Seed germination characteristics of *Phillyrea angustifolia* L. and *P. latifolia* L. (Oleaceae), two Mediterranean shrub species having lignified endocarp

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Abstract. The aim of this study was to determine the germination characteristics of *Phillyrea angustifolia* L. and *P. latifolia* L. seeds in order to develop an optimized propagation protocol for *Phillyrea* species. Seeds of *P. angustifolia* and *P. latifolia* were collected from wild plants growing in Cáceres province (CW Spain) and Andalucía (S Spain), respectively. Percentage of water uptake for *P. latifolia* seeds was calculated. Seeds with and without endocarp were germinated at different constant and alternating temperatures. Seeds without endocarp were soaked in distilled water or gibberellic acid, and then set to germinate. Seeds with endocarp of both species were stratified at 5 ºC for 30 or 90 days and then the endocarp was completely removed from the seeds before they were sowed. Chemical scarification with sulfuric acid and mechanical scarification were tested on *P. angustifolia* seeds with endocarp. *Phillyrea* endocarp was permeable to water, since *Phillyrea* seeds with endocarp imbibed water, but water uptake was faster when the endocarp was removed. Moreover, the encodarp could interfere mechanically in the emergence of the radicle, since seed germination of *Phillyrea* species was promoted by the complete removal of the lignified endocarp surrounding each seed. Optimal germination temperature for both species was 15 ºC, and lower temperatures produced secondary dormancy. Soaking in distilled water or gibberellic acid did not significantly enhance seed germination. Cold stratification and chemical scarification treatments were detrimental for seed germination.
Keywords cold stratification, *Phillyrea* species, treatments before sowing, seed germination, seed scarification, lignified endocarp.

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Introduction

Seed germination is the most sensitive stage in the plant ability for spatial colonization (Baskin & Baskin 1998). In Mediterranean ecosystems, evergreens repeatedly face important environmental constraints and cope with extreme summer droughts, extensive fires and soil erosion (Demmig-Adams & Adams 1996, Chaves et al. 2002, Dimitrakopoulos et al. 2013). Climate change models project a reduction in total precipitation and drier summers (Christensen et al. 2007) and associated increase in fire hazard in Mediterranean regions (Piñol et al. 1998, Pausas et al. 2008). Therefore, in the Mediterranean areas, plant regeneration strategies and environmental factors are closely interlinked, and knowledge on seed germination of Mediterranean species would be fundamental for the ecosystem conservation and for directing regeneration efforts (Varela 2000, Pardos et al. 2005).

Phillyrea L. is a genus of 2 species in the Oleaceae family: *P. angustifolia* L. and *P. latifolia* L. (Andrés 2011). They are evergreen shrubs very representative of the Mediterranean forest and with high importance in the ecological dynamics (Kutbay & Kilinç, 1994, Herrera et al. 1994). Many of the natural and semi-natural forests containing *P. angustifolia* are under decline due to the impact of anthropogenic disturbance, and restoration efforts to address this are underway in many countries. *Phillyrea* species are thermophilic and with low watering requirements, and have been recently considered suitable for landscaping purposes for their little maintenance needs (De Marco et al. 2005). *Phillyrea* species are, therefore, of growing economical importance. The species can be propagated by seed germination and vegetatively (Piotto & Di Noi 2003), but plant rooting is difficult (Catalán 1991). *Phillyrea* species produce fleshy-fruits (drupes), usually containing a single seed enclosed by a lignified endocarp. Typically, a lignified endocarp may be water-impermeable, thereby preventing im-

bibition and thus seed germination (Baskin & Baskin 1998, Baskin et al. 2002). Due to the presence of a lignified endocarp, it has been suggested that *Phillyrea* species may presents physical dormancy (García-Fayos et al. 2001, Takos & Efthimiou 2003), however, water absorption of the endocarp and dormancy break protocols have not been established. Within the Oleaceae family, the genus *Olea* is characterized by physiological dormancy and a woody endocarp that prevents the expansion of the embryo (Morales-Sillero et al. 2012), and even after total removal of the endocarp, seed germination is slow and erratic (Mitrakos & Diamantoglou 1984). Seed germination of *Phillyrea* species was showed to be poor and unreliable in nurseries (Catalán 1991). This erratic germination of *Phillyrea* could have an effect on the presence of plants at different physiological phases, complicating their management and commercialization.

The general aim of this work was to investigate the seed germination behavior of *Phillyrea* species in order to develop an optimized propagation protocol. The specific objectives of the present study were to determine (i) the optimal temperature requirement for germination of *P. angustifolia* and *P. latifolia* seeds, (ii) the effect of treatments before sowing on germination for both species, (iii) the water uptake capacity of *P. latifolia* seeds with and without endocarp and (iv) the effect of different scarification treatments on germination of *P. angustifolia* seeds.

Materials and methods

Plant material

Ripe fruits of *P. angustifolia* and *P. latifolia* were collected in 2011 from wild plants growing in Cáceres province (CW Spain) and Andalucía (S Spain), respectively. To carry out the trials in which seeds without endocarp were used, the endocarp of the fruit was completely

removed using pliers and a scalpel. Seeds were manually cleaned, kept in paper bags, and then stored dry under laboratory conditions (at ~23 ºC under darkness, 35% relative humidity) until the start of trials. Visible damaged seeds were excluded from experiments.

Effect of temperature regimes on seed germination

Seeds without endocarp of *P. angustifolia* and *P. latifolia* were tested for germination at different constant temperatures (5 ºC, 10 ºC, 15 ºC, 20 ºC, 25 ºC), with a 16-h light photoperiod (provided by cool white fluorescent tubes with an irradiance of 35 µmol m^2 s^{-1}), and the alternate temperature regimes of 20/7 ºC and 25/15 ºC (the highest temperature for 16 h in light and the lowest one for 8 h in dark). In each trial, four replicates of 25 seeds each for *P. angustifolia* and two replicates of 30 seeds each for *P. latifolia* were tested for germination on top of two sheets of filter paper (previously moistened with 3.5 mL of distilled water) in 7-cm-diameter glass Petri dishes. Filter papers were rewetted regularly with distilled water, as required. Dishes were checked three times a week and germinated seeds were counted and removed. Seeds were considered germinated on emergence of the radicle from the seed coat. The initial incubation period was 35 days for *P. angustifolia* and 55 days for *P. latifolia*, which showed a slower germination. To study the effect of high and low temperatures of incubation on seed germination at the optimum conditions, after incubation at different temperatures, non-germinated seeds were transferred to 15 ºC (which was the temperature that induced the highest germination percentages). Thus, non-germinated seeds of *P. angustifolia* after 35 days at 5 ºC, 25 ºC and 25/15 ºC were transferred to 15 ºC and then incubated for another 35-day period. Non-germinated seeds of *P. latifolia* after 55 days at 10 ºC, 20 ºC, 25 ºC and 25/15 ºC were transferred to 15 ºC and then incubated for another 137-day period.

P. latifolia assays were also performed with seeds having intact endocarp. *P. angustifolia* seeds with endocarp where not included on the assay due to the scarcity of seed material. In all trials, seeds that had not germinated at the end of the incubation period were opened to determine whether the seed was empty. If so, they were excluded from calculation of final germination percentages (Baskin & Baskin 1998). The number of empty seeds was always equal or less than 5% of the total seeds.

Effect of treatments before sowing on seed germination

3 treatments before sowing were applied by using seeds of *P. angustifolia* and *P. latifolia*. 4 replicates of 25 seeds each were tested for each treatment: (i) soaking in distilled water. Seeds without endocarp were soaked in distilled water at room temperature (~ 23 ºC) for 24 or 72 h, (ii) soaking in gibberellic acid (GA_3). Seeds without endocarp were soaked in a GA_3 solution (1000 mg L^{-1}) at room temperature (~ 23 ºC) for 24 h, (iii) cold stratification. Seeds with endocarp were stored in moist vermiculite under darkness at 5 ºC for 30 and 90 days. After the cold stratification period, the endocarp was completely removed from the seeds before they were sowed.

After these three treatments, seeds were tested to germinate at 15 ºC under a 16-h light photoperiod for 65 days. Untreated seeds without endocarp were sown in the same conditions and they were used as control.

Water uptake during seed soaking

To determine water uptake capacity during seed soaking, 2 replicates of 25 *P. latifolia* seeds (with and without endocarp) each were weighted and then placed in Petri dishes on filter paper moistened with distilled water. After 1, 2, 24, 48 and 72 h of imbibition, seeds were quickly surface-dried with filter paper and then reweighted. Percentage of water uptake (mean

± standard error) was calculated as the amount of water adsorbed by seeds relative to the initial seed weight. Due to the scarcity of seed material this assay could not be done with *P. angustifolia* seeds.

Effect of scarification treatments on seed germination

4 scarification treatments were compared on *P. angustifolia* seeds with endocarp before they were sowed at 15 ºC under a 16-h light photoperiod for 75 days. 4 replicates of 25 seeds each were tested for each scarification treatment: (i) endocarp totally removed. Endocarp was carefully removed using pliers and a scalpel, (ii) endocarp partially scarified. Endocarp was cracked with pliers but it was not completely removed from seed, (iii) sulfuric acid (6 h) + hot water (80 ºC). Seeds with endocarp were immersed in concentrated sulfuric acid for 6 h, then soaked in hot water (80 ºC) for 18 h, and then repeatedly washed with distilled water before sowing, (iv) sulfuric acid 30 min. Seeds with endocarp were immersed in concentrated sulfuric acid for 30 min and then repeatedly washed with distilled water before sowing.

Due to the scarcity of seed material this assay could not be done with *P. latifolia* seeds.

Statistical analysis

For all experiments, final germination percentage (mean value ± standard error) and mean germination time (MGT, mean value in days ± standard error) were calculated. The latter was determined according to the following formula: $MGT = \Sigma DN / \Sigma N$; where D is the number of days counted from the date of sowing and N is the number of seeds germinated on day D (Ellis & Roberts, 1981). The values of final germination percentages were arcsine square-root transformed and then subjected to analysis of variance (ANOVA) using SPSS (untransformed data appear in Tables). One-

way factorial ANOVA was used to test the effects of the different temperature regimes and treatments before sowing on seed germination capacity. In the same way, to determine differences among scarification treatments, data were analysed by means of one-way ANOVA. Where ANOVA indicated a significant effect, a comparison of mean values was carried out through the least significant difference test (LSD). The statistical analysis of MGT values was also carried out using one-way factorial ANOVA.

Results

Effect of temperature regimes

For each species, significant differences ($P < 0.001$) on the final germination percentages were noticed between the temperatures applied during the experiments (Table 1). The germination percentages reached by *P. angustifolia* seeds without endocarp after 35 days of incubation were equal to or less than 2% at the lower and higher temperatures (5 ºC, 10 ºC and 25 ºC), the temperatures with the best results being 15 ºC and 20 ºC (77% and 70%, respectively) (Table 1, Figure 1). The final germination percentages reached by *P. latifolia* seeds without endocarp were equal to or less than 5% for all temperatures assayed except for 15 ºC (52%) (Table 1, Figure 1). Regarding the speed of germination (MGT), for *P. angustifolia* seeds, germination velocity was not significantly ($P > 0.05$) affected by the temperature regimes (Table 1, Figure 1). Thus, MGT values were similar among temperatures, ranging from 18 to 24 days. Moreover, *P. angustifolia* seeds showed at 15 ºC a faster germination (lower MGT values) than *P. latifolia* seeds (20 and 29 days, respectively).

P. angustifolia seeds that did not germinate after being incubated at the temperature regimes of 5 ºC, 25 ºC or 25/15 ºC for 35 days, were transferred to 15 ºC. Germination percentage was increased in all cases, with final

Table 1 Final germination percentage and mean germination time (MGT) for *Phillyrea angustifolia* and *P. latifolia* seeds without endocarp incubated at different temperature regimes for 35 days. Means within a column followed by the same letter are not significantly different according to the LSD test at *P* > 0.05

Temperature (ºC)	*Phillyrea angustifolia*		*Phillyrea latifolia*	
	Germination (% ± SE)	MGT (days ± SE)	Germination (% ± SE)	MGT (days ± SE)
5	0[a]	-	NO	NO
10	0[a]	-	5 ± 3.53[a]	NC
15	77 ± 5.72[d]	20.35 ± 1.20[a]	52 ± 1.06[b]	29.00 ± 0.13
20	70 ± 5.92[d]	23.12 ± 0.44[a]	2 ± 1.41[a]	NC
25	2 ± 1.73[ab]	NC	0	-
20/7	10 ± 1.73[b]	26.50 ± 4.07[a]	NO	NO
25/15	39 ± 3.57[c]	18.22 ± 0.57[a]	0	-

Note. NC: MGT value was not calculated when the final germination percentage was equal to or less than 5%. NO: trial not carried out due to the scarcity of seed material.
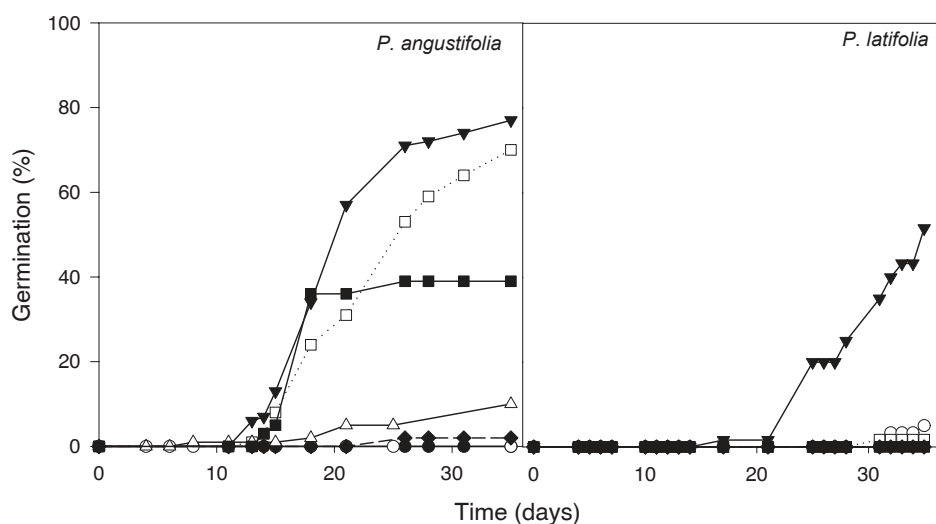

Figure 1 Germination time courses of *Phillyrea angustifolia* and *P. latifolia* seeds without endocarp incubated for 35 days at different temperature regimes: 5 ºC (●), 10 ºC (○), 15 ºC (▼), 20 ºC (□), 25 ºC (◆), 20/7 ºC (△) and 25/15 ºC (■).

values being registered from 30% to 83% (Figure 2).

Final germination percentages reached by *P. latifolia* seeds with and without endocarp at different temperatures are showed in Figure 3. For *P. latifolia* seeds with endocarp, germination occurred only at 15 ºC. However, when those seeds with endocarp incubated at 10 ºC, 20 ºC, 25 ºC and 25/15ºC for a 55-day period

were transferred to 15 ºC their germination increased greatly, with final values from 70% to 85%. *P. latifolia* seeds without endocarp that were moved to 15 ºC after a 55-day period of incubation at 10 ºC, 20 ºC, 25 ºC and 25/15 ºC increased their germination percentage from 60% to 87% after another 137-day period.
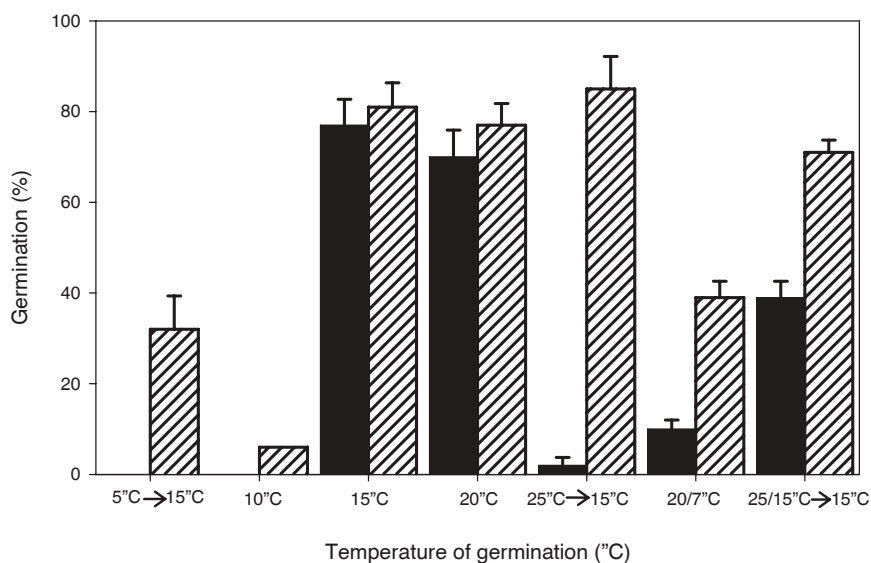
Figure 2 Final germination percentages of *Phillyrea angustifolia* seeds without endocarp incubated at different temperature regimes. Seeds were incubated for 35 days and then those at 5 ºC, 25 ºC and 25/15 ºC were transferred to 15 ºC for another 35-day period. Filled bars represent final germination (± standard error) at the indicated temperature after 35 days and striped bars after another 35-day period.
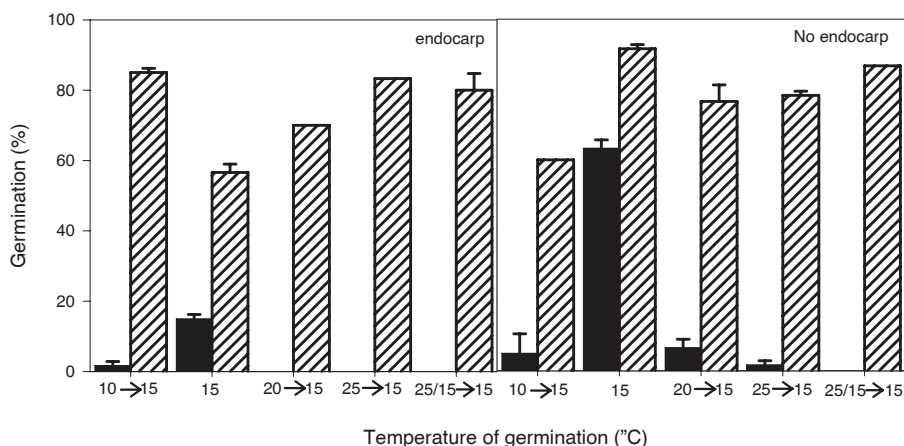

Figure 3 Final germination percentage of *Phillyrea latifolia* seeds with and without endocarp incubated at different temperatures for 55 days and then transferred to 15 ºC for another 137-day period. Filled bars represent final germination (± standard error) at the indicated temperature after 55 days and striped bars after another 137-day period.

Treatments before sowing

The effect of different treatments before sowing on germination of *P. angustifolia* and *P. latifolia* seeds is shown in Table 2 and Figure 4. For *P. angustifolia* seeds, no significant differences ($P > 0.05$) were found among the final germination percentages reached by control seeds (untreated seeds) and seeds soaked in distilled water or GA$_3$. However, stratification of *P. angustifolia* seeds at 5 ºC for 90 days significantly ($P < 0.001$) decreased the final germination percentage when compared to the control (9% vs. 81%, respectively). Germination speed was not significantly ($P > 0.05$) increased by the different treatments before

sowing assayed. For *P. angustifolia* seeds, all treatments significantly ($P < 0.001$) reduced the final germination percentages. Germination speed was higher for *P. latifolia* seeds soaked for 24 h and 72 h in distilled water than that of control seeds, but these differences were not significant ($P > 0.05$).

Water uptake

P. latifolia seeds without endocarp absorbed water quickly after 24 h immersion in distilled water (Figure 5), being registered seed mass increasing with 60% of the initial value. *P. latifolia* seeds with endocarp also absorbed water but at a lower rate. Thus, mean increase in mass for seeds without endocarp after 24 h was more than twice of that in seeds with endocarp (26%). After 48 h, seed mass increase was 67% in seed without endocarp, and 39% in seeds with endocarp. After 72 h of immersion in distilled water, seed mass increase was 68% in seed without endocarp, and 47% in seed with endocarp.

Scarification treatments

P. angustifolia seeds whose endocarp was cracked but not totally removed and seeds submerged in concentrated sulfuric acid showed a similar germination (22-26%), significantly

($P < 0.001$) lower than that of seeds without endocarp (81%) (Table 3). Germination speed was significantly ($P < 0.001$) higher for seeds scarified with sulphuric acid for 6 h than that of control seeds (3.4 days vs. 20.8 days, respectively).

Discussion

Regeneration potential of *Phillyrea* species depends on the effect of ambient conditions on its propagation process, and knowledge of the germination requirements would be fundamental for the conservation of this species. Our results indicate that *Phillyrea* species germination is slow. The highest germination percentage after a 35-day period was obtained at 15 ºC for both species, being maximum germination 77% for *P. angustifolia* and 52% for *P. latifolia*. Also, temperature regime for *P. latifolia* was very restrictive, since seeds did not germinate at either 25 ºC or 25/15 ºC. These results are in agreement with optimal germination temperature for most Mediterranean shrub species ranging between 15 ºC and 20 ºC (Thanos et al. 1992, 1995). Alternating temperatures (20/7 ºC and 25/15 ºC) resulted in low germination percentages, although previous works stated that *Phillyrea* seeds germinated a 90% after 60 days at 20/7 ºC (Herranz et al. 2006), or a 90%

Table 2 Final germination percentage and mean germination time (MGT) of *Phillyrea angustifolia* and *P. latifolia* seeds without endocarp after several treatments before sowing. Seeds were germinated at 15 ºC for 65 days. Untreated seeds without endocarp were used as control. Means within a column followed by the same letter are not significantly different according to the LSD test at $P > 0.05$.

Treatment before sowing	*Phillyrea angustifolia*		*Phillyrea latifolia*	
	Germination (% ± SE)	MGT (days ± SE)	Germination (% ± SE)	MGT (days ± SE)
Control	81 ± 5.36[b]	20.85 ± 0.86[a]	62 ± 5.20[b]	32.57 ± 2.19[a]
Soaking in distilled water for 24 h	84 ± 5.48[b]	16.20 ± 0.74[a]	14 ± 1.73[a]	31.12 ± 1.64[a]
72 h	86 ± 4.58[b]	12.67 ± 0.19[a]	10 ± 1.95[a]	29.00 ± 2.50[a]
Soaking in GA₃	79 ± 3.84[b]	16.17 ± 0.48[a]	21 ± 6.98[a]	39.62 ± 0.92[ab]
Stratification at 5 ºC 30 days	NO	NO	12 ± 0.65[a]	56.50 ± 0.56[c]
90 days	9 ± 2.17[a]	19.12 ± 6.32[a]	17 ± 2.98[a]	52.25 ± 4.42[bc]

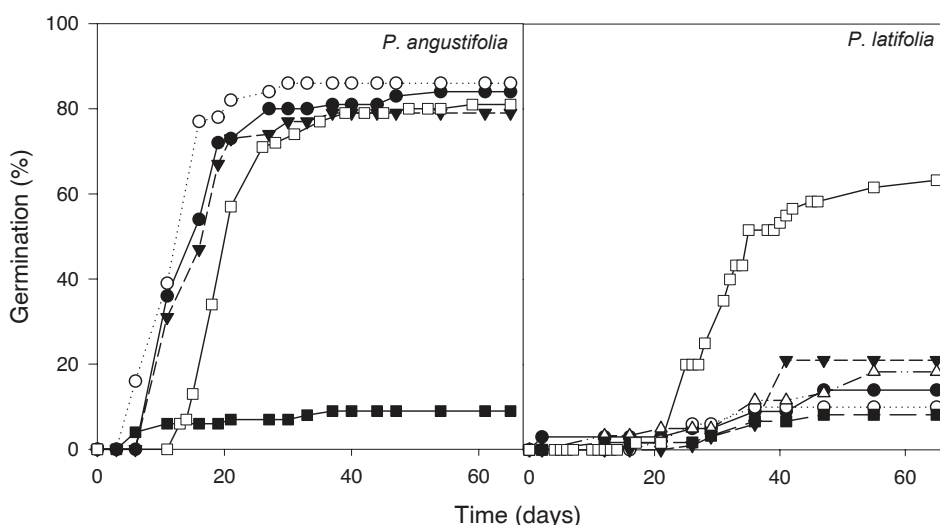Note. NO: trial not carried out due to the scarcity of seed material.

Figure 4 Germination time courses of *Phillyrea angustifolia* and *P. latifolia* seeds without endocarp after different treatments before sowing: control (□), soaking in distilled water for 24 h (●), soaking in distilled water for 72 h (○), soaking in a GA$_3$ solution (1000 mg L^{-1}) for 24 h (▼), cold stratification at 5 °C for 30 days (△) and 90 days (■). Seeds were incubated at 15 °C for 65 days
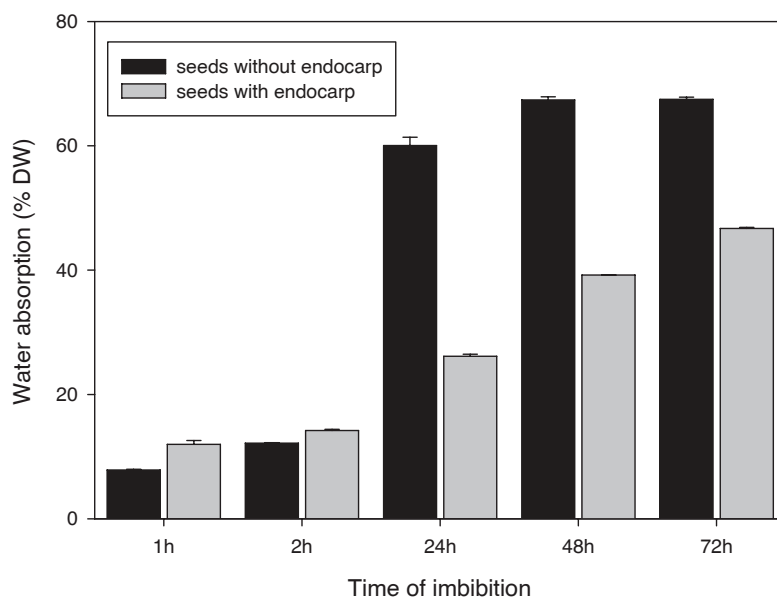

Figure 5 Mean (± standard error) increase in mass of *Phillyrea latifolia* seeds with and without endocarp placed on filter paper moistened with distilled water at ~23 °C.

after 15 days at 20/10 °C (García-Fayos et al. 2001). This could indicate a variation of the optimal germination temperatures for different populations of the same species. One of the most important survival adaptations for plant species growing under variable and unpredictable environmental conditions, as in the Mediterranean forests, is the intraspecific variation of germination requirements (Kigel 1995,

Baskin & Baskin 1998, Martínez-García et al. 2012), concerning the seed dormancy (Pérez-García et al. 2012), or seed longevity (Lazar et al. 2014, Mira et al. 2011a, 2011b, 2014). However, in some forest species, as *Populus euphratica* and *Pinus nigra,* it has been shown that source origin had minimal influence on seed germination or seedling quality (Ivetic & Skoric 2013, Soleimani et al. 2014).

It is known that some seeds with lignified endocarp germinate well following cold stratification (Baskin et al. 2002), however, cold stratification of *Phillyrea* seeds was detrimental for seed germination. Incubation at low temperatures (5 ºC for *P. angustifolia* and 10 ºC for *P. latifolia*) seemed to induce a secondary dormancy, since final germination did not achieve maximum values when seeds were transferred to 15 ºC. The inefficiency of a cold stratification treatment agrees with previous work in *Phillyrea* species (García-Fayos et al. 2001), but it is a especially interesting result since cold stratification is a common treatment for forestry species and some seed producing companies recommend it for these particular species. On the other side, seed incubation at high temperatures, 25 ºC and 25/15 ºC, resulted in a low germination percentage for both species but did not induce dormancy.

Treatments before sowing through soaking in distilled water or in a gibberellic acid solution did not significantly increase final germination for *P. angustifolia* seeds, and they were clearly detrimental for *P. latifolia*. Moreover, acid scarification with sulfuric acid registered very poor results in *P. angustifolia*, which is especially meaningful since this technique has been suggested as one of the best to promote germination in several species with lignified endocarp (Bonner & Karrfalt 2008), and it has been previously recommended for *Phillyrea* species by seed producing companies and previous reports (Piotto & Di Noi 2003, Bacchetta et al. 2008). These contradictory results could be explained by an inter- and intra-population

variability on the endocarp permeability and hardness in *Phillyrea* species, as has been previously suggested (Traveset et al. 2007, Arnal 2013). In our study, *P. latifolia* seeds with endocarp absorbed water during soaking, although more slowly than in seeds without endocarp. This results would indicate that *P. latifolia* seeds do not exhibit physical dormancy, according to the classification system of Baskin & Baskin (2004), were physical dormancy is defined as the result of a water-impermeable layer in the seed or fruit. Similar results had been reported in several species with lignified endocarp (Baskin et al. 2002) and some species within the Oleaceae family, like *Olea* sp. (Cuneo et al. 2010).

Total removal of the endocarp with pliers was the technique that showed best results regarding germination of *P. angustifolia*, while this morpho-physiological process was not promoted when endocarp was cracked. Moreover, *P. latifolia* seeds with endocarp incubated at 10 ºC, 20 ºC, 25 ºC or 25/15 ºC did not germinate for 55 days, but achieved high germination percentages when transferred at 15 ºC. Other authors found that seeds of *P. latifolia* collected from the soil in previous years showed a high germination (Yucedag & Gultekin 2011), and that *Olea europaea* seed germination in the soil did not occur until complete decomposition of the endocarp (Cuneo et al. 2010). Our results suggest that *Phillyrea* species may present a lignified endocarp that interferes mechanically in the emergence of the radicle but not in the absorption of water, and that complete removal of endocarp is necessary to achieve the full

Table 3 Final germination percentage and mean germination time (MGT) for *Phillyrea angustifolia* seeds after several scarification treatments. Seeds were incubated at 15 ºC for 75 days. Means within a column followed by the same letter are not significantly different according to the LSD test at *P* > 0.05.

Scarification treatment	Germination (% ± SE)	MGT (days ± SE)
Endocarp removed with pliers	81 ± 5.36[b]	20.85 ± 0.86[b]
Endocarp cracked	22 ± 2.24[a]	48.42 ± 3.32[d]
SO_4H_2 (6 h) + hot water (80 ºC)	22 ± 4.58[a]	3.37 ± 0.27[a]
SO_4H_2 (30 min)	26 ± 8.18[a]	34.75 ± 1.08[c]

germination. In nature, *Phillyrea* fruits spread in winter, their seeds remaining in the soil and with time the endocarp might degenerate allowing seeds to germinate during the period of early spring temperatures and rains.

Conclusions

Our data provided useful information in germination protocols for *Phillyrea* species propagation. Optimal germination temperature for *Phillyrea* species was 15 ºC, lower temperatures resulting in secondary dormancy. Treatments of cold stratification, soaking in distilled water or gibberellic acid did not improve seed germination. Our results proved that *P. latifolia* endocarp was permeable to water but could mechanically interfere with the radicle emergence, and total removal of the endocarp was the technique that showed best results for seed germination improving.

Acknowledgement

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