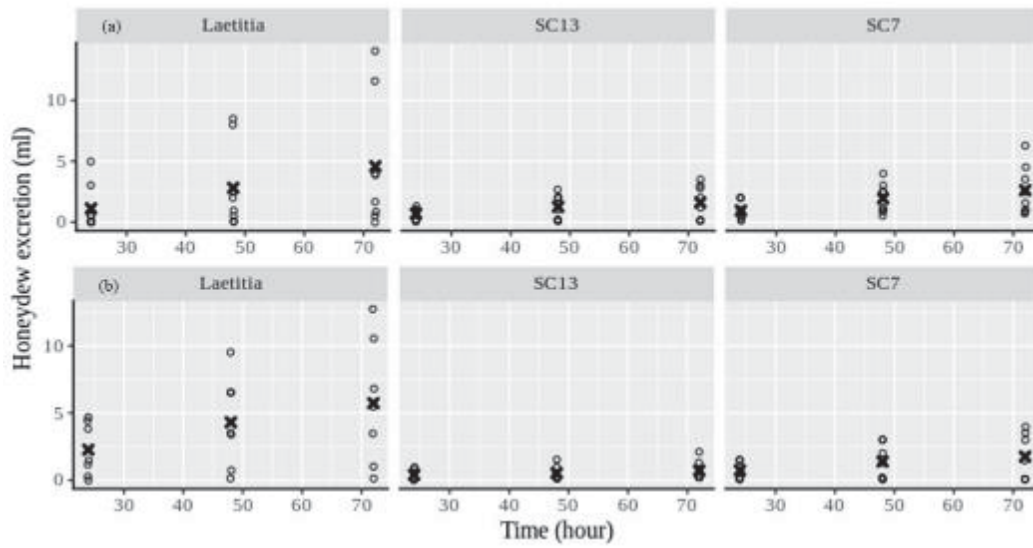
Supplementary Fig. S1. Half-normal plots with simulated envelopes for the deviance residuals from Poisson-Normal model fitted to the (a) *Bucephalagonia xanthophis* and (b) *Sibovia sagata* settling preference data.



Supplementary Fig. S2. Half-normal plots with simulated envelopes for the deviance residuals from Poisson-Normal model fitted to the (a) *Bucephalagonia xanthophis* and (b) *Sibovia sagata* honeydew excretion data.



Supplementary Fig. S3. Descriptive analysis to settling preference for (a) *Bucephalonia xanthophis* and (b) *Sibovia sagata* in plum genotypes ('Laetitia', 'SC7' and 'SC13'). 'Other' represents the incidence of dead insects or insects perched on the walls and basis of the cages throughout the time (hour).



Supplementary Fig. S4. Descriptive analysis to honeydew excretion for (a) *Bucephalagonia xanthophis* and (b) *Sibovia sagata* in plum genotypes ('Laetitia', 'SC7' and 'SC13') throughout the time (hour).

Supplementary Table S1. Parameter estimates by REML and their 95% simultaneous parametric bootstrap confidence intervals for settling preference of *Bucephalagonia xanthophis* and *Sibovia sagata*.

Parameters	<i>Bucephalagonia xanthophis</i>			<i>Sibovia sagata</i>		
	LB	Est.	UB	LB	Est.	UB
μ	1.442	1.589	1.731	1.653	1.933	2.156
$\alpha_{\text{Exp } 2}$	-0.095	0.127	0.322	0.512	0.777	1.039
$\alpha_{\text{Exp } 3}$	-0.196	0.031	0.234	-0.337	-0.046	0.217
τ_{SC7}	-0.635	-0.399	-0.206	-1.160	-0.774	-0.420
τ_{SC13}	0.545	0.717	0.895	0.179	0.497	0.795
τ_{Other}	1.311	1.469	1.643	0.382	0.752	1.036
θ_6				-0.526	-0.097	0.308
θ_9				-0.455	-0.064	0.291
θ_{24}				-0.404	-0.005	0.387
θ_{27}				-0.208	0.181	0.576
θ_{30}				-0.148	0.236	0.637
θ_{48}				-0.095	0.271	0.691
θ_{51}				-0.154	0.262	0.678
θ_{54}				-0.138	0.214	0.596
$(\alpha\tau)_{\text{Exp } 2 \times \text{SC7}}$	0.109	0.410	0.741	-1.145	-0.781	-0.424
$(\alpha\tau)_{\text{Exp } 2 \times \text{SC13}}$	-0.720	-0.474	-0.220	-1.206	-0.888	-0.538
$(\alpha\tau)_{\text{Exp } 2 \times \text{Other}}$	-0.349	-0.130	0.115	-1.820	-1.453	-1.090
$(\alpha\tau)_{\text{Exp } 3 \times \text{SC7}}$	-0.255	0.045	0.375	0.145	0.497	0.881
$(\alpha\tau)_{\text{Exp } 3 \times \text{SC13}}$	-0.126	0.136	0.400	-0.214	0.259	0.513
$(\alpha\tau)_{\text{Exp } 3 \times \text{Other}}$	-0.369	-0.147	0.124	-0.426	-0.077	0.301
$(\tau\theta)_{\text{SC7} \times 6}$				-0.236	0.237	0.843

$(\tau \theta)_{SC7 \times 9}$				-0.442	0.113	0.770
$(\tau \theta)_{SC7 \times 24}$				-0.175	0.333	0.957
$(\tau \theta)_{SC7 \times 27}$				-0.220	-0.277	0.823
$(\tau \theta)_{SC7 \times 30}$				-0.265	0.237	0.801
$(\tau \theta)_{SC7 \times 48}$				-0.323	0.260	0.828
$(\tau \theta)_{SC7 \times 51}$				-0.075	0.430	0.988
$(\tau \theta)_{SC7 \times 54}$				0.050	0.547	1.085
$(\tau \theta)_{SC13 \times 6}$				-0.235	0.259	0.843
$(\tau \theta)_{SC13 \times 9}$				-0.212	0.225	0.711
$(\tau \theta)_{SC13 \times 24}$				-0.266	0.184	0.741
$(\tau \theta)_{SC13 \times 27}$				-0.899	-0.389	0.139
$(\tau \theta)_{SC13 \times 30}$				-0.857	-0.352	0.199
$(\tau \theta)_{SC13 \times 48}$				-1.062	-0.602	-0.119
$(\tau \theta)_{SC13 \times 51}$				-1.156	-0.669	-0.107
$(\tau \theta)_{SC13 \times 54}$				-1.276	-0.799	-0.252
$(\tau \theta)_{Other \times 6}$				-0.514	-0.060	0.506
$(\tau \theta)_{Other \times 9}$				-0.578	-0.080	0.477
$(\tau \theta)_{Other \times 24}$				-0.804	-0.332	0.220
$(\tau \theta)_{Other \times 27}$				-0.805	-0.302	0.235
$(\tau \theta)_{Other \times 30}$				-1.079	-0.602	-0.001
$(\tau \theta)_{Other \times 48}$				-0.888	-0.397	0.153
$(\tau \theta)_{Other \times 51}$				-1.062	-0.571	-0.013
$(\tau \theta)_{Other \times 54}$				-0.812	-0.335	0.232
Σ_{cage}	0.0001	0.0003	0.0458	0.0007	0.0012	0.0945
σ_d	0.0726	0.1616	0.2001	0.3579	0.4549	0.4640

^a REML: Restricted maximum likelihood.

^b LB: Lower bound of confidence interval

^c Est: Parameter's estimate

^d UB: Upper bound of confidence interval

Supplementary Table S2. Parameter estimates by REML and their 95% simultaneous parametric bootstrap confidence intervals for honeydew excretion of *Bucephalagonia xanthophis* and *Sibovia sagata*.

Parameters	<i>Bucephalagonia xanthophis</i>			<i>Sibovia sagata</i>		
	LB	Est.	UB	LB	Est.	UB
μ (Intercept)	0.4681	0.8333	1.1985	0.6528	2.2714	3.8901
τ_{SC7}				-3.2251	-1.5286	0.1679
τ_{SC13}				-5.8747	-2.5896	-0.1827
θ_{48h}	0.4916	0.8107	1.1299	1.1027	2.0429	2.9830
θ_{72h}	0.6737	1.2415	1.8093	0.6681	3.4571	6.2461
$\theta_{SC7 \times 48h}$				-2.4511	-1.4000	-0.3489
$\theta_{SC7 \times 72h}$				-5.3834	-2.4428	-0.4977
$\theta_{SC13 \times 48h}$				-2.8725	-1.9143	-0.9560
$\theta_{SC13 \times 72h}$				-5.9258	-3.1286	-0.3313

$\sigma_{1\text{Laetitia}}$	1.1160	1.8883	3.1952	1.1667	2.0958	3.7649
$\sigma_{1\text{SC7}}$	0.4468	0.6916	1.0707	0.3812	0.6150	0.9920
$\sigma_{1\text{SC13}}$	0.4468	0.6916	1.0708	0.3812	0.6150	0.9920
$\delta_{1\text{Laetitia}}$	0.7509	1.1309	1.5109	0.6293	1.1315	1.6336
$\delta_{1\text{SC7}}$	0.3508	0.7208	1.0908	0.2615	0.7600	1.2585
$\delta_{1\text{SC13}}$	0.1825	0.5899	0.9974	-0.3001	0.2197	0.7394
σ_s	0.0708	0.1612	0.3673	0.0418	0.1225	0.3592

^a REML: Restricted maximum likelihood.

^b LB: Lower bound of confidence interval

^c Est: Parameter's estimate

^d UB: Upper bound of confidence interval

Supplementary Table S3. Intraclass correlation coefficient between times by treatment.

Treatment	θ_k	θ_{kj}	ICC
Laetitia	24	48	0.7477
	24	72	0.3542
	48	72	0.2834
SC7	24	48	0.6426
	24	72	0.3991
	48	72	0.3152
SC13	24	48	0.7604
	24	72	0.5632
	48	72	0.5440

^a Intraclass Correlation Coefficient (ICC) close to 1 indicates high similarity between values from the same group. A low ICC close to zero means that values from the same group are not similar.

Supplementary Table S4. Backward procedure based on LRT to test experiment, cultivar, time, and interactions effects for settling preference of *Bucephalagonia xanthophis* and *Sibovia sagata*.

Source	<i>Bucephalagonia xanthophis</i>				<i>Sibovia sagata</i>			
	Deviance	DF	LRT	<i>p</i> -value	Deviance	DF	LRT	<i>p</i> -value
Group (G)	2343.1	2	0.128	0.938	2330.7	2	0.690	0.708
Cultivar (C)	2353.0	3	449.12	<0.001	2282.8	3	47890	<0.001
Time (T)	1893.8	8	0.420	0.999	2282.4	8	0.392	0.999
G × C	1844.9	6	48474	<0.001	2222.0	6	60350	<0.001
G × T	1844.5	16	0.393	0.999	2220.3	16	1756	0.999
C × T	1827.0	24	17513	0.826	2178.8	24	41478	0.015
G × C × T	1779.6	48	47441	0.496	2135.5	48	43275	0.667

^a Group (G): groups of experiments were set up time, all with the same experimental framework. Each level of group was represented by an experiment whose design was randomized complete block with four blocks (replicate) in the first one and three blocks in the following two experiments for each sharpshooters species.

^bDF: degree of freedom

^c LRT: likelihood ratio test

Supplementary Table S5. Backward procedure based on LRT to test cultivar, time, and interaction effect for honeydew excretion of *Bucephalagonia xanthophis* and *Sibovia sagata*.

	<i>Bucephalagonia xanthophis</i>				<i>Sibovia sagata</i>			
Source	Deviance	DF	LRT	p-value	Deviance	DF	LRT	p-value
Cultivar (C)	253.42	2	1.522	0.4671	218.43	2	6.68	0.0355
Time (T)	251.90	2	26.36	<0.0001	211.75	2	18.82	<0.0001
C × T	243.55	4	8.347	0.0797	189.42	4	22.33	<0.0001

^a DF: degree of freedom^b LRT: likelihood ratio test**Supplementary Table 6.** Mean of cumulative honeydew excretion (mL) for *Bucephalagonia xanthophis* and *Sibovia sagata* and the respectively standard error of treatment (plum genotypes) within time (hours).

Sharpshooters species	Treatment	Time (hours)		
		24	48	72
<i>Bucephalagonia xanthophis</i>	Laetitia	1.12 (± 0.66) aA	2.81 (± 1.23) aBA	4.58 (± 1.89) aB
	SC7	0.95 (± 0.25) aA	1.95 (± 0.43) aB	2.62 (± 0.71) aB
	SC13	0.69 (± 0.17) aA	1.26 (± 0.37) aB	1.61 (± 0.50) aB
<i>Sibovia sagata</i>	Laetitia	2.27 (± 0.75) aA	4.31 (± 1.28) aB	5.73 (± 1.77) bAB
	SC7	0.74 (± 0.14) aA	1.39 (± 0.50) abA	1.76 (± 0.65) abA
	SC13	0.40 (± 0.24) aA	0.53 (± 0.20) aA	0.73 (± 0.27) aA

^a Means followed by the same letter (lower case in columns and upper case in lines) do not differ according to Tukey's pairwise comparison test with Hommel's correction, considering $\alpha = 0.05$.**Supplementary Model S1.** The intraclass correlation coefficient (ICC) under LME model (1)

is given by:
$$ICC_{jk,jk'} = \frac{\sigma_{1j}^2}{\sqrt{\left[\sigma_{1j}^2 + \sigma_{\epsilon}^2 + \exp\left(\frac{\delta_{1j}\theta_k}{12}\right) \right] \left[\sigma_{1j}^2 + \sigma_{\epsilon}^2 + \exp\left(\frac{\delta_{1j}\theta_{k'}}{12}\right) \right]}}$$

The ICC ranges from 0 to 1, where values close to 1 indicates a high similarity between measurements from the same treatment, while it is close to zero means that measurements from same treatment are not similar.

CHAPTER III. IDENTIFICATION OF VOLATILE COMPOUNDS FROM SIX PLUM GENOTYPES AS AN ATTEMPT TO UNDERSTAND THE RESISTANCE MECHANISM TO *Xylella fastidiosa* VECTORS³

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Abstract – Plum leaf scald is considered the main obstacle for the crop expansion in Brazil. This infection is caused by the bacterium *Xylella fastidiosa*, which colonizes the foregut of xylem-sap feeding insects and the xylem of contaminated plants. Management of this pathosystem is still ineffective. Breeding program of the Agricultural Research and Extension of Santa Catarina identified new plum genotypes that showed no leaf scald symptoms in the field and remained negative for *X. fastidiosa* in the molecular tests. However, the mechanisms that confer this resistance have not been revealed. The hypothesis would be that the host resistance could be determined by the interspecific relationship between plant-insect, particularly related to the emission of volatile organic compounds by the host. Therefore, here we investigated the chemical constituents of essential oil from leaves of six plum genotypes with different resistance levels to leaf scald disease. Thirty-nine compounds were identified. The major constituents in the essential oil were phytol (46.76%), butylated hydroxytoluene (11.26%), limonene (2.48%), (*E,E*)- α farnesene (1.95%), β -pinene (1.54%) and linalool (1.33%). ‘Laetitia’ presented the highest total alkane content. ‘Simka’ showed the highest alcohol content, while ‘Fortune’ and ‘SC13’ exhibited the highest concentration of ketones. Cedrol was not detected in susceptible genotypes, however was found in ‘SC7’ and ‘SC15’. (*E,E*)- α farnesene was verified in high amount in ‘Fortune’ (4.70%). According to principal components analysis, genotypes could be divided in four groups based on the resemblance of the volatile profile: 1: ‘Fortune’ and Laetitia’; 2: ‘Simka’ and ‘SC13’; 3: ‘SC7’; and 4: ‘SC15’.

Key Words – Plum leaf scald, insect, *Prunus salicina*, chemical constituents.

³ Prepared in accordance with the standards of Journal of Chemical Ecology.

INTRODUCTION

Plum is a very nutritious fruit, rich in vitamins A, C, E, phytochemical components and bioactive substances important to human health (Stacewicz-Sapuntzakis et al. 2001). The Japanese plum (*Prunus salicina* Lindl) compose the stone fruit group adapted to temperate and subtropical regions, being widely commercialized in the fresh market in America and Asia (Klabunde et al. 2014). In Brazil, this fruit has good acceptability by the consumers, however the internal supply does not attend the national demand, being necessary the importation from neighboring countries, like Chile and Argentina (Madail 2003). Despite the great productive potential, fruit growers have been discouraged because the occurrence of plum leaf scald (PLS), the main obstacle to the crop expansion in Brazil (Castro and Campos 2003; Ducroquet et al. 2001).

The PLS-associated causal agent *Xylella fastidiosa* (Wells et al. 1987) is a gram-negative bacterium, xylem-restricted (Raju et al. 1982) that obstructs the transport of water and nutrients from the roots to the aerial parts (Ducroquet et al. 2001). The disease symptoms appear only after several months of incubation, when the pathogen population increases progressively and is distributed systemically inside the plant (McGaha et al. 2007; Daugherty et al. 2011). Initially, the disease manifests as a slight irregular chlorosis on the edges of the leaves and may be confused with drought symptoms. As the disease progresses, leaf desiccation intensifies (Castro et al. 2008), leading to the plants' death. In addition, fruit from trees infected with PLS have reduced size, less pulp firmness and marked weight loss during the post-harvest period (Kleina et al. 2018).

This bacterium have a wide range of alternative hosts, including several mono and dicotyledonous species (Hopkins 1989), among them, crops of great economic importance, such as citrus (Rossetti et al. 1990), grapevine (Hopkins 1985) and coffee (Paradela-Filho et al. 1995). The transmission occurs through vectors (Hemiptera: Cicadellidae, Cicadellinae) (Hemiptera: Cercopoidea) (Redak et al. 2004; Coletta-Filho et al. 2016), by propagative techniques and by planting contaminated but asymptomatic vegetative material (Janse and Obradovic 2010).

The management for this pathosystem is still ineffective, even though PLS is considered the main plum disease in Brazil. The measures adopted are the use of bacterial-free propagative material, rouging and chemical insecticides applications to control hemiptera (Coletta-Filho et al. 2016), despite the development of resistance, environmental pollution and undesirable effects on non-target organisms (Dalbó et al. 2016, Khani et al. 2012). Cultivars

with a certain degree of resistance, such as 'Laetitia' and 'Fortune' in the colder regions and 'Gulfblaze' in the warmer areas has also been used. However, the useful life of the orchard is still very short (Dalbó et al., 2016) becoming unproductive within three to five years (Ducroquet et al. 2001).

Therefore, the Agricultural Research and Rural Extension of Santa Catarina (EPAGRI-Videira) has been directing efforts in the development of cultivars, aiming fruit quality and total resistance to PLS (Dalbó et al. 2018). The breeding program of this institution identified plum genotypes ('SC7', 'SC13' and 'SC15') that showed no leaf scald symptoms in the field and remained negative for *X. fastidiosa* in the molecular detection tests (PCR) for 10 years, indicating that these genotypes were not infected under natural conditions, even being close to contaminated plants. However, these genotypes were colonized by the pathogen when grafted on diseased plants, due to the connection between the xylem vessels (Dalbó et al. 2018).

Although laboratory and field studies have demonstrated that these new plum genotypes are more resistant to PLS, when compared to traditional cultivars, the mechanisms that confer this resistance have not been revealed. One hypothesis would be that the host resistance could be determined by the interspecific relationship between insect-plant, particularly related to the emission of vector-repellent volatile organic compounds (VOCs). Another assumption is that the bacterium would be present in these resistant plants, but in very low titers and with limited mobility, not being detected in molecular tests. This condition of restrictibility could be linked to the presence of compounds released by the host that would be deleterious to its establishment.

Plant volatiles are the metabolites that plants release into the air (Dorokhov et al. 2014). Plants have complex strategies to defend against the attack of insects and phytopathogens (Huot et al. 2013; Martins and Zarbin 2013), being able to emit VOCs from various organs (roots, leaves, flowers and fruits) (Maffei et al. 2007). Constitutively emitted VOCs also influence the behavior and physiology of some herbivores, having toxic, repellent, and deterrent effects (Holopainen 2004, Holopainen and Blande 2012). Different signaling pathways are induced by chewing insects (caterpillars) and piercing-sucking insects, such as aphids and whiteflies (Dorokhov et al. 2014). Piercing sucking-insects usually causes minimum mechanical damage in plants with the stylet penetration, but it may induce the defense apparatus by the emission of volatile compounds (Agrawal and Heil. 2012), such as terpenoids (Gosset et al. 2009), which in turn may explain the resistance or tolerance phenomena. For the glassy-winged sharpshooter *Homalodisca vitripennis* German (*X. fastidiosa* vector), volatiles released from *Prostanthera ovalifolia* are an effective deterrent to altering attraction cues and preventing

oviposition on this species (Rathé et al. 2014). Gajek et al. (1996) showed that the increased content of 3-carene and α -caryophyllene in black currant cultivar resistant to the mite *Cecidophyopsis ribis* had a repellent effect and caused vigour loss in the mite.

Identifying the volatile profile is the first step before studying and understanding the ecological roles of these compounds in interspecific interactions among plant-pathogen-vectors. Therefore, in the present study we investigated the chemical constituents of essential oil from leaves of six plum (*Prunus salicina*) genotypes with different resistance levels to leaf scald disease. Based on our knowledge, this is the first work involving the identification of the volatile profile from *Prunus salicina* leaves. In addition, no study has been reported previously for this pathosystem relating the volatile compounds activity with the plum leaf scald resistance.

MATERIAL AND METHODS

Plant Material and Sample Preparation.

Fully expanded leaves (n = 500) were randomly collected at middle third of healthy plum trees (ten years old) from six genotypes ('Fortune', 'Laetitia', 'Simka', 'SC7', 'SC13', 'SC15') with different degrees of resistance to leaf scald disease (Table 1). Leaf samples were collected from the germplasm collection orchard located at Agriculture Research and Rural Extension of Santa Catarina (EPAGRI), Videira (SC), Brazil (latitude 27° 00' S, longitude 51° 09' W, 779 meters of altitude). The climate of the region belongs to the Cfb type (Köppen classification), subtropical mesothermic climate, with average annual temperature between 15.8 °C to 17.9 °C. Annual rainfall varies from 1,460 mm to 1,820 mm, with annual rainfall days between 129 and 144 days (normal means). Samples were collected in late spring (December 05, 2017) from two plants of each genotype (n = 250 leaves per plant), representing one replicate. The mean temperature incident at collection time was 22.2 °C (13.3 °C – 34.7 °C) with 13 mm of precipitation (weather station: Videira, latitude 27° 00' S and longitude 51° 09' W) (EPAGRI CIRAM, 2019). In this work, three replicates were used for each treatment (genotype). The material were packed in plastic bags and placed in polystyrene boxes for the immediately transportation to the Chemistry Department (Federal University of Parana) located at Curitiba (PR), Brazil.

Table 1 – Plum genotypes, origin, parental of crosses and resistance level of plum leaf scald disease (PLS).

Genotypes	Origin	Parental of Crosses	Resistance to PLS ^a
Fortune	United States of America	‘Laroda’ x ‘B65-11’	Moderately susceptible
Laetitia	South Africa	‘Golden King’ x Unknown	Moderately susceptible
Simka	United States of America	Unknown	Highly susceptible
SC7	Brazil	‘Laetitia’ x ‘Piamontesa’ x ‘Fortune’	Resistant
SC13	Brazil	‘SC7’ x ‘Fortune’	Resistant
SC15	Brazil	‘Chatard’ x ‘Angelino’ x Unknow	Resistant

^a PLS: Plum leaf scald

VOCs Extraction.

The samples (100 g of fresh leaves) were placed in a 2000 mL round bottom flask with 1,000 mL of distilled water and adapted to a Clevenger apparatus. The hydrodistillation was performed for 5 hours. The essential oils were extracted 3 times with 10 mL of diethyl ether. Water traces in organic phase were removed by the addition of anhydrous magnesium sulfate and after filtration; the volatiles were concentrated by evaporating diethyl ether under argon gas (Podestá et al. 2011).

Volatile Analysis.

For the identification of the compounds present in the essential oils were used the Gas Chromatography - Mass Spectrometry (GC-MS) technique. The samples were dissolved in hexane HPLC grade (Merck - KGaA, Darmstadt – HE, Germany) and injected in a gas chromatograph Shidmazu® GC-2010 Plus, which were coupled to a Shimadzu® GC-MS/MS TQ8040 Plus mass spectrometer equipped with RTX-5MS capillary column (30 mx 0.25 mm x 0.25 µm). Injector was set in Split mode at 250 °C, interface and source of ions at 250 °C. The mass window was adjusted for analyzes in the range of 40-350 m/z, using helium as carrier gas, with column flow of 1.02 mL/min and column pressure of 59 KPa. Oven temperature program was initialized in 60 °C rising to 250 °C at a rate of 3 °C/min.

Standards of C₇H₁₆ – C₃₀H₆₂ of Saturated Alkanes (Supelco - Sigma-Aldrich. St. Louis - MO, USA) were injected in the CG-MS in the same programming described above. Chromatograms generated by the software GCMSsolution Shimadzu (Version 4.2, Kyoto, JP) were exported in NetCDF format for deconvolution and identification of the compounds using the software AMDIS_32 (Version 2.71, Gaithersburg, USA) under manual supervision.

Analytes were identified by comparing the mass spectra and retention time index with a data library previously made within the AMDIS, allowing practicality in the identifications of the compounds. RI values were calculated based on the analysis of an n-alkane mixture with carbon atoms between C₉H₂₀ – C₂₈H₅₈. For the determination of the relative percentage of each compound in the sample, the sum of all the areas produced by the major fragment of each compound with a signal: (S:R)> 40, identified or unidentified, were adopted as 100%, so the individual percentage of each compound was related to the whole.

Experimental Design and Statistical Analyses.

The trials followed a completely randomized design, comprising six treatments (genotypes) and 3 replicates. Data were transformed to $-\log_{10}$ for the chemometric and stepped calculations. Principal component analysis (PCA) was used to reduce the size of the data, using R program (Studio Server, *ggfortify* package). The variables were statistically tested for normality (Shapiro-Wilk) using R software with *multcomp*, *stats* and *lattice* packages (R Core Team 2018). Variables with normal probability distribution were analyzed using ANOVA with F test ($p > 0.05$). Comparisons between treatments and their significant interactions were evaluated by Tukey's 5% contrasts.

RESULTS

Among eighty-nine compounds extracted (Table 2 and Table 3) from the six plum genotypes, thirty-nine were identified by GC-MS/MS (Table 2), representing more than 50% of the total oil composition. Even with the low amount of essential oils obtained by this methodology, it was possible to classify the compounds in 12 alcohols, 1 aldehyde, 7 alkanes, 7 alkenes, 1 aromatic, 3 esters, 3 ethers and 5 ketones. Considering all genotypes, the major constituents identified in the essential oil over the entire period (5h) were phytol (46.76%), butylated hydroxytoluene (11.26%), limonene (2.48%), (*E,E*)- α farnesene (1.95%), β -pinene (1.54%) and linalool (1.33%), being more than 50% from the terpenes pathway. The terpenes were classified in 16 monoterpenes, 4 sesquiterpenes and 3 diterpenes. Other compounds, as alkanes, comes from fatty acid pathway and aromatic aldehydes comes from phenyl propanoic pathway.

Genotypes exhibited differences in some volatile groups. Alcohols, alkanes, alkenes and ketones were the major chemical families identified (Table 2). 'Laetitia' presented the highest total alkane content. 'Simka' showed the highest alcohol content, while 'Fortune' and 'SC13'

exhibited the highest concentration of ketones. ‘SC7’ presented intermediate relative contents of alcohols, alkanes and ketones, whereas, ‘SC15’ scored the lowest values for total alkane content.

Seven alcohols (*2E*-hexenol, linalool, *neo*-menthol, butylated hydroxytoluene, *iso*-phytol, phytol and *E,E*-geranyl linalool), two alkanes (pentadecane and octadecane), five alkenes (α -pinene, β -pinene, limonene, terpinolene and (*E, E*)- α farnesene) one aromatic (*p*-cymene), three esters (isopentyl acetate, 2-methyl butyl acetate and amyl acetate), one ether (1,8-cineole) and four ketones (camphor, menthone, *iso*-menthone and pulegone) were detected in all plum genotypes.

Cedrol was not detected in susceptible genotypes, however was found in ‘SC7’ (0.1%) and ‘SC15’ (0.12%) (Figure 1A). On the other hand, (*Z*)- β -damascenone was detected only in two susceptible genotypes (‘Fortune’ and ‘Simka’) (Figure 1B) and (*E,Z*)-geranyl linalool was just found in ‘Fortune’ (Figure 1E). Dendrolasin was higher in susceptible genotypes, but was not observed in the ‘SC7’ and ‘SC15’ genotypes (Figure 1C). (*E, E*)- α farnesene was observed in all plum genotypes, presented in high amount in ‘Fortune’ cultivar (4.70%) and less in ‘SC13’ (0.46%) and ‘SC15’ (0.46%) (Figure 1D). *cis*-Linalool oxide was identified in higher concentrations in the cultivar ‘Laetitia’ (0.072%), differing statistically from the genotype ‘SC7’, which did not present this compound on its volatile profile.

According to principal components analysis (PCA), PC1-PC2 account for 42.5% of the data. Score plot showed that in PC1 positive ‘Simka’, ‘SC7’ and ‘SC15’ were separate from ‘Fortune’, ‘Laetitia’ and ‘SC13’ (Figure 2A). However, PC2 negative separates ‘SC7’ from the other samples. At first analysis, the volatile compounds found in ‘Fortune’, ‘Laetitia’ and ‘SC13’ are closer than the other genotypes, forming a group. The inclusion of a third component (PC3) adds 13.7% to the explanation of the data, and helps to separate the samples contained in ‘SC13’, ‘Simka’ and ‘SC15’ (Figure 2C and 2E). In summary, the genotypes could be divided in four groups based on the resemblance of the volatile profile: Group 1: ‘Fortune’ and ‘Laetitia’; Group 2: ‘Simka’ and ‘SC13’; Group 3: ‘SC7’; and Group 4: ‘SC15’.

Loadings plots showed that the Compound 52 (ID 039), n-hexanol (ID 02), amyl acetate (ID 07), benzaldehyde (ID 012), myrcene (ID 014), Compound 051 (ID 015), *cis*-linalool oxide (ID 019), Compound 003 (ID 023), geraniol (ID 033), Compound 011 (ID 043), Compound 019 (ID 056), Compound 050 (ID 107), Compound 058 (ID 053), Compound 018 (ID 052) and cedrol (ID 075) are the ones that most influenced the data distribution. Compound 52 was closely related to the differentiation of the group formed by ‘SC15’. The compounds n-hexanol, Compound 003, *cis*-linalool oxide, Compound 002, benzaldehyde, Compound 051, n-dodecane

and Compound 019 were the ones that grouped the samples formed by 'Fortune', 'Laetitia' and 'SC13' (Figure 2B).

Considering PC1 with PC3, the Compound 043 (ID 059), Compound 017 (ID 051), Compound 003 (ID 023), Compound 028 (ID 080), Compound 040 (ID 72), (*E,E*)- α farnesene (ID 064), n-hexanol (ID 002) and n-dodecane (ID 030) are those that help to distinguish the samples formed by 'Simka' and 'SC13' from the others (Figure 2D and 2F).

Table 2 - Volatile compounds detected and quantified in plum leaves samples of six genotypes with different levels of resistance to leaf scald disease (*Xylella fastidiosa*) by GC-MS (Unit: relative area %).

ID	RT (min)	RI	Compound	Category	Fortune		Laetitia		Simka		SC7		SC13		SC15	
					Mean	CV (%)	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)
0002	3.74	877.6	<i>n</i> -hexanol ^a		0.05	55.18	0.12	33.13	0.03	21.85	ND	-	0.07	48.33	ND	-
0005	4.05	890.2	(2 <i>E</i>)-hexenol		0.09 ^{ns}	33.24	0.19 ^{ns}	39.35	0.07 ^{ns}	90.26	0.08 ^{ns}	63.38	0.16 ^{ns}	64.55	0.07 ^{ns}	71.26
0022	10.15	1100.5	Linalool		1.27 ^{ns}	83.49	1.03 ^{ns}	63.22	1.8 ^{ns}	72.2	0.99 ^{ns}	71.51	1.6 ^{ns}	43.39	1.31 ^{ns}	74.49
0028	13.03	1171.2	<i>neo</i> -menthol		0.2 ^{ns}	23.83	0.12 ^{ns}	26.46	0.09 ^{ns}	83.66	0.1 ^{ns}	77.71	0.14 ^{ns}	57.07	0.11 ^{ns}	42.32
0029	13.76	1189.1	<i>α</i> -terpineol		0.27 ^{AB}	32.59	ND ^B	-	0.66 ^A	70.92	0.09 ^{AB}	75.91	0.39 ^{AB}	13.1	0.23 ^{AB}	102.5
0033	16.42	1254.3	Geraniol ^a	Alcohol	0.24	39.18	ND	-	0.38	46.52	ND	-	0.22	41.04	0.21	94.17
0065	27.03	1515.2	Butylated hydroxytoluene		8.04 ^{ns}	86.24	7.77 ^{ns}	31.36	16.43 ^{ns}	20.36	12.83 ^{ns}	37.15	9.7 ^{ns}	17.49	12.84 ^{ns}	43.54
0075	30.39	1603	Cedrol ^a		ND	-	ND	-	ND	-	0.12	18.89	ND	-	0.1	37.1
0093	42.36	1950.9	<i>iso</i> -phytol		0.11 ^B	28.13	0.25 ^A	31.38	0.11 ^B	66.58	0.18 ^{AB}	15.6	0.09 ^B	43.9	0.13 ^{AB}	12.24
0096	44.94	2032.1	(<i>E,Z</i>)-geranyl linalool ^a		0.34	29.15	ND	-	ND	-	ND	-	ND	-	ND	-
0099	44.89	2030.5	(<i>E,E</i>)-geranyl linalool		1.25 ^{ns}	22.41	2.58 ^{ns}	30.52	0.35 ^{ns}	30.36	1.23 ^{ns}	150.48	0.83 ^{ns}	46.21	0.31 ^{ns}	80.95
0102	47.48	2118.2	Phytol		45.34 ^{ns}	23.82	58.81 ^{ns}	27.96	51.31 ^{ns}	2.64	44.77 ^{ns}	27.03	42.89 ^{ns}	16.80	37.42 ^{ns}	23.47
0012	5.84	960.9	Benzaldehyde ^a	Aldehyde	0.06	22.39	0.06	40.07	ND	-	ND	-	ND	-	0.1	39.18
0008	5.12	933.3	<i>α</i> -pinene		0.18 ^{ns}	57.94	0.36 ^{ns}	82.38	0.05 ^{ns}	74.23	0.08 ^{ns}	54.05	0.27 ^{ns}	134.97	0.02 ^{ns}	57.63
0013	6.22	977.6	<i>β</i> -pinene		1.22 ^{ns}	103.27	3.22 ^{ns}	79.8	0.62 ^{ns}	47.48	1.12 ^{ns}	53.34	2.76 ^{ns}	118.15	0.34 ^{ns}	58.66
0014	6.56	991.7	Myrcene ^a	Alkene	0.05	52.88	0.13	29.2	ND	-	0.03	100.76	0.09	54.26	ND	-
0017	7.74	1028.4	Limonene		2.07 ^{ns}	85.28	4.25 ^{ns}	76.96	1.25 ^{ns}	38.85	2.33 ^{ns}	50.71	4.22 ^{ns}	94.85	0.81 ^{ns}	58.2
0021	9.77	1089.4	Terpinolene		0.12 ^{ns}	22.01	0.09 ^{ns}	80.73	0.06 ^{ns}	45.89	0.06 ^{ns}	92.7	0.1 ^{ns}	101.81	0.05 ^{ns}	65.21
0062	26.32	1496.8	<i>α</i> -zingiberene ^a		0.38	38.31	ND	-	0.1	22.48	ND	-	ND	-	ND	-
0064	26.86	1510.7	(<i>E-E</i>)- <i>α</i> -farnesene		4.70 ^A	44.78	3.39 ^{AB}	76.81	1.14 ^{AB}	28.63	1.57 ^{AB}	90.53	0.46 ^B	53.97	0.46 ^B	69.57
0030	14.14	1198.4	<i>n</i> -dodecane ^a		0.17	22	ND	-	ND	-	0.02	173.21	0.04	19.31	ND	-
0035	18.28	1300	<i>n</i> -tridecane ^a	Alkane	0.11	32.1	ND	-	ND	-	ND	-	ND	-	ND	-

0048	22.43	1399.9	<i>n</i> -tetradecane	0.61 ^A	23.65	ND ^B	-	0.37 ^{AB}	91.2	0.28 ^{AB}	66.71	0.51 ^{AB}	46.01	0.16 ^{AB}	87.59	
0063	26.46	1500.4	<i>n</i> -pentadecane	0.33 ^{ns}	74	0.17 ^{ns}	56.25	0.34 ^{ns}	81.17	0.44 ^{ns}	29.73	0.53 ^{ns}	57	0.21 ^{ns}	43.48	
0074	30.33	1601.2	<i>n</i> -hexadecane	0.42 ^{ns}	22.52	ND ^{ns}	-	0.38 ^{ns}	42.02	0.35 ^{ns}	17.59	0.16 ^{ns}	82.96	0.21 ^{ns}	16.85	
0087	37.51	1802	<i>n</i> -octadecane	0.33 ^{ns}	86.96	0.2 ^{ns}	68.89	0.26 ^{ns}	16.69	0.6 ^{ns}	48.39	0.43 ^{ns}	43.61	0.32 ^{ns}	17.27	
0097	44.00	2001.5	<i>n</i> -eicosane ^a	0.19	21.89	ND	-	ND	-	0.16	32.95	ND	-	ND	-	
0016	7.59	1024.6	<i>p</i> -cymene	Aromatic	0.09 ^{ns}	34.12	0.13 ^{ns}	84.35	0.04 ^{ns}	20.02	0.06 ^{ns}	71.34	0.11 ^{ns}	103.78	0.02 ^{ns}	61.26
0003	3.88	883.1	Isopentyl acetate		0.71 ^{ns}	75.47	1.49 ^{ns}	81.5	0.16 ^{ns}	87.92	0.26 ^{ns}	52.04	1.08 ^{ns}	146.52	0.05 ^{ns}	60.43
0004	3.92	884.8	2-methyl butyl acetate	Ester	0.19 ^{ns}	75.63	0.43 ^{ns}	72.51	0.08 ^{ns}	66.35	0.08 ^{ns}	52.81	0.45 ^{ns}	82.43	0.02 ^{ns}	62.84
0007	4.59	911.9	Amyl acetate		0.3 ^{ns}	66.77	0.54 ^{ns}	75.81	0.11 ^{ns}	80.14	0.14 ^{ns}	59.61	0.41 ^{ns}	140.7	0.02 ^{ns}	61.64
0018	7.81	1031.1	(1,8)-Cineole		0.33 ^{ns}	79.87	0.55 ^{ns}	68.75	0.4 ^{ns}	64.98	0.39 ^{ns}	54.01	0.63 ^{ns}	60.09	0.16 ^{ns}	55.99
0019	9.16	1071.3	<i>cis</i> -linalool oxide	Ether	0.03 ^{AB}	37.99	0.07 ^A	24.11	0.06 ^{AB}	37.99	ND ^B	-	0.06 ^{AB}	75.33	0.03 ^{AB}	48.07
0071	29.58	1581.6	Dendrolasin ^a		0.72	22.91	0.61	72.63	0.16	20.82	ND	-	0.13	29.4	ND	-
0024	11.90	1143.6	Camphor		0.13 ^B	62.6	0.37 ^A	24.44	0.16 ^{AB}	35.36	0.24 ^{AB}	48.32	0.29 ^{AB}	31.61	0.15 ^{AB}	48.09
0025	12.26	1152.4	Menthone		0.6 ^{ns}	76.26	0.66 ^{ns}	66.82	0.56 ^{ns}	34.93	0.71 ^{ns}	53.42	0.9 ^{ns}	26.6	0.52 ^{ns}	57.8
0026	12.68	1162.8	<i>iso</i> -menthone	Ketone	0.2 ^{ns}	22.77	0.15 ^{ns}	82.94	0.15 ^{ns}	47.26	0.17 ^{ns}	28.75	0.19 ^{ns}	27.74	0.13 ^{ns}	61.77
0031	15.76	1238.3	Pulegone		1.01 ^{ns}	84.1	0.41 ^{ns}	75.24	0.78 ^{ns}	61.33	0.58 ^{ns}	74.9	0.98 ^{ns}	37.95	0.75 ^{ns}	66.98
0046	21.85	1386	(<i>Z</i>)- β -damascenone ^a		0.48	44.36	ND	-	0.08	54.62	ND	-	ND	-	ND	-

Means followed by the same letter in the line do not differ according to a Tukey test ($P < 0.05$). The results consist of the mean of three replicates. ^{ns} - not significant.

ID: Identity

RT: Retention Time in minutes

RI: Retention Index

CV: Coefficient of variation in percentage

ND: Not detected

^a Qualitative analysis

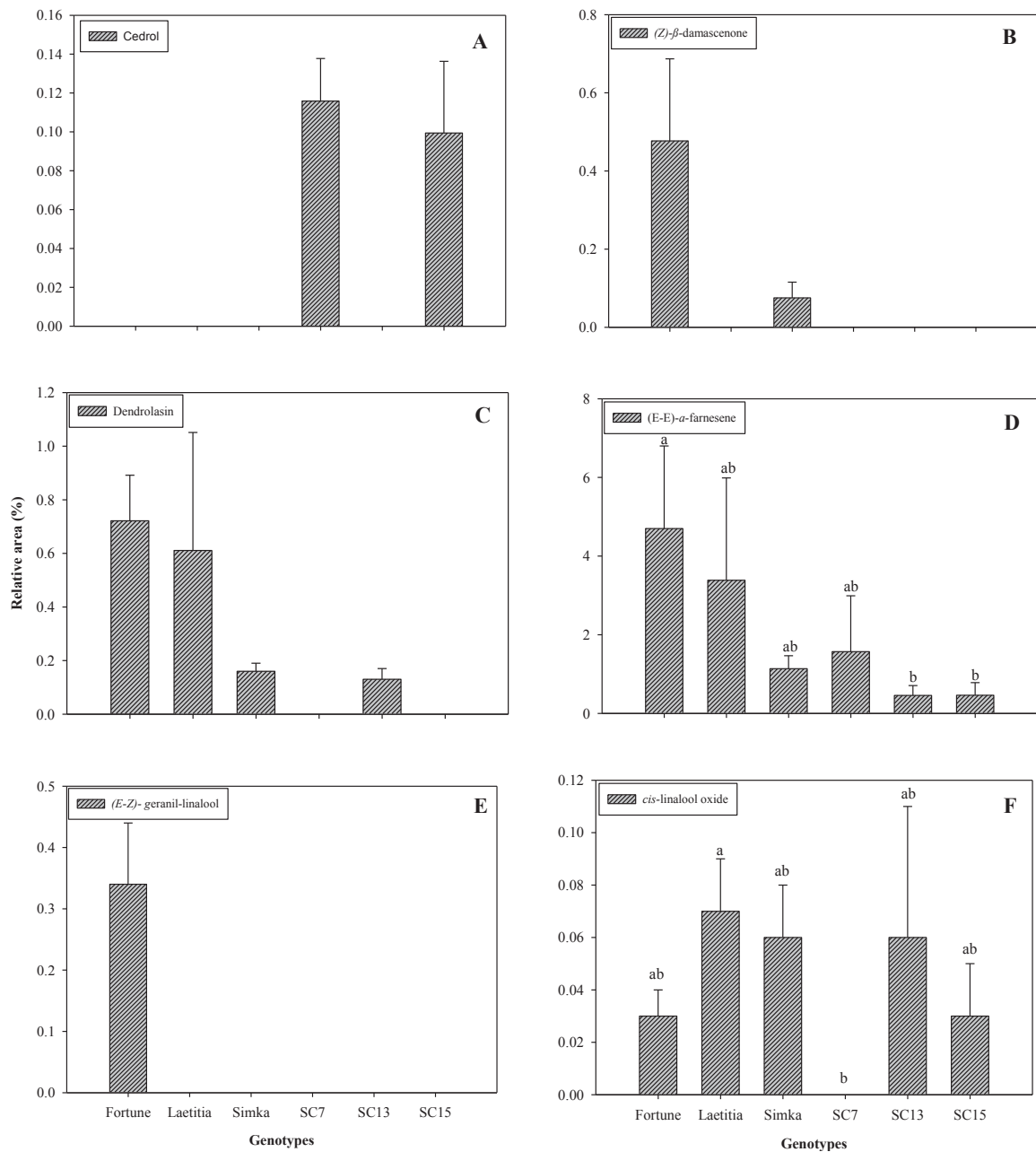


Figure 1 – Relative area (%) of the volatile compounds (cedrol^a, (Z)- β -damascenone^a, dendrolasin^a, (E,E)- α -farnesene, (E,Z)-geranyl linalool^a and *cis*-linalool oxide) emitted by six plum genotypes with different levels of resistance to leaf scald disease (*Xylella fastidiosa*). Means followed by the same letter do not differ according to a Tukey test (P < 0.05). The results consist of the mean of three replicates. ^{ns} - not significant. ^a Qualitative analysis

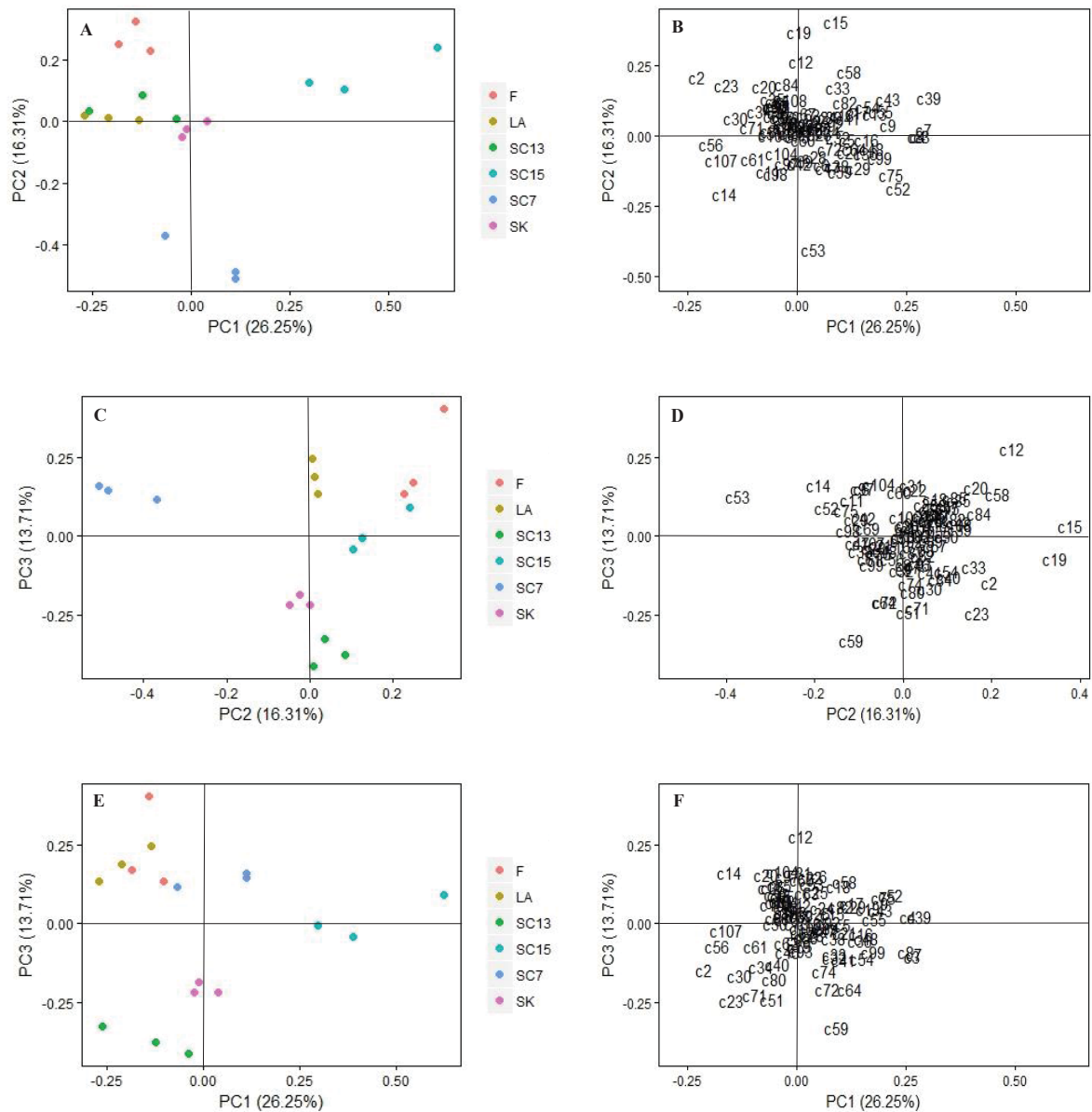


Figure 2 – A, C and E) Score plots of the samples. Principal components analysis (PCA) for six plum genotypes with different levels of resistance to plum leaf scald disease (*Xylella fastidiosa*). B, D and F) Loading plots of volatile compounds. Three analytical replicates are presented for each sample. The numbers indicate each compound shown in Table 2 and Table 3. The contribution ratios of the components are shown in parentheses on the corresponding axis. F: ‘Fortune’; LA: ‘Laetitia’; SK: ‘Simka’

DISCUSSION

Volatile compounds (VOCs) are a series of organic molecules from different classes (mainly terpenes and derivatives), produced as secondary metabolites that have, among others, the function of avoiding injuries promoted by external agents (Correia et al. 2008; Viegas Júnior 2003). Thus, these compounds have a recognized antimicrobial (De Martino et al. 2015) and insecticide activities (Khani et al. 2012). In this work, it was possible to observe that the genotypes resistant to leaf scald ('SC7' and 'SC15') showed a different volatile profile than the traditional cultivars ('Fortune', Laetitia 'and' Simka ') susceptible to *X. fastidiosa*.

Terpenoid pathway was the main route that express differences between the samples (Figure 3). Terpenoids have an important role in the plant defense and is expected that resistant responses include changes in the terpenoid fingerprint. As VOCs are synthesized due to different stress signals in the plant, resistant genotypes could modify the responses in order to have an efficient defense mechanism against insects and other pathogens. Greener parts of the plants have higher amounts of monoterpenes than sesqui and diterpenes (Kumari et al. 2014), and this fact can explain that less differences in monoterpenes constituents were found. On the other hand, compounds classified as sesquiterpenes and diterpenes draw a distinction between the six plum genotypes samples in PCA analysis.

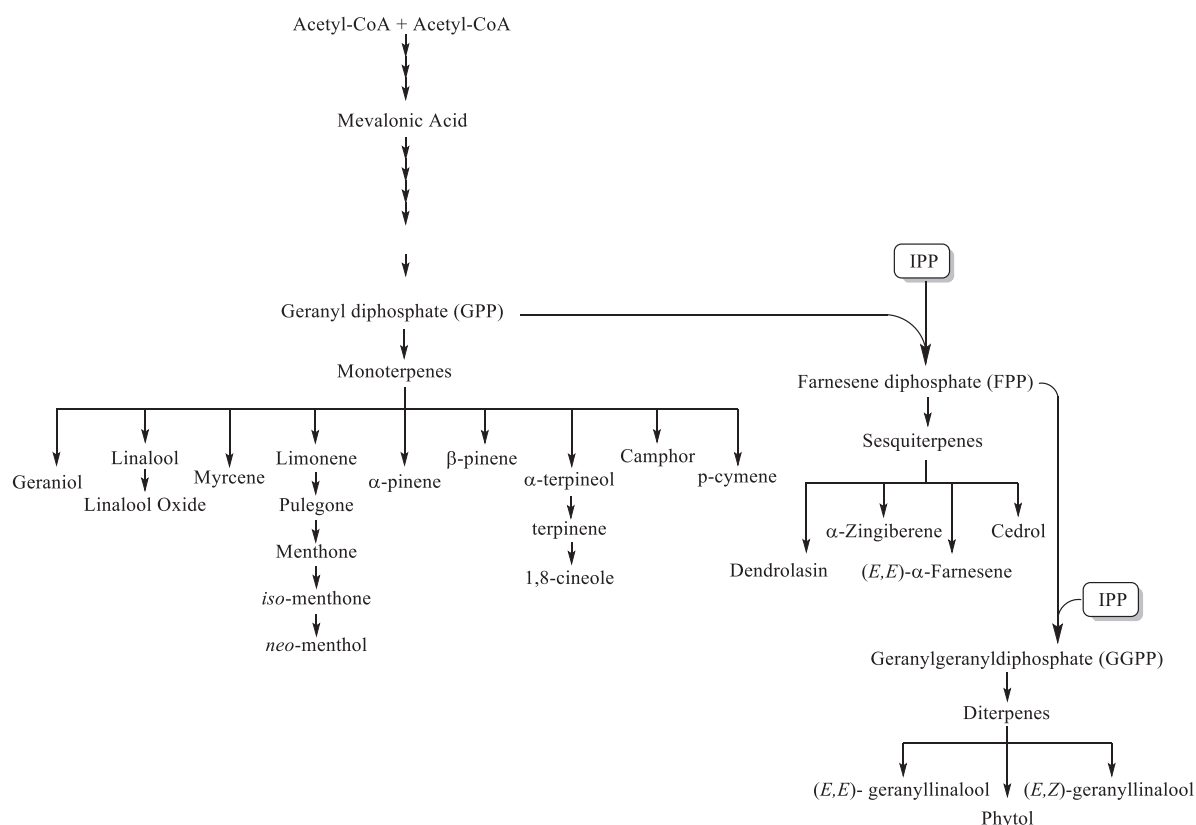


Figure 3 – Terpene pathway of the main volatile compounds found in six plum genotypes with different levels of resistance to plum leaf scald disease (*Xylella fastidiosa*)

The compounds found in the six genotypes with non-significative differences as: *n*-hexanol, linalool, neo-mentol, (*E,E*)-geranylinalool, phytol, α -pinene, β -pinene, limonene, terpinolene, pentadecane, hexadecane, octadecane, *p*-cymene, isopentyl acetate, 2-methylbutyl acetate, amyl acetate, (1,8)-cineole, menthone, *iso*-menthone and pulegone can be classified as constituents of the VOC profile intrinsic to the species (*Prunus salicina*). Phytol, the major compound found in the six cultivars, is part of chlorophyll molecule and is found in the leaves of green plants (Mastelic et al, 2006). The other compounds with significative differences could be classified as a VOC genotype characteristic. Gene expression studies must be load to understand if their occurrence is part of a gene expression or a phenotype response.

One of the most expressive differences was observed for cedrol (C₁₅H₂₆O), bioactive sesquiterpene compound, detected in two ('SC7' and 'SC15') of the three genotypes resistant to leaf scald, but not observed in susceptible genotypes ('Fortune', 'Laetitia', 'Simka'). Cedrol occurs naturally in several species of conifers, such as cypress (*Cupressus sempervirens*) and cedar (*Cedrus atlantica*, *Juniperus virginiana*) (Burdock 2004). Some studies indicate that cedrol presents insect repellency action. Yatagai et al. (2002) observed the control of the pest bark borer (*Cryptomeria japonica*) by this compound. Sabine (1975) verified that cedarwood oil (cedrene and cedrol) disrupted the reproductive and developmental cycle of peanut trash bug (*Elasmolomus sordidus*), the indian meal moth (*Plodia interpunctella*) and the forage mite (*Tyrophagus putrescentiae*). Similar observation has been reported for antimicrobial activity, controlling bacteria (*Flavobacterium*, *E. coli*, *Pseudomonas fluorescens*, *Bacillus subtilis* and *Saccharomyces cerevisiae*) (Tao et al. 2018) and plant pathogens, such as *Monilinia fructicola* (Liu et al. 2018). Kleina et al. (2018) verified that fruit removed from plants with leaf scald showed reduced size, greater weight loss, lower firmness and higher incidence of brown rot (*M. fructicola*) when compared with healthy plants, which lead us to believe that cedrol can act as a natural fungicide in these new plum genotypes.

(*E,E*)- α -farnesene, a sesquiterpene reported as a bioactive volatile released from apple (Huelin and Murray 1966), was detected in high concentrations in the susceptible genotype 'Fortune', but was not found in 'SC13' and 'SC15'. This compound was related to be attractive to host plant selection by adults of codling moth (*Cydia pommonella*) and to be able to stimulate oviposition of gravid females (Sutherland 1972; Sutherland and Hutchins 1972). Based on this assertion, we can hypothesized that the greater amount of this compound in the susceptible

cultivars could influence the host selection dynamics, attracting a greater number of leafhoppers in these plants, besides raising the oviposition and reproduction rate of these insects.

In conclusion, we believe that the release of specific volatile compounds by these new genotypes is a way of activating the defense mechanisms inherent to the host, conferring the resistance to leaf scald. Future research that tests the repellent action of these compounds on the vectors must be carried out. Studies involving possible modifications in the volatile profile of infected plums with *X. fastidiosa* should be conducted, since the presence of the bacterium can alter the compounds produced by the host. In the case of cucumber mosaic virus (CMV) infection in tobacco plants, it was observed that the pathogen induced quantitative and qualitative changes in the volatiles emission, altering the produced blend (Tungadi et al., 2017). The authors of this work indicate that the change in the volatile profile depends in part on the activity of the 2b counter-defense protein, which causes a larger number of aphids (*Myzus persicae*) to be attracted to these plants.

Another approach would be the identification of epicuticular waxes present in the leaf mesophyll of these new genotypes that could hinder the stylet penetration by the leafhoppers. Preliminary studies using scanning microscopy showed that the epicuticular layer of ‘SC7’ and ‘SC13’ contained depressions and a denser waxy layer, which may be an indicative of the existence of a physical barrier to transmission (Kleina et al. 2019, unpublished data).

ACKNOWLEDGEMENT

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001. The last author received research fellowships from National Council for Scientific and Technological Development (CNPq)/Brazil.

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SUPPLEMENTARY MATERIAL

Table 3 - Unidentified volatile compounds quantified in plum leaves samples of six genotypes with different levels of resistance to leaf scald disease (*Xylella fastidiosa*) by GC-MS (Unit: relative area %).

ID	RT	RI	Compound	Fortune		Laetitia		Simka		SC7		SC13		SC15	
				Mean	CV (%)	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)
0006	4.4590	906.4	Compound 001	0.11 ^{ns}	22.76	0.18 ^{ns}	34.75	ND ^{ns}	-	0.05 ^{ns}	76.31	0.17 ^{ns}	48.53	0.11 ^{ns}	38.49
0009	5.3580	942.7	Compound 038	0.23 ^{ns}	27.12	0.39 ^{ns}	61.83	0.27 ^{ns}	57.71	0.17 ^{ns}	33.47	0.44 ^{ns}	56.74	0.13 ^{ns}	113.25
0011	5.8070	952.5	Compound 055*	0.2	23.83	0.33	56.35	0.17	69.39	0.13	22.37	ND	-	ND	-
0015	7.2240	1013.4	Compound 051*	0.04	22.55	0.08	27.01	ND	-	ND	-	0.05	72.66	0.02	56.79
0020	9.4030	1078.4	Compound 002*	0.05	26.31	ND	-	ND	-	ND	-	ND	-	ND	-
0023	10.3545	1105.5	Compound 003*	0.11	28.55	ND	-	ND	-	ND	-	0.02	63.36	ND	-
0032	16.4125	1254.1	Compound 004*	0.16	28.06	ND	-	0.27	19.92	0.13	71.76	0.12	111.48	0.42	156.82
0034	18.2360	1298.9	Compound 005*	0.34	21.97	ND	-	ND	-	ND	-	0.11	29.44	ND	-
0036	18.5595	1306.7	Compound 006	0.64 ^A	22.96	ND ^B	-	0.27 ^{AB}	76.38	0.32 ^{AB}	101.67	0.45 ^{AB}	10.19	0.17 ^{AB}	83.46
0038	18.9175	1315.3	Compound 007	0.95 ^A	21.89	ND ^B	-	0.38 ^{AB}	74.91	0.39 ^{AB}	98.46	0.64 ^A	6	0.43 ^{AB}	39.64
0039	18.9710	1316.6	Compound 052*	ND	-	ND	-	ND	-	ND	-	ND	-	0.04	79.95
0040	18.9795	1316.8	Compound 008*	0.34	37.26	ND	-	ND	-	ND	-	0.12	29.41	0.04	173.21
0041	19.2305	1322.8	Compound 009	0.65 ^{ns}	24.32	ND ^{ns}	-	0.32 ^{ns}	56.15	0.38 ^{ns}	68.68	0.45 ^{ns}	13.73	0.28 ^{ns}	64.89
0043	19.7995	1336.5	Compound 011*	0.63	38.52	ND	-	ND	-	ND	-	ND	-	0.11	71.34
0045	21.8500	1385.9	Compound 013*	0.19	51.5	ND	-	ND	-	ND	-	ND	-	ND	-
0047	21.9905	1389.3	Compound 014	3.93 ^A	38.98	1.36 ^B	87.39	1.50 ^B	32.7	0.71 ^B	32.52	1.15 ^B	33.11	0.80 ^B	42.55
0050	22.9925	1413.9	Compound 016*	0.15	37.52	ND	-	0.13	33.5	ND	-	ND	-	ND	-
0051	23.1020	1416.6	Compound 017*	ND	-	ND	-	0.25	30.99	ND	-	0.15	53.94	ND	-
0052	23.1495	1417.8	Compound 018*	1.08	37.21	ND	-	ND	-	0.08	55.76	ND	-	0.1	72.54
0053	23.7905	1433.8	Compound 058*	ND	-	ND	-	ND	-	0.02	17.13	ND	-	ND	-
0054	23.9125	1436.8	Compound 049*	ND	-	ND	-	ND	-	ND	-	0.27	54.45	0.1	82.15
0055	24.2560	1445.4	Compound 042*	ND	-	ND	-	ND	-	ND	-	ND	-	0.12	50.45
0056	24.4490	1450.2	Compound 019	0.35 ^A	50.91	0.07 ^B	94.17	0.10 ^B	24.8	0.33 ^{AB}	19.74	0.09 ^B	47.51	ND ^B	-

0057	24.8880	1461.1	Compound 020*	0.18	47.22	ND	-	ND	-	ND	-	ND	-	ND	-
0058	25.1710	1468.1	Compound 059*	0.09	31.06	ND	-	ND	-	ND	-	ND	-	0.1	54.67
0059	25.3450	1472.5	Compound 043*	ND	-	ND	78.86	0.21	67.11	0.06	48.91	0.18	104.62		
0060	25.6570	1480.2	Compound 044*	ND	-	1.1	88.74	1.78	57.32	ND	-	3.29	ND	-	-
0061	25.7095	1481.6	Compound 021*	1.6	72.04	1.98	45.69	ND	-	1.55	68.59	ND	-	5.87	78.41
0066	27.0375	1515.3	Compound 022	6.06 ^{ns}	69.13	7.16 ^{ns}	57.69	8.68 ^{ns}	27.31	10.92 ^{ns}	8.3	9.73 ^{ns}	37.32	10.51 ^{ns}	39.66
0067	27.3705	1524	Compound 054	0.59 ^A	23.59	0.31 ^{AB}	26.05	0.32 ^{AB}	18.17	ND ^B	-	0.55 ^{AB}	38.65	0.33 ^{AB}	31.38
0068	27.7795	1534.6	Compound 023	0.42 ^{AB}	25.07	0.34 ^{AB}	23.98	0.28 ^{AB}	40.61	0.54 ^A	10.05	0.34 ^{AB}	12.76	0.26 ^B	59.12
0069	28.4185	1551.3	Compound 024*	ND	-	ND	-	ND	-	0.45	26.75	ND	-	ND	-
0070	29.2810	1573.7	Compound 025*	0.5	55.13	ND	-	0.61	22.11	ND	-	ND	-	ND	-
0072	30.2445	1598.8	Compound 040	0.20 ^A	39.6	ND ^B	-	0.06 ^B	39.71	0.11 ^{AB}	42.36	0.07 ^B	60.44	0.12 ^{AB}	24.24
0079	32.2700	1655.3	Compound 045*	ND	-	ND	-	ND	-	ND	-	ND	-	0.11	43.44
0080	32.3895	1658.7	Compound 028*	ND	-	ND	-	ND	-	ND	-	0.14	32.48	ND	-
0081	32.7245	1668	Compound 029*	0.25	36	ND	-	ND	-	ND	-	ND	-	ND	-
0082	33.4545	1688.4	Compound 046*	0.36	22.9	ND	-	ND	-	ND	-	ND	-	0.2	47.55
0084	35.3520	1741.4	Compound 030	0.37 ^{ns}	21.88	0.23 ^{ns}	50.25	0.18 ^{ns}	45.48	0.38 ^{ns}	2.16	0.28 ^{ns}	59	0.21 ^{ns}	38.14
0085	36.3625	1769.6	Compound 031	0.18 ^{ns}	103.42	0.28 ^{ns}	24.03	0.23 ^{ns}	37.62	1.28 ^{ns}	95.85	0.59 ^{ns}	47.17	0.4 ^{ns}	57.32
0089	39.0315	1848.6	Compound 041*	0.36	28.81	ND	-	0.21	25.41	0.79	33.17	ND	-	0.31	68.22
0091	39.8295	1873.1	Compound 032	0.32 ^{AB}	22.14	0.14 ^B	24.03	0.26 ^{AB}	36.62	0.35 ^{AB}	46.03	0.42 ^A	14.68	0.28 ^{AB}	40.47
0092	40.2420	1885.8	Compound 048*	ND	-	ND	-	ND	-	ND	-	ND	-	0.38	48.93
0098	44.0895	2004.3	Compound 034*	ND	-	0.27	33.65	ND	-	0.25	8.84	0.51	74.14	ND	-
0103	48.1180	2142.1	Compound 063*	0.71	61.96	ND	-	ND	-	ND	-	ND	-	ND	-
0104	53.8450	2360	Compound 056*	0.15	22.86	ND	-	ND	-	0.2	57.36	ND	-	ND	-
0105	54.7140	2393	Compound 064*	ND	-	ND	-	ND	-	ND	-	ND	-	0.69	72.5
0106	63.8175	2739.4	Compound 036	3.83 ^{ns}	37.95	3.49 ^{ns}	54.9	5.12 ^{ns}	72.1	5.24 ^{ns}	34.7	7.07 ^{ns}	63.97	6.81 ^{ns}	32.14
0107	64.8810	2779.9	Compound 050*	ND	-	ND	-	ND	-	ND	-	ND	-	13.12	114.54
0108	65.8585	2817.1	Compound 037*	0.74	43.89	ND	-	ND	-	4.99	134.97	1.84	46.98	ND	-

Means followed by the same letter in the line do not differ according to a Tukey test ($P < 0.05$). The results consist of the mean of three replicates. ^{ns} - not significant.

ID: Identity; RT: Retention time in minutes; RI: Retention index; CV: Coefficient of variation in percentage; ND: Not detected; *Qualitative analysis: presence and absence of the compounds.

CHAPTER IV. MECHANICAL TRANSMISSION OF *Xylella fastidiosa* TO *Prunus salicina* USING PRUNING SHEARS⁴

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Abstract

Plum leaf scald (PLS) is a systemic disease that causes great economic losses to the fruit growers. *Xylella fastidiosa*, causal agent, inhabit the xylem vessels and causes the dieback of plum trees. This bacterium is transmitted by sharpshooter leafhoppers and by propagative techniques, however, none information is available regarding the mechanical transmission by pruning equipment for this pathosystem. Therefore, this study aimed to evaluate the dissemination of *X. fastidiosa* subsp. *multiplex* to *Prunus salicina* by pruning shears. An adult plum tree (Reubennel cultivar) naturally infected by the bacterium *X. fastidiosa* represented the source-plant for the study. Ten non infected plants six-month old of 'Reubennel', 'SC7' and 'SC15' genotypes were taken to the orchard in cages with anti-aphid screen protection. For the transmission trial, one cut was made along the infected symptomatic branch of the source-plant, immediately followed by one cut on green shoots of the test-plant. This protocol was repeated for six times per each test-plant. Plants were placed in the greenhouse with ambient temperature and light. The *X. fastidiosa* detection was performed at 90, 270 and 450 days after pruning using polymerase chain reaction (PCR) and quantitative polymerase chain reaction (*q*PCR). No leaf scald symptoms were observed in the test-plants at 510 days after pruning. A sample of the 'SC7' genotype was detected as positive by *q*PCR 450 days after pruning. The sanitation of pruning tools is recommended, in order to prevent the spread of the disease within the orchards.

Key-words: plum leaf scald, stone fruit, molecular detection.

⁴ Prepared in accordance with the standards of Revista Brasileira de Fruticultura – Scientific Communications

Resumo

A escaldadura das folhas de ameixa (PLS) é uma doença sistêmica que causa grandes prejuízos econômicos aos fruticultores. A *Xylella fastidiosa*, agente causal, habita os vasos do xilema e causa a morte de ameixeiras. Esta bactéria é transmitida por cigarrinhas e por técnicas de propagação vegetativa, entretanto, não há informações disponíveis sobre a transmissão mecânica por equipamentos de poda para este patossistema. Portanto, este estudo teve como objetivo avaliar a transmissão de *X. fastidiosa* subsp. *multiplex* à *Prunus salicina* por tesouras de poda. Uma ameixeira adulta (cultivar Reubennel) naturalmente infectada pela bactéria *X. fastidiosa* representou a planta-fonte do estudo. Dez plantas não infectadas dos genótipos ‘Reubennel’, ‘SC7’ e ‘SC15’ foram levadas até o pomar em gaiolas com proteção anti-afídeos. Para o teste de transmissão, um corte foi feito ao longo de ramos sintomáticos da planta-fonte, imediatamente seguido por um corte nos brotos verdes da planta-teste. Este protocolo foi repetido por seis vezes para cada planta-teste. As plantas foram colocadas na estufa com temperatura e luz ambiente. A detecção da *X. fastidiosa* foi realizada aos 90, 270 e 450 dias após a poda, utilizando reação em cadeia da polimerase (PCR) e a reação em cadeia da polimerase quantitativa (*qPCR*). Nenhum sintoma de escaldadura foi visualizado nas plantas-teste nos 510 dias após a poda. Uma amostra do genótipo ‘SC7’ foi detectada como positiva por *qPCR* 450 dias após a poda. A sanitização dos instrumentos de poda é recomendada, a fim de prevenir a disseminação da doença dentro dos pomares.

Palavras-chave: escaldadura das folhas da ameixeira, frutas de caroço, detecção molecular.

Plum leaf scald (PLS) is the most important disease that occurs in Brazilian Southeast and Southern producing regions, decimating orchards since 1975 (KITAJIMA et al. 1975). PLS is caused by the xylem-restricted bacterium *Xylella fastidiosa* (WELLS et al. 1987), which is a recognized agent of several economically-important systemic diseases (PURCELL and HOPKINS 1996), including Pierce's disease (PD) in grapevines (HEWITT 1958; HOPKINS 1985), Citrus Variegated Chlorosis (CVC) (HE et al. 2000; ROSSETTI et al. 1990) and Olive Quick Decline Syndrome (OQDS) (SAPONARI et al. 2014). In other plant species, like weeds, *X. fastidiosa* causes no apparent symptoms (WISTROM and PURCELL 2005). Due to its phylogenetic diversity, *X. fastidiosa* is divided in five subspecies: *fastidiosa*, *morus*, *multiplex*, *pauca* and *sandyi* (NUNNEY et al. 2014; SCHAAD et al. 2004; SCHUENZEL et al. 2005).

This bacterium invade the water-conducting system of the hosts (DAVIS et al. 1981), leading the appearance of an extensive leaf scorch and dieback of plum trees within a few years from the onset of the symptoms. The disease causes great economic losses to the fruit growers, being a limiting factor for crop expansion in the country (DUCROQUET et al. 2001). Once infected, plants show symptoms only after a long incubation period (PURCELL 1979). However, asymptotically trees serve as a source of inoculum within the orchards, which can aggravate the spread and the disease control.

In the natural environment, *X. fastidiosa* is transmitted to short distances exclusively by infective sharpshooter leafhoppers (Cicadellidae and Cercopidae families) (HOPKINS 1989). The spread to long distances occurs by planting contaminated material and by propagative techniques, such as grafting (ROBERTO et al. 1996; LOPES et al. 1996). Another potential mode of dissemination that is deemed important is the transmission via contaminated pruning equipment. However, few studies have been conducted in this area. Krell et al. (2007) verified the transmission of Pierce's disease pathogen (approximately 5%) by pruning shears in grapevines. For citrus variegated chlorosis, Laranjeira et al. (2004) indicated low transmission probability to neighboring plants, excluding this form of dissemination. For ratoon stunting disease (RSD) was observed that the xylem-limited bacterium *Clavibacter xyli* subsp. *xyli*; sin: *Leifsonia xyli* subsp. *xyli* is readily transmitted through cutting equipment in sugarcane (DAMANN 1992).

Nevertheless, for PLS no information is available regarding this mode of dissemination. Cultural management practices adopted by the plum producers along the cycle, lead to the hypothesis that this bacterium could also be spread by pruning equipment. This idea becomes even more consistent, since spatial-temporal analysis showed that leaf scald symptoms appeared in trees along the same planting row in São Paulo (FERREIRA et al., 2016) and Paraná

orchards (FERREIRA, 2016). Another study involving spatial patterns of *X. fastidiosa* within vineyards showed that the disease often occurs in consecutive grapevines located in the same row (PARK et al. 2006), which could be a result of the transmission by pruning equipment.

Currently, there is no known cure for this deadly disease, since this pathosystem does not have an applied control proposal. In view of this, greater knowledge about the disease epidemiology and dissemination could guide producers to adopted more efficient management techniques. Therefore, this study aimed to evaluate the dissemination of *X. fastidiosa* subsp. *multiplex* to *Prunus salicina* by pruning shears.

In August 2016, thirty-six dormant nursery plum trees were obtained from the Agriculture Research and Rural Extension of Santa Catarina (EPAGRI) (Videira, SC, Brazil). The cuttings of 'Reubennel', 'SC7' and 'SC15' genotypes were grafted on 'A9' rootstocks and planted individually in plastic pots (Nutriplan® 17 cm x 21 cm; 4.9 L, Cascavel, PR, Brazil) containing the substrate mix composed of blasted pine bark (Mecplant® HF, Telêmaco Borba, PR, Brazil). 'Reubennel' is a cultivar moderately susceptible to PLS and widely cultivated in Southern Brazil (DALBÓ and FELDERG 2009). 'SC7' and 'SC15' are new plum genotypes which are not infected under field conditions, although, the transmission by grafting (xylem vessels connected) was already reported (DALBÓ et al. 2018).

Plants were grown in an isolated greenhouse with anti-aphid screen protection at the Federal University of Paraná (Curitiba, PR, Brazil) under ambient temperature and light. Plants were diary manually irrigated and the fertilized as recommended for the crop (SBCS/NEPAR, 2017). Due to the high incidence of mites (Acari: Tetranychidae) inside the greenhouse, the plants were sprayed with acaricide/insecticide Abamectin Nortox® (Nortox S/A, Arapongas, PR, Brazil) for control this pest. These potted plums served as test-plants for the study.

An adult plum tree (Reubennel cultivar) located at a commercial orchard in the municipality of Araucaria (Paraná, Brazil) and naturally infected by the bacterium *X. fastidiosa* represented the source-plant for the study. All plants (included the source-plant) were tested by polymerase chain reaction (PCR) before the onset of the assay to confirm the presence or absence of *X. fastidiosa*.

The transmission trial by pruning shears was performed on January, 2017. Ten non-infected six-month old plants of each genotype ('Reubennel', 'SC7' and 'SC15') were taken to the field in cages with anti-aphid screen protection in order to avoid the contact with leaf scald vectors. A 103 bypass pruning shear (Okatsune, Hiroshima, JP) was disinfected with 70% alcohol for 1 minute, sodium hypochlorite (1%) for 1 minute and washed in distilled water for 1 minute before the beginning of the experiment and among genotypes. One cut was made along

the infected symptomatic branch (source-plant), immediately followed by one cut on green shoots of the test-plant. This protocol was repeated for six times per each test-plant, totaling six cuts. The cutting sites were marked with colored ribbon to facilitate the identification of the budding point.

Plants were placed in the aforementioned greenhouse and conditions. Two noninfected plants of each genotype were placed in the greenhouse to serve as control plants. The *X. fastidiosa* detection was performed at 90, 270 and 450 days after pruning using two different molecular techniques (polymerase chain reaction (PCR) and quantitative polymerase chain reaction (*qPCR*)).

The leaves sprouted at each cut-off point were collected, forming a composed sample per plant. For PCR test, DNA was extracted from the petioles per the protocol based on the methodology adopted by Murray and Thompson (1980). The extracted DNA samples were amplified using a pair of specific primers, RST31 and RST33 (MINSAVAGE et al. 1994). The amplification was performed in a PTC-100 thermal cycler (Research Inc., Watertown, MA, USA), programmed with the following conditions: one initial cycle of 94 °C for 5 minutes; 32 denaturation cycles at 94 °C for 40 s; annealing at 50 °C for 40 s; an extension at 72 °C for 59 s followed by one stabilization cycle at 72 °C, indefinitely. Polymerase chain reaction (PCR) products were separated by agarose gel electrophoresis (1.5%) with a Tris/borate/EDTA (TBE) buffer (89 mM Tris, 89 mM boric acid, and 2 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0). The amplified fragments were visualized under ultraviolet light, and documented using the Eagle Eye II system (Stratagene, LaJolla, CA, USA).

For *qPCR* test, DNA was extracted from the petioles following a protocol based on the methodology adopted by Murray and Thompson (1980). The extracted DNA samples were submitted to quantitative polymerase chain reaction (*qPCR*), using the SYBR methodology. The standard amplification protocol was performed using a set of primers (XF16Sf and XF16Sr) described by Li et al. (2013), following the specific conditions: 95 °C for 10 minutes; 40 denaturation cycles at 95 °C for 15 s; and annealing at 62 °C for 45 s. Each reaction had a positive (source plant DNA) and negative control (Milli-Q water). A standard curve was constructed for the quantification of the bacteria in the samples (FRANCIS et al. 2006). The samples that presented Ct values (cycle threshold) equal to or less than 28 were considered positive. All samples were evaluated in duplicate.

The results showed that no leaf scorch symptoms were visualized in the test-plants until 510 days after pruning. The leaf samples analyzed by conventional and quantitative polymerase chain reaction were negative to *Xylella fastidiosa* in the first (90 days after pruning)

and the second (270 days after pruning) evaluations. However, one sample of 'SC7' genotype was detected as positive by *q*PCR in the third evaluation (450 days after pruning; cycle threshold ≤ 28), but was not detected by conventional PCR test (Table 1).

It is known that the *X. fastidiosa* transmission can occur through the dissemination of infected propagative material, grafting techniques (HE et al. 2000) and through the action of sucking insects (Cicadellidae and Cercopidae families) (LOPES 1996). However, this work has been proven that this bacterium is disseminated by pruning shears, being the first report of this dispersion form for this pathosystem. Even with a low rate of transmissibility (4%), contaminated pruning instruments were able to disseminate the disease in healthy seedlings, but without the appearance of leaf scald symptoms until 510 d.a.p. For citrus variegated chlorosis, Laranjeira et al. (2004) found a tendency of randomness in most evaluations, indicating low transmission probability to neighboring plants, which limits the possibility of human interference as a dispersing agent. On the other hand, Krell et al. (2007) observed 5% of *X. fastidiosa* transmission efficiency in grapevines using pruning shears. Unlike citrus, the management practices adopted for temperate fruit trees required a greater number of interventions during the productive cycle. For plum trees, at least four types of pruning (cleaning, summer, production and winter) are carried out annually, since three of them occur when there is an intense sap flow, which would potentiate the successful transmission by this method.

Although *q*PCR is considered the most sensitive molecular test used to confirm the presence of *Xylella fastidiosa* in plant tissues, its accuracy is affected by the period of sampling and irregular pathogen population distribution inside the plant (mainly at the early infection stages) (ZARCO-TEJADA et al. 2018), which can lead to false negative results. In this work, the pathogen detection was only possible after a long incubation period (approximately 450 days). 'SC7' was more sensitive to drought (more number of dead plants), when compared to the other genotypes. Water stress increased the onset of symptoms and, consequently, the bacterial concentration in the xylem vessels (MCELDRONE et al. 2001; HABBERMANN et al. 2003). For this reason, we believe that this feature may have facilitated the detection of *X. fastidiosa* in these plants. In view of this, the monitoring for a longer period of these plants will be continued, in order to give greater reliability to the generated data. Likewise, we intend to repeat this experiment using at least two detection methodologies.

Nevertheless, in consequence of these first results, the sanitation of pruning instruments using 70% alcohol, sodium hypochlorite and quaternary ammonia is recommended, as a way of prevent the disease dissemination within the orchards.

Table 1. Ct values (cycle threshold) for plum leaves samples from seedlings pruned after cut in infected tree with plum leaf scald.

Samples	Cycle Threshold (<i>Ct values</i>)					
	90 d.a.p		270 d.a.p		450 d.a.p	
	1	2	1	2	1	2
Reubennel Control 1	33.55	33.52	31.95	31.72	31.74	-
Reubennel Control 2	32.39	32.38	32.44	32.91	31.82	-
Reubennel 1	32.49	32.24	36.75	-	30.25	32.00
Reubennel 2	33.52	33.80	DP	DP	DP	DP
Reubennel 3	32.10	32.32	29.99	30.03	30.11	30.52
Reubennel 4	32.42	32.62	31.60	30.83	32.53	31.77
Reubennel 5	33.05	33.11	30.23	31.17	30.84	30.43
Reubennel 6	31.95	31.72	32.51	32.41	31.25	32.06
Reubennel 7	32.44	32.91	29.69	30.52	32.72	32.15
Reubennel 8	32.86	32.78	30.41	29.38	31.59	30.84
Reubennel 9	32.89	32.28	30.15	30.91	28.79	31.05
Reubennel 10	36.75	36.57	30.61	30.07	32.11	32.89
SC15 Control 1	31.60	30.83	30.39	30.36	30.42	-
SC15 Control 2	32.51	32.41	30.21	30.06	31.02	-
SC15 1	30.15	30.36	29.81	30.04	32.43	34.16
SC15 2	31.49	31.64	30.30	30.44	31.84	32.91
SC15 3	31.85	31.75	30.27	30.57	31.97	31.96
SC15 4	32.76	32.64	30.23	29.94	31.75	31.43
SC15 5	32.15	32.21	30.44	30.84	33.65	32.95
SC15 6	32.01	32.01	30.27	30.77	34.71	32.01
SC15 7	30.82	30.62	29.74	29.56	32.42	31.37
SC15 8	32.90	32.88	29.94	29.83	32.54	32.20
SC15 9	33.75	33.45	29.25	29.05	31.20	30.59
SC15 10	30.06	30.12	30.98	29.94	32.18	31.11
SC7 Control 1	31.25	31.35	DP	DP	DP	DP
SC7 Control 2	32.44	32.25	31.17	31.84	32.21	32.31
SC7 1	32.43	32.45	29.98	29.94	31.75	32.78
SC7 2	30.14	30.22	30.17	30.84	DP	DP

SC7 3	29.50	29.55	29.91	29.17	25.18	25.29
SC7 4	DP	DP	DP	DP	DP	DP
SC7 5	33.44	33.24	30.30	30.64	30.57	31.15
SC7 6	31.56	31.55	31.57	31.52	31.47	31.53
SC7 7	32.11	32.36	29.76	29.62	30.59	30.10
SC7 8	30.35	30.15	29.49	29.81	29.97	30.59
SC7 9	31.35	31.54	30.76	30.57	DP	DP
SC7 10	30.11	30.46	DP	DP	DP	DP
Positive control (SP)	21.56	21.45	19.50	19.73	22.02	22.11
Negative control	36.85	36.86	32.88	32.88	34.12	33.14

^ad.a.p: days after pruning.

^bDP: dead plant.

^cSP: source plant

^d - : Not assessed.

Acknowledgement

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001. The last author received research fellowships from National Council for Scientific and Technological Development (CNPq)/Brazil.

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GENERAL CONCLUSIONS

Fruit removed from plants with leaf scald showed reduced size, greater weight loss, lower firmness and higher incidence of brown rot when compared with healthy plants. In addition, colour attributes gradually decreased during the postharvest period in fruit removed from plants infected with *X. fastidiosa*. PME and PAL enzyme activity, respiration, and ethylene production was higher in plums from trees contaminated by PLS than in fruit from disease-free plants.

We have demonstrated the existence of variations in settling and feeding behavior by the vectors in plum genotypes with different levels of field resistance to leaf scald disease. These findings on vector-plant relationships contribute to our understanding of the mechanisms possibly involved in the field resistance of genotypes 'SC7' and 'SC13'.

Thirty-nine compounds were identified from the six plum genotypes by GC-MS/MS. The major constituents in the essential oil were phytol, butylated hydroxytoluene, limonene, (*E,E*)- α farnesene, β -pinene and linalool. Cedrol was not detected in susceptible genotypes, however was found in 'SC7' and 'SC15'. (*E,E*)- α farnesene was verified in high amount in 'Fortune'. Genotypes could be divided in four groups based on the resemblance of the volatile profile: 1: 'Fortune' and Laetitia'; 2: 'Simka' and 'SC13'; 3: 'SC7'; and 4: 'SC15'. We believe that the release of specific volatile compounds by these new genotypes is a way of activating the defense mechanisms inherent to the host, conferring the resistance to leaf scald.

In conclusion, we discovered that plum leaf scald disease (*X. fastidiosa*) was transmitted by pruning equipment. 'SC7' was positive for *X. fastidiosa* 450 days after pruning. The sanitation of pruning instruments is recommended for prevent the disease dissemination within the orchards.

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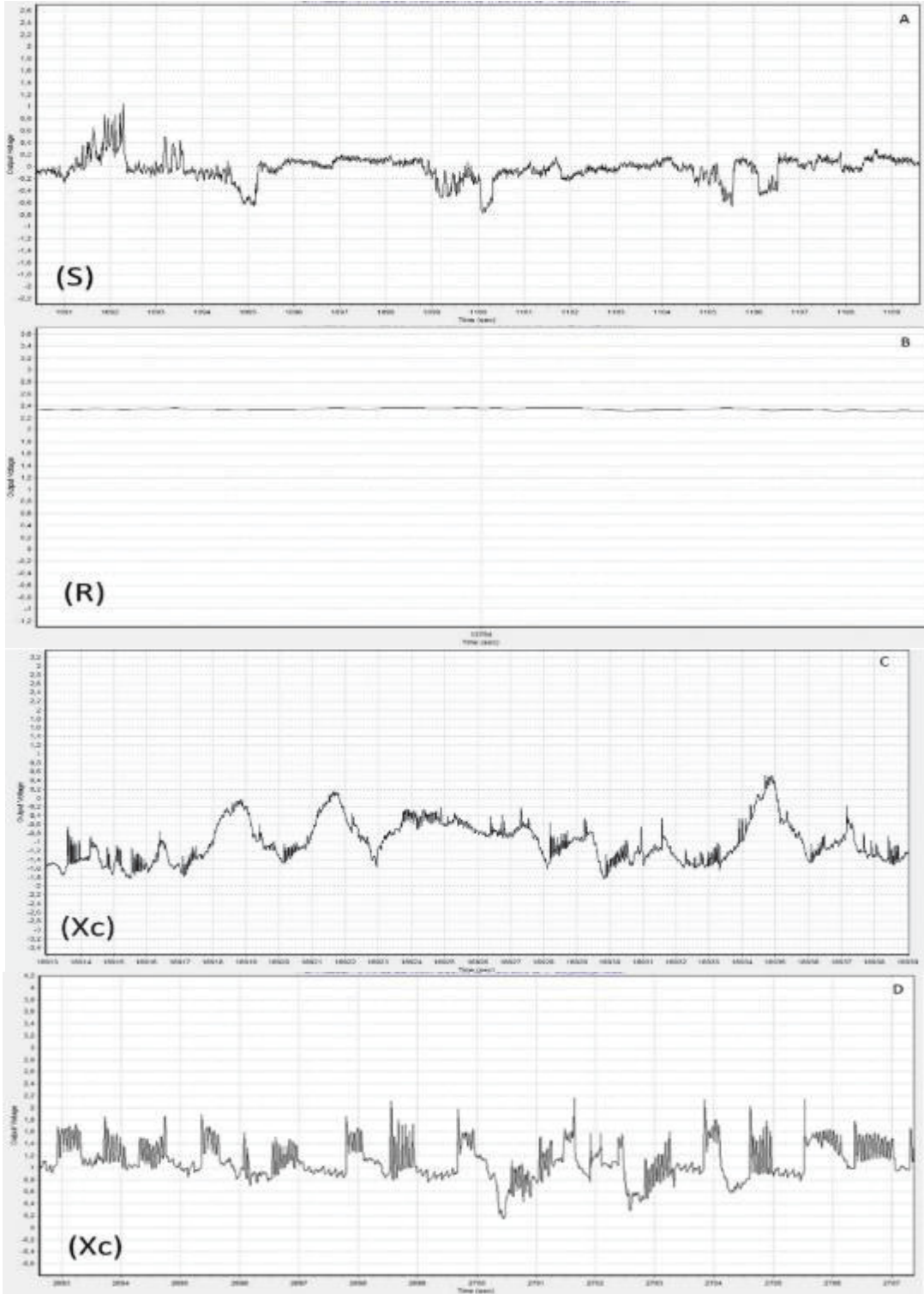
APPENDICES



Figure 1 – A) Chlorosis and necrosis on the leaf edges in infected plum trees by plum leaf scald disease (*Xylella fastidiosa*). B) Fruit removed from infected trees (smaller diameter) with plum leaf scald compared with fruit removed from healthy trees (larger diameter).



Figure 2 – Plum tree with leaf scald (*Xylella fastidiosa*) symptoms in orchard located at the municipality of Araucaria (Paraná, Brazil).



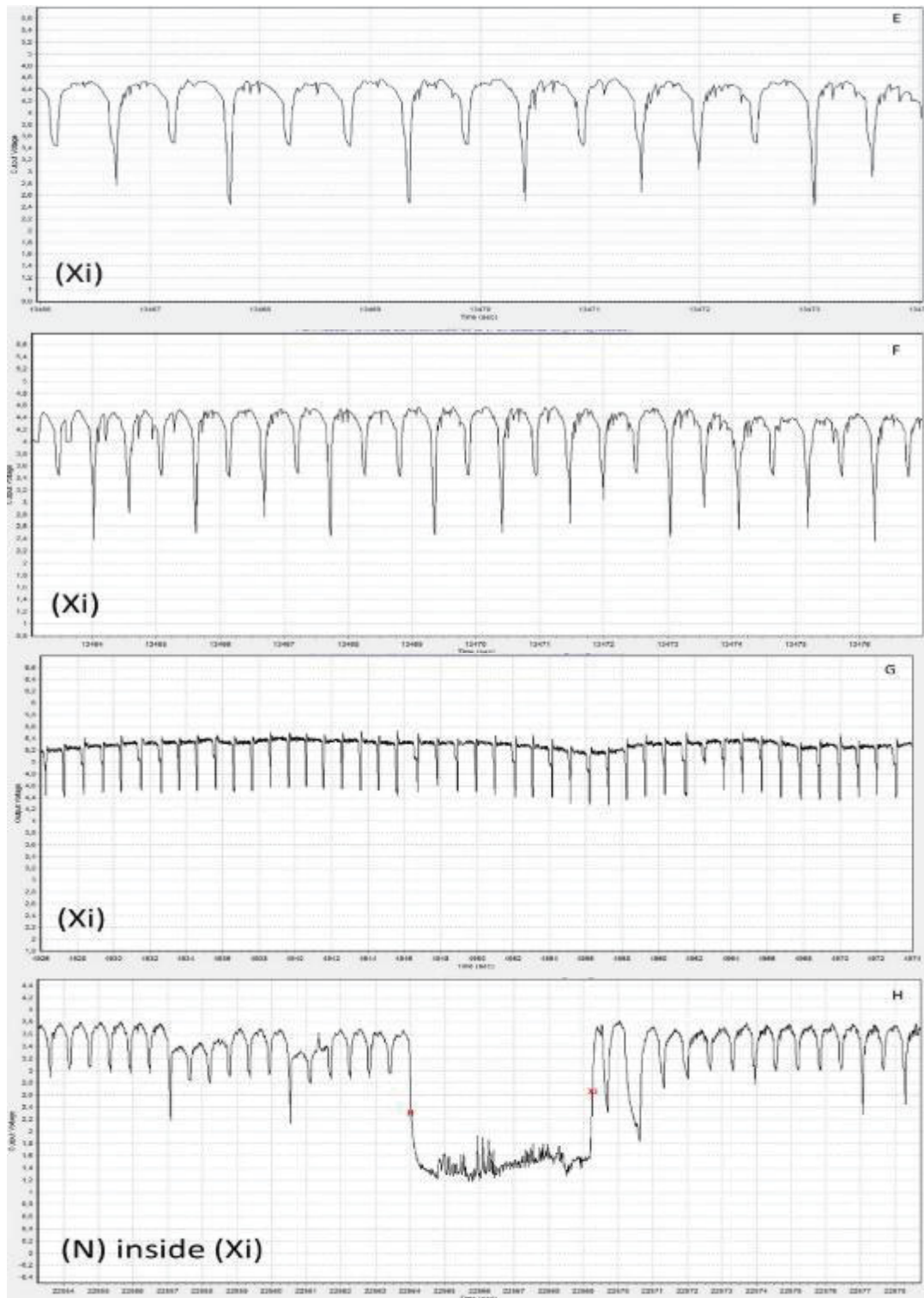


Figure 3 – Epg records and respective waveforms: S, pathway through epidermis and parenchyma (including both stylet penetration and withdrawal); R, resting during xylem activity; N, interruption of sustained ingestion during xylem phase; Xc, xylem contact/pre-ingestion; and Xi, active intake of xylem sap.