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1 **MICROORGANISMS IN THE PHYLLOPLANE MODULATE THE BVOC**
2 **EMISSIONS OF *Brassica nigra* LEAVES**

3
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15
16 **Abstract** - Numerous factors can affect the Biogenic Volatile Organic Compounds (BVOC)
17 emitted by plants. One of these factors is the microbial communities living on leaf surfaces
18 (phylloplane). Bacteria and fungi can use compounds produced and emitted by plants for their
19 own metabolism. Thus, microorganism communities can modulate BVOC emissions and
20 affect interactions between plants and other organisms.

21 The aim of this study was to evaluate the role of microbial communities on BVOC emissions
22 of *Brassica nigra* leaves. Therefore, we removed bacteria and/or fungi by using bactericide/
23 fungicide treatments in a factorial design experiment with *Brassica nigra* grown in pots.
24 BVOC emissions were sampled before and after the treatment application.

25 Our results showed that four new compounds (cyclohexanone, cyclohexyl cyanide and two
26 unknown compounds) were emitted after the removal of fungi, whereas no effect was
27 detected in response to the bactericide treatment. This suggests that fungi inhibit or reduce the
28 production of the above mentioned BVOCs from *Brassica nigra* leaves or use those
29 compounds for their own metabolism. The origin and the roles of the novel compounds
30 emitted requires further investigation.

31
32 **Key words** – Microorganisms, BVOC emissions, phylloplane.

INTRODUCTION

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Plants emit a large variety of Biogenic Volatile Organic Compounds (BVOC) belonging to several different chemical groups. These BVOCs include terpenes (e.g. isoprene, monoterpenes and sesquiterpenes), Green Leaf Volatiles (e.g. alcohols, aldehydes and acetates) and benzenoids (Dudareva *et al.* 2013). BVOCs have numerous different roles in plants, including (i) protecting plants against abiotic stress (e.g. water deficit, heat stress) through antioxidant properties (Vickers *et al.* 2009), (ii) attracting pollinators (Farré- Armengol *et al.* 2016), (iii) repelling herbivores and attracting their natural enemies, and (iv) protecting against pathogens (Trowbridge & Stoy 2013).

There are numerous factors that can modify BVOC emissions, including light and temperature, atmospheric pollution (Peñuelas & Staudt 2010), pathogen infection and herbivore attack (Trowbridge & Stoy 2013). There is also some evidence suggesting that the communities of microorganisms living on leaf surfaces, known as the phylloplane (Peñuelas & Terradas 2014), could alter the blend of BVOC emissions released by plants. This has been demonstrated for *Sambucus nigra* (Peñuelas *et al.* 2014). Numerous studies have shown that microorganisms (fungi and bacteria) can produce and emit a diverse range of BVOCs, including fatty acids, terpenes, alcohols and aldehydes (Kanchiswamy *et al.* 2015). Moreover, a relationship has been elucidated between the microorganisms within the phylloplane and their host plant, whereby the microorganisms use the different products produced and emitted by plants as substrates for their own metabolism (Vacher *et al.* 2016). For instance, methanol emitted by plants during the process of vegetative growth, can be used as a carbon source by microorganisms (Vorholt 2002). Therefore, it is likely that the quantity and/or the quality of BVOCs observed are modulated by microorganisms, though only a few studies have so far have addressed this issue (e.g. Peñuelas *et al.* 2014). It is important to understand the role of the phylloplane in the production and emission of BVOCs, since it could have a tangible role in all the biological and ecological functions attributed to BVOCs (Peñuelas & Terradas 2014). This has also practical relevance, since in modern agriculture, phylloplane microorganisms are commonly removed via pesticides from plant leaves, which could have a significant impact on plant's interactions with other organisms such as other plants, herbivorous insects or pollinators.

In this study, we investigated whether the removal of microorganisms from the surface of the phylloplane can change the blend and magnitude of BVOC emissions from *Brassica nigra* leaves. Using both a fungicide and a bactericide, we distinguished between the roles of

68 bacteria and fungi in BVOCs observed. Our hypothesis was that the BVOC emissions would
69 be modulated by the removal of microorganisms.

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METHODS AND MATERIALS

72 *Plant material.* Seeds of *Brassica nigra* were sown in 0.8L plastic pots containing a mix of
73 peat, soil and sand (3:1:1). Seeds were provided by E. Poelman (Wageningen University, the
74 Netherlands). A total of 20 plants were grown in plant growth chambers (Weiss Bio 1300m,
75 Germany) with a 16h/8h day/night cycle. Daytime conditions of 21°C and 60% relative
76 humidity and nighttime conditions 16°C and 80% RH were maintained. Plants were watered
77 every day and fertilized twice per week with a 0.1% solution containing nitrogen,
78 phosphorous and potassium in a 19:4:20 ratio (Kekkilä Oyj, Finland).

79

80 *Experiment.* *B. nigra* were separated into four groups of five plants, which were each assigned
81 to a different treatment and evaluated in a factorial design experiment: control (C), bactericide
82 (B), fungicide (F) and fungicide + bactericide (FB). An initial round of BVOC sampling was
83 performed 24h before the treatment application. The bactericide solution contained
84 streptomycin sulfate (Sigma-Aldrich, USA) at 300mg.L⁻¹, the fungicide solution contained
85 ciclopirox (Sigma-Aldrich, USA) at 50mg.L⁻¹ and the bactericide + fungicide treatment
86 contained both products at the same concentrations as above. Water was applied as a control.
87 Twenty mL of solution was sprayed to each plant. Twenty-four hours after the application of
88 treatments, BVOCs were sampled for a second time.

89

90 *BVOC collection and GC-MS analyses.* Each plant was enclosed in a polyethylene
91 terephthalate bag with dimensions of 35x43cm (Eskimo, Finland). Before use, each bag was
92 heated in an oven at 120°C for 1h. Pressurized inlet air was filtered through activated charcoal
93 and introduced into bags at 300mL.min⁻¹. After flushing the bags with clean air for 20mins,
94 BVOC emissions were collected for 1h into pre-conditioned cartridges containing Tenax TA
95 (Markes International, UK), which were positioned at the outlet of the bag. Cartridges were
96 connected to a vacuum pump (D-79112, KNF, Germany) via clean silicone tubing and
97 samples were pulled with a flow rate of 220mL.min⁻¹. Both inlet and outlet airflows were
98 calibrated with a mini Buck calibrator (mini Buck calibrator, AP Buck, USA). After
99 collection, cartridges were stored at 4°C before analysis.

100 Analysis of cartridges was performed by gas chromatography (GC, model 6890, Hewlett
101 Packard, USA) mass spectrometry (MS, model 5973, Hewlett Packard, USA) with samples

102 thermally desorbed with an autosampler (ATD 400 Perkin Elmer, USA). Samples were
103 desorbed at 250°C for 10 min, cryofocused at -30°C in split mode with a ratio of 1/20. The
104 column used to separate molecules was an HP5-MS (60m x 0.25mm x 0.5µm, Agilent, USA).
105 The chromatography program was set up as follows: 40°C at the beginning and with a hold
106 for 2min, 3°C.min⁻¹ temperature ramp to 210°C, and then a 5°C.min⁻¹ temperature ramp to
107 250°C. This final temperature was held for 5 min in order to clean the column. The carrier gas
108 was helium.

109 BVOC identification was corroborated with analytical standards (Sigma-Aldrich, USA),
110 which were injected as an external reference (and specified in Table 1 in supplementary files).
111 Kovats indices (calculated with a C8-C20 series of alkanes) as well as NIST and WILEY
112 libraries were used to further confirm compound identification. The analytical standards were
113 also used to quantify the BVOC emissions.

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115 *Statistical analyses.* Statistical analyses were performed with R software (v. 3.4.3). Partial
116 Least Squares – Discriminant Analyses (PLS-DA) were performed with the package
117 RVAideMemoire after log and auto-scaling transformation of the data. Pairwise tests were
118 performed based on the PLS-DA, with a cross validation with 50 submodels. The graphics for
119 the PLS-DA were built with metaboanalyst (<https://www.metaboanalyst.ca/>). The compounds
120 showing the highest scores in variable importance for projection (VIP > 2) were analysed for
121 each treatment with a paired Wilcoxon test to highlight the before and after treatment
122 differences.

123

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RESULTS

125 The emission rates for all individual compounds detected in the leaf blend of *B. nigra* are
126 available in Table S1 in supplementary files. PLS-DA analysis of the BVOC emissions prior
127 to application of the different treatments revealed no separation between the groups and
128 pairwise tests did not reveal significant differences for any individual compounds (Fig. 1, $P >$
129 0.05). Thus, the BVOC emissions from *B. nigra* leaves were the same between the different
130 groups before the application of treatments. After application of the antimicrobial treatments,
131 PLS-DA revealed a strong separation between both treatments with the fungicide (fungicide
132 and fungicide + bactericide) and the control and bactericide only treatments. Pairwise tests
133 confirmed this result and showed a marginally significant difference between the blends
134 emitted by plants treated with both fungicide treatments and the others ($0.1 < P < 0.05$).
135 Emission rates for all compounds did not significantly change though there was a trend

136 towards higher emissions with fungicide treatments, and are depicted in Fig. S1 (in
137 supplementary files).

138 Application of the fungicide resulted in four new compounds appearing in the blend:
139 cyclohexyl cyanide, cyclohexanone and 2 unknown compounds (Table 1; Fig. S2 and S3, in
140 supplementary
141 files). These four compounds were emitted in greater amounts after application of the
142 antifungal treatments than before the application ($0.1 < P < 0.05$). However, the appearance of
143 those four compounds did not significantly change the total emission rates between treatments
144 ($P > 0.05$, $22.1 \pm 6.9 \text{ ng.g}_{\text{DW}}^{-1}.\text{h}^{-1}$, $19.4 \pm 8.6 \text{ ng.g}_{\text{DW}}^{-1}.\text{h}^{-1}$, $85.2 \pm 44.2 \text{ ng.g}_{\text{DW}}^{-1}.\text{h}^{-1}$, 124.8 ± 74.8
145 $\text{ng.g}_{\text{DW}}^{-1}.\text{h}^{-1}$ for control, bactericide, fungicide and fungicide + bactericide, respectively, Fig.
146 S1 in supplementary files), though the standard deviation was high for the five replicates and
147 a tendency towards higher emissions for the fungicide treatments was noted, as mentioned
148 above.

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DISCUSSION

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152 In our study, four novel compounds were found in the blend of BVOCs emitted from leaves
153 after the application of the fungicide treatment. This observation could be the result of three
154 different mechanisms. Firstly, the results could suggest that, unlike bacteria (see below), fungi
155 may inhibit the emission of certain BVOCs. Secondly the novel compounds observed may
156 have been taken up and metabolized by fungi living in the phylloplane of the *B. nigra* leaves.
157 Indeed, it has earlier been shown that microorganisms have the ability to consume BVOCs
158 and metabolize them (Vacher *et al.* 2016). Thirdly, it is possible that the fungicide used in this
159 study could have disturbed the metabolism of plants, suggesting possible adverse effects of
160 pesticide treatments on plants in agriculture.

161 One of the novel compounds observed, cyclohexyl cyanide, could be directly emitted by *B.*
162 *nigra* leaves, as it is well documented that *Brassicaceae* species emit nitriles (Liu *et al.* 2018).
163 The function of cyclohexanone is still unclear, but previously it has been shown that the
164 emission of this compound is bound to bacterial activity (Perl *et al.* 2011). It is also plausible
165 that cyclohexyl cyanide has a bacterial origin, since a similar compound, 4-cyanocyclohexene
166 (known as cyclohexenyl cyanide), was found to be emitted by bacteria (Groenhagen *et al.*
167 2016). Both of the unknown compounds were difficult to identify based on compound
168 libraries and retention indices. The unknown compound 1 was annotated as β -thujaplicin
169 (mass spectra presented in supplementary files S4). However, the experimental Retention

170 Index (RI, 1229) for this compound did not match with published RIs for β -thujaplicin in
171 literature (1475, <https://webbook.nist.gov/chemistry/>). The unknown compound 3 was
172 annotated as 1,4-benzoquinone,2,6-diethyl-,1-imine. As for the unknown compound 1, this
173 annotation is not clear and should be confirmed by analytical standard injection.

174 Our results showed that bactericide treatment did not affect the quality or quantity of
175 emissions. These results differ from those of Peñuelas *et al.* (2014) where they showed a
176 decrease of *Sambucus nigra* floral emissions after bactericide treatment. In their study, they
177 used three different bactericides at relatively high concentrations that have a broad effect on
178 bacterial communities, whereas in our study we used a lower concentration of a single
179 bactericide. This difference in protocol could be responsible for the different results obtained.
180 Thus, the new compounds detected in the volatile blend after removal of the fungi could have
181 been emitted by the plant, but could also have been emitted by bacteria remaining on the plant
182 if the bactericidal treatment failed. However, it should also be noted that bacterial
183 communities of flowers and leaves are likely to be different and consequently the role of
184 bacteria in floral and leaf emissions could vary substantially (Junker *et al.* 2011).

185 It seems that there is a close relationship between the fungal communities existing on the
186 plant leaf surfaces of *B. nigra* and the composition of BVOC emissions (through inhibition
187 and/or metabolism). More studies are now required to determine if the new compounds
188 observed are emitted directly from the plant and are linked to microbial metabolism or if other
189 microorganisms emit them directly. Future studies should also focus on the microbes likely to
190 be controlling the BVOC emissions, e.g. by characterizing the microbial communities present
191 with and without the different treatments using modern molecular methods (Delmotte *et al.*
192 2009; Ryffel *et al.* 2016). It has also to be noted that this experiment was performed in
193 controlled conditions which could have resulted in impoverished microorganism communities
194 living on the phylloplane compared to natural conditions (Shaukat *et al.* 2014). Thus, it is
195 possible that different results could have been observed in the field. Performing this
196 experiment under more natural conditions would enable further elucidation of the ecological
197 roles of microorganism-induced changes in VOC blends.

198 As suggested by Peñuelas and Terradas (2014), microbial communities seem to play an
199 important role in the blend of BVOCs emitted by plants. Therefore, it is crucial to improve
200 our understanding of the relationships between phylloplane communities and plants,
201 especially in terms of BVOC emissions. Changes in those communities, by fumigation with
202 bactericides and fungicides that are largely used in modern agriculture, could lead to
203 modification of plant BVOCs. Consequently, the changes could disturb a plant's interactions

204 with other organisms such as other plants, herbivorous insects or pollinators. All these
205 hypotheses require further investigation.

206

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211

212 AUTHOR CONTRIBUTION

213 AS, JDB and CB designed the experiments. AS and PM conducted the research, collected and
214 analyzed the data. AS and JDB wrote the manuscript.

215

216 CONFLICT OF INTEREST

217 AS, PM, CB and JDB declare that they have no conflict of interest.

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261 **Table 1.** Four compounds indicated through VIP scores as separating fungicide treatments
 262 from other treatments. Paired Wilcoxon tests according to the time of sampling (before and
 263 after application) for fungicide (F) and fungicide+bactericidal (FB) treatments with n=5.

	Compounds			
	cyclohexanone	cyclohexyl cyanide	unknown compound 1	unknown compound 3
RT (min)	16.289	22.626	35.791	45.453
Kovats index	897	1013	1259	1466
Formula	C ₆ H ₁₀ O	C ₇ H ₁₁ N	C ₁₀ H ₁₂ O ₂	C ₁₀ H ₁₃ NO
Type of compound	Ketone	N-containing compound	Oxygenated monoterpene	N-containing compound
VIP scores	C1:2.5 ; C2: 2.4	C1:2.5 ; C2: 2.4	C1:2.5 ; C2: 2.4	C1:2.5 ; C2: 2.4
<i>P-value_F</i>	0.063	0.063	0.063	0.063
<i>P-value_{FB}</i>	0.063	0.063	0.063	0.063

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287 **Figure 1.** Partial Least Squared – Discriminant Analysis (PLS-DA) performed on all
288 compounds ($\text{ng.g}^{-1}.\text{h}^{-1}$) before (**left**) and after (**right**) the different treatments: control (C),
289 bactericide (B), fungicide (F) and bactericide + fungicide (FB). PLS-DA were performed after
290 auto-scaling and log transformation of the dataset. Pairwise tests indicate a difference between
291 treatments containing fungicide (fungicide, fungicide + bactericide) and the others (control
292 and bactericidal) after the application, with $n=5$ and $0.05 < \text{p-value} < 0.1$.

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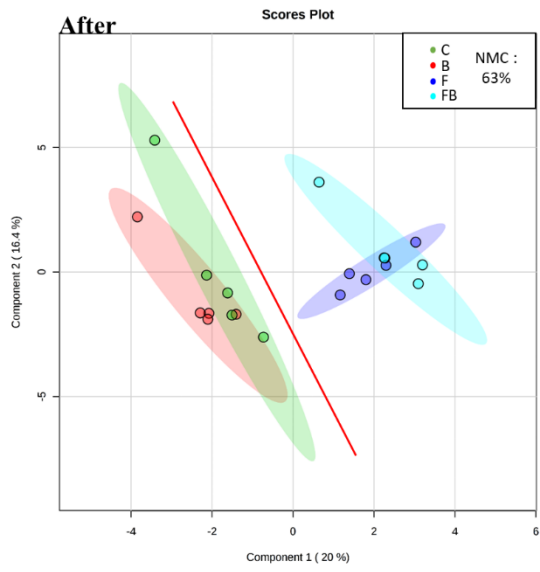
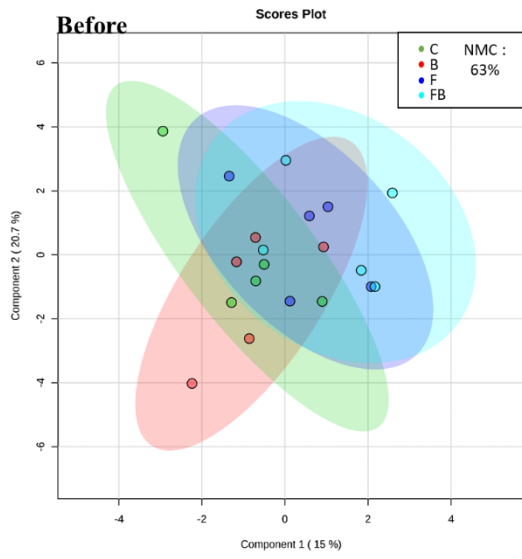
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312 **Figure 1.**

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