Seed physiological aspects of pushkarmool (*Inula racemosa*), a threatened medicinal herb: response to storage, cold stratification, light and gibberellic acid

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The seeds of *Inula racemosa* Hook. f. (Asteraceae), an economically and medicinally important threatened plant from northwest Himalaya, exhibited deep dormancy at harvest. Cold-stratification and gibberellic acid treatments effectively alleviated seed dormancy. Due to the storage of seeds in dry state (after-ripening), the dormancy level gradually declined; complete loss of dormancy was evident within 12 months of storage. Data indicate a clear requirement of light for seed germination in this species, as there was no germination in the dark and under green light. The viability loss coincided with diminished catalase activity, although lipid peroxidation status of the seeds remained generally unchanged, pointing to a possible role of oxidative stress in the observed changes. Periodic monitoring of seed viability in terms of 2,3,5-triphenyl tetrazolium chloride reduction ability during five years of storage revealed a gradual viability loss.

Keywords: After-ripening, catalase, *Inula racemosa*, photoblasticity, seed dormancy.

INULA RACEMOSA Hook. f. (Asteraceae), commonly known as pushkarmool or manu, is an economically and medicinally important perennial herb that grows in the northwest Himalayas[1] at altitudes ranging from 2000 to 3200 m. The roots have anthelmintic, antiseptic, diuretic, expectorant, anti-inflammatory and antipyretic properties, whereas the seeds are aphrodisiac. They are also used in veterinary medicine as a tonic and for relief in stomach ache[2]. Additionally, the roots are valued for their fragrance and aroma[3]. Roots contain beta-sitosterol, daucosterol and isoalantolactone. The latter, a major sesquiterpene lactone, has been reported to be active against various pathogenic fungi[4,5]. The pharmacological effects of root extract have been studied in rats, where they were found to have significant hypoglycaemic and hepatoprotective effects[6,7].

I. racemosa has limited distribution and is considered a rare species according to the Red Data Book of Indian plants[8]. The species was categorized as critically endangered in the Conservation Assessment and Management Prioritisation (CAMP) workshop[9]. In natural habitats, due to unsystematic exploitation, the reduction and fragmentation of plant population and habitats is occurring at a rapid and accelerating rate. These factors may result in extinction of species, especially those already with a threatened status. In recent years, *ex situ* cultivation is emerging as one of the possible solutions to the conservation of threatened medicinal plants[10]. Commercial cultivation of medicinal plants is expected to alleviate pressure on wild habitats while providing a substantial alternate source of income to the natives.

I. racemosa is commercially cultivated in some parts of the cold desert region of Lahaul valley in northwest Himalaya (Figure 1). However, in the last few years, cultivation has drastically declined due to the introduction of other cash crops like potato, pea and hops (*Humulus lupulus* L.), which provide greater economic returns. Their relatively shorter growth season is definitely advantageous. Small land holdings, lengthy cultivation cycle and fluctuating market prices are also some of the reasons associated with decline in *I. racemosa* cultivation. In order to fulfil the increasing demand from pharmaceutical industries and to conserve the natural populations of *I. racemosa*, *ex situ* cultivation needs to be encouraged. The optimum realization of the latter requires detailed information about the seed germination behaviour of the species. Despite its cultivation in the region, to the best of our knowledge, there is a dearth of information concerning the seed ecophysiological aspects of this species. Therefore, in the present study ecophysiological aspects of seeds of *I. racemosa* have been addressed in relation to storage-dependent changes and responses to certain physico-chemical treatments.

Materials and methods

Seed source

Mature seeds of *I. racemosa* were collected from the cultivated population (no wild population was found) at

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Patan Valley, Lahaul (2950 m amsl), Himachal Pradesh, India. The seeds were collected manually, air-dried under shade for a fortnight, and stored in polyethylene jars at room temperature (15–20°C) for subsequent studies.

Seed viability

Seed viability was tested using 2,3,5-triphenyl tetrazolium chloride (TTC) shortly after collection and at regular intervals during the subsequent storage period. Seeds (cut-off at one end to facilitate diffusion of TTC) were incubated in 0.1% TTC solution for 24 h and the staining intensity/pattern was examined. Seeds with completely stained embryo were considered viable. Formazan formed by seed tissue was quantified by homogenizing the seeds with 5 ml MetOH and the absorbance of clarified supernatant (10,000 *g* for 10 min) was recorded at 485 nm. TTC reduction was expressed in terms of A_{485}/50 seeds.

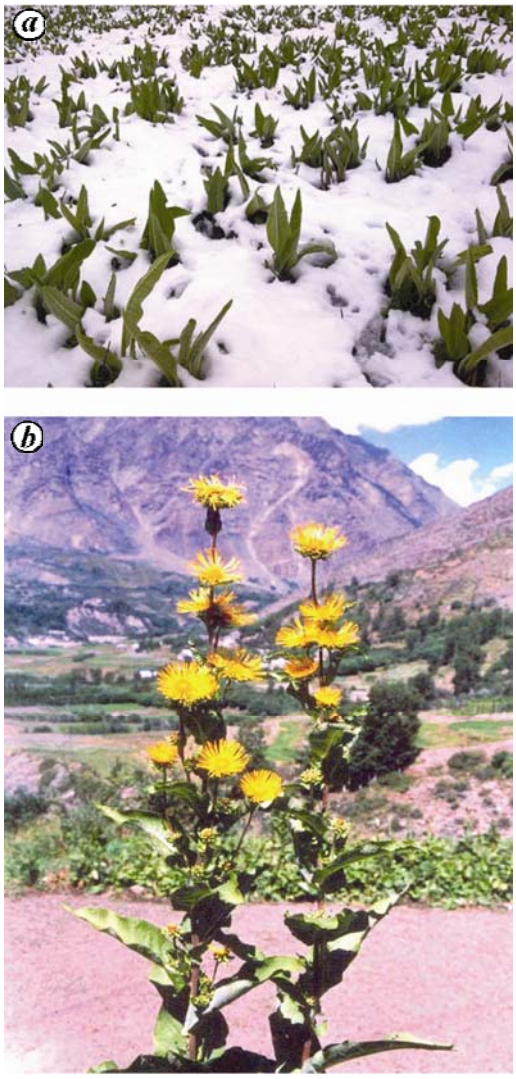

Figure 1. *Inula racemosa* in cultivation in Lahaul. One-year-old plants growing in snow-covered cultivated land (***a***) and three-year-old plants (***b***).

Seed germination assays

Seeds were surface-sterilized with 0.1% $HgCl_2$ for 5 min, washed thoroughly under tap water, soaked in distilled water for 24 h at 25 ± 1°C and transferred to petri dishes lined with three layers of filter paper moistened with distilled water in a seed germinator at 25 ± 1°C under continuous illumination (PAR: 40 μmol m^{-2} s^{-1}). Seeds were considered germinated upon the emergence of 2–5 mm radicle and scored periodically for germination.

The mean germination time (MGT) was calculated as follows[11]: MGT = $\sum (nd)/N$, where n is the number of seeds germinated after each incubation period in days, d, and N the total number of seeds emerged at the end of the test.

Light sources

To determine the effect of light on seed germination, different light treatments were given. White light was provided by 40 W fluorescent tubes (PAR: 40 μmol m^{-2} s^{-1}). Red and green lights were provided by 40 W fluorescent tubes in combination with two layers of red or green plastic sheet filters respectively. For dark treatment the petri dishes were kept in a box, wrapped in black sheet and seed germination was monitored every 2 days under dim-green light.

Physico-chemical and hormonal (gibberellic acid) treatments

Cold stratification: Surface-sterilized seeds soaked in distilled water for 24 h were placed on moist filter paper-lined petri dishes and subjected to low temperature (4°C) for 30 days and thereafter, petri dishes were transferred to 25°C for germination.

Acid scarification: Seeds were treated with concentrated H_2SO_4 for 30 s; this duration was worked out on the basis of preliminary experiments. Thereafter, the seeds were washed thoroughly under running tap water and soaked in distilled water for 24 h and transferred to germination conditions as described earlier.

Chemical and gibberellic acid treatments: Surface-sterilized seeds were soaked in aqueous solution of 0.2% potassium nitrate (KNO_3), or 0.1 and 1.0 mM gibberellic acid (GA_3) for 24 h and transferred to germination conditions as described earlier. For each treatment 25 seeds in triplicate (25 × 3) were used.

Measurement of lipid peroxidation

Lipid peroxidation was determined in terms of malondialdehyde (MDA) production following the method

described by Dhindsa *et al.*[12]. In brief, the seeds were homogenized with 0.1% trichloroacetic acid (TCA) and centrifuged at 10,000 *g* for 10 min. Then 1 ml supernatant was reacted with 0.5% thiobarbituric acid in 20% TCA and kept at 95°C in a water bath for 30 min. The reaction was terminated by cooling the reaction mixture in ice for 2 min. Absorbance was read at 532 nm. Measurements were corrected for nonspecific absorbance (turbidity) by subtracting the absorbance at 600 nm. The MDA contents were measured using the extinction coefficient at 155 $mM^{-1}\,cm^{-1}$.

Catalase assay

Catalase activity was measured polarographically by measuring the H_2O_2-dependent oxygen evolution at room temperature with an oxygen electrode unit (Hansatech, UK). In brief, the seeds (five) were homogenized with chilled 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 (0.1 M) pH 7.4 and 100 µl of 0.1 M H_2O_2. The reaction was initiated by the addition of 20 µl enzyme extract. The amount of O_2 evolved/min was recorded with the help of a chart recorder. The catalase activity was calculated through electrode calibration and slopes obtained on chart paper.

Protein content

Protein content in the crude extract was estimated according to the method of Lowry *et al.*[13].

Statistical analysis

All experiments were carried out in triplicate. Data are presented as arithmetic means and standard deviation.

Results

Seed viability

The freshly harvested and up to 6-month stored seeds exhibited 100% viability. Seed viability declined slightly in the case of 18-month stored seeds. Thereafter, the viability declined gradually. After 42 and 54 months of storage, seed viability was reduced to 80% and 32% respectively. A total loss of seed viability was observed after 60 months of storage (Figure 2). The TTC reduction ability of the seeds, expressed as A_{485}/50 seeds, increased substantially and gradually until 30 months and declined thereafter with the progression of storage until 60 months (Figure 2). The magnitude of TTC reduction was 1.98 and 0.05-fold after 30 and 60 months of seed storage respectively, relative to those of 6-month stored seeds.

Seed dormancy/germination status of freshly harvested seeds

Freshly harvested seeds exhibited dormancy; no germination occurred even after 60 days of subjecting to optimum germination conditions (data not shown beyond 30 days). Among the various physico-chemical (acid (H_2SO_4) scarification, cold stratification and KNO_3) and hormonal (GA_3) treatments tested, GA_3 and cold stratification proved effective in overcoming dormancy. The effect of GA_3 was concentration-dependent. Seed treatment with 0.1 and 1.0 mM GA_3 led to 17% and 100% germination respectively. Chilling treatment (30 days)-dependent dormancy removal was more rapid than that due to GA_3 (1 mM; Figure 3). Potassium nitrate (KNO_3) and acid (H_2SO_4) scarification caused a mere 3% and 9% germination respectively (data not shown).

Storage-dependent changes in seed dormancy/germination

Periodic testing done for seed germination during 60-months storage revealed the storage-dependent loss of

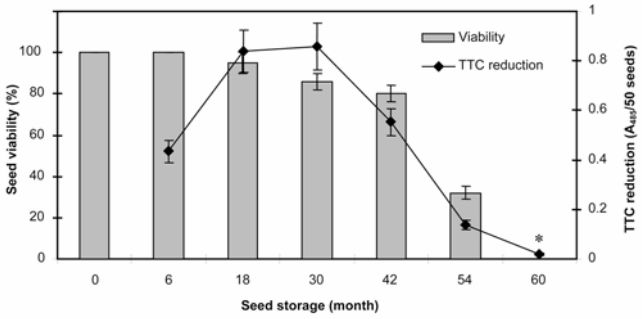

Figure 2. Storage-dependent changes in qualitative (bars) and quantitative (line) seed viability of *I. racemosa*. Data are average of three measurements each ± SD. *Seed viability zero.

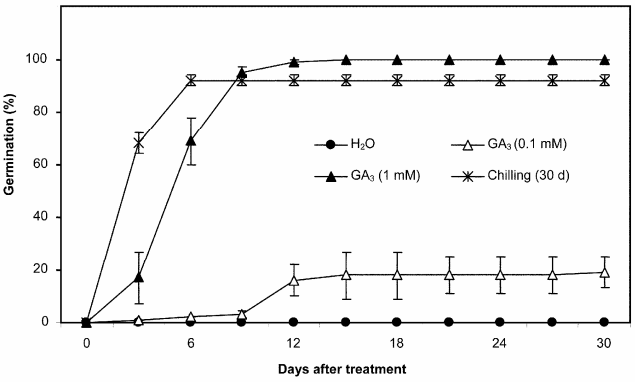

Figure 3. Time course of seed germination in *I. racemosa* as affected by different physico-chemical and hormonal treatments. Data are average of three replicates each ± SD.

dormancy in *I. racemosa* seeds. During 0, 6 and 12 months storage, 0%, 7% and 83% germination respectively was observed. Concomitantly, the MGT reduced from 21 days to 8 days in 6 and 12 months stored seeds respectively (Figure 4). Beyond 12-month storage, the germination status and MGT were maintained at least for 36 months; only minor quantitative fluctuations in a narrow range were observed. The seed germination started to decline after 30 months; the decline was steep as the storage period was prolonged. After 60 months storage no germination was observed (Figure 4 *a*).

GA_3 and chilling treatments strongly promoted germination. In freshly harvested seeds, 100% and 92% germination with MGT of 7 and 4 days was induced by 1 mM GA_3 and chilling (30 days) respectively. Although after 12 months storage, the seeds turned largely non-dormant, GA_3 and chilling treatments were still helpful as they reduced MGT by 40–50% (Figure 4). Acid scarification and KNO_3 were found ineffective in altering seed dormancy, germination performance and rate of germination (MGT) in freshly harvested as well as differentially stored seeds (data not shown)

Effect of light on seed germination

The seeds of *I. racemosa* exhibited photoblasticity as they invariably germinated only under illumination. Fully after-ripened or GA_3-treated or cold-stratified seeds, when subjected to light (white, red) exhibited 100% germination. No germination occurred in dark and green light (Table 1). This feature was evident in fresh as well as differentially stored seeds. The seed germination was also affected by seed-sowing depth; seed germination declined with increasing sowing depth. Seeds sown below 2 cm failed to germinate (data not shown).

Lipid peroxidation and catalase activity

The lipid peroxidation status of seeds, measured in terms of MDA content, did not change much during a 3-year storage period. Also, the MDA content remained generally unaltered in response to the pre-treatment of seeds with GA_3 and chilling (Figure 5 *a*). Freshly harvested seeds exhibited higher levels of catalase (CAT) activity, which declined gradually with the progression of storage. After storage for 3 years there was a 45% decrease in CAT activity. Seeds pre-treated with chilling showed lower CAT activity compared to the control during all periods of storage. GA_3 did not alter the CAT activity in freshly harvested as well as differentially stored seeds (Figure 5 *b*).

Table 1. Effect of different light conditions on seed germination of fully after-ripened seeds of *I. racemosa*. Data are average of three replicates each ± SD

Quality of light	White	Red	Green	Dark
Germination (%)	88 ± 8	92 ± 6	0	0

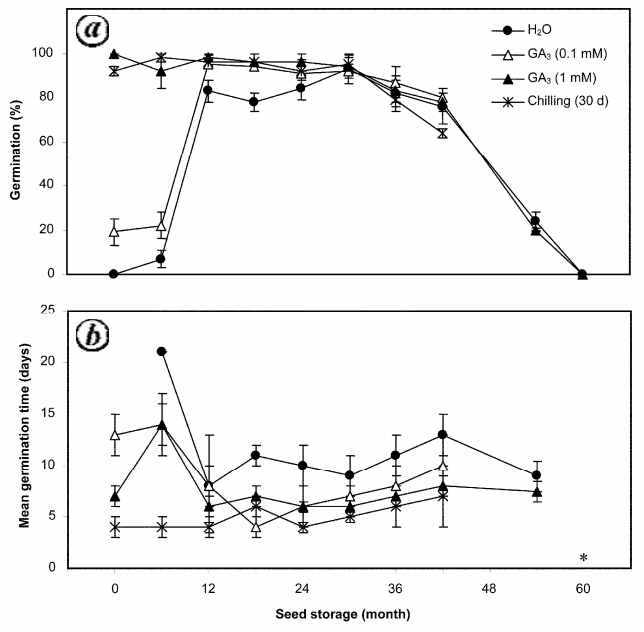

Figure 4. Storage-dependent changes in seed germination (***a***) and mean germination time (***b***) of *I. racemosa* as affected by different physico-chemical and hormonal treatments. Data are average of three replicates each ± SD. *No germination.

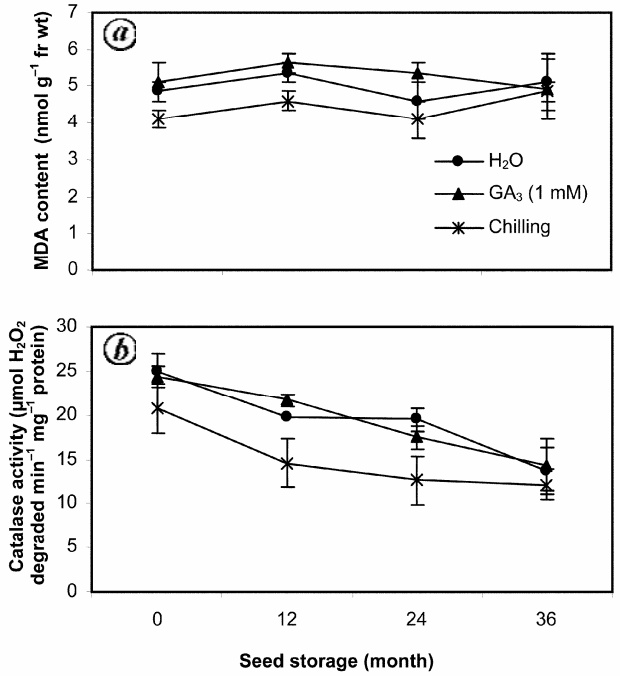

Figure 5. Storage-dependent changes in MDA content (***a***) and catalase activity (***b***) of seeds of *I. racemosa*. Data are average of three measurements each ± SD.

Discussion

Seed dormancy and poor germination are considered a barrier to the regeneration and *ex situ* cultivation of commercially important plant species. The freshly harvested seeds of *I. racemosa*, a medicinally and economically important perennial herb, were completely dormant; there was no germination despite 100% seed viability. GA_3 and chilling treatments effectively relieved the seed dormancy with a substantially reduced MGT. The effectiveness of GA_3 and chilling treatment in causing dormancy removal of freshly harvested seeds of *I. racemosa* has also been reported earlier[14]. The effects of gibberellin (GA) could be ascribed to stimulated activities of various hydrolytic enzymes and consequently increased availability of nutrients for embryo growth. GAs also regulate seed germination by loosening the mechanical restraints of the testa and endosperm to permit easy protrusion of the radicle. Generally, the micropylar endosperm acts as a barrier to radicle expansion and therefore contributes to seed dormancy. In several species, endosperm weakening has been found to be associated with induction of cell-wall remodelling enzymes, including endo-β-mannanase, β-1,3-glucanases, pectin methylesterases, polygalacturonase and others[15,16]. Such a role of GA has been shown in tomato[17] and *Arabidopsis thaliana*[18] through studies involving mutants. GAs also promote seed germination of dormant seeds by overcoming germination constraints existing in seeds requiring after-ripening (dry storage)[19], light[20] and cold. Low-temperature treatment is known to activate GA synthesis and/or increase the sensitivity of the embryo towards GA. Due to these changes the embryo is able to penetrate the covering structure[21–23].

Seed dormancy in *I. racemosa* declined with the progression of the storage period, leading to a complete loss after 12 months. Data suggest that an immature embryo could be one of the major causes of seed dormancy, which requires after-ripening. In the present study, GA_3 and chilling treatments seem to have substituted for the requirement of after-ripening. During after-ripening, biochemical changes occur that trigger embryo maturity/growth[24]. The increase in TTC reduction ability during the progression of early storage period reflected the increased metabolic status of seeds during the after-ripening process. This might have contributed to increased seed germination. On the other hand, prolonged storage of seeds (beyond 30 months) led to the decrease in seed viability reflected in reduced germination performance. During long-term seed storage, accumulation of reactive oxygen species (ROS) is known to occur causing oxidative stress and eventually cell death[25]. However, ROS are not invariably damaging; they are also considered as one of the key components of the signalling network involved in many aspects of seed physiology[26]. Oracz *et al.*[27] reported that in sunflower seeds (a member of the same family as *I. racemosa*), ROS accumulation during after-ripening was involved in dormancy alleviation. Thus, ROS accumulation appears to be a key signal governing cell activity during after-ripening. However, in the present study, lipid peroxidation (an indicator of oxidative stress) status of seeds remained generally unaltered during storage. Similar to our observations, certain studies on long-term storage also reported that there was no consistent association between lipid peroxidation and seed ageing[28,29].

In dry seeds, enzymatic reactions may play a small role in seed ageing, because dry seeds lack active enzymatic metabolism. However, certain non-enzymatic reactions, such as Amadori and Maillard reactions, are known to occur even at very low moisture content[30]. These are a series of complex reactions that occur following an initial simple non-enzymatic attack on amino groups of proteins and nucleic acid/protein complexes by reducing sugars or aldehydes. The affected proteins or enzymes result in loss of their function. These reactions are also known to inhibit the activities of several antioxidative enzymes[31], ribonuclease[32] and certain dehydrogenases[33]. Therefore, the protein or DNA damages by Amadori and Maillard reaction may accumulate during dry storage and eventually contribute to seed death. In the present study, however, such metabolic changes were not determined but they provide a reasonable explanation for the observed little or no change in lipid peroxidation status of seeds during dry storage.

The total phenolic content, measured as possible dormancy regulators, was not differentiable in dormant (fresh seeds) and non-dormant (fully after-ripened) seeds of *I. racemosa* (data not shown), allowing the exclusion of phenolic involvement in the regulation of dormancy.

Our data clearly point to the regulation of seed germination of *I. racemosa* by light. The seeds exhibited absolute requirement for light over and above any other factors. However, it could not substitute for after-ripening, cold stratification or GA_3. In a large number of species, light-dependent responses are mediated by phytochromes via appropriate conversions into photoconvertible-active (Pfr) and inactive (Pr) forms[34]. Phytochrome-dependent upregulation of active GA biosynthesis and degradation of abscisic acid is known in photoblastic lettuce seeds[35]. Similarly, the inability of seeds to germinate below 2 cm soil depth can be ascribed to the light requirement. Involvement of cryptochromes in seed germination processes has also been suggested, e.g. in *Arabidopsis*[36].

In conclusion, the study provides insight into the seed germination and dormancy behaviour of *I. racemosa*. Although the crop is largely grown through root cuttings in the cold desert region of Lahaul and Spiti, the findings would have implication for *ex situ* cultivation and conservation of the species.

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