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Emission of novel volatile biomarkers for wheat powdery mildew

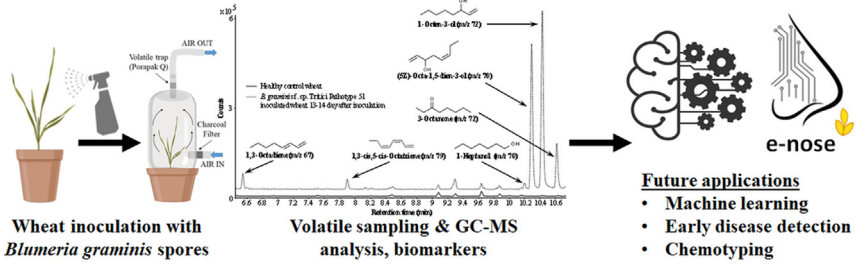
Kamirán Áron Hamow [a,b,1], Zsuzsanna Ambrózy [a,1], Katalin Puskás [b], Imre Majláth [b], Mónika Cséplő [b], Réka Mátyus [a], Katalin Posta [c], Péter Lukács [a,1], László Sági [b,*,1]

[a] Plant Protection Institute, Centre for Agricultural Research, Budapest, Hungary
[b] Agricultural Institute, Centre for Agricultural Research, Martonvásár, Hungary
[c] Institute of Genetics and Biotechnology, Hungarian University of Agriculture and Life Sciences, Gödöllő, Hungary

HIGHLIGHTS

- Agricultural ecosystems emit massive quantities of volatile organic compounds
- Volatile compounds identified by GC–MS during the wheat-powdery mildew interaction
- Six robust volatile biomarkers released by powdery mildew fungus during infection
- These biomarkers monitored for three years, various strains and symptomatic stages
- Baseline data for the combination of novel biosensors with machine learning

GRAPHICAL ABSTRACT



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ABSTRACT

Natural ecosystems including host-pathogen interactions produce a plethora of biogenic volatile organic compounds (BVOCs). Infections by phytopathogens change the volatile profile substantially and dynamically over a crop field or even on a larger scale. Despite their worldwide importance in agriculture little is known about the BVOC emissions of cereal crops, such as wheat, and their pathogens. The wheat-powdery mildew interaction is especially relevant, because powdery mildew is widespread on a global scale and is caused by an obligatory biotrophic pathogen (*Blumeria graminis* f.sp. *tritici*, *Bgt*). Since the BVOCs in this important fungus have still not been identified, the most frequent local natural pathotypes were studied by artificial inoculation and dynamic headspace collection followed by GC–MS analysis in three consecutive years. Of the 48 BVOCs identified in a total of 120 samples, six compounds (1,3-octadiene, 1,3-*cis*,5-*cis*-octatriene, 1-heptanol, (5Z)-octa-1,5-dien-3-ol, 1-octen-3-ol and 3-octanone) were detected only in *Bgt*-inoculated plants, and were hence quantitated. The latter three BVOCs were the most abundant in symptomatic plants with a characteristic distribution pattern. Additional wheat genotypes, different host plant development stages, and early and full disease progression in the presence of other pathogen mixes were also tested for the specificity of the proposed biomarkers. The three major biomarkers, namely (5Z)-octa-1,5-dien-3-ol, 1-octen-3-ol and 3-octanone were robustly applicable for differentiating the headspaces of healthy and mildew-infected plants even at early stages when disease symptoms are barely visible. These novel powdery mildew-related biomarkers are promising candidates for chemotyping and environmental monitoring in the field.

* Corresponding author.
E-mail address: sagi.laszlo@atk.hu (L. Sági).
[1] These authors contributed equally to this work.

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1. Introduction

Natural ecosystems are composed of a complex network of interactions between biotic components (vegetation, wildlife and microbial life) and abiotic factors, which constitute a self-sustaining system. In contrast, agro-ecosystems, created and managed by man, have relatively few species and, in the case of a farm field, primarily only one type of crop plants, a monoculture. The limited biodiversity and increased crop plant density make agro-ecosystems, in general, vulnerable to invading pests, zoo- and/or phytopathogens (Zavaleta and Hulvey, 2004; Keesing et al., 2010; Letourneau et al., 2011; Pagán et al., 2012; McDonald and Stukenbrock, 2016), including foliar fungal pathogens (Ullstrup, 1972; Mitchell et al., 2002, 2003; Rottstock et al., 2014). About 70% of biogenic volatile organic compounds (BVOCs) are emitted globally from terrestrial plant vegetation, with an estimated 10% from agricultural ecosystems (Guenther, 2013). However, this may be as high as 27% for the ten biogeographical regions of the Pan-European land (Karl et al., 2009). In addition to their biological role in essential plant processes such as chemical communication, defense signaling, stress adaptation and host-pathogen interactions (Spinelli et al., 2011; Dudareva et al., 2013; Niinemets et al., 2013; Xu and Turlings, 2018) BVOCs are also emitted by pathogens themselves (Hung et al., 2015; Kanchiswamy et al., 2015; Li et al., 2016). Pathogen-derived BVOCs can substantially and dynamically modify the VOC profile in and above a crop field or even on a larger scale, and may also function as biomarkers for the detection or forecasting of early infections (Li et al., 2019). However, despite their comparatively great abundance, surprisingly little is known at present about the composition and quantity of the BVOCs emitted from crop fields (Guenther, 2013; Bachy et al., 2016, 2020). Therefore, high priority should be given to the characterization and understanding of their precise composition, temporal and geographical distribution, and fluxes. To measure and characterize this process and its significance, the wheat-powdery mildew interaction was tested here.

Wheat is the most important cereal in the temperate climate, with a global production of approx. 750 million tonnes harvested on more than 200 million hectares (FAO, 2020). Powdery mildew (PM), caused by the fungus *Blumeria graminis* (DC.) Speer f.sp. *tritici* Marchal (*Bgt*, syn. *Erysiphe graminis* DC. f.sp. *tritici* Marchal), is one of the most widespread foliar diseases of wheat globally. It occurs practically everywhere wheat is grown, and may thus release BVOCs from millions of hectares worldwide (Basandrai and Basandrai, 2018). This pathogen can cause significant yield losses, especially where nitrogen fertilizers are routinely applied (Last, 1953; Rowaished, 1980; Tompkins et al., 1992). Though annual variations occur regionally depending on weather and other conditions (Murray and Brennan, 2009), without protective measures yield reductions may amount, in extreme cases, to 40–50% (Oerke, 2006; Savary et al., 2019), while grain quality is also affected (Gao et al., 2018). This is an obligate biotrophic pathogen, i.e. it grows only on the leaves of living plants and has a relatively minimalistic interaction with the host (Liang et al., 2018). As a result, fungal BVOCs will essentially be emitted from an active infection site, unlike other pathogens, which may be the source of additional emissions during their subsequent necrotrophic or saprophytic stages (Pusztahelyi et al., 2017).

Importantly, VOC emission from wheat (Bachy et al., 2020) appears to be weak and simple in profile compared to that of other crops (Gomez et al., 2019). This relatively "noise-poor" volatile background provides an as yet unexploited advantage and represents an excellent experimental system to screen for specific BVOCs that may be involved in and signal the progression of *Bgt* or other pathogen infections in wheat.

In order to fill the knowledge gap for the BVOCs emitted in relation to PM disease, comparative volatile profile analyses were performed in the headspace of healthy and pathogen-inoculated plants of the highly susceptible wheat cultivar 'Carsten V'. The 48 compounds identified by GC–MS included aliphatic and (a)cyclic hydrocarbons, aromatic hydrocarbons, polycyclic aromatic hydrocarbons, ketones, aldehydes, fatty alcohols, and terpenes. Six fungus-specific BVOCs were identified, four of which were quantitated at two time points during the disease process in three consecutive years. After confirming their robust applicability to different wheat cultivars infected with various *Bgt* strains, it appears that the six BVOCs could serve as biomarkers emitted from the onset of PM disease.

2. Materials and methods

2.1. Plant material and inoculation treatment

2.1.1. Greenhouse experiments

Lots of 20–30 seeds of the susceptible bread wheat cultivar 'Carsten V' (Mesdag and Donner, 2000) were sown in 1-liter clay pots containing garden soil with a 1 cm sand layer on the top. The bread wheat cultivar 'Carsten V' was used in all but one of the experiments (see Section 3.3), because it does not contain any known *Pm* resistance genes to powdery mildew (Nover, 1958; Vida et al., 2002) and was therefore expected to be susceptible to all *Bgt* pathotypes. The plants were grown in an automated greenhouse (Global Glasshouse Ltd., Szentes, Hungary) at a humidity of 60–90% and 12 h photoperiods with illumination provided by Groxpress 600 W E40 lamps at 2050 K color temperature (Sylvania, Budapest, Hungary). To simulate environmental temperature variations, three independent experiments were carried out in January–February of 2018 (28 days) and in February–March of 2019 (31 days) and 2020 (29 days). The temperature was continuously recorded at 10 min intervals both inside and outside the greenhouse compartment (Table S1).

The inoculum originated from a single colony and was maintained on 'Carsten V' plants under isolated circumstances (Supplementary Method). Inoculation was performed by manually shaking conidiospores of *Bgt* pathotypes 51 and 71 (Frauenstein et al., 1979) onto single leaves of 7-day-old test plants (stages 11–12 on the Zadoks scale, Zadoks et al., 1974) in a closed box. Control plants without inoculation and blank control pots with identical soil composition but without plants were also included in the experiments. Each treatment consisted of four biological replicates, except for the blank controls, which consisted of two individual pots. The experiments were executed three times (in 2018 with *Bgt* pathotype 51, and in 2019 and 2020 with pathotypes 51 and 71): in each experiment two repetitions of four pots per treatment were sampled simultaneously, except for plants inoculated with pathotype 71, which were represented by two individual pots (detailed setup in Supplementary Method).

2.1.2. Growth chamber experiment

During May 2020 two wheat cultivars ('Mv Suba' and 'Mv Kolompos') were grown in a PGR15 reach-in plant growth chamber (Conviron, Winnipeg, Canada) according to the T2 spring program (Tischner et al., 1997). The plants were inoculated at the beginning of flowering with a conidiospore mixture of *Fusarium graminearum*/*F. culmorum*, and slight symptoms of spontaneous PM infection were observed about 15 days later, but only on plants weakened by *Fusarium* disease (Fig. S1). To test the wider applicability and robustness of the identified VOCs in further cultivar-pathotype combinations, samples were taken from eight healthy control plants and eight *Fusarium*-inoculated and *Bgt*-infected plants of both wheat cultivars over an 8 h period (instead of 24 h, see below).

2.2. Dynamic headspace volatile collection and sample handling

The headspace of greenhouse plants was sampled 7 days after inoculation (DAI) when the first, barely visible symptoms emerged and at 14 DAI in the full-disease development stage in 2018 and 2020, but only at 14 DAI in 2019. To create a headspace the plants were covered with

specially crafted 2.5-liter glass cups (55 cm × 8 cm O.D.), which were carefully inserted into the soil inside the circumference of the pots to a few cm depth without damaging the plants or their roots. At the bottom of the cup there was an inlet for air with laboratory glass wool and an active charcoal filter (mesh 4–8, Alfa Aesar, Wardhill, MA, USA), and a PTFE tubing was connected to the top to serve as an air outlet (Fig. 1). Two volumes of air were sucked through the cup, which was then closed and left for 1 h prior to sampling. An adsorbent tube containing a load of 50 mg Porapak Q sorbent (mesh 80–100, Waters, Milford, MA, USA) between two layers of gas chromatographic grade deactivated glass wool and PTFE rings for fixation was connected to the PTFE tubing of the outlet. The adsorbent tube was covered with aluminum foil to prevent photodegradation or the alteration of adsorption capacity potentially caused by exposure of the sorbent to light for a prolonged period. A BA-4AR type flow meter (Kytola Instruments, Muurame, Finland) and a NMP 830 KNDC type pump (KNF-Micro AG, Reiden, Switzerland) were connected behind the adsorbent tube using silicone tubing. Sample collection from the headspace was done in each case for 24 h with a sampling speed of 0.8 L/min, each collection starting at 10 am on each sampling day. The flow meters were checked regularly during sample collection. After collection the adsorbent tubes were not thermally desorbed but instead eluted with 300 μL of PESTINORM® grade *n*-hexane, transferred into 1.5 mL GC injection vials with glass inserts closed with caps containing PTFE septum, then sealed with Parafilm and stored at −20 °C until analysis. Reproducibility and direct sorbent recovery, carryover and stability were tested and verified. After elution with *n*-hexane, the adsorbent tubes were cleaned by forced washing in 4 × 500 μL of each of the following solvents: methanol, methanol:chloroform 3:1, dichloromethane and finally *n*-hexane followed by drying under a gentle nitrogen stream. The glassware and PTFE tubing and connections were rinsed first with ultrapure water, then with acetone, and baked at 130 °C for 3–4 h, followed by wrapping with thick aluminum foil for storage prior to use for sampling. All solvents were at least HPLC-grade (VWR, Radnor, PA, USA).

In the growth chamber experiment, the headspaces of *Fusarium*-infected and control plants were created using PTFE bags with gastight sealing (instead of the glass apparatus in Fig. 1) and the adsorbents were handled after sampling as described above.

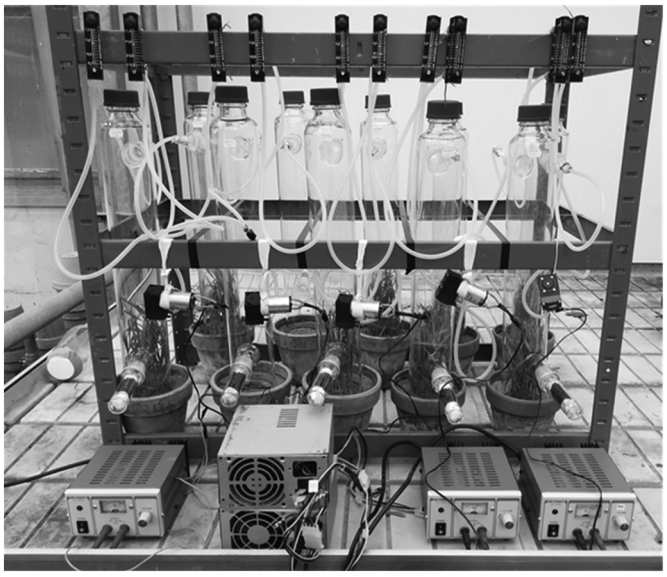

Fig. 1. Sampling setup consisting of eight pots of wheat plants and two blank pots under glass cups with air in- and outlets located in the bottom and top, respectively (for details see Section 2.2).

2.3. GC–MS VOC analysis

GC-EI-MS measurements were carried out on an Agilent (Santa Clara, CA, USA) 7890B GC coupled to a 5977B MS system. The GC was equipped with a CIS4 inlet with a septumless head installed (Gerstel GmbH, Mülheim a. d. Ruhr, Germany). Injection volume was 1 μL in splitless mode, septum purge flow was 3 mL/min, and purge flow was 50 mL/min starting from 3 min. Before each run the CIS4 inlet was cooled with liquid CO_2 to 20 °C and the temperature equilibrated for 0.5 min. To minimize compound degradation during column transfer, the injector temperature program was the following: 20 °C held for 0.25 min (initial time), then the CIS4 inlet was heated up to 270 °C at a rate of 12 °C/s with a hold time of 6 min. Chromatography was carried out on a J&W HP-5MS UI 30 m × 0.25 mm × 0.25 μm capillary column (Agilent). Helium was used as a carrier gas with a flow rate of 1 mL/min (36.26 cm/s) in constant flow mode. The oven temperature program was the following: 40 °C hold for 3.5 min, increased by 7 °C/min to 140 °C, then by 20 °C/min to 280 °C and held for 2 min. As a post-run function the column was flushed by heating it up to 325 °C with a column flow of 1.5 mL/min for 2 min before returning to the initial conditions. Total analysis time from injection to injection was 36 min.

For MS detection EI ionization was used with a standard 70 eV energy, and the MS was tuned and calibrated by perfluorotributylamine according to the manufacturer's instructions. A gain factor of two was applied for the scan and SIM events to maintain optimal sensitivity for both. The auxiliary heater was set to 250 °C, the MS source to 250 °C and the MS quad to 150 °C. Mass spectra were collected in the Scan & SIM combined acquisition mode, with a cut time of 5.2 min. For identification the scan event was set to monitor *m/z* 35–600 with a scan speed of 9 scan/s with 0.1 *m/z* step size. In the SIM event *m/z* 93 was monitored with a dwell time of 20 ms during the whole run, which is a characteristic fragment for most terpenoids. For the quantitation and confirmation of the important and abundant fungus-related alcohols and one ketone identified, *m/z* 57, 70, 72, 93 and 99 were acquired in SIM mode with 20 ms dwell time during 10.05 to 11 min.

2.4. Data evaluation, mining and statistical analysis

The Mass Hunter Workstation Qualitative Navigator B.08.00 and Quantitative Analysis B.09.00 programs (Agilent) were used for evaluation and quantitation. The identification of compounds was based on background subtracted mass spectra identified using the NIST (National Institute of Standards and Technology)/EPA/NIH Mass Spectral Library v17 (2017) and the Wiley Registry® of Mass Spectral Data, 10th edition (2014), and utilizing *n*-alkane retention indices with a C7-30 *n*-alkanes mix (Sigma-Aldrich, St. Louis, MI, United States). The best consistent library hit (min. 75% similarity with reverse search for mass spectra) and retention index score match was accepted for the identification of volatile compounds. Integration was carried out to the most abundant unique ion for each peak (Table 1). For unambiguous identification and quantitation commercially available reference materials were used for 1-heptanol, 1-octen-3-ol, 3-octanone (Sigma Aldrich) and (5Z)-octa-1,5-diene-3-ol (Toronto Research Chemicals Inc., North York, Canada). The reproducibility and linearity of the GC–MS method was verified by injecting a mixture of these reference materials. The relevant quality control tests and their results are summarized in Table S3 (sheet 1). For peak areas lower than the limit of quantification (LoQ) the background was always recorded with non-zero values for reliable statistical tests.

The distribution of the identified BVOCs was tested in the Metabolite Ecology database of the KNApSAcK Family databases (Afendi et al., 2012), and the mVOC 2.0 database (Lemfack et al., 2018). Genes encoding BVOC biosynthetic enzymes (dioxygenases, monooxygenases and lipoxygenases) were identified by basic local alignment search tool (BLAST) searches (Altschul et al., 1990) in *B. graminis* whole genome sequences maintained in the Ensembl Fungi database (release 48, August

Table 1
Characterization of volatile organic compounds identified from the headspace of control and *Bgt*-inoculated wheat 'Carsten V' in 2018 and 2019.

No.	Name[a]	CAS No.[b]	RT min[c]	RI calc.[d]	RI lit.[e]	*m/z* quant.[f]
1	Octane	111-65-9	5.91	800.0	800	71
2	Heptane, 2,4-dimethyl-	2213-23-2	6.45	821.2	821 ± 1 (41)	85
3	**1,3-Octadiene**	1002-33-1	6.54	824.9	827 ± 1 (9)	54
4	Ethylbenzene	100-41-4	7.43	860.3	855	91
5	Octane, 4-methyl-	2216-34-4	7.53	864.2	863	85
6	*m*-Xylene	108-38-3	7.63	868.2	866 ± 7 (170)	91
7	**1,3-*cis*,5-*cis*-Octatriene**	40087-62-5	7.90	878.7	879	79
8	3-Heptanone	106-35-4	8.09	886.5	887 ± 3 (33)	85
9	Styrene	100-42-5	8.18	890.1	893 ± 5 (91)	104
10	*o*-Xylene	95-47-6	8.22	891.7	887 ± 8 (178)	91
11	Nonane	111-84-2	8.43	900.0	900	71
12	α-Pinene	80-56-8	9.30	934.6	935 ± 7	93
13	Benzaldehyde	100-52-7	9.95	960.5	962 ± 3 (416)	106
14	Benzene, 1-ethyl-3-methyl-	620-14-4	9.98	961.9	957 ± 8 (67)	105
15	Benzene, 1,3,5-trimethyl-	108-67-8	10.16	969.1	972 ± 9	105
16	**1-Heptanol**	111-70-6	10.19	970.2	970 ± 2 (68)	70
17	**(5Z)-Octa-1,5-dien-3-ol**	50306-18-8	10.30	974.5	975 ± 2	57
18	β-Pinene	127-91-3	10.37	977.3	979, 974	93
19	**1-Octen-3-ol**	3391-86-4	10.43	979.8	980 ± 2 (355)	72
20	**3-Octanone**	106-68-3	10.62	987.4	986 ± 3 (101)	72
21	β-Myrcene	123-35-3	10.73	991.8	991	93
22	Benzene, 1,2,4-trimethyl- (Pseudocumene)	95-63-6	10.78	993.7	990 ± 6 (83)	105
23	Decane	124-18-5	10.94	1000.0	1000	71
24	3-Carene	13466-78-9	11.21	1011.5	1011 ± 2 (336)	93
25	*p*-Cymene	99-87-6	11.54	1025.6	1025 ± 2 (820)	119
26	(+)- Limonene	138-86-3	11.63	1029.6	1030 ± 2 (1004)	93
27	Indane	496-11-7	11.80	1036.7	1029 ± 11 (36)	117
28	Benzene, 1,2-diethyl-	135-01-3	12.14	1050.9	1045 ± 8 (22)	105
29	Acetophenone	98-86-2	12.51	1067.0	1065 ± 4 (134)	105
30	Benzene, 2-ethyl-1,3-dimethyl-	2870-04-4	12.83	1080.3	1080 ± 20 (12)	119
31	3-Octanol, 3,7-dimethyl-	78-69-3	13.25	1098.0	1100 ± 13 (8)	73
32	Undecane	1120-21-4	13.29	1100.0	1100	71
33	Nonanal	124-19-6	13.39	1104.3	1104 ± 2 (556)	70
34	Benzene, 1,2,3,4-tetramethyl-	488-23-3	13.68	1117.8	1116 ± 9 (32)	119
35	Benzene, 1,2,3,5-tetramethyl-	527-53-7	13.76	1121.5	1117 ± 9 (24)	119
36	Benzaldehyde, 3-ethyl-	34246-54-3	14.73	1165.7	1168 ± N/A (1)	134
37	Benzaldehyde, 4-ethyl-	4748-78-1	15.04	1180.0	1180 ± 16 (5)	134
38	Naphthalene	91-20-3	15.18	1186.4	1182 ± 8 (183)	128
39	Dodecane	112-40-3	15.48	1200.0	1200	71
40	Decanal	112-31-2	15.60	1206.0	1206 ± 2 (406)	70
41	Undecane, 2,6-dimethyl-	17301-23-4	15.76	1214.1	1210 ± 3 (18)	71
42	Ethanone, 1-(4-ethylphenyl)-	937-30-4	17.21	1284.8	1277 ± 4 (8)	133
43	Tridecane	629-50-5	17.52	1300.0	1300	71
44	Tridecane, 3-methyl-	6418-41-3	18.72	1374.4	1371 ± 1 (15)	71
45	Tetradecane	629-59-4	19.14	1400.0	1400	71
46	Longifolene	475-20-7	19.35	1418.7	1413 ± 5	93
47	β-Caryophyllene	87-44-5	19.52	1434.2	1423–1442	93
48	Pentadecane	629-62-9	20.25	1500.0	1500	71

Bold, identified biomarker VOCs.
[a] According to the NIST/EPA/NIH Mass Spectral Library v17 and the Wiley Registry of Mass Spectral Data, 10th edn.
[b] Chemical Abstracts Service registry number.
[c] Retention time in min.
[d] Kováts' Retention Index calculated (Kováts, 1958), experimentally determined using *n*-alkane retention indices.
[e] Retention Index literature, from corresponding data in NIST v17 and the PubChem repository (in brackets: no. of experimental records).
[f] Selected fragment ion (*m/z*) for quantitation.

2020) and the Joint Genome Institute Mycocosm Blugr2 database (Frantzeskakis et al., 2018; https://mycocosm.jgi.doe.gov/Blugr2/Blugr2.home.html) as well as by queries in the Universal Protein Resource (UniProt, https://www.uniprot.org/) database.

The significance of differences between controls and treatments (symptomatic stages, pathotypes and years) was analyzed by two-sample *t*-tests in MS Excel and by multivariate PERMANOVA using the 'adonis' function in the 'vegan' package (v. 2.5–7, Oksanen et al., 2020) of the R environment (v3.6.3, R Core Team, 2020). Besides calculating effect sizes (Cohen's *d*) and statistical power the obtained *p*-values were also controlled for False Discovery Rates (FDR) as described by Benjamini and Hochberg (1995). In order to identify potential BVOC biomarkers principal component analysis (PCA) was applied for the unsupervised reduction of data dimensions after standardization of the original data matrix by *z*-score normalization. Principal components, loadings and scatter (score) plots of the observations were made using the base R function 'prcomp'. The biplot illustration was performed using the 'pca' function of the packages FactoMineR (Lê et al., 2008) and 'factoextra' (Kassambara and Mundt, 2020). To reveal systematic patterns in BVOCs across various treatments colored heat maps were generated by the R packages 'ggplot2' and 'reshape2' (Wickham, 2007). The quantitated BVOC biomarkers were also statistically explored with boxplots (BoxPlotR, http://shiny.chemgrid.org/boxplotr/ Spitzer et al., 2014) and the corresponding basic parameters (Real Statistics Resource Pack software, Release 6.2) using Power Query in MS Excel.

3. Results

3.1. Volatile profile analysis from the headspace of wheat plants

In total, 48 BVOCs were identified by GC–MS scans in 2018 and 2019 in headspace samples collected at 7 days after infection (DAI) and 14

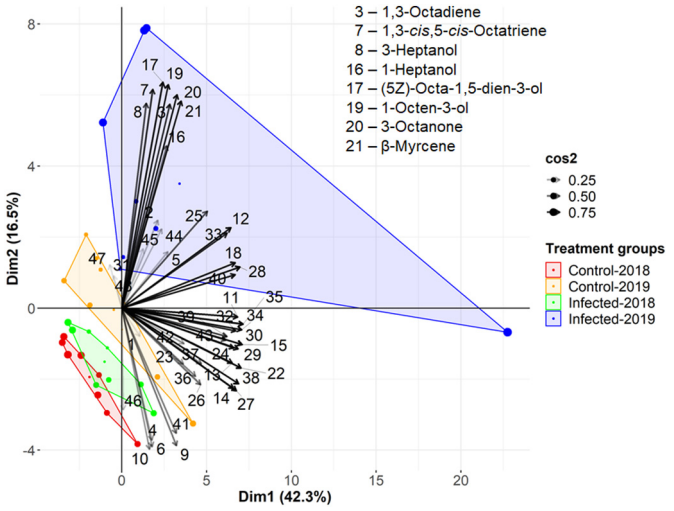

Fig. 2. PCA biplot analysis for all 48 BVOCs at 14 DAI in wheat headspace samples from 2018 and 2019 ($n = 16$ and 20, resp.). C, healthy control; I, *Bgt*-inoculated; see Table 1 for numbers of BVOCs.

DAI representing early (barely visible to the naked eye) and full symptomatic stages, respectively. These BVOCs belonged to the following chemical classes: aliphatic hydrocarbons, aromatic hydrocarbons, polycyclic aromatic hydrocarbons, aldehydes, ketones, fatty alcohols, and terpenes (Table 1). A more in-depth description of the compounds based on identifiers given in various standard databases and their reported occurrence in wheat and in *B. graminis* (KNApSAcK and mVOC 2.0 databases) is presented in Table S2. Out of the 48 BVOCs only 27% (13) were described previously in wheat seedlings and plants and none of them in *B. graminis*.

Significant differences between samples from uninoculated control (healthy) and *Bgt*-inoculated (diseased) plants were found for 36 compounds at various time points, but only six of them exhibited highly reproducible, significant quantitative differences for all time points and samples in all years (Table S3: sheets 2–4). Multivariate data analysis (PERMANOVA, Table S3: sheet 5; PCA loading plot in Fig. 2) and heat maps (Table S3: sheet 6) revealed a cluster of up to eight compounds, including the six significant ones, with strong, consistent effects as well as highly positive mutual correlations. This confirmed that the six BVOCs can be considered as diagnostic for PM infection.

3.2. Identification of diagnostic BVOCs for powdery mildew

Among the BVOCs detected, the most evident differences between control and *Bgt*-inoculated plants were observed for six compounds that were only present in the headspaces of inoculated plants. These seven- or eight-carbon (C7-C8) BVOCs were: 1,3-octadiene, 1,3-*cis*,5-*cis*-octatriene, 1-heptanol, (5Z)-octa-1,5-dien-3-ol, 1-octen-3-ol (OTL) and 3-octanone as demonstrated by the pooled extracted ion chromatogram (EIC) of a headspace sample from healthy and *Bgt*-inoculated plants (Fig. 3). The six BVOCs were abundant in the headspaces of *Bgt*-inoculated plants, while in the blanks and uninoculated controls their presence was below or around the LoQ.

The six BVOCs represent C7-C8 fatty alcohols (3), acyclic hydrocarbons (2) and a ketone (Table S2). The first key step in the biosynthesis of fatty alcohols in fungi and plants is the oxidation of α-linoleic acid by several types of oxygenase enzymes (Fischer and Keller, 2016) followed by further catalysis into volatile oxylipins, including various short-chain (C6-C8) fatty alcohols. As a bioinformatic proof of this pathway in the present experimental setup, a BLAST search in *B. graminis* genome sequences confirmed that several genes encoding two groups of linoleate diol synthases (belonging to the dioxygenase family) as well as numerous monooxygenases homologous to classical fungal enzymes are present in this pathogen. Further analysis revealed that all these genes are functional in two f.spp. of *B. graminis* based on evidence for both their transcription and their translation into proteins (Table S4).

There were significant differences in the emitted quantities of all six BVOCs between healthy control and inoculated wheat plants, irrespective of the *Bgt* pathotype (51 and 71), symptomatic stage (7 DAI and 14 DAI) and experimental year (2018-2019-2020) (Fig. 4, Table S3: sheets 2–5). In other words, control plants exhibited a concentration of around or below LoQ for all six BVOCs, whereas *Bgt*-inoculated plants produced massive, often several magnitudes higher quantities of these BVOCs. On the whole, there was no significant difference in the BVOC emission rates between plants inoculated with the two tested *Bgt* pathotypes when compared at any identical symptomatic stage or year.

A systematic comparison of the temperature profiles during the incubation periods and the sampling days revealed striking differences over the three experimental years (Table S1, Fig. S2): both the average temperature and its range were higher in each consecutive year for both time parameters. This seasonal or annual effect was readily confirmed when PCA was performed on the six diagnostic BVOCs after inoculation with only pathotype 51 of *Bgt* for three years and two symptomatic stages (Fig. 5). Control and inoculated treatments clearly

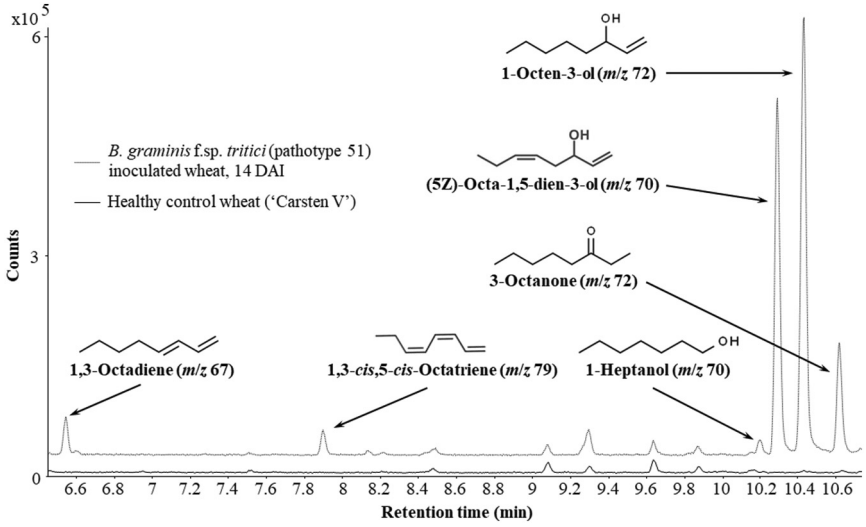

Fig. 3. Pooled extracted ion chromatograms of the optimal unique mass peaks to compare the six marker VOCs between samples collected from healthy (base line) and *Bgt*-inoculated (upper line) wheat plants.

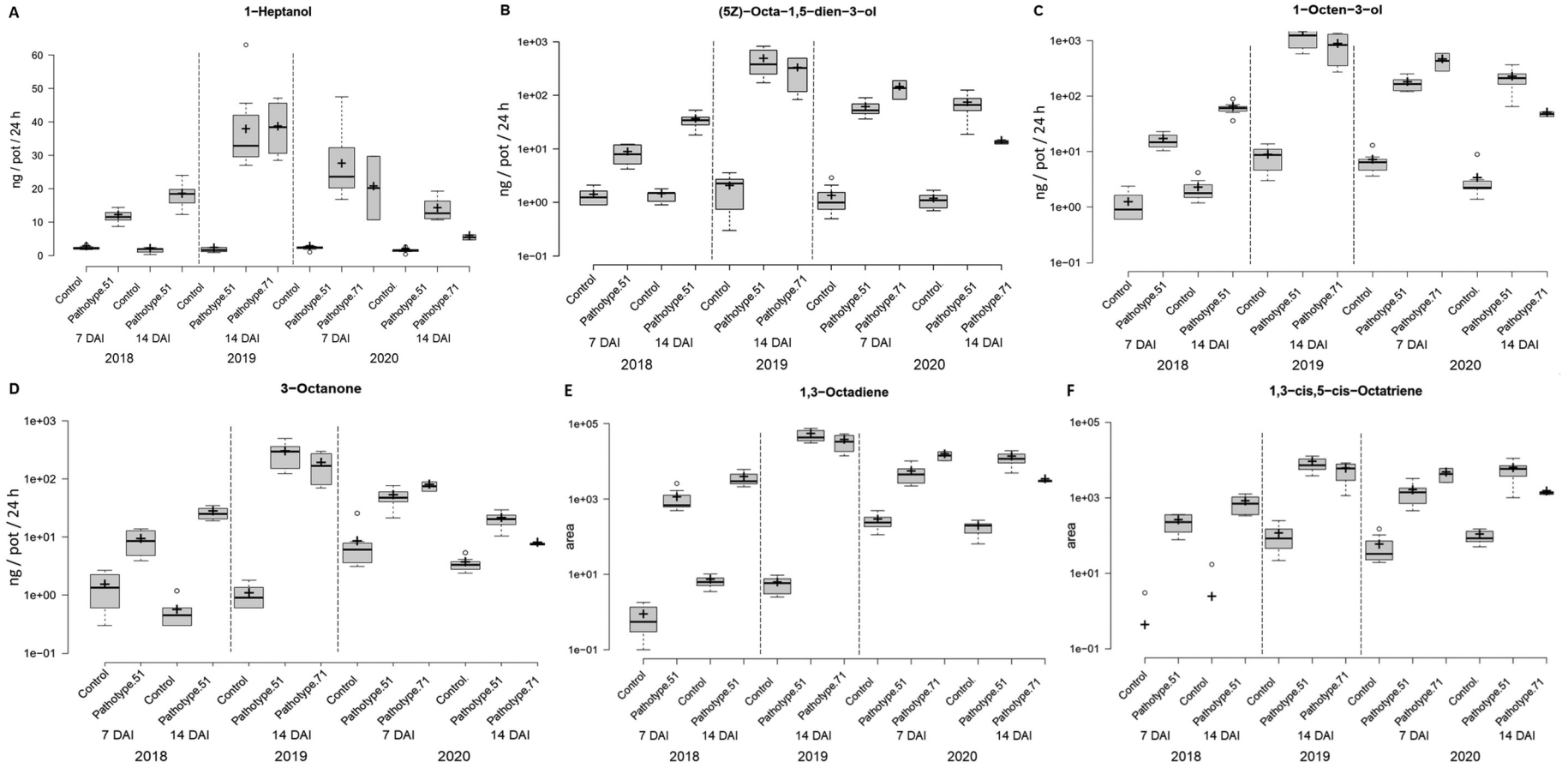

Fig. 4. Quantitative analysis of six diagnostic BVOCs at two time points (7 DAI and 14 DAI) in the headspace of 'Carsten V' wheat seedlings after inoculation in 2018–2020 with two pathotypes (51 and 71) of *Bgt* (powdery mildew fungus). (A) 1-heptanol, (B) (5Z)-octa-1,3-dien-3-ol, (C) 1-octen-3-ol, (D) 3-octanone, (E) 1,3-octadiene, (F) 1,3-*cis*,5-*cis*-octatriene. Quantitation by standards (A-D) and by area (*E*-F). Box = interquartile range (IQR), cross and bar within box = mean and median, whiskers = $\pm 1.5 \times$ IQR; widths of boxes are proportional to square-roots of the number of observations; n = 8 (for pathotype 71 $n = 4$); DAI, days after inoculation. All scales are logarithmic except for (A).

clustered separately on the scatter plot according to experimental years both at 7 DAI (Fig. 5A) and at 14 DAI (Fig. 5B) demonstrating that the selected BVOCs are reliable indicators of the onset and progression of PM disease in wheat.

3.3. Confirmation of VOC biomarkers in mixed pathogen background

In an independent experiment designed to evaluate the reaction of two further cultivars to *Fusarium* spp. in a growth chamber, mild PM symptoms appeared spontaneously about 2 weeks after *Fusarium* inoculation at the beginning of flowering (Fig. S1). This additional infection with unknown *Bgt* strain(s) provided an unexpected opportunity to test the more general utility of the diagnostic BVOCs identified above.

Of the six BVOCs, the three most abundant ones, namely (5Z)-1,5-octadiene-3-ol, 1-octen-3-ol, and 3-octanone (Fig. 3) were detected above LoQ and quantitated (Fig. 6) in the headspace of plants with early PM symptoms. Similarly to 'Carsten V' plants in the greenhouse (Fig. 4), all three BVOCs exhibited significantly higher emission in PM-symptomatic plants than in their parallel controls, in both, hitherto untested wheat cultivars (Fig. 6, Table S3**:** sheet 7). This consistent pattern further supports the fact that these BVOC combinations can be utilized as biomarkers for PM disease.

3.4. Calculation of aerial emission quantities

Based on the quantitative determination of the four most abundant biomarker BVOCs at two symptomatic stages in three consecutive years (2018–2020) we attempted to make an educated estimate of the aerial emission of biomarker BVOCs from infected wheat in the field. According to the experimental data the average release (in ng/pot/day) of *Bgt*-inoculated young wheat plants was: 22.2 (1-heptanol), 135.3 ((5Z)-octa-1,5-dien-3-ol), 389.0 (1-octen-3-ol) and 82.1 (3-octanone) (Table S5).

Extrapolating these data to a density of around 700 developed tillers/m^2 results in a total daily release of 31.5 μg/m^2 (or 0.5–0.6 μg/g dry weight per day) for these four BVOCs, which corresponds to a monthly emission of 9.4 g/ha. Assuming an annual 10% infection rate (Saunders and Doodson, 1970; Morgounov et al., 2012) on the global wheat production area of roughly 200 million ha, this figure amounts to 188 t (Mg) BVOCs/month worldwide, which can be attributed to PM disease alone. It should be noted, however, that adult plants may emit significantly higher quantities (cf. Figs. 4 and 6).

4. Discussion

4.1. Aerial release of BVOCs during the wheat-powdery mildew interaction

To the best of our knowledge, none of the 48 BVOCs has so far been identified in *B. graminis* (Table S2, columns 9 and 10). Based on a comprehensive statistical analysis six BVOCs turned out to be of diagnostic value at both sampling times in two consecutive years and could thus be considered as volatile biomarkers for PM disease in wheat. These six BVOCs are distributed in three chemical groups: three short-chain fatty alcohols (1-heptanol, (5Z)-octa-1,5-dien-3-ol and 1-octen-3-ol), two hydrocarbons (1,3-octadiene and 1,3-*cis*,5-*cis*-octatriene) and a ketone (3-octanone). Only 1-octen-3-ol (OTL) was previously found in wheat plants (Table S2, column 9), though proper controls for the exclusion of possible contamination sources were not shown in the references (Table S2). Possibly, even this BVOC is not produced by wheat plants. On the other hand, all six BVOCs have been described in a broad range of non-pathogenic and phytopathogenic fungi, though none of them in *B. graminis*. It is safe to conclude that these diagnostic BVOCs have not yet been characterized in the wheat-PM interaction.

As additional preliminary proof, when three standard algorithms (an artificial neural network, Naive Bayes and Random Forest) were applied to the results as a training and test dataset, the machine learning algorithms also separated three of the six identified BVOC biomarkers (1,3-octadiene, (5Z)-octa-1,5-dien-3-ol and 1-heptanol) between healthy and *Bgt*-inoculated wheat samples (unpublished results).

The three minor components (1,3-octadiene, 1,3-*cis*,5-*cis*-octatriene and 1-heptanol) exhibited very limited presence in plants (Suinyuy et al., 2013) or their pathogens (Börjesson et al., 1992; Kalalian et al., 2020). It is therefore quite unique and distinctive that they occur together during PM disease development. The three major BVOCs ((5Z)-octa-1,5-dien-3-ol, OTL and 3-octanone; Fig. 3), however, especially OTL, have long been known to occur in the soil (Jüttner, 1990), some plants (Naves, 1943; Andersson et al., 1963; Honkanen and Moisio, 1963) and molds (Kaminski et al., 1974). As indicated, the advantage of *Bgt* as an obligate biotrophic pathogen is that only active, 'live' infections exist and can be monitored for BVOCs. On the other hand, since the process of *Bgt* infection requires a living host and the fungus cannot be maintained separately it is difficult to ascertain, without proper controls, whether the detected BVOCs are produced by the plant and/or the pathogen itself.

Two types of controls were incorporated in the present experiments: (i) pots filled with identical soil but without wheat plants (blanks) to monitor baseline BVOC release (e.g. as a product of the aerobic and anaerobic metabolism of microbes) and (ii) pots with uninoculated, healthy wheat plants to check for background BVOC production. The two types of controls resulted in quantities at or below the detection limit of the diagnostic BVOCs and below the LoQ, respectively (Fig. 4), which was a significant difference compared to the inoculated plants, suggesting that *Bgt* was the source of these BVOCs. Theoretically, it is possible that the BVOCs were derived from the wheat plant upon induction by *Bgt*. However, the fact that wheat (and other) plants usually contain only a very low quantity of these BVOCs, if any (Table S2), whereas a broad range of fungi have massive quantities (Kaminski et al., 1974; Pyysalo, 1976; Börjesson et al., 1992; Mau et al., 1997; Fischer et al., 1999; Zawirska-Wojtasiak, 2004), makes this unlikely. For example, comparable levels of OTL and 3-octanone are only found in wheat grain or meal contaminated with molds (Sinha et al., 1988; Tuma et al., 1989). In addition, the precursor for these C8 oxylipins, 10-hydroperoxyoctadecadienoic acid (10-HPODE), is known to be present in several fungi (Wurzenberger and Grosch, 1984; Kermasha et al., 2002; Matsui et al., 2003; Akakabe et al., 2005), but not in plants. 10-HPODE might also be generated in *B. graminis* via a dioxygenase enzyme encoded by one of the genes identified by the BLAST search (Table S4). Taken together, these arguments strongly suggest that the six diagnostic BVOCs are all emitted by *Bgt* rather than by wheat.

Three explanations are offered here for the role some of the BVOCs may play in this or similar host-pathogen interactions. First, they may simply be the by-products of the lipase- and lipoxygenase-catalysed reactions required to degrade cellular lipid membranes during the adhesion and germination of conidiospores (Feng et al., 2009). Additionally, they may even be actively involved in the regulation of these processes. It is known that OTL (and to a lesser extent 3-octanone) is a self-inhibitor of spore germination (Chitarra et al., 2004) and mycelial growth (Okull et al., 2003) in *Penicillium* spp., *Trichoderma* (Nemčovič et al., 2008) and *Aspergillus* (Herrero-Garcia et al., 2011). OTL also inhibits mycelial growth in other fungi belonging to different genera (Chitarra et al., 2004), indicating that it may act as a general developmental signal for many species (Eastwood et al., 2013).

Finally, some of these BVOCs may function alone or in mixtures (Ndomo-Moualeu et al., 2016) as attractants for dwelling or visiting insects that can transmit the spores of the pathogen (Agrios, 1980). Indeed, OTL proved to be attractive for some plant-associated mites (Ozawa et al., 2000; Brückner et al., 2018), thrips (Zhang et al., 2015), beetles (Pierce et al., 1991; Malik et al., 2016) and flies (Birkett et al., 2004; Wu and Duncan, 2020) not only in closed laboratories, but even outdoors (Stevens et al., 2019). More specific evidence for the potential insect-mediated transfer of powdery mildews comes from the strong association of mildew-infected plants with thrips (Yarwood, 1943),

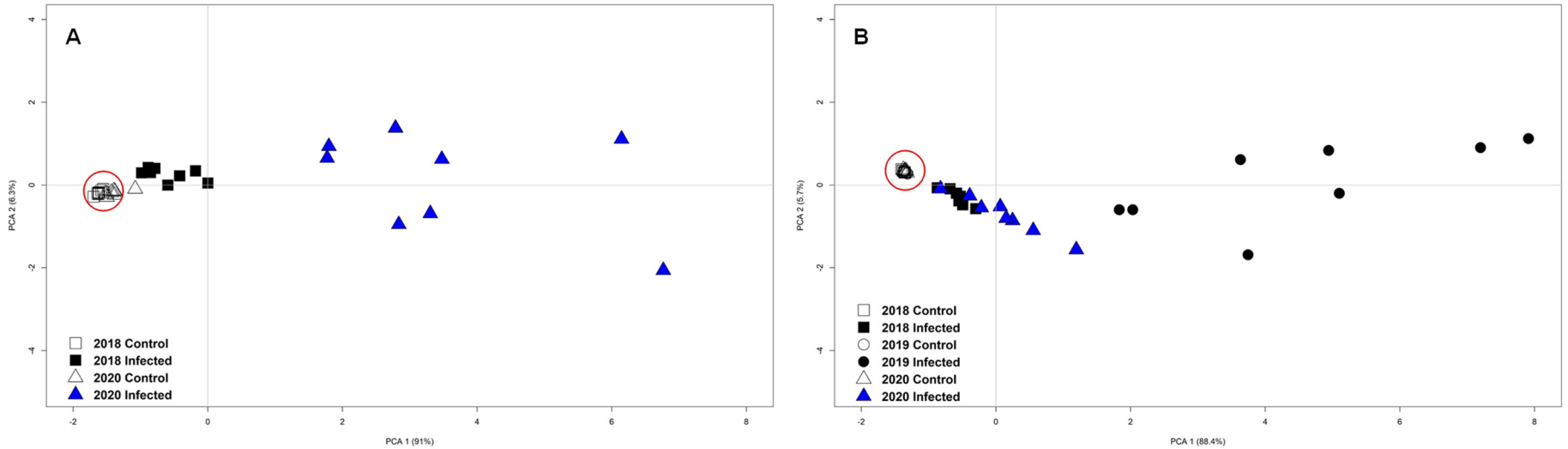

Fig. 5. PCA scatter plot of six diagnostic BVOCs at two time points, 7 DAI (A) and 14 DAI (B) in the headspace of 'Carsten V' wheat seedlings ($n = 8$) after inoculation with pathotype 51 of *Bgt* in 2018–2020. Circle: all controls group in a distinct cluster.

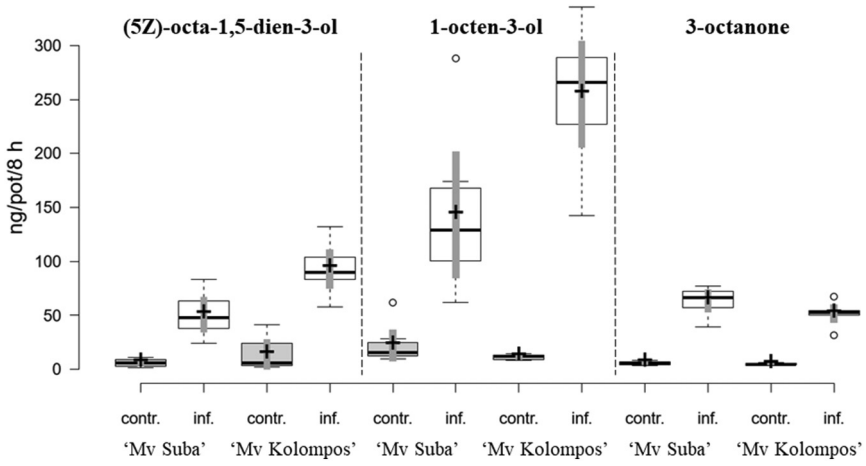

Fig. 6. Emission of three biomarker BVOCs from healthy control and spontaneously *Bgt*-infected early phase plants (n = 8) of two wheat cultivars. Gray stripes in boxes indicate 95% confidence intervals for the corresponding mean values. Further features of boxplots on Fig. 4, data in Table S3: sheet 4.

mites (Reding et al., 2001) and beetles (Tabata et al., 2011), in the latter case with the direct involvement of OTL. It is thus plausible that OTL and other BVOCs provide a chemical cue for insects that are then used as vectors for the mildew pathogen.

The source tissue of these important BVOCs could in general be both the spores (Chitarra et al., 2004; Nemčovič et al., 2008) and the mycelium (Schindler and Seipenbusch, 1990), which in the present case could explain their growing concentration during the infection process (7 DAI vs. 14 DAI, Fig. 4 and Table S3) as well as the massive quantities emitted (see Sections 3.4 and 4.3).

4.2. The diagnostic VOCs are biomarkers of PM disease

Out of the many criteria for a good biomarker (and any marker in general) sensitivity and specificity (Yerushalmy, 1947), reproducibility and robustness are deemed here to be the most relevant in relation to the six diagnostic BVOCs identified in the wheat-PM interaction.

Sensitivity (correct identification of all diseased plants) and specificity (no healthy plants are found to be false positive) were fulfilled for the six BVOCs in all the analyzed specimens (Figs. 4, 6), in total 120, which were collected from 56 healthy control and 64 diseased pots (Supplementary Method). Reproducibility was demonstrated by the consistently significant differences (Table S3**:** sheets 2–4) for all the BVOCs between 40 healthy control and 48 inoculated pots in three consecutive years (Fig. 4) representing different temperature conditions during the incubation period and sampling days (Table S1, Fig. S2).

Finally, robustness, i.e. the stability of detection in this BVOC set under non-optimal conditions, could be verified in the growth chamber experiment (Section 3.3). The spontaneous infection with *Bgt* of additional wheat genotypes originally inoculated with *Fusarium* spp. provided an unexpected opportunity to test these BVOCs in a complex background. Indeed, in a real-time scenario in the field the detection of a pathogen should happen in the presence of other microorganisms, pathogens and pests. The three most abundant *Bgt*-specific BVOCs (C8 oxylipins) were easily detected even in the early symptomatic phase (Fig. S1) compared to the control (Fig. 6), whereas plants only positive for *Fusarium* did not release these BVOCs, but primarily sesquiterpenes (unpublished results). Since the main potential sources, molds are associated with wheat during grain storage (Section 4.1) these data again indicate the *Blumeria*-specificity of these biomarkers in wheat plants. Another proof of the robustness of these BVOCs was the full-grown (flowering) stage of the plants in this experiment, which is far from optimal for PM disease development (Cunfer, 2002).

While in 2018 and 2019 untargeted analyses were performed in order to discover diagnostic BVOCs, in 2020 the targeted analysis of the six VOCs validated their utility in monitoring PM disease and *Bgt* pathotypes. According to the temperature records (Table S1, Fig. S2) there was a warming trend in the consecutive years, and despite the fact that 2020 was the warmest test period, especially during the 14 DAI samplings (Table S1C), the biomarkers performed as expected during these variable years (Fig. 4). The only systematic exception appeared at 14 DAI in 2020 when inoculation with pathotype 71 resulted in significantly lower quantities released for all six BVOCs (Fig. 4). This reaction coincided with great extremes in the day (max. 44.2 °C) and night (min. 10.3 °C) temperatures during the incubation period between 7 DAI and 14 DAI in 2020 (Table S1C).

It can be concluded that the diagnostic BVOCs meet the basic requirements for reliable biomarkers; however, a thorough confirmation is required under field conditions (in progress). In addition, the (positive or negative) predictive value of these volatile biomarkers also needs to be established in the field.

4.3. Environmental relevance and elucidation of future steps

The question is, whether a substantial part of the estimated 188 t of biomarker BVOCs/month released worldwide by PM-diseased wheat plants reaches the atmosphere? The detection of C6-C9 BVOCs (including OTL), called green leaf volatiles, by independent aircraft and satellite observations (Joutsensaari et al., 2015; Yli-Pirilä et al., 2016) suggests that the answer is a definite 'yes'. However, the fate and actual atmospheric concentrations of the biomarker BVOCs depend on the interaction and balanced effects of numerous factors. These factors include fluxes (Bachy et al., 2016, 2020) determined by the diffusion rate, stability and reactivity with atmospheric components, leading to the formation of secondary organic aerosols (SOA) – all of which are largely unknown for these BVOCs. In chamber experiments, OTL was found to be highly reactive with ozone and hydroxy radicals (Li et al., 2018; Fischer et al., 2020) as well as with chlorine (Grira et al., 2020), the major troposphere oxidants. Besides the internal alcoholic hydroxyl group, the terminal unsaturated double bond may be primarily responsible for this reactivity, which indicates that OTL is likely to form oxygenated products with various types of atmospheric nitrogen oxides (NO_x), too. The estimated half-life of OTL in the atmosphere appears to depend on the reaction type: due to its higher concentration reactions with the hydroxyl radical are predominant and result in a shorter half-life (about 3 h), whereas during ozonolysis it may amount to more than 1 day (Li et al., 2018). These data indicate that OTL and possibly other biomarker BVOCs may definitely contribute to the formation of SOA in agroecosystems.

On the basis of these observations, major BVOCs released in agroecosystems and especially by phytopathogenic fungi should be monitored in the future for their effects on the biodiversity of these

ecosystems. For the characterization and quantitation of these agro-BVOCs we develop a combination of analytical, machine learning and molecular biology approaches. As a first step, the identified biomarker BVOCs have already been detected under field conditions in wheat-*Bgt* interactions and more recently a different, but overlapping pattern of the same biomarkers has been observed in a vineyard infected with *Uncinula necator*, a related PM pathogen (unpublished data). Though wheat fields and vineyards are usually well separated spatially the specific biomarker patterns may allow a clear distinction even in adjacent areas. With respect to potential interference with BVOCs emitted by soil microbes there are limited field data available as opposed to odors from spoilage during storage of crops (including wheat and grape: Barkat et al., 2017; Lopez Pinar et al., 2017a, 2017b). To characterize this BVOC background, experiments are being performed on fallow land. However, monoculture farming at high plant densities in current cultivation systems is expected to produce relatively uniform and less complex signals.

Besides specific biomarkers non-invasive and more instantaneous detection tools are required for on-field monitoring. To this end, *Drosophila* olfactory receptors specific for single biomarker BVOCs have been expressed in cultured mammalian cell lines and tested for their response, sensitivity and specificity to our biomarkers. The most responsive receptor cell lines have been assembled into a sensor for pure BVOCs (manuscript in preparation). Preliminary results with this panel have shown differential reactions to healthy and infected plant samples. The analysis is now refined with the help of machine learning and deep learning techniques (briefly discussed in Section 4.1). It is envisaged that this type of bio(electronic) sensors will be able to detect the infection status of unknown samples and, after mobilization, in the field, too.

5. Conclusions

Despite the economic importance of wheat there is a clear gap in the knowledge on BVOC composition and release, especially during vegetative growth in the field, which is relevant to detect and elucidate the environmental effects of important fungal pathogens such as the obligatory biotroph *Blumeria graminis* f.sp. *tritici* (*Bgt*) causing powdery mildew disease worldwide. A total of 48 different BVOCs were detected and identified, six of which, namely 1,3-octadiene, 1,3-*cis*,5-*cis*-octatriene, 1-heptanol, (5Z)-octa-1,5-dien-3-ol, 1-octen-3-ol and 3-octanone, were found to be present only in the headspace of *Bgt*-inoculated plants. These six BVOCs showed a unique, highly reproducible pattern in their presence and quantities during a three year follow-up period. The latter three, which were the most abundant BVOCs, were robustly applicable for differentiation between healthy and *Bgt*-inoculated wheat plants, as early as 7 days after inoculation, in a number of wheat genotypes at various developmental phases, in two symptomatic stages and even for unidentified *Bgt* strains. These BVOCs are therefore proposed as novel biomarkers for the chemical monitoring of powdery mildew disease in wheat. To the best of our knowledge, this is the first study to systematically assess specific BVOC emission patterns during the interaction of a cereal plant with a foliar fungal pathogen. Powdery mildew-related BVOC biomarkers should also be useful for early disease detection in the agroecosystem for the purposes of plant protection, precision agriculture and environmental monitoring in the field (in progress). The above results are currently being utilized in machine learning as well as for the development of a novel e-nose biosensor for BVOC-based detection and chemotyping.

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CRediT authorship contribution statement

Kamirán Áron Hamow: Conceptualization, Methodology, Formal analysis, Investigation, Software, Validation, Visualization, Project administration, Supervision, Writing – original draft. **Zsuzsanna Ambrózy:** Conceptualization, Formal analysis, Investigation, Visualization, Project administration, Writing – review & editing. **Katalin Puskás:** Methodology, Formal analysis, Validation, Writing – review & editing. **Imre Majláth:** Formal analysis, Software, Validation, Visualization, Writing – review & editing. **Mónika Cséplő:** Methodology, Formal analysis, Validation, Writing – review & editing. **Réka Mátyus:** Formal analysis, Software, Project administration, Writing – review & editing. **Katalin Posta:** Investigation, Writing – review & editing. **Péter Lukács:** Conceptualization, Investigation, Project administration, Resources, Supervision, Funding acquisition, Writing – review & editing. **László Sági:** Conceptualization, Methodology, Formal analysis, Investigation, Validation, Visualization, Supervision, Writing – original draft and finalization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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