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Research article

The volatile metabolome of grapevine roots: First insights into the metabolic response upon phylloxera attack

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ABSTRACT

Many plant species respond to herbivore attack by an increased formation of volatile organic compounds. In this preliminary study we analysed the volatile metabolome of grapevine roots [Teleki 5C (*Vitis berlandieri* Planch. × *Vitis riparia* Michx.)] with the aim to gain insight into the interaction between phylloxera (*Daktulosphaira vitifoliae* Fitch; Hemiptera: Phylloxeridae) and grapevine roots. In the first part of the study, headspace solid phase microextraction (HS-SPME) coupled to gas chromatography – mass spectrometry (GC–MS) was used to detect and identify volatile metabolites in uninfested and phylloxera-infested root tips of the grapevine rootstock Teleki 5C. Based on the comparison of deconvoluted mass spectra with spectra databases as well as experimentally derived retention indices with literature values, 38 metabolites were identified, which belong to the major classes of plant volatiles including C6-compounds, terpenes (including modified terpenes), aromatic compounds, alcohols and *n*-alkanes. Based on these identified metabolites, changes in root volatiles were investigated and resulted in metabolite profiles caused by phylloxera infestation. Our preliminary data indicate that defence related pathways such as the mevalonate and/or alternative isopentenyl pyrophosphate-, the lipoxygenase- (LOX) as well as the phenylpropanoid pathway are affected in root galls as a response to phylloxera attack.

1. Introduction

Grape phylloxera, *Daktulosphaira vitifoliae* Fitch (Hemiptera: Phylloxeridae), is one of the most devastating grapevine pests worldwide, causing organoid galls (nodosities) on the root tips. After its introduction to Europe in the second part of the 19th century phylloxera caused major economic losses to the wine industry [1]. The spreading of this pest could be prevented by grafting susceptible European grape varieties onto tolerant rootstocks. However, in the last decades the appearance of more aggressive phylloxera biotypes has been reported (e.g., [2,3]). Thus, understanding the interaction between phylloxera and the grapevine root in more detail would be of utmost interest.

The number of studies, which investigated the physiological and molecular response of grapevine to phylloxera root-infestation are sparse and mainly focus on primary metabolites on detached root tissue. So far, increased concentrations of mono- and disaccharides [4,5], starch [4–6] and amino acids [6] and amides [7] have been found to be present in nodosities compared to uninfested root tips. Additionally, recent studies evaluating the metabolic response of grapevine leaves to a phylloxera root infestation, reported a reduction in the ratio of linoleic acid to linolenic acid [8] as well as the chlorophyll content but an increase in xanthophyll-cycle related pigments [9].

Volatile compounds constitute another important class of metabolites known to be involved in the response of many plant species to various types of abiotic (e.g., [10,11]) and biotic stress (e.g., [12,13]), and it is further well known that plant herbivory is associated with an increased formation of volatile metabolites in leaves [14] or roots [15].

Surprisingly, no reports on the involvement of volatile metabolites in the interaction between phylloxera and grapevine have been published so far. While several publications described the detection and identification of volatiles in leaves (e.g., [16,17]) and berries (e.g., [18,19]) of grapevine plants, to the best of our knowledge, there is only a single report on volatile metabolites produced by grapevine roots. Du et al. [20] investigated the volatile metabolites produced by one phylloxera resistant [5BB (*Vitis berlandieri* × *Vitis riparia*)] and one susceptible [Kyoho (*Vitis*

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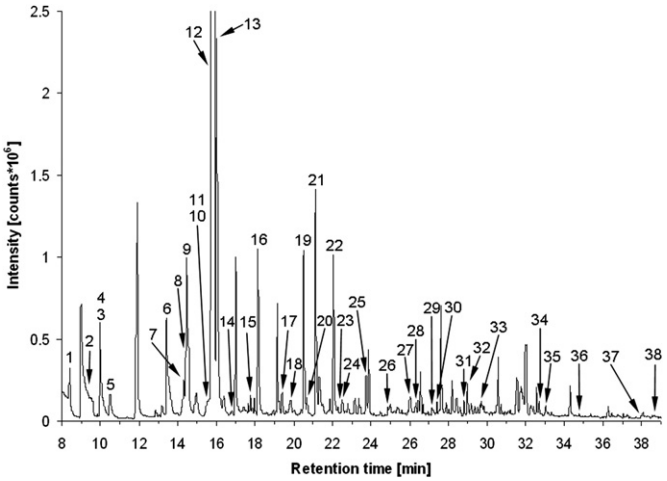

Fig. 1. Typical GC–MS chromatogram obtained after HS-SPME extraction of phylloxera-infested root sample (nodosities). Numbers correspond to substances in Table 1.

vinifera × *Vitis labrusca*)] cultivar and tried to correlate the nymphal preference for the susceptible cultivar Kyoho with the volatile metabolites.

In the present study, the volatile metabolome of grapevine roots of the cultivar Teleki 5C (*V. berlandieri* × *V. riparia*) was investigated by GC–MS. This is the first report on a differential comparison of volatile metabolites of uninfested and phylloxera-infested root tips of grapevine plants.

2. Results and discussion

2.1. Identification of root metabolites

A typical GC–MS total ion current chromatogram obtained after analysis of a phylloxera-infested root tip sample from cultivar Teleki 5C is shown in Fig. 1. This cultivar was chosen due to its phylloxera tolerance [21,22] and its widely use as a model rootstock cultivar in different experiments (e.g., [23,24]).

Manual inspection of the chromatograms resulted in assignment of at least 100 different substances. The automated deconvolution of

Table 1
Volatile substances identified in grapevine root tissue [phylloxera-infested (nodosities) and uninfested]. Numbering of first column corresponds to elution order on DB-5 column, see Fig. 1.

Number in Fig. 1	CAS-Number[a]	Identified substance (trivial name in parentheses)	LTPRI				Formerly described in *Vitis* sp.[e]			
			DB-5MS		Optima-WAX					
			Sample	Reference value	Sample	Reference value	roots	leaves	flowers	berries
1	66-25-1	Hexanal[c]	805	805	1091	1087	x	x	x	x
2	98-01-1	Furan-2-carbaldehyde[c]	842	837	1482	1479				x
3	6728-26-3	(E)-Hex-2-enal[c] (leaf aldehyde)	858	858	1232	1229		x		x
4	928-95-0	(E)-Hex-2-en-1-ol[c]	858	862	1424	1422		x		x
5	111-27-3	Hexan-1-ol[c]	874	873	1370	1370		x		x
6	100-51-7	Benzaldehyde[c]	967	967	1548	1546		x		x
7	123-35-3	beta-Myrcene[c]	994	993	1152	1151				x
8	3777-69-3	2-Pentylfuran[c]	995	994	1227	1223				
9	124-13-0	Octanal[c]	1007	1005	1296	1294				
10	104-76-7	2-Ethylhexan-1-ol[c]	1032	1031	1505	1504				x
11	5981-54-8	Limonene[c]	1034	1032	1192	1191	x		x	x
12	100-51-6	Phenylmethanol[c]	1042	1042	1908	1905		x	x	x
13	122-78-1	2-Phenylacetaldehyde	1048	1047[d]	1668	1648[d]		x		
14	111-87-5	Octan-1-ol[c]	1073	1073	1576	1575			x	x
15	124-19-6	Nonanal[c]	1105	1106	1404	1403	x	x	x	x
16	60-12-8	2-Phenylethanol[c]	1119	1120	1946	1944		x	x	x
17	18 829-56-6	(E)-Non-2-enal	1162	1161[d]	1556	1536[d]		x	x	x
18	124-07-2	Octanoic Acid[c]	1178	1177	2124	2115				
19	119-36-8	Methyl 2-hydroxybenzoate[c] (Methyl salycilate)	1201	1202	1806	1805		x	x	x
20	112-31-2	Decanal[c]	1207	1208	1511	1510	x	x		
21	67-47-0	5-(Hydroxymethyl)furan-2-carbaldehyde[c]	1236	1238	2550	2551		x		
22	106-24-1	(2E)-3,7-dimethylocta-2.6-dien-1-ol[c] (Geraniol)	1257	1258	1870	1869		x	x	x
23	112-05-0	Nonanoic acid[c]	1273	1274	2233	2234		x		
24	141-27-5	(2E)-3,7-dimethylocta-2.6-dienal[c] (Geranial)	1274	1275	1759	1758				x
25	7786-61-0	4-Ethenyl-2-methoxyphenol[c]	1322	1322	2232	2221				x
26	97-53-0	2-Methoxy-4-prop-2-enylphenol[c] (Eugenol)	1364	1365	2202	2195				x
28	87-44-5	beta-Caryophyllene[b,c]	1432	1433	1617	1619	x	x	x	x
29	3796-70-1	(5E)-6,10-Dimethylundeca-5.9-dien-2-one[c] (Geranyl acetone)	1456	1456	1877	1876			x	x
30	6753-98-6	Humulene (alpha-Caryophyllene)	1466	1453[d]	1691	1654[d]			x	
31	39 029-41-9	gamma-Cadinene	1526	1513[d]	1780	1759[d]			x	
32	483-76-1	delta-Cadinene	1533	1524[d]	1773	1747[d]				x
33	143-07-7	Dodecanoic acid[c]	1568	1565	2557	2564		x		
34	629-78-7	Heptadecane[c]	1696	1700	1699	1700	x	x	x	x
35	2765-11-9	Pentadecanal	1716	1714[d]	2050	2041[d]				
36	593-45-3	Octadecane[c]	1797	1800	1800	1800		x		
37	57-10-3	Hexadecanoic acid[c]	1962	1962	2986	2986		x	x	
38	112-95-8	Eicosane[c]	1999	2000	2000	2000		x	x	

[a] Chemical Abstracts Service, SciFinder Scholar 2007.
[b] Only detected in infested samples.
[c] Confirmed with standard.
[d] LTPRI corresponds to the median from NIST Chemistry Webbook.
[e] Substances described in former studies are marked with "x", references see text (2.1).

mass spectra [25] and comparison with MS databases together with evaluation of linear temperature programmed retention index (LTPRI) values [26] on two types of stationary phases led to the positive identification of 38 metabolites (Table 1). Thirty two of these metabolites were additionally confirmed with authentic standards. It shall be noted here that in case of terpenes special caution has to be taken. These substances comprise a very large and diverse class of natural compounds with closely related chemical structures, many of which show similar mass spectra and retention indices. Hence, there is a strong need for the use of authentic standards to avoid false positive identifications.

The identified volatiles comprise numerous chemical substance classes. More precisely, ten aldehydes, nine terpenes five C6-compounds, five alcohols, four acids, three alkanes, one ether and one ester were detected in the investigated samples (Table 1). The chromatograms also contained peaks which have been assigned to dibutyl phthalate and 2,4-di-tert-butylphenol (data not shown). Since these compounds are frequently used as plasticizers and stabilizers of synthetic polymers, they have not been further considered in this study as they represent most likely artefacts.

The majority of the identified substances have already been found with GC–MS in other parts of *Vitis* plants such as leaves (e.g., [12,16,17,27–29]), berries (e.g., [16–18,30–32]) or flowers (e.g., [16,33]). These findings have been summarized in Table 1. To the best of our knowledge, 32 of the metabolites are described for *Vitis* root tissue for the first time, whereas four of those, namely pentylfuran, octanal, octanoic acid and pentadecanal have not been described for *Vitis* spp. at all.

In a recent study Du et al. [20] investigated root volatiles in phylloxera resistant (5BB) and susceptible (Kyoho) rootstock cultivars and detected in total 79 substances. For the resistant 5BB they reported 56 volatile metabolites, whereas 47 volatiles were assigned in total to the cultivar Kyoho. Partly different volatiles were found for the cultivars 5BB and Kyoho with fatty acid methyl esters being the dominating volatile substance classes for both cultivars. In our study, no fatty acid methyl esters were detected. Unfortunately, Du et al. [20] did not describe their extraction method in detail, therefore a direct comparison with our findings is not feasible. Nevertheless, we also found hexanal, limonene, nonanal, decanal, beta-caryophyllene and heptadecane.

2.2. Comparison of volatile profiles obtained for uninfested root tips and nodosities

As presented in Fig. 2 significant differences ($p < 0.05$) in peak areas were found for 14 metabolites. Remarkably, all but one substance (dodecanoic acid) occurred at elevated levels in nodosity samples, whereas beta-caryophyllene was exclusively found in mature nodosity samples. Interestingly, relative standard deviations of peak areas were significantly lower in uninfested root samples compared to mature nodosities (Fig. 2). Furthermore, we detected significant differences for concentration levels of geraniol, eugenol, vanillin and delta-cadinene in roots infested by one 2nd nymphal stage phylloxera (young nodosities) compared to uninfested root tips and elevated levels of phenylmethanol, methyl salicylate, 4-ethenyl-2-methoxyphenol as well as eugenol in mature nodosities compared to young ones (data not shown). Those findings indicate that the metabolic response appears to be highly dynamic and requires closer investigation in the future.

Considering relevant metabolic routes acting on the formation of volatile organic compounds after herbivore attack, we found that at least three different pathways might be affected in phylloxera-infested root tips. The detected and/or elevated terpenoids (beta-caryophyllene, geraniol, beta-myrcene) allow the assumption that the mevalonate (MEV) and/or alternative isopentenyl pyrophosphate

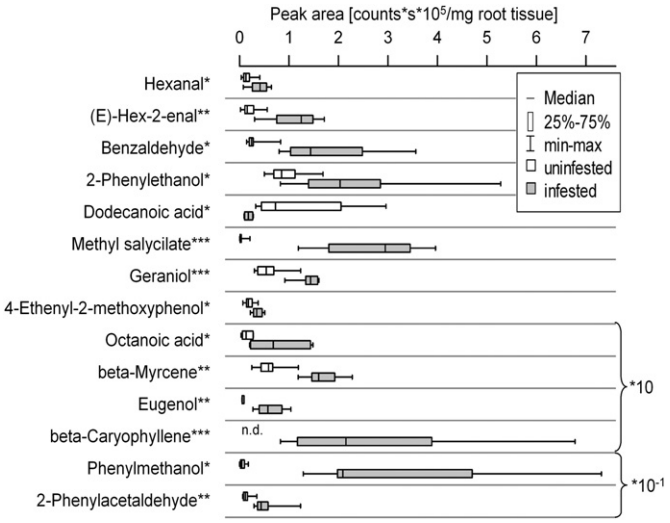

Fig. 2. Overview of differentially expressed volatiles detected in uninfested root tips and mature nodosities (infested by one adult phylloxera producing maximal 5 eggs). Intensities of some metabolite peaks were multiplied by a factor of 10 or 10^{-1} respectively (see graph). Asterisks indicate significant differences between the two sample types: $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$; n.d.: not detected.

(alt. IPP) pathway are modified as a consequence of phylloxera damage. Beta-caryophyllene for example, is well known to be associated with the response of various plants to herbivore root attack and has been reported to attract natural enemies [15]. Geraniol [34] and beta-myrcene [35] are also known to be produced by the green parts of plants after hemiptera attack such as aphids or stink bugs. Further, it can be suggested that the phenylpropanoid pathway is triggered due to a phylloxera infestation. The aromatic compounds, phenylacetaldehyde, methyl salicylate and eugenol, which have been found at elevated levels in phylloxera-infested samples compared to uninfested root tips, have also been described to be produced by plants after leave herbivory [36] or pathogen attack [37]. Mallinger and colleges, for example further suggest that methyl salicylate attracts natural enemies of soybean aphids [38]. Moreover, elevated levels of the C6-compounds (E)-hex-2-enal and hexanal were observed in mature nodosities compared to uninfested root tips which indicates that the lipoxygenase (LOX) pathway might be initiated after phylloxera infestation. C6-compounds result from the oxidative cleavage of linoleic- and linolenic acid. Both substances are well known to be released after herbivore attack [39] as well as C6-compounds from *V. vinifera* [40].

3. Conclusion

Applying strict identification criteria we identified 38 volatile metabolites in grapevine root samples, including C6-compounds, terpenes, aromatic compounds, aldehydes, alcohols and *n*-alkanes. Comparing in a second step mature nodosities with uninfested root tips we identifying twelve substances at significantly increased concentration levels in infested root samples, while dodecanoic acid was found at decreased levels and beta-caryophyllene exclusively in mature nodosity samples. Our preliminary data indicate that several defence related metabolic pathways, namely the MEV and/or alt. IPP, the phenylpropanoid as well as the LOX pathway might be affected as a consequence of phylloxera attack. However, more detailed studies are required to gain deeper insight into the metabolic processes induced upon phylloxera attack e.g., on nodosities still attached to the plant evaluating which volatiles might be involved in plant–plant signalling as well as direct or

indirect defence responses against phylloxera. Furthermore, comparing the metabolic response of rootstocks with different levels of susceptibility to phylloxera and *V. vinifera* will provide a better understanding of the molecular mechanisms mediating resistance against root-feeding phylloxera.

4. Material and methods

4.1. Insect and plant material

Leaf-galling *D. vitifoliae* Fitch (Hemiptera: Phylloxeridae) were collected in Grosshoeflein, Austria in 2007 and maintained since then as a single founder lineage in the greenhouse on the grapevine rootstock Teleki 5C (*V. berlandieri* Planch. × *V. riparia* Michx.).

Samples from different vegetatively propagated cuttings of this rootstock clone were collected during several independent runs during June–October 2009 in the greenhouse. Further details on the experimental setup are given in Lawo et al. [41]. We sampled uninfested root tips and mature nodosities, which were infested by one adult phylloxera producing maximal five eggs. In case of nodosities, the phylloxera was removed and both, uninfested root tips and mature nodosities were immediately cooled with liquid nitrogen and stored at −80 °C until further sample preparation and analysis.

4.2. Sample preparation and HS-SPME–GC–MS analysis

Cooled root tissues were homogenized via a pre-cooled pestle and 25–50 mg of the homogenized sample were weighted into a 20 ml screw cap headspace (HS) vial, sealed with 1.3 mm silicone/PTFE septa (Supelco, distributed by Gerstel, Mühlheim a.d. Ruhr, Germany). Subsequently, samples were incubated for 30 min and extracted for 60 min at 90 °C by headspace solid phase microextraction (HS-SPME) (fibre coating: DVB/CAR/PDMS 50/30 μm, 2 cm stableflex fibre, Gerstel). Thereafter, samples were analyzed by GC–MS according to Stoppacher et al. [42], with the following modifications: apolar column: DB-5MS (Agilent, Waldbronn, Germany); polar column: Optima-WAX (Machery-Nagel, Germany), dimensions for both columns: length 30 m, inner diameter 0.25 mm, film thickness 0.25 μm, oven program: 35 °C (hold 2 min), 5 °C min^{-1} to 260 °C (hold 5 min), no solvent delay, *m/z* scan range: 35–500 amu.

4.3. Analysis of standards and determination of retention indices

Standard substances were purchased from Sigma–Aldrich (Vienna, Austria) (summarised by brand, minimum purity in parentheses): SAFC: (2E)-3,7-dimethylocta-2,6-dien-1-ol (97%), (5E)-6,10-dimethylundeca-5,9-dien-2-one (97%), 4-ethenyl-2-methoxyphenol (98%), beta-caryophyllene (80%), 2-pentylfuran (97%), hexadecanoic acid (98.9%), octanal (92%), Aldrich: (E)-hex-2-en-1-ol (96%), (E)-hex-2-enal (98%), 5-(hydroxymethyl)furan-2-carbaldehyde (99%), dodecanoic acid (98%), furan-2-carbaldehyde (98%), (−)-limonene (96%), nonanal (95%), Sigma–Aldrich: 4-hydroxy-3-methoxybenzaldehyde (99%), benzaldehyde (99%), hexan-1-ol (98%), phenylmethanol (99%), Sigma: decanal (98%), nonanoic acid (97%), Riedel de Haën: octanoic acid (99%), Supelco: octan-1-ol (99.9%), Fluka: 3,7-dimethylocta-2.6-dienal (cis + trans, 95%), 2-ethylhexan-1-ol (99.5%), 2-methoxy-4-prop-2-enylphenol (99.8%), 2-phenylethanol (99%), beta-myrcene (95%), hexanal (97%), methyl 2-hydroxybenzoate (99.5%), alkane standards C8–C20 (40 mg L^{-1} each in hexane), C21–C40 (40 mg L^{-1} each in toluene). Additionally, a C5–C10 alkane standard was mixed from the pure substances (pentane 99% Sigma–Aldrich, hexane Supra-Solv Merck, heptane 99.5% J.T. Baker, octane 99% Sigma Aldrich, nonane 99% Sigma Aldrich, decan p.a. Promochem) in a ratio resulting in narrow peak shapes.

From the original standards (liquids and solids) stock solutions with a concentration of 100 mg L^{-1} in acetonitrile (HPLC gradient grade, VWR, Vienna, Austria) were prepared and stored at 4 °C. Standards and dilutions were always handled with gastight Hamilton syringes.

For determination of linear temperature programmed retention indices (LTPRI, [26]) the standards were combined in a mixture resulting in a concentration of 25 μL L^{-1} in MilliQ-water (in-house device, Millipore, Molsheim, France). Twenty μL of the mix were transferred in a 20 mL HS vial and measured with the same SPME–GC–MS method as the root samples. The alkane standards were measured with the following SPME-methods to achieve narrow peak shapes: C5–C10: 1 μL in 20 mL HS vial, sampling out of tray (10 °C) for 0.01 min, C8–C20: 10 μL in 20 mL HS vial, extraction for 10 min at 90 °C, C21–C40: 30 min equilibration and 60 min extraction both at 120 °C.

4.4. Data evaluation

4.4.1. Detection and identification of metabolites

For identification of metabolites, two pooled samples (one uninfested and one infested root sample) were used. Peak detection, spectra deconvolution, comparison of MS spectra against Wiley/NIST 08 spectra library [43] and LTPRI calculation were carried out with the AMDIS software (version 2.65, www.amdis.net, [25]) with default settings for deconvolution. Putative metabolites found on both columns with a match factor ≥90 and a LTPRI deviation of ≤15 (if determined from a standard) or ±2% (if derived from literature) were put to the results list according to Stoppacher et al. [42]. Median values of those LTPRIs listed in the NIST Chemistry Webbook [44] which corresponded to the same column diameter and film thickness as well as a comparable stationary phase material were used as reference. Substances known to originate from the fibre coating or the stationary phase of the GC-column (e.g., silicium containing substances) were removed from the results list.

4.4.2. Differential comparison of uninfested root tips and nodosities

Metabolites identified according to the criteria listed in 4.4.1, were considered for further comparison of volatile profiles associated with uninfested root tips ($N = 8$) and nodosities ($N = 7$). For this purpose, a sub library containing mass spectra of all identified substances was created. The well defined uninfested and nodosity samples were measured on the DB-5MS column and data were evaluated again with AMDIS with the following settings: minimum match factor = 60, RI-window = 5, match factor penalties: level infinite, maximum penalty and “no RI in library”: 100.

The data were grouped according to sample type. For those metabolites detected in ≥6 out of 7 (nodosities) or in ≥6 out of 8 (uninfested) samples the arithmetic means of each sample type were compared by an unpaired two-sided Welch’s *t*-test with a confidence level of 0.95 (R statistic software version 2.9.2).

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