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COMPARATIVE ECOPHYSIOLOGY OF SEED GERMINATION IN TWO PAIRS OF CLOSELY RELATED PINES OF MEDITERRANEAN AND HIMALAYAN ORIGINS

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Abstract

In this study we have examined the comparative ecophysiology of seed germination in two pairs of related pines of Mediterranean and Himalayan origins. One pair comprises *Pinus canariensis* and *Pinus roxburghii,* and the second *Pinus peuce* and *Pinus wallichiana*. Each pair has close taxonomic and morphological affinities. We have observed considerable differences among these pines about their germination behaviour. Seed germination of *Pinus canariensis* was feasible within a range of 15-20°C both under light and darkness, while the optimum germination of *Pinus roxburghii* was achievable at 25°C under white light. Germination rate of *Pinus roxburghii* was significantly enhanced under white light. A brief red light pulse resulted in promoting germination in *Pinus roxburghii*; however, the enhancing effect of red light was reversed by a brief far-red pulse; which indicated the mediation of phytochrome system in seed germination. *Pinus peuce* seeds were deeply dormant and required an extended period of stratification in order to come out of dormancy. *Pinus wallichiana* seeds were less dormant as compared to *Pinus peuce,* 30 d stratification or incubation under white light resulted in overcoming the dormancy.

Key words: Dormancy, Ecophysiology, Germination, Light, Phytochrome, Stratification.

Introduction

There are eleven reported pine species naturally growing in Europe (Keeley, 2012). Among the European pines the Mediterranean pines represent an important group since it includes the species which have morphological and taxonomic similarities with Himalayan pines. The resemblances among them endorse their close relationship. The Mediterranean pines namely *Pinus canariensis* C. Smith. (Canary Island Pine) and *Pinus peuce* Griseb. (Balkan pine) have close relationship with Asian pines *Pinus roxburghii* Sarg. (*Chir* Pine) and *Pinus wallichiana* A. B. Jacks. (Blue Pine) respectively (Mirov, 1967). There are several taxonomic and morphological similarities between *P. canariensis* and *P. roxburghii*, for example both have three leaves per fascicle, the resemblance in cone and apophyses, loss of basal cone scales, and possession of adnate seed wings. Taxonomically the two pines share a common place into subgenus *Pinus,* section *Tarnatae* and subsection *Canariensis.* Likewise *P. peuce* and *P. wallichiana* share numerous morphological traits, for example both possess five leaves per fascicle, articulate seed wings, and soft cone scales. Taxonomically this pair has been placed into subgenus *Strobus,* section *Strobus* and subsection *Strobi* (Critchfield & Little 1966, Klaus, 1989).

Molecular phylogenetic studies have shown that *Pinus canariensis* and *P. roxburghii* belong to the same subsection *Canarienses* within Eurasian clade of section *Pinus* (Gernandt *et al*., 2005). Nevertheless, Grotkopp *et al.* (2004) have identified the Himalayan *P. roxburghii* to be a divergent taxon from all the remaining Eurasian pines; and Wang *et al.* (1999) have suggested its association with North American pines. According to molecular phylogenetic studies *P. peuce* has been resolved as a solitary member of subsection *Strobi* within the subgenus *Strobus* and has been shown as a sister taxon to the remainder of the subsection *Strobi* and *Cembrae* to which *P. wallichiana* belongs (Grotkopp *et al*., 2004; Eckert & Hall, 2006; Kovacevic *et al.,* 2012).

In the present study we have examined the comparative ecophysiology of seed germination in two pairs of related pines of Mediterranean and Himalayan origins. The first pair comprises *Pinus canariensis* and *P. roxburghii,* and the second *P. peuce* and *P. wallichiana*. Within each pair the individual species occupy different biogeography. *P. canariensis* is a subtropical montane species found on Canary Islands located near Africa between 28° and 29° north and between 15° and 18° west on five main islands of the archipelago: Tenerife, La Palma, Gomera, Hierro, and Gran Canaria. The altitudinal range of *P. canariensis* is 900 to 2000 m above sea level (Gil *et al*., 2002). Its counterpart in Himalayas is *P. roxburghii*, distributed in the monsoon belt of outer Himalayas from Pakistan to Bhutan at the elevation of 900-2000 m above sea level (Critchfield & Little, 1966).

Pinus peuce is confined to the high mountains of south-eastern Europe, Southern Yugoslavia, Western Bulgaria, Northern Greece, and Albania. *P. peuce* is separated by more than 4800 km (3000 miles) from its Himalayan counterpart (Critchfield & Little, 1966). *P. wallichiana* ranges throughout the Himalayan Mountains, extending beyond them to eastern Afghanistan north-eastern Balochistan, north-western Khyber Pakhtunkhwa province of Pakistan, northern Burma and Yunnan province, China. Some key characteristics of these pines are presented in Table1.

Table 1. Key characteristics of *Pinus canariensis, Pinus roxburghii, Pinus peuce* and *Pinus wallichiana.* Data has been derived from Critchfield and Little (1966), Jesse and Perry (1990), and Price *et al*. (1998).

	P. canariensis	*P. roxburghii*	*P. peuce*	*P. wallichiana*
Common name	Canary Islands pine	*Chir* pine	Balkan Pine	Blue Pine
Natural Distribution	Confined to central and western Canary Islands, off the coast of West Africa	Monsoon zone on the edge of the Himalayas from Bhutan to the North-East Pakistan	South Balkan Peninsula (limited distribution)	Throughout the Himalayas, eastern Afghanistan, eastern Balochistan (Pakistan), North Burma and Yunnan Province (China)
Altitude (m)	900-2000	900-2000	~1700	1600-4000
Mature tree height (m)	40	55	20 - 30	45
Seed bearing age (y)	15-20	15-40	12-30	15-20
Seed bearing intervals (y)	3-4	2-4	3-5	1-2
Flowering dates	April-May	February-April	May-June	April-June
Cone ripening dates	September	Winter	September-October	August-October
Seed production (years after flowering)	2 years	2 years	2 years	2 years
Seed dispersal dates	September-October	April-May	September-October	September-November
Average seed weight, mg	111	107	50	59

The knowledge of germination characteristics plays a role in understanding of the regeneration and establishment of a species in its environment. The knowledge of the light and temperature conditions required for seed germination of these pine species is scarce. International Seed Testing Association (Anon., 2007) describes the standard laboratory condition for some of the pines. Seed germination of *P. canariensis* has been studied by some researchers (Escudero *et al*., 2002; Calvo *et al*., 2013) as well as there are field observations of ecophysiology of regeneration of *P. canariensis* (Pardos *et al*., 2005). Limited research work has been conducted on seed germination of *P. roxburghii* (Rao & Singh, 1985, Sharma *et al*., 1999; Ghildiyal *et al*., 2009). The scientific work carried out on high altitude montane species *P. peuce* and *P. wallichiana* is even rarer, and there are some sparse studies dealing with the germination of *P. peuce* (Skordilis & Thanos, 1997; Alexandrov & Andonovski, 2011), while Singh and Thapliyal (2012) have examined the seed germination of *P. wallichiana*.

In an effort to understand the ecophysiology of seed germination, we have examined the effect of light, temperature and cold stratification on germination. We have compared the behaviour of each pair to examine whether germination behaviour is determined by the genetics or by the biogeography of a species. Each pair of pines species in our study is spatially segregated but has common phylogeny.

Materials and Methods

The seeds were either collected from natural stands or procured from forest departments of the respective countries of the origin. Seed germination at constant temperatures and alternating day and night temperatures was accomplished inside the incubator (Heraeus, BK 5060 EL, W.C. Hanau, Germany); while a growth Bench (GB48 Conviron, Canada) was used for seed germination under programmable light and temperature conditions. Daily light and temperature conditions in the Bench were programmed to simulate the estimated conditions encountered during the time of germination of *Pinus roxburghii* in nature.

The red and far-red broad band irradiations were produced by a bank of tubes and the light was filtered through a combination of coloured Plexiglas sheets (each 3 mm thick, Rohm GmbH, Germany). Red light (11.0 µmol m^{-2} s^{-1}) was produced by 10 red fluorescent tubes (TL 20W/15 Philips) and 1 layer of red Plexiglas, 501. Far-red light (18.2 µmol m^{-2} s^{-1}) was obtained by 12 white incandescent tubes (Philinea 6276, 60 W, Philips) filtered through 3 layers of Plexiglas (2 blue, 627, and 1 red, 501). The dim green safe light (0.05 µmol m^{-2} s^{-1}) was achieved by 1 green fluorescent tube F 15T8.G.6, 15 W Green-Photo, General Electric, USA and 2 Plexiglas sheets (1 red-orange, 478, and 1 green, 700). Photon flux rates were measured by Licor 1800 USA.

The role of phytochrome in seed germination was determined by illuminating the seeds either with a single or with intermittent pulses of red and far red lights. In case of single pulse illuminations, one set of seeds was illuminated with a single pulse of red light for 15 min; the other set of seeds was illuminated with 15 min of red light followed by a pulse of 15 min far-red light. While in case of intermittent pulse illuminations, one set of seeds was illuminated with a pulse of red light for 15 min every 24 h for 7 consecutive days, the other set of seeds was illuminated with 15 min of red light followed by a pulse of 15 min far-red light every 24 h for 7 consecutive days. The seeds were illuminated 24 h after the onset of imbibition. The illuminated seeds were imbibed in the darkness for germination. The dark imbibed seeds were manipulated under the green safe light.

The cold moist stratification was accomplished by imbibing seed at 5°C under darkness. The germination tests were performed with 5 replicates of 20 seeds per Petri dish of 9 cm diameter. Each Petri dish was lined with 2 layers of filter paper and moistened with 8 mL of distilled water. The criterion of seed germination was visible radicle protrusion.

Results

The seed germination of *Pinus canariensis* representing three provenances, namely Papagou, Greece; Gran Canaria, Canary Island; and Tenerife, Canary Island was investigated under various constant temperatures. The seeds were incubated at the range of 5-25°C under white light and darkness. The germination of *P. canariensis* took place within a narrow range of 15-20°C (Fig. 1). The final percent germination values at 15°C under darkness ranged 52-68%; at 20°C the corresponding values ranged 58-68%. Constant white light enhanced the germination of *P. canariensis* at 20°C, where final germination values were 92% and 78% for Papagou and Tenerife seed lots respectively.

The final seed germination of *Pinus canariensis* was significantly reduced at 10°C where final germination values were recorded as 11, 37, and 20% under darkness for Papagou, Gran Canaria and Canary Island provenances respectively. At this temperature the onset of germination was delayed and speed of germination was reduced. Seed germination did not take place at the marginal temperatures of 5 and 25°C. The imbibition for 30 d at 25°C caused necrosis in the seeds hence no further experiments were carried out with these seeds. While soaking seeds at lower temperature did not induce dormancy; as the seeds from Tenerife provenance when initially imbibed at 5°C for 30 days resumed germination upon their subsequent incubation at 15°C.

The preliminary tests performed on *Pinus roxburghii* seeds revealed that dark imbibed seeds did not germinate to their optimum capacity at constant temperatures ranging from 5-25°C. The germination time course of *P. roxburghii* under changing light and temperature conditions is shown in Fig. 2. The light and temperature in the germination chamber were programmed to simulate the approximate conditions met in its natural habitat during rainy season. The figure 2 represents the results of two seed germination test carried out with the same seed lot at an interval of two years. Dry storage at room conditions did not affect seed germinability as the final germination values of fresh *versus* stored seeds were similar when seeds were incubated in the dark and under white light (14% *vs.* 19% and 77% *vs.* 75%, under white light and darkness respectively). The light resulted in a significant increase of germination rate and final germination percentage. The germination under light started on the 6th day after imbibition and final germination was achieved on the 21st day for both fresh and stored seeds.

The role of phytochrome in seed germination of *P. roxburghii* was determined by exposing them to single as well as to intermittent doses of red and red followed by far-red irradiations (Fig. 3). The germination tests were carried out at a constant temperature of 25°C under darkness. The effect of a single and intermittent red light irradiation was almost similar. The red pulses promoted the final germination which was 41 and 46% after single and intermittent red light irradiation respectively. The exposure to far-red light reversed the promotional effect induced by the red light, which suggested the involvement of phytochrome in the seed germination of *P. roxburghii*. Nevertheless, the far-red irradiation did not totally reverse the promotional effect of red light as the final germination was higher as compared to germination under dark control.

Pinus peuce seeds were deep dormant and required an extended period of stratification for the release of dormancy. Their dormancy could be overcome by more than 300 d of cold moist stratification. The stratified seeds were imbibed at constant temperature of 20°C (Fig. 4). Final germination of stratified seeds for 210, 240, and 270 d was less than 30%, while 300 and 330 d of cold stratification resulted in a significant increase in final germination (43, and 53% after 300 d, and 330 d stratification respectively). The seeds were able to achieve maximum germination capability after 360 d stratification (59, 68, and 63% under light and 48, 54, 69% under darkness after 360, 390, and 420 d respectively). There was no significant difference in final germination of dark imbibed seeds and those imbibed under light.

The germination time course of *Pinus wallichiana* seeds stratified for 30 and 60 d is presented in Fig. 5. The seeds were imbibed for germination under constant light and temperature at 20°C. *P. wallichiana* seeds were less dormant as compared to *P. peuce* and 30d of stratification resulted in overcoming the dormancy. The stratification resulted in enhancement of both rate and final germination. Optimum germination was attained after 60 d stratification where final germination was 85 and 67% under light and darkness respectively. While for 30 d stratification final germination values were 79 and 60% under light and darkness respectively. The stratification also offset the requirement of light as the stratified seeds of *P. wallichiana* were able to germinate under darkness.

Discussion

The germination behaviour of each species varied in response to different factors like, temperature, light, and stratification. Seed germination of *Pinus canariensis* is feasible within a range of 15-20°C and it takes place both under light and darkness which is a successful adaptive trait of Mediterranean plants (Skordilis & Thanos, 1995). Relatively low temperatures associated with the winter rainy season favour germination of most of the Mediterranean species (Trabaud, 1995; Baskin & Baskin, 2014). Seed germination in *P. canariensis* is restricted to a short period as germination is not attainable at suboptimal temperatures of 5 and 25°C. Our results regarding inability of seeds to germinate under suboptimal temperatures are in agreement with Escudero *et al*. (2002). Our experiments show that seed germination in *P. canariensis* is dependent more on temperature than on light, although at 20°C light results in a slight enhancement in final germination.

Pinus roxburghii is photophilous in nature and germination does not take place under darkness. The seeds of *P. roxburghii* are capable of germinating within a range of 20-25°C under white light. Our results show that the light is absolute requirement for the seed germination of the species. These findings are in agreement with earlier studies (Rao & Sigh, 1985), while Ghildiyal *et al*. (2009) have shown to optimally germinate *P. roxburghii* seeds at 25°C in the laboratory without elaborating precise light conditions.

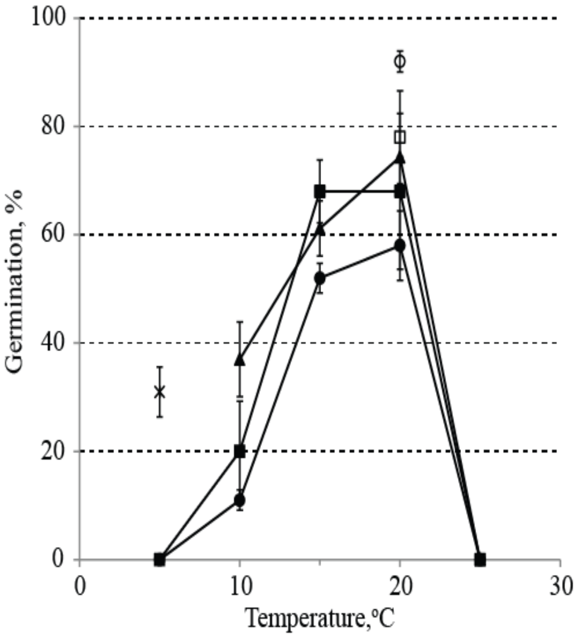

Fig. 1. Final germination of *Pinus canariensis* seeds from three provenances as a function of temperature. The curves correspond to Papagou, Greece seed lot under white light (○) and darkness (●), Gran Canaria, Canary Island seed lot under darkness (▲), and Tenerife, Canary Island seed lot under white light (□) and darkness (■). The (×) represents the seed germination of Tenerife, Canary Island seed lot initially incubated at 5°C for 30 d and subsequently transferred to 15°C under darkness.

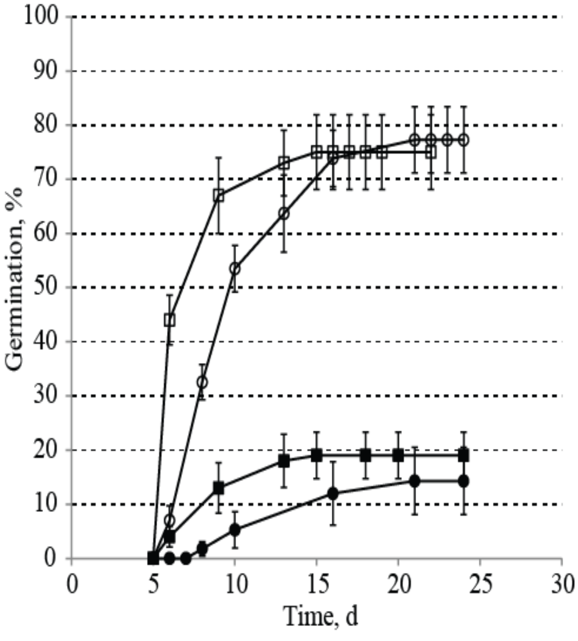

Fig. 2. Germination time course of *Pinus roxburghii* seeds stored at room temperature for 30 months. Fresh seeds of the same seed lot were used as a control. Germination tests were carried out under fluctuating light and temperature conditions simulating those encountered in their natural habitat. The temperature range during the course of experiments was 15-21°C, with an average value of 18°C. The (□) and (■) represent seed germination of stored seeds under light and darkness respectively, while (○) and (●) represent germination of fresh seeds under light and darkness respectively.

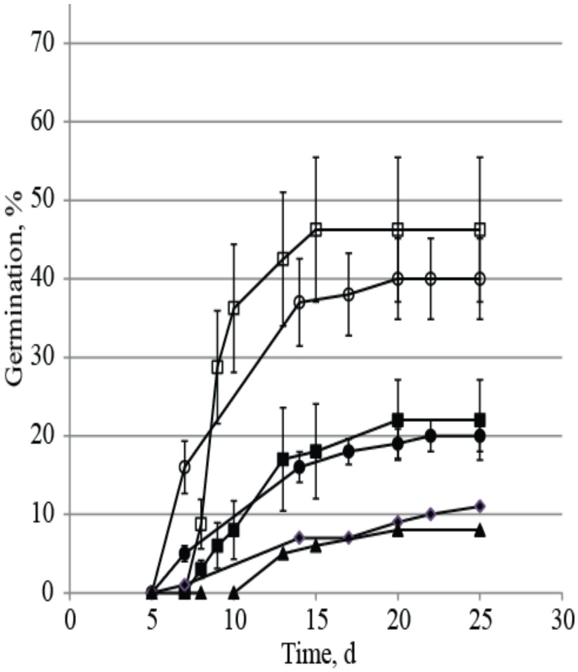

Fig. 3. The time course of seed germination of *Pinus roxburghii* at constant temperature of 25°C after a single pulse of red light for 15 minutes (○), a 15 min single pulse of red light followed by a 15 min far-red light pulse (●). And after intermittent pulses of red light every 24 h for 7 consecutive days for 15 minutes (□), and a 15 min intermittent pulses of red light followed by a 15 min far-red light pulse every 24 h for 7 consecutive days (■). The seeds were the illuminated after 24 hours of soaking.

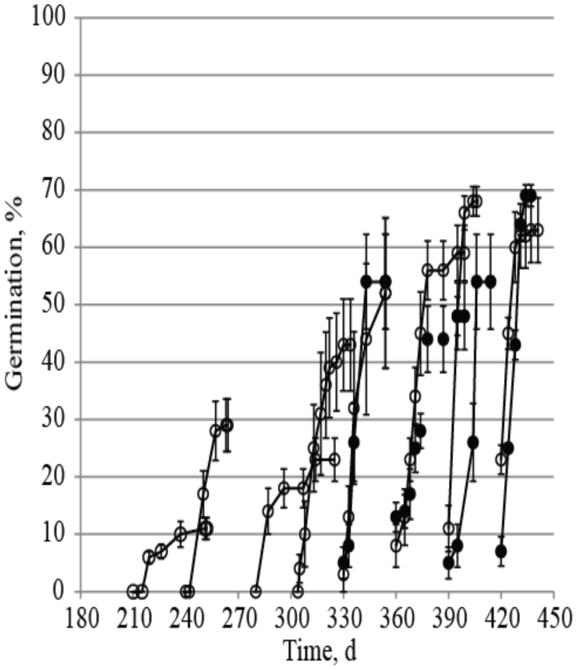

Fig. 4. Germination time course of *P. peuce* seeds after various periods of cold and moist stratification. The seeds were checked every 30 days for germination. The seeds were imbibed at a constant temperature of 20°C under constant light (○) or darkness (●).

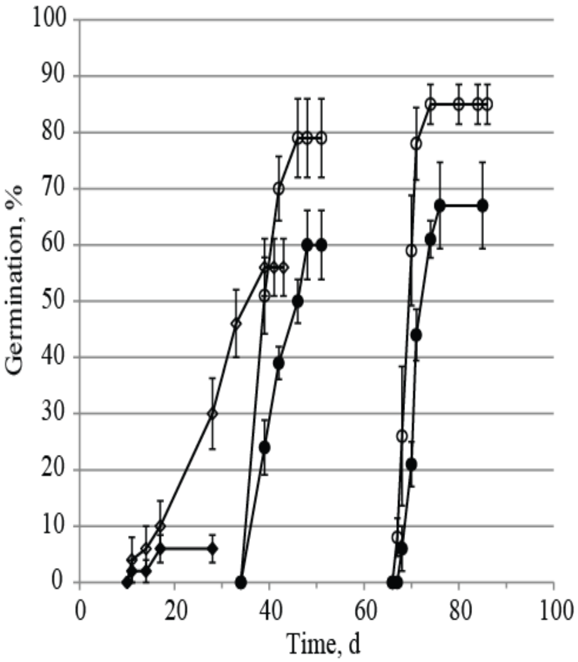

Fig. 5. Germination time course of stratified seeds of *Pinus wallichiana*. Seeds were stratified for 30 and 60 d. The seeds were soaked in a constant temperature 20°C in constant light (○) and darkness (●). The germination of non-stratified seeds is represented by ◊ and ♦, under white light and darkness respectively

Pinus canariensis and *P. roxburghii* are both sub-tropical montane species and morphologically very identical to each other. However, both species vary in their seed germination characteristics, the diverse reproductive strategies by these species are adapted to suite their respective geographical distribution. The seed germination of *P. canariensis* is adapted to the Mediterranean climate where its seeds are dispersed in late summer just before the onset of rainfall (Young & Young, 1992). Similar to the many pine habitats, the conditions suitable for *P. canariensis* seed germination are met within months of its seed dispersal (Pratt *et al*., 1984). The seed germination in *P. roxburghii* on the other hand coincides with monsoon in July and August in rather higher temperature. The seed dispersal in nature precedes the monsoon rainfall and occurs in April to May (Young & Young, 1992). Most of the rainfall occurs during the month of July until September. Considering the climatic data it is worth mentioning that seeds acquire temperature suitable for germination during July until September, and the peak germination in field is also observed during these months. The rainfall does not seem to be a limiting factor in the establishment of seedlings after germination; however mean winter temperature lower than 20°C may be considered as a factor restraining the seedling development.

Pinus roxburghii occupies the lands opened due to cutting or fire therefore requirement of light for its germination is understandable. The light sensitivity of a species indicates the 'invasive' potential of colonizing open habitats (Skordilis & Thanos, 1995). Germination in *P. roxburghii* is controlled by light; this control is exerted through the mediation of phytochrome system. Brief red light pulses are adequate to promote germination while the effect of red light can be reversed by a brief far-red pulse. The far-red induced reversion of germination suggests the involvement phytochrome in the seed germination of *P. roxburghii*. Nevertheless, the far-red irradiation does not totally reverse the incremental effect of red light; this indicates high sensitivity of seeds to small increases in Pfr. The capacity of *P. roxburghii* to invade barren places and its immunity from fire damage has extended its range (Champion *et al*., 1965; Sharma *et al*., 1999). Fire resistance is another common ground shared by *P. canariensis* and *P. roxburghii,* they possess fire resistant characteristics for example thick bark, long needles, thick buds, tall growth habit, deep rooting, and longevity (Klaus 1989, Climent *et al*., 2004).

The difficulty in germinating deep dormant seeds of *Pinus peuce* is well known (Savill, 2013). The research work conducted to remove the dormancy and to find the causes of the dormancy is fairly old (Djordjeva, 1967; Djordjeva *et al*., 1970; Mason *et al.,* 1994), while relatively recent research work reports the *in vitro* regeneration of *P. peuce* (Stojicic *et al.*, 2012). Some authors have suggested that the seed dormancy in *P. peuce* is physical and not physiological, which they have attributed to the poor permeability of seed coat (Djordjeva *et al*., 1970). In their experiments the seed coat treatments have resulted in germination up to 60%, which is not in accordance with the present study, where various seed coat treatments failed to break the dormancy in the pine, thus dormancy seems to be physiological. The various seed coat treatments which we employed included removal of seed coat, scarification on micropylar end, and seed coat cracking. Mason *et al*. (1994) have applied alternating cold and warm stratification for 24 weeks and were able to achieve about 50% final germination in *P. peuce*.

The seeds of *Pinus peuce* are deeply dormant; they do not respond well to a shorter periods of cold moist stratification. To completely recover from the deep dormancy they need cold moist stratification of more than 1 year. The length of stratification required for germination is related to the degree of dormancy and may exist as an ecological adaptation to a longer winter (Bewley *et al*., 2013). Under natural conditions dormancy may prohibit seed germination of the Balkan pine in first spring season after seed dispersal that occurs on late autumn. It may be possible that the germination in nature occurs after the seeds have over-wintered for 2 years. Germination in *P. peuce* and *P. cembra* may take place the second or even third year following seed dispersal (Young & Young, 1992).

Another interesting observation regarding the germination behaviour of *Pinus peuce* seeds is their ability to germinate at 5°C after very prolonged stratification. Seeds are capable of germination to their full capacity (>90%) after 400 d at 5°C in darkness. This phenomenon has also been noted in other pine species, for example Papaioannou (1975) achieved approximately 60% germination of *P. heldreichii* at 5°C after 25 weeks in darkness.

The marked effect of light and stratification on seed germination suggests the presence of dormancy in *Pinus wallichiana*. However, the seeds of *P. wallichiana* are less dormant as compared to *P. peuce*. In non-stratified seeds this dormancy can be overcome by soaking the seeds under white light. The effect of light is profound on germination of non-stratified seeds however, it decreases as seeds are stratified; this effect is further declined with increased duration of stratification. Pre-germination stratification is known to alter the light response a phenomenon that has been observed in other pine species like *P. taeda* and *P. virginiana* (Toole, 1973). Stratification may increase seed germination in darkness and when the duration is long enough, it may even eliminate beneficial effect of light (Toole, 1973). In our experiments it is observed that light plays a role in the germination of stratified seeds of *P. wallichiana* as it results in enhanced rate and final germination. Working on seed storage and germination experiment on *P. wallichiana* seeds Siddiqui and Parvez (1981) have also shown that 120 days of cold stratification of blue pine seeds was required for satisfactory germination in the nursery.

Conclusion

Germination of *Pinus canariensis* takes place within a range of 15-20°C and germination takes place both under light and darkness. While *P. roxburghii* is photophilous in nature and germination does not take place under darkness. The optimal germination temperature is 25°C under white light, although germination can take place at 20°C under light.

Pinus peuce seeds are deep dormant and need a much extended period of stratification before they germinated in the laboratory. Their dormancy can be broken by more than 300d of stratification. After a very prolonged stratification the seeds of *P. peuce* can even germinate at 5°C. *P. wallichiana* is less dormant as compared to *P. peuce* and 30d of stratification and light can break the dormancy. The effect of light is profound on germination of non-stratified seeds but decreases as the seeds are stratified. Stratification in *P. wallichiana* increases the final germination.

The differences observed in the germination behaviour of the pines may be attributed to varying ecophysiological strategies evolved in response to the particular geographical distribution of a species. Such strategies are implemented through the modification in phenology of seed maturity and seed dispersal, as well as in seedling emergence.

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