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Full Length Research Paper

Effects of different treatments on the germination of wild pear (*Pyrus glabra*) seeds and their peroxidase, amylase, and catalase reactions

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Wild pear (*Pyrus glabra*) is an endemic and medicinally important endangered plant species of Zagros Mountain. Interventions on behalf of conservation are necessary to protect this species. The seeds show very low germination (%), and these low rates represent a major obstacle to successful regeneration. In this study, the viability, germination (%), germination rate, pattern of germination and enzymes activities during seed germination under cold stratification were assessed. The experimental treatments used were cold stratification (CS), CS with cool water for 24 h, CS with gibberellic acid at 1000 ppm and at 1500 ppm for 48 h. The mean seed viability was 42.25 ± 1.9%. The highest germination rate was obtained under cold stratification for a period of 50 days. The activity of catalase was low during germination. Peroxidase activity increased with increasing periods of CS. Amylase activity was always high compared to the other enzymes, which showed a limited range of variation. The best time for germination could be inferred by determining the time at which the enzymatic activities are equal. The study found that the seed dormancy of the wild pear is of the morphological type. The embryo needs a 50-day cold period at a temperature of 5°C to begin germination. The study demonstrated that the low regeneration rate of this species is caused by the low percentage of viable seeds.

Key words: Seed germination, viability, treatments, catalase, peroxidase, amylase.

INTRODUCTION

Seed dormancy is controlled by the physiological or structural properties of a seed and by the prevailing external conditions. It is induced as part of the genetic program of seed development and maturation (Pawłowski, 2009). Dormancy is common in seeds of species that grow in environments in which conditions are unfavorable for successful plant establishment immediately following seed dispersal. Five classes of seed dormancy are now recognized (Nikolaeva, 1977; Baskin and Baskin, 1998, 2004; Baskin et al., 2006). Seeds with physiological dormancy (PD) have a water-permeable seed coat, a fully developed embryo and a physiological inhibiting mechanism that prevents radicle emergence. Morphological dormancy (MD) involves an under-developed embryo that requires time to grow (that is, a dormancy period) before the seed can germinate, and morphophysiological dormancy (MPD) involves an underdeveloped embryo that is physiologically dormant. Physical dormancy (PY) is caused by a water-impermeable seed or fruit coat and combinational dormancy (PY + PD) is caused by a water-impermeable seed or fruit coat and a physiologically dormant embryo. The embryo is fully developed in seeds

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Abbreviations: CAT, Catalase; **H_2O_2,** hydrogen peroxide; **POD,** peroxidase; **PD,** physiological dormancy; **MD,** morphological dormancy; **PY,** physical dormancy; **MPD,** morpho-physiological dormancy; **CS,** cold stratification.

with PY and in those with PY + PD. Seeds with deep physiological embryo dormancy can be stimulated to germinate by a variety of treatments, including cold stratification. Hormonal imbalance with growth promoters (for example, gibberellins) is the main cause of the breaking of seed dormancy (Pawłowski, 2009).

The development of an organism from embryo to adult is accompanied by the differential expression of enzymes that constitute the biochemical pathways present in different cell types, tissues and organs (McMillin, 1983). Moreover, different phases of vegetative and reproductive development are characterized by changes in enzyme activities. Thus, seed germi-nation, seedling growth, flowering, seed maturation and seed ageing are defined by the changes in the status of their enzyme activities (Mitrović, 2007). Catalase has a high reaction rate but a low affinity for H_2O_2 and thereby removes the bulk of H_2O_2. Conversely, peroxidase (POD) has a higher affinity for H_2O_2 and allows the scavenging of small amounts of H_2O_2 in more specific locations (Dat et al., 2000). Changes in CAT activity are linked to desiccation during seed maturation (Bailly et al., 2004), seed germination (Bailly et al., 2002; Prodanović et al., 2007; Bogdanović et al., 2008) and plant growth and development (Mitrović and Bogdanović, 2008). PODs are the most frequently investigated enzymes because they have a role in very important physiological processes such as seed germination and seedling growth (Dučić et al., 2003, 2004; Prodanović et al., 2007; Bogdanović et al., 2008; Mitrović et al., 2010). Amylase is an enzyme that breaks starch down into sugar.

Wild pear (*P. glabra*) is an endemic and important tree species in the western oak forests of Iran (Khatamsaz, 1992). *P. glabra* fruits, leaves and flowers have different medicinal properties. The plant's medicinal compounds include 6% hydroquinone glycoside in the leaves and phloridzine in the stem, bark and roots. The seeds contain amygdaline and 12 to 21% of other oil compounds (Zargari, 1996; Hunter, 2007). The germination rate of the seeds of this species and its rate of natural regeneration are both very low. A key issue tied to dormancy and germination is the lack of proper regeneration of *P. glabra* seeds in natural habitats. Indeed, there is limited information concerning the seed dormancy of this species in Iran. The aims of this study were;

(1) to estimate the percentage of seed viability in *P. glabra*;
(2) to test the effects of various treatments (that is, cold stratification, cold stratification with cool water for 24 h and cold stratification with GA3 (1000 or 1500 ppm, 48 h) on dormancy breaking in seeds of *P. glabra*;
(3) to examine the changes in enzymatic activity during the best treatments identified; and
(4) to study the relationship of enzymatic activity to germination rate in seeds of *P. glabra.*

MATERIALS AND METHODS

Seed source

Mature seeds of *P. glabra* were collected in 2009 from Zagros Mountain, located in the Delfan region in the west of Iran (32° 08′ N, 50° 08′ W, 2320 m above sea level with a slope of > 40%). The mean annual air temperature was 22°C (winter: -1.7°C; summer: 24°C), and the mean annual precipitation was 315 mm. Seeds were collected randomly from different trees in a pure stand. After collection, immature seeds and seeds damaged by insects were removed. The seeds were surface sterilized by soaking in 1% sodium hypochlorite (NaOCl) solution for 5 min and subsequently rinsed thoroughly with sterilized water prior to applying any treatment. All germination experiments were conducted using four replications of 100 seeds per treatment. Seeds were placed on double-layered Whatman No.1 filter paper moistened with 5 ml of distilled water in sterilized Petri dishes.

Seed viability determination

Prior to the start of the experiments, seeds were tested for viability using standard tetrazolium testing procedures (n = 100 seeds) (Grabe, 1970). The test involved incubation of transversely cut seed halves for 24 h and subsequent examination to determine the staining intensity/pattern. Seeds with completely stained embryos were considered viable.

Seed germination assays

Cold stratification

After being moisturized with distilled water, seeds were maintained at a temperature of 5°C for 120 days.

Soaking in cool water and cold stratification

Seeds were soaked in cool water for a period of 24 h and subsequently kept at 5°C for 120 days.

Soaking in GA3-1000 and cold stratification

Seeds were soaked in 1000 ppm GA3 for a period of 48 h and subsequently kept at 5°C for 120 days.

Soaking in GA3-1500 and cold stratification

Seeds were soaked in 1500 ppm GA3 for a period of 48 h and subsequently kept at 5°C for 120 days. After each treatment, the seeds were transferred to germinators. The following conditions were maintained in the germinators: continuous darkness, a constant temperature of 20°C and a relative humidity between 70 and 75%. Germinated seeds were counted and removed once a week until the end of the experiment (Wiese and Binning, 1987; Auld et al., 1988).

Enzyme extraction and assays

All enzyme assays were performed with seeds during the cold stratification treatment. For the enzyme extraction and assay, 1 g of seeds was rubbed and plugged in extraction buffer (pH = 7.5) for 24 to 72 h (Ebermann and Stich, 1982). The homogenate was centrifuged at 3000 rpm for 15 min, and the supernatant was

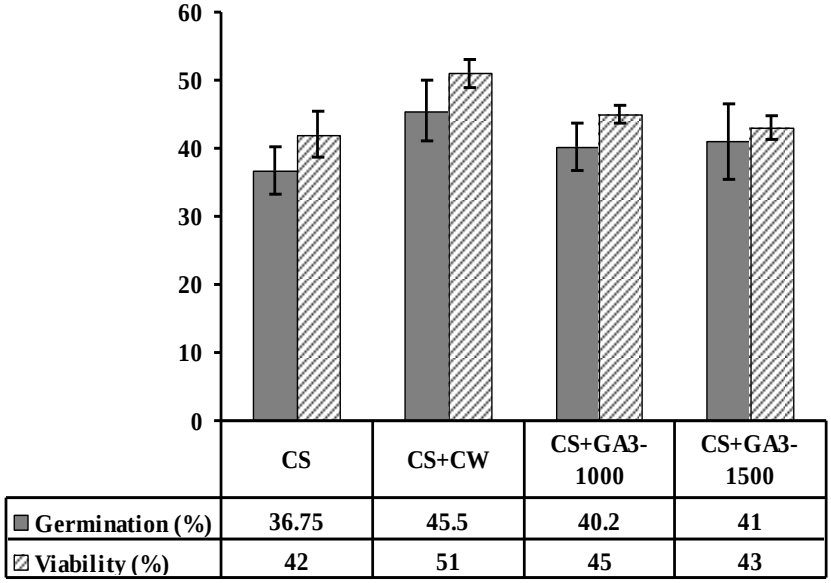

Figure 1. Effect of cold stratification, cold stratification with cool water treatment and cold stratification with GA3 (1000 and 1500 ppm, 48 h) on percent germination of seeds of *Pyrus glabra* collected in 2009. Duration of experiment, 120 days.

collected and used for enzyme assays. The assay for POD activity was based on the method of Chance and Maehly (1955) with phosphate buffer (pH = 6.0). The determination made using this method involves the oxidation of guaiacol as an electron donor to tetraguaiacol in the presence of H_2O_2 at 420 nm. CAT activity was determined according to the method described by Eising and Gerhardt (1989). This method uses a modified phosphate buffer (pH = 7.0) that measures the decrease in absorbance at 240 nm for 1 min, following the decomposition of H_2O_2. Total amylase activity was measured according to Goggin and Colmer (2007).

Analysis

The data were graphically explored by plotting the patterns of germination curves for the treatments (SigmaPlot, Systat Software Inc., 2004). Germination percentages were corrected for seed viability by dividing germination percentage by the possible number of germinated seeds (that is, the number of viable seeds based on the initial tetrazolium tests). For calculation of the germination rate, determinations were made of the maximum germination percentage, the lag time to germination (in days), the time for 50% germination (t50) and the maximum germination rate (percent of seeds germinating per week). The lag time to germination was defined as the number of days between the start of the experiment and the time at which germination first occurred. The time to 50% germination (t50) and the maximum germination rate for treatments were calculated. We also used ANOVA and Tukey's HSD to evaluate the effect of the treatments on the maximum percentage of germination (Analytical Software, 2003). P-values of < 0.05 were considered significant.

RESULTS

Seed viability

Seed viability was slightly variable. The values of the percent viability (mean ± S.E.) of *P. glabra* seeds under cold stratification with GA3 (1000 and 1500 ppm, 48 h) were 45 ± 1.3% and 43 ± 1.7, respectively (Figure 1). The values of seed viability for cold stratification and for cold stratification with cool water for a period of 24 h were 42 ± 3.4 and 51 ± 2.1%, respectively (Figure 1). The average seed viability across all treatments was 42.25 ± 1·9%. Percent viability did not differ significantly among treatments ($p < 0.05$).

Germination percentage

Percent germination did not differ significantly among treatments ($p < 0.05$) (Figure 1). Cold stratification with GA3 (1000 and 1500 ppm, 48 h) resulted in 40.2 and 41% germination, respectively. Cold stratification and cold stratification with cool water for a time period of 24 h resulted in 45.5 and 36.75% germination, respectively (Figure 1).

Germination pattern

Germination of seeds treated with cold stratification, cold stratification with cool water (24 h) and cold stratification with 1000 ppm GA3 began on the 50th day of the experiment. However, germination began on the 64th day for seeds treated with cold stratification and with 1500 ppm GA3 (Figure 2). The fastest germination occurred with cold stratification and with cold stratification with cool water (24 h). Maximum germination in all treatments was attained by day 85 (Figure 2).

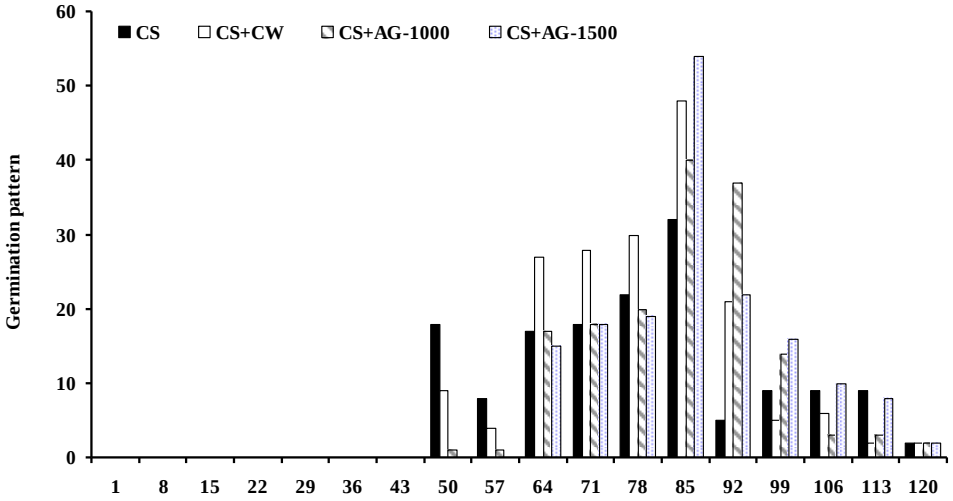

Figure 2. Germination pattern during cold stratification, cold stratification with cool water treatment and cold stratification with GA3 (1000 and 1500 ppm, 48 h).

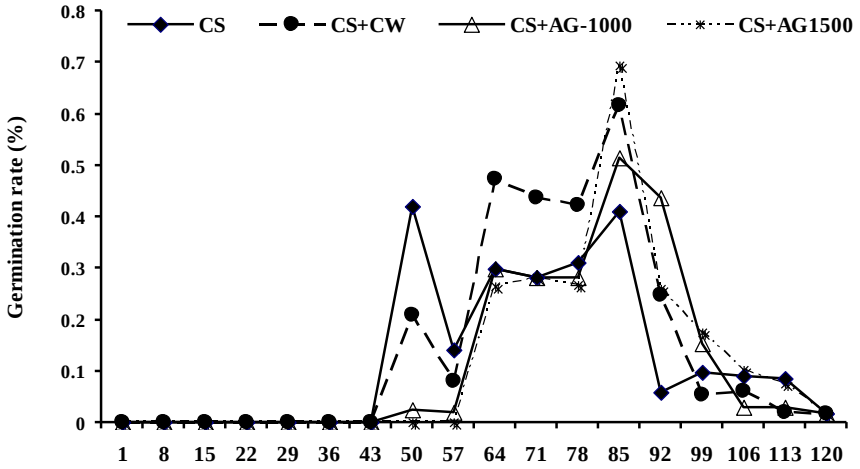

Figure 3. Percentage germination rate (± S.E.) for cold stratification, cold stratification with cool water treatment and cold stratification with GA3 (1000 and 1500 ppm, 48 h).

Germination rate

The treatments differed significantly at the 50th day of germination ($p < 0.05$). The germination rate resulting from the cold stratification treatment was significantly higher than the rates obtained in the other treatments. Germination to 50% occurred after 50 days in the cold stratification treatment, whereas germination to 50% occurred after 70 days in the other treatments. The highest germination rate occurred for cold stratification (GRI = 0.41 ± 0·08) (Figure 3). The time until the first germination was lowest for *P. glabra* seeds treated with cold stratification (50th day), but the values for the other treatments did not differ significantly.

Changes in enzyme activities during cold stratification treatment

The effect of cold stratification on the activities of the antioxidant enzymes peroxidase and catalase in relation to the germination rate of *P. glabra* is shown in Figure 4. The highest amount of antioxidant activity occurred under cold stratification by day 85, at precisely the time associated with the highest germination rate and highest germination percentage. The activity of catalase, an important antioxidative enzyme present in the seeds, was low under cold stratification. Peroxidase activity increased with increases in the length of the period of cold stratification during the germination process. The POD

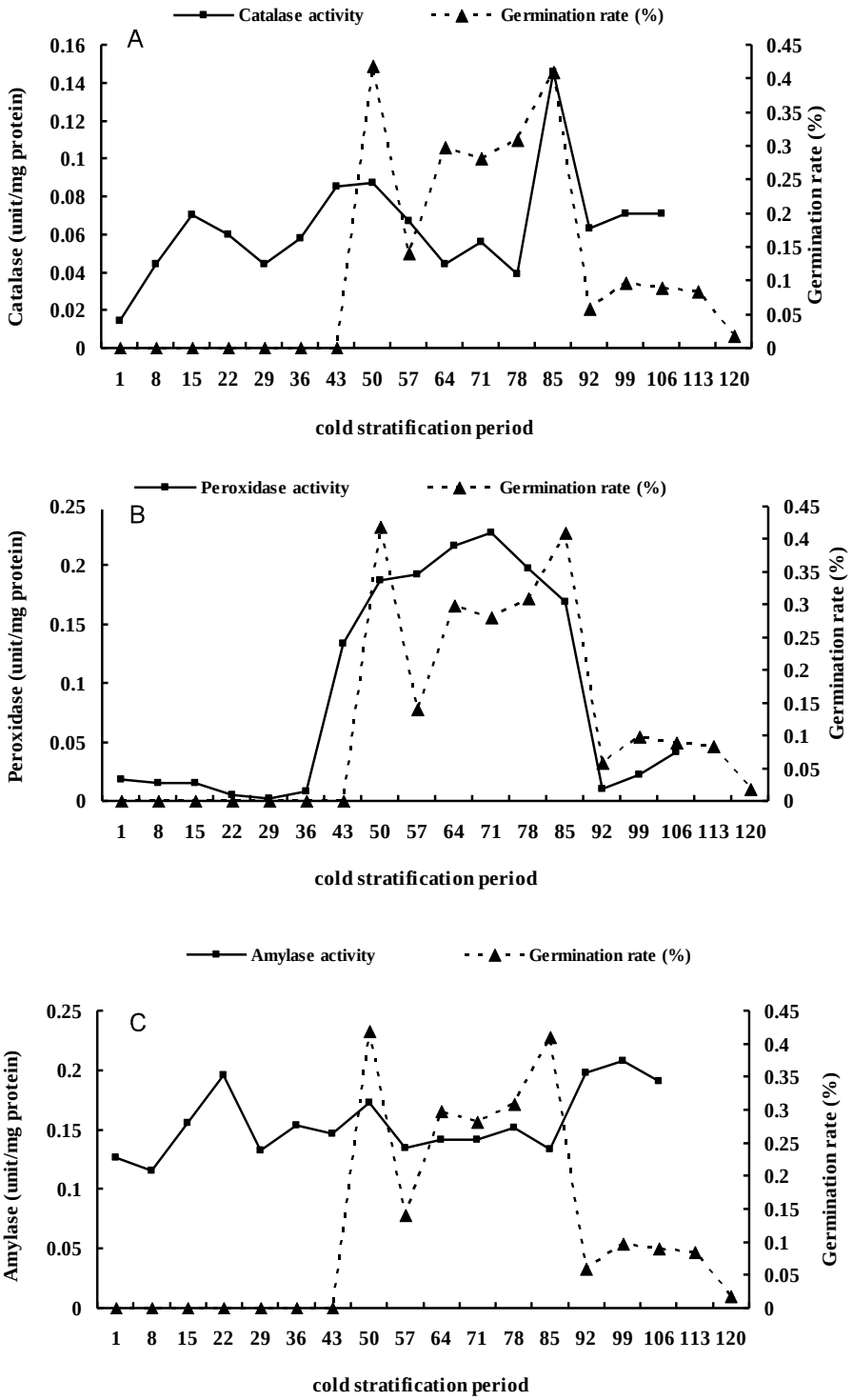

Figure 4. Changes in catalase (A), peroxidase (B) and amylase (C) enzyme activity and the germination rate (%) of *P. glabra* seed during the cold stratification treatment.

activity increased from day 36 through day 92. Germination started at the beginning of this period, and the germination rate attained its highest value at the end of this period. The lowest amounts of POD activity were found to occur during two periods. The first of these periods occurred from day 1 to day 36, and the second began after day 92. The activity of amylase during the period of high germination rates was high, compared with

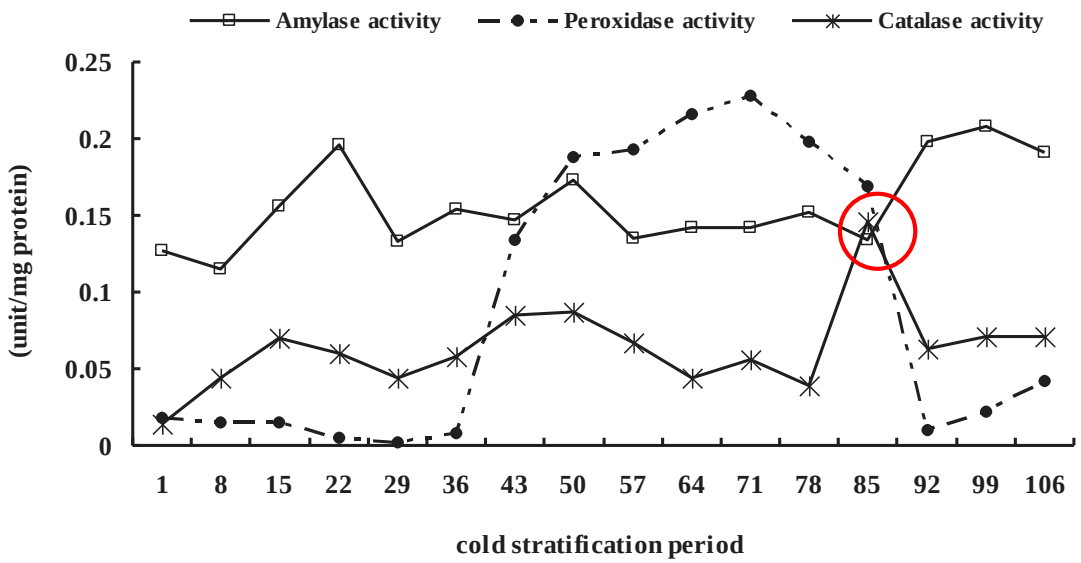

Figure 5. Changes in the activity of peroxidase, catalase and amylase in *P. glabra* seeds during the cold stratification treatment.

the activity of the other enzymes, but it fell within the same range of values during the entire period of seed germination under the cold stratification treatment. Moderate changes in the activity of this enzyme occurred during the study period.

Comparison of enzyme activities in seeds treated with cold stratification

The results of the study indicated that sudden changes of peroxidase activity occurred during germination under cold stratification, compared with other enzyme activities, and that these changes persisted until the 85th day. At approximately the 85th day, the activities of all three enzymes were approximately equal (Figure 5). More than 85% of the seeds had germinated by that time.

DISCUSSION

Wild pear (*P. glabra*) trees, endemic to the forests of western Iran, belong to a threatened and endangered species. They are characterized by a low rate of regeneration and are not well established. To address this problem, we characterized the seeds of *P. glabra* physiologically by calculating percent viability and by studying seed dormancy and enzymatic changes. Neither percent viability nor percent germination differed among treatments ($p < 0.05$). Both values were low, 42.25 ± 1.9 and 40.86 ± 1.8%, respectively (Gendreau and Corbineau, 2009). In all the treatments, the value of percent germination reached a peak on the 85th day, decreased until the 120th day, and then remained at near-zero levels.

The study found that the seed germination rate under cold stratification was significantly higher than the corresponding rates for the other treatments at the 50th day. It has previously been shown that treatment with different concentrations of GA3 increases the percent germination of *Pyrus* spp. (Khan, 1982; Shawky et al., 1988). The results of the current study suggest that to obtain the best germination rate in the shortest possible time, cold stratification is the best treatment (Suszka, 1989; Lin et al., 1994; Maeda et al., 1997; Al-Bukhari et al., 2002; Pio et al., 2007). Moreover, the cold stratification treatment corresponds to the conditions present in the plant's natural habitat. It may therefore be concluded that the cold stratification treatment is preferable for this species. This study demonstrated that seed dormancy of pear trees is of the MD type, in which the embryo must be exposed to a 50-day cold period at a temperature of 5°C to begin germination. However, Bao and Zhang (2010) have demonstrated that the seed dormancy of pear trees is of the MD + PD type. All of the seeds required an 85-day period to reach their highest rate of germination. This finding is consistent with the conditions present in nature. In fact, seeds on the forest floor during the cold period of autumn and winter are exposed to the conditions that they require to germinate during the spring of the following year. Nevertheless, the low percent germination rate of seeds could result from the high frequency of empty seeds and pests and diseases attack immature embryos. The results of this experiment suggest that poor, low regeneration is the result of low seed viability and that this low level of

viability is the chief threat to the species in its habitat. Finally, it could be demonstrated that getting model from natural regeneration has more succeeding results for rehabilitation of this species in the disturbed forests.

The second part of the study considered physiological aspects of the seeds under the best treatment (cold stratification) identified in the first part of the study. The physiology of the seeds was studied by investigating the activities of the enzymes amylase, catalase and peroxidase. Activity of peroxidase has been found to increase with increasing periods of cold stratification at 5°C during germination (Tao and Khan, 1979; Zhang et al., 2010). Peroxidase activity chiefly reflects the influence of the germination process. Accordingly, changes in peroxidase and changes in germination rates followed the same trends. For example, the peroxidase activity increased just 7 days before the germination rate improved. Likewise, the germination rate decreased some days after the peroxidase activity decreased. The activity of catalase, an important antioxidative enzyme found in the seeds, was consistently low under cold stratification. This result suggests that oxidative stress is involved in seed viability and in the response to low temperatures (Sharma and Sharma, 2010; Zhang et al., 2010). However, the highest catalase activity occurred on the same day that percent germination peaked (the 85th day) (Mei and Song, 2010). Amylase activity increased with the progress of germination (Al-Helal, 1996; Winstead and Belk, 1968; Richa and Vikas, 2010; Mallick et al., 2010), but the overall amount of variation was limited. By plotting the activities of all three enzymes on the same graph, we found the surprising result that the graphs of the activity of all three enzymes intersected on the 85th day, when over 85% of the seeds had germinated. This result implies that the best time for germination can be inferred by studying enzymatic activities, which vary across different tree species. These findings may provide a new approach to identifying the suitable time for maximum seed germination based on enzyme activity, and the system used in this study may represent an excellent model system to solve the problem of maximum germination percentage.

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