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## Ailanthone from *Ailanthus altissima* (Mill.) Swingle as potential natural herbicide

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1 **Ailanthone from *Ailanthus altissima* (Mill.) Swingle as potential natural herbicide**

2

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15

16 **Abstract**

17

18 Ailanthone (Ail) is the most phytotoxic quassinoid in plant extracts of *Ailanthus altissima*  
19 (Mill.) Swingle, an invasive tree of Simaroubaceae with allelopathic activity. Ail has raised  
20 attention as a potential biological herbicide in weed management to reduce the impact on the  
21 environment and human health. However, high costs for its extraction and purification, and low  
22 persistence in the soil have been considered so far limits for its development as herbicide for open  
23 field applications. In this study we explored its phytotoxic activity and persistence, through five  
24 experiments, to evaluate its potential for the weed management in the horticulture sector and in  
25 urban green areas, where lower herbicide amounts are needed. Ail inhibition activity on

26 germination and growth was evaluated on two model species (garden cress - *Lepidium sativum* L. -  
27 and radish - *Raphanus sativus* L.). Firstly, the dose-response curve between Ail concentration and  
28 index of germination was calculated; Ail persistence along 30 days was also assessed. Afterwards,  
29 Ail bioactivity and persistence were evaluated in a non-sterile urban soil and a horticultural  
30 substrate. Ail inhibited by 80 to 90 % the plant growth already at low doses ( $7.5 \text{ mg L}^{-1}$ ) in paper  
31 and soil, while higher concentrations ( $\geq 30 \text{ mg L}^{-1}$ ) were necessary in the cultivation substrate to  
32 obtain similar results. Regarding the phytotoxic persistence, the two species were similarly inhibited  
33 at the first evaluation (10 days after treatment) both in paper and cultivation substrate, whereas on  
34 the longer period (20 and 30 days after treatment), radish was more affected, with growth inhibition  
35 higher than 45 % until 30 days. Results of these experiments implement the knowledge on Ail  
36 phytotoxic activity, envisioning its potential use as a biological solution for weed management in  
37 urban areas and protected cultivation environments.

38

39 **Keywords:** allelopathy; degradation kinetics; phytotoxicity; quassinoids; seed germination; weed  
40 management

41

#### 42 **Abbreviations**

43 Ail: ailanthone

44 DAT: days after treatment

45 IGe: index of germination

46 IGr: index of growth

## 47 **1. Introduction**

48        Researches to improve the control of weeds are mainly related to agroecosystems (Duke et al.,  
49 2000; Kohli et al., 1997; Narwal, 1994; Westwood et al., 2018). This issue, however, is a  
50 particularly serious problem in many other productive sectors, such as in horticultural nurseries for  
51 the production of vegetable crops or ornamental plants (Altland et al., 2003; Case et al., 2005;  
52 Stewart et al., 2017). In this industry, weeds can negatively affect the growth of cultivated species,  
53 but also the marketability of the final product, which must be weed-free (Altland et al., 2003; Case  
54 et al., 2005; Stewart et al., 2017). Weed control is a challenge in urban environment as well, albeit  
55 the undesirability of these plants is attributable to factors not related to plant production, for  
56 instance the negative aesthetic effect, the damage to walls and hard surfaces, the reduced visibility  
57 on the streets or the diffusion of allergenic pollen. The combination of different control techniques  
58 is necessarily required in urban areas, where chemical measures may represent a risk for the  
59 population and should be replaced with alternative methods (Benvenuti, 2004; Monaco et al., 2002;  
60 Rask and Kristoffersen, 2007), as required also by current legislation in the EU (e.g. Regulation  
61 (EU) No 1107/2009 and Directive 2009/128/CE). Studies on weed control in the abovementioned  
62 two sectors, i.e. urban areas and horticulture, are thus topical and mandatory in the attempt to  
63 answer legislation and both horticulturists and citizen needs.

64 A promising perspective on weed management is the development of herbicides based on natural  
65 compounds to cope with the dangerous exposure to humans and the environmental concern on  
66 synthetic products (Kohli et al., 1997; Westwood et al., 2018). Among natural compounds, plant  
67 by-products have gained increasing attention for their benefits over synthetic compounds for an  
68 eco-friendly control of weeds (Benvenuti, 2004; Bhowmik and Inderjit, 2003; Kohli et al., 1997;  
69 Narwal, 1994), since they are perceived as safer for the environment and human health (Abbas and  
70 Duke, 1995). However, few of them have been developed into commercial herbicides, namely  
71 triketones, cinmethylin, bialaphos and glufosinate (Cutler and Cutler, 1999; Duke et al., 2000).

72 Higher plants produce several secondary metabolites to compete with or defend from surrounding  
73 species, namely terpenoids, tannins, saponins, flavonoids, and lactones (Ferreira and Aquila, 2000).  
74 In particular, the release of chemicals into the environment by one plant to affect another plant is  
75 called allelopathy and often results in the germination and growth inhibition of the target plant  
76 (Rice, 1984). Mergen (1959) firstly observed that extracts from *Ailanthus altissima* (Mill.) Swingle,  
77 a medium-sized tree of the Simaroubaceae family, caused phytotoxicity to other species. This plant  
78 is native to China, Japan, Vietnam, and Taiwan and was introduced in Europe as ornamental plant  
79 (Sladonja et al., 2015). It has become invasive in all continents, colonising a wide range of habitats,  
80 with allelopathy having contributed to its competitiveness and invasiveness, together with its  
81 abundant production of seeds and root suckers, a fast growth and high tolerance to pollutants  
82 (Benvenuti, 2004; Gómez-Aparicio and Canham, 2008; Kowarik and Säumel, 2007; Sladonja et al.,  
83 2015). Plant extracts and essential oils from different organs of *A. altissima* contain alkaloids,  
84 terpenoids, steroids, flavonoids, phenolic derivatives, and quassinoids (Albouchi et al., 2013; El  
85 Ayeb-Zakhama et al., 2014; Ni et al., 2019), that are sesquiterpene lactones abundantly present in  
86 all organs of Simaroubaceae plants. The first quassinoid identified in *A. altissima* was ailanthone  
87 (Ail) (Heisey, 1996; Kowarik and Säumel, 2007; Sladonja et al., 2015) that is used to treat  
88 ascariasis, diarrhea, spermatorrhea, bleeding and gastrointestinal diseases and has also recently  
89 showed antiproliferative activity (Daga et al., 2019). As far as concerns herbicidal activity, Ail  
90 showed phytotoxic activity on monocots and dicots both in pre- and post-emergence, causing a  
91 strong germination and growth inhibition (De Feo et al., 2003; Heisey, 1996, 1999). Despite  
92 showing the potential for the development as a natural-product herbicide, Ail is not commercially  
93 used (Bhowmik and Inderjit, 2003), since its application in large agrosystems appears limited by  
94 various constraints. In particular, Ail separation and purification costs are remarkably high and the  
95 molecule seems not stable and persistent in soil, even at high concentrations (Heisey, 1996, 1999).  
96 However, the development of Ail as a natural herbicide could be addressed to protected or limited

97 environment applications rather than to the open field, where large amount of herbicide are often  
98 required and multiple environmental factors can lead to a quick breakdown of the product (Ashton,  
99 1982).

100 This study aimed to provide new insights in Ail herbicidal activity, persistence and kinetics  
101 and to assess its application perspectives for weed control in urban green areas and horticulture. To  
102 this aim, the phytotoxic activity was, evaluated on two indicator species, i.e. garden cress  
103 (*Lepidium sativum* L.) and radish (*Raphanus sativus* L.), chosen for their different sensitiveness to  
104 toxins and their rapid growth (De Feo et al., 2003; Heisey, 1990, 1996, 1999; Heisey and Heisey,  
105 2003; Molinaro et al., 2016). Trials, performed at first on filter paper, were then carried out both on  
106 soil from urban environment and on a horticulture cultivation substrate in the attempt of  
107 reproducing the substrate conditions of two application sectors of interest. The duration of  
108 phytotoxic activity in these substrates and Ail degradation kinetics were also examined to  
109 understand stability and persistence of this natural herbicide.

110

## 111 **2. Material and methods**

112 Four experiments, summarised in Table 1, were conducted in the DISAFA facilities  
113 (45°03'58.5" Lat. N; 7°35'29.1" Long. E) during 2016-2017. Ailanthone was purchased from  
114 Herbest (Baoji Herbest Bio-Tech Co., Ltd. Baoji, China). The phytotoxic activity of Ail  
115 (*Experiments 1 to 4*) was assessed through bioassays in controlled laboratory conditions on garden  
116 cress (*Lepidium sativum* L. 'Inglese') and radish (*Raphanus sativus* L. 'Tondo Rosso BIO'),  
117 purchased from Fratelli Ingegnoli Spa (Milano, Italy). Treatments were provided by means of  
118 aqueous solutions and deionised water was used as control.

119

### 120 **2.1. Experiment 1. Ailanthone dose-response curve in filter paper**

121 The phytotoxicity of Ail was evaluated by assessing its influence on germination and on root  
122 length of garden cress and radish. These parameters were determined in 9 cm diameter Petri dishes  
123 (Supplementary Figure S1) by placing randomly ten seeds on filter paper disk (Whatman No. 1,  
124 Maidstone, UK). Then, 5 mL of seven aqueous solutions of Ail (1, 1.5, 2, 2.5, 5, 7.5 and 10 mg L<sup>-1</sup>)  
125 were added. Dishes were maintained in a growth chamber, at 25°C, in dark conditions. The number  
126 of germinated seeds (n) in each dish and their root (r) length (mm) were measured 96 hours after the  
127 treatment (ISTA, 2011) in treated (t) and control (c) seeds. These data were used to calculate the  
128 Index of Germination (IGe) with Eq. (1) (Molinaro et al. 2016):

$$129 \quad IGe\% = \frac{n_{(t)} \times r_{(t)}}{n_{(c)} \times r_{(c)}} \times 100 \quad (1)$$

130

### 131 **2.2. Experiment 2. Phytotoxic activity persistence in filter paper**

132 Phytotoxicity dynamics of 1.5 and 7.5 mg L<sup>-1</sup> of Ail on germination, root length and  
133 hypocotyl length of garden cress and radish were evaluated on filter paper in 100 mL plastic flasks  
134 to allow seedling growth. Five seeds were placed randomly on the filter paper and 1.7 mL of  
135 treatment or deionised water were added. Flasks were maintained in a growth chamber at 25°C,  
136 with a 12h-light photoperiod. In order to evaluate the persistence of Ail effects, the seeds and the  
137 obtained seedlings were removed and new seeds were positioned every 10 days after treatment  
138 (DAT), till 30 DAT. Deionised water only (without Ail) was added to all flasks to prevent dryness.  
139 The number of germinated seeds (n), their root (r) and hypocotyl (h) length (mm) were measured in  
140 each evaluation (10, 20, and 30 DAT) (ISTA, 2011) in treated (t) and control (c) plants. These data  
141 were used to calculate the Index of Growth (IGr%) with Eq. (2):

$$142 \quad IGr\% = \frac{n_{(t)} \times r_{(t)} \times h_{(t)}}{n_{(c)} \times r_{(c)} \times h_{(c)}} \times 100 \quad (2)$$

143

### 144 **2.3. Experiment 3. Phytotoxic activity of ailanthone in cultivation substrate and non-sterile soil**

145 The response of garden cress and radish to Ail was also determined in a cultivation substrate  
146 suitable for containerized production of ornamentals (Floradur<sup>®</sup> B Seed, Floragard Vertriebs-  
147 GmbH) and in non-sterile soil (Table 2) sampled in the DISAFA Campus (Grugliasco, TO, Italy).  
148 One-hundred millilitres plastic flasks (Supplementary Figure S2) were filled with 20 g of substrate  
149 or soil and moistened with 5 mL of deionised water. Five seeds were then placed randomly in each  
150 flask and 1.7 mL of treatment (7.5, 30, 60, and 90 mg L<sup>-1</sup> of Ail) were added. Flasks were  
151 maintained in a growth chamber, at 25°C, in dark conditions and the parameters to calculate Eq. (1)  
152 were recorded 96 hours after the treatment.

153

#### 154 **2.4. Experiment 4. Phytotoxic activity persistence in cultivation substrate and non-sterile soil**

155 Phytotoxicity dynamics of 30, 60 and 90 mg L<sup>-1</sup> of Ail was assessed in cultivation substrate  
156 and non-sterile soil (Table 2), with the same experimental conditions used with filter paper  
157 (*Experiment 2*). Data were collected at 10, 20 and 30 DAT to calculate Eq. (2).

158

#### 159 **2.5. Experiment 5. Degradation kinetics of ailanthonone in cultivation substrate and non-sterile** 160 **soil**

##### 161 **2.5.1. Extraction method**

162 The method was developed by spiking non-sterile soil and cultivation substrate samples with  
163 known amounts of an Ail aqueous solution. Different extraction solvents were tested, the best one  
164 being methanol/water (10:90, v/v) in the following conditions: suspensions of 20 g soil or substrate  
165 in 50 mL methanol/water (10:90, v/v) were stirred on a mechanical shaker (shaker mod. M102-OS,  
166 MPM Instruments, Milan, Italy) at 100 rpm for 30 min. The extraction was repeated twice with 25  
167 mL of the same solution. The reunited extracts were filtered through a Whatman no. 1 filters  
168 (Whatman, Maidstone, UK). A 10 mL aliquot of the extract was eluted on a BAKERBOND<sup>™</sup> SPE  
169 C18 (1 g, 6 mL) column (J.T.Baker<sup>®</sup> Avantor Performance Materials, Center Valley, PA, USA) at a

170 rate of about 1–2 drops/second. At last, the analyte was eluted with 5 mL of liquid  
171 chromatography–mass spectrometry (LC/MS) grade methanol, filtered with a syringe filter in PP  
172 (0.45 µm), then diluted (1:5, V/V) with LC/MS grade water and analysed by LC-MS/MS, according  
173 to the method described below.

#### 174 **2.5.2. LC-MS/MS determination**

175 LC-MS/MS analysis was carried out on a Varian 310 triple quadrupole mass spectrometer  
176 (Agilent, Milan, Italy) equipped with an electrospray ionization ESI source, a 212 LC pump, a  
177 ProStar 410 AutoSampler and dedicated software. LC separation was performed on a Pursuit 5 C18  
178 column, 5 µm particle size, 50 × 2.1 mm (Agilent, Milan, Italy). The mobile phase consisted of  
179 water (A) and methanol (B), both containing 0.1% (v/v) acetic acid. The gradient was 90 to 10 % A  
180 in 3 min with a flow rate of 0.2 mL/min. ESI conditions used in negative polarization were: needle  
181 potential – 4000 V, shield -450 V, capillary – 54 V. Gas conditions were set with 25 psi of N<sub>2</sub> as  
182 nebulizing gas and 25 psi at 250°C N<sub>2</sub> as drying gas. The respective ion transitions were  $m/z$  375 →  
183 300.7 (collision energy 13 V) and 375 → 150.6 (collision energy 24 V). The  $m/z$  150.6 was used for  
184 quantification.

#### 185 **2.5.3. Degradation studies**

186 Sub samples of freshly collected soil and substrate were supplemented with 1.7 mL of an  
187 aqueous solution of Ail (30 mg L<sup>-1</sup>) in order to obtain a 2.5 mg kg<sup>-1</sup> soil concentration. The flasks  
188 were incubated in the same conditions described in *Experiment 3*. Evaporation of water was  
189 periodically (4-5 days) compensated by addition of deionized water. The quantification of Ail was  
190 performed 0, 1, 2, 3, 7, 11 and 18 DAT by determination of its concentration in three independent  
191 flasks.

192

#### 193 **2.6. Statistical analyses**

194 Dishes in *Experiment 1* had three replicates (30 seeds in total), while flasks of *Experiment 2, 3*  
195 and *4* had six replicates (30 seeds in total) per treatment, arranged in a completely randomized  
196 design. All the studies were conducted in triplicate. Arcsine transformation was performed on  
197 percentage data prior to analysis. All presented values are means of untransformed data. Data were  
198 tested for the homogeneity of variance (Levene test), then mean comparison and one-way ANOVA  
199 were performed to analyse the phytotoxic effect of Ail on model species and means were separated  
200 according to Tukey post-hoc test ( $p < 0.05$ ). The analyses were performed with SPSS software  
201 (SPSS Inc., version 25, Chicago, Illinois). To define the dose-response curve, for each species a  
202 separate regression analysis was performed between Ail concentration (independent variable) and  
203 the index of germination IGe (dependent variable) fitting the following three parameters log-logistic  
204 model, Eq. (3):

$$205 \text{ IGe} = \frac{d}{1 + \exp[b(\log(x) - \log(e))]} \quad (3)$$

206 where *IGe* is the index of germination expressed as a percentage of the control, *b* and *d* are the  
207 curve parameters, with *b* being the relative slope at the point of inflection *e* and *d* the upper limit,  
208 while *x* is the Ail concentration (in mg L<sup>-1</sup>). The regression analysis was performed using the  
209 function *drm* of the add-on package *drc* of the *R* software (RCoreTeam, 2017). The effective  
210 concentration required to reduce by 10%, 50% and 90% the IGe index (ED<sub>10</sub>, ED<sub>50</sub>, ED<sub>90</sub>) was  
211 calculated using the function *ED* of the package *drc*. The function *EDcomp* of the package *drc* was  
212 used to calculate the ratio between the ED values of the two species (ED ratio) and to test the  
213 significance of differences of ED<sub>10</sub>, ED<sub>50</sub>, and ED<sub>90</sub> between the two species. Differences were  
214 considered significant when the confidence interval of ED ratio at  $p \leq 0.05$  did not included one.

215

## 216 **3. Results**

### 217 **3.1. Experiment 1**

218 The dose-response curve showed that at increasing rates of Ail the Index of germination  
219 (IGe%) gradually reduced (Figure 1). In particular, at lower Ail concentrations, garden cress was  
220 more sensitive compared to radish; for example, at 1.5 mg L<sup>-1</sup> of Ail the average IGe values were 32  
221 and 45 for garden cress and radish, respectively (Figure 1). The higher sensitiveness of garden cress  
222 was also demonstrated by its ED<sub>10</sub> (Ail concentration able to reduce the index of germination of  
223 10%), which was significantly lower compared to that of radish (Table 3). However, at higher rates  
224 the behaviour was opposite as radish showed a strong reduction of IGe, with values of about 1 or  
225 less at concentrations above 7.5 mg L<sup>-1</sup>, while for garden cress the recorded values were about 10 or  
226 higher at the same concentrations. The ED<sub>50</sub> and ED<sub>90</sub> for index of germination were not  
227 significantly different between the two tested species due to data variability.

228

### 229 **3.2. Experiment 2**

230 Generally, Ail was more effective at the highest concentration (7.5 mg L<sup>-1</sup>) in both species  
231 (Figure 2), because significant differences in their IGr were recorded in each evaluation. The  
232 concentration of 1.5 mg L<sup>-1</sup> reduced the IGr of both species by 80% compared with control at 10  
233 DAT, but its efficacy was gradually lost in the following evaluations. Conversely, the herbicidal  
234 effect of the highest concentration strongly persisted until 20 DAT in garden cress (Figure 2a), and  
235 30 DAT in radish (Figure 2b), with a growth reduction of about 95% compared with control in both  
236 evaluations.

237

### 238 **3.3. Experiment 3**

239 The lowest concentration tested (7.5 mg L<sup>-1</sup>) was not markedly effective on both species in  
240 cultivation substrate (Figure 3a), while the growth was reduced by more than 95% in soil compared  
241 with control (Figure 3b). The herbicidal activity was very strong at higher concentrations (30, 60,  
242 and 90 mg L<sup>-1</sup>) in both substrates, with no significant differences between species.

243

### 244 **3.4. Experiment 4**

245 Moulds started to develop after the first measurement (10 DAT) in non-sterile soil in growth  
246 chamber conditions, therefore data refer solely to the cultivation substrate. Although little  
247 stimulation effects were observed in garden cress at 30 DAT, data were not significantly higher than  
248 control. A growth inhibition was instead mostly observed and garden cress (Figure 4a) appeared  
249 less sensitive than radish (Figure 4b), especially at 20 and 30 DAT. Every Ail treatments  
250 significantly affected seedlings compared with control. Moreover, 60 and 90 mg L<sup>-1</sup> had stronger  
251 herbicidal activity than 30 mg L<sup>-1</sup> at 10 and 30 DAT in radish, which displayed an IGr lower than  
252 10% (10 DAT) and 60% (30 DAT), respectively.

253

### 254 **3.5. Experiment 5**

255 Notwithstanding different polarity extraction solutions were tested at different pH, the highest  
256 recovery from the spiked non-sterile soil, obtained with the procedure described above, was 65.6 ±  
257 5.9 %. In the case of the spiked cultivation substrate, the recovery dropped to 46.0 ± 5.8 %. The  
258 degradation rate of Ail, expressed in µg of Ail recovered at each sampling time, is illustrated in  
259 Figure 5. The degradation curves were fitted by an exponential equation ( $R^2 > 0.97$ ), indicating that  
260 the degradation followed a first order kinetics. The calculated half-life times (DT<sub>50</sub>) were 1.5 and  
261 1.4 days for soil and substrate respectively, while the DT<sub>90</sub> values were 7.0 days in the soil and 6.1  
262 days in the substrate.

263

## 264 **4. Discussion**

265 Data obtained in this study deepen the knowledge on the quassinoid Ail derived from *A.*  
266 *altissima*, which already proved to have numerous biological activities, not only herbicidal but also  
267 antiproliferative, antiplasmodial, antituberculosis, antimicrobial, insecticidal, antioxidant, anti-

268 inflammatory, and algaecide (Albouchi et al., 2013; De Feo et al., 2005; El Ayeb-Zakhama et al.,  
269 2014; Gu et al., 2014; Lü and He, 2010; Meng et al., 2015; Okunade et al., 2003; Rahman et al.,  
270 2009). In controlled conditions, Ail was extremely efficient on indicator species in filter paper,  
271 suggesting that it is an already active compound and does not need to be modified to reach its active  
272 form (Soltys et al., 2013). Very low concentrations (2.5-10 mg L<sup>-1</sup>) were sufficient to depress by  
273 more than 80% the IGe of garden cress and radish in Petri dishes in 96 hours under controlled  
274 conditions (Figure 1Figure 1), confirming previous results of Heisey (1996) on garden cress at  
275 similar concentrations (2 mg L<sup>-1</sup>). On the whole, garden cress had a lower ED<sub>50</sub> and ED<sub>90</sub> compared  
276 to radish. At increasing Ail doses, a different behaviour between species was observed. A lower  
277 amount of Ail was needed to reduce the growth of radish and this was mainly due to a strong total  
278 germination decrease in this species, while in garden cress the germination was only partially  
279 affected (Demasi et al., Data in brief). Conversely, the root length was markedly inhibited in both  
280 species at increasing Ail concentrations. If seedlings were let to grow in paper, radish displayed a  
281 high sensitivity (Figure 2) to a very low amount of Ail (1.5 mg L<sup>-1</sup>) and for 10 days more than cress.  
282 Again, data showed that germination of cress was only partially affected, while root and hypocotyl  
283 growth were mostly inhibited (Demasi et al., Data in brief).

284 Most of the information on herbicide activity and degradation derive from field soil trials, and  
285 are often assumed to be the same in other substrates. However, horticulture and urban green areas  
286 use soilless substrates which consist almost entirely of organic matter (Stewart et al., 2017). The  
287 effectiveness of Ail can be deeply influenced by the presence of an organic substrate (Heisey,  
288 1996). In this study, the amount of 7.5 mg L<sup>-1</sup> of Ail, which was very effective on filter paper (IGe  
289 about 2.5%) and soil (IGe about 5%), was almost ineffective in cultivation substrate (IGe about  
290 75%). This evidence was fundamental to assess the suitable amount of Ail (i.e.  $\geq 30$  mg L<sup>-1</sup>) to  
291 obtain herbicidal efficacy in substrate on garden cress (IGe lower than 20%) and radish (IGe lower  
292 than 10%). When bioassays were conducted in cultivation substrate, radish confirmed its higher

293 sensitiveness than cress to Ail, even at 20 and 30 DAT (Figure 4), even if both species showed a  
294 slight reduction of germination (Demasi et al., Data in brief). These data confirm that radish is more  
295 prone to be affected by *A. altissima* allelochemicals compared to other species (De Feo et al., 2003).  
296 Natural compounds can inhibit the germination or growth of plants through different modes of  
297 action (Macías et al., 2003), thus the diverse responses of one species to toxic compounds can be  
298 possibly due to the different mechanisms of inhibition of the active ingredient. However, in this  
299 regard little is known about ailanthone (Duke et al., 2000) but, similarly to other quassinoids, can be  
300 envisaged that Ail might act as mitosis inhibitor (Dayan et al., 1999).

301 In literature, the only studies on Ail toxicity duration (Heisey, 1996, 1999) reported that the  
302 effect was rapidly lost (in 3 days) when incubated at 25°C in presence of non-sterile soil. The  
303 breakdown of an herbicide in soil depends on several factors, including degradation acted by  
304 microbes, which presence is usually supported by organic matter. This latter, in turns, can give  
305 suitable surface for sorption of the herbicide, limiting its bioavailability. Both of the  
306 abovementioned mechanisms are plausible explanations for the loss of phytotoxicity displayed in  
307 this study in the cultivation substrate. Indeed, when the trial was performed on paper (*Experiment*  
308 2), Ail was deeply active at low doses until 30 DAT on radish and 20 DAT on garden cress, while  
309 on cultivation substrate lasted 30 DAT on radish, but using eight-times higher concentration (60 mg  
310 L<sup>-1</sup>). Ail remained highly toxic throughout 21 days if the soil was sterilised (Heisey, 1996). This  
311 study also provided for the first time information on Ail degradation in soilless substrate, compared  
312 to that in non-sterile soil. The half-life times for Ail degradation (DT<sub>50</sub>) were similar in both  
313 substrates, while the DT<sub>90</sub> value was higher in the soil, meaning that longer time was needed to  
314 degrade Ail. This is probably due to the lower amount of organic material in the soil tested. The  
315 short persistence of the active principle could be an advantage for the environment and human  
316 safety, but at the same time can be a hindrance, leading to a rapid loss of the herbicidal activity

317 (Bhowmik and Inderjit, 2003; Cutler and Cutler, 1999; Duke et al., 2000; Kohli et al., 1997;  
318 Narwal, 1994; Sladonja et al., 2015).

319

## 320 **Conclusions**

321 Ail showed a high phytotoxic activity, supporting evidence of *A. altissima* allelopathy, which  
322 contributes to the high invasiveness of the species. The phytotoxic activity was different based on  
323 the tested species, thus its efficacy can be variable and related to the species sensitivity. Further  
324 researches in this regard are thus needed to confirm Ail potential use in horticulture and urban green  
325 areas as natural herbicide.

326

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442 **Tables**

443 **Table 1.** Summary of the bioassays performed to evaluate the phytotoxic activity of ailanthone  
 444 (Ail).

Experiment No.	Ail concentration (mg L <sup>-1</sup> )	Container	Substrate <sup>a</sup>	Conditions <sup>b</sup>	Duration	Evaluation
1	1, 1.5, 2, 2.5, 5, 7.5, 10	Petri dish	P	D	96 h	IGe% <sup>c</sup>
2	1.5, 7.5	Plastic flask	P	L	10, 20, 30 DAT <sup>e</sup>	IGr% <sup>d</sup>
3	7.5, 30, 60, 90	Plastic flask	C and S	L	96 h	IGe%
4	30, 60, 90	Plastic flask	C and S	L	10, 20, 30 DAT	IGr%

445 <sup>a</sup>P=paper; C=cultivation substrate; S=soil. <sup>b</sup>D=dark, growth chamber, 25°C; L=12h photoperiod, growth chamber, 25°C. <sup>c</sup>IGe%= Index of Germination. <sup>d</sup>IGr%= Index of Growth. <sup>e</sup>DAT= days after  
 446 treatment  
 447  
 448

449 **Table 2.** Physical and chemical characteristics of the cultivation substrate and non-sterile soil used  
 450 in the *Experiments 3 and 4*.

Floradur® B Seed			Non-sterile soil		
pH (CaCl <sub>2</sub> )		5.6	pH <sup>a</sup>		8.0
Salinity	g L <sup>-1</sup>	0.8	Carbonates <sup>b</sup>	%	12.02
N (CaCl <sub>2</sub> )	mg L <sup>-1</sup>	140	C tot <sup>c</sup>	%	2.257
P (P <sub>2</sub> O <sub>5</sub> )	mg L <sup>-1</sup>	80	N tot <sup>c</sup>	%	0.069
K (K <sub>2</sub> O)	mg L <sup>-1</sup>	190	CEC <sup>d</sup>	meq 100g <sup>-1</sup>	4.61
			Exchangeable Ca	mg L <sup>-1</sup>	1012
			Exchangeable Mg	mg L <sup>-1</sup>	27
			Exchangeable K	mg L <sup>-1</sup>	30
			Available P <sup>e</sup>	mg L <sup>-1</sup>	9.5
			Clay	%	3.69
			Silt	%	7.60
			Sand	%	88.71

451 <sup>a</sup>ISO 10390; <sup>b</sup>ISO 10693; <sup>c</sup>ISO 10694; <sup>d</sup>cation exchange capacity, ISO 11260; <sup>e</sup>Olsen.

452  
 453 **Table 3.** Concentration required to reduce by 10%, 50% and 90% (ED<sub>10</sub>, ED<sub>50</sub> and ED<sub>90</sub>) the IGe  
 454 index and their ratio with lower and upper limits of the confidence interval for garden cress and  
 455 radish (*Experiment 1*).

	Garden cress	Radish	ED ratio <sup>a</sup>	ED ratio lower limit	ED ratio upper limit
ED <sub>10</sub>	0.16	0.41	0.40*	0.06	0.74
ED <sub>50</sub>	1.06	1.19	0.89ns	0.63	1.16
ED <sub>90</sub>	6.93	3.46	2.00ns	0.99	3.01

456 <sup>a</sup>ED ratio: calculated as EDx garden cress/EDx radish. The statistical relevance is provided (ns,  
 457 non-significant; \*  $p \leq 0.05$ ).

458 **Figure captions**

459 **Figure 1.** Dose-response curve of index of germination (IGe%) of garden cress (*Lepidium sativum*)  
460 and radish (*Raphanus sativus*) in response to different concentrations of ailanthonone on filter paper  
461 after 96 hours under controlled conditions (*Experiment 1*).

462 **Figure 2.** Index of growth (IGr%) of a) garden cress (*Lepidium sativum*) and b) radish (*Raphanus*  
463 *sativus*) in response to 1.5 (grey) and 7.5 mg L<sup>-1</sup> (black) of ailanthonone in filter paper 10, 20, and 30  
464 days after treatment (DAT) under controlled conditions (*Experiment 2*). Similar upper case letters  
465 along the same treatment and similar lower case letters within the same group denote no significant  
466 differences according to Tukey post-hoc test ( $p < 0.05$ ). Bars indicate the standard error.

467 **Figure 3.** Index of germination (IGe%) of garden cress (*Lepidium sativum*) and radish (*Raphanus*  
468 *sativus*) in response to different concentrations of ailanthonone on a) cultivation substrate and b) non-  
469 sterile soil in controlled conditions (*Experiment 3*). Similar lower case letters in garden cress and  
470 similar upper case letters in radish denote no significant differences among concentrations,  
471 according to Tukey post-hoc test ( $p < 0.05$ ). Bars indicate the standard error.

472 **Figure 4.** Index of growth (IGr%) of a) garden cress (*Lepidium sativum*) and b) radish (*Raphanus*  
473 *sativus*) in response to 30, 60 and 90 mg L<sup>-1</sup> of ailanthonone on cultivation substrate after 10, 20, and  
474 30 days after treatment (DAT) (*Experiment 4*). Similar upper case letters along the same treatment  
475 and similar lower case letters within the same group denote no significant differences according to  
476 Tukey post-hoc test ( $p < 0.05$ ). Bars indicate the standard error.

477 **Figure 5.** Degradation rate of ailanthonone in non-sterile soil and cultivation substrate.