

Volatiles emitted by resistance inducer-treated grapevines affect *Hyalesthes obsoletus* behavioural responses

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Abstract

The planthopper *Hyalesthes obsoletus* Signoret (Hemiptera Cixiidae) is the main vector of 'Candidatus Phytoplasma solani', that cause the grapevine Bois noir disease. Amongst the alternatives to synthetic pesticides, many studies have investigated the use of resistance inducers to control plant pathogens and phytophagous insects. In this study, the planthopper behavioural responses to volatiles emitted by grapevine shoots treated with three commercial chemical elicitors were investigated in a dual-choice olfactometer. Each formulation was applied at one of three different times, as 0, 2, and 7 days before the bioassays. Insect behavioural responses toward grapevine volatiles differed significantly across formulations and application times. In particular, *H. obsoletus* were significantly ($\chi^2 = 7.258$, $df = 1$, $P = 0.005$) repelled by volatiles emitted by grapevines sprayed with a benzothiadiazole-based formulation 7 days before the bioassays. The two other formulations were based on oligosaccharides and glutathione and gave contrasting results. The planthoppers were significantly ($\chi^2 = 3.571$, $df = 1$, $P = 0.044$) attracted to volatiles emitted by grapevines treated with one of these formulations on the same day of bioassays, but not subsequently. This suggested a response to the product rather than an elicited response from the plants, so we conducted headspace analysis on samples of the three products to identify differences in volatile constituents for future experimentation. The remaining formulation had no effect at any of the post-treatment intervals. Our results indicate that the benzothiadiazole-based formulation may contribute to a novel sustainable integrated management strategy for the control of *H. obsoletus*, the main vector of the Bois noir phytoplasma.

Key words: phytoplasma vector, Bois noir, elicitors, Y-olfactometer, integrated pest management.

Introduction

Bois noir (BN) is a grapevine (*Vitis vinifera* L.) disease that can represent a limiting factor for grapevine production in Europe and the Middle East (Maixner, 2011; Zahavi *et al.*, 2013). It is associated to the pathogen 'Candidatus Phytoplasma solani' (16SrXII-A subgroup) (Quaglino *et al.*, 2013). Characteristic symptoms of this disease include chlorosis and downward rolling of leaves, stunting of the shoots, and shriveling of berries (Belli *et al.*, 2010).

The main vector of 'Ca. Phytoplasma solani' is *Hyalesthes obsoletus* Signoret (Hemiptera Cixiidae), a phloem-feeding planthopper present in southern and central European vineyards (Maixner *et al.*, 1994; Sforza and Boudon-Padiou, 1998). Nymphal stages are linked to the root systems of their host plants where they feed and overwinter (Sforza *et al.*, 1999). Planthopper adults are the aerial stage involved in phytoplasma transmission through their feeding activity (Bressan *et al.*, 2007). *H. obsoletus* males show greater flight activity than females, suggesting that they play a key role in the diffusion of the phytoplasma (Bressan *et al.*, 2007; Mazzoni *et al.*, 2010; Minuz *et al.*, 2013). This planthopper feeds on many different wild and cultivated plants that can also host the phytoplasma (Sharon *et al.*, 2005; Riolo *et al.*, 2007; 2012; Murolo *et al.*, 2010; Landi *et al.*, 2013; Kosovac *et al.*, 2015; Landi *et al.*, 2015) and act as reservoirs for the disease.

For BN, the application of roguing to symptomatic plants does not contribute to any reduction in its epidemiology, because the infected grapevines are seldom if ever a source of infection (Osler *et al.*, 1993). The use

of synthetic insecticides and herbicides have been suggested for the management of this disease, through the control of the insect vectors on the wild vegetation within the vineyard or on the vineyard borders (Maixner, 2010; Mori *et al.*, 2015), or through the control of alternative host plants. However, the life history of *H. obsoletus*, its relationship to wild host plants, and its casual feeding behaviour on grapevines impede the efficient control of this vector (Maixner, 2010). In addition, widespread pesticide applications can have severe impacts on non-target invertebrates (Gill and Garg, 2014). Alternative and sustainable integrated pest management strategies are thus strongly needed.

Among the alternatives to synthetic pesticides, many studies have investigated the use of plant resistance inducers to control plant pathogens and phytophagous insects (Vallad and Goodman, 2004). One approach involves chemical analogs (elicitors) to plant signals that are applied directly to the plant tissues. Elicitors include chemicals that mimic the actions of salicylic acid (e.g., benzothiadiazole), and compounds that can be released from plant or microbial cell walls (e.g., oligosaccharides, glutathione) (Wingate *et al.*, 1988; Hadwiger, 1999; Mou *et al.*, 2003; Lyon, 2014). Systemic acquired resistance is a well-known mechanism of induced plant defence. In most of the cases, systemic acquired resistance is associated with an increase in the activity of the salicylic acid pathway that plays a role in the expression of genes that code for pathogenesis-related proteins (Pieterse *et al.*, 2014; Landi *et al.*, 2017).

Induced responses cause phenotypic plasticity that can affect the three-way interactions between plants, herbivorous insects and pathogens (Dicke and Hilker,

2003). However, the literature contains contradictory data on the effects of chemical elicitors on herbivorous insects. In the majority of cases, the salicylic acid pathway associated with systemic acquired resistance has shown only minimal effects on herbivorous insects, although detrimental effects (e.g., on survival, development, reproduction, feeding) after elicitor applications have been reported also for some phloem-feeding insects such as leafhoppers, aphids, and whiteflies (Bi *et al.*, 1997; Inbar *et al.*, 2001; Moran and Thompson, 2001; Cooper *et al.*, 2004; Bressan and Purcell, 2005; Boughton *et al.*, 2006; Civolani *et al.*, 2010). Furthermore, elicitor applications can reduce leafhopper phytoplasma transmission (Bressan and Purcell, 2005; Miliorios *et al.*, 2017).

In addition to increase the direct plant defences, elicitor applications may also affect the emission of plant volatile compounds (Dicke and Hilker, 2003; Sobhy *et al.*, 2014). Volatiles provide important cues for insect 'evaluation' of their host plants (e.g., differentiation between host and non-host plants, indicator of host-plant status) (Gripenberg *et al.*, 2010; Bruce and Pickett, 2011; Riolo *et al.*, 2012; Ruschioni *et al.*, 2015; Gross, 2016; Germinara *et al.*, 2017; Riolo *et al.*, 2017).

Romanazzi *et al.* (2013) evaluated the activities of some elicitors for the induction of recovery of BN-infected grapevines, and they reported encouraging data for the use of benzothiadiazole and two different formulations that contained glutathione plus oligosaccharides. Therefore, the aim of the present study was to analyse the behavioural responses of *H. obsoletus* males to volatiles emitted by grapevine that had been treated with a commercial formulation containing benzothiadiazole or with one of two distinct formulations containing oligosaccharides plus glutathione.

Materials and methods

Planthoppers

Adult planthoppers were sampled on nettle (*Urtica dioica* L., Urticaceae) plants in fallow farmlands of the Ancona province (Marche region, central Italy) using a modified leafblower (THB-2510, Tanaka Kogyo Co., Japan). Then, the planthoppers were housed into collapsible insect rearing cages (BugDorm-1, MegaView Science Co., Taiwan) and provided with nettle shoots. One day prior to the start of the bioassays, the males were placed in cleaned cages and provided with a mixed sucrose-sorbitol solution. The planthoppers were maintained at 26 ± 1 °C, $60 \pm 10\%$ relative humidity, and 16 hours photophase.

Plants and treatments

The chemical elicitors were applied in a commercial vineyard of 'Chardonnay' grapevines in the Ancona province. The commercial products used were: a formulation containing benzothiadiazole (Bion®, 50 WG; 50% active ingredient; Syngenta Crop Protection, Switzerland), and two different formulations containing glutathione plus oligosaccharides (Kendal®; 3% active ingredients; Valagro, Italy [GO1]; Olivis® B2; 3% active

ingredients; Agrisystem, Italy [GO2]). Each formulation was diluted with deionized water at the highest registered rates (0.2 g/L for Bion®, 4 mL/L for Kendal® and Olivis®), according to the manufacturer instructions. These were used at 1,000 L/ha for application as foliar treatments to the canopy of the grapevine plants to runoff, using a motorized pump backpack liquid sprayer (GX 25; Honda, Tokyo, Japan) (Romanazzi *et al.*, 2013).

Each diluted formulation (i.e., Bion®, Kendal® and Olivis®) was applied at one of three different times, as 0, 2, and 7 days before the bioassays, for a total of nine treatments ($n = 4$ plants for each treatment). Control plants were sprayed with deionized water (1,000 L/ha) up to the point of the runoff, at the same treatment dates ($n = 12$ plants).

Terminal shoots (approximately 30 cm long with three leaves) were tested immediately after being collected from the control and treated grapevine plants. The cut stems were wrapped in cotton-wool soaked with distilled water, putted into a 6-mL vial and sealed with Teflon tape.

Y-tube olfactometer bioassays

The responses of planthoppers to volatiles emitted by the shoots treated with water (control) and the chemical elicitors (Bion®, Kendal® and Olivis®) were studied in a glass Y-tube (stem length, 25 cm; arm length, 20 cm; arm angle, 75°; internal diameter, 4 cm). Each arm of the Y-tube was connected to a glass chamber (9 × 18 cm). Control and treated shoots were then placed in one of the holding chambers. The airflow (flow rate 1.0 L/min⁻¹) passed through activated charcoal and distilled water before entering the holding chambers and the arms. The Y-tube was positioned with a slope of 10° from the horizontal plane. One insect at a time was observed until it had moved 13 cm up one of the olfactometer arms, or for a total of 5 minutes.

The treatments were randomly assigned at the beginning of each bioassay, and changed after every four planthoppers. To avoid any location effects and environmental factors the Y-tube was cleaned with acetone (Sigma-Aldrich, Italy) and reversed after every four insects. The trials were carried out at the same time of day (between 12:00 and 17:00), and environmental conditions (of 26 ± 1 °C and a relative humidity of $60 \pm 10\%$) (Riolo *et al.*, 2012). The number of planthoppers tested was 32 for each treatment (total $n = 288$ specimens).

Headspace analysis of formulations containing chemical elicitors

In order to understand the basis for the attraction of *H. obsoletus* to plants treated with Olivis® immediately before the bioassays (see results), we looked at the volatile constituents of each of the formulations, since at day 0 the leafhopper response was presumably triggered directly by the formulation rather than by elicited compounds, which would take longer to be synthesised by the plant. The volatile constituents were collected from pure samples of each plant resistance elicitor formulation using headspace solid-phase microextraction, with divinylbenzene/ carboxen/ polydimethylsiloxane

(DVB/CAR/PDMS) 50/30 μm fibres (Supelco, Bellefonte, PA, USA). The analysis of the volatile constituents was by gas chromatography (3900 GC; Varian Analytical Instruments, Walnut Creek, CA, USA) coupled to an ion trap mass detector (Saturn 2100T; Varian Analytical Instruments). The gas chromatography was run with a fused silica capillary column (ZB-5; length, 30 m; internal diameter, 0.25 mm; film thickness, 0.25 μm ; Phenomenex, Torrance, CA, USA). The injector was operated in splitless mode for 0.1 min at a constant temperature of 220 °C. The oven temperature was held at 40 °C for 5 minutes, then increased to 140 °C at a rate of 4.0 °C/min, then increased again to 220 °C at a rate of 40 °C/min, with a final hold time of 10 minutes. The carrier gas (He) was set to constant flow mode at 1.0 mL/min, the transfer line was set at 220 °C, and the ion trap was set at 220 °C. Both electronic impact fragmentation (70 eV) and chemical ionization (reagent gas: methanol) were carried out. The full scan mass spectrometry data were acquired in the mass range of 31 amu to 250 amu. Identification of the chromatographic peaks was by (i) careful interpretation of the mass spectra and chromatographic behaviour, (ii) comparisons with Kovats retention indexes and mass fragmentation patterns of pure analytical standards, and (iii) comparisons with data published in the National Institute of Standards and Technology/ Environmental Protection Agency/ National Institutes of Health (NIST/EPA/NIH) Mass Spectral Library Version 2.0a, build July 1, 2002.

Data analysis

Olfactory responses of planthoppers were analysed using Chi-squared tests with Yates corrections (H_0 : the planthoppers choose the arms of the olfactometer in a 1:1 ratio) (Systat Software Inc.). Differences among treatment formulations and application times were tested by contingency table analysis based on Chi-square (Zar 1999). The significance level of the statistical tests was set at $P < 0.05$. Planthoppers that did not respond (i.e. insects stayed below 13 cm of the Y-tube arm for the whole 5-min period) were excluded from the statistical analysis.

Results

Y-tube olfactometer bioassays

The planthopper behavioural responses toward the grapevine shoot volatiles differed significantly among the elicitor formulations and application times. In particular, *H. obsoletus* males were significantly ($\chi^2 = 7.258$, $df = 1$, $P = 0.005$) repelled by volatiles emitted by the Bion®-treated grapevine shoots when the formulation was applied 7 days before the bioassays, compared to the control (figure 1). Plants sprayed with Bion® 7 days before the bioassays was also significantly ($\chi^2 = 7.459$, $df = 2$, $P = 0.024$) repellent for the planthoppers when compared to plants sprayed with Bion® both 2 days before and on the same day as the bioassays. Moreover, among all of the formulations sprayed 7 days before the

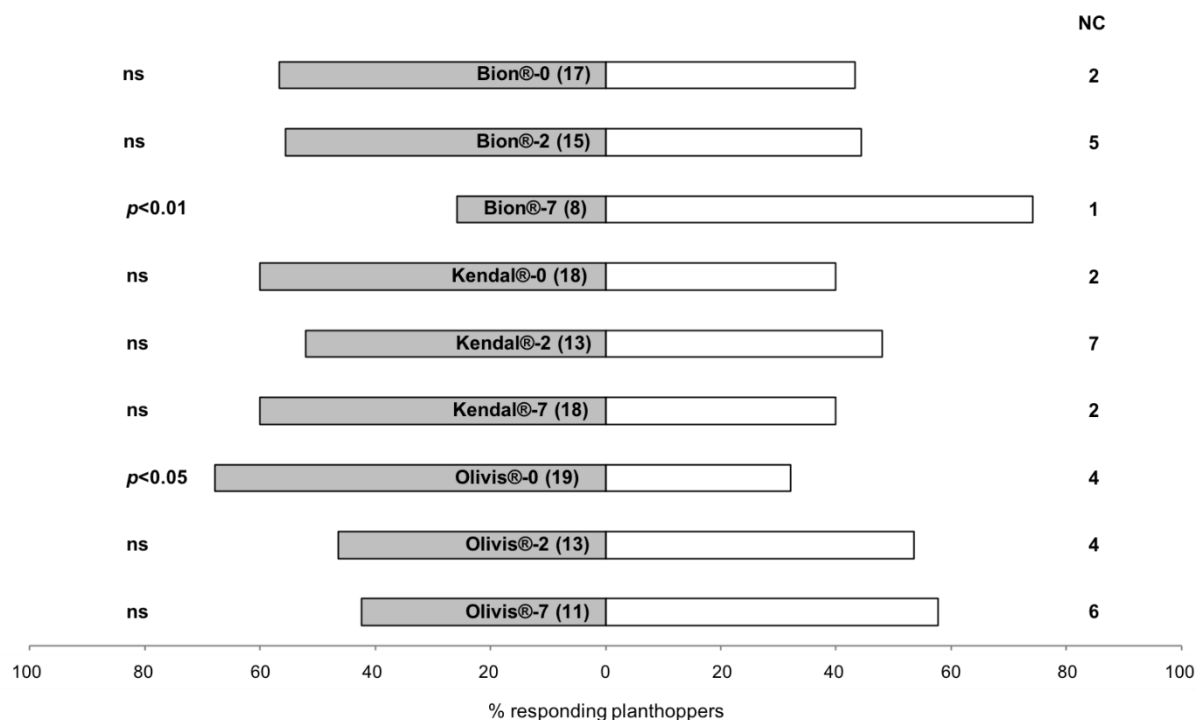


Figure 1. Behavioural responses of *H. obsoletus* to volatiles emitted by grapevine shoots sprayed with commercial formulations containing benzothiadiazole (Bion®) or one of two different formulations containing glutathione plus oligosaccharides (Kendal® and Olivis®), applied at 0, 2, and 7 days before bioassays (as indicated). Numbers in brackets indicate number of planthoppers that made a choice. Significance levels of treatment *versus* control are indicated left (Chi-square analysis: ns, nonsignificant). NC, number of planthoppers that did not make a choice.

Table 1. Volatile compounds identified in the headspace of commercial formulations containing benzothiadiazole (Bion®) or one of two different formulations containing glutathione plus oligosaccharides (Kendal® and Olivis®).

RT ^(a)	MS Base peak (m/z)	ID ^(b)	Compound name	Peak Area (×104)		
				Bion®	Kendal®	Olivis®
4.6	94 [M] ⁺	MS, RI	dimethyl disulphide	9459	tr	6398
6.9	43 [M – COOH] ⁺	MS, RI, CI	2-methyl-propanoic acid		839	1858
7.1	60 [M – CH ₃ C] ⁺	MS, CI	butanoic acid		736	1152
10.4	60 [M – CH ₃ CCH ₃] ⁺	MS, CI	3-methyl-butanoic acid		2670	1443
10.8	74 [M – CH ₃ C] ⁺	MS, RI, CI	2-methyl-butanoic acid		1897	2987
11.3	107 [M – 1] ⁺	MS, RI	dimethyl-pyrazine		tr	3514
11.6	107 [M – 1] ⁺	MS, RI	dimethyl-pyrazine			1841
13.5	126 [M] ⁺	MS, RI	dimethyl trisulphide	1518	tr	3173
14.9	121 [M – 1] ⁺	MS, RI	ethyl-methyl-pyrazine			953
15.2	122 [M] ⁺	MS, RI	trimethyl-pyrazine			1914
18.2	135 [M – 1] ⁺	MS, RI	diethyl-pyrazine			246
18.5	136 [M] ⁺	MS	tetramethyl-pyrazine		tr	408
21.3	149 [M – 1] ⁺	MS, RI	ethyl-trimethyl- / diethyl-methyl-pyrazine			5
23.2	158 [M] ⁺	MS	dimethyl tetrasulphide	39		172

^(a) RT = retention time (min). ^(b) ID = identification method: MS, identification by comparison with mass spectra stored in NIST library; RI, identification by comparison with tabulated Kovats Retention Indexes; CI, identification by interpretation of chemical ionization experiments. tr < 1 × 10.

bioassays, Bion®-treated plants were significantly ($\chi^2 = 7.294$, df = 2, $P = 0.026$) repellent. The planthoppers were also significantly ($\chi^2 = 3.571$, df = 1, $P = 0.044$) attracted to volatiles emitted by grapevine shoots treated with one of the two commercial formulations containing glutathione plus oligosaccharides (Olivis®) on the same day as the bioassays (figure 1).

Headspace analyses of formulations containing chemical elicitors

The headspace analysis of Olivis® formulation was characterized by dimethyl polysulphides (table 1). Mass spectra of these compounds showed an intense molecular ion peak (base peak) and a fragment ion at [M-15]⁺, due to loss of methyl groups, which was gradually less visible as the number of sulphur atoms increased. The sulphur atoms were identified by their characteristic isotopic ratio [M]⁺/[M+2]⁺. This analysis identified dimethyl disulphide ([M]⁺ = 94), trisulphide ([M]⁺ = 126), and tetrasulphide ([M]⁺ = 158) at retention times under the chromatographic conditions adopted of 4.6, 13.5, and 23.2 min, respectively. Moreover, seven alkyipyrazines were identified. Their electronic impact fragmentation mass spectra showed a strong base peak at [M-1]⁺ (in some cases at [M]⁺) and no significant fragmentation. Short-chain carboxylic acids were also identified. The two fronting peaks at retention times of around 10 minutes were identified as 3-methyl-butanoic acid (iso-valeric acid) and 2-methyl-butanoic acid. Their electronic impact fragmentation mass spectra showed the base peak at m/z 60 for 3-methyl-butanoic acid (due to loss of CH₃CCH₃), with no ions at m/z 74, and the base peak at m/z 74 for 2-methyl-butanoic acid (due to loss of CH₃C), with no fragments at m/z 74. In both of these cases, chemical ionization produced ions at m/z 103 [M+1]⁺, 85 [M+1 – (H₂O)]⁺, and 117 [M-15]⁺. The same ion types were observed in the chemical ionization spectra of the two fronting peaks at

retention times of around 7 minutes, at m/z 89 [M+1]⁺, 71 [M+1 – (H₂O)]⁺, and 103 [M-15]⁺. These were identified as butanoic acid and 2-methyl-propanoic acid (iso-butanoic acid). Comparisons of the electronic impact fragmentation patterns with the NIST library confirmed these identifications. In contrast, the headspace analysis of the Kendal® formulation (table 1) did not show similar levels of dimethyl polysulphides (with only traces of disulphide, trisulphide) and alkyipyrazines (with only traces of dimethylpyrazines, tetramethylpyrazines). The short-chain carboxylic acids 3-methyl-butanoic, 2-methyl-butanoic, butanoic, and 2-methyl-propanoic were the main constituents of the Kendal® volatile fraction. For the headspace analysis of the Bion® formulation, neither alkyipyrazines nor volatile fatty acids were identified, while the dimethyl polysulphides were again identified (disulphide, trisulphide, small amount of tetrasulphide) (table 1). The Bion® volatile fraction was characterized by compounds with retention times of 6.54, 11.56, and 30.05 minutes, where their mass spectra indicated that they contained a nitrogen atom and one or two sulphur atoms. Their fragmentation patterns suggested substituted benzothiazole-like structures. There were also small levels of aromatics identified, including toluene, naphthalene, ethyl benzoate, and methoxy phenols.

Discussion and conclusions

This is the first study to evaluate the responses of the phloem-feeding planthopper *H. obsoletus* to volatiles emitted by resistance inducer-treated grapevines. The planthopper behavioural responses toward the grapevine shoot volatiles differed significantly among the elicitor formulations and application times. In particular, *H. obsoletus* were significantly repelled by volatiles emitted by the benzothiadiazole-treated grapevine shoots when

the treatment was applied 1 week before the bioassays. Here, an indirect mechanism of action can be suggested, as manipulation of salicylic acid responses might influence the planthopper behaviour due to changes in the emission of the grapevine volatiles. Benzothiadiazole has been described as an exogenous elicitor of plant defence responses, and a set period of time is needed for acquired resistance establishment (Vallad and Goodman, 2004; Landi *et al.*, 2017).

The systemic response induced by benzothiadiazole on phloem-feeding insects would be expected to differ among plant and insect species (Maleck and Dietrich, 1999). Repeated applications of benzothiadiazole to grafted grapevine cuttings have been shown to reduce phytoplasma transmission by the leafhopper *Scaphoideus titanus* Ball (Miliordos *et al.*, 2017). Similarly, Bressan and Purcell (2005) reported that application of benzothiadiazole to arabidopsis [*Arabidopsis thaliana* (L.)] 7 days before phytoplasma transmission bioassays reduced the survival of *Colladonus montanus* (Van Duzee), with inhibition of feeding of this leafhopper vector, and reduced phytoplasma transmission efficiency. In contrast, D'Amelio *et al.* (2010) reported that application of benzothiadiazole to the painted daisy [*Chrysanthemum carinatum* (Schousboe)] 7 days before the bioassays did not reduce the transmission efficiency of phytoplasma by the leafhopper *Macrosteles quadripunctulatus* Kirschbaum. Tomato (*Lycopersicon esculentum* Miller) treated with benzothiadiazole 4 days before the probing behaviour assays for the aphid *Myzus persicae* (Sulzer) reduced probing behaviour and reduced the total duration and number of phloem ingestions (Civolani *et al.*, 2010). Benzothiadiazole applications 1-2 days before cohort establishment reduced nymph development and reproduction by *M. persicae* on both arabidopsis and tomato, and by the aphid *Macrosiphum euphorbiae* Thomas on tomato (Moran and Thompson, 2001; Cooper *et al.*, 2004; Boughton *et al.*, 2006). Finally, treatment of cotton (*Gossypium hirsutum* L.) with benzothiadiazole 14 days before trials had no effects on the whitefly *Bemisia tabaci* (Gennadius) host-plant preference, although the benzothiadiazole-treated older leaves showed a reduction in egg density (Inbar *et al.*, 2001).

Unexpectedly, in the present study, *H. obsoletus* was attracted by the grapevine shoots treated on the same day as the bioassay with one of the two commercial formulations containing glutathione plus oligosaccharides, Olivis®. In contrast, the grapevine shoots treated with the other formulation, Kendal®, did not elicit any behavioural responses in *H. obsoletus*. Here, it would appear that volatile compounds present inside the Olivis® formulation attracted the planthoppers. Different polysulphides and alkylpyrazines were detected in the headspace volatiles from the Olivis® formulation that were absent from the Kendal® formulation. Moreover, alkylpyrazines were not found in the headspace volatiles from Bion® formulation. Alkylpyrazines have been identified as volatile organic compounds emitted by microorganisms. Intraspecific, interspecific and interkingdom chemical communication can be mediated by microbial volatile compounds (Ryu *et al.*, 2003; Effmert

et al., 2012; Rybakova *et al.*, 2016). Recently, it was reported that the bacterium *Serratia marcescens* associated to the ant *Atta sexdens rubropilosa* Forel is able to produce pyrazines formerly identified as trail pheromones of this ant (Silvia-Junior *et al.*, 2018) and alarm pheromones of other ant species (Wheeler and Blum, 1973; Morgan *et al.*, 2006; Showalter *et al.*, 2010). What the role of the compounds present inside the Olivis® formulation is in the attraction of *H. obsoletus* toward treated grapevine shoots remains unknown. Additional studies are necessary to identify which compounds within Olivis® are responsible for the attraction of *H. obsoletus*, what compounds are emitted by grapevines when treated with elicitor formulations, and to define the roles of these compounds as olfactory cues using electrophysiological and behavioural studies.

These findings provide evidences that grapevine foliar applications of particular commercial formulations containing chemical elicitors affect the olfactory responses of the main planthopper vector of Bois noir phytoplasma. These preliminary results indicate that the benzothiadiazole-based formulation may have the potential to contribute to a novel sustainable integrated management strategies for the control of *H. obsoletus*, the main vector of the Bois noir phytoplasma.

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