International Journal of Botany

ISSN: 1811-9700

science alert

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International Journal of Botany 6 (2): 151-156, 2010
ISSN 1811-9700

Effect of Storage and Cold-Stratification on Seed Physiological Aspects of *Bunium persicum:* A Threatened Medicinal Herb of Trans-Himalaya

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Abstract: Kalajira (*Bunium persicum*) is an economically and medicinally important threatened plant species of cold desert region of trans-Himalaya that needs appropriate conservation interventions. The seeds exhibit very deep dormancy which is a major restriction for its cultivation/expansion. Therefore, we have analysed the seed physiological aspects of a *B. persicum* population from Lahaul (Himachal Pradesh, India) with an aim to alleviate seed dormancy and/or improve germination. The freshly harvested seeds exhibited high viability (93%) as determined by tetrazolium test. The seeds, however, were completely dormant. Seed storage under ambient conditions for 42 months did not alter the dormancy/germination status. But, the viability declined gradually 18 months onward leading to about one-third loss after 42 months. Of the various dormancy breaking treatments tested (H_2SO_4-scarification, stratification at 4°C, leaching, KNO_3, SNP, NaN_3 and GA_3), only the moist-stratification at 4°C (continuous) effectively released the dormancy in freshly harvested as well as differentially stored seeds. Remarkably, the seeds germinated only at low temperature (4°C); germination ceased upon shifting the seeds from 4 to 25°C. Further, the response of seeds to this treatment declined consistently with the progression of storage period. The activity of catalase, an important antioxidative enzyme in the seeds, also declined with the progression of seed storage suggesting the involvement of oxidative stress in storage-dependent changes in seed viability and responsiveness to low temperature. Involvement of phenolics in observed changes might be excluded. The findings are of potential significance for seed-based propagation of *B. persicum*.

Key words: Catalase, cold desert, dormancy, germination, seed longevity

INTRODUCTION

Bunium persicum (Boiss.) Fedtsch. (Apiaceae), commonly called Kalajira is one of the economically and medicinally important plant species from the cold desert region of trans-Himalaya. The ripe fruits are rich in essential oil and are used as carminative, lactagogue, diuretic, expectorant, antispasmodic and as valuable spice for flavouring foods (Chauhan, 1999; Kala, 2003). In addition, the essential oil is reported to exhibit significant antioxidative (Shahsavri *et al*., 2008), antibacterial (Moghtader *et al*., 2009; Talei and Mosavi, 2009) and antifungal (Sekine *et al*., 2007) activities. The species is a native of west Asia and has a limited distribution (Valizadeh *et al*., 2006). In Himachal Pradesh, the species is found only in some parts of cold desert area (Chamba, Kinnaur, Lahaul and Spiti). A large quantity of *B. persicum* seeds are collected by the local people and sold at Rs. 800-1000 kg^{-1} and is a good source of earning for the hill people of cold desert area. However, the ruthless collection of seeds resulted in its threatened population status (Ved *et al*., 2003) and requires cultivation and/or conservation interventions. In the natural habitat, plants generally regenerate/multiply through seeds. However, the seeds are reported to have very deep dormancy which represents one of the greatest restrictions for cultivation and expansion of this species. The release from dormancy through cold-stratification of seeds has been demonstrated in different *B. persicum* populations (Sharifi and Pouresmael, 2006; Sharma *et al*., 2006). A narrow range of low temperature (5-10°C) was found to be effective for germination induction (Durrani *et al*., 1997). These pretreatments apparently constitute a close simulation of conditions actually experienced by the wild populations.

Seeds following harvest undergo numerous metabolic changes that are eventually reflected in altered status of viability and germination. Such storage-dependent seed physiological changes are strictly species-specific and their understanding has definite implications for propagation and multiplication of the species concerned.

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Despite the availability of some reports about germination behavior of certain *B. persicum* populations (Sharma *et al*., 2006), there is a dearth of information concerning the storage-dependent alteration in the status of seed viability and germination. We here report the findings on these aspects of a *B. persicum* population from the cold desert area of Lahaul and Spiti (Himachal Pradesh).

MATERIALS AND METHODS

Seed source: The fully ripened seeds of *B. persicum* were collected during August 2003 from Lahaul in the district of Lahaul and Spiti (altitude: 3150 m above mean sea level), Himachal Pradesh, India. The seeds were air-dried in shade for a fortnight. Thereafter, they were stored in polyethylene jars at room temperature for subsequent studies (42 months).

Seed viability determination: Seeds were subjected to 2,3,5-triphenyl tetrazolium chloride (TTC) test for viability evaluation shortly after collection and at regular intervals during the subsequent storage period. The test involved incubation of transversely cut seed halves for 24 h and examination for staining intensity/pattern. Seeds with completely stained embryo were considered viable. Furthermore, the TTC reduction by seed tissue was quantified by measuring the formazan formed. For this the seed halves, at the end of incubation with TTC, were homogenized with 5 mL MetOH and centrifuged at 10,000 g for 10 min; absorbance was recorded at 485 nm. TTC reduction was expressed in terms of A_{485}/25 seeds.

Seed germination assays: Seeds were surface sterilized with 0.1% $HgCl_2$ for 5 min, washed thoroughly under tap water and soaked in distilled water for 24 h at 25±1°C. Thereafter, the seeds (25, in triplicate) were transferred to Petri dishes lined with three layers of filter paper moistened with distilled water and allowed to germinate in a seed germinator at 25±1°C under continuous illumination provided by the fluorescent white light (PAR: 40 μmol/m^2/sec). Seeds were considered germinated upon radicle emergence (≥2 mm); germinated seeds were counted periodically. The Mean Germination Time (MGT) was calculated as follows (Airi *et al*., 2005):

$$MGT = \Sigma (nd)/N$$

where, n is number of seeds germinated after each incubation period in days, d and N is total number of seeds emerged at the end of the test.

Physico-chemical and hormonal (GA_3) treatments

Cold stratification: Surface-sterilized seeds soaked in distilled water for 24 h were transferred to moist filter papers and subjected to low temperature (4°C) of stated durations (30, 45, 60 day) after which they were allowed to germinate at 25°C. Alternately, the seeds were continuously incubated at 4°C.

Chemical and GA_3 treatments: Surface-sterilized seeds were soaked in aqueous solution of 0.2% potassium nitrate (KNO_3), 1 mM sodium nitroprusside (SNP), 0.1 mM sodium azide (NaN_3) or 0.1 and 1.0 mM gibberellic acid (GA_3) for 24 h followed by germination on moist filter paper.

Catalase assay: Catalase activity was assayed polarographically with an oxygen electrode unit (Hansatech, UK). In brief, the seeds were homogenized with chilled 100 mM Na-phosphate buffer (pH 7.4) and centrifuged at 10,000 g for 5 min at 4°C. The supernatant served as crude enzyme. Assay (1 mL) included 880 μL phosphate buffer (100 mM) pH 7.4 and 100 μL of 0.1 M H_2O_2. The reaction was initiated by addition of 20 μL enzyme extract. The amount of O_2 evolved was calculated through electrode calibration and slopes obtained on chart paper.

Protein contents: Protein contents were estimated according to the method of Lowry *et al.* (1951).

Total phenolic content estimation: Total phenolic content was determined by the method of Goldstein and Swain (1963). The seeds were homogenized in 0.3 N HCl in methanol and centrifuged at 10,000 g for 10 min. The supernatant was collected and the pellet was extracted again in 0.3 N HCl in methanol and centrifuged. The supernatants were pooled and evaporated on a hot water bath. The residue obtained was dissolved in distilled water and final volume made to 5 mL. Subsequently, 0.5 mL of Folin-phenol reagent was added to this solution and shaken vigorously. After 3 min, 1 mL of 35% sodium carbonate solution was added, shaken and allowed to stand for 1 h. Absorbance was recorded at 630 nm. Amount of total phenols was determined using a calibration curve prepared with gallic acid.

Data analysis: All experiments were carried out in triplicate. Data are presented as arithmetic mean with standard deviation.

RESULTS

Seed viability: Freshly harvested seeds of *B. persicum* exhibited 93% viability; this status was maintained at least

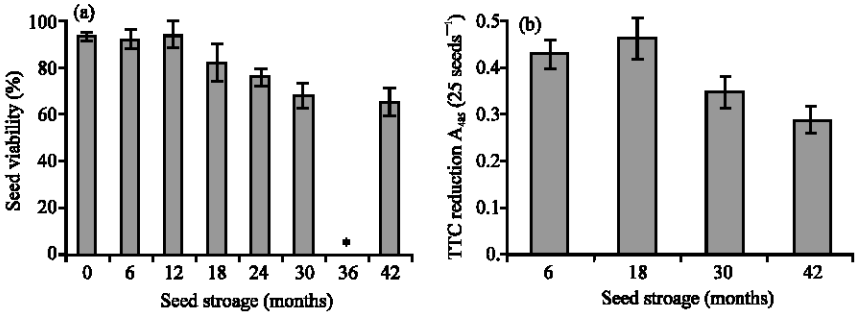

Fig. 1: (a) Storage-dependent changes in seed viability and (b) quantitative ability of seed tissue to reduce TTC to formazan of *B. persicum*. Data are average of 3 measurements each ±SD. *Not determined

for 12 months of storage. Thereafter, the seed viability gradually declined; 30% decline as compared to freshly harvested seeds was evident after 42 months of storage (Fig. 1a). Similarly, the quantitative ability of seed tissue to reduce TTC was high until 18 months of storage but declined consistently thereafter. A 19 and 33% decline was evident after 30 and 42 months, respectively as compared to 6-month stored seeds (Fig. 1b).

Seed dormancy/germination status of freshly harvested seeds: Freshly harvested seeds of *B. persicum* exhibited deep dormancy; no germination occurred in control until 120 days under optimum germination conditions (25°C). Of the various physico-chemical (H_2SO_4-scarification, stratification at 4°C, leaching, KNO_3, SNP and NaN_3) and hormonal (0.1 and 1.0 mM GA_3) treatments tested, only moist-stratification at 4°C (continuous incubation) was effective at dormancy removal. Under this treatment seed germination started after 45 days of incubation and increased gradually until 120 days when 75% germination was evident (Fig. 2). Even when the seeds stratified for 30, 45 and 60 days were shifted from 4 to 25°C the seed germination ceased to proceed further. For example, seeds kept at 4°C for 60 days exhibited 24% germination. A shift from 4 to 25°C led to 36% germination after 15 days of incubation but there was no change beyond 15 days (Fig. 2). The other seed pre-treatments namely, acid-scarification, leaching, KNO_3 (0.2%), SNP (1 mM), NaN_3 (0.1 mM) and GA_3 (0.1 and 1 mM) were altogether ineffective in removal of seed dormancy (data not shown).

Storage-dependent changes in seed dormancy/ germination: During a 42 months storage under ambient conditions, seeds exhibited complete dormancy. Also, none of the dormancy breaking treatments tested namely, acid (H_2SO_4)-scarification, leaching, KNO_3, SNP, NaN_3 and GA_3 proved effective in breaking dormancy. The moist-stratification at 4°C (continuous incubation)

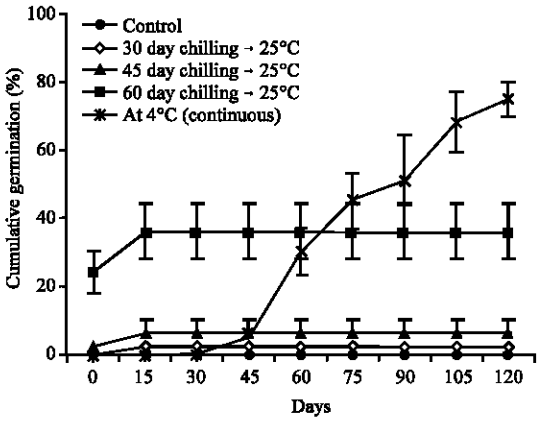

Fig. 2: Time-course of seed germination in *B. persicum* as affected by different durations of cold stratification. Data are average of 3 replicates each±SD

remained the only effective treatment. However, the response of seeds to this treatment declined with the progression of storage period. Due to this treatment after 0 (freshly harvested), 12, 24 and 36 months storage, the seeds exhibited 75, 47, 40 and 33% germination with an MGT of 80, 102, 92 and 89 days, respectively (Fig. 3).

Catalase activity and total phenolic contents: To get an idea about antioxidative status of seeds and changes therein due to storage, the catalase (CAT) activity was measured. The freshly harvested seeds exhibited higher levels of CAT activity which declined gradually with the progression of storage period. Due to storage of 12, 24 and 36 months, a 40, 67 and 78% decline in catalase activity, respectively was observed (Fig. 4).

The freshly harvested seeds of *B. persicum* contained 19.93 mg total phenolics g^{-1} seed which did not change much during the storage period. Even after 36 months only 8% decline was observed (Fig. 5).

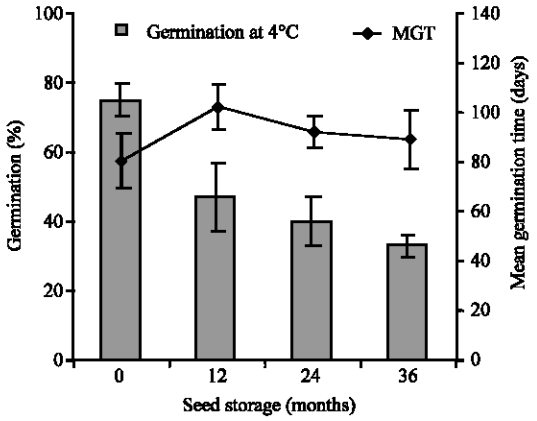

Fig. 3: Storage-dependent changes in seed germination and mean germination time of *B. persicum* at low temperature (4°C). Data are average of 3 replicates each±SD

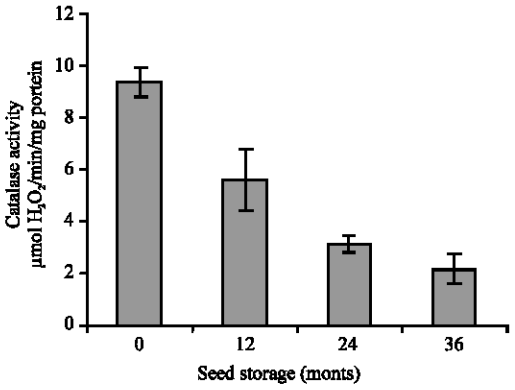

Fig. 4: Storage-dependent changes in catalase activity of seeds of *B. persicum*. Data are average of 3 measurements each±SD

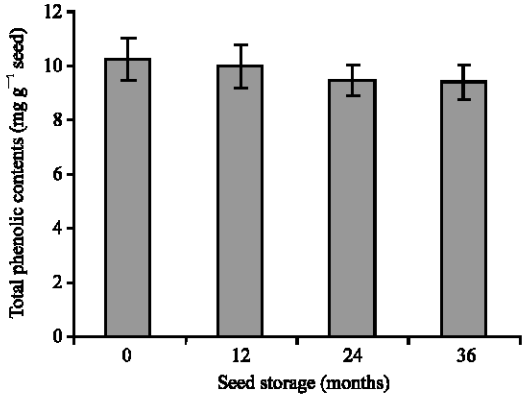

Fig. 5: Storage-dependent changes in total phenolic contents of seeds of *B. persicum*. Data are average of 3 measurements each±SD

DISCUSSION

The poor and erratic germination of *B. persicum* in natural conditions is one of the major restrictions to the cultivation/expansion of this valuable plant species. In the present study, seeds of a *B. persicum* population from Lahaul in cold desert area of Himachal Pradesh, India exhibited deep dormancy which did not change even after 42 months of storage. Of a number of seed pre-treatments tested, only a prolonged moist stratification at 4°C (continuous) was effective at dormancy removal. The seeds germinated only at low temperature (4°C); germination ceased upon shifting the seeds from 4 to 25°C. We observed this earlier also when *B. persicum* seeds were stratified for 60 days prior to shifting to 25°C (Sharma *et al*., 2006). In this study, stratification of variable duration (30, 45, 60 days) yielded similar responses. In the light of these data it appears that germination and a certain stage of seedling growth need to have occurred before the temperature rise begins where further plant growth is possible. Such a temperature regime actually prevails in the area of region of the population studied. The degree and duration of low temperature exposure would be expected to vary in a population-specific manner. Obviously, the findings obtained with a specific population might not be necessarily generalized.

The requirement of cold stratification for dormancy removal has previously been observed in a number of other species belonging to family Apiaceae (Baskin *et al*., 1995; Walck and Hidayati, 2004; Vandelook *et al*., 2007). Low temperature treatment is known to activate GA_3 synthesis which ultimately enhances the embryo growth potential to a level sufficient to penetrate the covering structure (Finch-Savage and Leubner-Metzger, 2006). Enhanced gibberellin contents of the seeds in response to chilling have been demonstrated in several species (Gianfagna and Rachmiel, 1986; Halinska and Lewak, 1987). Karssen and Lacka (1986) demonstrated that chilling-dependent removal of dormancy in GA_3-deficient mutants of *Arabidopsis thaliana* L. Heynh. was due to an increase in sensitivity to GA_3 and not due to the biosynthesis of GAs. In the present study, endogenous GAs were not determined in the cold stratified seeds. Therefore, it could not be ascertained whether cold stratification-induced germination in dormant *B. persicum* seeds occurred due to accumulation of GAs or an increase in sensitivity towards GA or both. However, the pretreatment of seeds with exogenous GA_3 did not seem to substitute for cold stratification.

Interestingly, the responsiveness of seeds to low temperature declined consistently during storage. Such a

change might be associated with storage-dependent alteration in the ability of seeds to synthesize the germination inducing hormonal (e.g., GA_3) or other factors. The sensitivity of target molecules/sites to low temperature might also change over a period of time causing a disruption of signal transduction events involved. Like the seed responsiveness to low temperature, seed viability also declined 18 months onward. Nevertheless, seeds retained 65% viability even after 42 months storage. A reasonably high viability status even after substantial storage of seeds under ambient conditions suggests the suitability of seeds for regeneration/cultivation of this species. Although, not attempted here, the seed longevity is likely to be further enhanced by appropriate manipulation of storage conditions (temperature/RH regimes). The prolonged storage of seeds in dry state is reported to be associated with the production of Reactive Oxygen Species (ROS) (Hendry, 1993; Bailly, 2004). Various ROS react with all biological molecules including lipids, DNA and proteins leading to oxidative stress and related deteriorative events (Pukacka and Ratajczak, 2005). Involvement of oxidative stress in the observed storage dependent decline in seed viability and responsiveness to low temperature is likely as catalase (an antioxidative enzyme) activity was found to decline with the progression of seed storage period. Phenolics, measured as possible dormancy regulators (Phartyal *et al*., 2003; Chien *et al*., 2004; Finkelstein *et al*., 2008) did not appear to be associated with seed dormancy or germination of *B. persicum*.

Present data on storage-dependent changes in seed viability and their responsiveness to low temperature in a high altitude *B. persicum* population from cold desert area of Himachal Pradesh are of potential implication for propagation of this plant species. Further work is needed towards the understanding of the factors/treatments that might enhance the seed longevity and similarly modify the requirement of low temperature treatments.

ACKNOWLEDGMENTS

The financial assistance in the form of Senior Research Fellowship and Research Associateship by CSIR, New Delhi (9/237(74)2002 EMR-1, 9/237(124)/2008-EMR-1) to Rajender K. Sharma is thankfully acknowledged.

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