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SEED GERMINATION CHARACTERISTICS OF RATANJOT, *ARNEBIA EUCHROMA* (ROYLE) I.M. JOHNSTON, A THREATENED MEDICINAL PLANT SPECIES FROM COLD DESERT OF SPITI (HIMACHAL PRADESH, INDIA): IMPROVEMENT BY GA_3, SODIUM NITROPRUSSIDE AND PROLINE

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Arnebia euchroma (Royle) I. M. Johnston, commonly known as Ratanjot, is a threatened, medicinally important plant species. It is used to cure an array of ailments including cough, lung problems, hair fall, dandruff and menorrhagia etc. The roots are also used to colour food preparations by the inhabitants of Lahaul and Spiti. We have characterized the seed germination aspects of *A. euchroma* from the cold desert region of Spiti (Himachal Pradesh, India) in W. Himalaya with an emphasis on achieving improved seed germination performance through pre-treatment of seeds with GA_3 and certain physico-chemical effectors. The freshly harvested seeds exhibited poor germination (30%). Of the tested effectors, GA_3 was most effective in promoting the seed germination. Besides, sodium nitroprusside (SNP) and proline proved nearly as effective as GA_3; these effectors have not been commonly employed for germination improvement in high altitude medicinal plants. KNO_3 was marginally promotory but acid scarification of seeds had virtually no effect. Analyses of certain biochemical parameters such as free proline contents, total phenolic contents, MDA contents and α-amylase activity provided insight into the mechanism of stimulation of seed germination by the stated effectors. The findings have implications for the conservation and cultivation of *A. euchroma*.

Keywords: *Arnebia euchroma*, GA_3, Proline, Seed germination, Sodium nitroprusside

The Indian Himalayan region (IHR) is a part of Himalayan centre of biodiversity, one of the mega hot spots of biological diversity (Myers *et al*. 2000). It harbours numerous endemic, threatened and rare plants which the local inhabitants use for different purposes including medicine, food, fodder, fuel, timber, making agricultural tools and religious ceremonies (Samant *et al*. 1998). Spiti, a well-known cold desert, an integral part of North Western Himalaya, is home to many medicinal plants used in local Amchi as well as in other traditional healing systems. Although the vegetation in entire Spiti valley is sparse owing to dry, arid and harsh climate, it is varied and diverse. The unique tiny populations of medicinal plants in the region are prone to overexploitation due to ever increasing local and market demands. Indeed, a significant depletion of the medicinal plant populations in natural habitats has occurred due to unscientific collection and habitat destruction (Kala 2000). The prevalent extreme climatic conditions significantly restrict the seed germination and propagation of these plants. Apparently, the conservation and cultivation issues of threatened and rare medicinal plants need to be addressed on priority basis.

Although many of these plants also propagate by vegetative means, propagation through seeds comprises by far the most convenient and cost effective means for the purpose. The seeds of many medicinal plant species possess dormancy and exhibit erratic germination (Baskin and Baskin 1988, Sharma *et al.* 2006). These features hold significance in an ecological and plant survival context but they become a disadvantage for seed-based cultivation and propagation of the concerned species. Therefore, a clearer understanding of the seed germination behaviour is critically important for developing the species-specific conservation and cultivation strategies. Three species of genus *Arnebia* are reported from

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Spiti (Aswal and Mehrotra 1994). Of these, *A. euchroma* (syn. *Macrotomia perennis*, Boiss) is commercially very important. It is known as Dimok or Khamed in the Spiti valley and as Ratanjot in trade. It is distributed in dry areas of alpine Western Himalaya, Western Tibet and Nepal, usually between 3300-4500 m asl. (Polunin and Stainton, 1984). It is found growing on dry, sandy slopes near the villages of Demul (4500 m asl) and Kibber (4200 m asl). *A. euchroma* is a threatened plant species that has been categorised as critically endangered (Singh *et al.* 2007) and later as endangered on the basis of IUCN criteria (Barik *et al.* 2018).

A. euchroma is a perennial herb with multiple erect stems (30-40 cm in height) covered with trichomes and emerging from axils of basal leaves. The insect pollinated hermaphroditic purplish-white flowers appear during June to August while the seeds mature from July to September. The roots yield a purple dye used as spice in food preparations. The powdered roots are used to cure cough, lung problems and menorrhagia. Root powder mixed with mustard or apricot oil is used to impart red colour to hair and also to prevent dandruff and hair fall.

Root extracts of *A. euchroma* yield a shikonin derivative which has significant anti-microbial, anti-inflammation and anti-tumour activities (Ghorbani 2005). The plant also exhibits a potent anti-HIV activity (Kashiwada *et al*. 1995). In view of the immense medicinal value and threatened status (Singh *et al*. 2007, 2012, Barik *et al.* 2018), there is an urgent need to develop realistic strategies for conservation of *A. euchroma.* As such, there is a dearth of information concerning the seed germination behaviour of *A. euchroma* and underlying metabolic basis thereof. The present study aimed at determining the seed germination characteristics of *A. euchroma* from Spiti with an emphasis on achieving improved germination through physico-chemical and hormonal pre-treatments and getting insight into the underlying metabolic mechanism(s). The findings are expected to have implications for cultivation as well as conservation of the Spiti population of *A. euchroma*.

MATERIALS AND METHODS

Study Area: The present work concerns a population of *Arnebia euchroma* from Spiti, a sub-division of Lahaul and Spiti district of Himachal Pradesh (H.P.). It is bordered by Ladakh on the North, Lahaul on the West, Kinnaur on the South and Tibet on the East. With an average elevation of 4000 m above sea level, Spiti is a typical cold desert of middle and great Himalayan ranges. It predominantly remains cool and dry throughout the year except the period of May to September when it receives very little rain. The temperatures typically range from sub-zero during winters to quite high during summers.

Seed source: The ripened fruits/mature seeds of *Arnebia euchroma* were collected from Kibber village (4200 m asl) of Spiti sub-division. The manually separated seeds from the fruits were air dried for at least a fortnight at room temperature and stored in airtight polyethylene containers for further studies.

Seed germination assays: The seeds were surface sterilized with 0.1% $HgCl_2$ followed by thorough washing with water. After soaking in distilled water for 24 h, they were transferred to Petri plates lined with 2 layers of filter paper made wet with distilled water. The Petri plates were placed in a seed germinator/incubator at $25 \pm 2^{\circ}$ C under continuous illumination (PAR: 40 µmol photons m^{-2} s^{-1}) provided by fluorescent white light. The seed germination was recorded at periodic intervals until the final germination count. Emergence of 2 mm of radicle was considered as seed germination (ISTA, 1999). The experiments were performed in triplicate with 30 seeds each (10x3). In addition to germination percentage, following parameters were calculated:

A. Emergence index (EI) $= \frac{dn}{n}$ Where, *dn* is day in

which emergence ends and *n* is day when emergence starts

B. Germination Speed $= \frac{Percentage\ germination}{Day\ of\ completion\ of\ germination}$

C. Mean Germination Time (MGT)= $\sum(nxd)/N$

Where, n=the number of seeds which germinated after each period in days (*d*) and *N* = the total number of seeds germinated at the end of experiment (Hartman and Kester, 1989).

Physico-chemical and GA_3 pre-treatment of seeds: The surface sterilized seeds of *A. euchroma* were pre-treated with GA_3 (0.1 and 1 mM), sodium nitroprusside (SNP, a nitric oxide donor: 1 and 10 mM), proline (1 and 10 mM) and KNO_3 (2 mM), by keeping them submerged in test solutions for 24 h. Seeds simultaneously imbibed with water constituted the control. For acid scarification, the seeds were treated with 98% H_2SO_4 for 1 min. after which they were washed thoroughly under tap water and soaked in distilled water for 24 h. Following the pre-treatments, the seeds were placed in Petri plates lined with two layers of filter paper moistened with distilled water and incubated in a seed germinator under the above stated conditions. Different germination parameters as stated above were measured.

Measurement of biochemical parameters: The seeds pre-treated with different effectors for 24 h were subjected to determination of α -amylase activity and contents of certain metabolites.

α -amylase was assayed following the method of Filner and Varner (1967). In brief, the seeds were homogenised with Tris-HCl (pH 7.2) buffer and the homogenate centrifuged. The supernatant served as the enzyme extract. One ml substrate (0.15% starch) was incubated with 1 ml enzyme extract for 10 min at 25°C after which 3 ml IKI reagent was added and absorbance read at 620 nm. The amount of starch degraded was calculated with the help of a calibration curve and enzyme activity expressed as μg starch degraded min^{-1} mg^{-1} protein. Protein contents in the extract were measured using Bradford reagent (Bradford, 1976).

Free proline contents were determined following the method of Bates *et al*. (1973). The seeds were homogenised with 3% sulfosalicylic acid and the obtained homogenate was centrifuged. One ml supernatant was reacted with 1 ml freshly prepared ninhydrin reagent and 1 ml glacial acetic acid for 1 h at 95°C. Thereafter, the reaction mixture was cooled, chromophore

Table 1. Effect of different seed pre-treatments on seed germination (%), emergence index, germination speed and mean germination time (MGT) in seeds of *A. euchroma.* Data are arithmetic means ± SE., n=3.

Seed Treatments	% Germination on 15th day	Emergence index	Germination speed	Mean Germination Time, MGT (days)
Control	30±10.0	1.29	3.33	8
KNO_3 2 mM	40±10.0	2.2	3.64	8
SNP 1 mM	70±5.77	2.25	5.83	7
SNP 10 mM	60±10.0	2.2	5.45	8
GA 0.1 mM	66.67±3.33	3.33	6.67	6
GA 1 mM	86.67±3.33	4.67	6.19	6
Proline 1 mM	60±15.3	2.75	5.45	8
Proline 10 mM	46.67±8.82	2.8	3.33	9
H_2SO_4 1 min.	30± 2.16	2.8	2.14	10

Figure 1. *Arnebia euchroma* growing in natural habitat in Spiti valley (altitude: 4200 masl)

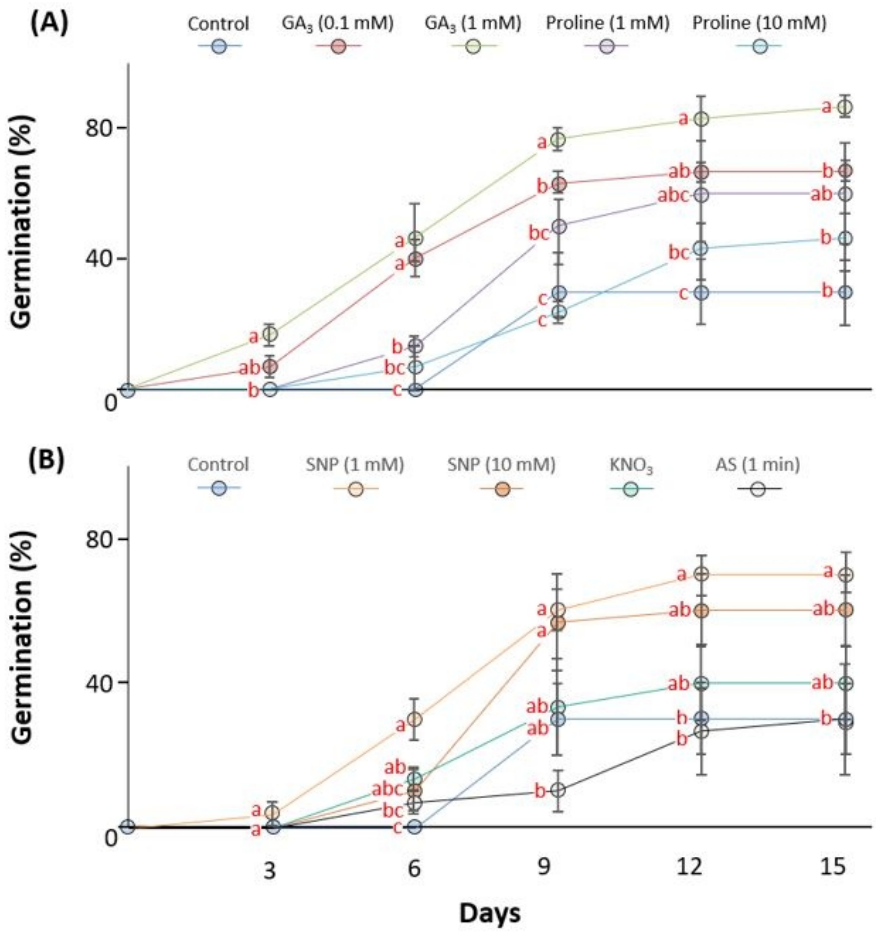

Figure 2. Time course of seed germination of *Arnebia euchroma* as affected by pre-treatment of GA3 and proline (A);SNP, KNO3 and acid scarification (AS) (B). Data are arithmetic means of three replicates each comprising 30 seeds ± SE.Different letters represent significant differences among different pre-treatments (ANOVA; Tukey's test, $p<0.05$).

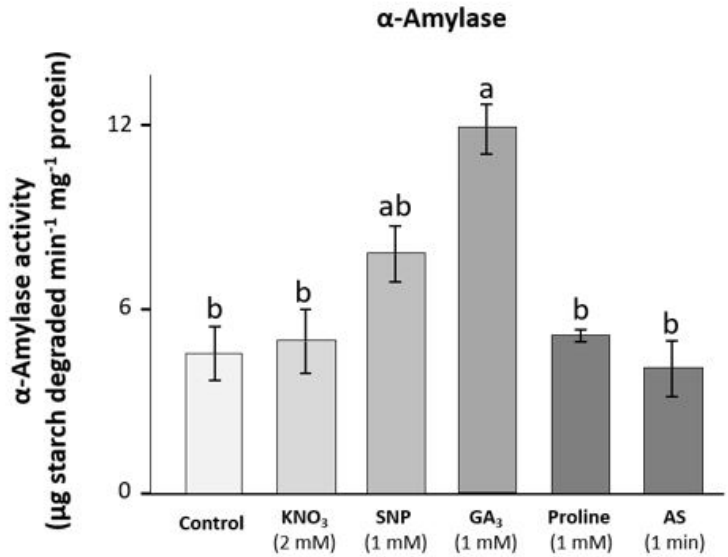

Figure 3. Effect of GA_3 and certain chemical pre-treatments (24 h) on α-amylase activity in the seeds of *Arnebia euchroma.* Data are arithmetic means ± SE, n=3. Different letters represent significant differences among different pre-treatments (ANOVA; Tukey's test, $p<0.05$).

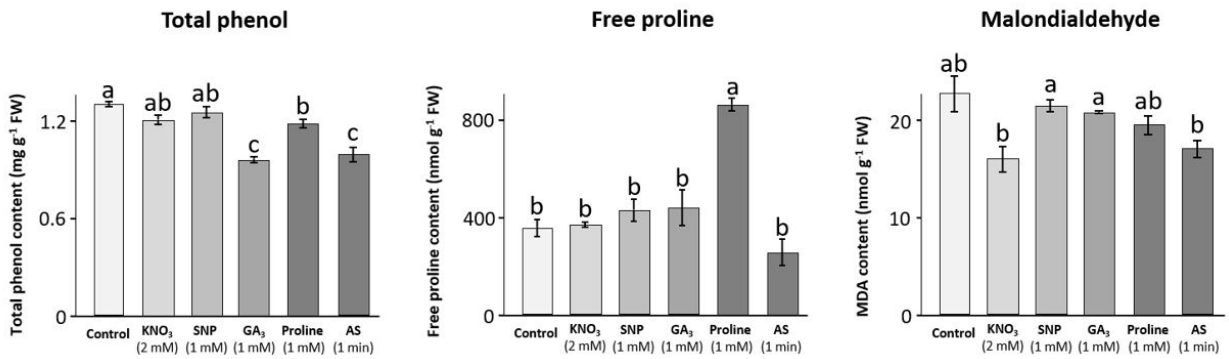

Figure 4. Effect of GA_3 and certain chemical pre-treatments (24 h) on total phenol (A), free proline (B) and malondialdehyde (C) contents of *Arnebia euchroma* seeds. Data are arithmetic means ± SE, n=3. Different letters represent significant differences among different pre-treatments (ANOVA; Tukey's test, $p<0.05$).

extracted with 2 ml toluene and absorbance read at 520 nm. The proline contents were determined with the help of a calibration curve.

Lipid peroxidation was estimated in terms of malondialdehyde (MDA) contents following the method described by Dhindsa *et al*. (1981). The seeds were homogenised with 0.1% trichloroacetic acid and the homogenate was centrifuged. The supernatant was reacted with 0.5% thiobarbituric acid in 20% trichloroacetic acid at 95˚C for 1 h. After cooling, the absorbance was read at 532 nm. Measurement corrections for unspecific turbidity were made by subtracting the absorbance at 600 nm. The MDA contents were determined using the extinction coefficient of 155 mM^{-1} cm^{-1}.

Total phenol contents were determined according to Goldstein and Swain (1963). The seeds were homogenised with 0.3 N HCl in methanol and the homogenate was centrifuged. The supernatant was evaporated and the residue dissolved in water which was reacted with 1N Folin-Ciocalteau reagent and 35% sodium bicarbonate. After 1 h, absorbance was

recorded at 630 nm. The phenol contents were determined using a calibration curve prepared with gallic acid.

Statistical analysis: All experiments were carried out in triplicate and data are presented as arithmetic means ± SE. The data were subjected to one-way analysis of variance (ANOVA) followed by Tukey's test for significant differences between individual means.

RESULTS

Seed germination in response to various pre-treatments

The germination assays were performed on freshly harvested seeds (within two months after harvest) of *A. euchroma*; a plant growing in natural habitat is shown in figure 1. In control, seed germination started after 7 d of incubation. A maximum of 30% germination was evident on 15 d. With the aim of achieving improved seed germination performance, the seeds were subjected to various physico-chemical and GA_3 pre-treatments. Among all the treatments applied, GA_3 (1 mM) was most effective (Fig. 2A). With this, germination started after 2 d of incubation that increased gradually to 87% after 15 d. The lower concentration (0.1 mM) was also promotory and caused 67% germination. Like GA_3, SNP (1 mM) pre-treatment induced early seed germination and strongly promoted the same causing 70% germination after 15 d (Fig. 2B). The higher SNP concentration (10 mM) was not as effective. Proline (1 mM) pre-treatment also promoted the seed germination substantially leading to 60% after 15 d; effect of 10 mM proline was lower in magnitude (47%) (Fig. 2A). In contrast to GA_3, SNP and proline, KNO_3 (2 mM) promoted the seed germination only marginally (Fig. 2B). Acid scarification in fact somewhat suppressed the seed germination particularly during initial period although the final count was comparable to that in control (Fig. 2B). Other germination characteristics namely, emergence index and germination speed were consistent with the seed germination data. Thus, their values were highest in case of pre-treatment with GA_3 followed by that with SNP, proline, KNO_3 and acid scarification (Table 1).

Biochemical changes due to various seed pre-treatments: To get insight into the possible mechanism(s) underlying the germination enhancement induced by different effectors, certain biochemical parameters namely, α-amylase activity, free proline contents, lipid peroxidation levels (MDA contents) and phenol contents were measured in the seeds after pre-teatment of 24 h. The α-amylase activity in the seeds was strongly promoted by 1 mM GA_3 (2.62-fold) and SNP 1 mM (1.73-fold). In contrast, proline and KNO_3 had no effect and AS marginally suppressed the activity (Fig. 3). Seed phenolic contents declined due to GA_3 (1 mM), AS and proline (1 mM) treatment by 34, 28 and 12%, respectively. However, SNP (1 mM) and KNO_3 (2 mM) did not alter the same (Fig. 4A). Due to pre-treatment with exogenous proline (1 mM), the endogenous seed proline contents increased 2.35-fold. They increased by 18 and 17% due to SNP (1 mM) and KNO_3 (2 mM), respectively but remained unchanged under GA_3 (1 mM) treatment (Fig. 4B). The seed MDA contents tended to be lower than the control levels due to KNO_3, GA_3, proline and AS; lowest levels were observed in KNO_3-treated seeds. SNP had no effect (Fig. 4C).

DISCUSSION

The aim of present study was to characterize the seed germination behaviour of *Arnebia euchroma,* a threatened species, from Spiti (Himachal Pradesh, India) cold desert in W. Himalaya. Such information holds significance for its seed-based conservation and cultivation; the latter could potentially ease

the pressure on natural populations. The freshly harvested seeds of *A. euchroma* exhibited poor germination; maximum of only 30% seeds germinated after 15 d of incubation under favourable conditions. Therefore, we explored the possibility of improvement of seed germination performance through the pre-treatment of seeds with a couple of uncommon effectors namely, SNP, a donor of nitric oxide (NO) and proline in addition to the common ones including GA_3, acid (H_2SO_4) scarification, and KNO_3. Strongest stimulation of seed germination, in terms of both advancement and germination percentage (87%, 12 d), was evident in case of GA_3 pre-treatment. Such an influence of GA_3 on seed germination has been reported in case of several high altitude populations of medicinal plants e.g. *Podophyllum hexandrum*, *Inula racemosa, Hyoscymus niger, Bunium persicum, Carum carvi, Saussurea costus and Rheum australe* (Sharma et al. 2006, 2010, 2014). Improvement in germination performance has also been reported in *Arnebia benthamii* (Kandari *et al.* 2008). The role of GA_3 in alleviation of seed dormancy and enhancement of seed germination is well established (Debeaujan and Koornneef 2000, Finch-Savage and Leubner-Metzger 2006; Finkelstein 2008). Dormancy release and germination enhancement involve increase in GA biosynthesis and ABA degradation leading to low ABA/GA ratio (Cadman *et al.* 2006). GA_3 induces amylase activity in germinating seeds that hydrolyses starch into sugars to sustain the seed germination (Kaneko *et al.* 2002). Consistent with this, amylase activity in GA_3 pre-treated *A. euchroma* seeds was strongly enhanced (Fig. 3). GA_3 could replace the requirement of after ripening by increasing its endogenous level (Bewely and Black 1994).

Quite interestingly, SNP, an NO donor and proline strongly promoted the seed germination of *A. euchroma*. SNP was demonstrated to break seed dormancy in *Arabidopsis thaliana* (Bethke *et al.* 2006). NO promoted seed germination and seedling growth in rice (*Oryza sativa* L.) under cadmium stress (He *et al*. 2014). The reversal of NO dependent dormancy removal in *Lactuca sativa* by cPTIO, an NO scavenger, confirmed the role of endogenous NO in the regulation of the process (Beligni and Lamattina 2000). NO alleviated the dormancy of apple seeds via alteration of ABA and JA biosynthetic or transduction pathways (Andryka-Dude *et al.* 2019). Recently, the seed germination acceleration by NO in chickpea has been demonstrated to be a consequence of the regulation of respiration and ROS accumulation (Pandey *et al.* 2019).

ROS production occurs in germinating seeds (Bailly *et al.* 2004). The MDA contents, however, were not affected by SNP pre-teatment in *A. euchroma* seeds. Due to SNP pre-treatment, amylase activity was enhanced that might have contributed to enhanced seed germination in the present study. In germinating wheat seeds, NO stimulated the α - amylase activity (Zhang *et al.* 2005). SNP promoted imbibition and caused storage reserve degeneration finally leading to stimulated seed germination (Zhang *et al*. 2007). The stimulation of *A. euchroma* seed germination by proline could be ascribed to multiple actions of proline. Thus, being an osmoprotectant (Kishor *et al.* 1995), it would be expected to appropriately contribute to the maintenance of water relations in the seed tissue and seedlings. There is a positive correlation between proline contents and cell elongation. It is quite likely to act as an N source (Claussen 2005). Proline promoted seed germination in wheat seeds by stimulating α-amylase activity (Sultana *et al*., 2000). Besides, proline could scavenge a variety of free radicals in vitro (Kaul *et al*. 2008) and in turn is likely to be a component of cellular antioxidant defence network. Through this property, proline might help the redox regulation of germinating seeds in view of the

generation of ROS during the process (Bailly *et al.* 2004). As a consequence of seed pre-treatment with exogenous proline, the endogenous contents increased by a factor >2 that supports the involvement of proline in stated processes. Proline accumulated in the seeds pre-treated with SNP and GA_3, albeit to a much lower magnitude than that in proline treated ones, pointing to its likely contribution towards germination stimulation. KNO_3 has been reported to have a positive effect on seed germination and seedling growth of many species (Ahmadvand *et al*. 2012). KNO_3 stimulated germination by enhancing the water uptake in dormant caryopsis of *Avena fatua* (Hilton 1984). Nitrate induced seed dormancy removal was reported to be accompanied by reduction in ABA levels (Matakiadis *et al*. 2009; Fredes et al. 2019). In contrast to other pre-treatments, acid scarification did not alter the seed germination of *A. euchroma* implying the lack of a major role of seed coat hardness in poor germination ability of seeds.

Total phenolic contents of *A. euchroma* seeds decreased as a consequence of most of the treatments; maximum decrease was evident in GA_3 (1 mM) treated seeds. Phenols are categorized as plant growth regulators and are involved in regulation of seed germination and dormancy (Chien *et al*. 2004). Mayers and Evenari (1952) observed simple phenols to be the potent germination inhibitors. Thus, the enhanced magnitude of seed germination might partly be due to lowered phenolic levels. In *Arnebia benthamii,* the seed coat contains the chemical inhibitors which have an inhibitory effect on seed germination (Ganaie *et al*. 2011). MDA is an indicator of membrane injuries and cellular metabolic deterioration (Fan *et al*. 2012). In *A. euchroma*, a cold desert plant species, MDA contents were found to be highest in control showing 30% germination. No effect of acid scarification indicates that the seeds of *A. euchroma* from Spiti (H.P.) possess only physiological dormancy. The parameters such as emergence index, germination speed and MGT support the findings of present study. For example, the lower values of MGT in case of GA_3 pre-treatments signify an acceleration of germination process.

CONCLUSIONS

In brief, the present study represents the first characterization of seed germination behaviour of threatened, high value *Arnebia euchroma* from the cold desert region of Spiti in W. Himalaya. The findings revealed a strong stimulation of seed germination by SNP and proline, two uncommon effectors, besides that by Ga_3. Diverse biochemical parameters provide insight into the metabolic basis of the observed stimulation. The observations have implications for conservation and cultivation of the species.

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