BOSQUE 38(2): 347-355, 2017 DOI: 10.4067/S0717-92002017000200012

Effect of pre-treatments on the germination of jasmin box (*Phillyrea latifolia*) seeds in Greece

Efecto de tratamientos previos sobre la germinación de semillas de *Phillyrea latifolia* en Grecia

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SUMMARY

The major problem in seedlings production of jasmin box (*Phillyrea latifolia*) is the poor and irregular germination due to seed dormancy. The objective of the present study was to evaluate methods of seed pre-treatments to increase germination. The effect of different solutions of KOH and NaOH, wood ash lye, GA_3 and cold or warm stratification on germination were evaluated. Sets of four replicates of 25 seeds were soaked for 24 hours in KOH and NaOH solutions of 0.2, 0.5, 1 and 2 %, in wood ash lye solutions of 33, 66, 133, 166, 532 g L^{-1} and in GA_3 solutions of 250, 500, 1000, 2,000, 3,000 mg L^{-1}. A control set of seeds was imbibed in water for 48 hours. All sets went through zero, two, four and six months of cold (2-4 °C) or warm (15 °C) stratification. The seeds were incubated in petri dishes filled with moistened sand, at two alternating temperatures (20/15 and 30/20 °C) with a light period of 16 hours. From the results we conclude that: Chemical pre-treatments with KOH and ash lye and warm stratification increased germination to 96 %. Treatment with water and warm stratification also increased germination to 79 %. Treatment with 250 mg L^{-1} GA_3 and six months warm stratification increased germination to 70 % while higher concentrations had a negative effect. Cold stratification did not break dormancy. Optimum germination temperature in a growth chamber was 15-20 °C, while temperature at 20-30 °C inhibited germination.

Key words: dormancy breaking, seed germination, stratification, lye solutions, gibberellic acid.

RESUMEN

Un problema en la producción de plantas de labiérnago (*Phillyrea latifolia*) es la irregular y baja germinación debido al letargo de las semillas. El objetivo del presente estudio fue evaluar tratamientos previos a las semillas para aumentar su capacidad germinativa. Se evaluó el efecto de soluciones de KOH y NaOH (0,2, 0,5, 1 y 2 %), lejía de ceniza de madera (33, 66, 133, 166, 532 g L^{-1}) y GA_3 (250, 500, 1.000, 2.000, 3.000 mg L^{-1}) y estratificación en frío o en caliente. Conjuntos de cuatro repeticiones de 25 semillas se sumergieron durante 24 horas en las soluciones. Adicionalmente, se aplicó un tratamiento control conjunto de semillas embebidas 48 horas en agua. Todos los conjuntos pasaron por 0, 2, 4 y 6 meses de estratificación en frío (2-4 °C) o temperatura ambiente (15 °C). Las semillas, en placas de Petri con arena húmeda, se incubaron a dos temperaturas alternantes (20/15 y 30/20 °C) con un periodo de luz de 16 horas. Se concluyó que: los tratamientos con KOH y lejía de ceniza de madera y la estratificación cálida aumentaron significativamente la germinación hasta el 96 %. Tratamientos con agua y estratificación caliente también aumentaron la germinación a 79 %. El tratamiento con 250 mg L^{-1} GA_3 y seis meses estratificación cálida aumentó la germinación hasta el 70 %, pero las concentraciones más altas tuvieron efecto negativo. La temperatura de germinación en la incubadora fue 15-20 °C. La alta temperatura de incubación (20-30 °C) y la estratificación fría (2-4 °C) resultaron en nula germinación.

Palabras clave: ruptura del letargo, germinación de semillas, estratificación, soluciones de lejía, ácido giberélico.

INTRODUCTION

One of the typical species of the Mediterranean maquis is the jasmin box (*Phillyrea latifolia* L.). It is an evergreen broad-leaved bush or small tree up to 5 m high (Dimopoulos *et al.* 2013). It grows in the peri-Mediterranean countries and, specifically in Greece, in dry maquis sites and as understory shrub in Aleppo pine and oak forests (Dimopoulos *et al.* 2013). As all evergreen, sclerophyllus shrubs play an important role in the ecology of maquis vegetation. The genus *Phillyrea* belongs to the Oleaceae family and comprises two species, *P. latifolia* and *P. angustifolia* (L.). The first species grows across the eu-Mediterranean zone, all over Greece, while the second does not occur in Greece (Dimopoulos *et al.* 2013).

Phillyrea latifolia is a species of ecological and economic importance. It can be used as plant for soil protection in reforestations, suitable for landscape restorations because of its drought tolerance and easy resprouting after fires, as an ornamental shrub or small tree and finally it can be cultivated for the medicinal use of the leaves and fruits (Gucci *et al.* 1997, Yücedağ and Gültekin 2011).

The seeds of *P. latifolia* germinate very irregularly and with long delay if they are not submitted to some pre-treatment (Bachiller 1991). Traditional methods in nurseries usually give bad results in terms of low percentage and long-time germination (Cantos *et al.* 2001). They usually present double dormancy due to the impermeability of the seed coats because of hardness of the coat, the oily substances that may prohibit the water penetration and embryo physiological dormancy (Bachiller 1991, Takos and Efthimiou 2002). However, Mira *et al.* (2015) found that the *Phillyrea* spp. seeds imbibe water and thus do not present morphological dormancy. This type of double dormancy can be overcome by combining immersion in different chemical solutions, such as acids and pre-chilling at 2-4 °C for a certain period (Gordon *et al.* 1991, García –Fayos *et al.* 2001, ISTA 2008). In nature the fruits of *P. latifolia* are dispersed by birds and other fruit eating animals (Herrera *et al.* 1994). With the ingestion of the fruits the seeds are subjected to a chemical pre-treatment by the hydrochloric acid and other enzymes of the animal's ingestion system, hence the hard seed coat structure changes (Traveset *et al.* 2001). The inhibitor substances that are contained in the endosperm can be removed by stratification on forest floor and seeds germinate more easily in the spring.

There is a number of studies that are concerned with the ecology and germination of the seeds of the genus *Phillyrea*. Most of them have been carried out in Spain and few in Turkey. Traveset (1994) studied the effect of galling insects on the reproductive output of *P. angustifolia* in South-eastern Spain. Herrera *et al.* (1994) studied the fruit removal by birds, seed rain, post dispersal seed predation, seed germination and seedlings emergence, survival and establishment of *P. latifolia* in South-eastern Spain. The effect of high temperatures (70 and 120 °C) on the germination of *P. latifolia* was investigated by Raimon and Lloret (1995) in Barcelona, Spain. Cantos *et al.* (2001) reported that traditional methods in nurseries gave bad results in terms of low germination percentages and long germination time. In contrast, in vitro cultures of isolated embryos improved the germination of *P. latifolia* seeds collected in Malaga, Spain. Traveset *et al.* (2001) studied the effect of the fruits of bird dispersed trees that passed through bird guts and found interspecific differences in seed germination characteristics of *P. latifIolia* growing in Balearic Islands. More recently, Mira *et al.* (2015) studied the seed germination characteristics of both *P. angustifolia* and *P. latifolia* growing in Cáceres and Andalucía, Spain. The effect of fruit collection date on the germination of *P. latifolia* growing in Erdigir, Southwestern Turkey, was conducted by Yücedağ and Gültekin (2011). Aygün *et al.* (2011) used chemical scarification with concentrated sulphuric acid, gibberellins and cold stratification to evaluate the germination ability of different shrubs, including *P. latifolia* growing in Eskişehir province, Turkey.

Phillyrea latifolia, as a species belonging to Oleaceae family, produces fruits (drupes) that contain oily substances such as fat acids in the endocarp as well as in the endosperm similar to the fruits of olive trees (Barclay and Earle 1974, Royal Botanic Gardens Kew 2016). In the past, fruits of this species were harvested and consumed by people as wild olives (Pieroni and Pachaly 2000). These substances, among other inhibitors, might also be responsible for the physiological dormancy the seeds present and prevent germination. As it was mentioned before, *P. latifolia* is a species of growing ecological and economic importance. Recently, it has been suggested to be included, among other broadleaves, in the reforestations for biodiversity enhancement; although the difficult and irregular germination it presents makes it ineligible by the nurseries. Having this in mind as a general aim, we thought that in order to break dormancy and to improve the germination capacity of this species, it would be useful to treat these seeds with different lye solutions in order to neutralize the fat acids contained in the endocarp and subsequently, with cold at 2-4 °C or warm at 15 °C stratification in moist sand to remove the inhibitors that might be present in the embryo.

More specifically, the aims of this study are to evaluate the effect of lye solutions, of hormone GA_3, as well as of cold or warm stratification and incubation temperatures on dormancy breaking and to improve seed germination of *P. latifolia* seeds for successful plant production.

METHODS

Seed collection. The fruits of *P. latifolia* were collected from Kassandra, Chalkidiki, Northern Greece, (39° 59' 50'' N, 23° 30' 14''E and 190 – 320 m a.s.l.) by hand in November 2012. The fruits were collected from 50 different mother trees and from all sides of the crown, packed in plastic bags and transferred to the nursery where cleaning and drying was carried out. The fruits were soaked in water for 24 hours and afterward beaten in a mixer. Using sieves and water under pressure the fleshy pulp was removed and seeds with endocarp were obtained. It must be mentioned here that the term seed is referred to the true seed plus endocarp. Subsequently the seeds were left to dry to a moisture content of 6-8 % and stored in hermetically closed glass containers in the refrigerator at 2-4 °C.

Chemical seed pre-treatments. Various chemical pre-treatments were applied to enhance the germination of *P. latifolia* seeds:

- Soaking in deionized water. Seeds were soaked in deionized water at room temperature for 48 hours (control).
- Soaking in KOH. Seeds were soaked in 0.2, 0.5, 1 and 2 % KOH for 24 h at room temperature for 24 hours.

- Soaking in NaOH. Seeds were soaked in 0.2, 0.5, 1 and 2 % KOH for 24 h at room temperature for 24 hours. Percent concentrations were used instead of Molarity (M) in KOH and NaOH solutions because we did not want to compare the solutions, rather, to study their potential impact on the germination of *P. latifolia* seeds.
- Soaking in ash lye solutions. Seeds were soaked in five different ash lye solutions for 24 hours. These solutions were obtained using fuelwood ash as solute and dissolving 33.25, 66.5, 133, 266 and 532 g L^{-1} of oak fuelwood ash in water for 24 hours at room temperature and getting only the solution that had formed above the sediment of the fuelwood ash.
- Soaking in GA_3. Seeds were soaked in 250, 500, 1,000, 2,000, 3,000 mg L^{-1} for 24 h at room temperature for 24 hours. Specifically, for GA_3 concentrations of 1,000, 2,000, 3,000 mg L^{-1} the GA_3 salt was dissolved in phosphate buffer solution as it is recommended by the International Seed Testing Association (ISTA 2008).

Seed stratification and germination tests. After each one of the above pre-treatments, seeds were stratified in moistened sand for zero, two, four and six months at 2-4 °C (cold stratification) and at 15 °C (warm stratification).

To determine the effect of temperature on germination, seeds were incubated at two different alternating temperatures: (a) 16 hours light at 30 °C and 8 hours dark at 20 °C and (b) 16 hours light at 20 °C and 8 hours dark at 15 °C. Four replicates of 25 seeds were used for each combination of pre-treatments. The seeds were placed in 9 cm plastic petri dishes that were filled with sand as a substrate and moistened with deionized water except the GA_3 pre-treatments where the substrate was moistened with the respective GA_3 solutions (ISTA 2008). Radical emergence was taken as the criterion of seed germination. Germination counts were made for the first time on the seventh day and subsequently, three times per week and the last, on the fifty sixth day.

Data analysis. Germination percentages were calculated for each pre-treatment. Differences among means were tested by the one way analysis of variance and GLM. Duncan's multiple range test was used for the identification of the homogenous groups, when significant differences were identified. A significance level of 5 % was used for all statistical analyses. SPSS statistical software was used for the analyses (Norusis 2002).

RESULTS

Effects of incubation temperature. The set of high germination temperature conditions in growth incubator (20-30 °C) resulted to zero germination for all seed pre-treatments. For this reason data and statistics are not shown for this treatment. Despite the duration of cold stratification, once the seeds were placed in the incubator at 30/20 °C, some of them seemed to have already started to germinate by the end of the first week (figure 1). Four weeks later, the situation remained the same and no seed developed a regular root of a normal seedling. The second set of low germination temperature conditions in growth incubator 20/15 °C resulted in high germination percentages for *P. latifolia* seeds for all pre-treatments.

Effect of chemical seed pre-treatment. The effect of the different chemical solutions in which the seeds were pretreated, regardless of the solution concentrations, type and duration of stratifications and for incubation temperatures (20/15 °C), appears to be significant ($F = 3.643$, $P = 0.009$). Seeds pre-treated with KOH, ash lye and NaOH resulted in mean germination of 55.4 %, 52.9 % and 42.9 %, respectively (figure 2). Although the seeds pre-treated with NaOH showed smaller mean germination compared to the other two solutions, there is no statistical significant difference among them. Seeds pre-treated with GA_3, regardless of concentrations and water, resulted in 24.7 % and 21.17 % germination respectively with no statistical significant difference among them.

The water pre-treatment exhibited mean germination 21.2 % for the four durations of stratification. Taking into account that the "no" stratification pre-treatment resulted to zero germination, the above mean germination confirms the positive effect of the stratification on the germination of *P. latifolia* seeds.

Effect of stratification. The effect of the different stratification durations the seeds have passed, regardless of the chemical pre-treatments and concentrations and for incubation temperatures (20/15 °C), appears to be very significant ($F = 17.887$, $P < 0.000$). It is clear that without stratifica-

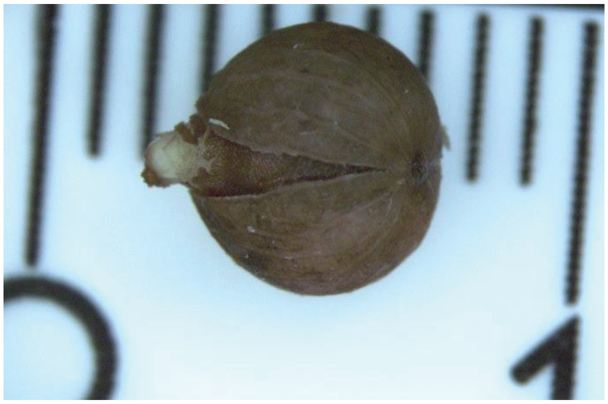

Figure 1. *Phillyrea latifolia* seed with emerged and stopped growing radicle after four weeks in the incubator at 20-30 °C (Scale: 0-1 cm).

Semilla de *Phillyrea latifolia* germinada con crecimiento radicular después de cuatro semanas dejadas en condiciones de incubadora a 20 – 30 ºC.

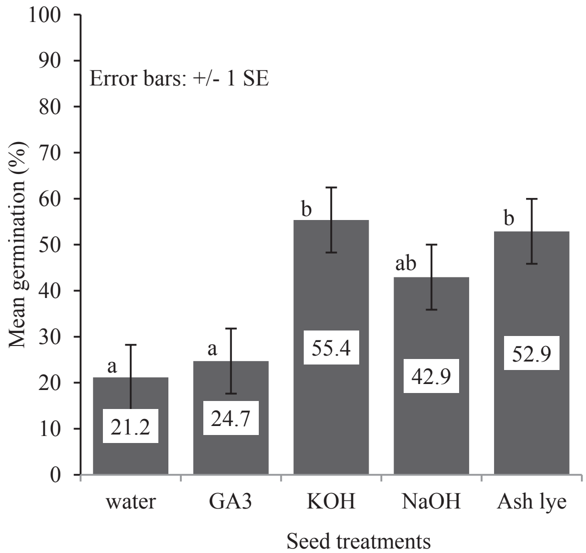

Figure 2. Effect of different chemical solutions on the mean germination percentage of *Phillyrea latifolia* seeds incubated at 20/15 °C. Means are statistically different when they share no common letter (Duncan, $P < 0.01$).

Efecto de diferentes soluciones químicas sobre el porcentaje de germinación de semillas de Phillyrea latifolia a 20/15 °C. Letras diferentes en cada columna indican diferencias significativas (Duncan, $P < 0{,}01$).

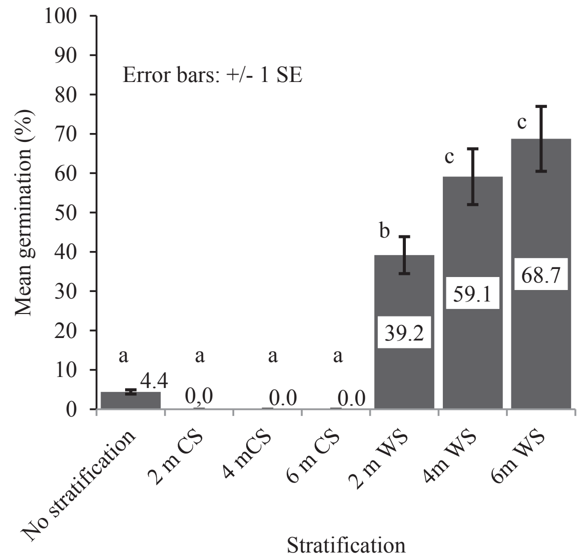

Figure 3. Effect of stratifications on the mean germination percentage of *Phillyrea latifolia* seeds incubated at 20/15 °C. Means are statistically different when they share no common letter (Duncan, $P < 0.01$). CS: Cold stratification, WS: Warm stratification.

Efecto de las estratificaciones en la capacidad germinativa media de semillas de *Phillyrea latifolia* a 20/15 °C. Letras diferentes en cada columna indican diferencias significativas (Duncan, $P < 0{,}001$). CS: estratificación fría, WS: estratificación caliente.

tion, seeds of *P. latifoli*a do no germinate. However, the general mean germination of 4.4 % means that the chemical pre-treatments themselves had a marginal positive effect on germination.

Two months stratification resulted in 39.2 % germination. The stratification periods of four and six months showed mean germination rates of 59.1 % and 68.7 % respectively across all seed pre-treatments and they appear as a homogenous group with no statistical significant difference (figure 3). A potentially negative effect of the six months warm stratification, which is considered long time, was the formation of long roots by several seeds, some of them being as long as 10 cm.

The effect of cold stratification at 2-4 °C was detrimental for all seeds of *P. latifolia*, irrespectively from the chemical pre-treatments that they were subjected to.

Effect of chemical solutions and warm stratification pre-treatment combinations on germination. The highest arithmetic germination percentage mean (96 %) was recorded for seeds pre-treated with 0.2 % KOH solution, followed by six months warm stratification at 15 °C (table 1, figure 4). In general, this pre-treatment resulted to be the second, after ash lye, more homogenous performance of germination across all concentrations and stratification periods.

The second highest germination (91 %) was recorded for 266 g L^{-1} wood ash lye solution and six months warm stratification at 15 °C (table 1, figure 4). The germination percentage was homogenous and directly analogous to the increase of concentration and duration of stratification. For NaOH solution the best germination (89 %) was recorded in 0.5 % concentration and six months warm stratification at 15 °C (table 1, figure 4). The NaOH pre-treatment shows a different pattern of germination compared to KOH pre-treatment. It is clear that the 2 % concentration of both hydroxides had a negative effect on germination in all stratification durations. The 250 mg L^{-1} GA_3 solution followed by six months warm stratification at 15 °C resulted in 68 % germination (table 1, figure 4). For the long stratification periods, it was observed that the higher the concentration of GA_3, the lower the germination percentage. The fact that the substrate of all GA_3 pre-treatments was moistened by the respective solution resulted in negative effect on germination for high GA_3 concentrations. The smallest germination (11 %) recorded for 3,000 mg L^{-1} GA_3 after six months of stratification.

An interesting result from this study was that the highest germination of the seeds pre-treated with water was 79 % after six months of warm stratification (table 1, figure 4).

DISCUSSION

Four interesting findings were derived from this study. First, the incubation temperatures of 30 °C for 16 hours light, alternate with 20 °C for 8 hours dark not only pro-

Table 1. Effects of chemical pre-treatments combined with warm stratification at 15 °C on mean germination percentage of *Phillyrea latifolia* seeds incubated at 20/15 °C [mean (± SD)].

Efectos de los pre-tratamientos químicos, y estratificación caliente en el porcentaje de germinación de las semillas de *Phillyrea latifolia* que se incubaron a 20/15 °C [Media (± desviación estándar)].

Treatment	Warm stratification months	Concentrations				
		0.2 %	0.5 %	1.0 %	2.0 %	
KOH	0	0 (0.00)	1 (0.5)	2 (0.58)	0 (0.00)	
	2	63 (1.71)	56 (2.71)	68 (3.83)	47 (0.96)	
	4	79 (1.71)	83 (2.63)	88 (0.82)	62 (1.91)	
	6	96 (1.41)	89 (1.71)	90 (1.73)	62 (2.65)	
NaOH	0	0 (0.00)	0 (0.00)	2 (0.58)	0 (0.00)	
	2	55 (1.71)	55 (1.71)	50 (1.29)	11 (1.26)	
	4	84 (1.83)	80 (1.83)	68 (2.45)	23 (3.77)	
	6	75 (2.87)	89 (2.22)	76 (2.45)	19 (2.06)	
		33.25 g L^{-1}	66.5 g L^{-1}	133 g L^{-1}	266 g L^{-1}	532 g L^{-1}
Ash lye	0	1 (0.5)	1 (0.5)	0 (0.0)	1 (0.5)	3 (0.96)
	2	36 (2.16)	35 (2.06)	54 (1.73)	63 (1.89)	54 (3.11
	4	59 (3.95)	66 (2.52)	75 (2.5)	90 (1.73)	84 (3.46)
	6	79 (3.5)	90 (1.00)	90 (1.29)	91 (2.63)	86 (1.29)
		250 mg L^{-1}	500 mg L^{-1}	1,000 mg L^{-1}	2,000 mg L^{-1}	3,000 mg L^{-1}
GA_3	0	2 (0.58)	8 (1.41)	21 (2.36)	22 (2.08)	15 (1.26)
	2	23 (0.96)	21 (4.19)	15 (1.71)	8 (1.41)	17 (4.03)
	4	49 (2.06)	32 (1.83)	32 (2.94)	18 (2.08)	16 (1.83)
	6	68 (2.16)	48 (6.48)	43 (3.86)	25 (0.5)	11 (0.96)
Water	0	0 (0.00)				
	2	13 (1.71)				
	4	35 (4.43)				
	6	79 (3.59)				

moted germination (figure 1), but also induced secondary dormancy on *P. latifolia* seeds, and seeds failed to germinate. The second set of incubation temperatures of 20 °C for 16 hours light, alternate with 15 °C for 8 hours dark proved to be the appropriate germination temperature for all seed pre-treatments. Mira *et al.* (2015) tried different incubation temperatures, from 5 to 25 °C for the germination of *P. angustifolia* and *P. latifolia* seeds in Spain and found that the optimum germination temperature for both species was 15 °C. Temperatures approaching 30 °C are common during the summer months in the Mediterranean zone and this fact sets the seeds automatically at dormant stage, in order to overcome the summer high temperatures. In contrast, in winter and spring time, the prevailing mean temperatures are usually above 7-10 °C in the vegetation zones where *Phillyrea* species grow. It is worth mentioning that after the end of the germination test under the set of high germination temperature conditions in growth incubator, the petri dishes with the seeds were left in a room with temperature between 8 and 15 °C. After two months they started to germinate. Other studies on germination temperatures for most Mediterranean shrub species suggest 15-20 °C as optimal temperature (Thanos *et al.* 1992, 1995). Concerning stratification, the cold stratification at 2-4 °C for all the combinations of chemical pre-treatments resulted in zero germination, while warm stratification at 15°C significantly increased the germination. Generally, stratification temperatures at 2-4 °C, which are suitable for breaking seed physiological dormancy of many forest species growing in high mountains or high latitudes, for the evergreen broadleaved species of Mediterranean region, are considered very low temperatures, even for winter time and seeds remain dormant as long as stratified in these temperatures. In the case of *P. latifolia*, which grows

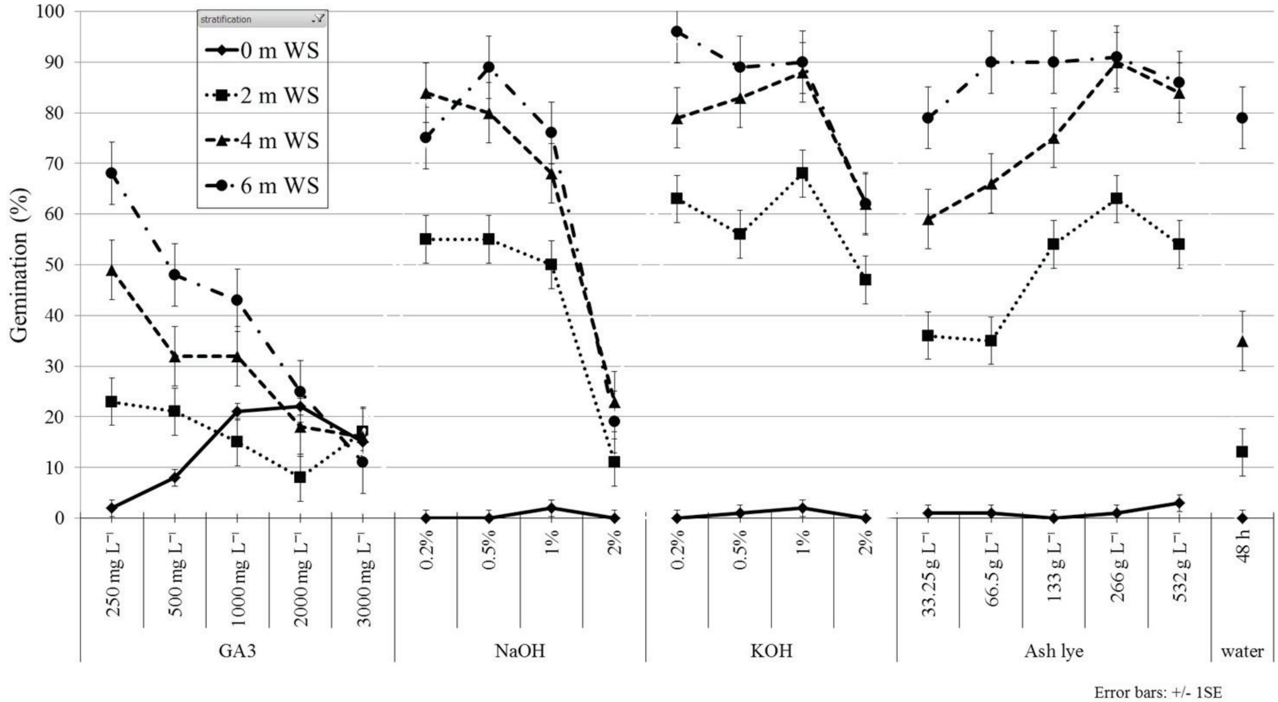

Figure 4. Germination percentage of *Phillyrea latifolia* seeds pre-treated with different concentrations of GA_3, NaOH, KOH, wood ash lye, water (control) and warm stratification (WS) at 15 °C for zero, two, four and six months.

Porcentaje de germinación de semillas de *Phillyrea latifolia* tratados con diferentes concentraciones de GA3, NaOH, KOH, lejía de ceniza de madera, agua (control) y estratificación caliente (WS) a 15 °C para cero, dos, cuatro y seis meses.

in Mediterranean maquis where the weather conditions are dry summers and mild winters, the warm stratification at 15 °C should be considered more as cold stratification than as warm, because this temperature is closer to real natural winter conditions than is this other temperature of 2-4 °C. Mira *et al.* (2015) found that cold stratification at 5 °C had a negative effect on seed germination of *P. angustifolia* and *P. latifolia* seeds in Spain, probably inducing secondary dormancy. In contrast, 30.1 % germination after two months of cold stratification at 3-4 °C for *P. latifolia* seeds is reported by the Aygün *et al.* (2011), which directly contradicts our results. However, the low incubation temperatures (10 °C in dark and 22 °C in light for 60 days) have created the necessary conditions for overcoming the dormancy induced by the cold stratification and the seeds germinated. Relatively low germination percentages (58 %) were obtained in another study that was performed in Turkey by Yücedağ and Gültekin (2011). The autumn sowing in the nursery at the 926 m a.s.l. worked as if the seeds had been subjected to cold stratification. Another experiment of autumn sowing of *P. latifolia* seeds that was performed in Northern Greece resulted in zero germination in the next spring (Takos and Efthymiou 2002). The meteorological data of the nearby station that was reported in the study show that the winter temperatures were below 5 °C. This is a possible explanation for the germination failure. Raimon and Lloret (1995) reported that subjecting *P. latifolia* seeds to cold stratification at 6 °C for one month did not improve significantly the germination percentage of seeds exposed to temperatures of 70 and 120 °C.

The positive effect of lye solutions on the germination of *P. latifolia* seeds is illustrated through the second finding of this study. According to figure 2, the effect of KOH, NaOH and wood ash lye solutions on the germination was higher than that of GA_3, which, in statistical sense, did not behave differently from the water, control pre-treatment. The assumption that fat acids may prevent water molecules to penetrate the endosperm and start the germination processes stands true, at least for the seeds collected in the present study. Ellis *et al.* (1985) report as dormancy breaking treatments for olive tree seeds the immersion in KOH or NaOH 0.5 -0.75 % for 6 hours. The low performance of GA_3 solutions, irrespectively from concentrations, is attributed to two facts; firstly, that the substrate was moistened by the GA_3 solution and thus there was a constant effect on the seeds during the stratification periods, as well as during incubation time and secondly, that concentrations above 1,000 mg L^{-1} are too high and may have been harmful to the seeds. Results of studies for other tree species indicate that the seed germination and growth rate is hastened to maximum by the application of 750 mg L^{-1} GA_3 for 24 hours (Dhupper 2013).

The third finding concerns the stratification time the seeds were subjected to in this study. It is clear that without stratification, germination of *P. latifolia* seeds is roughly close to zero (figure 3). The marginal mean germination percentage of 4.4 % that was recorded can be attributed to the pre-treatment with GA_3 solutions, but in any case, stratification at 15 °C is the main driving factor for breaking dormancy. Finch-Savage and Leubner-Metzger (2006) in their review on dormancy and control of germination classify the seeds of the oleaceae family into those which exhibited morphophysiological dormancy. Piotto and Di Noi in APAT (2003) reported that embryos of the Chalkidikis olive cultivar seeds achieved germination levels up to 73 %, while the ones previously undergoing cold stratification (+10 °C) for at least two weeks germinated more completely (96 %). Our findings indicate that two months stratification, irrespectively from chemical pre-treatments, is the critical time necessary for the inhibitor substances concentrations to start reducing and allow the process of germination to initiate. Further stratification to four months significantly enhances the germination, reaching a mean value of almost 60 %. Six months of stratification increases germination even more, although this is not as statistically significant as that of four months. A problem of treating the seeds with six months stratification is the development of long roots from the seeds that germinated early within the stratification. This might have a potential negative effect of handling the germinated seeds by the nurseries at the time of transplanting them to planting containers. Treating seeds with two- or four-month stratification reduces the germinated seeds in the stratification, without losing much of the seed's germination potential.

The combined effects of solutions, concentrations and durations of stratification gave significant results. The best germination percentage was exhibited by the 0.2 % of KOH concentration, followed by six months of stratification (figure 4). The second best germination percentage was achieved by the 266 g L^{-1} wood ash lye solution after six months of stratification (figure 4). It must be noted here that ash lye solutions presented the most homogenous germination percentages among the solution concentrations and stratification periods, compared with the other pre-treatments. The third best germination was achieved by the 0.5 % NaOH solution pre-treatment after six months of stratification. The large difference (47 % vs. 11 %) in the germination between 2 % w/v KOH and 2 % w/v NaOH is attributed to the fact that the two solutions are not chemically equivalent, because 2 % w/v KOH corresponds to 0.36 M solution, while 2 % w/v NaOH is equivalent to 0.5 M solution. Despite the higher reactivity of Potassium, due to the higher molecular weight of KOH, the solution is weaker compared to the same w/v NaOH solution. The difference of 0.14 M in the concentrations of the two solutions was responsible for the significantly lower germination performance of seeds pre-treated with NaOH. The use of alkali hydroxides as chemical scarification pre-treatments is reported by APAT (2003) for the germination of *Olea cuspidata* (Wall ex G. Don) Cif. seeds, a species similar to *Olea europaea* L., which is used as rootstock in many countries. Scarification with alkalis (KOH, NaOH) has also been shown to be effective in breaking seed dormancy in grasses (Perveen *et al.* 2008, Hu *et al.* 2014).

Soaking *P. latifolia* seeds in GA_3 solutions followed by warm stratification also produced satisfactory germination results, nonetheless in a rather irregular pattern, compared with alkali hydroxides and wood ash lye. The seeds that were stratified for four and six months had higher germination percentages (figure 4) only at the lowest concentration (250 mg L^{-1}) of the GA_3, compared with higher concentrations and shorter stratification durations. The same pattern of the hormone behavior combined with stratification found by Smiris *et al.* (2006) on the germination of strawberry tree (*Arbutus unedo* L.) seeds pre-treated with GA_3 and cold stratification. The fact that the substrate of each GA_3 pre-treatment was moistened by the respective solution might have resulted in this low germination percent for the highest GA_3 concentration. The interesting finding of GA_3 pre-treatment was the fairly high germination percentages (15 % to 23 %) achieved by 1,000, 2,000 and 3,000 mg L^{-1} concentrations without stratification. This clearly shows a certain positive effect of GA_3 on the germination of *P. latifolia* seeds, which is not present in all other pre-treatments (figure 4). Similar results (21 %) of the effect of GA_3 at 1,000 mg L^{-1} on the germination are reported by Mira *et al.* (2015). The positive effect of high GA_3 concentrations on seeds germination is reported in studies for other forest species such as the *Arbutus unedo* (Smiris *et al.* 2006).

The surprising finding in this study was the water or control pre-treatment performance (figure 4). *Phillyrea latifolia* seeds reached 13 % after two months, 35 % after four months and finally 79 % germination after six months of warm stratification, meaning that very satisfactory results can be obtained by just giving a good hydration to the seeds that are going to be germinated and submitting them to six months of stratification at 15 °C without using expensive or corrosive substances such as Gibberellic acids and alkali hydroxides. Mira *et al.* (2015) reported 14 % germination after 24 h soaking in water. The use of wood ash lye however, could be a good and more environmentally friendly alterative.

Although seeds of shrub species growing in the maquis vegetation zone have no morphological dormancy (underdeveloped embryos), our results showed that stratification at 15 °C for up to six months was the main driving factor for the germination of *P. latifolia* seeds. These seeds may present a combination of morpho-physiological dormancy (Hartmann *et al.* 2011). The cold stratification itself may have broken down the physiological dormancy, however has also prohibited the embryo development. The stratification at 15 °C for at least four months allowed the embryo development, because the temperature was not so low (near freezing) and at the same time the four or six months stratification time was sufficient to break the physiological dormancy. Since the seed coat of *P. latifolia* is permeable to water and not hard enough to prevent germination mechanically, further research on the embryo maturation may explain the long stratification time needed for germination.

CONCLUSIONS

From the present study the following conclusions can be drawn: (1) The wood ash lye solutions, alkali hydroxides, gibberellic acid and warm stratification have a strong positive effect on the germination capacity of *P. latifolia* seeds. (2) Stratification at 15 °C improved significantly the germination capacity. (3) Stratification treatment at 2-4 °C and incubation temperature at 20-30 °C prevent seeds from germination inducing secondary dormancy. (4) High concentrations of GA_3 and NaOH reduce the germination potential. (5) The control method (pre-treatment with water and stratification at 15 °C for six months) improved significantly the germination capacity and constitutes a cheap and safe germination method easily applied by nurseries.

ACKNOWLEDGEMENTS

This research received funding from the VERENIKE LIFE09 NAT /GR/000326 project.

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Recibido: 11.11.16
Aceptado: 18.04.17