1 # *Carum carvi* essential oil: a promising candidate for botanical
2 herbicide against *Echinochloa crus-galli* in maize cultivation

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17 **Highlights (3 to 5 bullets, max 85 caracters per point including spaces**

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19

20 **Abstract**

21 In this study we tested the possibility that foliar-applied caraway or peppermint essential oils

22 (EOs) can selectively inhibit the growth of *Echinochloa crus-galli* (a typical maize weed) but

23 not that of maize plants, attempting to develop an eco-friendly botanical herbicide.

24 We tested the phytotoxic potential of oil-in-water emulsions of each EO with addition of

25 commercial adjuvant mainly composed of fatty acids methyl esters, studying their effect on

visible plants injuries, biomass accumulation, chlorophyll *a* fluorescence and changes to biochemical patterns of both the main crop (maize) and the weed (*E. crus-galli*) via an untargeted metabolomic approach. We found that oil-in-water emulsion containing 2.5% of adjuvant and of caraway EO did not affect significantly the growth of maize plants, did not induce foliar symptoms and did not alter the status of the photosynthetic apparatus, as revealed by chlorophyll *a* fluorescence. On the contrary, this emulsion exerted significantly negative effects against *E. crus-galli* growth, inducing foliar injuries and reducing the photosynthetic efficiency of photosystem II. We also found that the studied emulsions caused a series of biochemical changes in the plant tissues, with caraway emulsion being more phytotoxic, as compared to the peppermint EO-emulsion. We conclude that oil-in-water emulsion containing 2.5% of caraway EO could be used in future as a foliar-applied botanical herbicide against *E. crus-galli* in maize cultivation.

***Keywords*:** Bio-herbicide; biochemical process; chlorophyll *a* fluorescence; leaf injury; metabolomics; phytotoxicity

41 **1. Introduction**

42 Numerous studies shown that essential oils (EOs), especially those for which the main
43 compounds are monoterpene alcohols or oxygenated monoterpenes, are promising substances
44 for production of botanical herbicides, since they can cause significant inhibition of weed
45 germination and growth (Benvenuti et al., 2017; Synowiec et al., 2017, Rolli 2014, Vokou et
46 al., 2003), as well as they provoke, severe leaf burns in foliar applications (Bainard et al., 2006,
47 Stokłosa et al., 2012). Considering the chemical composition of their EOs, peppermint (*Mentha*
48 x *piperita* L:) and caraway (*Carum carvi* L.) can be promising candidates for the production of
49 bioherbicides in a temperate European climate (Synowiec et al., 2017). Both species are widely
50 cultivated in Europe (Oroian et al., 2017; Seidler-Łożykowska and Bocianowski 2012) and are
51 characterized by high EO yields. In particular, in plants cultivated in Poland, EOs extraction
52 from peppermint leaves and caraway seeds could give a yield around 2.3% and 3.4-4.8%,
53 respectively (Pisulewska et al., 2010; Seidler-Łożykowska et al., 2013). Both EOs are rich in
54 oxygenated monoterpenes (> 80 % for peppermint and > 60 % for caraway EO) (Seidler-
55 Łożykowska et al., 2013; Fejér et al., 2017). The main chemical compounds of these EOs are
56 menthol and menthone in peppermint (Guidi and Landi, 2014), and carvone and limonene in
57 caraway EOs (Chemat et al., 2017).

58 However, physical and chemical properties of EOs, such as high volatility or poor water
59 solubility make difficult a wider use of them as natural herbicides. These disadvantages can be
60 overcome by creating appropriate emulsions. As shown by Hazrati et al. (2017), garden savory
61 (*Satureja hortensis*) EOs applied as oil-in-water (o/w) nanoemulsion, with 2% (v/v) Tween 80,
62 displayed adequate physical properties and posed a strong phytotoxic potential on germination
63 and early growth of *Amaranthus retroflexus* and *Chenopodium album*. In turn, Synowiec et al.
64 (2017) showed satisfactory effectiveness of o/w emulsion of peppermint EO (2.5%) applied
65 with the addition of oilseed rape fatty acid methyl esters (1.5 L ha^{-1}) against *E. crus-galli*.

66 Foliar-applied EOs display a contact action and induce visible injuries caused as early as
67 few hours following their application (Hazrati et al., 2017; Synowiec et al., 2015). In general,
68 foliar-applied EOs mixture leads to a general impairment of plant metabolism due to multi-
69 spectrum targets (Synowiec et al., 2015). Conversely, application of a single or a few
70 compounds isolated from EO may act selectively via inhibition of a specific metabolic pathway
71 (Araniti et al., 2017a; Araniti et al., 2016; Graña et al., 2013), but this approach is often more
72 important for obtaining a total herbicide rather than a selective herbicide. Many experiments
73 showed that one of the main effects of EOs is the inhibition of photosynthesis, resulting from a
74 decrease in the chlorophyll content (Hazrati et al., 2017) and alterations of the light phase of
75 photosynthesis (Synowiec et al., 2015). In some cases, EOs lead to the production of
76 uncontrolled level of reactive oxygen species, thereby promoting oxidative stress and oxidative
77 burst (Ahuja et al., 2015) as well as loss of the efficiency of cellular respiration (Kaur et al.,
78 2010). Recently, Araniti et al. (2018), through a physiological and metabolomic approach,
79 described in detail the physiological response of *Arabidopsis thaliana* seedlings to the EO of
80 oregano. The authors observed a reduction of plant growth and leaf chlorosis of *A. thaliana*
81 seedlings as a result of series of metabolic alterations, including principally the inability to
82 incorporate assimilated nitrogen into amino acids, especially the nitrogen devoted to the
83 biosynthesis of one of the first precursors of other amino acids, namely glutamine.

84 The metabolomic approach allows to analyze simultaneously hundreds of metabolites in a
85 given biological sample (Nicholson and Lindon, 2008), yielding a comprehensive picture of
86 changes in the metabolism of plants under different types of stresses (Mosa et al., 2017, Ghatak
87 et al., 2018). Therefore, untargeted metabolomics could consent to characterize the phytotoxic
88 effects of foliar application of EOs emulsion on key metabolic pathways, in order to understand
89 the main biochemical/physiological processes altered in the plant. For this reason, this research
90 aimed at: i) assessing the phytotoxic potential of foliar-sprayed peppermint or caraway EOs,

applied as o/w emulsions with addition of a commercial adjuvant in maize (*Zea mays* L.) and barnyardgrass [*Echinochloa crus-galli* (L.) P.Beauv.], and ii) employ the imaging of chlorophyll *a* fluorescence and an untargeted metabolomic approach to dissect plant responses to foliar application of EOs emulsions.

2. Materials and Methods

2.1. Characteristic of essential oils and adjuvant

The EOs isolated from caraway (*Carum carvi* L.) seeds was purchased from the Avicenna-Oil company (Wrocław, Poland), whereas the essential oil of peppermint (*Mentha ×piperita* L.) was steam-distilled for 2 h in the laboratory conditions using Deryng-type apparatus (Baj et al., 2015) from the air-dry mass of herbs collected from the production fields in Michałowice, Poland (50°37’45’’N, 20°48’03’’E), in summer 2015.

Commercial multifunctional adjuvant ATPOLAN BIO 80 EC (Producer: AGROMIX Niepołomice, Poland) was chosen as adjuvant and emulsifier of EOs. This adjuvant is mainly composed of fatty acid methyl esters of oilseed rape oil (80%), surfactants and a pH buffer (according to the product label provided by the producer). It was chosen as in previous experiments this adjuvant displayed good herbicidal potential as an emulsifier of peppermint or caraway EOs (Synowiec and Drozdek, 2016).

2.2. Chemical analysis of essential oils

The chemical composition of the EOs was analyzed by gas chromatography coupled with mass spectrometry (GC-FID-MS). After dilution in diethyl ether (10 µL in 1 mL), the EOs were analyzed using a Trace GC Ultra gas chromatograph coupled with DSQ II mass spectrometer (Thermo Electron Corporation). The operating conditions were as follows: non-polar capillary column Rtx-1ms (60 m x 0.25 mm, 0.25 mm film thickness), programmed temperature: 50 (3

116 min)-300ºC, 4ºC/min, injector (SSL) temperature 280ºC, flame ionization detector temperature
117 300ºC, transfer line temperature 250ºC, carrier gas - helium, flow with constant pressure 200
118 kPa, split ratio 1:20. The mass spectrometer parameters: ion source temperature 200°C,
119 ionization energy 70 eV (EI), scan mode: full scan, mass range 33-420. The percentages of
120 constituents were computed from the GC peak area without using a correction factor.
121 Identification of EO components was based on a comparison of their mass spectra and linear
122 retention indices (RI, non-polar column), determined with reference to a series of n-alkanes C8-
123 C26, by comparing with those reported by Adams (2007) as well as in computer libraries: NIST
124 2011, and MassFinder 4.1. Percentages were obtained from the FID response without the use
125 of correction factors.
126

127 *2.3. Preparation of o/w emulsions*

128 The emulsion (250 mL) based on 2.5% or 5% of each of EO was prepared at room
129 temperature. For the emulsion, 6.25 g or 12.5 g of selected essential oil, respectively were
130 weighted into a vial. Then, 6.25 g (for 2.5% solution) or 12.5 g (for 5% solution) of ATPOLAN
131 BIO 80 EC was added. This mixture was stirred vigorously on magnetic stirrer (300 rpm) using
132 3 cm stir bar. Afterwards, while constant mixing 237.4 mL or 225 mL of distilled water was
133 added in small portions. Then the stirring was increased to 500 rpm for 5 min. Prepared
134 emulsion was homogenized using handheld homogenizer (Ingenieurbüro CAT M. Zipperer
135 GmbH, Unidrive D, rotation speed 5000 rpm). The emulsions were stored at room temperature
136 until use.
137

138 *2.4. Pot experiment*

139 A pot experiment was settled up in the foil tunnel (16 m long, 6 m wide and 3 m high) in
140 Krakow-Mydlniki, south of Poland (N 50° 08’54’’, E 19° 85’21’’), with daily temperature

141 monitoring. The experiment was established in the period of 6th April – 8th June 2017. There
142 were ten replications (plants) per each species and emulsion treatment.
143 Seven seedling palettes (0.154 m^2 with 24 pots in 4 rows with a single pot size: 46 x 46 x 70
144 mm per each palette), were filled up with a sieved layer (0-15 cm) of a sandy brown soil (pH
145 6.3; P_2O_5 18.2; K_2O 7.5; MgO 6.9 [mg 100 g^{-1} of soil]). Two seeds of maize (cv. 'Wilga') or a
146 few seeds of barnyard grass (*Echinochloa crus-galli* (L.) Beauv) were sowed into the 10 of pots
147 per palette per species and after emergence the number of plants was thinned to one per pot.
148 The four middle pots per palette were left empty to avoid shading between plants of maize and
149 barnyard grass and to optimize the spraying process. Then the palettes were positioned
150 alternately. During growth the plants were watered according to their needs.
151 When maize reached the growth stage of 4-6 leaves and barnyard grass the stage of 3-4 leaves,
152 the plants were hand-sprayed with one of the following emulsions:
153 i) Water only (control; W);
154 ii) Water + 2.5 % adjuvant (Control; WA2.5);
155 iii) Water + 5.0 % adjuvant (Control; WA5.0);
156 iv) Water + 2.5 % caraway EOs + 2.5 % of adjuvant (WAC2.5);
157 v) Water + 5.0 % caraway EOs + 5.0 % of adjuvant (WAC5.0);
158 vi) Water + 2.5 % peppermint EOs + 2.5 % adjuvant (WAP2.5);
159 vii) Water + 5.0 % peppermint EOs + 5.0 % of adjuvant (WAP5.0).
160 Each palette was sprayed with 10 cm^3 of one of the emulsions (i-vii), using a 1 L volume hand
161 pressure sprayer Kwazar Venus Super 360 PRO+ (Producer: Kwazar Corporation Sp. z o.o.,
162 Poland). The calculated amounts of the EOs in the spraying solutions were equal to 1.5 g m^{-1}
163 (solutions iv and vi) and 3.0 g m^{-1} (solutions v and vii).
164 Seven days after spraying the plants were visually assessed (by one person) for the percentage
165 of aboveground injuries (0-100%) caused by the foliar-application of emulsions (i-vii). Next,

166 the plants were cut at the ground level. For each plant, the aboveground parts were placed in
167 envelopes and dried in the temperature of 50 °C in a lab oven for 3 days. After that, their dry
168 mass was recorded.
169

170 *2.5. Chlorophyll a fluorescence imaging*

171 The analyses were carried out on 5 plants (replications) of each species and for selected
172 treatments:
173 i) Water (Control; W). The two other control treatments, namely: water plus 2.5% of
174 adjuvant and water plus 5% of adjuvant, showed results similar to water, so for this
175 analysis only water as a control is presented;
176 ii) Water + 2.5 % caraway EOs + 2.5 % of adjuvant (WAC2.5);
177 iii) Water + 5.0 % caraway EOs + 5.0 % of adjuvant (WAC5.0);
178 iv) Water + 2.5 % peppermint EOs + 2.5 % adjuvant (WAP2.5);
179 v) Water + 5.0 % peppermint EOs + 5.0 % of adjuvant (WAP5.0).
180 Plants of maize and barnyard grass were grown and sprayed with the emulsions similarly as in
181 the pot experiment. A second leaf was cut 48 hours after spraying and placed flat on filter paper
182 moistened with distilled water, and immediately placed in a lightproof measurement chamber
183 FluorCam FC 800C (Photon Systems Instruments, Czech Republic) for 20 min of dark. The
184 measurement was taken right after a pulse of saturation actinic light (4,000 μmol $m^{-2}s^{-1}$ PAR,
185 800 ms), according to Lichtenthaler et al. (2005). The following parameters were analyzed: 1)
186 Fv/Fm (*aka* QYmax) – maximum quantum yield of photosystem II photochemistry, 2) NPQ-
187 non-photochemical quenching and 3) Rfd-fluorescence decrease ratio (Kalaji et al., 2014). All
188 of the fluorescence parameters were graphically imaged using FluorCam 7, ver 1.0.20.4
189 software (www.psi.cz/downloads/). The color scale presents conventional values of the studied
190 parameters of leaves subjected to treatments with the emulsions. The numeric comparisons

191 between the treatments were performed based on a calculation of a "mean gray value", which
192 is the sum of the gray values of all the pixels in the selection, by converting each RGB pixel to
193 grayscale in ImageJ software ver. 1.52a (http://imagej.nih.gov/ij).
194

195 *2.6. The identification and quantification of primary metabolites in the aboveground parts of*
196 *plants*

197 The selected three plants (replications) sprayed with the higher doses of emulsions for both
198 EOs were selected for these analyses, as they displayed significant increases in injuries as well
199 as significant reductions in plants' biomass, following their foliar applications.

200 i) Water + 5.0 % adjuvant (control; WA5.0);
201 ii) Water + 5.0 % caraway EOs + 5.0 % adjuvant (WAC5.0);
202 iii) Water + 5.0 % peppermint essential oil + 5.0 % adjuvant (WAP5.0).

203 Fourty eight hours after spraying the aboveground parts of plants for each species, samples
204 sprayed with the emulsions i), ii) or iii), were collected and freeze-dried. Each plant was frozen
205 separately in liquid nitrogen, homogenized using a laboratory mortar and immediately
206 lyophilized using the Freeze Dry System (Freezone 4.5, Labconco, USA).

207 The metabolome extraction and derivatization, as well as metabolite identification and relative
208 quantification of maize and *E. crus-galli* plants treated with caraway and peppermint EOs, were
209 carried out as previously described by Araniti et al. (2017c). Derivatized samples were injected
210 into a gas chromatograph apparatus (Thermo Fisher G-Trace 1310), equipped with a capillary
211 column TG-5MS (30 m×0.25 mm×0.25 μm), coupled to a single quadrupole mass spectrometer
212 (ISQ LT). Helium at high purity (6.0) was used as a carrier.

213 Injector and source were settled at the temperature of 250°C and 260°C, respectively. Samples
214 (1 μL) were injected in splitless mode with a helium flow of 1 mL min^{-1} and chemical separation
215 was achieved using the following programmed temperature: isothermal 5 min at 70 °C, from

216 70° to 330°C with a ramp of 5°C min^{-1}, isothermal at 330°C for 5 min. Mass spectra were
217 recorded in electronic impact (EI) mode, scanning at 45–500 amu.
218 The identification of the metabolites was carried out comparing the unknown mass spectra with
219 reference spectra of several commercial libraries (NIST 2005, Wiley 7.0, Fiehn library etc.).
220 Metabolites relative quantification was based on a pre-added internal standard (adonitol at 0.02
221 mg mL^{-1}), which was added during the extraction process.
222
223 *2.7. Experimental design and statistical analysis*
224 The experiments were carried out in a completely randomized design with different number
225 of replications depending on the parameter evaluated. In particular, 10 replications for leaf
226 injuries and dry biomass, 5 replications for chlorophyll *a* fluorescence and 3 replications for
227 metabolomic experiments were used.
228 Estimation of plant injuries, biomass production and chlorophyll fluorescence parameters were
229 analyzed with R software (R Core Team, 2014), using 'dplyr' package. The percentage values
230 were Bliss transformed prior analyses. All data were tested for their homogeneity of variance
231 (Levene test) and the normality of distribution (Kolmogorov-Smirnoff test). Data were then
232 analyzed through one-way ANOVA using the Tukey's HSD test as post-hoc ($P \leq 0.05$)
233 ('multcomp' package).
234 A completely-randomized sampling, was applied for metabolomic analyses, for which
235 three independent replicates where analyzed. Metabolite concentrations were checked for
236 integrity and missing values were replaced by a small positive value (the half of the minimum
237 positive number detected in the data). Data were successively normalized by a reference sample
238 (adonitol), transformed through "Log normalization" and scaled through Pareto-Scaling. Data
239 were then classified through Principal Component Analysis (PCA) and metabolite variations

240 were presented as heatmap. Significant differences among the treatments were highlighted
241 through ANOVA using LSD test as post-hoc ($P \leq 0.05$).

242 The analysis of the pathways perturbed by the treatments was carried out using MetPA, a
243 web-based tool that combines the results from pathway enrichment analysis with the pathway
244 topology analysis. Pathway analysis was carried out using the pathway library built on the
245 metabolome of *Oryza sativa japonica* since the two plants studied (*Z. mays* and *E. crus-galli*)
246 are monocots. All the metabolomic analysis were carried out using the software Metaboanalyst
247 3.0 (Xia et al., 2015).

248

249 **3. Results and discussion**

250 *3.1. The chemical composition of essential oils*

251 In Table 1 the chemical composition of caraway and peppermint EOs is reported. In
252 caraway EO the 99.7% of the total ion chromatogram was identified. In particular, the main
253 compounds were limonene (32.5%) and carvone (66.4%), whereas in peppermint EO the main
254 components were represented by menthol (42.7%) and menthone (25.5%) (Table 1). The
255 process of emulsification did not change the chemical composition of EOs (data not shown).
256 These results are in agreement with Synowiec et al. (2017) who observed a similar chemical
257 profile of EOs isolated from both species.

258 The phytotoxicity of the major molecules identified is largely known in literature. Menthone
259 and carvone strongly affected germination and growth of several crops and weeds, including
260 monocotyledonous *Triticum aestivum* and *Zea mays*, *Lolium multiflorum* and *Digitaria*
261 *sanguinalis* and also dicotyledonous *Lactuca sativa* (Vaughn et al., 1993; Sunohara et al.,
262 2015). Among monoterpenes, limonene is one of the most phytotoxic. In fact, recent studies
263 reported that this molecule strongly affected carrot and cabbage growth and development
264 causing leaf injuries and affecting the photosynthetic apparatus (Ibrahim et al., 2004). Similarly,

265 Vaid et al. (2011) observed an inhibition of germination, root growth, pigment content and
266 respiration on *Amaranthus viridis*. Finally, Schults et al. (2007) observed that menthol was
267 affecting *Arabidopsis* growth, dewaxed the leaf cuticular layer and altered stomatal anatomy
268 and function. Moreover, it has been demonstrated that also minor compounds in the EO blend
269 could act synergistically improving the phytotoxicity of the major chemicals (Araniti et al.,
270 2013).

271

272 *3.2. Effect of the emulsions on maize and E. crus-galli plant injuries and biomass*

273 Thermal conditions in the foil tunnel during the course of experiment, both before and after
274 spraying the plants with emulsions (April-June) were similar and in a range of 19-23 °C.
275 Spraying maize with emulsions containing only water and adjuvant did not cause any injuries
276 of maize leaves (Fig. 1A). On the other hand, addition of EOs to the emulsions caused injuries
277 of maize leaves (in the form of necroses) by 8-40 %, as compared to control (W), with
278 significant injuries caused by the emulsions containing 5.0 % of caraway or peppermint oil (Fig.
279 1A). The leaf-spraying of emulsions containing EOs caused a significant reduction of maize
280 dry mass, especially with treatments containing 5% of caraway (57% reduction) or peppermint
281 EO (41% reduction) (Fig. 1B). Should be noted that treatment WAC2.5 did not affect both leaf
282 integrity and plant biomass.

283 *E. crus-galli*, plants sprayed with emulsions, which contained only water and adjuvant
284 (both WA2.5 and WA5.0), did not cause any visible injury (Fig. 2A). Addition of caraway EO
285 to the emulsion caused a significant increase of leaf injuries, as compared to W and WA2.5
286 controls, ranging from 20 to 42% for WAC2.5 and WAC5.0, respectively. Both WAC2.5 and
287 WAC5.0 induced a decrease of plant biomass with respect to W controls and WA2.5 (Fig. 2b).
288 Noteworthy, the use of the adjuvant alone at the higher dose, WA5.0, promoted a reduction of
289 *E. crus-gallis* biomass with a similar extent to those observed with WAC2.5 and WAC5.0,

290 suggesting a negative effect of the adjuvant alone. However, the adjuvant alone did not cause
291 visible injuries at any concentration(Fig. 2A).. On the contrary, WAP2.5 and WAP5.0
292 treatments similarly affected leaf integrity causing leaf injuries on the 30% of the leaf surface
293 (no statistical differences were observed between the two treatments) (Fig. 2). Among
294 treatments, the emulsion containing 5% of caraway oil was the most harmful for *E. crus-galli*
295 leaves (Fig. 2A). Concerning plant biomass, the most significant decrease was observed on
296 plant sprayed with both emulsions containing peppermint oil, which caused a 50% reduction in
297 dry biomass compared to control (W) (Fig. 2B).

298 Therefore, biometric analyses revealed that water emulsions of EOs and a commercial
299 adjuvant composed of FAME of oilseed rape caused a significant reduction of biomass of maize
300 and *E. crus-galli*, with emulsions containing EO of peppermint being more toxic than those of
301 caraway. Notably, a significant reduction of biomass was observed in maize for the dose of EO
302 in emulsions as high as 5% by using caraway oil, whereas for *E. crus-galli* the negative effects
303 occurred even when the lower dose was applied (WAC2.5). For the sack of the truth, reduction
304 of plant biomass could be partially attributable to the effect of the adjuvant alone (see WA5.0
305 biomass reduction) but that WAC5.0 did not cause any visible injury and plant damage (whilst
306 WAC2.5 and WAC 5.0 did) is a valuable result of the effectiveness of caraway EO. Our dataset
307 cannot explain the physiological reasons behind the side effect of adjuvant on *E. crus-gallis*
308 biomass reduction and further research is needed to clarify this point. In any case, visible
309 injuries were only detected in WAC2.5 plants of *E. crus-galli* and not in maize plants, which is
310 a promising result in the attempt to develop a selective botanical herbicide. One should also
311 consider that the selective 20% damage over *E. crus-galli* leaves (which is seems not a
312 negligible result for an ecofriendly botanical herbicide bioassayed at such low concentrations)
313 can strongly reduce the competition between the crop and the pest in the field in an early
314 developmental stage, thus allowing maize to be more competitive and to grow faster. After the

315 first stage, *E. crus-galli* would suffer for the fast-growing maize developing, this resulting in a
316 further reduction of the possibility to compete with maize plants. In addition, our experiment
317 describes the use of a single treatment with WAC2.5 EO, but repetitive treatments could further
318 increase the effectiveness of this EO at 2.5 concentration. Finally, addition of other co-
319 formulants could also increase the effect of WAC EO. For all these reasons, we proposed that
320 the use of WAC 2.5 lead to interesting results which could be exploited proficiently for
321 developing an eco-friendly herbicide. About possible mechanism of action, below we propose
322 the physiological and biochemical reasons on the base of our data. For example, it has been
323 demonstrated that essential oils might act as a desiccant herbicide which alters the leaf cuticular
324 wax layer causing alterations in leaf membrane integrity, dehydration and death (Bainard et al.,
325 2006). Moreover, considering that some species are affected more than others by the same EOs,
326 varying the concentration of a given EOs might be useful to increase/reduce its selectivity
327 allowing weed control without damaging crop growth and production. Recent studies
328 demonstrated that the impairment of photosynthetic process is one of the main cascade effects
329 of multi-target EOs (Araniti et al., 2018). Therefore, in principle we decided to investigate the
330 effects of peppermint and caraway EOs in relation to changes induced in chlorophyll
331 fluorescence parameters.
332

333 *3.3. The effect of emulsions on chlorophyll a fluorescence*

334 In Figures 3-6 the detailed images of chlorophyll *a* fluorescence parameters monitored on
335 both maize and *E. crus-galli* leaves after spraying are displayed.
336 In particular, three specific fluorescence parameters were monitored: i) Fv/Fm – maximum
337 quantum yield of photosystem II, that is the most common fluorescence parameter to measure
338 response of plants to different kinds of stress (Kalaji et al., 2014) ii) NPQ – non-photochemical
339 quenching, a parameter which is principally associated with the dissipation of the excess of

340 excitation energy in the form of heat (Müller et al., 2001), and iii) Rfd – fluorescence decrease
341 ratio, which can be considered as a measure of the photosynthetic activity of a whole leaf
342 (Lichtenthaller et al., 2005).

343 False color images of chlorophyll fluorescence suggest a decline of all these parameters in
344 leaves of maize when treated with peppermint EO at 5% and with caraway EO at both 2.5 and
345 5%, as revealed by the reduced surface of the leaves which still emits a fluorescence signal (Fig.
346 7). It is conceivable that this is related to the occurrence of symptoms over the leaf and to the
347 pre-symptomatic reduction of photosynthetic efficiency in areas of the leaf laminae where
348 symptoms will consequently appear. Similar results can be observed in *E. crus-galli* leaves,
349 which differently from maize leaves, had a lower chlorophyll fluorescence signal, even when
350 sprayed with 2.5 % of peppermint EO (Fig. 8). A conversion of these false color-RGB images
351 into the "mean grey value" enabled a statistical comparison between treatments (Figs 7-8) that
352 included both the area of a living leaf-tissue and the intensity of color. The statistical analysis
353 in "mean grey values" confirms our previous observation made by false color images revealing
354 that photosystem II performances, a key indicator of photosynthetic efficiency, was less
355 affected by the treatment with caraway as compared to peppermint EO. Moreover, it confirms
356 that the lower dose of caraway emulsion (WAC2.5) did not alter two out of the three
357 fluorescence parameters in maize leaves (Fig. 7). On the contrary, Fv/Fm and Rfd declined in
358 a dose-dependent manner in *E. crus-galli*, whose photosynthetic apparatus seems much more
359 susceptible than that of maize to peppermint EOs emulsions (Fig. 8). A reduction in Fv/Fm as
360 well as alteration of other chlorophyll fluorescence parameters have been observed by several
361 authors on plants treated with both EOs or their pure constituents. Araniti et al. (2017c, 2018)
362 reported that oregano essential oils as well as *D. viscosa* volatiles strongly affected the
363 photosynthetic machinery of Arabidopsis and lettuce, principally reducing the efficiency of
364 both dark and light adapted PSII and NPQ. Similar results were observed by Graña et al. (2013)

365 and Araniti et al. (2017b) on Arabidopsis plants treated with the terpenoids citral and *trans*-
366 caryophyllene, respectively. Finally, Synowiec et al. (2015) highlighted that leaf-application of
367 clove oil and its main constituents caused a significant alteration of fluorescence parameters.
368 Therefore, the results of both experiments showed that fluorescence parameters are not only
369 indicative of the early reaction of the photosynthetic apparatus to the stress caused by the foliar
370 application of EOs, but also allow to display differences in the sensitivity of plant species to the
371 individual EOs.

372 As a next step, a more detailed metabolomic analyses revealed differences in key
373 biochemical pathways altered by the foliar-applied emulsions of caraway or peppermint EOs.

374

375 *3.4. Differential effects of essential oils on plant metabolism*

376 The GC-MS analysis was performed to identify differentially produced metabolites,
377 following leaf spraying with EO emulsions and adjuvant. In particular, we screened the effect
378 of the highest concentration of both EOs (5%), which caused significant increase of leaf injury,
379 and in consequence the reduction of biomass, in order to inspect the main compounds as well
380 as the pathway differentially affected by EOs treatments in both maize and *E. crus-galli* (Tables
381 2-5 and Figures 9-10).

382 In order to assess the influence of the treatments on overall metabolites, raw data were
383 analyzed through principal component analysis (PCA) and successively significant features
384 were identified through the univariate analysis ANOVA (analysis of variance). Finally, to get
385 more insights into the metabolic pathways affected by the treatments, data were analyzed
386 through the "pathway analysis" (Tables 2-5 and Figures 9-10).

387 GC-MS analysis led to the identification of 51 and 52 compounds in *Z. mays* and *E. crus-*
388 *galli*, respectively (Table 2, 4 and Figures 9, 10). In particular (out of the parenthesis are
389 reported the number of metabolites annotated in *Z. mays*, whereas in the parenthesis those in *E.*

390 *curs-galli*), 10 (13) amino acids, 13 (12) organic acids, 12 sugars, 3 (1) sugar acid, 5 (4) sugar
391 alcohols, 3 (4) amines, 2 (4) fatty acids, 1 glycan, 1 glycoside and 1 lactone for maize and 1
392 inorganic acid for *E. crus-galli* have been annotated (Tables 2, 4 and Figures 9, 10).
393
394 *3.4.1. Metabolic characterization of treated and non-treated maize*
395 Concerning the results obtained from *Z. mays*, the PCA analysis pointed out a clear
396 separation among all treatments and the combination of the two principal components PC1
397 (55.2%) *vs* PC2 (26.3%) explained a total variance of 81.5% (Fig. 9A).
398 In *Z. mays* experiments the PCA loading plot highlighted that sample separation was
399 mainly due to lactose, erythritol, ribono-1,4-lactone, glyceryl-glicoside, 2-oxoglutaric acid,
400 silanamine, sedoheptulose, tagatose and fructose for the PC1, whereas in PC2 it was due to
401 malonic acid, galactinol, acotinic acid, tagatose, maltose, valine, aspartic acid and arabitol (Fig.
402 9B). Both *Z. mays* PCA and heatmap visualization of metabolomic data showed distinct
403 segregation between control and treated seedlings (Fig. 9C). At a higher level the metabolome
404 of WAC5.0-treated seedlings and the peppermint treatment (WAP5.0) clustered together,
405 suggesting that the treatments completely changed the metabolic profile of treated plants
406 compared to control plants (Fig. 9C). The univariate analysis of variance (ANOVA), carried
407 out on *Z. mays* seedlings treated with both caraway (WAC5.0) and peppermint (WAP5.0) EOs,
408 pointed out several statistical and contrasting differences among control and treatments (Table
409 2). In particular, in seedlings treated with WAC5.0 three amino acids were significantly
410 stimulated (glutamate, serine and L-alanine), whereas aspartic acid and norvaline were
411 significantly reduced (Table 2). On the contrary, in WAP5.0 treated plants only the aspartic
412 acid and valine were significantly reduced, whereas serine was accumulated (Table 2). A
413 similar trend was also observed in organic acids and sugars contents which were differentially

414 affected by the treatments (Table 2), suggesting that the two EOs were able to interfere with
415 different metabolic pathways.

416 This hypothesis was confirmed by the pathway analysis, which highlighted that caraway
417 EOs significantly affected more metabolic pathways than peppermint EO (11 *vs* 7) (Table 3).
418 In particular, both treatments significantly interfered with the amino acid metabolism (e.g.
419 alanine, aspartate and glutamate metabolism as well as glycine, serine and threonine
420 metabolism). On the contrary, the citrate cycle, the inositol phosphate metabolism, the starch
421 and sucrose metabolism as well as the glycerolipid metabolism were only affected by caraway
422 treatment (Table 3).

423 The highest accumulation of metabolites observed in maize plants treated with caraway
424 EOs suggests that plants were less affected by this treatment as compared to those treated with
425 peppermint EOs, which is in agreement with chlorophyll fluorescence data and the biomass
426 changes observed in this study. In fact, the increase in glutamic acid, serine and alanine as well
427 as the increments in sugars has been reported as an adaptation strategy adopted by resistant
428 plants to cope with abiotic stress since they act as osmoprotectants (Kovàcs et al., 2012; Good
429 and Zaplachinski, 1994; Rhodes et al., 1986). Moreover, the accumulation of maltose might
430 hint at an enhanced potential for starch mobilization in plants when exposed to EO stress. It has
431 been suggested that in plants β-amylase induction during biotic stress could lead to starch-
432 dependent maltose accumulation, and that maltose might contribute in protecting proteins and
433 the electron transport chain in the chloroplast stroma during acute stress (Kaplan and Guy,
434 2005).

435 The high accumulation in maltose only observed in maize plants treated with caraway EOs
436 suggests that this species has a higher ability to cope with the stress induced by this EOs-
437 formulation. This hypothesis is strongly supported also by the weaker effects (in terms of leaf

438 injuries, reduction in biomass, impact to PSII efficiency etc.) induced by WAC treatment when
439 assayed at the lower concentration (WAC2.5).
440
441 *3.4.2. Metabolic characterization of treated and non-treated E. crus-galli*
442 The score plot of the unsupervised PCA (Fig. 10a) highlights a clear separation among
443 control and treatments. In the experiments carried on *E. crus-galli* (Fig. 10a) the separation was
444 achieved using the principal components (PCs) PC1 vs PC2, which explained a total variance
445 of 80.2%. In particular, PC1 explained the highest variance (54.3 %) while PC2 explained
446 25.9% of the total variance. In Fig. 10b is reported the PCA loading plot which highlighted that
447 the PC1 was dominated by maltose, asparagine, glucose, mannose, fructose, tagatose, arabinose
448 and tyrosine, whereas PC2 was dominated by turanose, galactinol, lactic acid, malonic acid,
449 quinic acid and glutamine.
450 In the heatmap reported in Fig. 10c visualization of metabolomic data showed distinct
451 segregation and a peculiar clusterization among control and treatments WAC5.0 and WAP5.0.
452 Agglomerative hierarchical clustering begins with each sample as separate cluster and then
453 proceeds to combine them until all samples belong to one cluster. At a higher level the
454 metabolome of control seedlings (WA) and the treatment WAC5.0 clustered together.
455 As for *Z. mays*, the ANOVA pointed out a high number of statistically affected metabolites in
456 *E. crus galli* WAP5.0-treated plants. Interestingly, in plants treated with caraway EO a general
457 reduction in both amino acids and sugars content was observed, whereas in plants treated with
458 caraway EO an opposite behavior was observed, which was also characterized by a general
459 reduction of both classes of compounds (Table 4). The strong downregulation of different
460 metabolic pathways leading to the biosynthesis of amino acid and sugars are likely on the bases
461 of the strong effectiveness of the caraway EO treatment, confirming again the multitarget nature
462 of this EO. Concerning the organic acids, in both treatments it was observed a reduction in

463 acotinic acid, malate and malonate as well as an accumulation of citric acid and glycolic acid
464 (Table 4). In addition, in WAP5.0-treated plants it was also observed an increment in galactinol
465 and glycerol content, while myo-inositol was reduced by both treatments and sorbitol content
466 was increased only in WAC5.0-treated plants (Table 4).

467 Finally, the pathway analysis highlighted that treatments carried out on the plants of *E.*
468 *crus-galli* differentially affected several pathways (Table 5). In particular, both emulsions with
469 caraway and peppermint EOs affected alanine, aspartate and glutamate metabolism as well as
470 the galactose metabolism (Table 5). On the other hand, glycine, serine and threonine
471 metabolism as well as isoquinoline alkaloid biosynthesis was significantly affected only by the
472 emulsions with peppermint EOs (Table 5).

473 Interestingly, both EOs had a quite completely different effects on *E. curs-galli* seedlings.
474 In fact, in WAP5.0 treatment a high accumulation of almost all the amino acids and sugars was
475 observed, whereas in WAC5.0 treated plants an opposite behavior was observed. On the
476 contrary, organic acid content followed a similar trend in both treatments. As previously
477 reported amino acid and sugar accumulation plays a pivotal role in protecting plants from
478 oxidative stress acting as osmoprotectants (Kovàcs et al., 2012; Good and Zaplachinski, 1994;
479 Rhodes et al., 1986). Moreover, in plants treated with peppermint EOs (WAP5.0) a higher
480 increase in sucrose, galactinol and glycerol was observed. These molecules are important plant
481 protectors during several abiotic stress such as salinity, heat- and cold-shock stress (Nishizawa
482 et al., 2008; Eastmond, 2004; Taji et al., 2002; Santarius, 1992). Satarius (1992) reported that
483 sucrose and glycerol, which easily penetrate across chloroplast membranes, strongly protected
484 isolated thylakoid membranes from cold shock preventing membrane damages and stabilizing
485 protein complex. In addition, Eastmod (2004) demonstrated that *Arabidopsis* mutants, which
486 accumulate glycerol, were more resistant to abiotic stresses associated with leaf dehydration.
487 Concerning galactinol, it has been suggested that this molecule not only acts as osmoprotectant

488 and stabilizer of cellular membranes, but is also a pivotal ROS scavenger playing a novel role
489 in the protection of cellular metabolism, in particular the photosynthetic apparatus, from
490 oxidative damages caused by several abiotic stress factors (Nishizawa et al., 2008; Taji et al.,
491 2002).

492 These results suggest that *E. curs-galli* plants exposed to peppermint EOs were able to cope
493 with EO-promoting stress by activating some metabolic strategy aiming to enable plant
494 protection. These results are in agreement with leaf injuries and plant biomass results. In fact,
495 leaf injuries induced by WAP5.0 treatment were significantly lower compared to those
496 exhibited by WAC5.0-treated plants. On the other hand, biomass in plants treated with
497 peppermint EO was significantly lower than that of WAC5.0-treated plants. Probably, as also
498 suggested by Good and Zaplachinsky (1992) plants underwent a series of reaction which finally
499 lead to a reduction of protein synthesis in order to increase the amino acid content to cope and
500 protect themselves from EOs-induced osmotic and oxidative stress.

501

502 5. Conclusions

503 The dataset presented here offers clear evidence that foliar-applied oil-in-water (o/w)
504 emulsions containing peppermint EO and fatty acid methyl esters strongly affect both species,
505 maize and barnyard grass at the growth phases of leaves development, from both a
506 physiological and biochemical point of view. On the contrary, o/w emulsions containing
507 caraway EOs (WAC) were more effective on *E. crus-galli* at both concentrations, causing leaf
508 injuries and reduction in biomass as well as significant alterations on the photosynthetic
509 apparatus and plant metabolism, whereas biomass as well as photosynthetic apparatus of *Z.*
510 *mays* seedlings were not affected by WAC2.5, as compared to control plants. Moreover, despite
511 the presence of some injuries on leaf blades after WAC5.0 treatment, maize seedlings were able
512 to activate metabolic mechanisms, such as amino acids and sugars accumulation, to protect

themselves from EOs-induced stress. Taken together these results suggests that the o/w emulsion based on caraway EO and fatty acids methyl esters represents a potential candidate for the development of a commercial botanical herbicide against *E. crus-galli* in maize cultivation.

Conflict of interest statement

The authors declare no conflicts of interest.

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Authors contribution

AS designed and performed the pot experiment, statistically analyzed results and wrote part of the manuscript; FA designed, performed and statistically analyzed the results of metabolomic analyses, and wrote part of the manuscript; KM and ML performed and analyzed the chlorophyll fluorescence analysis and wrote part of the manuscript; AK performed the chemical analyses of essential oils and wrote methodological part for this analysis.

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672 **Table 1**

673 Main constituents (%) of caraway seed and peppermint herb essential oils distilled from

674 plants grown in the temperate climate.

Caraway oil (WAC5.0)				Peppermint oil (WAP5.0)			
RI	**RI_{lit}**	**Constituent**	**%**	**RI**	**RI_{lit}**	**Constituent**	**%**
927	934	α-Pinene	0.1	927	924	α-Thujene	0.3
963	970	Sabinene	0.1	939	934	α-Pinene	t
966	974	β-Pinene	0.1	962	970	Sabinene	0.2
982	983	Myrcene	0.1	965	974	β-Pinene	0.7
1001	1006	Car-3-ene	t	982	983	Myrcene	0.1
1011	1016	p-Cymene	t	994	998	α-Phellandrene	t
1023	1025	Limonene	32.5	1006	1006	Car-3-ene	0.1
1086	1086	Linalool	t	1009	1015	p-Cymene	0.2
1101	1103	p-Mentha-2,8-dien-1-ol	t	1016	1025	1.8-Cyneol	5.2
1112	1116	cis-Limonen oxide	0.1	1018	1025	Limonene	2.1
1117	1121	trans-Limoene oxide	t	1026	1025	(Z)-β-Ocimene	t
1168	1172	cis-Dihydrocarvone	0.1	1046	1055	γ-Terpinene	0.2
1174	1177	trans-Dihydrocarvone	0.1	1049	1053	trans-Sabinene hydrate	0.3
1201		Dihydrocarveol (isomer)	t	1076	1081	Terpinolene	0.1
1206	1210	cis-Carveol	0.1	1080	1082	cis-Sabinene hydrate	t

1224	1218	Carvone	66.4	1084	1087	Linalool	0.1
1413	1421	(E)-β-Caryophyllene	t	1087	1091	2-Methylbutyl-2-methyl isobutyrate	t
1446	1446	(E)-β-Farnesene	t	1091	1094	2-Methylbutylisovalerate	t
1565	1546	β-Caryophyllene oxide	t	1105	1108	cis-p-Menth-2-en-1-ol	t
				1125	1129	trans-Sabinol	t
				1134	1139	Menthone	25.5
				1140	1146	Isomenthone	4.0
				1144	1150	Menthofuran	2.0
				1148	1056	Neomenthol	3.3
				1163	1163	Menthol	42.7
				1167	1176	Isomenthol	0.4
				1171	1176	α-Terpineol	0.4
				1188	1176	Neoisomenthol	t
				1210	1215	Pulegone	0.8
				1223	1226	Piperiton	0.4
				1253	1259	Isopulegol acetate (Isomer I)	t
				1256	1263	Neomenthyl acetate	0.3
				1276	1280	Menthyl acetate	5.9
				1289	1298	Isomenthyl acetate	0.2
				1372	1380	α-Copaene	t
				1379	1386	β-Bourbonene	0.2
				1384	1389	β-Elemene	0.1

		1414	1421	(E)-β-Caryophyllene	2.2
		1422	1430	β-Copaene	t
		1436	1445	Isogermacrene D	t
		1444	1446	(E)-β-Farnesene	0.1
		1446	1455	α-Humulene	0.1
		1466	1474	γ-Muurolene	t
		1471	1479	Germacrene D	0.9
		1486	1494	Bicyclogermacrene	0.1
		1489	1496	α-Muurolene	t
		1491	1497	α-Cuprenene	0.1
		1509	1507	γ-Cadinene	t
		1561	1572	Spathulenol	t
		1565	1578	Caryophyllene oxide	0.1
		1576	1589	Globulol	0.1
Sum of constituents	99.7			Sum of constituents	99.5

675

676 **Table 2**

677 Effects of water plus adjuvant (WA), caraway (WAC5.0) and peppermint

678 (WAP5.0) essential oil on *Zea mays* metabolites content.

Feature	WA	WAC5.0	WAP5.0	Class
Isoleucine	1.32	0.81	0.71	
Aspartic acid	8.65[b]	7.96[b]	4.88[a]	
Glutamic acid	15.15[a]	20.68[b]	13.78[a]	
Valine	0.82[b]	0.88[b]	0.48[a]	
Serine	4.07[a]	9.44[b]	10.72[b]	
L-Alanine	9.94[a]	17.82[b]	10.00[a]	**Amino acid**
Norvaline	1.22[b]	0.72[a]	1.16[b]	
Threonine	2.32	4.16	2.07	
Glycine	3.76	3.49	3.46	
Pyroglutamic acid	5.24	7.67	6.35	
Aconitic acid	217.97[c]	170.33[b]	90.75[a]	
Cinnamic acid	2.51	2.65	2.49	
Carbamate	17.69[b]	9.82[a]	15.81[b]	
Citric acid	16.13[b]	13.23[a]	12.26[a]	
cyclohexanecarboxylicacid	108.71	116.03	71.46	
Itaconic acid	1.68	5.81	2.83	
Malic acid	105.73	110.11	112.04	
Malonic acid	1.09[b]	0.33[a]	0.19[a]	**Organic acid**
Oxalic acid	50.38[a]	66.47[b]	73.63[b]	
2-Oxoglutaric acid	0.77[a]	3.00[b]	1.94[b]	
Quinic acid	282.40	249.73	250.44	
Succinic acid	3.41	6.79	5.51	
Threonic acid	18.48	18.13	12.98	
Glycolic acid	2.25[b]	0.93[a]	0.93[a]	
Lactate	15.75[a]	24.15[b]	15.32[a]	
Arabinose	4.35[a]	8.30[b]	9.29[b]	
Fructose	763.56[c]	223.89[a]	479.78[b]	**Sugar**
Glucose	1128.24[c]	352.92[a]	907.93[b]	

Inosose	6.96	7.49	5.40	
Lactose	3.23[a]	21.00[c]	5.44[b]	
Lyxose	1.95[b]	0.86[a]	2.52[b]	
Maltose	10.77[a]	29.16[b]	7.54[a]	
Mannobiose	4.20[a]	8.43[b]	3.44[a]	
Sedoheptulose	3.88[a]	12.89[b]	4.09[a]	
Sucrose	409.95	283.04	363.48	
Tagatose	23.08[b]	4.97[a]	3.78[a]	
Threose	76.50[c]	41.85[a]	65.47[b]	
Glyceric acid	6.45	11.80	7.86	
Threonic acid	18.48	18.13	12.98	**Sugar Acid**
Erythronic acid	7.57[a]	15.37[b]	8.65[a]	
Arabitol	1.58[a]	1.47[a]	2.98[b]	
Dithioerythritol	2.69[a]	14.42[c]	6.69[b]	
Galactinol	2.32[b]	3.03[b]	0.93[a]	**Sugar alcohol**
Glycerol	2.76	4.86	2.84	
Myoinositol	59.06[a]	70.17[b]	53.75[a]	
Ethanolamine	6.12	7.00	5.26	
Silanamine	1.82[a]	6.30[b]	5.54[b]	**Amine**
Hydroxylamine	91.68	110.88	93.80	
Octadecanoic acid	9.75[a]	16.62[b]	14.78[b]	**Fatty acid**
Palmitic acid	17.72[a]	22.34[b]	20.79[b]	
Galacturonic acid	20.35[b]	16.31[a]	17.48[a]	**Glycan**
Ribono-1,4-lactone	0.21[a]	1.08[b]	0.70[b]	**Lactone**
Glyceryl-glycoside	10.05[a]	58.02[c]	34.41[b]	**Glycoside**
Phosphoric acid	21.85	22.32	21.88	**Inorganic acid**

679 Different letters along the rows indicate statistical differences with $P \leq 0.05$ (LSD's test). N=3.

680 **Table 3**

681 Pathway analysis: result from "Pathway Analysis" carried on the concentrations of metabolite
682 identified in Zea mays treated with water plus adjuvant (WA), caraway (WAC5.0) and
683 peppermint (WAP5.0) essential oils. In the table are reported the results obtained through the
684 ingenuity pathway analysis carried out with MetPa.

			WA *vs* WAC5.0	**WA *vs* WAP5.0**	
Pathways	**T Cmpd**	**Hits**	**Raw p**	**Raw p**	**Impact**
Alanine, aspartate and glutamate metabolism	21	5	0.014344	0.034342	0.66439
Glycine, serine and threonine metabolism	29	4	0.0034682	0.012128	0.53477
Citrate cycle (TCA cycle)	20	4	0.012643	//	0.24667
Inositol phosphate metabolism	17	1	0.039874	//	0.24503
Glyoxylate and dicarboxylate metabolism	17	4	0.0057461	0.0054114	0.23944
Galactose metabolism	26	6	0.0003485	0.0030181	0.1668
Methane metabolism	11	2	0.0052073	0.0089587	0.16667
Arginine and proline metabolism	37	2	0.053811	0.0056003	0.1268
Starch and sucrose metabolism	25	4	3.59E-01	//	0.11156
Glycerolipid metabolism	14	2	0.053595	//	0.09402
Aminoacyl-tRNA biosynthesis	67	8	0.0052887	0.013395	0.09302

685 T Cmpd: the total number of compounds in the pathway; Hits: is the actually matched number
686 from the uploaded data; Raw P: is the original p value calculated from the enrichment analysis;
687 Impact: is the pathway impact value calculated from pathway topology analysis.

688 **Table 4**

689 Effects of caraway (WAC5.0) and peppermint (KWAP5.0) essential oil on

690 *Echinocloa curs-galli* metabolites content.

Feature	WA	WAC5.0	WAP5.0	Class
Asparagine	1.53[b]	0.61[a]	9.66[c]	
Aspartic acid	18.66	21.13	14.78	
GABA	4.79[a]	7.18[a]	16.91[b]	
Glutamic acid	46.32[b]	24.34[a]	25.91[a]	
Glutamine	3.7[a]	12.09[b]	24.30[c]	
Glycine	2.60[a]	2.80[a]	5.90[b]	
L-Alanine	24.22	20.72	35.24	**Amino acid**
Leucine	2.50	4.06	6.62	
Proline	19.69[a]	32.08[a]	53.85[b]	
Serine	12.21[a]	15.94[a]	34.27[b]	
Threonine	5.45[a]	4.59[a]	7.10[b]	
Tyrosine	2.37[a]	1.88[b]	8.52[c]	
Valine	19.30	17.49	19.24	
Aconitic acid	380.50[b]	219.48[a]	219.13[a]	
Carbamate	16.72	12.20	13.76	
Citric acid	8.63[a]	11.19[b]	16.45[c]	
Malic acid	37.11[b]	21.64[a]	24.30[a]	
Malonic acid	0.93[b]	0.39[a]	0.50[a]	
Methylmaleic acid	1.01	0.89	1.11	
Oxalic acid	105.06[b]	90.40[b]	71.51[a]	**Organic acid**
Quinic acid	91.96[b]	15.23[a]	124.41[c]	
Succinic acid	6.68[a]	4.10[a]	9.42[b]	
Threonic acid	8.21	4.13	7.09	
Glycolic acid	0.77[a]	1.43[b]	2.12[c]	
Lactic acid	159.93	27.43	34.14	
Arabinose	10.11[b]	5.79[a]	28.41[c]	
Cellobiose	1.46	0.16	0.85	**Sugar**
Fructose	150.39[b]	46.45[a]	391.27[c]	

Galactose	0.64^{b}	0.42^{a}	1.05^{c}	
Glucose	120.19^{b}	38.83^{a}	428.70^{c}	
Lactose	5.45^{b}	2.16^{a}	7.26^{c}	
Maltose	1.90^{a}	2.29^{a}	31.11^{b}	
Mannose	66.59^{b}	28.87^{a}	240.91^{c}	
Sucrose	404.63^{b}	243.74^{a}	792.80^{c}	
Tagatose	179.38^{b}	52.78^{a}	410.22^{c}	
Turanose	1.23^{c}	0.27^{b}	0.09^{a}	
Levoglucosan	8.54^{a}	19.95^{c}	11.24^{b}	
Glyceric acid	5.89^{a}	4.15^{a}	9.95^{b}	**Sugar acid**
Galactinol	7.48^{b}	0.75^{a}	14.71^{c}	**Sugar alcohol**
Glycerol	8.38^{a}	9.41^{a}	31.16^{b}	
Myoinositol	52.02^{c}	26.33^{a}	45.41^{b}	
Sorbitol	59.40^{a}	137.32^{b}	60.22^{a}	
Urea	5.97	2.61	4.85	**Amine**
Silanamine	0.47^{a}	2.06^{b}	8.94^{c}	
Hydroxylamine	120.87^{a}	181.72^{b}	126.36^{a}	
Urea	5.97	2.61	4.85	
Palmitoleic acid	4.10^{b}	2.64^{a}	6.20^{c}	**Fatty acid**
Palmitic acid	30.66	32.40	29.09	
Oleic acid	1.72^{a}	1.45^{a}	3.18^{b}	
Stearic acid	18.08^{b}	18.41^{b}	15.47^{a}	
Glucuronic acid γ-lactone	10.60^{b}	4.72^{a}	22.54^{c}	**Glycan**
Glyceryl-glycoside	11.20^{a}	15.50^{b}	44.31^{c}	**Glycoside**
Phosphoric acid	32.98	48.08	40.04	**Inorganic acid**

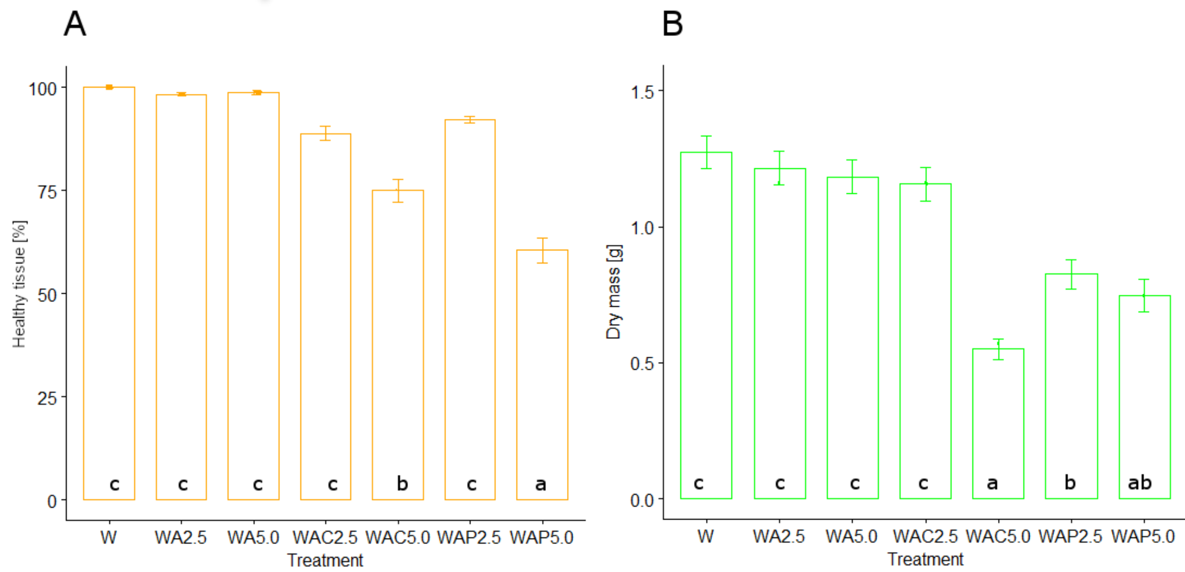
691 Different letters along the rows indicate statistical differences with $P \leq 0.05$ (LSD's test). N=3.

692 **Table 5**

693 Pathway analysis: result from “Pathway Analysis” carried on the concentrations of metabolite
694 identified in *Echinochloa curs-galli* treated with water plus adjuvant (WA), caraway (WAC5.0)
695 and peppermint (WAP5.0) essential oils. In the table are reported the results obtained through
696 the ingenuity pathway analysis carried out with MetPa.

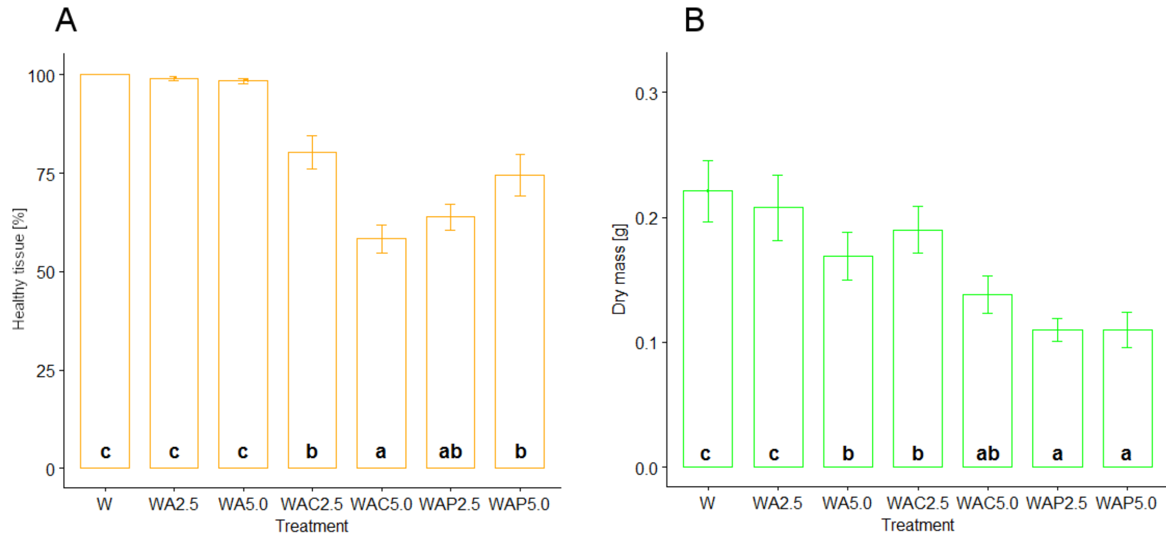
			WA *vs* WAC5.0	WA *vs* WAP5.0	
Pathways	**T Cmpd**	**Hits**	**Raw p**	**Raw p**	**Impact**
Alanine, aspartate and glutamate metabolism	21	7	0.021001	2.72E-01	0.74658
Glycine, serine and threonine metabolism	29	4	//	6.20E-01	0.53477
Galactose metabolism	26	9	1.04E-01	0.0001071	0.51278
Isoquinoline alkaloid biosynthesis	6	1	//	1.65E-01	0.5
Tyrosine metabolism	18	2	//	4.41E-01	0.27273
Inositol phosphate metabolism	17	1	0.0001203	0.048818	0.24503
Glyoxylate and dicarboxylate metabolism	17	4	0.0032152	0.00069149	0.23944
Citrate cycle (TCA cycle)	20	3	0.0093208	8.05E-01	0.17418
Methane metabolism	11	2	//	0.00056205	0.16667
Arginine and proline metabolism	37	6	0.0089378	2.58E-01	0.14946
Starch and sucrose metabolism	25	4	1.51E-02	4.42E-01	0.11156
Glycerolipid metabolism	14	2	//	0.014229	0.09402
Aminoacyl-tRNA biosynthesis	67	12	0.043696	0.00037297	0.09302
Valine, leucine and isoleucine biosynthesis	26	4	//	//	0.03645
Glutathione metabolism	26	2	0.003328	7.52E-01	0.03345

697 T Cmpd: the total number of compounds in the pathway; Hits: is the actually matched number
698 from the uploaded data; Raw P: is the original p value calculated from the enrichment analysis;
699 Impact: is the pathway impact value calculated from pathway topology analysis.



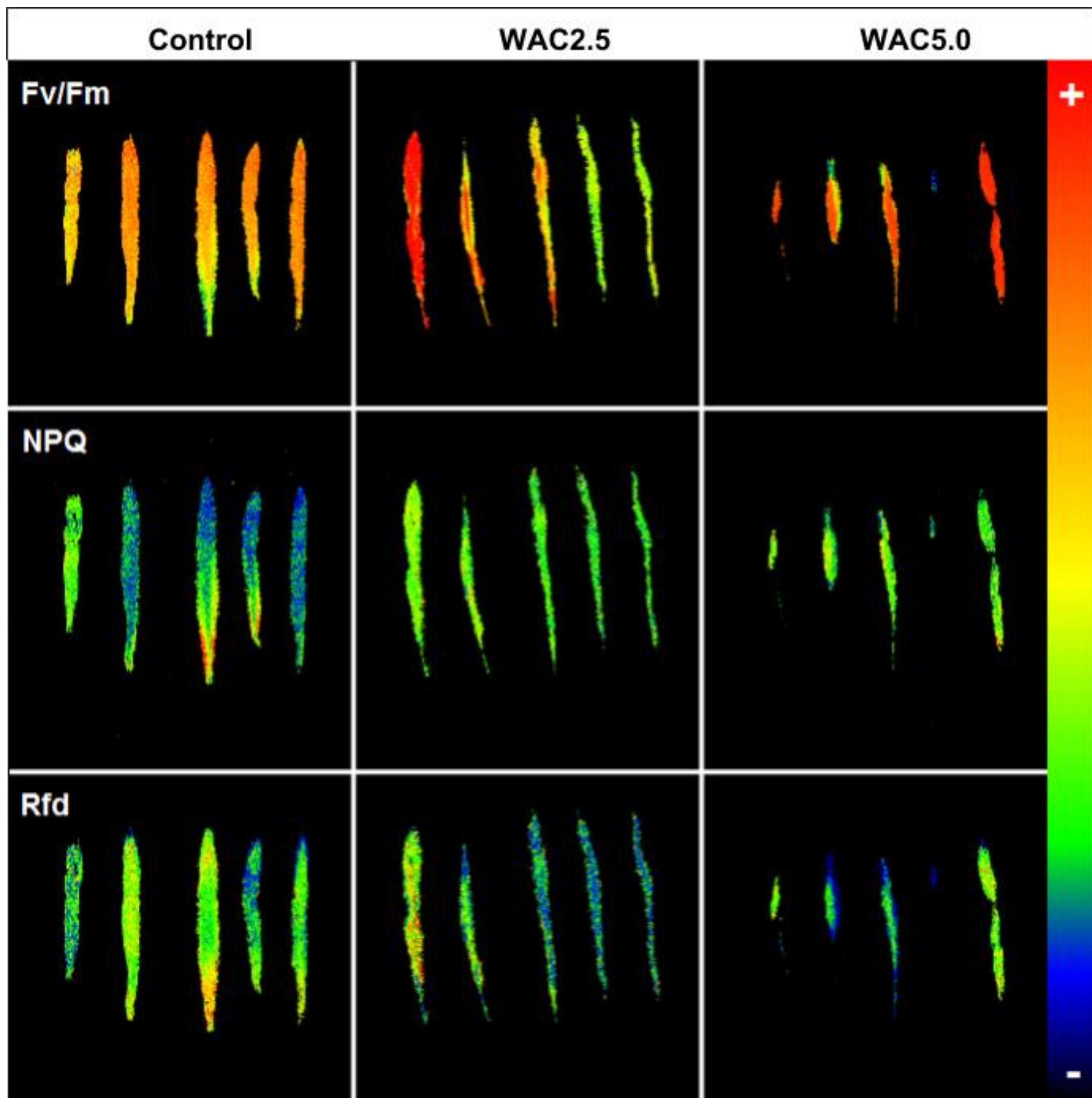
700

701 **Fig. 1. Leaf injuries and effects of different doses of adjuvant and essential oils on maize**
702 **biomass:** The average leaf injuries (A) and plant biomass (B) of maize sprayed in the stage of
703 4-6 leaves with the oil-in-water emulsions containing caraway or peppermint essential oil and
704 commercial adjuvant in the concentrations of 2.5 % or 5.0 %. Different letters refer to
705 significant differences between means, as separated by post-hoc Tukey HSD test.
706 Abbreviations: W – water; A – adjuvant; C – caraway oil; P – peppermint oil. The bars represent
707 mean value ± standard error; N = 10.



708

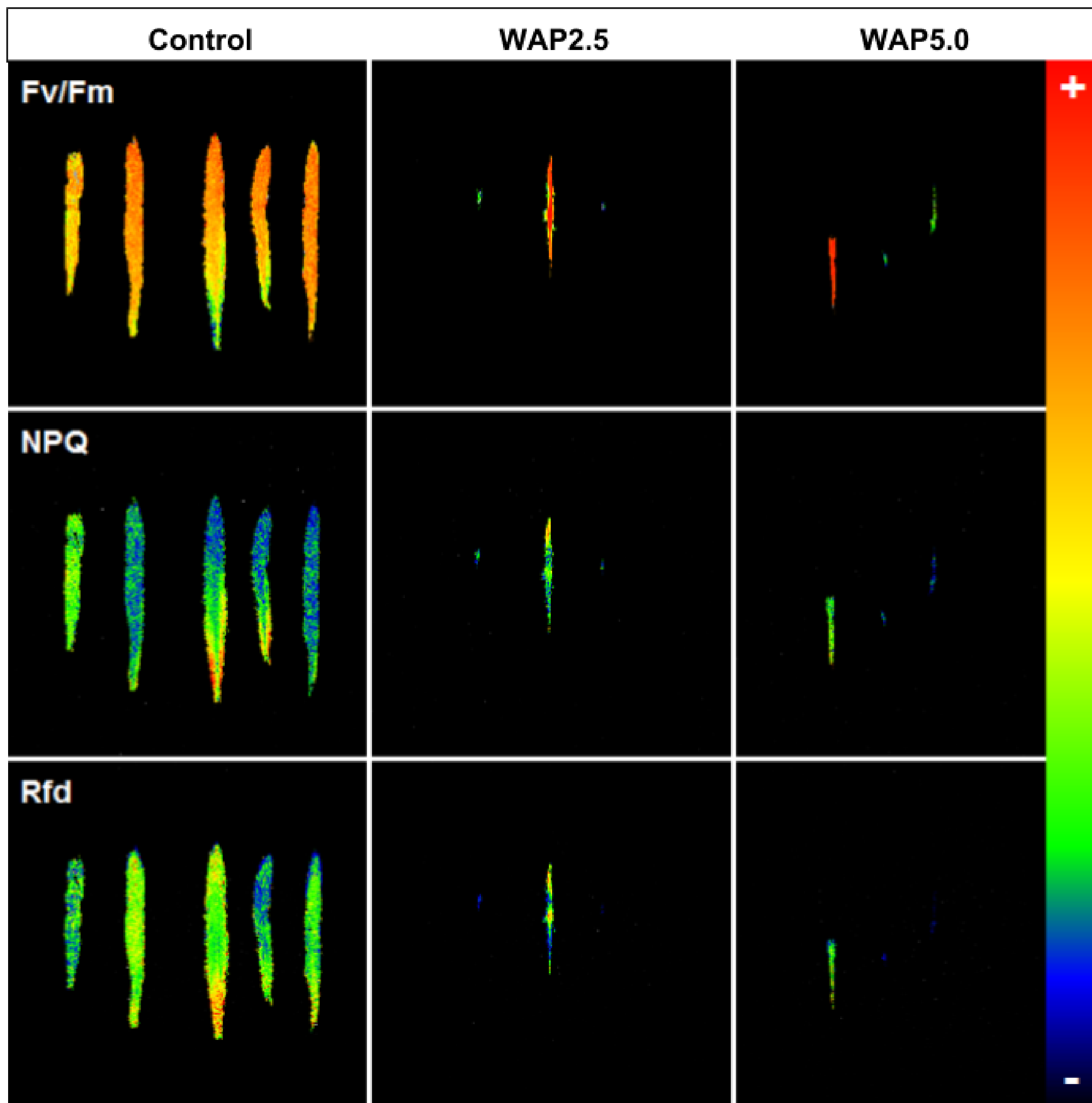
709 **Fig. 2. Leaf injuries and effects of different doses of adjuvant and essential oils on *E. crus-***
710 ***galli* biomass:** The average leaf injuries (A) and plant biomass (B) of *E.crus-galli* sprayed in
711 the stage of 3-4 leaves with the oil-in-water emulsions containing caraway or peppermint
712 essential oil and commercial adjuvant in the concentrations of 2.5 % or 5.0 %. Different letters
713 refer to significant differences between means, as separated by post-hoc Tukey HSD test.
714 Abbreviations: W – water; A – adjuvant; C – caraway oil; P – peppermint oil. The bars represent
715 mean value ± standard error; N = 10.



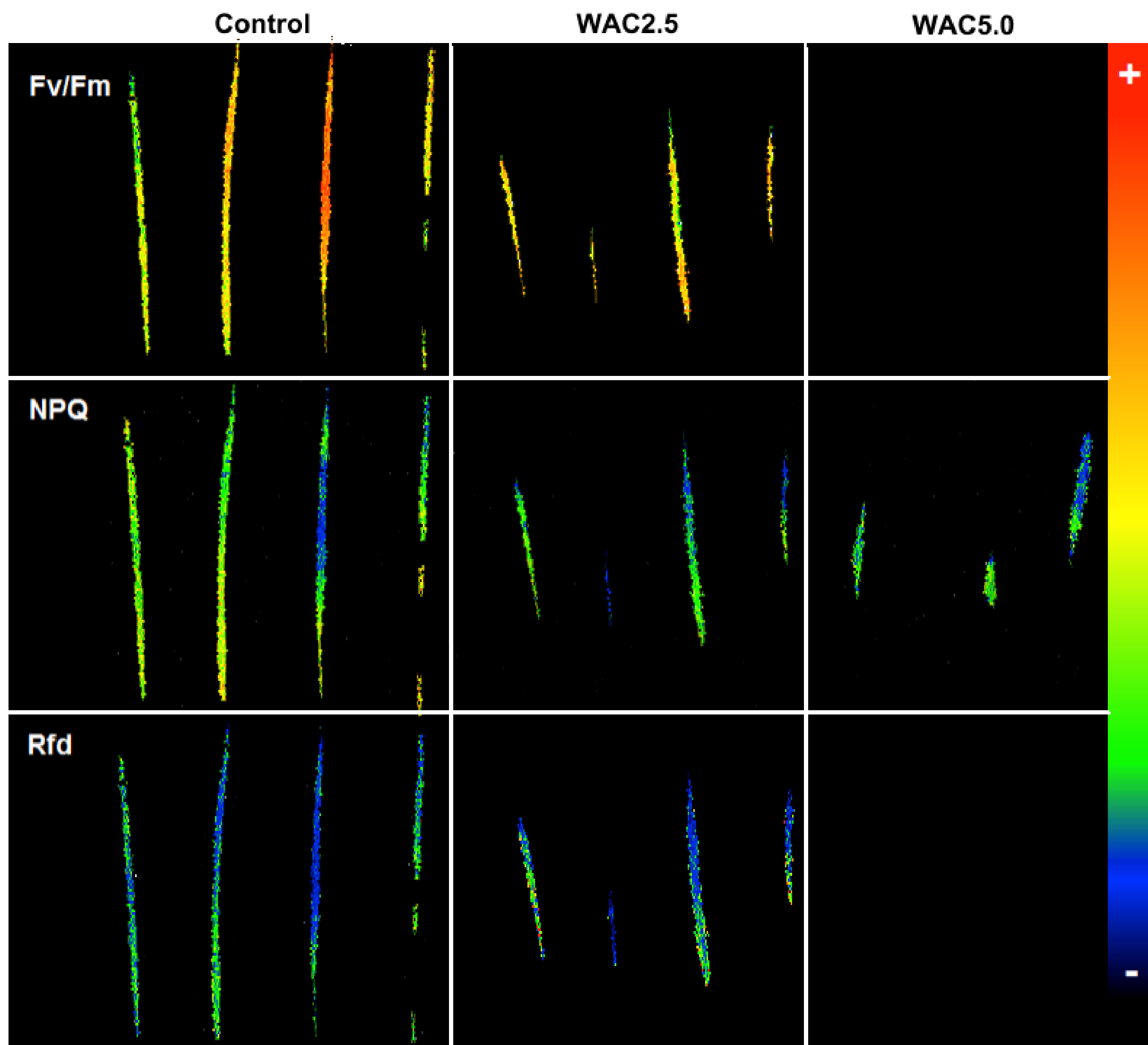
716

717 **Fig. 3. Pseudo-colour images of PSII parameters after the treatments of *Z. mays* with**
718 **essential oils:** The selected parameters of chlorophyll *a* fluorescence of maize second leaf 48
719 hours after spraying with water (control) or with the oil-in-water emulsions containing caraway
720 essential oil and commercial adjuvant in the concentrations of 2.5 % or 5.0 %. A conventional
721 color scale for the comparisons is on the right. Abbreviations: W – water; A – adjuvant; C –
722 caraway oil; Fv/Fm - maximum quantum yield of photosystem II photochemistry parameter;
723 NPQ – non-photochemical quenching; Rfd –fluorescence decrease ratio. N = 5.

724



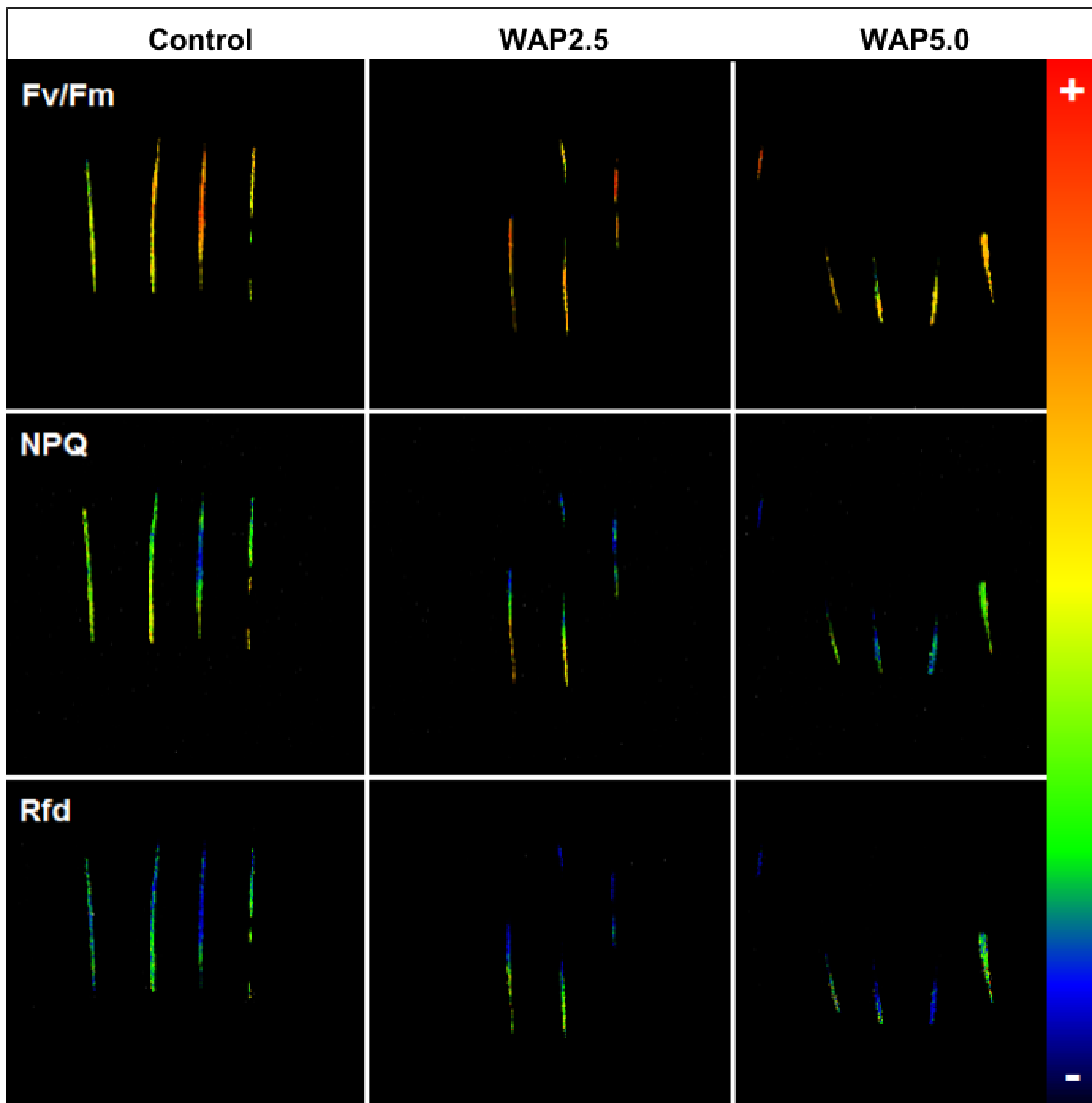
725 **Fig. 4. Pseudo-colour images of PSII parameters after the treatments of *Z. mays* with**
726 **essential oils:** The selected parameters of chlorophyll *a* fluorescence of maize second leaf 48
727 hours after spraying with water (control) or with the oil-in-water emulsions containing
728 peppermint essential oil and commercial adjuvant in the concentrations of 2.5 % or 5.0 %. A
729 conventional color scale for the comparisons is on the right. Abbreviations: W – water; A –
730 adjuvant; P – peppermint oil; Fv/Fm - maximum quantum yield of photosystem II
731 photochemistry parameter; NPQ – non-photochemical quenching; Rfd – fluorescence decrease
732 ratio. N = 5.



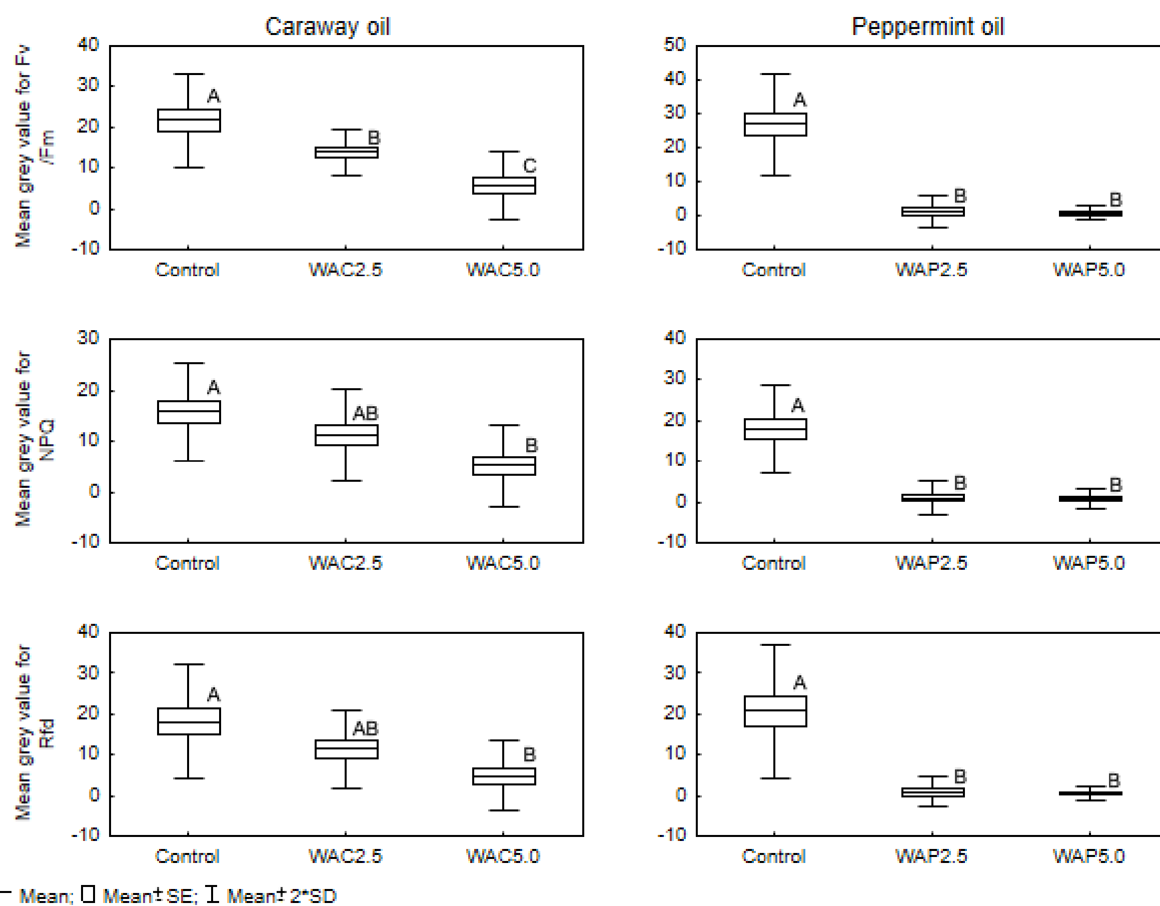
733

734 **Fig. 5. Pseudo-colour images of PSII parameters after the treatments of *E. crusgalli* with**
735 **essential oils:** The selected parameters of chlorophyll *a* fluorescence of *E. crusgalli* second leaf
736 48 hours after spraying with water (control) or with the oil-in-water emulsions containing
737 caraway essential oil and commercial adjuvant in the concentrations of 2.5 % or 5.0 %. A
738 conventional color scale for the comparisons is on the right. Abbreviations: W – water; A –
739 adjuvant; C – caraway oil; Fv/Fm - maximum quantum yield of photosystem II photochemistry
740 parameter; NPQ – non-photochemical quenching; Rfd –fluorescence decrease ratio. N = 5.

741

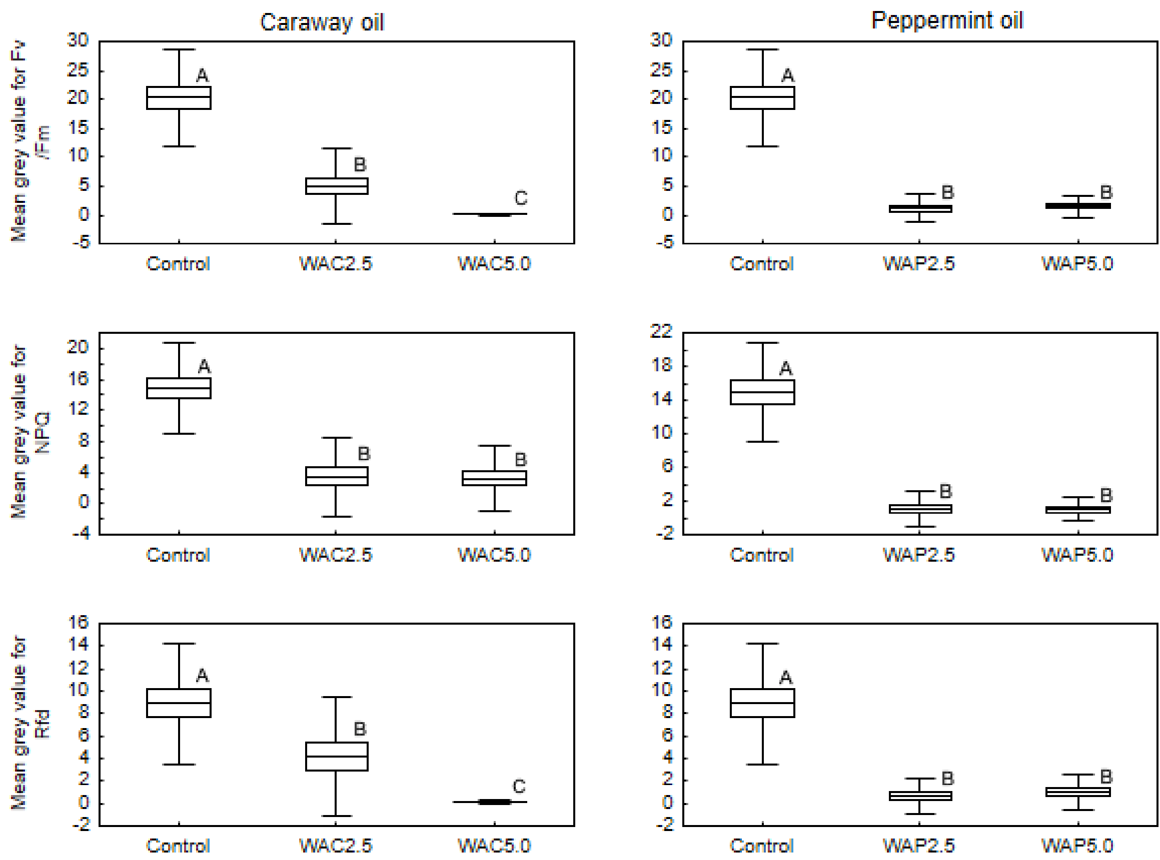


742 **Fig. 6. Pseudo-colour images of PSII parameters after the treatments of *E. crus-galli* with**
743 **essential oils:** The selected parameters of chlorophyll *a* fluorescence of *E. crus-galli* second
744 leaf 48 hours after spraying with water (control) or with the oil-in-water emulsions containing
745 peppermint essential oil and commercial adjuvant in the concentrations of 2.5 % or 5.0 %. A
746 conventional color scale for the comparisons is on the right. Abbreviations: W – water; A –
747 adjuvant; P – peppermint oil; Fv/Fm - maximum quantum yield of photosystem II
748 photochemistry parameter; NPQ – non-photochemical quenching; Rfd – fluorescence decrease
749 ratio. N = 5.



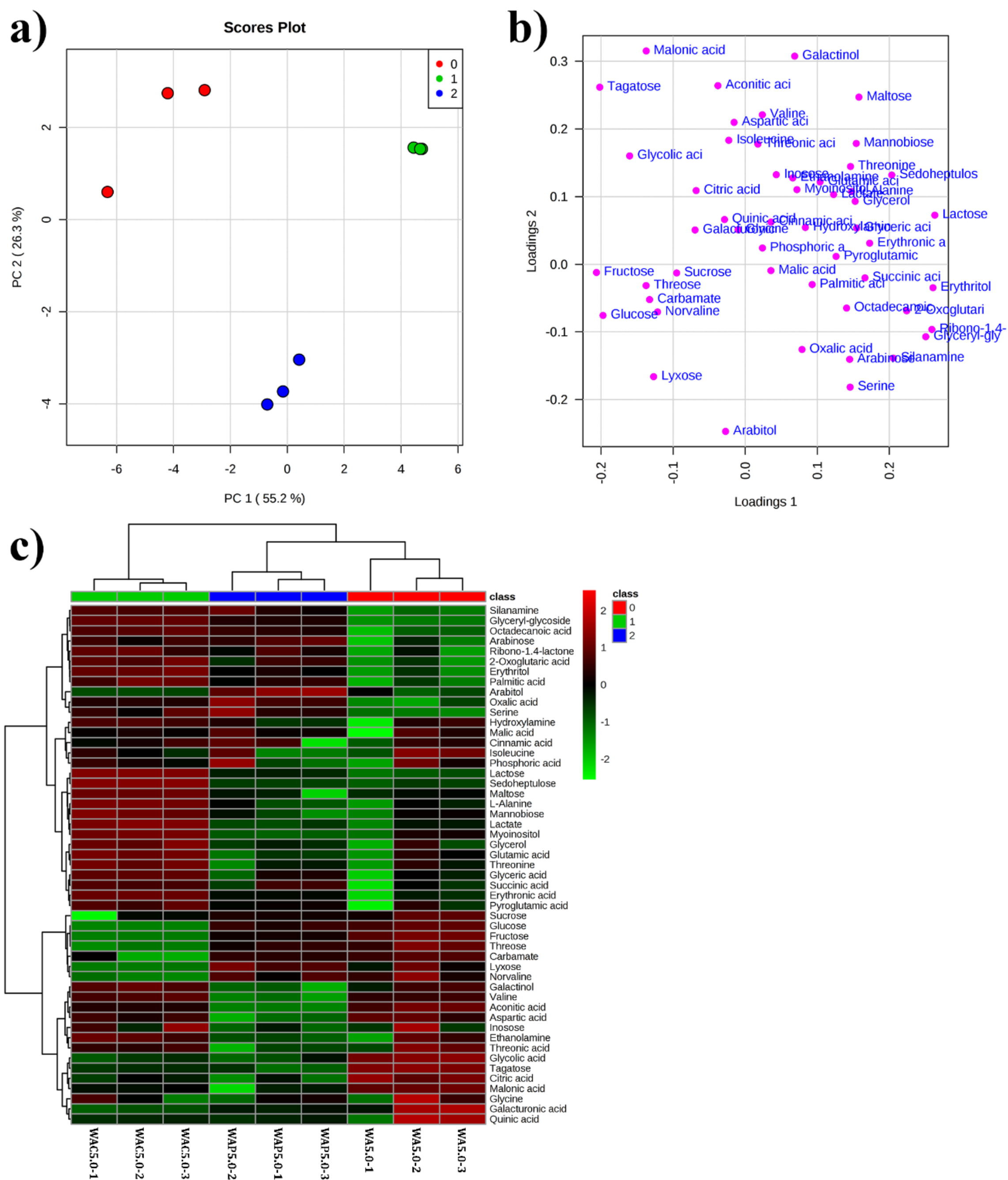
750

751 **Fig. 7. Effects of essential oils on *Z. mays* photosystem II parameters:** A mean gray values
752 for the selected parameters of chlorophyll *a* fluorescence of maize. Different letters refer to
753 significant differences between means, as separated by post-hoc Tukey HSD test. Line
754 represents a mean value, box – a mean value ± standard error, and whiskers – a mean value ±
755 2*standard deviation. N = 5. Abbreviations: W – water; A – adjuvant; C – caraway oil; P –
756 peppermint oil; Fv/Fm - maximum quantum yield of photosystem II photochemistry parameter;
757 NPQ – non-photochemical quenching; Rfd – fluorescence decrease ratio.



758

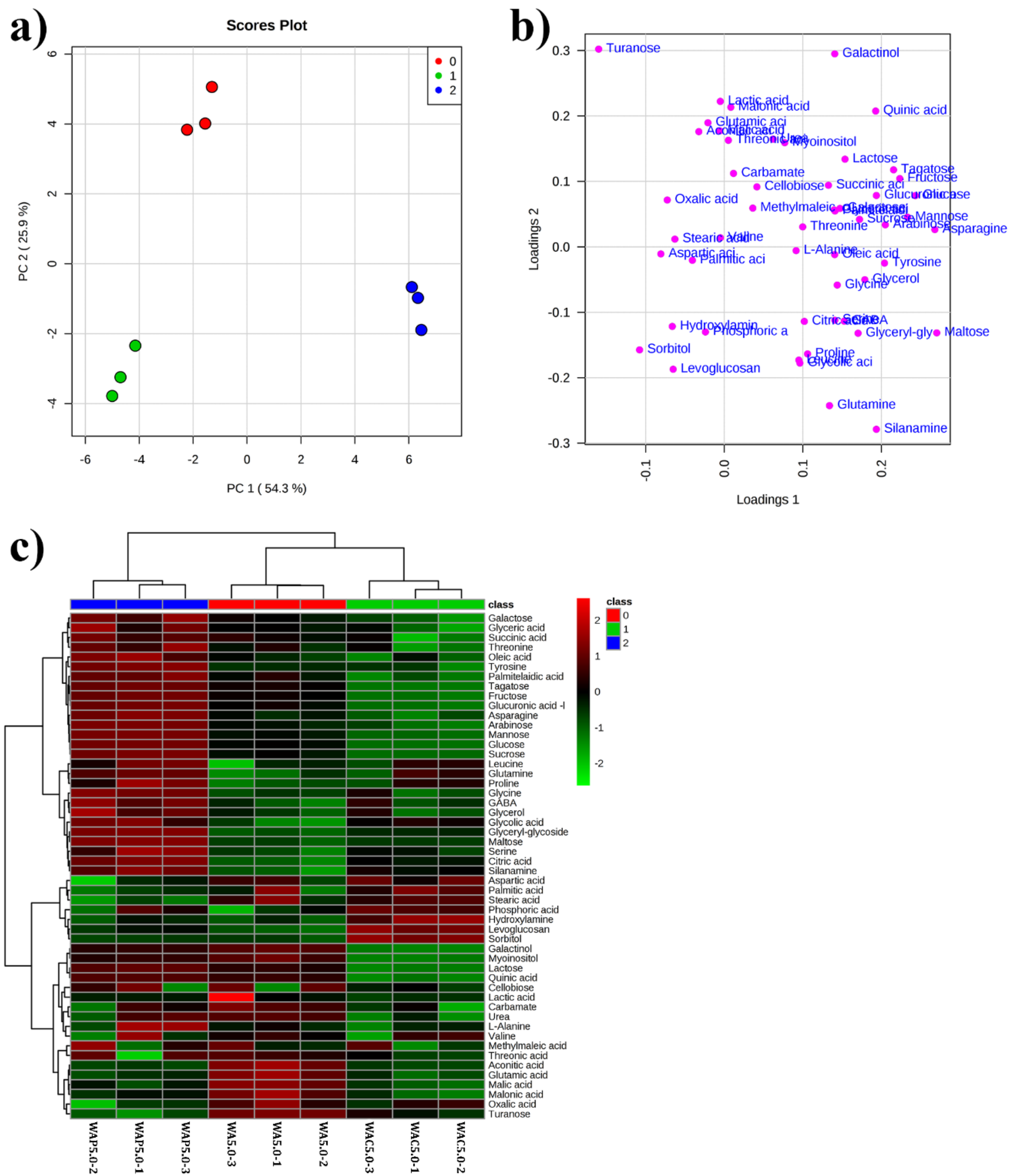
759 **Fig. 8. Effects of essential oils on *E. crus-galli* photosystem II parameters:** A mean gray
760 values for the selected parameters of chlorophyll *a* fluorescence of *E. crus-galli*. Different
761 letters refer to significant differences between means, as separated by post-hoc Tukey HSD test.
762 Line represents a mean value, box – a mean value ± standard error, and whiskers – a mean value
763 ± 2*standard deviation. N = 5. Abbreviations: W – water; A – adjuvant; C – caraway oil; P –
764 peppermint oil; Fv/Fm - maximum quantum yield of photosystem II photochemistry parameter;
765 NPQ – non-photochemical quenching; Rfd – fluorescence decrease ratio.



766

767 **Fig. 9. Effects of essential oils on *Z. mays* metabolome:** Principal component analysis (PCA)
768 carried on the metabolite identified in maize plants 48 hours after spraying with water (control)
769 or with the oil-in-water emulsions containing caraway (WAC5.0) or peppermint (WAP5.0)
770 essential oil and commercial adjuvant in the concentrations of 5.0 %. A) PCA analysis model
771 scores A) and loading plot B) of metabolite profile of control plants [red dots (0) - WA; green
772 dots (1) – WAC5.0; blue dots (3) – WAP5.0]. Both score and loading plots were generated
773 using the first two PCs, PC1 vs PC2, with the explained variances shown in brackets; C)

774 Overlay heat map of metabolite profiles in seedlings exposed to caraway (WAC5.0) and
775 peppermint (WAP5.0) essential oils. Each square represents the effect of the essential oils on
776 the amount of every metabolite using a false-color scale. Red and green regions indicate
777 increase or decrease of metabolite content, respectively. WA (1-3), indicate control replicates;
778 WAC5.0 (1-3) indicate the replicates of seedlings treated with caraway essential oils; WAP5.0
779 (1-3) C-24 and C-48 indicate the replicates of seedlings treated with peppermint essential oils.
780 N=3.
781



782

783 **Fig. 10. Effects of essential oils on *E.curs-galli* metabolome:** Principal component analysis

784 carried (PCA) on the metabolite identified in *E. crus-galli* plants 48 hours after spraying with

785 water (control) or with the oil-in-water emulsions containing caraway (WAC5.0) or peppermint

786 (WAP5.0) essential oil and commercial adjuvant in the concentrations of 5.0 %. A) PCA model

787 scores A) and loading plot B) of metabolite profile of control plants [red dots (0) - WA; green

788 dots (1) – WAC5.0; blue dots (3) – WAP5.0]. Both score and loading plots were generated

789 using the first two PCs, PC1 vs PC2, with the explained variances shown in brackets; C)

790 Overlay heat map of metabolite profiles in seedlings exposed to caraway (WAC5.0) and
791 peppermint (WAP5.0) essential oils. Each square represents the effect of the essential oils on
792 the amount of every metabolite using a false-color scale. Red and green regions indicate
793 increase or decrease of metabolite content, respectively. WA (1-3), indicate control replicates;
794 WAC5.0 (1-3) indicate the replicates of seedlings treated with caraway essential oils; WAP5.0
795 (1-3) C-24 and C-48 indicate the replicates of seedlings treated with peppermint essential oils.
796 N=3.