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Stimulation of germination in dormant seeds of *Juniperus polycarpos* by stratification and hormone treatments

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Abstract Dormancy in *Juniperus polycarpos* (K. Koch) seeds is a major hurdle for production of the desired quantity of seedlings in nurseries for restoration purpose. Thus, the aim of this study was to develop an optimal dormancy breaking treatment and subsequent stimulation of germination. The treatments applied were cold stratification at 1 °C for 4, 8, 12, and 16 weeks; warm stratification at 20 °C for 4, 8, 12, and 16 weeks followed by 12 weeks of cold stratification; exogenous application of 250, 500, and 1000 ppm gibberellic acid (GA_3) and 6-Benzylamino purine (BAP); and a combination of 500 ppm hormones and cold stratification. The results show significant differences in germination between cold and warm-cold stratification, length of stratification and their interaction ($p < 0.01$). Warm stratification for 16 weeks followed by 12 weeks of cold stratification induced 72 % of the seeds to germination in 12 days compared with 16-week cold-stratification alone (42 %) and the control (8 %). Exogenous application of GA_3 and BAP alone or in combination with cold stratification resulted in less than 50 % germination though significantly ($p < 0.01$) higher than the control. Apparently, the hormone treatments alone or in combination with cold stratification are not effective in completely

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breaking dormancy and stimulation of germination. The findings suggest that *J. polycarpos* seeds may possess intermediate simple morpho-physiological dormancy, which could be an adaptive mechanism for relatively warmer autumn temperature during seed maturation and the subsequent cold winter temperature under natural conditions.

Keywords Iran · Juniper · Morpho-physiological dormancy · Warm-cold stratification

Introduction

Seed dormancy is a phenomenon in which a seed fails to germinate and produce a normal seedling under favorable germination conditions—moist substrate, sufficient oxygen supply and optimal temperature. It has been evolved as an adaptation for persistence in unpredictable environments, so as to escape from competition and for optimizing reproductive success in a randomly varying environmental condition by dispersing germination in space and time (Baskin and Baskin 2001). The cause of dormancy can be due to impermeable seed coat, restricting imbibition (physical dormancy); underdeveloped embryo (morphological dormancy); chemical and physiological inhibitory mechanisms (physiological dormancy); or combinations of the above factors (Baskin and Baskin 2004).

Many tree seeds show one or combination of these dormancy types, and juniper seeds are opined to have physiological or morpho-physiological dormancy (Scianna 2001; Al-Refai et al. 2003; Tigabu et al. 2007; Tilki 2007; Tylkowski 2009, 2010). Physiological or morpho-physiological seed dormancy can be broken by cold or warm-cold stratification for a certain period, accompanied by light and hormonal treatments (Baskin and Baskin 2001; El-Juhany et al. 2009; Afroze and ÓReilly 2013). However, the specific treatment conditions to achieve maximum germination vary between species.

Juniperus polycarpos (K. Koch) is one of the dominant juniper species with a wide geographic distribution from Central and Western Asia and as far as near East and Western China (Adams 2014). In Iran, juniper forests once occupied large mountainous terrain at altitudes ranging from 750 to 3400 m above sea level in the north, north-west, central, and southeast highlands but today their populations are highly fragmented due to anthropogenic disturbance (Korouri et al. 2012). Its ability to grow on poor soil conditions where no other species can grow coupled with its tolerance to harsh climatic conditions make *J. polycarpos* a candidate species for reforestation/restoration of degraded arid and semi-arid areas of Iran with mean annual rainfall range of 100–550 mm (Korouri et al. 2012). However, poor seed quality coupled with seed dormancy is a major hurdle for raising the desired quantity of *J. polycarpos* seedlings in the nursery (Ahani et al. 2013). Germination of freshly collected seeds of *J. polycarpos* is very low and erratic, ca. 10 %. Hitherto, there is no well-defined treatment regime for breaking dormancy in *J. polycarpos* seeds.

Thus, developing an effective dormancy-breaking treatment that enables complete and synchronous germination of *J. polycarpos* seeds is highly needed to produce the desired quantity of seedlings in the nursery for restoration planting. The objectives of this study were to examine (1) the efficacy of cold stratification or warm-cold stratification on the release of seed dormancy; (2) the effects of exogenously applied phytohormones, gibberellic acid (GA_3) and 6-Benzylamino purine (BAP), on dormancy release; and (3) whether dormancy release and subsequent germination is stimulated more by treatments involving a combination of phytohormones and cold stratification.

Materials and methods

Study site and seed collection

The *J. polycarpos* stand from which cones were collected is located in North-east Iran near the Gorgan city (capital of Golestan province), with altitudes ranging from 2150 to 3150 m above sea level. The average annually precipitation is 305 mm and the forest belongs to the Iranian Northern vegetation Hyrcanian Zone (Korouri et al. 2012). Cones of *J. polycarpos* were collected from 20 randomly selected trees collected at Chaharbagh on Alborz mountain chain located between 36°36′–36°41′N and 54°28′–54°35′E at the end of October 2012, when it was a seed mast year. For maximizing genetic variation among seed trees, cones were collected from trees that were 100 m apart from each other within the same altitudinal range. To get representative seed samples and to avoid the confounding effect of cone position in the crown on seed viability, cones were picked from all accessible branches of each sample tree (2 kg cones per tree).

In the laboratory, the fleshy part of cones was removed, and resin was cleaned off the extracted seeds by carefully washing with acetone (2 min) and running water. Since *J. polycarpos* seed lots tend to have a large quantity of empty, insect-infested and dead seeds, the seeds were first sorted to remove dead seeds following the incubation, drying and separation protocol developed for this species (Daneshvar et al. 2016). The viability of the sunken fraction was 82 % based on a topographical tetrazolium (TTC) test, which is a rapid biochemical test for assessing seed viability, particularly for dormant seeds (International Seed Testing Association 2010). The seeds were then packed in plastic bags, sealed and stored in the refrigerator at 5 °C for 2 months until the experiments were conducted.

Stratification treatments

A series of experiments was conducted to identify the best stratification treatment for breaking dormancy in *J. polycarpos* seeds. The first experiment involved cold stratification at controlled moisture content in a refrigerator at 1 °C for 4, 8, 12, or 16 weeks. Cold stratification at controlled hydration (ca. 30 % moisture content) and without medium has been found to reduce the risk of precocious germination during the treatment period (Soltani et al. 2005; Afroze and ÓReilly 2013). The amount of water needed to reach 30 % moisture content was determined using the following formula:

$$\text{Water to be added (g)} = \left\lceil \frac{(100 - A)}{(100 - B)} \times W1 \right\rceil - W1$$

W1 = initial weight of seeds per replication; A = initial seed moisture content and B the desired moisture content.

A total of 50 seeds per treatment, with four replication, were then put in polyethylene tubes (5 cm long and 2 cm in diameter), and the desired amount of water added. Thereafter the polyethylene tubes were capped with a one-way polytetrafluoroethylene membrane (Trade mark GORE-TEX) that allows gas exchange but not water. The polyethylene tubes were then left in a glass bowl filled with moist sand and then covered with a lid to maintain close to 100 % relative humidity around the tubes thereby reducing the risk of moisture loss by seeds during stratification. It should be noted that the moisture content might be slightly higher than 30 % during stratification because the Gore-Tex allows water vapour

to pass through although the uptake of water by the seeds is regulated by the water potential gradient between the seeds and the tubes. After each stratification period, the seeds were sown for germination test.

The second experiment involved warm-cold stratification where seeds (50 seeds per treatment, replicated four times) with 30 % moisture content were put in polyethylene tubes, and then warm-stratified at 20 °C for 4, 8, 12, or 16 weeks followed by 12 weeks of cold stratification at 1 °C in a refrigerator (as described above). After each treatment period, seeds were sown for germination evaluation. The third experiment involved cold-warm and warm-cold-warm stratification, but these treatments resulted in high precocious germination. Therefore, results of the third experiment are not reported.

Exogenous application of hormones

The effects of gibberellic acid (GA_3) and 6-Benzylamino purine (BAP) on the release of dormancy in *J. polycarpos* seeds were tested using 50 seeds per treatment with four replications. For this purpose, seeds were soaked in three concentrations of GA_3 or BAP (250, 500, and 1000 ppm) solutions for 72 h at 20 °C to reach 30 % of moisture content. The amount of hormone solutions added to increase the moisture content of the seeds to 30 % was determined by the same formula used for adjusting the moisture content for stratification treatments above. Afterwards, seeds were washed with tap water for 5 min before sowing for germination tests.

Combination of hormones and cold stratification

Based on the findings of the above two experiments, a combination of 500 ppm GA_3 or 500 ppm BAP solutions and 12 weeks cold stratification were tested to examine their effect on breaking dormancy. For this purpose, 50 seeds per treatment, replicated four times, were soaked in 500 ppm GA_3 or BAP solutions for 72 h at 20 °C to reach 30 % moisture content. Afterwards, seeds were washed for 5 min under tap water and cold-stratified in the polyethylene tubes in the refrigerator at 1 °C as described previously.

Germination test

For each experiment, four replicates of 50 seeds from each treatment were sown on standard moistened filter paper in petri dishes (Munktell filter paper; diameter 95 mm) and placed on Jacobsen's apparatus that was set at 20 ± 1 °C constant temperature during day and night with a continuous illumination of ca. 20 μE m^{-2} s^{-1} (fluorescent lamp F 40 W/33 RS cool white light) for 30 days as per the recommendation of the international seed testing for dormant seeds (International Seed Testing Association 2010). To serve as a control, four replicates of 50 untreated seeds were also sown. The germination process was monitored daily and germinated seeds were counted when the radicle reached 2 mm long and had a normal appearance.

Data analysis

For all experiments, germination capacity (GC) and mean germination time (MGT) of each treatment replicate were calculated as:

$$GC\,(\%) = \left(\frac{\sum n_i}{N}\right) \times 100$$

$$MGT\,(days) = \frac{\sum (t_i \times n_i)}{\sum n_i}$$

where $\sum n_i$ is the number of germinated seed after 30 days, and N is the total numbers of seed sown; t_i is the number of days starting from the date of sowing and n_i is the number of seeds germinated at each day (Bewley and Black 1994). All percentage data sets were arcsine- transformed prior to statistical analysis to approximate the normality assumption for analysis of variance (Zar 1996). Analysis of variance (ANOVA) following General linear model (GLM) for factorial design was performed to examine significant differences in GC and MGT in response to stratification treatments (stratification type × duration). Similarly, ANOVA was performed to examine significant differences in germination response to hormone treatments (type × concentration). One-way ANOVA was performed to determine significant differences in germination response among the combined treatments of hormones and 12 weeks of cold-stratification, and untreated control. Means that showed significant differences were compared by Tukey's honestly significant test at 5 %. All statistical analyses were performed with Minitab 17.0 statistical software (Minitab Inc., State College, PA, USA).

Results

Germination responses to stratification

There was a significant interaction effect of type and duration of stratification on GC ($F_{(4,\ 30)} = 16.95$; $p < 0.01$). Warm-cold stratification resulted in 40–72 % germination capacity compared to 12–42 % germination for cold stratification, depending on the duration of stratification treatments (Fig. 1a). Among all durations of stratification treatments tested in the present study, germination capacity was higher after 12 and 16 weeks than 4 and 8 weeks of stratification, which in turn resulted in significantly higher germination capacity than the control, particularly for warm-cold stratified seeds. For cold-stratified seeds, 4 weeks appeared to be insufficient to release dormancy and stimulate germination compared to the control.

The speed of germination, as determined by MGT, also varied significantly in response to the interaction effect of type and duration of stratification ($F_{(4,\ 30)} = 4.69$; $p = 0.005$). Warm-cold stratification resulted in faster germination than cold stratification across all durations of stratification treatment although both stratification treatments shortened the germination time compared with the control, except 4 weeks of cold stratification (Fig. 1b). Overall, MGT tended to decrease for stratification periods longer than 8 weeks.

Germination responses to hormones

Significant interaction effect was detected in GC between type and concentrations of hormones ($F_{(3,\ 24)} = 11.15$; $p < 0.01$). While high concentration of GA_3 (1000 ppm) totally inhibited germination (Fig. 2a), application of 500 ppm GA_3 stimulated

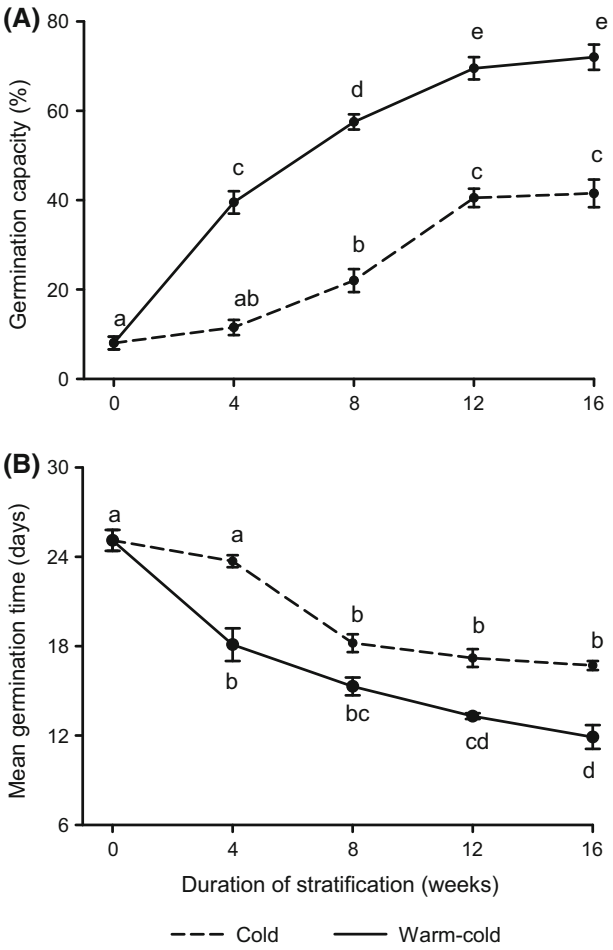

Fig. 1 Germination capacity (**a**) and mean germination time (**b**) of *J. polycarpos* seeds in response to different lengths of cold and warm-cold stratification period (mean ± SE). Note that the length of cold stratification for the warm-cold stratification was 12 weeks

germination (25 %) better than 250 ppm GA_3 (11 %) and the control (6 %). Application of 500 ppm BAP also stimulated germination (16 %) compared with 6 % for the control; however, GC did not differ statistically across all concentrations of BAP tested (Fig. 2a).

Similar to GC, there was a significant interaction effect ($F_{(3, 24)} = 118.98$; $p < 0.01$) for MGT between hormone type and concentration. The MGT was shorter for seeds treated with hormones than the control, except application of 1000 ppm GA_3 that totally inhibited germination (Fig. 2b). Among hormone treatments, the fastest germination was observed for seeds treated with 250 ppm BAP.

Germination responses to combination of hormones and cold stratification

Results showed significant differences in GC ($F_{(2, 9)} = 126.36$; $p < 0.01$) and MGT ($F_{(2, 9)} = 123.02$; $p < 0.01$) among GA_3 + cold-stratification, BAP + cold stratification, and the control. Exogenous application of 500 ppm GA_3 followed by 12 weeks of cold stratification

resulted in higher GC than seeds treated with 500 ppm BAP, which in turn resulted in higher germination than the control (Table 1). The MGT did not differ between combined treatment of hormones and cold stratification but was shorter than the control (Table 1).

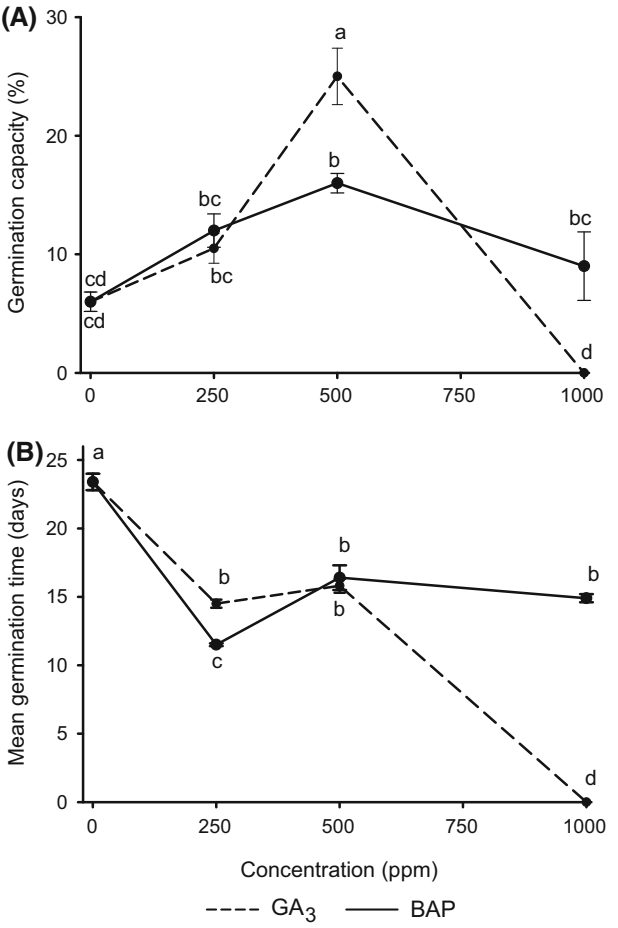

Fig. 2 Germination capacity (**a**) and mean germination time (**b**) of *J. polycarpos* seeds in response to exogenous application of GA_3 and BAP (mean ± SE)

Table 1 Germination capacity (GC) and mean germination time (MGT) of *J. polycarpos* seeds in response a combined treatment of hormones and cold stratification for 12 weeks (mean ± SE)

Treatments	GC (%)	MGT (days)
Contro	6 ± 1a	23.4 ± 0.6A
500 ppm BAP + cold stratification	31 ± 2b	15.5 ± 0.3B
500 ppm GA3 + cold stratification	48 ± 2c	14.9 ± 0.2B

Means followed by the same letter across the column for each stratification treatment are not significantly different using Tukey's test ($\alpha = 0.05$)

Discussion

The present study revealed that cold stratification for 16 weeks induced 42 % of the seeds to germinate compared with 8 % germination for untreated seeds. This indicates that there may be two groups of seeds within the seed lot—one with non-deep dormancy and the other with deep dormancy for which cold stratification alone might not be sufficient. Cold-stratification is a well-established pre-sowing treatment for breaking physiological dormancy in many plant species (Teketay 1997; Baskin and Baskin 2001; Soltani et al. 2005; Tigabu et al. 2007). Advances in seed biology research have shown that low temperature treatment of dormant seeds triggers a cascade physiological processes that promote germination (Pawłowski 2010), including decline in the level of ABA in the embryonic axis, decrease in expression of protein phosphatase gene that appears in dormant seeds (Lorenzo et al. 2001); and regulation of the ABA/GA balance (Finch-Savage et al. 2007), which in turn influence embryo growth potential (Alvarado et al. 2000). Furthermore, cold stratification induces energy metabolism, as observed, for example, in European beech in which the fumarase activity (a key respiratory enzyme in the tricarboxylic acid cycle) doubles in cold stratified than dormant seeds (Shen and Odén 2002).

However, the length of the cold stratification period required for dormancy release largely depends on the level of dormancy (Baskin and Baskin 2001) and varies between and within populations (Tigabu et al. 2007). Within seed lot variability in dormancy level and germination can occur as a result of genetic and environmental effects during seed development (Mamo et al. 2006). It should be noted that juniper seeds for the present study were collected from 20 randomly selected mother trees from a natural stand that were separated by 100 m, thus it is reasonable to expect a maternal effect on the level of dormancy and germination. The stratification temperature is another important factor that determines the dormancy release. It is generally in the region of 5 °C but in some cases the termination of dormancy can be achieved by temperatures as high as 12–15 °C or as low as 1 °C (Baskin and Baskin 2001). In our case, cold stratification at 5 °C resulted in considerable amount of precocious germination (data not shown), thus stratification at 1 °C seems to be reasonable, although may not be optimal. For some species, alternating the stratification temperature regime between 1 and 5 °C is more effective in breaking dormancy than constant low temperature regime (Soltani et al. 2005).

Exogenous application of phytohormones, such as GA_3 or BAP, alone or in combination with cold stratification, induces dormancy release and germination in some species, depending on the concentration and length of incubation (Hidayati et al. 2000; Baskin and Baskin 2001; Sivakumar et al. 2006; Ahmadloo et al. 2015). The mechanism of dormancy release and germination stimulation by GA_3 treatments is often attributed to the mobilization of stored reserves (Bewley and Black 1994) and weakening of the mechanical resistance of the endosperm cells around the radicle tip due to increased activities of cell wall degrading enzymes (Downie et al. 1997). Cytokinins (BAP) are also opined to contribute to the promotion of dormancy release and germination by enhancing ethylene biosynthesis, which is implicated in the promotion of germination in some species (Matilla 2000; Kucera et al. 2005).

In the present study, warm-cold stratification was more effective in breaking dormancy and stimulating germination of *J. polycarpos* seeds than other treatments; suggesting that *J. polycarpos* seeds possess complex morpho-physiological dormancy. Seeds exhibiting this type of dormancy often need up to 4 months of stratification at 18–20 °C followed by 4 months of cold-stratification at 0–3 °C for maximum germination (Baskin and Baskin

2001; Baskin et al. 2002; Afroze and ÓReilly 2013). This is evident in the present study where germination was substantially improved (72 %) by warm stratification for 16 weeks followed by 12 weeks of cold stratification compared with cold-stratification alone (42 %) and the control (8 %). Prolonged warm stratification promotes sufficient embryo growth while the subsequent cold stratification breaks physiological dormancy. For instance, Santiago et al. (2015) reported an increase in embryo length from 1.3 mm in fresh *Viburnum lantana* seeds to 3.0 mm after 20 weeks of warm stratification while the embryo hardly grew after 24 weeks of cold stratification. Similarly, Chen et al. (2014) reported that *Sambucus chinensis* seeds require warm stratification for dormancy break and germination.

The germination response to warm-cold stratification observed in the present study accords with the fruiting phenology and environmental conditions during seed maturation and dispersal in the natural habitat of *J. polycarpos*. Normally, *J. polycarpos* berries ripen and disperse in September (Ahani et al. 2013) when the autumn temperature is adequately warm (>15 °C) to stimulate embryo growth while the subsequent low winter temperature from December to March (<0 °C) breaks the physiological dormancy; thus the seeds germinate in spring and/or early summer. Given that *J. polycarpos* seeds appear to have morpho-physiological dormancy; presumably intermediate simple morpho-physiological dormancy (sensu Baskin and Baskin 2004), this is likely an adaptive mechanism for relatively warmer autumn temperature during seed maturation and the subsequent cold winter temperature under natural conditions. Overall, the germination response to warm-cold stratification treatment reported here is a major improvement compared to the current nursery practice where seeds are sown in nursery beds for 2 years to achieve ca. 10 % germination.

Conclusions and recommendations

In an attempt to break dormancy and stimulate germination of *J. polycarpos* seeds, stratification, hormones and their combined treatments were tested. Based on the results, the following conclusions can be drawn: (1) cold stratification at 1 °C up to 16 weeks is not effective to completely break dormancy; (2) exogenous applications of GA_3 or BAP plays a minor role in dormancy release compared to a combined treatment of hormones and cold-stratification, but the combined treatment is still not effective in breaking dormancy; (3) warm stratification for 16 weeks at 20 °C followed by 12 weeks of cold stratification at 1 °C induces 72 % of the seeds to germinate in 12 days for a seed lot with 82 % initial viability; and (4) the findings suggest that morpho-physiological dormancy is the most likely dormancy type in *J. polycarpos* seeds, opined to be intermediate simple morpho-physiological dormancy. This class of dormancy could be an adaptive mechanism for relatively warmer autumn temperature during seed maturation and the subsequent cold winter temperature under natural conditions.

Further studies are recommended to determine the critical embryo length, examine whether GA substitutes for cold stratification, and if alternating the temperature regime shortens the effective duration of warm-cold stratification, as these treatments have been opined to alleviate intermediate morpho-physiological dormancy (Baskin and Baskin 2001). Further study should also be conducted to determine optimum warm-cold stratification treatments for seeds from different elevation of each region and from different provenances among the natural distribution of species around the country (mid-north, northwest, central, and southern highlands).

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