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The effect of germination temperature on antioxidant enzymes activities and seed germination of *Prangos ferulacea* Seeds harvested at different seed moisture content

Ali Moradi[1*], Tahereh Bazi[2], Asad Masoumiasl[1] and Tayebeh Zaree[3]
1, 2, 3. Assistant Professor, Former M. Sc. Student and Ph.D. Candidate of Crop Physiology, Department of Agronomy and Plant Breeding, Yasouj University, Yasouj, Iran, P.O. Box, 7591874934
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ABSTRACT

Prangos ferulacea is a plant from the *umbeliferae* family that is propagated by seed germination. In order to investigate the effects of seed ripening and germination temperature on germination and some antioxidant enzymes activity of this plant seed, a two-factor factorial experiment was carried out in a completely randomized design with three replications in the laboratory of Seed Sciences and Technology (Yasouj University, Yasouj, Iran) in 2012-2013. Experimental factors were including harvest seed moisture content (SMC) (67, 51, and 17% based on fresh weight) and germination temperature at two levels (15 and 20°C). Seeds pre-chilled at 4°C before germination test. The α-amylase, dehydrogenase, catalase, and ascorbate peroxidase enzymes activities were measured in dormant (non-chilled) and nondormant (pre-chilled) seeds. The germination results showed that with the progress of ripening, seed physiological quality and germination also increased such that compared to harvest at SMC of 67%, the germination percentage of seeds collected at SMC of 17% rose from 19.68% to 43.2%. Also, it was observed that the pre-chilled seeds incubated at 15°C germinated more (36.6 %) than compared to those at 20°C (24.4%). Moreover, it was observed that α-amylase, dehydrogenase, and ascorbate peroxidase activities increased with the ripening progress.

Keywords: α-amylase, ascorbate peroxidase, chilling, germination percentage.

تأثیر دمای جوانه‌زنی بر فعالیت آنزیم‌های آنتی اکسیدانی و جوانه‌زنی بذر جاشیر (*Prangos ferulacea*) برداشت شده در محتوای رطوبت بذری متفاوت

علی مرادی[1*]، طاهره بازی[2]، اسد معصومی اصل[1] و طیبه زارعی[3]
۱، ۲ و ۳. استادیار، دانشجوی سابق کارشناسی ارشد و دانشجوی دکتری، گروه زرعت و اصلاحات نباتات، دانشکده کشاورزی، دانشگاه یاسوج
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چکیده

جاشیر (*Prangos ferulacea*) گیاهی است از خانواده چتریان که تکثیر آن در طبیعت از طریق رویش بذر صورت می‌گیرد. به منظور بررسی اثر رسیدگی بذر و دمای جوانه‌زنی بر جوانه‌زنی و فعالیت برخی آنزیم‌های آنتی‌اکسیدانی بذرهای این گیاه آزمایشی به صورت فاکتوریل دو عاملی در قالب طرح کاملاً تصادفی با سه تکرار در آزمایشگاه علوم و تکنولوژی بذر دانشکده کشاورزی دانشگاه یاسوج در سال ۹۲-۹۱ انجام گرفت. فاکتورهای آزمایشی شامل محتوای رطوبت بذر برداشتی (٦٧/٥١ و ١٧ درصد بر مبنای وزن تر) و دمای جوانه‌زنی در دو سطح (١٥ و ٢٠ درجه سانتی‌گراد) بودند. بذرها قبل از آزمون جوانه‌زنی در دمای ٤ درجه سانتی‌گراد سرمادهی شدند. فعالیت آنزیم‌های آلفا آمیلاز، دهیدروژناز، کاتالاز و آسکوربات پراکسیداز در بذرهای خفته (بدون سرمادهی) و غیر خفته (سرمادهی شده) اندازه‌گیری شد. نتایج جوانه‌زنی نشان داد که با پیشرفت رسیدگی، کیفیت فیزیولوژیکی بذر و جوانه‌زنی افزایش یافت، به‌طوریکه درصد جوانه‌زنی بذرهای جمع‌آوری‌شده در محتوای رطوبت بذری ١٧ درصد در مقایسه با بذرهای جمع‌آوری‌شده در محتوای رطوبت بذری ٦٧ درصد، از ١٩/٦٨ درصد به ٤٣/٢ درصد افزایش یافت. همچنین، مشاهده شد که بذرهای سرمادهی شده در دمای ١٥ درجه سانتی‌گراد (٣٦/٦ درصد) بیشتر از دمای ٢٠ درجه سانتی‌گراد (٢٤/٤) جوانه زدند. فعالیت آنزیم‌های آلفاآمیلاز، دهیدروژناز و آسکوربات‌پراکسیداز با پیشرفت رسیدگی افزایش یافت.

واژه‌های کلیدی: سرمادهی، آلفا آمیلاز، آسکوربات پراکسیداز، درصد جوانه‌زنی.

* Corresponding author E-mail: amoradi@yu.ac.ir

Introduction

Prangos ferulacea is a forage and medicinal pasture plant from Apiaceae family that grows mainly in the western and southwestern parts of Iran, where it provides a significant share of livestock winter forage (Freidooni *et al*., 2012). This plant often grows in moderate to cold areas that experience significant cold periods. Seeds of many plant species that grow in the cooler and often in moderate climates have some levels of physiological dormancy and need a chilling period before start germination (Baskin & Baskin, 2004).

Seeds must be harvested at full maturity, which is the time at the end of the development period and after physiological maturity. At this period, grain filling and the transfer of materials from the maternal plant to seeds are completed, the maximum dry weight is achieved (Elias *et al*., 2006), and seed moisture content gradually decreased to less than 30%. Meanwhile, studies conducted on millet, barley, and wheat showed that the maximum vigor and viability of seeds is achieved just immediately after physiological maturity (Gharine *et al*., 2004). Also, studies on the effect of different harvesting times on the germination of soybean seeds showed that the delay in the harvest time results in a 1000-seed weight increase and, therefore, a better seed quality (Akbari *et al*., 2004).

Seed dormancy is defined as a period in which, despite favorable conditions for germination, the healthy and intact seeds do not germinate. During the dormancy period, germination will not happen even if suitable environmental conditions such as humidity and temperature for germination are provided (Baskin & Baskin, 2004). Seed dormancy, a survival mechanism that supports the reproduction and distribution of the plants, is useful in adverse growing conditions. However, it is limiting when there is a need for germinating and growing after harvest (Savage *et al*., 2006). The synthesis and activation of hydrolyzing enzymes of storage tissue such as alpha-amylase are one of the first processes that occur during germination. This enzyme is the first enzymes active during germination and involved in the breakdown of starch (Doroth & Naofumi, 2002).

Reactive oxygen species (ROS) play a key role in the process of germination. The amount of reactive oxygen species, particularly H_2O_2 increases, in the embryo of the dormant seeds. When dormancy is broken, the amount of these compounds is reduced (Tejera *et al*., 2007). Dehydrogenase, one of the pentose phosphate cycle enzymes, plays an important role in supplying cell energy during seed germination. Dehydrogenase enzyme transfers hydrogen and electron to oxygen, which is the last electron acceptor through electron transport chain. Ascorbate peroxidase enzyme eliminates superoxide radicals, in ascorbate glutathione cycle, using ascorbate as an electron donor. Catalase directly causes the breakdown of hydrogen peroxide (H_2O_2) (Marini *et al*., 2013). In order to solve the germination problem of seeds in different areas, it is necessary to consider the condition that resembles the conditions of their natural habitats. Seeds of many plant species that grow in moderate and cold climates need a cool period for dormancy breaking. Research on the *Prangos ferulacea* seeds showed that they have deep dormancy and germination does not occur without using seed dormancy breaking treatment. Chilling at low temperatures (5℃) has resulted in the germination of this species; moreover, the optimum temperature for germination of this species has been reported 15℃ (Razavi, 2012).

The aim of the present study is to

investigate the changes in dormancy and germination of the seeds at harvested at different stages of seed development (based on seed moisture content) on the mother plant. Also, an attempt was made to investigate the relationship between the enzyme activity with seed dormancy and maturity of *Prangos ferulacea* in chilling and non-chilling conditions.

Materials and methods

To study the changes in some enzyme activities in dormant and nondormant seeds of *Prangos ferulacea* at different maturity stages, an experiment was done in the laboratory of Seed Sciences and Technology, Faculty of Agriculture, Yasouj University, Yasouj, Iran. Seed samples were collected from their natural habitats (Vezg county, Kohgiluyeh and Boyer-Ahmad province, Iran with the coordinates of latitude 30°34″ North and 51° 39″ east, elevation of 2224 m.a.s.l) on three seed moisture contents. The seeds with these moisture contents (67, 51 and 17 %) were collected 15 days after pollination and 30 days after pollination and physiological maturity, respectively. The collected seeds were dried at room temperature. Before starting the test, the seeds were sterilized using 1% sodium hypochlorite solution for 5 min. Then, they were washed with distilled water to completely eliminate their disinfectant agents. The two-factor factorial experiment was done in a completely randomized design with three replications. The first factor was harvest stage based on seed moisture content in three levels (above mentioned) and the second factor was germination temperature in two levels (15 and 20℃). Each experimental unit consisted of 25 completely healthy seeds laid down on a layer of the paper filter with 0.5 cm distance from one another in a petri dish. To supply moisture, 5 ml of distilled water was added to each petri dish. Before germination, the seeds were moist-chilled at 4℃ for 8 weeks. Then, germination was conducted in an incubator at 15 and 20℃. Petri dishes were observed on a daily basis and germination changes were recorded at optimal moisture level. The seeds were considered germinated when the radicle was protruding at least 2 mm from the seed coat.

Germination percentage (%) was calculated using the following formula:

$$GP(\%) = \frac{n}{N} \times 100$$

Where GP is the germination percentage, n is the number of germinated seeds at the end of the experiment, and N is the total number of planted seeds (Ghaderi-Far & Soltani, 2011).

The activities of enzymes such as alpha amylase, catalase, dehydrogenase, and ascorbate peroxidase were measured from freshly harvested seeds (stored at -40℃) and the moist-chilled (8 weeks storage at 4℃) seeds. To assess the activity of catalase, 0.1 g seed sample was extracted using 3 mL of sodium phosphate buffer 25 mM with pH=6.8. The resultant homogeneous mixtures were centrifuged at 15,000 rpm at 4℃ and the supernatant was used to measure the activity of catalase (Cakmak & Horst, 1991). The reaction absorption of the mixture, which contains 2.5 ml sodium phosphate buffer at pH 6.8, 25 mm, 0.5 ml of H_2O_2, and 100 ml enzyme extract, was measured by a spectrophotometer at a wavelength of 240 nm. To assess ascorbate peroxidase activity, 0.1 g of the seed sample was extracted using 3 ml potassium phosphate buffer 0.1 M with pH=7.2. The resulting extract was vortexed for 1 minute until a homogeneous liquid was obtained. The resultant homogeneous then was centrifuged for 15 minutes at 11,000 rpm. The supernatant was used to measure enzyme activity. In this method, the

enzyme activity is based on absorption reduction in 290 nm. To calculate the concentration of ascorbic acid, the extinction coefficient of 2.8 $m\mu^{-1}cm^{-1}$ was used (Nakano & Asada, 1981).

To assess dehydrogenase activity 0.1 gram of seed sample was homogenized using 3 ml of sodium phosphate buffer 0.1 M contains 2, 3, 5 tetrazolium chloride with pH=7.2. The homogenate was incubated at 25℃ for 24 hours. Samples were taken out from incubator after 24 hours and centrifuged for 6 minutes at 12,000 rpm at 4℃. The resultant pellets were extracted with 7 ml of acetone. The supernatant was used to measure enzyme activity. The light absorption was measured by spectrophotometer at 510 nm. To assess alpha-amylase activity, 0.3 grams of seed sample was weighed and grounded using liquid nitrogen in a Chinese mortar and then homogenized using 10 ml phosphate buffer 1 M with pH=7.2 and then the homogenized sample was centrifuged at 10,000 rpm for 25 minutes at 4℃. The supernatant was used to measure the α-amylase activity. The light absorption was measured by a spectrophotometer at 540 nm (Tabatabaei, 2013). Data analysis was performed using SAS statistical software, means compared using the Least Significant Difference (LSD) test at 5% probability level and excel software was used to plot the corresponding graphs.

Results and Discussion

Germination percentage

Analysis of variance (ANOVA) showed that the significant effect of harvest seed moisture content (SMC) and germination temperature ($P \leq 0.01$) on the germination percentage of *Prangos ferulacea* seeds (Table 1).

Comparing the average of germination percentage in the seeds harvested at different SMC showed a significant and increasing germination with maturity progress; with the highest germination percentage for the seeds harvested in a seed moisture content of 17% (43.2 %) (Figure 1-a). The results show that *Prangos ferulacea* seeds can germinate before full ripening. However, due to the inadequacy of accumulated reserves during these stages, harvesting may lead to the production of the low-quality seeds that also have low vigor and low germination percentage (Elias *et al*., 2006).

Among the two temperatures examined, 15℃ showed the highest percentage of germination (Fig 1-b). Studying the effects of treatments such as washing, scarification, chilling, temperature, and gibberellin on seed dormancy breaking and germination of the *Prangos ferulacea*, it was found that chilling at 5 and 12℃ increased germination up to 35 and 40%, respectively (Razavi & Hajiboland, 2009). Moreover, studies conducted on *Prangos ferulacea* in its natural habitats show that the growth period of the seed of this plant in cold regions continues from late March to late April as the temperature during this period is low (Hasani & Shahmoradi, 2007). Chilling can reduce the amount of abscisic acid and increase gibberellic acid hormones by creating a balance between these two hormones break seed dormancy (Tajbakhsh, 1996).

Several studies on different species of this plant also reveal the effective role of stratification treatment on breaking the seed dormancy. Also, studies conducted on *Erythronium* and *Osmorhiza* species from the Apiaceae family showed that these species have some degrees of physiological dormancy that can be broken by applying appropriate chilling periods (Baskin & Baskin, 2004). A study conducted on Prangos ferulacea showed that germination of its seeds at 15℃ is more than the germination at 20℃ (Razavi, 2012).

Table 1. Mean squares analysis of variance of seed moisture content and germination temperature for germination percentage of *Prangos ferulacea*

Source of Variation	df	Germination percentage
Seed Moisture Content	2	829.72**
Germination temperature	1	665.67**
Seed Moisture Content × Germination Temperature	2	6.90 ns
Error		3.82
CV		6.40

**: significant at 1% probability level. ns: Non-significant.

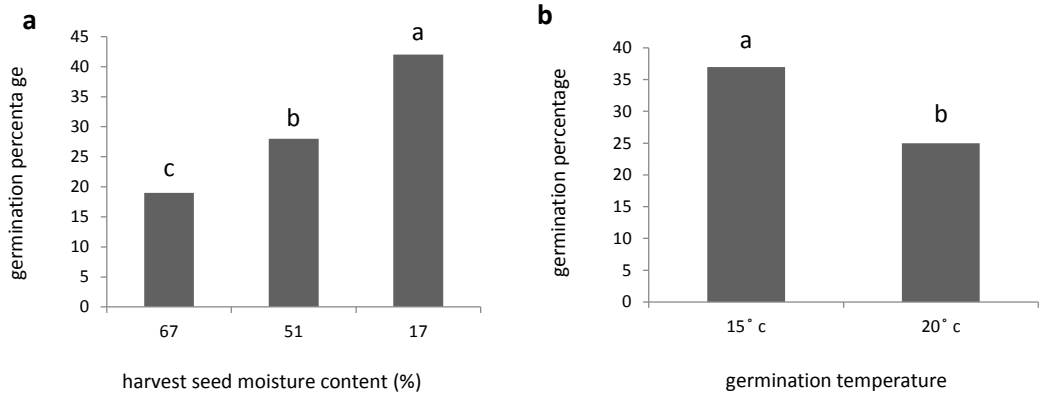

Figure 1. Mean comparison of the effect of seed moisture content (a) and germination temperature (b) on germination percentage of *Prangos ferulacea*. Before transferring to germination media the seeds pre-chilled for 8 weeks at 4 ° C. Each column is the mean of 3 replicates and columns with the same letters are not significantly different at the 5% probability level.

Enzyme activity

Alpha Amylase

The ANOVA results showed that the simple effect of chilling and interaction of chilling and seed moisture content were significant on alpha-amylase enzyme at a probability level of 1% (Table 2). Interaction of seed moisture content and chilling treatment showed that the activity of the alpha-amylase enzymes at each of the three seed moisture contents was higher in chilling treatment compared to non-chilled seeds. The highest and lowest rates of enzyme activity were observed in the seed moisture content 17% in chilling conditions and seed moisture content 67% in non-chilled seeds, respectively (Figure 2).

The increase in the activity of this enzyme in the final stages of maturity can be explained by the fact that this enzyme is mainly synthesized during seed maturation (Kruger, 1972; Briggs, 1973). Regarding the chilling treatment, it seems that chilling causes the biosynthesis of gibberellic acid in the embryo and then is transferred to aleurone layer and induces the expression of alpha-amylase enzyme. The α-amylase, secreted from the aleurone layer to the endosperm, provides the energy needed for the root growth by decomposition of the starch molecules stored in the endosperm. Therefore, the increase in the germination percentage of chilled seeds could be explained by the increased activity of this enzyme in breaking seed dormancy. Such an increase, in turn, is due to the fact that the seeds stored at low temperatures have a higher accumulation of sugars in comparison with seeds stored at high temperatures and, therefore, they have high starch decomposing activities at low temperatures (Nielsen *et al*., 1997). A study on the impact of chilling and hormonal treatments on seed germination of Taxus chinensis showed that alpha- and beta- amylase enzymes activities increased after chilling and reached a maximum after the end of post-ripening period (Zhang & Gao, 2012).

Table 2. Analysis of variance for effect of seed moisture content and temperature on activity of some enzymes in *Prangos ferulacea*

SOV	df	Alpha amylase	Catalase	Ascorbate peroxidase	Dehydrogenase
Seed moisture content	2	0.31^{ns}	4.61^{**}	0.183^{*}	1.97^{**}
Chilling	1	2.68^{**}	1.05^{ns}	5.55^{**}	10.28^{**}
Seed moisture content × chilling	2	0.29^{**}	1.95^{**}	0.073^{ns}	0.415^{ns}
Error	7	0.0202	0.256	0.0592	0.158
CV	8	16.96	27.708	13.10	17.5

* and **: Significant at 5% and 1% probability level, respectively. ns: Non-significant.

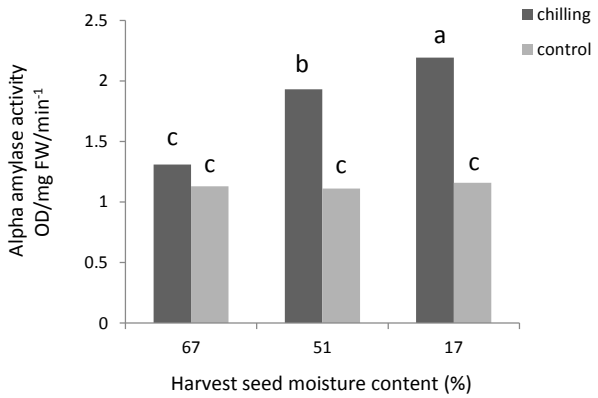

Figure 2. Changes of alpha-amylase enzyme activity (OD mg FW / min^{-1}) in chilled and non-chilled (control) seeds of *Prangos ferulacea* collected by different of seed moisture content. Each column is the mean of 3 replicates and columns with the same letters are not significantly different at the 5% statistical level.

Catalase

The ANOVA results showed that the simple effect of seed moisture content and interaction of seed moisture content and chilling was significant on catalase activity at a probability level of 1% (Table 2). At seed moisture contents of 67 and 51%, catalase activity was not significantly different between the chilled and non-chilled seeds, but in the seed moisture content 17%, this difference was statistically significant. At this stage, the chilled seeds have lower catalase activity compared to non-chilled seeds (Figure 3). Catalase as one of the most important enzymes causes the elimination of reactive oxygen species in the plant cells. The increased activity of this enzyme at the seed moisture content is indicative of an increase in the amount of free oxygen radicals during maturity and reduction water content of the seeds. In fact, during the reduction of the water of seed, active oxygen species such as hydrogen peroxide regulate catalase genes expression and therefore increase catalase activity; and cause hydrogen peroxide to be converted to water and oxygen. Therefore, the activity of catalase is essential since it eliminates the potential toxicity of hydrogen peroxide under various stresses (Willekens *et al*., 1995). A study showed that 29 days after pollination in the freshly collected seeds catalase activity was low, but during seed development, to 50 days after pollination it has increased and remained almost at a stable level. The authors of this research reported a negative correlation between the measured catalase activity in freshly harvested seeds and seed moisture content (Bailly *et al*., 2004).

It seems that the catalase activity is

low due to higher moisture content in immature seeds. In sunflower seeds, the activity of this enzyme increased during maturation that depended on the seed moisture content (Bailly *et al*., 2004). In another study, it was reported that the catalase activity increases in dehydrated bean seeds (Bailly *et al*., 2001).

Ascorbate peroxidase

The ANOVA results showed that the single effects of seed moisture content and chilling factors are significant on ascorbate peroxidase activity at probability levels of 5% and 1%, respectively (Table 2). The activity of ascorbate peroxidase increased by reducing the seed moisture content and the greatest amount of this enzyme activity was observed in the seed moisture content of 17% (physiological maturity); besides, there was not the significant statistical difference between seed moisture content 67 and 51% (Figure 4-a). As a result of chilling treatment, the activity of this enzyme increased compared to the non-chilled (Figure 4-b).

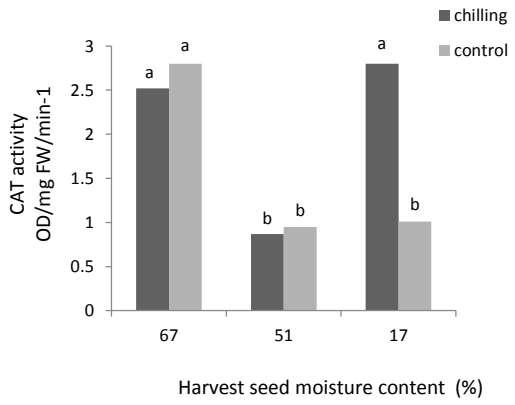

Figure 3. Changes of catalase enzyme activity (OD mg FW / min^{-1}) in chilled and non-chilled (control) seeds of Prangos ferulacea collected by different of seed moisture content. Each column is the mean of 3 replicates and columns with the same letters are not significantly different at the 5% level.

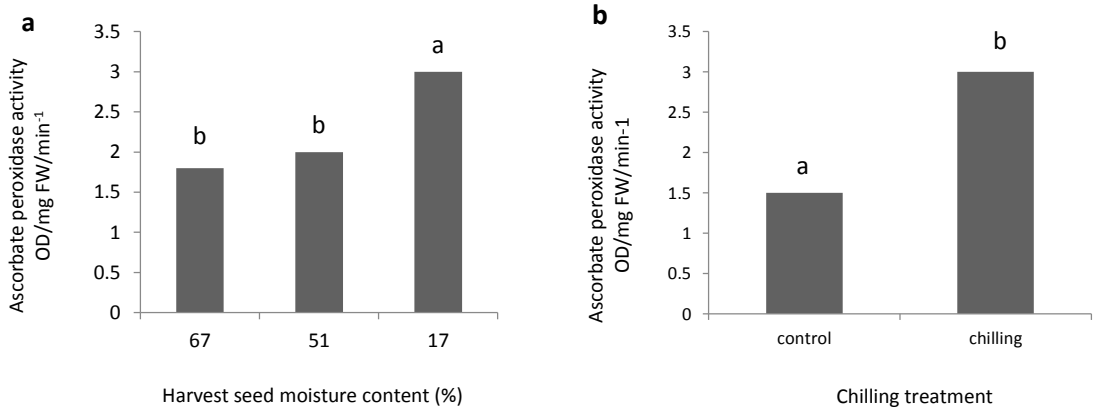

Figure 4. Changes in APX (OD mg FW / min^{-1}) activity of *Prangos ferulacea* seeds collected by different seed moisture content (a) and in chilled and non-chilled (control) seeds (b). Each column is the mean of 3 replicates and columns with the same letters are not significantly different at the 5% level.

In fact, along with seed maturity progress and reduction in seed water content, active oxygen species increased and, as a defense system, the activity of ascorbate peroxidase also increased. Pukacka & Ratajczak (2007) studied the seed enzymes of the two maple species, orthodox and recalcitrant with different desiccation sensitivity. They reported that during seed desiccation, the production of reactive oxygen species increased and, at the same time, the activity of ascorbate-glutathione enzyme cycle began to increase while the production of ascorbate peroxidase in embryonic axis increased. It has been stated that ascorbate system can play an important role in maintaining metabolic homeostasis in orthodox seeds compared to recalcitrant during desiccation and also in protecting against reactive oxygen species. In another study, it has been reported that during maturation of sunflower seeds, the activity of ascorbate peroxidase increased depending on the seed moisture content (Bailly *et al*., 2004).

Dehydrogenase

The ANOVA results showed that the simple effect of seed moisture content and chilling were significant ($P \leq 0.01$) on dehydrogenase activity enzymes (Table 2). By reducing the seed moisture content and seed maturity progress, the activity of dehydrogenase increased; along with a reduction in seed moisture content the highest enzyme activity was observed in the seed moisture content 17% that had no significant difference with seed moisture contents of 67 and 51% (Figure 5-a). Compared with the non-chilled seeds, in chilling treatment, the activity of this enzyme demonstrated a significant increase (Figure 5-b).

It seems that chilling could participate in breaking seed dormancy by increasing the activity dehydrogenase enzyme. In a study on *Corylus avellana*, along with seed dormancy breaking, a significant increase in the activity of the pentose phosphate pathway enzymes was reported during stratification (Gosling & Ross, 1980).

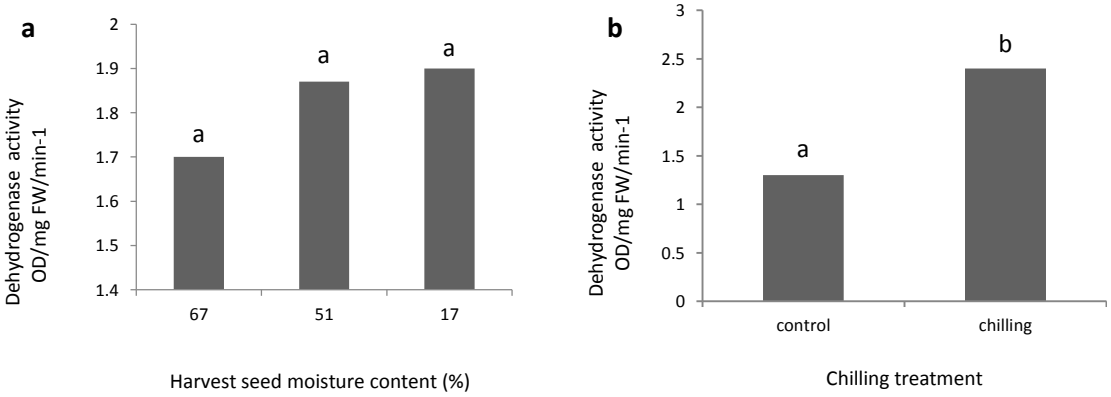

Figure 5. Changes in activity of dehydrogenase enzyme (absorbance at 480 nm) in *Prangos ferulacea* seeds collected by different of seed moisture content (a) and in chilled and non-chilled (control) seeds (b). Seeds treated (imbibed) at 4 °C for 8 weeks before transferring to germination. Each column is the mean of 3 replicates and columns with the same letters are not significantly different at the 5% level.

Conclusion

The results of this experiment showed that seed moisture content has a significant effect on seed germination of seeds *Prangos ferulacea*. The highest growing and germination power was obtained from the seeds harvested at seed moisture content of 17%, which is consistent with the physiological maturity. With maturity progress, germination increased mainly because of the accumulation of food reserve during seed development that was mobilized during the germination. Stratification (moist chilling at 4℃) plays a key role in stimulating the seed germination of this plant. Considering that *Prangos ferulacea* is from a cold climate and experiences cold winters, it can be assumed that as an ecological compatibility mechanism, this plant has some level of physiological dormancy that is broken by chilling.

Also, the results of this study show that activity of alpha-amylase and dehydrogenase enzymes increased with moist chilling; actually, chilling has a significant role in the seed dormancy release associated with these enzymes activity. Therefore, we can conclude that these enzymes play an important role in seed germination of *Prangos ferulacea*. The results also show an important role of ascorbate and catalase enzymes in the removal of reactive oxygen compounds produced in the later stages of maturity or during desiccation tolerance stage.

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