1 **Terracrepolo (*Reichardia picroides* (L.) Roth.): wild food or new horticultural crop?**

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9 ABSTRACT

10 The extreme adaptability of *Reichardia picroides* to stressful environments motivated experiments

11 aimed to investigate the genotype-environment interactions on the nutraceutical parameters of this

12 ancient food. The concentrations of antocyanins, flavonol glycosides, carotenoids and total phenols

13 and the antioxidant capacity were significantly higher in the inland "Agnano" ecotype than in the

14 coastal "Calafuria" ecotype. As expected, the cultivation of *R. picroides* generally led to a decrease

15 in the compositional parameters except the content of carotenoids. A sodium chloride solution was

16 sprayed onto the cultivated plants to simulate the stress caused by marine aerosols. However, the

17 hypothesis that salt stress could act as an elicitor for nutraceutical substances was not validated,

18 particularly in the Calafuria ecotype that evolved close to the sea shore. The nutraceutical

19 performances of the wild ecotypes could be retained in cultivation through a chronic stress, which

20 could allow the activation of the physiological response.

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22 Keywords: antioxidant; nutraceutical; ethnobotany; wild species; human health

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24 HIGHLIGHTS

25 Terracrepolo showed different nutraceutical performances depending on the ecotype.

26 Cultivation lowered the levels of nutraceuticals found in the wild-grown plants.

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29 Salt stress in cultivation did not restore the nutraceutical levels of wild-grown plants.

30 Further studies could focus on the chronic effects of abiotic stress in cultivation.

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33 1. INTRODUCTION

34 The growing need for nutraceutical foods (Ozen et al., 2013) has elicited an increasing interest for
35 ethnobotanical studies (Tardío et al., 2006), which have been addressed mostly to edible wild herbs
36 (Pieroni, 2000; Guarrera and Savo, 2016). Indeed, important health benefits of the plant kingdom
37 are mainly provided by the wild species, for their richness in secondary metabolites such as
38 polyphenols (Hättenschwiler and Vitousek, 2000). Overall, secondary metabolites are the result of
39 evolutionary processes in natural ecosystems, especially related to self-defence from both biotic and
40 abiotic adversity (Jwa, 2006), and have a crucial role as a source of nutraceuticals. Paradoxically,
41 the rediscovery of ancient local foods represents a promising healthy innovation of the daily
42 Mediterranean diet (Heinrich et al., 2005).

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43 Terracrepolo (*Reichardia picroides* (L.) Roth.), belonging to the Asteraceae botanic family, is a
44 steno-Mediterranean herb of high ethnobotanic interest as medicinal food, since it was traditionally
45 used as a depurative (Pieroni, 2000) or tonic (Loi et al., 2004) agent. In Sardinia it was even used
46 as a popular treatment against heart diseases such as angina pectoris (Atzei et al., 1991). This
47 species, utilized row or cooked (Nebel et al., 2006) was found to be a valuable source of
48 antioxidants (Vanzani et al., 2011), probably due to its richness in phenolics (Recio et al., 1992).
49 Terracrepolo is spread throughout the climatic area of olive grove (Pignatti, 1982), and grows in
50 dry, rocky and calcareous soils in open space. It is also very common on buildings in the urban
51 environment (Benvenuti, 2004), even on ancient monuments such as the Colosseum (Caneva et al.,
52 2002). Moreover, its multiple stress tolerance allows it to be commonly present among the sand-
53 dune vegetation in the saline environment of the Mediterranean coast (Sýkora et al., 2003).

55 The annual regrowth dynamics of this perennial species occurs through: i) the sprouting of basal
56 buds (life cycle of hemicryptophyte) and/or ii) autumnal and/or spring seed germination (Benvenuti
57 and Pardossi, 2016). Dispersal is carried out by anemocory, due to a white plumose pappus able to
58 be moved by the wind (Andersen, 1993).
59 On account of this attitude to spatial dispersal, this species is a good example of "pioneer" flora
60 belonging to the *Reichardia* botanic *Genus* (Parraga-Aguado et al., 2013), typically able to colonize
61 biologically inhospitable areas and allow a floristic transition to other successive, more exigent
62 species. The survival of this invasive species in new environments is also favoured by a genetic
63 variability able to select the desired characters in the various colonized habitats (Lee, 2002). Plant
64 species are often characterized by both phenotypic plasticity and large genetic variation. Indeed, the
65 successful occupation of many ecological niches depends on the occurrence of many genotypes
66 (Joshi et al., 2001) specialized to co-evolve in particular environmental conditions (Van Tienderen
67 1990), and this could be the case also for some ecotypes of *R. picroides* (number of chromosomes
68 n=7; Siljak-Yakovlev, 1981). However, although it is clear that the abiotic stresses are elicitors of
69 secondary metabolites (Zhao et al., 2005) necessary for plant survival (Namdeo, 2007), such as
70 flavonoids (Treutter, 2006), anthocyanins (Chalker-Scott, 1999), or total phenolics (Michalak,
71 2006), and carotenoids (Young, 1991), it is not known whether this metabolic over-expression
72 could be genetically retained even in different ecotypes that do not have to endure the same stress
73 conditions. On the other hand, it is not even known which is the most effective environmental stress
74 for the elicitation of secondary metabolites in *R. picroides*, since this species can colonize
75 diversified environments (inland or immediately near the sea). In addition to the typical poor
76 fertility, calcareous matrix and water stress, some ecotypes adapted to grow near the sea may
77 withstand salt stress (Mittler, 2002), due to the periodic deposition of marine aerosol on the coastal
78 vegetation (O'Dowd and De Leeuw, 2007). Information about the genotype-environment interaction
79 (Lila, 2006) could assume a crucial role in the agronomic perspective of cultivating this species as a

80 new “nutraceutical crop”. Anyway, it is not clear whether and to what extent cultivation could
81 imply changes in the nutraceutical performances typical of the plants from the native environment.
82 Based on the above considerations, the aim of this study was: i) to quantify some important
83 nutraceutical parameters (anthocyanins, chlorophylls, carotenoids, flavonol glycosides, total
84 phenols, antioxidant capacity) of two different ecotypes of *R. picroides*, ii) to verify whether the
85 cultivated progeny retains the same nutraceutical performances as the mother plants, iii) to
86 artificially elicit the synthesis of secondary metabolites by a simulated marine aerosol.
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88
89 2. MATERIALS and METHODS
90 *2.1. Plant material and sampling*

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91 *2.1.1. Germplasm collection*
92 Wild plants of *R. picroides* belonging to different ecotypes were collected in Tuscany (central
93 Italy), in the inland (Agnano) and close to the coast (Calafuria). Table 1 reports some details on the
94 two different areas, while Figure 1 shows the ecotypes from Agnano and Calafuria, respectively.
95 Seed collection was carried out in September 2015, by removing the whole inflorescences from the
96 senescent tissues in the laboratory. The seeds were cleaned, dried in dry room, and kept in glass
97 containers at 20°C.
98 *2.1.2. Greenhouse cultivation*
99 The plants were cultivated during winter-spring 2015 in a greenhouse at the Department of
100 Agriculture, Food and Environment of the University of Pisa, Italy (43°70’ N 10°43’ E). The seeds
101 were sown in alveolar polystyrene containers (50 holes) commonly used in horticultural nurseries.
102 Each hole (3 cm diameter, 5 cm depth) was filled with a peat-perlite substrate (1:1 v/v) and hosted
103 one seed, which was placed on the surface and covered with an additional substrate layer (1 mm).
104 Irrigation was carried out daily by water nebulization (about 3 mm day^{-1}).

106 After 3 weeks from emergence, 30 seedlings per each ecotype were transplanted in plastic pots (10
107 cm height, 9 cm diameter) filled with the same substrate enriched with 3 g l^{-1} of controlled-release
108 fertilizer (Osmocote Plus Organics® 13.1N-2.3P-4.0K, Everris, Australia). Daily irrigation was
109 carried out through a 2 l m^{-2} over-head (boom) at each application. The growing conditions were: 20
110 °C average temperature, 70–80 % humidity, approximately 12/12h photoperiod, 300 µmol m^{-2} s^{-1}
111 light intensity.

112 *2.1.3. Sampling of cultivated plants*

113 For each ecotype, plant sampling was carried out 4 weeks after transplantation (6 weeks from
114 seedling emergence), at the vegetative phenological stage, when the plants had produced a basal
115 rosette of leaves. Completely developed young leaves were collected for the laboratory analyses
116 during the first light hours (8.00 – 9.00 a.m.). Four samples (1g) were prepared by pooling the leaf
117 tissues of seven distinct plants. The samples were immediately wrapped in aluminium foil, placed in
118 refrigerator bags and stored at -80 °C. They were analyzed within 3-4 weeks from collection. An
119 aliquot of the fresh material was kept one week in ventilated oven at 60°C for dry weight
120 determination.

121 *2.1.4. Sampling of wild-grown plants*

122 Wild plants were sampled at the same time as the cultivated ones, in the same environments where
123 seeds had been collected the previous year (Calafuria rocky coast, and the drystone walls of
124 Agnano). For each ecotype, leaf samples from plants in the same phenological stage as the
125 cultivated ones were prepared as described in the previous subsection and kept in refrigerated bags
126 (0 °C) during the short way to the laboratory (about 30 minutes), where they were immediately
127 freezed at -80 °C, or oven-dried at 60 °C . The samples were analyzed together with those from the
128 cultivated plants.

129 *2.1.5. Salt stress*

130 The experiment aimed at evaluating the effect of a saline aerosol was performed twice, using a
131 completely randomized experimental design with four replicates, each composed of the leaves of

132 seven greenhouse cultivated plants. For each of the two ecotypes, the pot plants were grown under
133 the above described conditions (unstressed) or were subjected to a simulated marine aerosol
134 treatment (salt stressed). A 3.5 g l^{-1} sodium chloride solution was sprayed onto the latter plants (14
135 ml m^{-2}) by means of a microairbrush after 3 weeks from transplanting. To ensure that the desired
136 amount of solution was entirely conveyed onto a known surface, a plastic shield was pierced on the
137 airbrush at the base of the nozzle insertion. The resulting salt dose of 0.049 g m^{-2} could resemble a
138 deposition left by marine aerosol after wind events (Franzén, 1990). Leaves sampling was carried
139 out as described in the previous subsection, after 10 days of salt spraying.
140
141 *2.2. Reagents and apparatus*
142 HPLC grade methanol was purchased from Sigma–Aldrich (Milano, Italy). Reagent grade
143 chemicals were purchased from the same manufacturer or from Carlo Erba Reagents (Cornaredo,
144 Milano, Italy). All the determinations were performed by spectrophotometric assays, measuring the
145 absorbance of the solutions with a Lambda35 UV-Vis double beam spectrophotometer (Perkin
146 Elmer, Waltham, Massachusetts, USA).
147
148 *2.3.Plant analyses*
149 *2.3.1. Extraction*
150 Acidified 80% methanol (containing 1% hydrochloric acid) was used for the extraction of total
151 anthocyanins and flavonolglicosides; pure methanol was used for all the other determinations. The
152 extraction protocol reported by Maggini et al. (2013) was used with modifications. The leaf samples
153 (1g) were soaked with 5 ml extraction solvent, ground with mortar and pestle, and transferred in 10-
154 ml test tubes. The tubes were sonicated 15 minutes in ice bath four times, stored overnight at -20°C
155 and centrifuged 5 minutes at 2700*g*. After separation of the supernatant, the extraction was repeated
156 on the pellet with 5 ml fresh extraction solvent. The two supernatant aliquots were pooled and used

157 for the subsequent analyses within a few days. All the parameters were expressed on a fresh weight
158 (FW) basis.

159 *2.3.2. Chlorophylls and carotenoids*

160 For the determination of chlorophylls and carotenoids, the methanol extracts were diluted 1:10 with
161 methanol. The absorbance of the diluted extracts was read at 665.2, 652.4 and 470 nm, and the
162 concentrations of the pigments (μg g^{-1} FW) were calculated according to Lichtentahler and
163 Buschmann (2001).

164 *2.3.3. Anthocyanins and flavonol glicosides*

165 The determinations of total anthocyanins and flavonol glicosides were accomplished following
166 Hrazdina et al. (1982). For the evaluation of the content of total anthocyanins, the absorbance of the
167 acidic extract was read at 530 nm, and the results were expressed as mg cyanidin-3-glucoside g^{-1}
168 FW, using the value 38000 M^{-1} cm^{-1} for the molar absorptivity. The total concentration of flavonol
169 glycosides was determined on the same extracts after proper dilution by absorbance readings at 360
170 nm, using the molar absoptivity of quercetin-3-glucoside at the working wavelength (20000 M^{-1} cm^{-}
171 ^{1}), and expressing the results as mg quercetin-3-glucoside g^{-1} FW.

172 *2.3.4. Total phenols*

173 The determination of total phenols was carried out both by the Folin-Ciocalteu phenol reagent, and
174 by absorbance readings at 320 nm, as reported by Kang and Saltveit (2002). For the former assay,
175 100 μl methanol extract, 2.0 ml distilled water and 300 μl Folin-Ciocalteu phenol reagent were
176 mixed in plastic test tubes. After four minutes, 7.5% sodium carbonate (1.6 ml) was added into the
177 tubes and the solutions were kept 2 hours at room temperature. The concentration of total phenols
178 was determined by measuring the absorbance of the solutions at 765 nm, using standard gallic acid
179 (0 – 500 mg L^{-1}) for calibration, and expressing the results as mg gallic acid g^{-1} FW. For the
180 absorbance readings at 320 nm, the methanol extracts were diluted 1:100 with methanol. The results
181 were expressed as absorbance units of the pure extract at 320 nm per gram leaf tissue, A(320nm) g^{-1}
182 FW.

183 *2.3.5. Antioxidant capacity*

184 The antioxidant capacity was determined by both the ferric reducing antioxidant power (FRAP) and
185 the 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH) assays. The FRAP
186 determination was carried out according to Benzie and Strain (1996). The FRAP reagent was
187 freshly prepared immediately before the analyses and contained 2mM ferric chloride and 1 mM
188 TPTZ (2,4,6-tris(2-pyridyl)-s-triazine). The following solutions were mixed in a spectrophotometric
189 cuvette: 0.25 M acetate buffer pH 3.6 (2.0 ml); FRAP reagent (900μl); diluted 1:4 methanol extract
190 (100 μl). A calibration curve was prepared with standard solutions containing ferrous ion (Fe(II); 0
191 – 1000 μM), obtained from ferrous ammonium sulfate. The absorbance was read at 593 nm and the
192 results were expressed as μmol Fe(II) g^{-1} FW. The DPPH assay was performed following Dudonné
193 et al. (2009) with slight modifications. 2.97 ml methanol DPPH solution (20 mg L^{-1}) and 30 μl
194 methanol extract were mixed in a spectrophotometric cuvette. A blank solution was also prepared
195 by replacing the plant extract with methanol. The cuvettes were kept 45 minutes in the dark at room
196 temperature, and the absorbance was read at 515 nm. The percentage inhibition of the DPPH radical
197 per gram tissue was calculated from the absorbance values of the blank (A_{blank}) and of the sample
198 (A_{sample}) as follows:

199

200 $$\% \text{ Inhibition } g^{-1} \text{ FW} = 100 \cdot [(A_{blank} - A_{sample})/A_{blank}] / \text{g FW}$$

201

202 *2.4. Statistical analyses*

203 The mean value and standard deviation of four samples of each type were evaluated in all the
204 assays. For all the parameters under investigation, normal distribution and variance homogeneity of
205 the data were verified by means of Kolmogorov-Smirnov and Levene tests, respectively. For the
206 evaluation of the effect of cultivation, the data concerning wild or cultivated samples were
207 subjected to pairwise comparisons by means of four distinct t-tests: Agnano wild versus Calafuria

208 wild plants; Agnano cultivated versus Calafuria cultivated plants; Agnano wild versus Agnano
209 cultivated plants; Calafuria wild versus Calafuria cultivated plants. For the Agnano ecotype, t-tests
210 were also performed to compare wild and salt treated samples. For the evaluation of the effect of a
211 saline aerosol on cultivated plants, the data were subjected to two-way ANOVA with the ecotype
212 (Agnano or Calafuria) and the treatment (unstressed or salt stressed) as the sources of variation. The
213 Bonferroni post-test was used for means comparisons. The linear regression analysis was applied to
214 the results of distinct assays (FRAP versus DPPH or Folin-Ciocalteu versus absorbance at 320 nm).
215 The Statgraphics Centurion Version 17 software (Statpoint Technologies, Warrenton, Virginia,
216 USA) was used for the statistical analyses.
217
218
219 3. RESULTS
220 Both wild and crop plants were collected under optimal turgidity conditions, and had 80 - 82%
221 moisture content.
222 Figure 2 shows that, although all the samples contained similar concentrations of total chlorophylls,
223 the two wild grown ecotypes showed distinct levels of anthocyanins, carotenoids and flavonol
224 glycosides, the plants from Agnano containing higher amounts than those from Calafuria. The
225 concentrations of anthocyanins and flavonol glycosides were higher in the wild-collected samples
226 than in the cultivated plants. In contrast, the concentration of carotenoids was significantly
227 (Calafuria) or tendentially (Agnano) higher in the cultivated plants than in the wild-collected ones.
228 The antioxidant power of our samples was determined by means of both the FRAP and the DPPH
229 assays (Figure 3), and the results obtained with the two independent methods were linearly
230 correlated (correlation coefficient $r^2 = 0.886$); however, the differences among the samples were
231 more evident with the latter assay. The antioxidant capacity was higher for the wild grown plants
232 from Agnano than for those from Calafuria, and was generally higher for the spontaneous plants
233 than for the cultivated ones. The only exception to this trend was the Calafuria cultivated ecotype,

234 whose antioxidant power in the FRAP assay was close to that of the corresponding wild grown
235 plants.
236 Two independent methods were employed also for the determination of the concentration of total
237 phenols (Figure 3). In addition to the commonly used Folin-Ciocalteu assay, we measured the
238 absorbance of the extracts at 320 nm according to Kang and Saltveit (2002). The linear correlation
239 coefficient between the results of two assays was $r^2 = 0.989$. According to the former, the
240 concentration of total phenols in the wild plants was higher for the ecotype from Agnano, and with
241 both methods the cultivated samples of this ecotype contained less phenolics compared to the plants
242 at the spontaneous state. In contrast, the wild or cultivated plants from Calafuria contained similar
243 concentrations of phenolics.
244 Table 2 reports the results of the two-way ANOVA concerning the salt stress experiment, which
245 show that the effect of the ecotype was significant for all the parameters under investigation. Figure
246 4 shows the variation of the individual constituents in both ecotypes consequent to the saline
247 aerosol application to the cultivated plants. For each parameter, the difference between the values in
248 stressed (S) and unstressed (U) plants is expressed as a percentage, according to the formula:
249
250 $\%$ Difference from unstressed plants = 100 * (S - U) / U
251
252 The saline aerosol caused a general decrease of the parameters in the Calafuria ecotype. On the
253 other hand, the concentration of total phenols and the antioxidant capacity tended to increase in the
254 Agnano ecotype.
255
256 4. DISCUSSION
257 Despite the large diffusion of *R. picroides* all over Tuscany, a distinction could be made between
258 the two ecotypes from the inland (Agnano) and the coast (Calafuria), based on their nutraceutical
259 performances (Figure 2 and Figure 3). At the spontaneous state, the plants from Agnano showed

260 overall higher contents of the metabolites of interest, which included important classes of plant
261 pigments such as anthocyanins, carotenoids, chlorophylls, along with flavonol glycosides, whose
262 concentrations were obtained by simple readings of the absorbance of the extracts. Both
263 anthocyanins and flavonols belong to the widespread class of flavonoids. Specifically, flavonols are
264 one of the largest subclasses of flavonoids, which in turn are the largest group of plant phenolics
265 (Chang et al., 2002; Balasundram et al., 2006).The antioxidant capacity of our samples was
266 determined both as the reducing ability towards the ferric ion (FRAP assay) and as the scavenging
267 ability towards the DPPH free radical (DPPH assay). The use of independent methods is generally
268 recommended because the antioxidant capacity of a complex mixture such as a plant extract is due
269 to the contribution of its individual constituents. These are chemical compounds which often have
270 very different structures and show distinct reactivity in dependence of the experimental conditions
271 that they undergo. As a consequence, distinct assays could measure slightly different antioxidant
272 properties. However, with our samples a good linear correlation was evidenced for the two
273 methods.
274 In a similar way, a strong linear correlation was apparent also for the two independent assays for the
275 determination of the concentration of total phenols (Folin-Ciocalteu assay or absorbance readings at
276 320 nm). This outcome was in total agreement with those obtained in lettuce with the same assays
277 (Kang and Saltveit, 2002). Moreover, our results on the antioxidant power and the concentration of
278 total phenols as obtained through the FRAP and the Folin-Ciocalteu assays, respectively, were in
279 full agreement with those found with the same methods by Vanzani et al. (2011).
280 The reasons for the differences in the compositional parameters between the spontaneous plants
281 from the two sites could be due to a different mechanisms of adaptation to environmental stress
282 conditions. In particular, the reaction of the plants from Calafuria to their draughty, windy and
283 saline environment might involve non-phenolic antioxidants such as vitamin C, proline or
284 glutathione, or different classes of phenolics than those that have been examined in this work.
285 Alternatively, in the coastal ecotype adaptation could be based mainly on antioxidant enzymes such

286 as ascorbate peroxidase, catalase or superoxide dismutase, rather than on antioxidant molecules
287 (Das and Roychoudhury, 2014; Demidchik, 2015).
288 Different response mechanisms to the natural environment could be explained by the strong
289 adaptation attitude of *R. picroides*, which was able to develop ecotypes that can endure particular
290 environmental stress conditions. In a recent paper, the genus *Reichardia* has been reported as an
291 appropriate model to investigate on genome evolution (Siljak-Yakovlev et al., 2017).
292 In contrast with the spontaneous plants, significant differences between the two ecotypes were not
293 apparent in cultivation, except for the contents of anthocyanins and carotenoids (Figure 2).
294 As expected, the cultivated plants of both ecotypes, which had grown in a less stressful environment
295 compared to those at the spontaneous state, contained lower concentrations of anthocyanins and
296 phenol glycosides. The opposite trend that was observed for the content of carotenoids could be due
297 to the much higher light intensity in the native environment than in the greenhouse, leading to a
298 higher rate of carotenoid oxidation in the wild grown plants.
299 Anyway, although similar results were obtained in cultivation for both ecotypes, only the cultivated
300 plants from Agnano contained a significantly lower concentration of total phenols and showed a
301 significantly lower antioxidant capacity than the spontaneous ones (Figure 3). These findings
302 suggest that in this ecotype both anthocyanins and phenol glycosides could bring a relevant
303 contribution to the pool of phenolics, and that phenolic antioxidants could play an essential role in
304 determining the overall antioxidant activity. In contrast, in the Calafuria ecotype, cultivation did not
305 have a strong overall effect on the content of total phenols or in the antioxidant power, since only
306 the DPPH assay revealed a significant reduction of the radical scavenging activity.
307 Also the salt stress affected the two ecotypes in a different way, especially concerning the
308 concentration of total phenols and the antioxidant capacity (Table 2 and Figure 4), suggesting that
309 the coastal grown plants and those from the inland could have developed distinct salt tolerance
310 mechanisms.

311 In the Agnano ecotype, both the concentration of total phenols and the antioxidant capacity tended
312 to increase in reaction to the saline aerosol. Although only a slight variation was observed, this
313 could indicate a possible role of phenolics in the physiological response to salt stress. On the other
314 hand, a similar trend was not observed for the other parameters, including anthocyanins and
315 flavonol glycosides. This outcome suggests that different classes of phenolic substances could be
316 involved in the mechanism of defence against salinity, such as phenolic acids or different subclasses
317 of flavonoids. According to t-test comparisons, the salt treated plants of the inland ecotype
318 contained lower levels of bioactive compounds than the corresponding wild samples, with the only
319 exceptions of total chlorophylls and carotenoids, indicating that the application of a saline spray
320 was not effective in restoring the nutraceutical properties of the spontaneous plants from Agnano.
321 In the ecotype from Calafuria, the concentrations of chlorophylls, flavonol glycosides and
322 carotenoids tended to decrease with salt stress, and all the other parameters under examination were
323 strongly lowered. This unexpected behaviour may be indicative of a salt tolerance mechanism not
324 involving antioxidant compounds, or could be ascribed to a slow physiological response, not yet
325 apparent after only ten days from the beginning of the salt treatment. Alternatively, a sudden salt
326 stress during optimal plant growth could be ineffective in triggering a stress response in this
327 ecotype, since the activation of the metabolic pathways of salt stress tolerance might require the
328 adverse conditions to occur already during germination or in the early phenological phases.
329 By an overall comparison between the wild plants from the two sites, those from Agnano appeared
330 more promising for ex situ cultivation, because they were naturally richer in important bioactive
331 components and showed a higher antioxidant capacity, which tended to increase in cultivated plants
332 with the application of salinity conditions. Anyway, even in this ecotype an acute stress caused by
333 foliar treatment was not effective to stimulate a significant accumulation of antioxidant molecules,
334 especially phenolics. In order to observe a marked effect on the compositional parameters, more
335 severe conditions could be required, such as a chronic stress induced by root uptake.
336

337 5. CONCLUSIONS

338 Genotype, environment and their interaction may significantly affect the chemical composition of
339 *R. Picroides*, as already found for common crops (Shaw et al., 2016). Our results showed that only
340 the chlorophyll content of the leaf tissues was not influenced by the ecotype or the growing
341 environment. In contrast, the concentrations of antocyanins, flavonol glycosides, carotenoids and
342 total phenols, along with the antioxidant capacity, were strongly dependent on both factors. This
343 adaptable richness of health-friendly metabolites could arouse interest toward future collection and
344 selection of *R. picroides* ecotypes evolved in different environments. Moreover, the nutraceutical
345 performances of this “new vegetable” could be improved through appropriate cropping systems. In
346 our experiments, the hypothesis that nutraceuticals may be elicited by a sudden salt stress on the
347 leaf canopy was not validated. However, further work is in progress to test the chronic long-term
348 effect of tolerable salt doses and investigate the influence of different types of abiotic stress on the
349 phytochemical composition of this species.

350

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514

515 **Table 1.** Geographical and environmental information on the two different localities of *Rheicardia*

516 *Picroides* germplasm collection.

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Site of germplasm collection	Tuscany province	Geographical coordinates	Environment	Substrate type	Altitude	Distance from the sea
Agnano	Pisa	43°73'N 10°48' E	Drystone wall in open spaces of Mediterranean chaparral	Calcareous soil	75 m a.s.l.	18.000 m
Calafuria	Livorno	43°47'N 10°33' E	Coastal rocky and arid environment	Calcareous rocks	10 m a.s.l.	10 m

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519

520 **Table 2**. The statistical effects of treatment (unstressed or salt stressed), ecotype (Agnano or

521 Calafuria) and their interaction on the contents of anthocyanins, flavonol glycosides, carotenoids,

522 total chlorophylls, total phenols and antioxidant capacity (FRAP and DPPH) in the leaf tissues of

523 cultivated *Reichardia picroides*, according to two way ANOVA. Four replicates were analyzed,

524 each one consisting of seven plants. Asteriscs: significant at $P<0.05$ (*), $P<0.01$ (**) or $P<0.001$

525 (***); ns: not significant.

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	Anthocyanins	Flavonol Glycosides	Carotenoids	Total Chlorophylls	Total Phenols (Folin-Ciocalteu)	Total Phenols (A 320 nm)	Ferric Reducing Antioxidant Power (FRAP)	DPPH-radical Scavenging Activity (DPPH)
Treatment	**	ns	ns	ns	ns	ns	ns	ns
Ecotype	***	**	**	*	**	*	*	**
Interaction	ns	ns	ns	ns	***	**	**	***

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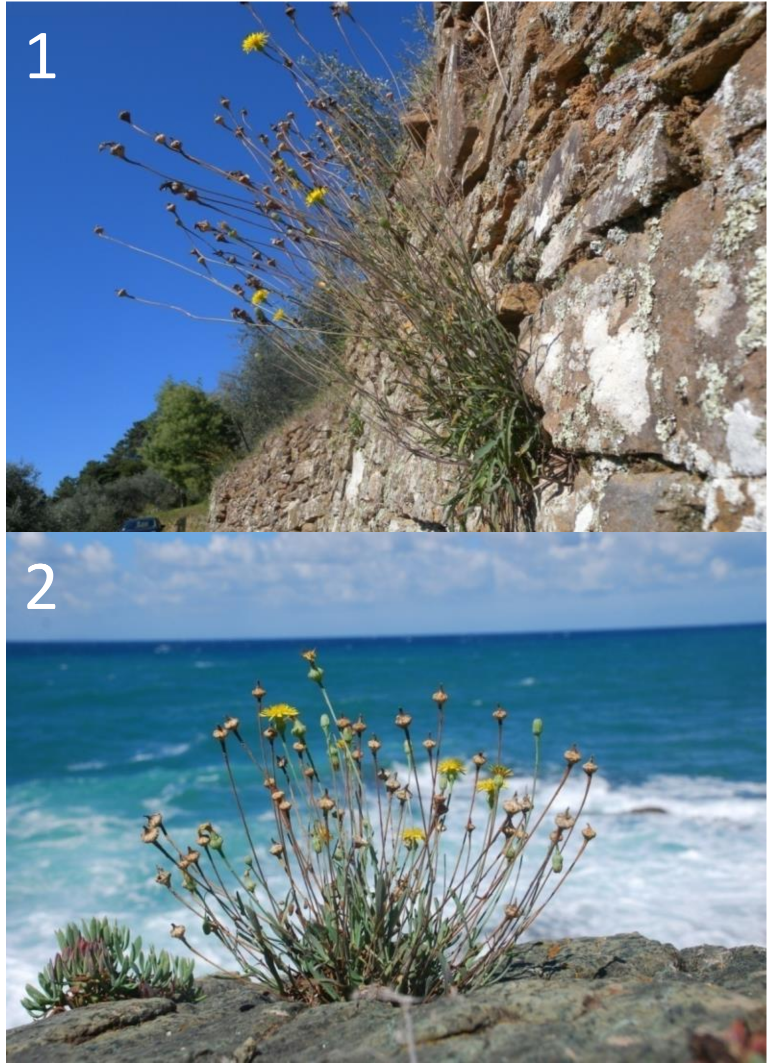

548

549 **Figure 1.** The two different *Rheicardia picroides* ecotypes in their respective environments: 1)
550 drystone wall of Agnano (Pisa) and 2) coastal rocks of Calafuria (Livorno).

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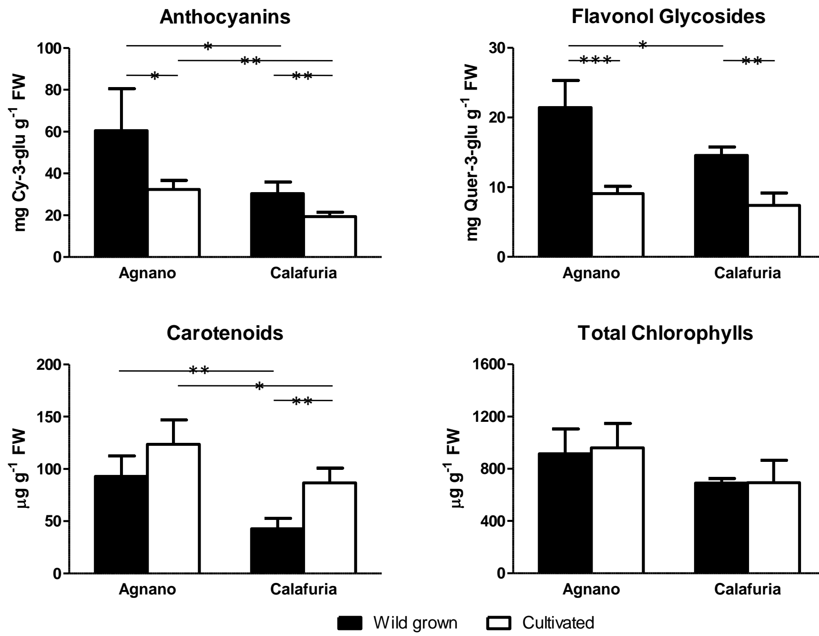

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553 **Figure 2.** The concentration of anthocyanins (mg cyaniding-3-glucoside g^{-1} FW), flavonol

554 glycosides (mg quercetin-3-glucoside g^{-1} FW), carotenoids and total chlorophylls (µg g^{-1} FW) in the

555 leaves of *Reichardia picroides* from different ecotypes (Agnano or Calafuria) and growing

556 environments (wild-collected or cultivated). Mean values and standard deviation of four samples.

557 Data were subjected to pairwise means comparisons by t-test. Only significant differences at $P<0.05$

558 (*), $P<0.01$ (**) or $P<0.001$ (***) are indicated.

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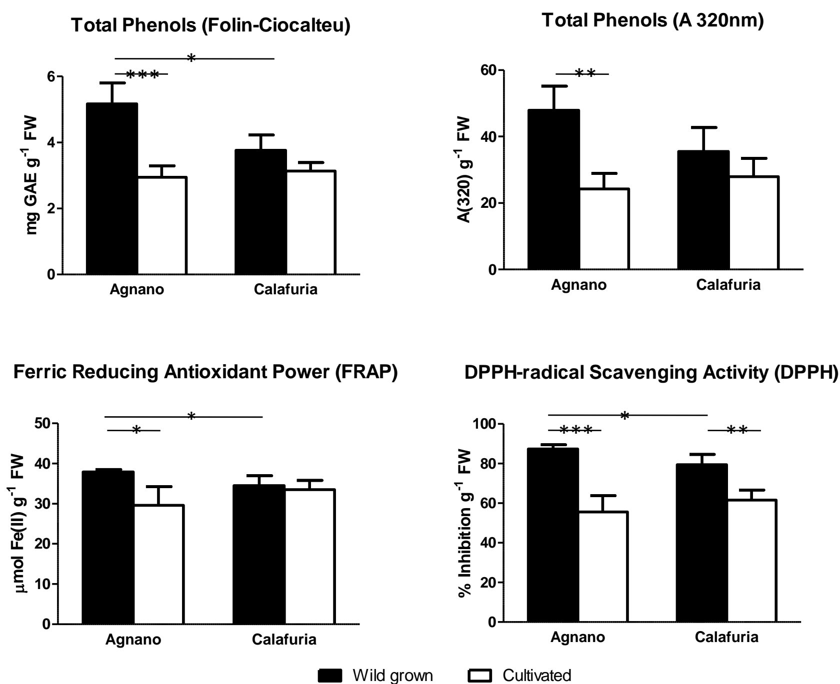

562
563 **Figure 3.** The concentration of total phenols (mg gallic acid equivalents g^{-1} FW or absorbance at
564 320 nm g^{-1} FW) and the antioxidant capacity as determined by the FRAP (µmol Fe(II) g^{-1} FW) or
565 the DPPH (percentage inhibition of the DPPH radical g^{-1} FW) assays, in the leaves of *Reichardia*
566 *picroides* from different ecotypes (Agnano or Calafuria) and growing environments (wild-collected
567 orcultivated). Mean values and standard deviation of four samples. Data were subjected to pairwise
568 means comparisons by t-test. Only significant differences at $P<0.05$ (*), $P<0.01$ (**) or $P<0.001$
569 (***) are indicated.
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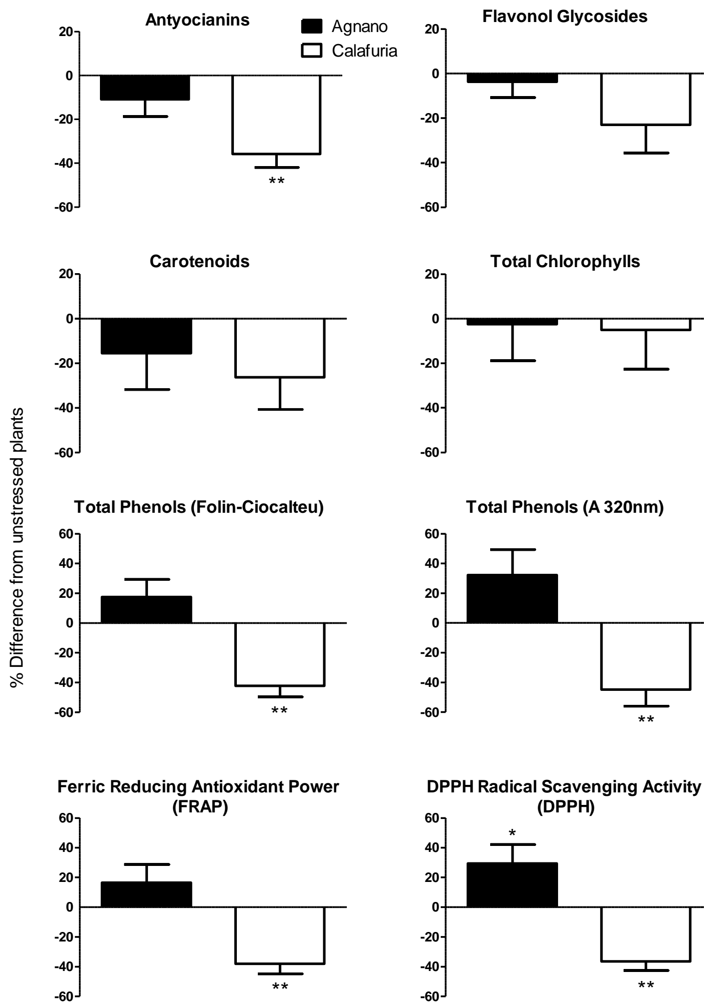

572

573 **Figure 4.** The percentage variation of the contents of anthocyanins, flavonol glycosides,
574 carotenoids, total chlorophylls, total phenols and antioxidant capacity (FRAP and DPPH) in the leaf
575 tissues of cultivated *Reichardia picroides* of different ecotypes (Agnano or Calafuria), after 10 days
576 spraying of sodium chloride solution (0.049 g m^{-2}). Mean values and standard deviation of four
577 replicates. Only significant differences relative to the unstressed control are indicated (*: $P<0.05$,
578 **: $P<0.01$), according to Bonferroni post-test following two way ANOVA.