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SEED GERMINATION BEHAVIOUR OF *BERBERIS LYCIUM* ROYLE FROM ANNI KHAD WATERSHED AREA, KULLU (HIMACHAL PRADESH)

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Berberis lycium (Berberidaceae), commonly known as Kasmal or Indian barberry is an economically and medicinally important plant species. It is extensively used for the treatment of various liver, abdominal and skin diseases. All parts of the plant are used in various Ayurvedic preparations. According to the IUCN, it is categorized as vulnerable. In order to fulfill the demand of plant material of local populations and pharmaceutical companies it is required to cultivate the concerned plant species. It is generally propagated through seeds. The seeds of this plant species possess dormancy which hampers the seed germination. Therefore, understanding of seed physiological aspects would be of great significance in plant regeneration through seeds. Thus, we have examined the eco-physiological aspects of seeds of *B. lycium* from Anni Khad Watershed area from Kullu (Himachal Pradesh). Freshly harvested seeds of *B. lycium* exhibited poor germination (40% after 24 d). The effect of various physico-chemical (acid scarification (AS) 5 min., SNP 1 mM, KNO_3 2 mM and proline1 mM) and GA_3(1 mM) treatments were examined in order to achieve improved germination. Seed germination was enhanced by all the treatments. Among all the treatments applied, proline (1 mM) was found to be most effective in enhancing seed germination performance. It caused 78% germination as compared to 40% germination in control. All other treatments were also effective in improving seed germination percentage, germination speed and emergence index, when applied individually or after acid scarification. The magnitude of enhancement in seed germination was higher with individual treatments as compared to combined treatments. Enhanced germination with all the treatments applied individually was consistent with the increased α - amylase activity. In contrast, acid scarified seeds treated with various effectors exhibited decreased α-amylase. It might be due to the fact that AS alone inhibited the α - amylase activity. Data having implications for seed-based multiplications of *B. lycium*

Key words: Seed germination, *Berberis lycium,* GA_3, α -amylase, Kullu (HP)

Berberis lycium (Berberidaceae) commonly known as Indian barberry or kasmal, is an economically and medicinally important plant species in Indian Himalayan Region growing at an altitudinal range between 1200-2400 m amsl. It is an evergreen, erect shrub upto 3 mts height. The stem and branches are pale whitish to greyish and contain spines arranged alternately on the stem. Leaves are brightly coloured, thick and entire or toothed with oblanceolate to oblong-obovate shape. Inflorescence is corymbose raceme with 11- 16 flowers per cluster and the flowers are hermaphrodite. Fruits (berries) are about 8 mm long which acquire bright red colour or purplish colour on ripening (Fig.1). Flowering and fruiting occurs from March to July.

B. lycium is extensively used for the treatment of various human diseases. The roots contain a number of alkaloids; berberine is the major alkaloid universally present in the rhizomes of *Berberis* species (Khosla *et al.* 1992). It has wide application in pharmaceutical industries. It is used in the preparation of drugs for cholera, diarrhoea, dysentery and eye troubles (Chandra and Purohit 1980). In Unani system of medicine, it is used for the treatment of leprosy (Chopra *et al.* 1981). Every part of the plant is used for medicinal purpose. Its root, bark, stem and fruits are used in various Ayurvedic preparations (Bhattacharjee 2004). *Berberis* is used extensively for liver disorders, skin, abdominal disorders and cough (Ahmed *et al.* 2009). Besides, berberine other chemical constituents are, berbamine, gilgitine, sindamine, malic acid and ascorbic acid (Khare 2004). Berries of *B. lycium* are generally consumed raw especially by the rural population and are a rich source of vitamin C and anthocyanins (Sood *et al*. 2010). Dried leaves are used as tea (Ahmed *et al*. 2009). It is also an important source of dye and tannin and is used for dyeing clothes and for tanning leather. Herbal wine is prepared from *Berberis* in Himachal Pradesh. It is also used for fencing and fuel purposes by local inhabitants. According to International union for conservation of nature (IUCN), *B. lycium* is

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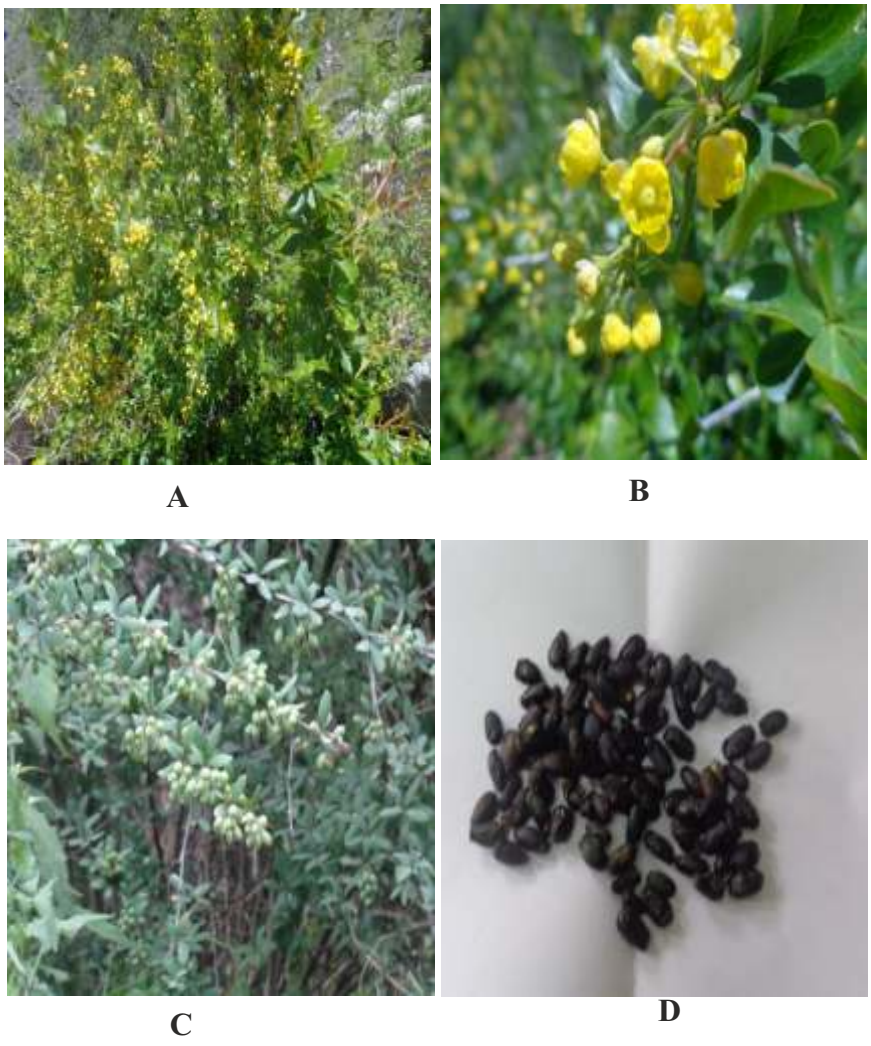

Figure 1: Photographs of *B. lycium* showing plant in natural habitat (A), Flowering (B), Fruiting (C) Mature fruits (D)

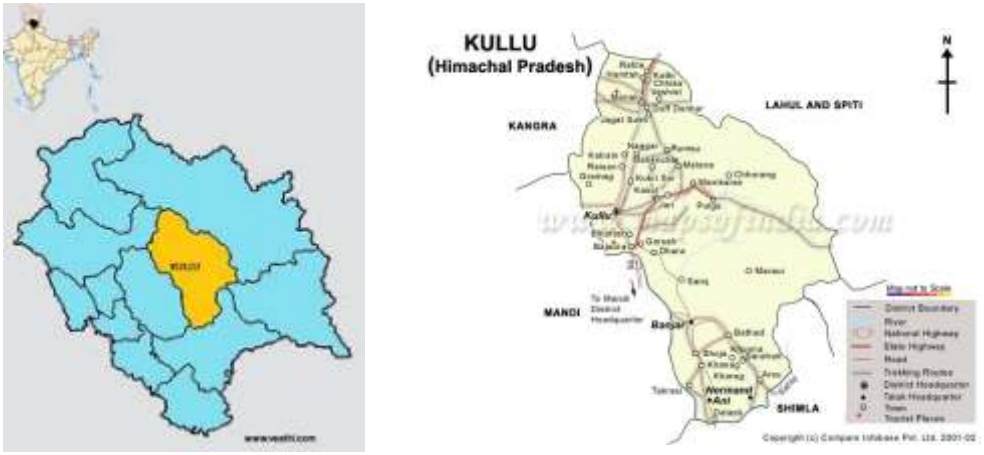

Figure 2: Map of Himachal Pradesh showing the location of the study area (Kullu district)

categorized as vulnerable (Waseem *et al.* 2006, Hamayun *et al*. 2006). *B. lycium* being a valuable multipurpose plant is under consistent demand by local population and pharmaceutical companies. In the prevailing scenario it is required to cultivate and conserve the concerned plant species. It is generally propagated through seeds but its germination capacity is poor. The understanding of its seed germination behaviour would be of significance for seed-based propagation. Therefore, in the present study seed physiological aspects of *B. lycium* have been examined.

MATERIALS AND METHODS

Study area

The study area, Anni Khad Watershed is located in district Kullu (HP). It is situated approximately 140 kms away from the district headquarters of Kullu. Anni is situated between 31^0 58' 88" N latitudes and 77^0 25' 4" E longitudes. The altitudinal range varies between 700-4000 m above mean sea level. Climate of the region is sub-tropical to alpine type. Temperature shows a great variation from season to season and along an altitudinal gradient. Majority of the portion of Anni tehsil is covered by Anni Khad Watershed which receives an annual rainfall of 800 -700 m.

Seed collection and storage

Mature fruits (ripe and dark purple in colour) were collected in May/June 2014 from the wild in the AnniKhad Watershed area. The flesh was removed and the seeds were washed, dried and stored in air-tight containers for subsequent studies.

Viability test

Seed viability was determined qualitatively by International seed testing association topographic tetrazolium chloride (TTC) test (ISTA 1996). The seeds were surface sterilized with 0.1% aqueous solution of $HgCl_2$ for 5 min., washed thoroughly under running tap water and soaked in distilled water for 24 h at 25±2°C. The seeds were then cut off 1/3 rd at the broad end opposite the radicle in order to expose the embryos. Thereafter, the seeds were soaked in 0.1% aqueous solution of TTC at 25±2°C in dark for 24 h. Viability was determined by counting the coloured embryos. Seeds having completely red stained embryos were considered viable. The experiments were carried out in triplicate.

Seed germination assays

The seed germination tests were performed as prescribed in the International seed testing association (ISTA 1996) rules. Seeds were surface sterilized with 0.1% $HgCl_2$ for 5 min. followed by thorough washing with distilled water. They were soaked in distilled water (control) or with solutions of different effectors for 24 h at 25±2°C. Thereafter, the seeds were transferred to petri-dishes lined with three layers of filter paper moistened with distilled water and allowed to germinate in the seed germinator at 25±2°C under continuous illumination provided by the fluorescent white light (PAR:40 μ mol m^{-2} sec^{-1}). Emergence of 2-5 mm radicle was taken as seed germination. The day of radicle emergence was recorded and thereafter seed germination was recorded at regular intervals until the final count. Experiments were performed in triplicate. The treatments included SNP (1 mM), GA_3 (1 mM), Proline (1 mM), KNO_3 (2 mM)) that were applied individually or following acid (H_2SO_4) scarification of seeds.Acid scarification was also applied individually.

Germination characteristics

Certain seed germination characteristics namely, mean germination time (MGT), emergence index (EI) and germination speed (GS) were calculated asfollows:

Mean germination time (MGT)

Mean germination time was calculated by the method given by Hartmann and Kester (1989)

MGT= $\sum$ nd/N, Where, n = number of seeds which germinated after each incubation period in days (d) N = total number of seeds emerged at the end of test.

Emergence index (EI)

EI = dn/n

Where,

dn = emergence end

n = day of emergence

Germination speed

$$\text{Germination speed} = \frac{\text{Germination percentage}}{\text{Day of completion of germination}}$$

α- amylase activity measurement

α- amylase activity was determined by the method given by Filner and Varner (1967). Seeds treated as described above for 24 h were homogenized in 0.05 M Tris- HCl buffer at pH 7.2 at 4°C. The homogenate was centrifuged at 10,000 rpm for 10 min. at 4°C and the supernatant served as enzyme extract. 1ml of enzyme extract was added to 1 ml of the substrate (0.15% starch, 0.2 mM $CaCl_2$ in Tris-HCl buffer at pH 7.2) and incubated for 10 min. at 25°C. Thereafter, 3 ml IKI reagent (0.6% iodine in 6% KI; 1 ml of this diluted to 50 ml with 0.05 N HCl) was added to the reaction mixture to quench the reaction and absorbance was recorded at 620 nm. In control set, IKI reagent was added prior to adding the enzyme. The amount of starch degraded was determined with the help of a calibration curve.

Determination of protein contents

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

Statistical analysis

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

RESULTS

Seed viability

Qualitative seed viability was monitored in freshly harvested seeds of *B. lycium* by using 2, 3, 5- triphenyltetrazolium chloride (TTC) reduction test. Seeds of *B. lycium* exhibited 80 % viability at the time of harvest.

Seed dormancy/germination

The freshly harvested seeds of *B. lycium* started germinating on 7 d (2.2%) of incubation which eventually increased to 40% on 24 d of incubation when subjected to favourable germination conditions. Thereafter, it remained constant. Various physico-chemical treatments namely, acid scarification (5 min.), SNP (1 mM), KNO_3 (2 mM) and proline (1 mM) and GA_3 (1 mM) treatments were applied to enhance seed germination. All the treatments applied individually or in combination were effective in improving germination performance except acid scarification for 5 min. (Fig.3). In general, the individual treatments were more effective in enhancing seed germination than the combined treatments (treatments applied to acid scarified seeds). Among all the pretreatments applied, proline (1 mM) was found to be most effective which caused 78 and 62% germination with 2.9 and 2.6 germination speed in non scarified and acid scarified seeds, respectively as compared to 40% germination with 1.7 germination speed in control. No substantial effect of proline was found on MGT (Fig. 3 A, B; Plate 1, 2 ; Table 1).

Other treatments e.g., SNP, KNO_3 and GA_3 were also effective in enhancing seed germination and caused 69, 60 and 53% germination with 4.2, 4.0 and 3.6 emergence index and 2.8, 2.5 and 2.1 germination sPeed, respectively in non scarified seeds. Acid scarified seeds treated with GA_3 (1 mM), SNP (1 mM) and KNO_3 (2 mM) exhibited 49, 44 and 53% germination with 3.0, 3.1 and 2.8 emergence index and 2.0, 1.6 and 2.0 germination speed, respectively as compared to 40% germination with 3.4 emergence index and 1.7 germination speed in control (Fig. 3 A, B; Table 1). In contrast, acid scarification applied individually inhibited the seed germination. MGT in seeds varied with various pretreatments. It was reduced with SNP, KNO_3 and GA_3 applied individually as compared to control. Whereas it was enhanced with acid scarification and combined

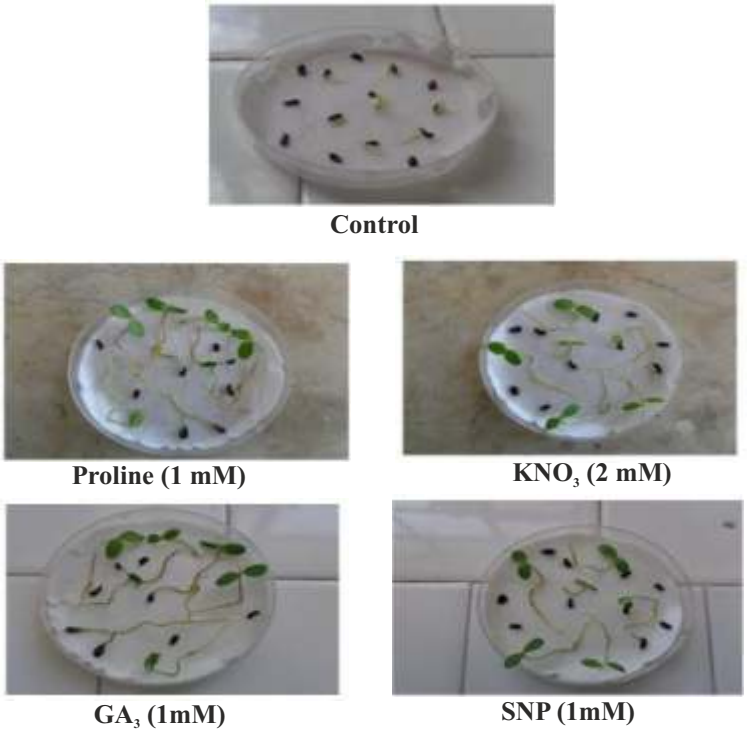

Plate 1:Photographs showing the effect of various chemical (Proline, SNP, KNO_3) and GA_3 treatments on seed germination in freshly harvested seeds of *B. lycium* after 20 d of incubation

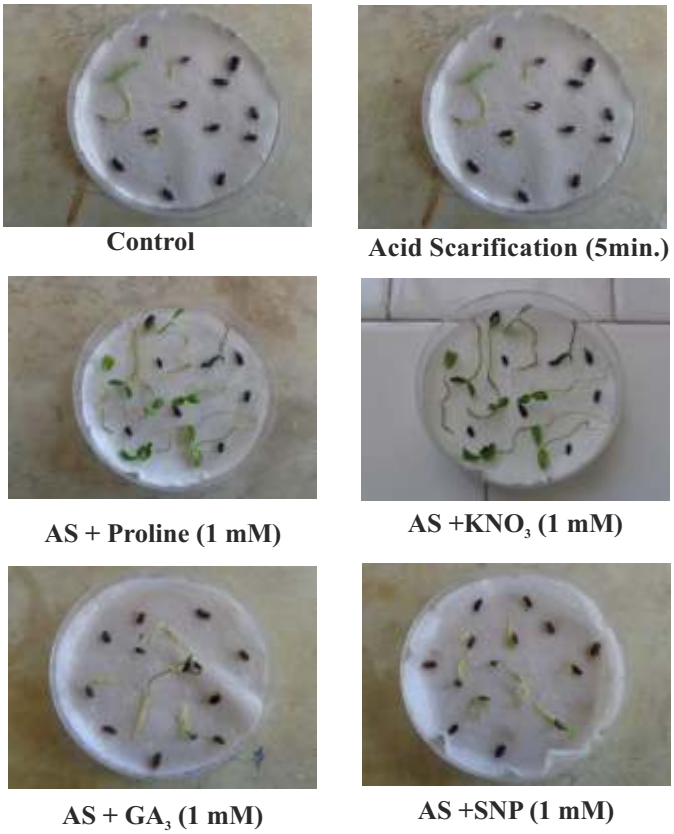

Plate 2: Photographs showing the effect of proline, KNO_3, SNP and GA_3 treatments on seed germination in freshly harvested seeds of *B. lycium* following acid scarification after 20 d of incubation

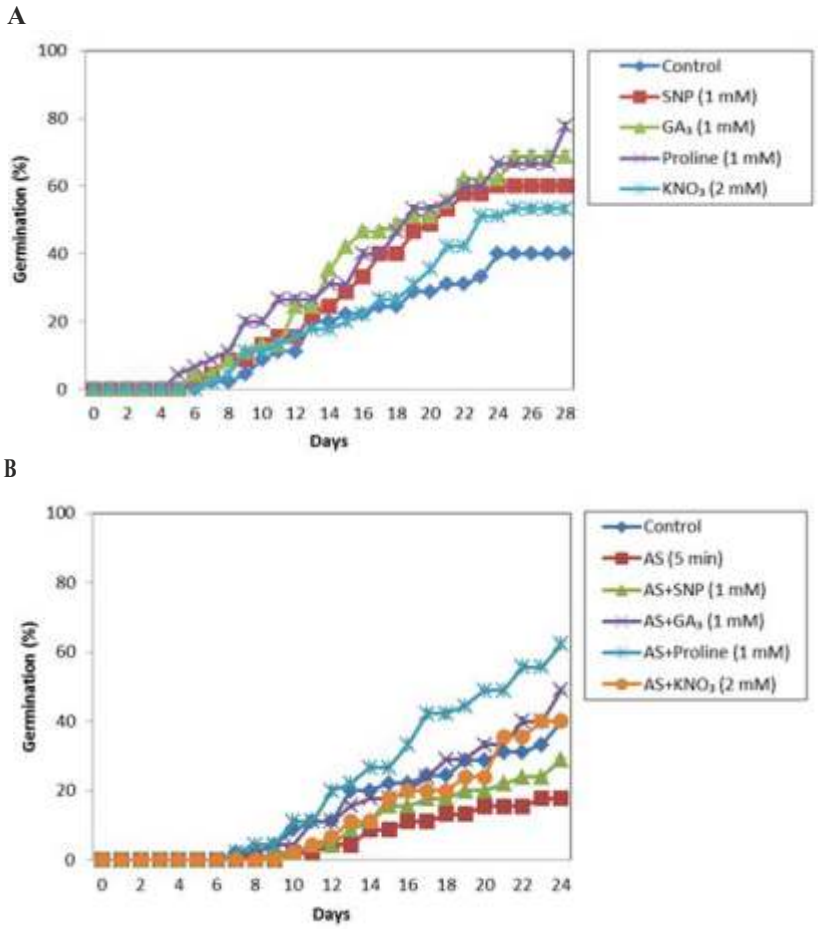

Figure 3: Time- course of germination of freshly harvested seeds of *B. lycium* as affected by different chemical and GA_3 treatments applied individually (A) or following acid scarification (B). Data are arithmetic means n=3 ± S.D.

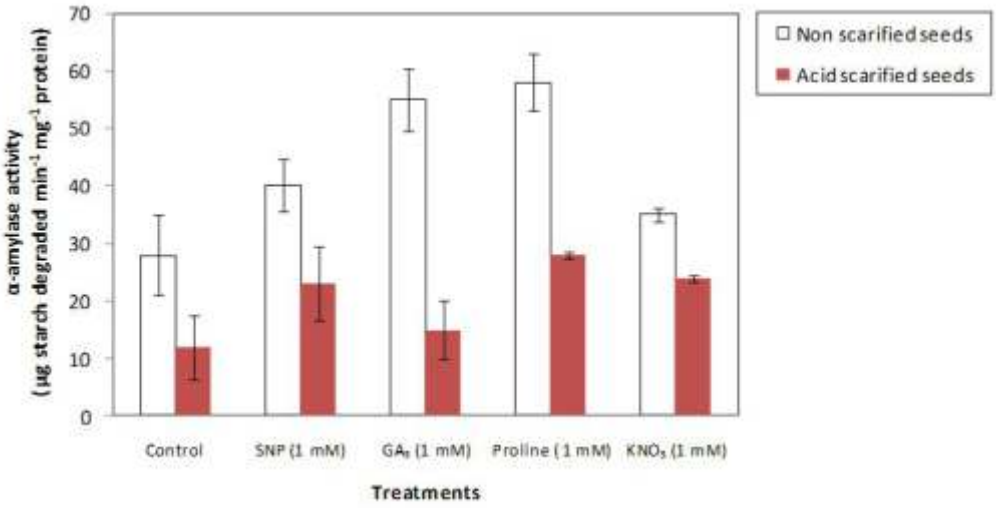

Figure 4: α-amylase activity in *B. lycium* seeds as affected by different chemical and GA_3 treatments (24 h) applied individually or following acid scarification. Data are arithmetic means n=3 ± S.D

Table 1: Effect of various physico-chemical and hormonal treatments on germination (%, final count), mean germination time (MGT), emergence index (EI) and germination speed (GS) in seeds of *B. lycium*

Treatment	Germination (%)	MGT (days)	Emergence index	Germination speed
Control	40	16.94	3.4	1.7
SNP (1 mM)	60	15.29	4.0	2.5
GA_3(1 mM)	68.8	15.48	4.2	2.8
Proline (1 mM)	77.7	16.42	5.4	2.9
AS (5 min)	29	20.1	2.5	1.2
KNO_3 (1 mM)	53.3	15.9	3.6	2.1
AS+SNP (1 mM)	44.4	19.7	3.1	1.6
AS+GA_3 (1 mM)	49	17.1	3.0	2.0
AS+Proline (1 mM)	62.2	16.46	3.4	2.6
As+KNO_3 (1 mM)	53.3	18.1	2.8	2.0

treatments (SNP, KNO_3 and GA_3 applied to acid scarifiedseeds as compared to control (Table 1).

To understand the possible mechanism underlying the germination enhancement by various effectors, activity of α-amylase in seeds was determined. The α-amylase activity was enhanced by proline (1mM), GA_3 (1 mM), SNP (1 mM) and KNO_3 (2 mM) treatment and inhibited by acid scarification and all other combined treatments as compared to control. The enzyme activity was increased 2.1, 2, 1.4 and 1.2 fold with proline (1 mM), GA_3 (1 mM), SNP (1 mM), and KNO_3 (2 mM), treatments, respectively. It was found to be decreased 2.3, 1.3, 1.9, 1 and 1.1 fold with acid scarification, AS + SNP (1 mM), AS+GA_3 (1 mM), AS+ proline (1 mM) and AS+ KNO_3treatment, respectively as compared to control (Fig. 4). In control the α -amylase activity was 28 µg starch degraded /min/ mg protein.

DISCUSSION

The present study was carried out to determine the viability and germination characteristics in freshly harvested seeds of a population of *B. lycium* from Anni (Kullu, H.P.). The results revealed the freshly harvested seeds of *B. lycium* to be highly viable (80%) with about 40% germination (24 d) in control. Different pretreatments (proline 1 mM, SNP 1 mM, KNO_3 2 mM, AS and GA_3 1 mM)) applied individually or following acid scarification were effective to varying degrees in germination improvement. Among them proline was found to be most effective. The role of proline on seed germination has been mainly studied under stress conditions where it is known to alleviate different types of stresses like water stress, salinity stress, heavy metal stress etc. (Kubala *et al*. 2015, Ali *et al*. 2007, Tabatabaei 2013, RezwanMolla *et al*. 2014). However, its role in improving seed germination under non stress conditions is very less known. Poljakoff- Mayber *et al.* (1994) reported that in seeds proline contents increase during germination. Catabolism of proline in stress free conditions generates ATP to provide energy for seed germination. One molecule of proline yields 30 ATP equivalents. (Hare and Cress, 1996). Proline has also been reported to scavange different free radicals (Kaul *et al*. 2008). It acts as pro-oxidant as well as antioxidant and maintains a redox balance in the cell which is crucial in seed germination.

Stimulation of growth by proline is usually attributed to the nitrogen content of this amino acid.

Improved seed germination by GA_3 might be due to the activation and synthesis of α-amylase, a hydrolytic Enzyme that breaks down starch into simple sugars which are transported to the embryo, where it promote the growth of seedlings. GA_3 increases the growth potential of embryo and promotes germination. It is also involved in reducing mechanical restraint conferred by the seed covering layers by weakening of the tissues surrounding the radicle (Finch- Savage and Leubner-Metzger 2006). SNP is a nitric oxide (NO) donor. NO is a reactive gaseous free radical that functions as a signaling molecule. Enhancement of seed germination by SNP might be due to the enhanced synthesis and secretion of α-amylase through its GA signal transduction or independent of GA. SNP causes storage reserve degeneration and promotes imbibition, finally leading to stimulated seed germination (Zhang et al. 2003).

In the present study KNO_3 also enhanced the seed germination performance. A reduction in the average time of germination in seeds primed with KNO_3 was observed in other species e.g., *Gladiolous alatus* (Musthaq *et al*., 2012). It might be due to KNO_3 causing a shift in respiratory metabolism to pentose phosphate pathway (Robert and Smith 1977). The positive effect of KNO_3 has been reported on seed germination and seedling growth of many species (Soltani et al. 2006, Ahmadvand *et al.*, 2012, Abdolahpour and Lotfi 2014). It has been suggested that KNO_3 increases nitric oxide (NO) synthesis by activating the nitrate reductase (NR) (Lara *et al.* 2014) and NO synthase (NOS), key enzymes for NO synthesis. It has also been proposed that nitrate stimulates germination by acting as an osmoticum thus enhancing the water uptake in dormant caryopses of *Avena fatua* (McIntyre 1997). NO breaks dormancy or stimulates germination through the interaction with the phytochrome signaling pathways, the ethylene biosynthesis and interplays with reactive oxygen species. Nitrate broke the seed dormancy in *A. thaliana* seeds by reducing ABA levels. (Matakiadis et al., 2009). Scarification with sulfuric acid showed a slight inhibitory effect on the seeds of *Berberis*. Inhibitory effect of acid treatment on seed germination has been reported in many cases (Suthar *et al*. 2009). These findings support our result. However, the combined effects of various treatments promoted germination. This may be due to removal of physical barrier for water uptake and gaseous exchange for initiating the germination. Availability of enhanced effector molecules enhances the seed germination. The present data revealed that the seeds of *B. lycium* possess physiological dormancy. These findings would be helpful in formulating cost- effective and simple methods for seed based conservation and propagation.

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