VOLATILE ORGANIC COMPOUNDS: A ROLE IN ELICITOR-INDUCED RESISTANCE OF GRAPEVINE AGAINST PATHOGENS?

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Abstract

As Vitis vinifera varieties are susceptible to fungal diseases, numerous chemical treatments are generally required to ensure the quantity and quality of the harvest. However, in the context of sustainable viticulture, there are increasing societal request, political incitation, and winegrowers' awareness to reduce the use of pesticides. Among possible solutions the use of elicitors that could be included in integrated pest management or biocontrol strategies might be very promising. These bioactive compounds are able to trigger plant defences, leading to induced resistance (IR) against pathogens. Despite IR can be elicited very successfully in controlled environments; it is in most cases not effective enough in practically controlling disease in the vineyard. To obtain a comprehensive understanding of IR and to identify molecular markers enabling the identification of factors (physiological, environmental…) that can impact IR efficacy in the vineyard we performed a transcriptomic analysis under controlled conditions. The results indicated that among highly up-regulated genes associated to IR, one was annotated as terpene synthase, suggesting that terpenes could be emitted following elicitor treatment. This prompted us to investigate whether IR elicitors actually induce the production of volatile organic compounds (VOCs). Applying online analysis (PTR-QMS) of VOC emissions in dynamic cuvettes and passive sampling in gas tight bags with solid phase micro extraction (SPME / GC-MS), we followed the emission of VOCs of vines in response to elicitor-IR against downy mildew under controlled greenhouse conditions. The results obtained point out some of them as potential markers of elicitor-IR (as trans *a-farnesene) whereas MeSA is rather a marker of downy mildew infection.*

Keywords: grapevine, elicitor, induced resistance, downy mildew, VOCs

1 INTRODUCTION

Most of the commercially cultivated *Vitis vinifera* cultivars are susceptible to fungal diseases such as downy mildew caused by *Plasmopara viticola*. In consequence these impacts cause important qualitative and quantitative losses and huge amounts of fungicides are generally required to supress disease symptoms and to ensure a satisfactory yield and the quality of the harvest. Despite fungicides are generally effective, some of them are potentially harmful for the environment and human health, and also important contribute to the selection of resistant pathogen strains. Nowadays, in an objective of sustainable viticulture, there is increasing social request, political incitation, and winegrower wishes to develop alternative or integrated strategies of plant protection

allowing a reduced use of pesticides. Among strategies already developed or investigated are organic farming, biodynamics, and resistant hybrids. Another way is the induction of grapevine resistance to disease by the use of molecules able to stimulate its natural defences. Such molecules are called elicitors (Ebel and Cosio, 1994). Elicitor perception by the plant induces a signalling cascade involving ion fluxes, H_2O_2 and NO production, Mitogen Activated Protein Kinase activation (Garcia-Brügger *et al.* 2006), leading to the activation of the expression of defence genes encoding PR proteins, enzymes involved in phytoalexin production, and cell-wall strengthening. Phytohormones, especially salicylic acid, jasmonic acid, ethylene, and abscisic acid are also involved in defence signalling and their role depends on plant/pathogen interactions (Bari and Jones, 2009). Elicitors therefore lead to induced resistance (IR) of plants against a set of pathogens. Despite IR is successful under controlled conditions, in most cases it is not effective enough in the vineyard (Adrian et al, 2012). Previous transcriptomic analyses initiated to better understand the IR response of vines indicated that one of the most up-regulated gene in elicitor-treated leaves was annotated as terpene synthase (Daire, unpublished), suggesting that volatile organic compounds (VOC) could be emitted following elicitation. VOCs are secondary metabolites including terpenes emitted from above and belowground plant tissues and they have long and short distance effects on different interacting organisms (Dudareva et al. 2006). The role of VOCs in plant / herbivore interactions has been well established (Dicke and Baldwin 2010; Junker et al. 2010; Raguso 2008). They are also active on microorganisms (Kalemba and Kunicka 2003; Tripathi et al. 2011) and seem to play a role in structuring plant-microbe interactions (Del Giudice et al. 2008; Huang et al. 2012; Junker et al. 2011; Karamanoli et al. 2005, Junker and Tholl, 2013). Overall plants VOCs clearly emerge as the most important factor allowing communication and interaction between plants, microbes, animals, neighbouring plants and the environment in all terrestrial agro-ecosystems (Dicke et al. 2014). This prompted us to study the elicitor-induced VOC production in grapevine and to check whether induced VOCs could be good candidates to monitor elicitor-IR in *V. vinifera* under field conditions.

2 MATERIALS AND METHODS

Plant material

Grapevine (*Vitis vinifera* L. cv. Marselan) herbaceous cuttings were cultivated as previously indicated (Gamm et al., 2011) under greenhouse conditions at 23 ± 4 and 18 ± 7 °C (day and night, respectively) until they developed 6-8 leaves (ca 7 weeks).

Elicitor treatment

Sulfated laminarin (PS3, provided by Goëmar laboratories, France) was prepared at a concentration of 2.5 mg ml^{-1} in distilled water and added or not with 0.05% adjuvant (Adrian, personnel communication). The elicitor solution was applied to both the upper and lower sides of leaves using a hand-held sprayer. Plants were maintained in the greenhouse in the conditions described below for Gas Chromatography Mass Spectrometry (GC-MS) analysis or inserted into dynamic cuvettes for online analysis of VOC emission by Proton Transfer Reaction Mass Spectroscopy (PTR-QMS) as previously described (Ghirardo et al., 2012).

Plant inoculation

Plasmopara viticola (Berk. & Curt. ex de Bary) Berl. & de Toni inoculation was performed 1 or 2 days post-elicitor treatment (dpt) using a sporangia suspension at a concentration of 10^4 sporangia ml^{-1} as previously described (Trouvelot et al. 2008). Plants were placed at 90-100% relative humidity at 6 days post inoculation (dpi), to provoke sporulation.

VOC analysis

For online analysis of VOC emissions with proton-transfer-reaction quadrupole mass spectrometry (PTR-QMS; Hansel et al. 1995) 7-week-old plants were subjected to 4 conditions: untreated (control) and mock-inoculated, treated by the surfactant alone and *P. viticola* inoculated at 1 day post-treatment (dpt), treated by the elicitor PS3 [2.5 g l^{-1} plus 0.02 % (v/v) surfactant] and mock-inoculated, treated by PS3 and *P. viticola* inoculated 1 dpt. The aboveground parts of the plants were enclosed in the cuvette system (two similar cuvettes) described in Ghirardo *et al.* (2012). Always two randomly selected treatments where analyzed in parallel. Plants were kept under a day-night regime of 16/8 h (day/night) and a CO_2 concentration of 400 ppb. Details of VOC analysis, instrument parameters and VOC quantification are given in Ghirardo *et al*. (2012). VOCs were collected dynamically during 4 dpt switching between the 2 cuvettes every 15 minutes. Each treatment was analyzed with 3 biological replicates.

For GC-MS analysis, plants were sprayed by water (control) or PS3 (2,5 g.l-1 without surfactant), and *P. viticola*-inoculated. Each treatment included 3 biological replicates and the same plants were used from the beginning to the end of an experiment. VOCs were collected at 1 day before treatment, 6 hours post treatment (hpt), 2 dpt, and 2 dpi (4dpt) by the non-destructive static headspace-sampling mode using fibers coated with DVB/CAR/PDMS 50/30 µm (Supelco, Bellefonte PA). For this, plants were enclosed in commercial oven plastic bags (50 x 25 cm, Albal, France) at 11 h a.m. After 1 hour of equilibration, SPME (Solid Phase Micro Extraction) fibers were inserted into the bag and left for 3 hours. Analysis were performed using an Agilent 6890 GC system coupled with a mass spectrometer (Agilent MSD 5973) (Agilent, Palo Alto CA). SPME fibers were desorbed in the GC injector held at 270℃. Compounds were separated using a DB-5MS column (Agilent J&W), (30 m x 0.25mm i.d., film thickness 1.0 µm). The GC oven was programmed as follow: 40°C held for 2 min, then increase at 6°C/min to 80°C held for 3 min, at 3.4°C/min to 170°C and 12°C/min to 300°C. Helium was used as carrier gas. The mass spectrometer was used in positive electron impact mode (EI) with ionization energy of 70eV at 230°C with a quadruple at 150°C and a transfer line at 280°C. The MS detector was set in full scan mode in the mass range 29-300 *m/z*. Each VOC detected was identified according to the retention time, the retention index and the mass spectrum and by calculation and comparison of the GC retention index of a series of alkanes (C8-C30) with retention index from published data calculated under the same conditions. A set of VOCs provided by INRA aroma library was injected as standards and their retention times were compared to those of SPME samples. The total emission of each compound was calculated and express as cumulative histograms of peak areas (in arbitrary units) normalized to the leaf area. Three mass spectra databases were used for comparisons: NIST08 library, INRA mass library and Wiley 138 database.

Statistical analyses

Student t-test and correlation coefficient were calculated using Past software (University of Oslo).

3 RESULTS AND DISCUSSION

The VOC emissions, especially of methyl salicylate (MeSA; m/z^+ 153) and the sums of monoterpenes (m/z^+ 137) and sesquipterpenes (m/z^+ 205) were first analyzed dynamically during the day course by PTR-QMS. Plants were subjected to 4 conditions: untreated (control) and mock-inoculated, treated by the surfactant alone and *P. viticola* inoculated at 1 day post-treatment (dpt), treated by the elicitor PS3 [2.5 g l^{-1} plus 0.02 % (v/v) surfactant] and mock inoculated, treated by PS3 and *P. viticola* inoculated 1 dpt. The oligosaccharide PS3 was chosen as elicitor as it is efficient against downy mildew in controlled conditions (Trouvelot *et al*., 2008). Surfactant was added as we have shown (unpublished results) that it facilitates the cuticular penetration of PS3 and therefore the level of induced resistance. For MeSA (m/z^+ 153), a first peak of emission appeared at 1 dpt in all treatments containing the surfactant, irrespectively the other compounds, followed by a progressive decline of emission in the next 2

days. However, the emission MeSA emission started to increase again specifically in *P. viticola*-inoculated samples at 3 dpi (4dpt) not yet reading is maximum during the time of analysis. Even when the measurements where stopped before MeSA reached its maximal emission rates, the induction indicates that MeSA might be used as a non-invasive signature of downy mildew (or even more general other fungal infections) infection in grapevine leaves. Grapevines are known for their monoterpene emission (Lund and Bohlmann, 2006 ; Swiegers et al. 2005). Since monoterpene emissions from leaves with no storage pools (Ghirardo *et al.* 2010) are closely related to photosynthesis, the overall emission of monoterpenes (m/z^+ 137) showed the typical day / night rhythm. In general monoterpene emission rates increased during the time course of the experiment for all samples. Nevertheless the emission rate slightly more increased in PS3 treated samples (both inoculated and non-inoculated) at 4 dpt than in the surfactant control-treated plants. Also the emission of sesquiterpenes (m/z^+ 205) followed a diurnal course though the differences in emission rates between surfactant and PS3-treated samples became more prominent at 4dpt.

As the PTR-QMS analysis of the grapevine emissions were restricted regarding the number of plants and the duration of the experiment, additional experiments were conducted with plants enclosed in static bags and passive sampling of VOCs using SPME with subsequent GS-MS analysis to analyze the emission pattern of mono- and sesquiterpenes and confirm the initial induction pattern of MeSA and volatile terpenes. As we have observed that the presence of surfactant in the spraying-solution induces VOCs irrespectively of other constituents, we now applied the PS3 elicitor alone to stimulate IR. In this set of experiment leaves were treated with water or PS3 and *P. viticola* inoculated at 2 dpt. The GC analysis revealed the presence of 49 compounds, from which 19 could be identified as monoterpenes, 26 as sesquiterpenes, 1 sequiterpene alcohol, 1 green leaf volatile (breakdown product of unsaturated lipid oxidation), MeSA and methyl jasmonate (MeJA). After 6 hours post treatment (6 hpt), the VOC emissions were still unchanged compared to the initial situation. 24 h later we observed that the emission of 17 terpenes out of the 45 we overall detected was already in PS3-treated plants compared to the control Overall monoterpene concentrations increased sharply in control leaves at 1 dpt, probably as a response of the spraying and enclosure in bags (e.g. by increase of air temperature, reduction of CO_2, etc…). Then the concentration of monoterpenes went down at 3 dpt followed by a second in increase again at 5dpt. In elicitor-treated leaves, VOC concentrations in the bags increased from 1 to 3 dpt and exceeded that of the control treatment. Among monoterpenes, the concentration of some compounds such as α-pinene remained steady in both conditions. *Trans*-β–ocimene was the most abundant one and its concentration in the bag slightly increased during the experiment despite a high variability. The sesquiterpene concentrations in controls remained steady throughout the experiment, in spite of inoculation, whereas their abundance increased during the time course of the experiment in PS3-treated samples. *Trans*-α-farnesene was the most abundant sesquiterpene detected. This sesquiterpene might be therefore a good candidate marker of grapevine in response to PS3.

Similar to monoterpenes, the concentration of MeSA also had its maximum at 1 dpt and decreased at 1 dpi both in control and PS3 treated leaves, probably as a stress response to spraying treatment and bagging. However, it increased significantly in response to *P. viticola* inoculation as we have also seen by our online analysis using the PTR-QMS. We could therefore consider this compound a marker of infection rather than marker of elicitor-IR. MeJA was detected in low amounts and did not seem to be affected either by PS3 treatment or by infection.

Altogether, our data indicate that PS3 elicits VOC production. Other elicitors from different chemical families should be tested in order to conclude about the non-specificity of the response. The emission of *trans*-α-farnesene might be a good marker for IR but its suitability has to be confirmed both in controlled and field conditions. MeSA could be a good marker of *P. viticola* infection. Once again, it would be interesting to follow MeSA in grapevine

organs (leaves and berries) infected by other biotrophic (*i.e. Erysiphe necator*) or necrotrophic (*i.e. Botrytis cinerea*) pathogens.

4 CONCLUSION

A growing body of evidence shows that VOC are part of direct or indirect plant defense against pathogens. This study suggests that they are involved in vine response to elicitor-induced resistance and in downy mildew infection process. Further studies are required to precise their role.

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