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Original

Seed germination and antioxidant pattern in *Lavandula multifida* (Lamiaceae): A comparison between core and peripheral populations / Panuccio, M., Fazio, A., Musarella, C.m., Mendoza-fernández, A.j., Mota, J.f., Spampinato, G.. - In: PLANT BIOSYSTEMS. - ISSN 1724-5575. - 152:3(2018), pp. 398-406. [10.1080/11263504.2017.1297333]

Availability:

This version is available at: <https://hdl.handle.net/20.500.12318/4613> since: 2020-11-15T19:43:19Z

Published

DOI: <http://doi.org/10.1080/11263504.2017.1297333>

The final published version is available online at: <https://www.tandfonline.com/doi/full/10.1080/11263504>.

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(Article begins on next page)

13 **Seed germination and antioxidant pattern in *Lavandula multifida* (Lamiaceae): A**
14 **comparison between core and peripheral populations.**

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24

25 Running title: **Comparison between two populations of *Lavandula multifida***

26 **Abstract**

27 We evaluated the environmental adaptability of *Lavandula multifida* L., a plant species
28 presents in the Western Mediterranean Basin with a threatened peripheral population in
29 Southern Italy.

30 Germination capacity, activities of some hydrolyzing enzymes associated with
31 germination and antioxidative pathway of Calabrian *L. multifida* were quantified in
32 comparison with a Spanish core population.

33 The Calabrian population showed a lower germination ability and it can be related to the
34 small size of the population and associated inbreeding depression. Differences between
35 two populations in enzymatic assays and antioxidative pattern during germination and
36 early seedling development may in part explain a different ability of these two
37 populations to respond to external cues and a diverse environmental adaptability.

38 The study on germination strategies of isolated populations is important to define
39 possible programs for to preserve genetic biodiversity of autochthon plants populations.

40

41 Key words: Almeria; biodiversity; Calabria; conservation; endangered species;

42 *Lavandula*; peripheral population; seed germination.

43

44 **Introduction**

45 Plant populations of isolated species at the edge of the distribution range are an
46 interesting object of study with regard to the ecology, evolution and conservation of
47 biodiversity. Many characteristics of peripheral plants populations as morphology,
48 breeding system, demography, ecology, vary within the range of distribution of a
49 species, with more favourable conditions in the centre of the distribution.

50 The peripheral populations may differ greatly from the central populations for a
51 number of performance (Abeli, 2014). They have different common features: are small,
52 isolated, and occur in ecologically marginal habitats (Lawton, 1993; Hoffmann &
53 Blows, 1994). In several cases this resulted to a reduced genetic diversity (Lesica &
54 Allendorf, 1992, 1995) and the conservation value of these populations has been
55 questioned. The peripheral populations are often the most vulnerable and susceptible to
56 extinction. The conservation of rare and endangered plants requires considerable
57 research and adequate knowledge on their biology (Leppig & White, 2006.).

58 This study is focused on a threatened plant species of Italian flora, *L. multifida*, a
59 species present in Italy with a peripheral plant population, located in Southern Italy, to
60 evaluate the environmental adaptability of this threatened and rare plant which needs to
61 be preserved and valorised. In this study the germination capacity, the activities of some
62 hydrolyzing enzymes associated with germination and the antioxidative pathway were
63 evaluated in comparison with a core population from South Western of Iberian
64 Peninsula. These two populations of *L. multifida*, belonging to two geographically
65 distant regions, Capo dell'Armi (Reggio Calabria, Italy) and Almeria (Spain), are also
66 characterized by a different environmental adaptability, as the Spanish species is very

67 widespread on different types of substrates and it is part of matorral and xerophilous
68 pastures plant communities.

69 The aim was to study germination strategies of isolated populations of Calabrian *L.*
70 *multifida* to define possible conservation programs through the population
71 reinforcement and reintroduction in Italy. The research of biological traits associated
72 with reproduction is considered in several studies as essential for the development of
73 guidelines for the conservation and management of endangered species (Evans *et al.*,
74 2003).

75

76 **Materials and methods**

77 *Study species*

78 *L. multifida*, is a suffruticose chamaephyte with woody stems, but modest in size, native
79 of South Western Mediterranean region. It is distributed in North Africa (Morocco,
80 Algeria, Tunisia, Egypt), Southern Spain and Portugal, Sicily and Southern Italy
81 (Guinea, 1972; Morales Valverde, 2011; Euro-Med PlantBase, 2015). *L. multifida* is one
82 of the rarest plant species in Italy, where this plant occupies the northernmost part of its
83 distribution range. It is distributed in few small and fragmented populations between
84 Calabria and Sicily (Pignatti, 1982; Conti *et al.*, 2005). In Calabria is located only in
85 Capo dell'Armi, in the province of Reggio Calabria (Spampinato, 2014), in Sicily is in
86 three localities (Capo S. Alessio, M. Pellegrino and Brucoli), whereas it is disappeared
87 from some historic Sicilian sites such as Capo Scaletta and Taormina (Galesi *et al.*,
88 2005; Giardina *et al.*, 2007). In Calabria this plant spontaneously grows in garigue on
89 poorly-evolved limestone soils (Brullo *et al.*, 2001), between 10 and 200 m a.s.l., in the
90 most arid climatic conditions of Italian peninsula characterized by "mediterranean

91 pluvio-oceanic” bioclimate with thermo-Mediterranean thermotype and dry ombrotype
92 (Rivas-Martinez, 2008). It blooms from February to April, although this period is often
93 more extended.

94 Since *L. multifida* populations appear to be reduced and fragmented due to the human
95 impact on its natural habitat, this plant has been included in the “Regional Red Lists of
96 Italian Plants” under the IUCN status of “critically endangered” in Calabria region and
97 “endangered” in Sicily (Conti *et al.*, 1997).

98

99 *Germination experiments*

100 Seeds were collected in 2014, immediately after maturation from two different sites,
101 where the plant spontaneously grows: Almeria (Southern Spain) and Capo dell’Armi
102 (Reggio Calabria, Southern Italy). The sampling involved randomly throughout the
103 subpopulation examined.

104 Clean seeds were stored dry at 4°C. Germination experiments were carried out after 6
105 months of collection to ensure that the seeds were not dormant. First of all, steps were
106 taken to test for the viability of the seeds. They have been selected 50 seeds of each lot
107 and were treated with a 1% solution of 2,3,5-triphenyltetrazolium chloride (ISTA,
108 2006). From this viability test, it was found that 80% of the seeds selected was viable.

109 Seeds were selected and surface-sterilized with freshly-prepared 30% (v/v) commercial
110 bleach solution for 15 minutes, followed by washing for several times with distilled and
111 autoclaved water. Twenty seeds were placed on filter papers in Petri dishes (9 cm
112 diameter) and then stratified at dark at 4°C for 72 hours, in order to synchronize
113 germination process. Stratification procedure allows the break of dormancy, so
114 improving the germination process (Būdvytytė, 2001).

115 After the stratification process, Petri dishes were placed in a growth chamber at
116 different temperatures (15°C, 20°C, 25°C and 30°C), under a photoperiod of 12 hours-
117 light/12 hours-dark, to assess possible differences in germination percentage due to
118 different temperature conditions. Some experiments were also performed at dark, at
119 15°C, 20 °C and at 25°C, to evaluate the importance of the light on germination process.
120 For all experimental conditions, five Petri dishes were prepared. The number of
121 germinated seeds was assessed every day over a period of 14 days. Seeds were
122 considered germinated when the radicle was at least 0.5 cm long.
123 At a temperature of 15 ° C no seed has germinated. For this reason, this temperature
124 value was not included in the graphs.

125

126 *Sample extraction*

127 Seeds at the end of stratification (Time 0) and 4 days after sown (4 days) were
128 homogenized using chilled mortar and pestle, with 0.1 M K-phosphate buffer (pH 7.0)
129 (1:5 w/v), 1 mM Na₂-EDTA, 10 mM MgCl₂, and 1% (w/v) PVPP. The extracts were
130 centrifuged at 12.000 rpm for 20 minutes at 4°C. The resulting supernatant was used for
131 all assays. All enzyme activities were measured at 25°C on a UV, visible light
132 spectrophotometer.

133

134 *Determination of total proteins*

135 Total proteins were determined according to method of Bradford (1976) using bovine
136 serum albumin as standard. The concentration of protein was obtained by reading the
137 absorbance at 595 nm against blank and related to the calibration curve. Total proteins
138 were expressed as mg proteins/gr fresh weight.

139

140 *Enzyme assays*141 Alpha-Amylase (EC 3.2.1.1) was determined at 546 nm as described by Coombe *et al.*

142 (1967). At 0.3 ml of 1% starch solution were added to 0.3 ml of seed-extract; the

143 mixture was incubated in water bath at 30°C for 30 minutes. After 0.6 ml of Colour

144 reagent was added and the mixture was boiled for 5 minutes. At the end, 2.5 ml of

145 distillate water were added and the final mixture was cooled for 30 minutes.

146 Isocitrate-lyase activity (ICL EC 4.1.3.1) was determined according to Bajracharya and

147 Schopfer (1979). At 0.8 mL of buffer K-phosphate 0.1M (pH 7.6) were added: 0.15 ml

148 of phenylhydrazine 33 mM, 0.15 ml of dithiothreitol 50mM, 0.15 ml of magnesium

149 chloride 220 mM and 0.1 ml of seed extract. The mixture was incubated at 25°C for 5

150 minutes, and after 0.15 ml of isocitrate 175 mM was added. The kinetic was recorded at

151 334 nm for 180 seconds.

152 Glucose 6-Phosphate Dehydrogenase (G6P-DH EC.1.1.1.49) activity was determined at

153 340 nm as reduction of NADP to NADPH. At 0.8 ml of Tris HCl were added 0.1 ml of

154 seed-extract, and 0.1 ml of NADP (freshly prepared) and of 0.1 ml of glucose 6-

155 phosphate (De Meillon *et al.*, 1990).

156 Superoxide dismutase (SOD EC 1.15.1.1) activity was estimated by recording the

157 decrease in absorbance of formazan produced from NBT, at 560 nm (Dhindsa *et al.*,

158 1981). The mixture reaction contained: 1.5 ml of 0.1 M potassium-phosphate buffer (pH

159 7.5) containing EDTA, 15 µl of 13 mM methionine, 15 µl of 50 µM nitroblue

160 tetrazolium, 50 µl enzyme. Reaction was started by adding 150 µl of 2 mM riboflavin

161 and placing the tubes under two fluorescent lamps for 15 minutes. A complete reaction

162 mixture without enzyme gave the maximal color after the irradiation, and served as

163 control; while a non-irradiated complete reaction mixture served as a blank. Reaction
164 was stopped by switching off the light and putting the tubes into dark at room
165 temperature.

166 Catalase activity (CAT, EC 1.11.1.6) was determined according to Beaumont *et al.*
167 (1990) by monitoring the disappearance of H₂O₂ at 240 nm and calculated by using its
168 extinction coefficient (ϵ) = 0.036 mM⁻¹ cm⁻¹. The reaction mixture contained 1 ml
169 potassium phosphate buffer (50 mM, pH 7.0), 40µl enzyme extract and 5 µl H₂O₂.

170 Peroxidase activity (POX, EC 1.11.1.7) was determined as reduction in guaiacol
171 concentration by reading the absorbance at 436 nm continuously for 90 seconds. The
172 reaction mixture contained 1 ml potassium phosphate buffer (0.1 M, pH 7.0), 20 µl
173 guaiacol, 40 µl enzyme extract and 15 µl H₂O₂. POX activity was quantified by the
174 amount of tetraguaiacol formed using its extinction coefficient (ϵ) = 25.5 mM⁻¹ cm⁻¹
175 (Panda *et al.*, 2003).

176 Dehydroascorbate reductase (DHA-Rd, EC 1.8.5.1) activity was assayed following the
177 increase in absorbance at 265 nm owing to the reduced glutathione (GSH) dependent
178 production of ASC (Doulis *et al.*, 1997). The reaction mixture contained 0.1 M K-
179 phosphate buffer pH 6.5, 1 mM GSH and 1 mM DHA (ϵ = 14 mM⁻¹cm⁻¹).

180 Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined from the decrease
181 in absorbance at 290 nm, due to oxidation of ascorbate. The reaction mixture was 0.1 M
182 K-phosphate buffer pH 6.5, 90 mM H₂O₂ and 50 mM ascorbate (Amako *et al.*, 1994).
183 The decrease in absorbance was recorded continuously for 90 seconds (extinction
184 coefficient 14 mM⁻¹cm⁻¹).

185 Glutathione Reductase (GR, EC 1.6.4.2) activity was assayed following the oxidation
186 rate of NADPH at 340 nm (ϵ = 6.2 mM⁻¹ cm⁻¹). The reaction mixture contained 0.1 M

187 potassium phosphate buffer (pH 7.0), 20 mM GSSG, 2 mM NADPH₂, 350 µl H₂O and
188 50 µl enzyme extract.

189 Ascorbate (ASC) and dehydroascorbate (DHA) were determined by Kampfenkel
190 (1995). Seeds (1 g) were homogenized in 5% metaphosphoric acid. The homogenate
191 was centrifuged at 13 000 g and the supernatant was used for ASC and DHA analysis.

192

193 *Data analysis*

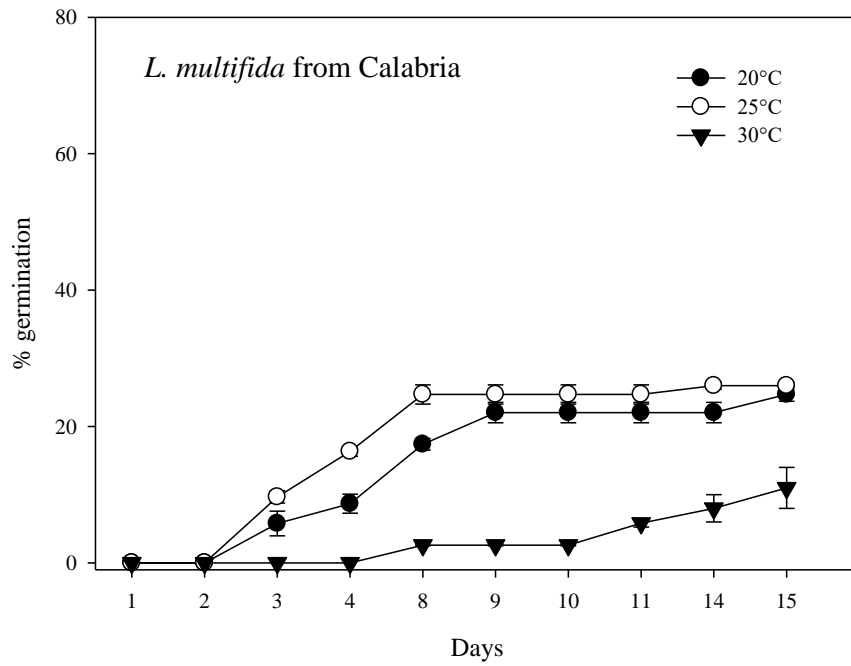
194 We analysed the effects of treatments on final germination by fitting factorial
195 generalized linear model (GLM) to the germination data. A statistical analysis was
196 performed with the software SYSTAT v. 8.0 software (SPSS Inc.) using one-way
197 Anova, followed by LSD test to evaluate significant differences in enzymatic activities
198 and antioxidant pathway within species.

199

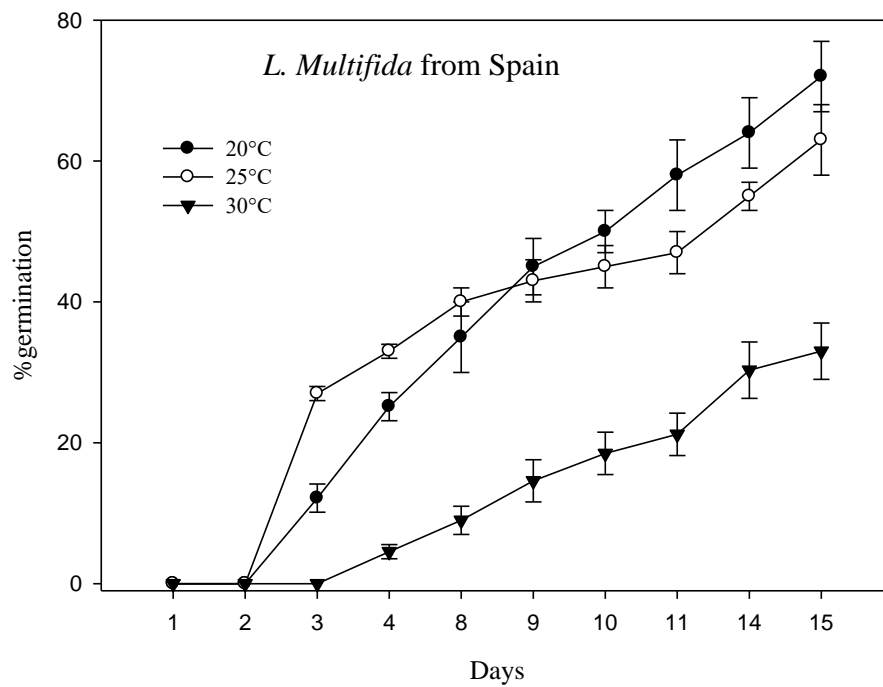
200 **Results**

201 Germination percentage of Calabrian *L. multifida* seeds did not show significant
202 differences between 20°C and 25°C; but it strongly decreased at 30°C (Figure 1).
203 Spanish *L. multifida* seeds showed a similar trend under the same temperature
204 conditions (Figure 1), even if the maximum percentage of germination of Calabrian *L.*
205 *multifida* (about 25%) was significantly lower than that of *L. multifida* from Spain
206 (about 70%), and also the speed of germination was significantly lesser in Calabrian *L.*
207 *multifida* seeds if compared to Spanish *L. multifida*, in all treatments.

208



209



210

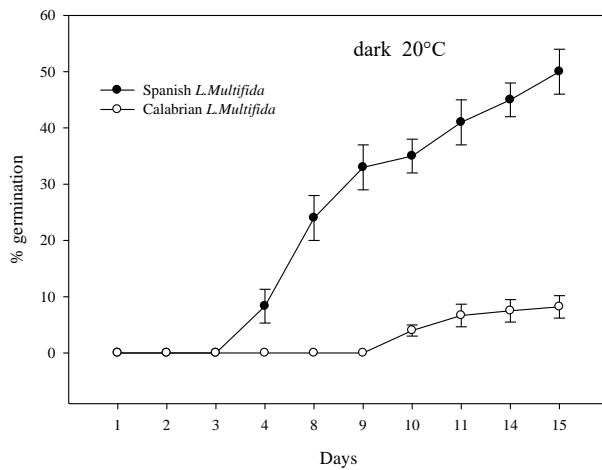
211 Figure 1. Germination percentages of Calabrian *L. multifida* and Spanish *L. multifida*
 212 seeds incubated at 20°C, 25°C and 30°C under a photoperiod of 12 hours light/12 hours.
 213 Data were expressed as mean \pm standard error.

214

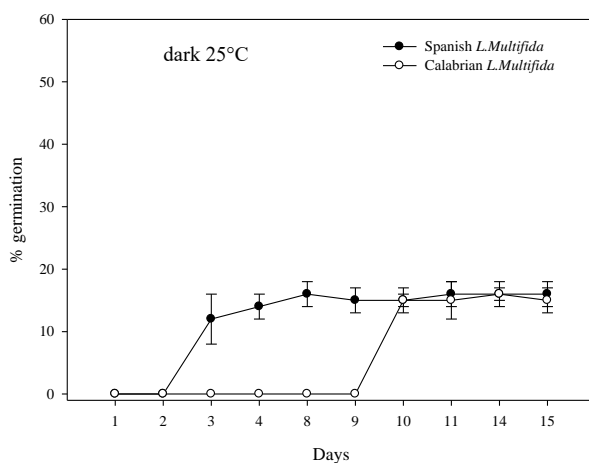
215

216

217 Germination in both species was strongly affected when the seeds were incubated in the
 218 dark. Particularly, a delay in germination process until the 9th day of incubation was
 219 observed in Calabrian *L. multifida*. (Figure 2) and also total germination was reduced,
 220 with a maximum germination percentage of about 13% for the seeds incubated at 25°C
 221 and about 6% for the seeds incubated at 20°C. Conversely, no delay in the germination
 222 process was observed in Spanish *L. multifida* seeds (Figure 2), and germination
 223 percentage was significantly higher than that of Calabrian *L. multifida* seeds at 20°C, in
 224 the dark.



225



226

227 Figure 2. Germination percentages of Calabrian *L. multifida* and Spanish *L. multifida*
 228 seeds incubated at 20°C and 25°C under dark conditions. Data were expressed as mean
 229 \pm standard error.

230

231 GLM analysis (Table I) showed that the differences in germination performance
 232 between two species are very significant (P value) and the positive coefficient (1.48)
 233 confirmed a higher germination ability of Spanish *L. multifida*. The temperature (20 and
 234 25°C) did not produce significant effects even if the negative coefficient value indicated
 235 a progressive lowering of germination by increasing the temperature.

236 Table I. Generalized linear model fitted to germination data comparing population,
 237 mean temperature and light presence. AIC, Akaike Information Criterion; SE, standard
 238 error; Z, Wald statistic . ***0.001 **0.01 *0.05.
 239

Model	Effect	Coefficient	SE	Z	P value
AIC = 57.41	Intercept	-0.68990	0.95298	-0.724	0.469
	Population [T. <i>L. multifida</i> Spagnola]	1.48621	0.21627	6.872	6.33e-12 ***
	Mean temperatures	-0.06090	0.04183	-1.456	0.145
	Light	1.22128	0.21438	5.697	1.22e-08 ***

240
 241 The activities of some hydrolyzing enzymes like alpha-amylase and isocytate lyase
 242 were assayed on seed extracts of both *L. multifida* populations at the end of stratification
 243 (0 day) and four days after sown (4 days). The activity of α -amylase declined during
 244 four days in Spanish *L. multifida* while it did not change in the Calabrian lavandula
 245 seeds (Table II). The activity of Isocytate lyase (ICL), a key-enzyme involved in
 246 glyoxylate cycle, was very low and showed an opposite trend between two species
 247

248 Table II Activities of hydrolyzing enzymes in seeds at 0 and 4 days after sown. ^a
 249 **a.** α -amylase activity was expressed as μg maltose per mg of protein. ICL and G6P-DH
 250 activities were expressed as enzyme units (U) per mg of protein. One unit of enzyme
 251 was defined as the amount of enzyme necessary to decompose 1 nmol of substrate per
 252 min at 25°C. Data were expressed as mean \pm standard error. Small letters indicate
 253 significant differences in Calabrian *L. multifida*,
 254
 255

	Calabrian <i>L. multifida</i>		Spanish <i>L. multifida</i>	
	0 days	4 days	0 days	4 days
α -amylase	178.12 \pm 6.7 ^a	164.35 \pm 8.4 ^a	349.04 \pm 10.2 ^a	160.35 \pm 7.5 ^b
ICL	0.75 \pm 0.03 ^a	0.40 \pm 0.02 ^b	0.32 \pm 0.08 ^b	0.64 \pm 0.01 ^a
G6P-DH	0.63 \pm 0.02 ^b	4.71 \pm 0.03 ^a	n.d.	2.65 \pm 0.02 ^a

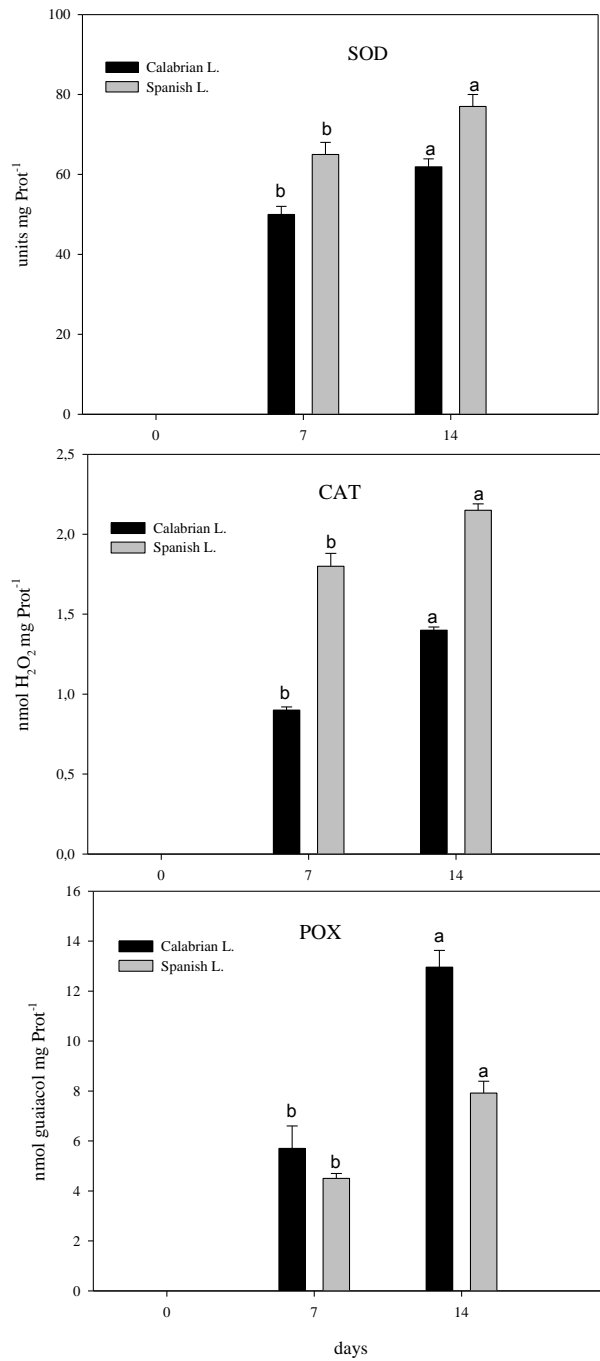
256
 257 ICL activity lowered during 4 days, while in Spanish *L. multifida* seeds, this activity
 258 doubled at time 4 compared to time 0. Glucose 6-phosphate Dehydrogenase (G6PDH) is
 259 an enzyme of the oxidative pentose-phosphate pathway (OPPP) and it plays an
 260 important role in the regulation of germination, at resumption of respiratory activity
 261 (Come *et al.*, 1988; Perino & Come, 1991). In both populations, G6PDH activity
 262 increased during germination, with the highest values in Calabrian one, 4 days after
 263 sown (Table I). The reactivation of metabolism may provide an important source of
 264 reactive oxygen species (ROS) (Garnczarska & Wojtyla, 2008) and the results showed
 265 that ROS-scavenging systems are activated in both populations by means different
 266 responses of antioxidant molecules and enzymes. SOD and CAT activities increased
 267 and the highest activities were detected in Spanish *L. multifida*, after 14 days differences
 268 within species. Significance level was set at $P < 0.05$.
 269

270 Table III. Ascorbic acid (ASC) and Dehydroascorbate (DHA) content in dry
 271 seeds. ASC was expressed as μg ascorbic acid $\text{g}^{-1}\text{F.W.}$; DHA was expressed as
 272 μg dehydroascorbic acid $\text{g}^{-1}\text{F.W.}$. Small letters indicate significant differences
 273 between species.
 274

	Calabrian <i>L. Multifida</i>	Spanish <i>L. Multifida</i>
ASC	23.12 ± 0.96^b	34.87 ± 9.10^a
DHA	120.59 ± 4.35^b	212.15 ± 29.68^a
ASC/DHA	0.19	0.16

275
 276 Seeds, at the end of stratification, showed a significant DHA-Rd activity, in particular
 277 the Spanish population (Figure 4).
 278 This may confirm the role postulated for DHA reduction in producing an initial ascorbic
 279 acid supply during germination (De Tullio & Arrigoni, 2003; Hameed *et al.*, 2014).
 280 DHA-Rd and APX activities increased, for both populations, until 14 days (Figure 4).
 281 Glutathione Reductase activity (GR) was undetectable in seeds and then increased over
 282 time, and the values were similar for both Calabrian and Spanish populations of *L.*
 283 *multifida* (Figure 4).
 284

285



286

287 Figure 3. SOD, CAT and POX activity in Calabrian and Spanish *L. multifida* seeds at 0,

288

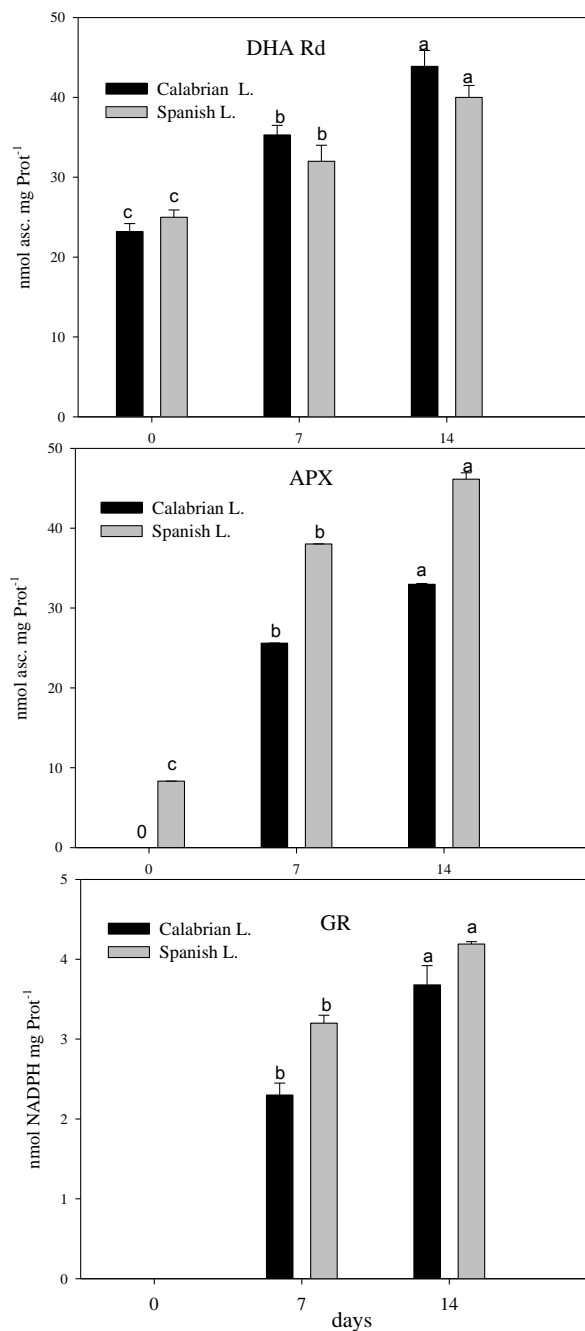
7 and 14 days after sown. Data were expressed as mean \pm standard error. Small letters

289

indicate significant differences within species. Significance level was set at $P < 0.05$.

290

291



292
 293 Figure 4. DHA-Rd, APX and GR activity in Calabrian and Spanish L. multifida seeds at
 294 0, 7 and 14 days after sown. Data were expressed as mean \pm standard error. Small letters
 295 indicate significant differences within species. Significance level was set at $P < 0.05$.

296

297

298 Discussion

299 Temperature and photoperiod differently affect germination process in the two
300 populations of *L. multifida*. In particular Calabrian *L. multifida* showed to be more
301 sensitive than Spanish and this different resistance may be important for the
302 environmental adaptability and the distribution of this species. Both *L. multifida*
303 provenances have the maximum germination at 20° and 25°C and with 12 hours
304 light/dark alternating and these findings are in agreement with what has been observed
305 for the Spanish populations of this species (Estrelles *et al.*, 2004). Instead, there are
306 significant differences in germination percentages. These different germination
307 performances can be linked to specific characteristics of the populations, as reported by
308 Menges (1991) that pointed out that seeds of small populations, at limit of their
309 distribution area, have fewer germinative capacity.

310 Utilization of seed reserves is one of the important physiological and biochemical
311 process associated with germination and α -amylase enzyme is responsible for initiating
312 the mobilization of starch in germinating seeds. The development of this activity is
313 important for providing sugars, the main source of energy during the early development
314 of the plant. In Spanish population, the activity of α -amylase decreased over time
315 suggesting a more efficient mobilization and use of reserves compared to Calabrian *L.*
316 *multifida*. Isocitrate lyase (ICL) represents a key-enzyme involved in glyoxylate cycle
317 which is essential to convert lipids into carbohydrates during the germination. Lipids are
318 not generally considered to be quantitatively important respiratory substrate in plants
319 (Eastmond *et al.*, 2000). Also for both *L. multifida* populations, the low activities of ICL
320 suggest that, during germination, carbohydrates are mainly originated from starch

321 degradation, through amylase action, rather than from malate produced by glyoxylate
322 cycle through gluconeogenesis.

323 One of the first changes upon imbibition is the resumption of respiratory activity that
324 reflects the oxidation of carbohydrates via the respiratory pathway. In both species,
325 G6PDH activity increased over time, providing the cell with reducing power (NADPH).
326 The balance between glycolysis and the OPPP ensures that the seed, during
327 germination, is supplied with the necessary levels of reducing power, ATP and carbon
328 skeletons.

329 The significant differences of enzyme activities involved in the mobilization of
330 energetic reserves between the two *L. multifida* populations may explain the diverse
331 germination speed and germination percentages. However, the relationship between
332 enzyme activity and germination is not always well defined, in fact is difficult to
333 identify how much of the metabolism occurring during germination is actually
334 necessary for the radicle emergence *per se*, rather than in preparation for post-
335 germinative events and this means a limited mobilization of reserves during germination
336 compared to reserve utilization following germination (Nonogaki *et al.*, 2010;
337 Garnczarska & Wojtyła, 2008).

338 Germination and ROS accumulation appear to be linked and seed germination success
339 may be closely associated with internal ROS contents and activities of ROS-scavenging
340 systems (Gomes & Garcia, 2013). ROS regulation might be achieved by changes in
341 concentration of low molecular mass antioxidants, such as ascorbate and glutathione,
342 and by different responses of antioxidative enzymatic systems. As reported for dry
343 orthodox seeds of several angiosperms, the activities of two ROS scavenger enzymes,
344 SOD and CAT, are very low in seeds of both *lavandula* populations but they suddenly

345 raised during germination (Wojtyla *et al.*, 2006). The coupled activities of SOD and
346 CAT are of particular importance in maintaining intracellular redox homeostasis of
347 seeds during germination; SOD acts as the first line of defense converting the toxic
348 superoxide radical into H_2O_2 and CAT detoxify H_2O_2 to H_2O . Peroxidases are a group
349 of nonspecific enzymes that catalyse the oxidation of a wide variety of substrates, using
350 hydrogen peroxide as electron donor, they are involved in several metabolic processes
351 and not exclusively in mechanisms of defense (Biles & Martyn, 1993). Ascorbate
352 peroxidase (APX) and dehydroascorbate reductase (DHA Rd) are enzymes of ascorbate-
353 glutathione cycle. The results showed an increase of DHA-Rd activity, for both
354 populations, over time and this means a high supply of ascorbate for APX. In fact, the
355 activity of APX was very low in seeds, but gradually increased contributing in H_2O_2
356 removal and catalyzing the DHA production. According with APX and DHA Rd
357 activities, also GR activity increased over time to reduce oxidized GSSG to GSH. In
358 seeds glutathione (GSH) could be involved as an antioxidant in direct reactions with
359 free radicals, or in cooperation with ascorbate in the ascorbate-glutathione cycle (Szalai
360 *et al.*, 2009; Tommasi *et al.*, 2001). In all pathways GSH is oxidized to GSSG and must
361 be rapidly reduced by GR activity.

362 Results showed that to prevent oxidative damage, the two populations of *Lavandula*
363 possess a battery of antioxidant enzymes and also antioxidant compounds that are
364 differently involved in seed germination and after emergence of radicle (Smirnoff &
365 Wheeler, 2000; Miller *et al.*, 2010; Foyer & Noctor, 2011). Although ROS were long
366 considered hazardous molecules, their function as cell signalling compounds is now
367 well established. In seeds ROS have important roles in endosperm weakening,
368 mobilization of seed reserves and may also function as messengers or transmitter of

369 environmental cues during germination (Kwak *et al.*, 2006). Slightly enhanced levels of
370 oxidative stress have been observed to stimulate germination (Lefevre *et al.*, 2009). It is
371 important that antioxidant systems maintain intracellular redox homeostasis, preventing
372 the accumulation of toxic amounts of ROS while allowing ROS-signaling function.
373 Therefore, the different activation of antioxidative systems during germination between
374 Calabrian and Spanish populations of *L. multifida* can be also considered as metabolic
375 adaption preparing the seeds to conditions occurring after emergence of radicle and the
376 different behaviour may in part explain the different ability of these two species to
377 respond to external cues.

378 Calabrian population of *L. multifida* shows lower germination ability in comparison
379 with Spanish population. Results from enzymatic assays further confirmed the different
380 behaviour of seeds of the two *L. multifida* populations during germination and early
381 seedling development. Differences between two populations in antioxidative molecules
382 and enzymes and their activation during germination perform a crucial function in
383 regulation of ROS concentration, but can be also considered a metabolic adaption
384 related to high germination capacity and vigorous seedling development and then can
385 also explain the difference in environmental adaptability of two populations of *L.*
386 *multifida*.

387 The lower germination ability of peripheral populations, such as the Calabrian *L.*
388 *multifida*, can be related to the small size of populations and associated inbreeding
389 depression (Lammi *et al.*, 1999). *L. multifida*, in its northern-most distribution site, is a
390 rare and endangered plant, because all Italian populations are small and isolated.
391 Specific actions are therefore needed for the conservation of these populations.
392 Propagation from seed is a viable method for the ex situ conservation of peripheral

393 populations of *L. multifida*, and considering germination requirements, it can be
394 profitably realized only by research facilities or specialized centres.

395

396 **Funding**

397 This work was supported by Regione Calabria [PSR Calabria 2007/2013 Misura 214
398 Azione 6 Sviluppo Rurale “Salvaguardia, diffusione e valorizzazione della *Lavandula*
399 *multifida* L. in Calabria”].

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