13 **Seed germination and antioxidant pattern in *Lavandula multifida* (*Lamiaceae*): A**

14 **comparison between core and peripheral populations.**

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

**Abstract**

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

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

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

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

Key words: Almeria; biodiversity; Calabria; conservation; endangered species; Lavandula; peripheral population; seed germination.

## Introduction

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

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

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

widespread on different types of substrates and it is part of mattoral and xerophilous pastures plant communities.

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

## Materials and methods

### *Study species*

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

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

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

*Germination experiments*

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

Clean seeds were stored dry at 4°C. Germination experiments were carried out after 6 months of collection to ensure that the seeds were not dormant. First of all, steps were taken to test for the viability of the seeds. They have been selected 50 seeds of each lot and were treated with a 1% solution of 2,3,5-triphenyltetrazolium chloride (ISTA, 2006). From this viability test, it was found that 80% of the seeds selected was viable. Seeds were selected and surface-sterilized with freshly-prepared 30% (v/v) commercial bleach solution for 15 minutes, followed by washing for several times with distilled and autoclaved water. Twenty seeds were placed on filter papers in Petri dishes (9 cm diameter) and then stratified at dark at 4°C for 72 hours, in order to synchronize germination process. Stratification procedure allows the break of dormancy, so improving the germination process (Būdvytytė, 2001).

After the stratification process, Petri dishes were placed in a growth chamber at different temperatures (15°C, 20°C, 25°C and 30°C), under a photoperiod of 12 hours-light/12 hours-dark, to assess possible differences in germination percentage due to different temperature conditions. Some experiments were also performed at dark, at 15°C, 20 °C and at 25°C, to evaluate the importance of the light on germination process. For all experimental conditions, five Petri dishes were prepared. The number of germinated seeds was assessed every day over a period of 14 days. Seeds were considered germinated when the radicle was at least 0.5 cm long.

At a temperature of 15 ° C no seed has germinated. For this reason, this temperature value was not included in the graphs.

*Sample extraction*

Seeds at the end of stratification (Time 0) and 4 days after sown (4 days) were homogenized using chilled mortar and pestle, with 0.1 M K-phosphate buffer (pH 7.0) (1:5 w/v), 1 mM $Na_2$-EDTA, 10 mM $MgCl_2$, and 1% (w/v) PVPP. The extracts were centrifuged at 12.000 rpm for 20 minutes at 4°C. The resulting supernatant was used for all assays. All enzyme activities were measured at 25°C on a UV, visible light spectrophotometer.

*Determination of total proteins*

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

*Enzyme assays*

Alpha-Amilase (EC 3.2.1.1) was determined at 546 nm as described by Coombe *et al.* (1967). At 0.3 ml of 1% starch solution were added to 0.3 ml of seed-extract; the mixture was incubated in water bath at 30°C for 30 minutes. After 0.6 ml of Colour reagent was added and the mixture was boiled for 5 minutes. At the end, 2.5 ml of distillate water were added and the final mixture was cooled for 30 minutes.

Isocytrate-lyase activity (ICL EC 4.1.3.1) was determined according to Bajracharya and Schopfer (1979). At 0.8 mL of buffer K-phosphate 0.1M (pH 7.6) were added: 0.15 ml of phenylhydrazine 33 mM, 0.15 ml of dithiothreitol 50mM, 0.15 ml of magnesium chloride 220 mM and 0.1 ml of seed extract. The mixture was incubated at 25°C for 5 minutes, and after 0.15 ml of isocytrate 175 mM was added. The kinetic was recorded at 334 nm for 180 seconds.

Glucose 6-Phosphate Dehydrogenase (G6P-DH EC.1.1.1.49) activity was determined at 340 nm as reduction of NADP to NADPH. At 0.8 ml of Tris HCl were added 0.1 ml of seed-extract, and 0.1 ml of NADP (freshly prepared) and of 0.1 ml of glucose 6-phosphate (De Meillon *et al.*, 1990).

Superoxide dismutase (SOD EC 1.15.1.1) activity was estimated by recording the decrease in absorbance of formazan produced from NBT, at 560 nm (Dhindsa *et al.*, 1981). The mixture reaction contained:1.5 ml of 0.1 M potassium-phosphate buffer (pH 7.5) containing EDTA, 15 µl of 13 mM methionine, 15 µl of 50 µM nitroblue tetrazolium, 50 µl enzyme. Reaction was started by adding 150 µl of 2 mM riboflavin and placing the tubes under two fluorescent lamps for 15 minutes. A complete reaction mixture without enzyme gave the maximal color after the irradiation, and served as

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

Catalase activity (CAT, EC 1.11.1.6) was determined according to Beaumont *et al.* (1990) by monitoring the disappearance of $H_2O_2$ at 240 nm and calculated by using its extinction coefficient (ε) = 0.036 $mM^{-1}$ $cm^{-1}$. The reaction mixture contained 1 ml potassium phosphate buffer (50 mM, pH 7.0), 40µl enzyme extract and 5 µl $H_2O_2$.

Peroxidase activity (POX, EC 1.11.1.7) was determined as reduction in guaiacol concentration by reading the absorbance at 436 nm continuously for 90 seconds. The reaction mixture contained 1 ml potassium phosphate buffer (0.1 M, pH 7.0), 20 µl guaiacol, 40 µl enzyme extract and 15 µl $H_2O_2$. POX activity was quantified by the amount of tetraguaiacol formed using its extinction coefficient (ε) = 25.5 $mM^{-1}$ $cm^{-1}$ (Panda *et al.*, 2003).

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

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined from the decrease in absorbance at 290 nm, due to oxidation of ascorbate. The reaction mixture was 0.1 M K-phosphate buffer pH 6.5, 90 mM $H_2O_2$ and 50 mM ascorbate (Amako *et al.*, 1994). The decrease in absorbance was recorded continuously for 90 seconds (extinction coefficient 14 $mM^{-1}cm^{-1}$).

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

potassium phosphate buffer (pH 7.0), 20 mM GSSG, 2 mM $NADPH_2$, 350 µl $H_2O$ and 50 µl enzyme extract.

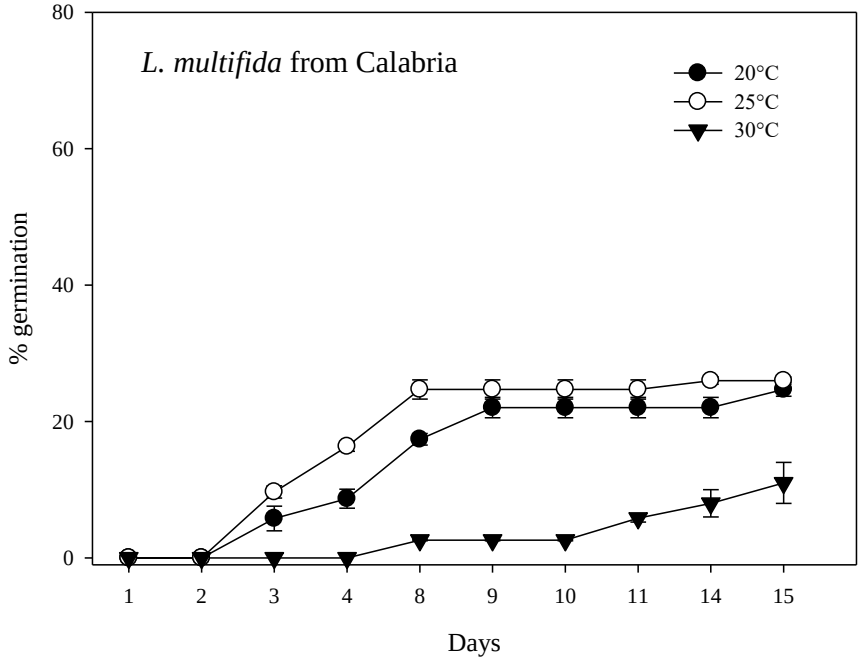
Ascorbate (ASC) and dehydroascorbate (DHA) were determined by Kampfenkel (1995). Seeds (1 g) were homogenized in 5% metaphosphoric acid. The homogenate was centrifuged at 13 000 *g* and the supernatant was used for ASC and DHA analysis.

*Data analysis*

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

**Results**

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

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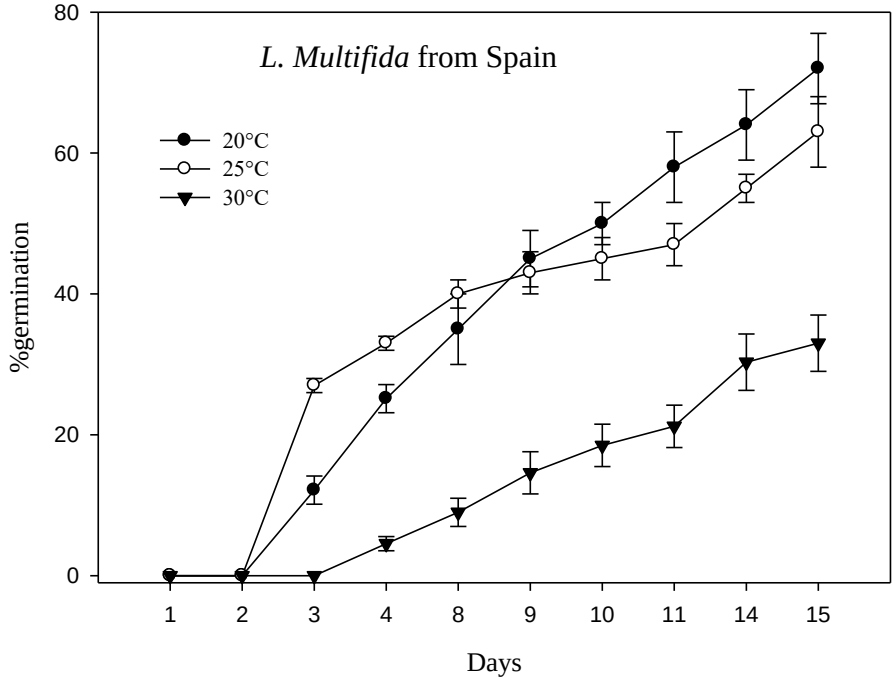


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211 Figure 1. Germination percentages of Calabrian L. multifida and Spanish L. multifida
212 seeds incubated at 20°C, 25°C and 30°C under a photoperiod of 12 hours light/12 hours.
213 Data were expressed as mean ± standard error.
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215 .

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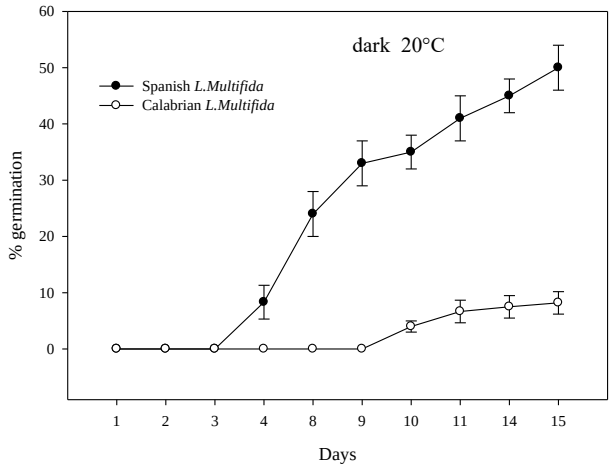
Germination in both species was strongly affected when the seeds were incubated in the dark. Particularly, a delay in germination process until the 9th day of incubation was observed in Calabrian *L. multifida*. (Figure 2) and also total germination was reduced, with a maximum germination percentage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



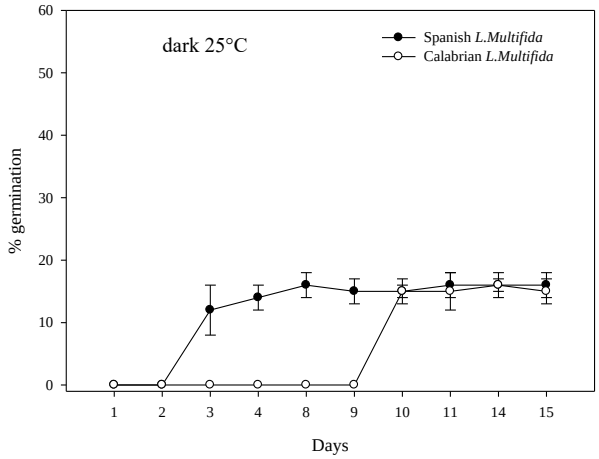




Figure 2. Germination percentages of Calabrian *L. multifida* and Spanish *L. multifida* seeds incubated at 20°C and 25°C under dark conditions. Data were expressed as mean ± standard error.

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

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

| **Model** | **Effect** | **Coefficient** | **SE** | **Z** | **P value** |
|---|---|---|---|---|---|
| AIC = 57.41 | Intercept | -0.68990 | 0.95298 | -0.724 | 0.469 |
| | Population [T. *L. multifida* 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 *** |

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

Table II Activities of hydrolyzing enzymes in seeds at 0 and 4 days after sown. [a]
**a.** α-amylase activity was expressed as µg maltose per mg of protein. ICL and G6P-DH activities were expressed as enzyme units (U) per mg of protein. One unit of enzyme was defined as the amount of enzyme necessary to decompose 1 nmoL of substrate per min at 25ºC. Data were expressed as mean ± standard error. Small letters indicate significant di In Calabrian *L. multifida,*

| | Calabrian *L. multifida* | | Spanish *L. multifida* | |
|---|---|---|---|---|
| | 0 days | 4 days | 0 days | 4 days |
| α-amylase | 178.12±6.7[a] | 164.35±8.4[a] | 349.04±10.2[a] | 160.35±7.5[b] |
| ICL | 0.75±0.03[a] | 0.40±0.02[b] | 0.32±0.08 [b] | 0.64±0.01[a] |
| G6P-DH | 0.63±0.02[b] | 4.71±0.03[a] | n.d. | 2.65±0.02 [a] |

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

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

| | Calabrian L.*Multifida* | Spanish L. *Multifida* |
|---|---|---|
| ASC | $23.12 \pm 0.96^{b}$ | $34.87 \pm 9.10^{a}$ |
| DHA | $120.59 \pm 4.35^{b}$ | $212.15 \pm 29.68^{a}$ |
| ASC/DHA | 0.19 | 0.16 |

Seeds, at the end of stratification, showed a significant DHA-Rd activity, in particular the Spanish population (Figure 4).

This may confirm the role postulated for DHA reduction in producing an initial ascorbic acid supply during germination (De Tullio & Arrigoni, 2003; Hameed *et al.*, 2014).

DHA-Rd and APX activities increased, for both populations, until 14 days (Figure 4).

Glutathione Reductase activity (GR) was undetectable in seeds and then increased over time, and the values were similar for both Calabrian and Spanish populations of *L. multifida* (Figure 4).

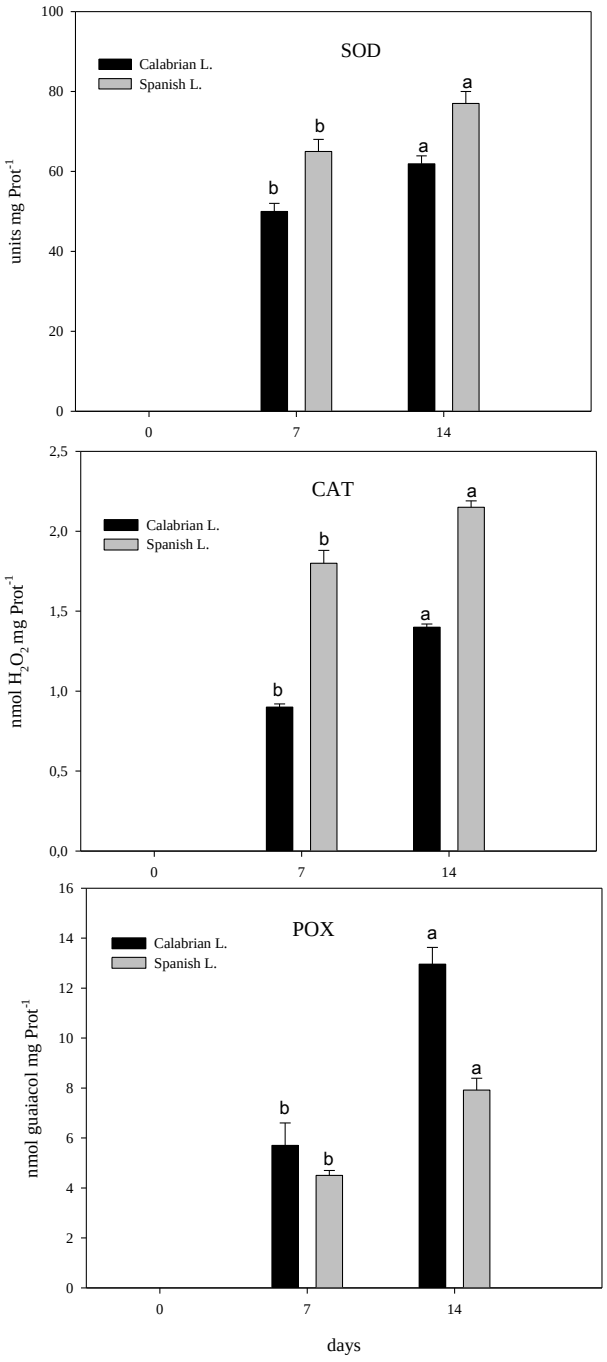



Figure 3. SOD, CAT and POX activity in Calabrian and Spanish *L. multifida* seeds at 0, 7 and 14 days after sown. Data were expressed as mean ± standard error. Small letters indicate significant differences within species. Significance level was set at $P<0.05$.

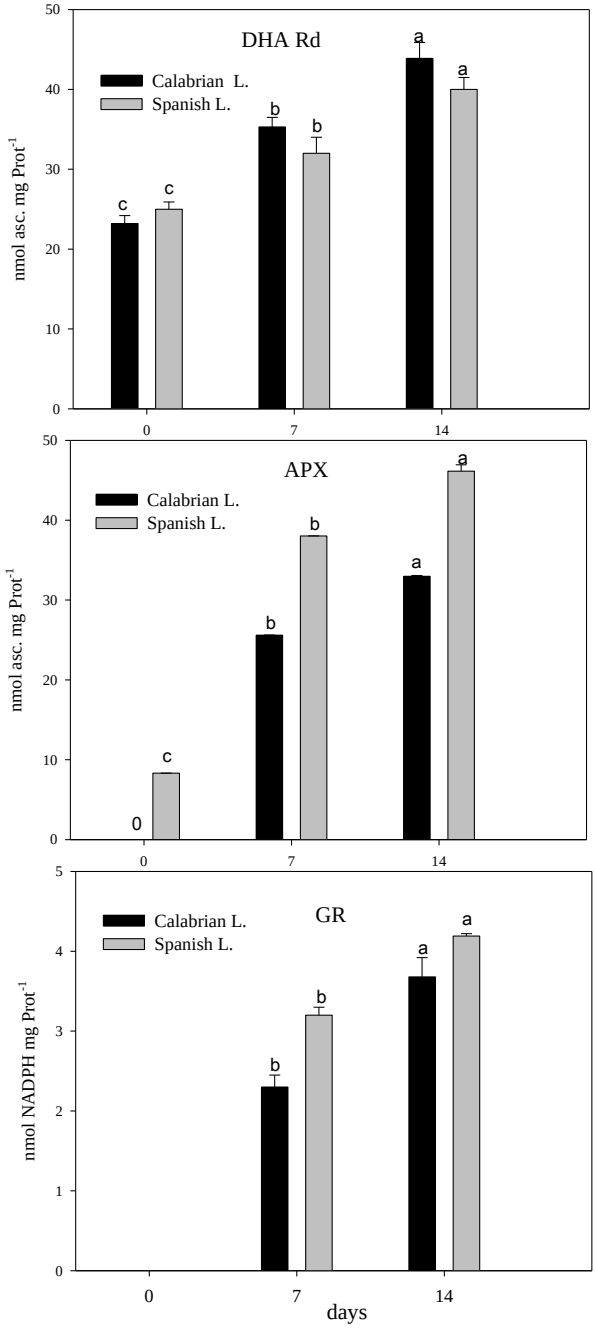



Figure 4. DHA-Rd, APX and GR activity in Calabrian and Spanish L. multifida seeds at 0, 7 and 14 days after sown. Data were expressed as mean ± standard error. Small letters indicate significant differences within species. Significance level was set at $P<0.05$.

## Discussion

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

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

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

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

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

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

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

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

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

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

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