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Assessment of the efficacy of some physico-chemical and hormonal treatments for dormancy removal & germination improvement in *Bunium persicum* (Kala Zeera)

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Abstract

The aim of this study was to determine the efficacy of some physico-chemical and hormonal treatments for dormancy removal / germination improvement. In laboratory experiment, seeds were subjected to various hormonal treatments like Gibberellic acid (0.01mM & 0.1mM), Indole Acetic acid (.01mM & 0.1mM) & Kinetin (0.01mM & 0.1 mM) for 24h. Seeds were also subjected to 24h, 48h running water for leaching and seed were kept for moist chilling at 4 °C for 2 months. Seeds were also treated with hormones (48h L +Kn (0.01 & 0.1mM); 48h L+GA₃ (0.1mM) for 24h after 48h leaching. Seed germination was promoted by GA₃ (0.1mM), IAA (0.01mM) and Kinetin (0.01mM). Kinetin (0.01 mM) was found to be most effective caused 77.33% germination within 150 days at 4 °C as compared to 60% in control. Leaching (24h & 48h) alone was not found to be effective, rather it was inhibitory for seed germination. Hormonal treatments (Kinetin 0.01 & 0.1 mM) when applied to seeds after 48h leaching enhanced seed germination. Lower concentration was found to be more effective, around 72% & 68% germination was observed at 48hL + Kn 0.01mM and 48h L + Kn 0.1mM, respectively as compared to 60% in control. Based on dormancy breaking requirements, it was confirmed that seeds failed to germinate under favourable conditions throughout the study period (8 months). However seeds germinate only at low temperature (4 °C). Pretreated seeds shifted from 4 °C to 25 °C got deteriorated within 10 days. The data indicate that seeds of *Bunium persicum* exhibit morpho physiological dormancy.

Keywords: Germination, dormancy, hormones, leaching, medicinal, stimulants

Introduction

Bunium persicum (Kala zeera) is a major spice crop distributed in dry temperate zone at higher elevation ranging from 1850m to 3100 m in Lahaul spiti and Kinnour, districts of H.P. *Bunium persicum*. The genus *Bunium* contains about 166 species, *B. persicum*, *B. carum*, *B. bulbocastenum*, *B. copticum*, *B. flexuosum*, *B. elegans*, *B. cylendricum* and *B. chaerophyllocides* were occurred in Central Asia, Caucasus crimea and Europe (Vasilava *et al.*, 1985) [47]. Plant species had been reported to grow wild in North Himalayan region (Chopra *et al.*, 1956) [12]. It was also found in dry temperate regions of Jammu Kashmir, Afghanistan and Baluchistan (Sarin, 1967; Dutt *et al.*, 1972; Raina and Jamwal, 1989) [34, 14, 32]. *B. persicum* has possessed various chemical compounds like p-cymene and cuminaldehyde had been isolated through Gas Liquid Chromatography (GLC). The flavour of kala zera is due to γ -terpinene and p-cymene (Thappa *et al.*, 1991) [43]. It was found cuminaldehyde, p-mentha-1, 3-dien-7-al and p-mentha-1, 4,7-al were the main components (Panwar, 2000) [29] of kala zeera. Volatile components were also isolated from *B. persicum* through supercritical fluid extraction method (Pourmorta *et al.*, 2005) [30]. Various species of *Bunium* had different compounds which are responsible for essential oil as mentioned in (Table2). The essential oil of *B. persicum* is used in the perfume industry and in confectionery (Sormaghi *et al.*, 2002) [35]. Commercially, *B. persicum* is confronted with two major constraints including long juvenile period (3-4 years) and seed dormancy. Baskin and Baskin (2004) [9] have classified dormancy into five major groups as mentioned in (Table 2). Earlier an attempt at somatic embryogenesis and organogenesis in *B. persicum* was conducted (Wakhlu *et al.*, 1990) [49]. But due to cost effective nature of micro propagation technique, seed germination was hampered.

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Seed germination was mostly restricted due to dormancy. Researchers suggested that seed coat dormancy can be overcome by mechanical and chemical stratification in *Melilotus* and *Trigonella* (Baskin *et al.*, 2003) [8]. In contrast, evidences shows that stratification at 3-5 °C for 20d resulted in seed germination in *B. persicum* but the best germination occurred at 46 d (Banyanpour and Khui, 2001) [7]. Enhanced seed germination in black zeera population from Iran has been observed by Kinetin or benzyladenine treatment and increase duration of stratification (Sharefi, 2006) [37]. Some researchers believe that dormancy is removed by leaching over a period of time (Villiers and Wareing, 1965; Hamilton and Carpenter, 1976) [46, 18] and GA application. Reports showed that seeds of certain plant species such as *Lythyrus salicaria*, *Nicotiana tobaccum* exhibit dormancy due to specific light requirement. It could be overcome by treatment with KNO₃ and GA₃. Kinetin enhanced the effect of red light in breaking dormancy ((Panneerselvam, 1998) [26]. Evidences showed that seeds of plants like *Trollius* possessed morpho-physiological dormancy which can be broken by combination of warm (> 18°C) and cold (0-10 °C) stratification of GA₃ treatment (Hepher and Roberts, 1985) [19]. In some species for e.g.,

Polygonum convolvulus (Timeson, 1966) [39] and *Stachys alpinum* (Pinifield *et al.*, 1972) [27] GA₃, Thiourea and kinetin are the substitute for the cold stratification. Seeds of *Impateins parviflora* (Nikolaeva, 1969) [24], *Acer tartarticum* and *Acer platanoides* (Pinifield *et al.*, 1974) [28] have possessed deep dormancy which can be overcome by colder warm stratification (Baskin *et al.*, 2005) [10]. The other natural inhibitors are coumarin, parascorbic acid, ammonia, phthalides, ferulic acid and abscisic acid. Tetcyclasis, uniconazol, ancymidol and paclobutrazol are highly active GA synthesis inhibitors (Rademacher, 1991) [33].

At some time seed dormancy is due to chemical inhibitors in apple and cheery can be overcome by after ripening i.e., storing the dormant seed in moist, well aerated and low temperature conditions (Villiers and Wareing 1965; Chien *et al.*, 1993) [46]. Low temperature treatment often enhances the germination in many seeds possibly due to degradation of ABA present in seeds and activation of gibberalic acid synthesis (Bewley and Black, 1982) [4]. Various physico-chemical (KNO₃, SNP and NaN₃) and hormonal (GA₃) treatment alleviated the dormancy (Sharma *et al.*, 2006; Kumar, 2008) [38, 23].



Plate 1: Photograph of *B. persicum* showing flowering and seeds

Material and Methods

Seed Collection

Seeds of *Bunium persicum* were collected from Lahaul, Himachal Pradesh (altitude: 2900-3850 m) during Aug-Sept from the wild populations. The district Lahaul and Spiti lies in the state of H.P., between 31°44' 57" and 32°59' 59" north latitude and 76°46' 29" and 78°41' 34" east longitude (Fig 1.1). The threshold of the Lahaul plateau is nourished by the

Chandra and Bhaga river (Balokhara, 2006) [11]. The summers are clear and cloudless. The precipitation during winter months (December to middle of April) is three times than during the monsoon period. The typical cold desert of Lahaul and Spiti encompasses the rich trans-Himalayan Flora. Seeds were separated manually, air dried for about a fortnight at room temperature and stored in air tight plastic jars at room temperature for subsequent studies.

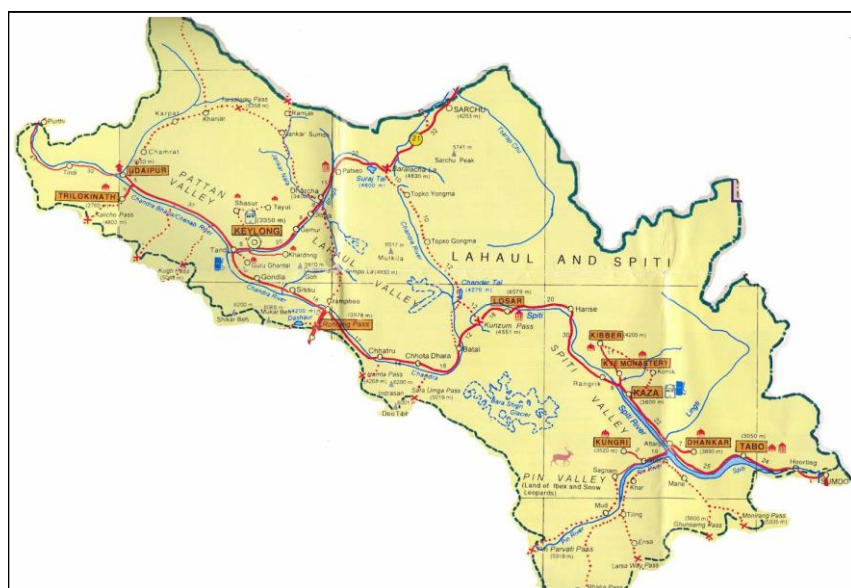


Fig 1: Map showing the location of the study area in Lahaul-Spiti

Chemicals and Reagents

Table 1: The following chemicals were used in the present study

I	Gibberellic acid (GA ₃)	Sigma Chemicals, Co. Mo USA
ii	2,3,5-triphenyltetrazolium chloride (TTC)	Sisco Research Laboratories, Mumbai
iii	Starch	S.D. Finechem., Mumbai
iv	Sulphuric acid (H ₂ SO ₄)	S.D. Finechem. Mumbai
v	Sodium carbonate (Na ₂ CO ₃)	S.D. Finechem. Mumbai
vi	Tris buffer	Loba-Chemie, Bombay
vii	Calcium chloride (CaCl ₂), Mercuric chloride (HgCl ₂), Iodine	Ranbaxy Lab., SAS Nagar, Punjab
viii	Indole acetic acid (IAA)	Sisco Research Laboratories, Mumbai
ix	Kinetin	Sigma Chemicals, Germany

All other chemicals and reagents used were of analytic grade and were procured from either Sigma or Ranbaxy Co.

Seed Viability: Seed viability was determined, qualitatively as well as quantitatively, using 2,3,5-triphenyl tetrazolium chloride (TTC) reduction assay

<p>Qualitative test The seeds were surface sterilized with 0.1% aqueous solution of HgCl₂ for three minutes, washed thoroughly under running tap water and soaked in distilled water for 24 h at 25 ± 2 °C. Thereafter, the seeds were cut off 1/3rd at the broad end opposite the radicle in order to expose the embryos. Then the seeds were soaked in 0.1% aqueous solution of TTC at 25 ± 2 °C in dark. After 24 h, qualitative viability as determined by counting the coloured embryos. Seeds having a completely stained (pink/red) embryo were considered viable. The experiment was done in triplicate</p>	<p>Quantitative Test The TTC assay conducted as above was extended to get an idea about the amount of TTC reduction by embryo containing half seeds. Thus, the embryos containing half seeds were treated with 0.1% solution of TTC for 24 h in dark. Thereafter, they were homogenized in methanol and the homogenate was centrifuged at 10,000 rpm for 10 minutes to remove the debris, volume was made to 5 ml with methanol and absorbance was read at 485 nm. The TTC reduction was expressed as A_{485/5} embryos in <i>Bunium persicum</i>. The experiment was done in triplicate.</p>
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Seed germination assays

The germination tests were performed according to the rules prescribed in the International seed testing association (ISTA, 1966). The seeds selected for uniformity (on the basis of colour and size as far as possible) were surface sterilized with 0.1% HgCl₂ for 5 minutes, washed thoroughly under the running tap water and soaked in distilled water for 24 h at 25 ± 2 °C. Thereafter, the seeds were transferred to petridishes lined with three layers of filter paper moistened with distilled water and allowed to germinate in a seed germinator under continuous illumination provided by the fluorescent white light (PAR: 40 μmol m⁻² s⁻¹). Seeds were considered germinated upon the emergence of 2.5 mm radicle (ISTA, 1966) and recorded periodically for germination until the final count. Germination percentage, was calculated as follow:

$$\% \text{ Germination} = \frac{\text{Number of seeds germinated (final count)}}{\text{Total number of seeds}} \times 100$$

Physical and hormonal treatment for dormancy removal / germination improvement

The seeds were subjected to the following physical and hormonal treatments in order to find out their efficacy in dormancy alleviation/germination improvement.

Stratification: Surface sterilized seeds soaked in distilled water for 24 h were subjected to low temperature (4 °C) treatment in a refrigerator for variable periods (2 to 3 months). Thereafter, the seeds were subjected to germination conditions as described in seed germination assay.

Hormonal treatment (Gibberellic acid (GA₃), Indole acetic acid (IAA) and Kinetin (Kn) Seeds were soaked in aqueous solution of gibberellic acid (0.01 and 0.1 mM), Indoleacetic acid (0.01 and 0.1 mM) and Kinetin (0.01 and 0.1 mM) for 24 h. These concentrations were chosen on the basis of a set of preliminary experiments by examining/monitoring the response to a range of concentrations. Thereafter, the seeds were subjected to germination assays.

Leaching + Chilling treatment: Seeds were subjected to 24 h, 48 h running water for leaching. Then the seeds were kept for moist chilling at 4 °C for 2 months. Thereafter, seeds were kept for germination at 25 °C.

Leaching + Hormonal treatment (48 h L + Kn (0.01 and 0.1 mM); (48 h L + GA₃ (0.1 mM): Seeds were treated with hormones for 24 h after 48 h leaching. Seeds soaked in distilled water for 24 h served as control. Thereafter, seeds were transferred to moist (distilled water) substratum for germination at 25 ± 2 °C.

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

Result

Viability status: The seeds of *B. persicum* exhibited 100% viability as tested by 2, 3 5-triphenyl tetrazolium chloride (TTC) reduction method qualitatively.

Germination/dormancy status: The seeds of *B. persicum* exhibited very deep dormancy as the seeds failed to germinate when provided with favourable germination conditions throughout the study period (8-months). At low temperature (4 °C) no deterioration was observed throughout chilling period (3 months) but as soon as the seeds were shifted to normal temperature (25 ± 2 °C) in seed germinator for germination, the stratified seeds (3 months) also got deteriorated within 10 d of incubation (Fig. 3.1). In control (dw) seed germination started after 75 d of continuous chilling treatment at 4 °C and showed 18.66% germination which increased gradually up to 60% after 150 d. Seed germination was promoted by GA₃ (0.1 mM), IAA (0.01 mM) and kinetin (0.01 mM) (Plate 3.1). Kinetin (0.01 mM) was found to be most effective caused 77.33% germination within 150 d at 4 °C (Fig. 3.2 A) as compared to 60% in control. Other treatments tested namely, 24 h, 48 h leaching and combined treatments 48 h + Kn (0.01 mM, 0.1 mM) and 48 h

L + GA₃ (0.1 mM) exhibited varied effect. Leaching (24 and 48 h) alone was not found to be effective, rather it was inhibitory for seed germination. For example, 40 and 41% germination was observed at 24 and 48 h leaching, respectively as compared to 60% in control (Fig. 3.2 B). Hormonal treatments (Kinetin, 0.01 and 0.1 mM) when applied to seeds after 48 h leaching enhanced seed germination. Lower concentration was found to be more

effective, around 72% and 68% germination was observed at 48 h L + Kn 0.01 mM and 48 h L + Kn 0.1 mM, respectively as compared to 60% in control. GA₃ treatment was not found to be effective in improving the germination percentage as only 56% germination was exhibited at GA₃ 0.1 mM concentration when applied after 48 h leaching as compared to 60% in control (Fig. 3.2 B). The effectiveness of treatment can be mentioned in (Table 3).

Table 2: Types of various seed dormancy

S. No	Types of dormancy	
1	Physical dormancy (PY)	Seeds of many plants possess hard seed coat which is impermeable to water and gases. The impermeability is due to the presence of palisade layer of lignified cells which interfere with water uptake and gaseous exchange. It also acts as a barrier against the escape of inhibitors from embryo and may contain chemical inhibitors. Seed coat impermeability may also be due to the presence of phenolics.
2	Morphological dormancy (MD)	At the time of dispersal, seeds of some species contain immature embryos. They are not physiologically dormant but need time to grow e.g., Celery (Jacobson and Pressman, 1979). Morphological dormancy occurs in two types of embryos. (i) Differentiated embryos (ii) Undifferentiated embryos
3	Physiological dormancy (PD)	Physiological dormancy is associated with the physiological state of embryo. In this case, embryo is fully developed but fails to germinate under suitable germination conditions due to reasons such as hormonal imbalance or presence of chemical inhibitors in seed coat. As most of the chemical inhibitors are water soluble, they are translocated to embryo and inhibit its growth. PD can be divided into 3 levels. (i) Non - deep PD (ii) Intermediate PD (iii) Deep PD
4	Morpho-Physiological dormancy (MPD)	In this case, seeds possess both morphological as well as physiological dormancy having physiological dormant embryos. It is reported in a number of plant families e.g., apiaceae and lillaceae.
5	Combinational dormancy	In combinational dormancy, the seeds possess both physical (hard seed coat) and physiological dormancy e.g., <i>Geranium trifolium</i> (Ransom, 1935).

Source: (Baskin and Baskin, 2004) [9]

Table 3: Chemical compounds in various species of *Bunium*.

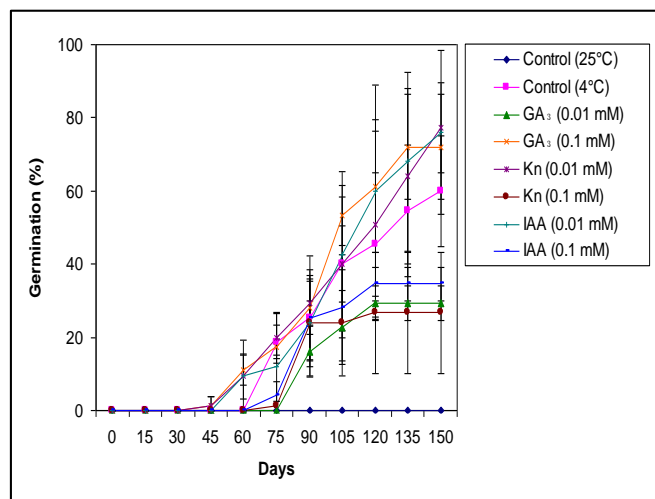
S. No	Name of species	Chemical composition	References
1	<i>B. elegans</i>	Germacrene-d (24.1%), E-caryophyllene (38%)	Jassbi <i>et al.</i> , (2005) [21]
2	<i>B. caroides</i>	Germacrene-d (22.1%), E-caryophyllene (26.6%)	Jassbi <i>et al.</i> , (2005) [21]
3	<i>B. cylindrum</i>	myristin (43.1%), b-phellandrene (20%), b-pinene (15.6%) and a-pinene (10.7%).	Shiva <i>et al.</i> (2005) [36]

Table 4: Effect of various treatments on seed germination of *B. persicum*

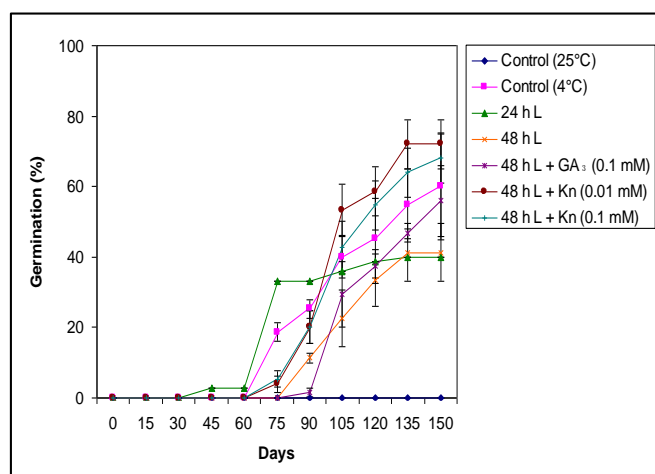
S. No	Treatments	Nature of treatment	% Germination
1	Stratification (4°C)		
1a	At 75 d of incubation	effective	18.66%
1b	At 150 d of incubation (control)	effective	60%
2	Hormonal treatment		
2a	GA (0.01Mm)	Least effective	—
2b	GA (0.1mM)	Least effective	—
2c	IAA(0.01mM)	effective	—
2d	IAA(0.1mM)	Least effective	—
2e	Kn(0.01mM)	Most effective	77.33%
2f	Kn(0.1mM)	Least effective	—
3	Leaching +Chilling Treatment		
3a	24h L	Effective	40%
3b	48h L	Effective	41%
4	Leaching +Hormonal treatment		
4a	48h L + Kn (0.01mM)	Most effective	72%
4b	48h L+Kn (0.1mM)	Most effective	68%
4c	48h L GA ₃ (0.1mM)	Effective	56%



Plate 1: Effect of hormones (GA₃, IAA and Kinetin) at 4°C, 25°C on seed germination of *Bunium persicum*.



A



B

Fig 2: Time-course of seed germination in *Bunium persicum* pretreated with different hormones directly (A) or following 24 h or 48 h leaching in water (B). Data are the arithmetic means. n = 3 ± s.e.m. 24 h L, 48 h L: 24 h and 48 h leaching in water.

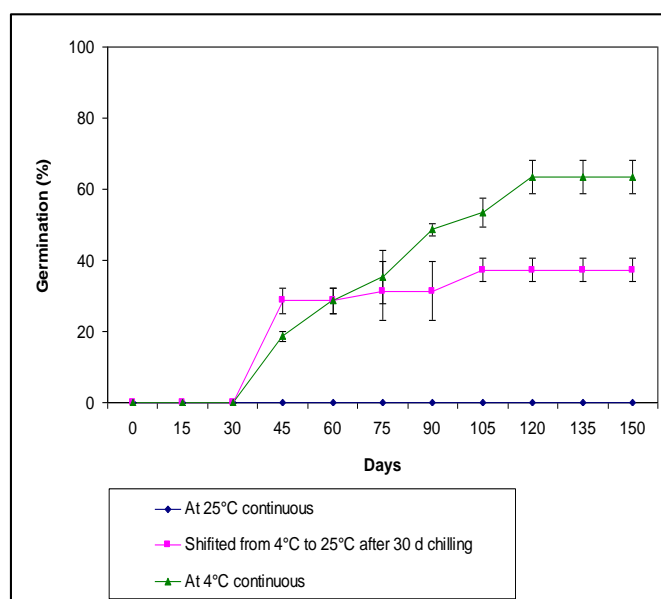


Fig 1: Time-course of seed germination in *Bunium persicum* as affected by temperature. Seeds were either incubated continuously at 4°C/25°C or shifted from 4°C after 30 d to 25°C. Data are the arithmetic means. n = 3 ± s.e.m.

Discussion

The present study was undertaken to explore some physico-chemical and hormonal treatments to alleviate the dormancy in *B. persicum* seeds. *B. persicum* is an important medicinal plant belonging to the family Apiaceae. Botanical description has been mentioned in introduction section. It is a perennial herb of cold desert regions of Himachal Pradesh. It is used as stimulant, carminative and is useful in diarrhoea and dyspepsia (Abduganiew *et al.*, 1997) [2]. The extract of *B. persicum* due to its hypoglycemic activity is commonly used to prevent diabetes and obesity (Giancarlo *et al.*, 2006) [17]. Besides being used for medicinal purpose, it is also used for flavoring food and beverages. It generally grows wild in scattered pockets in forests and grassy slopes. This species is now being cultivated in few areas of H.P. only. Due to relentless collection of seeds by rural populations and difficulties in cultivation of *B. persicum* through seeds, it has become a threatend species in H.P. (Ved *et al.*, 2003) [43]. Seeds of *B. persicum* have been reported to be deeply dormant.

Germination behaviour/dormancy status of a *B. persicum* population from Lahaul (H.P.) has been previously studied in our laboratory (Sharma *et al.*, 2006; Kumar, 2008) [38, 23]. The seeds were found to be deeply dormant. The germination could be induced only at low temperature, various physico-

chemical (SNP, KNO₃, NaN₃ and leaching alone) and hormonal (GA₃) treatments were effective in removing the seed dormancy. In all cases, the germination occurred only at low temperatures. The present study is an extension of the previous work done in our laboratory on seed germination/dormancy removal in *B. persicum* seeds. Some additional treatments namely, IAA, kinetin and combined treatments with leaching have been tested for alleviation of seed dormancy in *B. persicum*. Indole acetic acid (IAA) plays an important role in cell elongation whereas kinetin (Kn) regulates the cell division. Both processes are associated with seed germination. As in earlier studies (Sharma *et al.*, 2006, Kumar, 2008) [38, 23], the seeds of *B. persicum* exhibited deep dormancy; there was no germination under favourable germination conditions throughout the study period (8-months). Among all the treatments tried to remove the dormancy, only continuous moist stratification at 4 °C (chilling treatment) was found to be effective. A very peculiar feature of seeds of *B. persicum* was that the seeds of *B. persicum* germinated only at low temperature (4 °C); germination ceased when seeds were shifted from 4 °C to 25 °C. Furthermore, the seeds got deteriorated when incubated at 25 °C within 10 d.

The exogenous application of GA₃ did not substitute for cold stratification. All the treatments were effective only at 4 °C. GA₃ (0.01 and 0.1 mM), IAA (0.01 and 0.1 mM) and Kn (0.01 and 0.1 mM) effectively alleviated the seed dormancy at low temperature (4 °C). No improvement in germination or growth was observed when the seeds were shifted to 25 °C after chilling treatment (30 d). The stimulating effect of GA₃ has been reported in many medicinally important plant species (Grappin *et al.*, 2000; Nadeem *et al.*, 2000; Sharma *et al.*, 2006) [15, 25, 38]. GA₃ induced seed germination is largely on account of the promotion of α -amylase activity which in turn facilitated the availability of sugars for embryo growth. IAA is an important phytohormone involved in regulation of many growth processes in plants including cell elongation (Taiz and Zeiger, 2006) [44]. The GA₃ and IAA levels have been shown to gradually increase during germination (Atici *et al.*, 2007) [3]. Improved seed germination with IAA has been reported in *Phaseolus vulgaris* (Tillberg, 1974) [40], *Zea mays* (Ueda *et al.*, 1969) [45] and *Pinus silvestris* (Alden, 1971). Enhanced germination by kinetin in the present study may be due to the fact that cytokinins probably penetrate the testa and neutralize the inhibitors present in the embryo, thus enabling the embryo to rupture the seed coat (Khan, 1971) [22]. Sharefi and Pouresmail (2006) [37] showed that increased duration of stratification and kinetin or benzyladenine enhanced seed germination in black zeera population from Iran. However, it is not always necessary that a factor effective to one population would act similarly on other population. Cytokinins do not affect germination directly, it appears to be essential for completion of gibberellin induced germination processes (Khan, 1971) [22]. GA₃ are permitted to reach their active site through the modifying influences of cytokinins on transport across membranes and are then able to initiate the biochemical processes necessary for germination to occur (Thomas *et al.*, 1975) [42].

The combined treatment of GA₃ and Kn along with 48 h leaching inhibited the seed germination as compared to the individual treatments. Reduced germination through leaching may be due to the fact that the naturally occurring growth promoters (GA₃, IAA and Kn etc.) and inhibitors e.g., aldehyde, alkaloids, phenols, essential oils, organic acids, cyanide releasing compounds and ammonia releasing

compounds both might have been leached out from the seeds. The amount of growth promoters leached out may be higher than the inhibitors. Thus, caused inhibition in germination of *B. persicum* seeds through leaching. All the above treatments tested were effective only at low temperature (4 °C). The requirement of cold stratification for dormancy removal has previously been observed in a number of other species belonging to family Apiaceae for example, in *Osmorhiza* and *Erythronium* (Baskin *et al.*, 1995) [5]. Bungard *et al.* (1997) [6] showed that stratification resulted in an increase in germination percentage in seeds of *Clematis vitalba*. Banyanpour and Khui (2001) [7] reported that stratification at 3-5 °C for 20 d resulted in seed germination in *B. persicum* but the best germination occurred at 46 d. Increase in germination by stratification and GA₃ has been shown in *Leymus arenarius* seeds (Greipsson, 2001) [16] and in seeds of black spruce (Wang and Berjak, 2000) [50].

Conclusion

Bunium persicum is an economically and medicinally important plant belonging to the family Apiaceae. It is a perennial herb of cold desert regions of Himachal Pradesh and is used as carminative, diuretic and expectorant. It is also used as spice for flavoring foods. The ripe fruits of *B. persicum* are rich in secondary metabolites like alkaloids, phenols, flavonoids, terpenoids and various essential oils. The antioxidant properties of *B. persicum* seeds are due to the presence of essential oils in seeds. *B. persicum* generally grows wild in scattered pockets. Due to cultivation on small scale on few areas of H.P. and large extraction of the plant species from wild by rural populations, the status of *B. persicum* has become threatened in H.P. (Ved *et al.*, 2003) [43]. The seeds of *B. persicum* exhibit very deep dormancy which hampers the seed germination, thus restricting its cultivation. Some ecophysiological aspects of seed germination of a *B. persicum* population from Lahaul (H.P.) have already been studied in our laboratory (Sharma *et al.*, 2006; Kumar, 2008) [38, 23]. Seeds of *B. persicum* exhibited deep dormancy. All the tested seed treatments (GA₃, IAA, kinetin, leaching and combined treatments) were effective in alleviating dormancy. However, seeds germinated only at low temperature (4 °C). Pretreated seeds shifted from 4 °C to 25 °C got deteriorated within 10 d. The data indicate morpho-physiological dormancy (MPD) in seeds of *B. persicum*.

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