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Effect of Various Physico-chemical and Hormonal (GA_3) on Seed Germination of *Dioscorea deltoidea* Wall. ex Kunth.

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ABSTRACT: *Dioscorea deltoidea* Wall. ex Kunth. (Dioscoreaceae) is commonly known as singli-mingli, Kins and Ganj. It grows in the North-Western Himalayas at altitudes ranging 1,000-3,500 m amsl. This medicinally important plant species is categorized as endangered. The seed viability status was almost maintained at least for 6 months. As the storage period progressed, a consistent decline in seed viability was observed. Of the various pre-treatments applied, GA_3 induced an early germination. Other seed effectors were also effective in promoting the seed germination. A consistent decline in seed germination was observed during the 3 years of storage period. Although there was a marked reduction in germination percentage under various physico-chemical and GA_3 pre-treatments, the responsiveness of seeds towards various effectors appeared to be enhanced as the storage period progressed.

Keywords: Seed Germination; Sodium nitroprusside (SNP); Storage period, Viability and GA_3.

INTRODUCTION: *Dioscorea deltoidea* Wall. ex Kunth. (Dioscoreaceae) is commonly known as singli-mingli, Kins and Ganj. *Dioscorea* tubers are also known as wild yam. It is a perennial climber growing upto 3 m. It grows in the North-Western Himalayas from Kashmir, Punjab and Himachal Pradesh, eastward to Nepal and China at altitudes ranging 1,000-3,500 m amsl[4].

D. deltoidea has been categorized as endangered due to uncontrolled exploitation from the wild habitat. Out of 600 species of *Dioscorea*, so far reported globally, only ten species are in commercial cultivation. Yam tubers are rich in essential dietary nutrients and are used as staple food in China[13, 2]. Diosgenin is the main active principle found in the rhizomes of *D. deltoidea.* A rhizome of good quality is reported to contain 4.8-8% diosgenin, a valuable steroid sapogenin. The latter is used in the partial synthesis of modern drugs like cortisone and other steroids. The main intermediate isolated from diosgenin is 16-DPA (16, dihydropregenolone acetate), which can further be synthesized to any desired steroid hormone used in various formulations including oral contraceptive pills. The phenolic compounds, widely present in *D. deltoidea* plants are reported to have multiple biological effects, including antioxidant activity, antitumour, antimutagenic and antibacterial properties[15]. There are two ways of plant propagation namely, by seeds and by vegetative parts (rhizome cuttings) of the plants. The fruit is a three winged pod, capsule or samara and seeds are rounded, flat and winged all around the margin. Seed treatment with chilling and GA_3 has been found particularly effective in stimulating the seed germination of *Dioscorea* species[12]. This, however, has not been reported specifically in *D. deltoidea*.

MATERIALS AND METHODS:

Seed source: Seeds of the *D. deltoidea* were collected from Nichar (2110 m amsl) in Kinnaur district (Himachal Pradesh) during August-October 2007 and same duration of subsequent years.

The seeds were collected from wild populations. The seeds were separated manually from the fruits, air-dried for about a fortnight at room temperature and stored in polyethylene jars under ambient conditions (room temperature) until they were used in described studies. The seeds were subjected to various analyses following harvest and subsequently at regular intervals during a storage period of three years.

Chemicals and reagents: Gibberellic acid (GA_3), 2, 3, 5-triphenyl tetrazolium chloride (TTC) and sodium nitroprusside (SNP) were procured from Sigma chemical Co., USA and Merck, Mumbai, respectively. All other chemicals and reagents used were of analytical grade and were procured from Ranbaxy, Himedia, s.d. fine chemicals and Sisco Research Laboratories.

Seed viability test: Qualitative as well as quantitative (ability to reduce TTC) viability was determined by using 2, 3, 5-triphenyl tetrazolium chloride (TTC) reduction assay. In brief, the uniform seeds were surface sterilized with 0.1% aqueous solution of $HgCl_2$

for 5 min. Thereafter, they were washed thoroughly with tap water and kept submerged in distilled water for 24 h at 25 ± 2ºC. The imbibed seeds were cut off at one end (depending upon the seed morphology) so as to facilitate the diffusion of TTC solution to exposed embryos and other seed parts. The seeds cut in above manner were incubated with 0.1% aqueous solution of TTC at 25 ± 2ºC in dark for 24 h. The seeds with completely stained embryos were considered as viable. The tests were performed in triplicate (10 seeds x 3). Data are presented as % viability.

The TTC assay conducted as above was extended to determine the amount of TTC reduced by embryos/seeds. Thus, the seeds/embryos treated with TTC were homogenized with a small volume of MetOH. The homogenate was centrifuged at 10,000 g for 10 min to remove the debris; volume was made to 5 ml with MetOH and absorbance read at 485 nm. The TTC reduction was expressed as A_{485}/seeds or A_{485}/embryos, the number of seeds/embryos depending upon the species.

Seed germination assays: The seeds selected for uniformity (on the basis of colour and size) as far as possible were surface sterilised with 0.1% $HgCl_2$ for 5 min, washed thoroughly under tap water and soaked in distilled water for 24 h at 25 ± 2ºC. Thereafter, the seeds were transferred to Petri-plates lined with three layers of filter paper moistened with distilled water and allowed to germinate in an incubator at 25 ± 2ºC under continuous illumination provided by the fluorescent white light (PAR: 40 µmol m^{-2} s^{-1}). The radicle emergence (> 2 mm) was taken as seed germination[7]. Seed germination was recorded at periodic intervals until the final count. 20 seeds in triplicate were used for each treatment described in the study.

Physico-Chemical and hormonal (GA_3) treatments for dormancy removal/germination improvement: The seeds of the stated five species were subjected to the following physico-chemical and hormonal treatments with the aim of assessing their efficacy to remove dormancy and/or improve seed germination:

(i) Stratification (chilling): The surface sterilized seeds, soaked in distilled water for 24 h, were subjected to low temperature (2-4ºC) treatment in a refrigerator for one month or more as stated. Thereafter, the seeds were subjected to germination conditions as described above. The treatment is designated as chilling in the figures (result section).

(ii) Scarification with sulphuric acid (H_2SO_4) and sodium hypochlorite ($NaHClO_3$): The plant species were treated with 50% diluted H_2SO_4 for 5 min. These concentrations were selected on the basis of seed size, morphology and trial experiments. Sodium hypochlorite solution was used as such (commercial) for all the plant species for 5 min. Thereafter, the seeds were washed thoroughly under tap water and soaked in distilled water for 24 h and then transferred to germination conditions as described above.

(iii) Sodium nitroprusside (SNP): The surface sterilized seeds were incubated with aqueous solutions of SNP (1 and 10 mM) for 24 h at 25 ± 2ºC and transferred for germination to the substratum with distilled water.

(iv) Potassium nitrate (KNO_3): The surface sterilized seeds were soaked in 0.2% aqueous solution of KNO_3 for 24 h in an incubator at 25 ± 2ºC. Thereafter, they were transferred to moist substratum for germination. For moistening the substratum, distilled water was used.

(v) Gibberellic acid (GA_3): The surface sterilized seeds were incubated with 0.1 and 1 mM GA_3 for 24 h at 25 ± 2ºC and transferred for germination to the substratum moistened with distilled water.

(vi) GA_3, SNP and KNO_3 treatment of acid (H_2SO_4) scarified seeds: The seeds were washed with conc. or dil. (50%) H_2SO_4 for 5 min. as described earlier for various plants. Thereafter, they were washed thoroughly with tap water and soaked in aqueous solutions of GA_3 (0.1, 1 mM), SNP (1, 10 mM) or KNO_3 (0.2%) for 24 h at 25 ± 2ºC. The seeds were then transferred to moist substratum for germination. For moistening the substratum, distilled water was used.

The methods of seed viability measurement and germination assays have been optimized in our laboratory and are routinely used[14, 8].

RESULTS AND DISCUSSION:

Salient features of seeds: The seeds of *D. deltoidea* are dark black in colour and ovate, winged (1-2 mm) unequally all around (Plate 1). The average seed weight of air-dried or 24 h H_2O-soaked seeds was 25 and 32 mg, respectively. Thus, a 28% increase in seed mass was observed after soaking (24 h) of seeds in distilled water.

Plate 1: Seeds of *Dioscorea deltoidea* collected from natural habitat.

A. Seed viability: The viability of the seeds of *D. deltoidea* from Nichar in district Kinnaur (H.P.) (altitude: 2110 m amsl) was assessed periodically during 3 years of storage on the basis of 2, 3, 5-triphenyl tetrazolium chloride (TTC) test.

Qualitative viability assessment: The freshly harvested seeds of *D. deltoidea* were highly viable. When incubated with 0.1% TTC (24 h), seed halves containing embryo took up stain in 100% seeds. The seed viability status was almost maintained at least for 6 months. Thereafter, a consistent decline in seed viability was observed as the storage period progressed. Thus, the viability was reduced by 12, 38 and 57% after 1, 2 and 3 years of seed storage, respectively (Figure 1 A).

Quantitative viability assessment: The ability of *D. deltoidea* seed tissue for TTC reduction was not affected much until seed storage of 1 year; only 7% reduction was evident. However, with further storage, a 19 and 31% decline in TTC reduction ability of the seed tissue was observed in 2 and 3 years stored seeds, respectively (Figure 1 B).

B. Seed dormancy/germination and responses to various physico-chemical effectors and GA_3 in freshly harvested seeds: The freshly harvested seeds of *D. deltoidea* were non-dormant as was apparent from their germination performance when subjected to the optimum germination conditions. Seed germination started on 10 d of incubation (5%) and increased gradually to 73% after 70 d of incubation (Figure 2 A; Plate 2).

In freshly harvested seeds, various physico-chemical and GA_3 pretreatement improved seed germination to some extent. Of all the treatments, GA_3 (1 mM) was most effective; the magnitude of effect was comparable in seeds treated directly or following acid scarification. For example, GA_3 (1 mM) and A S + GA_3 (1 mM) treatment led to 92 and 90% seed germination (after 70 d) in *D. deltoidea* seeds, respectively (Fig. 2 A; Plate 2). Besides, GA_3 also induced an early germination. With GA_3 (1 mM) and A S + GA_3 (1 mM), 8 and 12% germination was observed on 5 d of incubation whereas there was no germination in control seeds. Other seed pretreatments were also effective in promoting the seed germination to varying magnitudes. Thus, 88, 87, 85, 83, 83, 82 and 80% germination was observed with A S + SNP (10 mM), A S + GA_3 (0.1 mM), $NaHClO_3$ (5 min.), GA_3 (0.1 mM), KNO_3 (0.2%), A S + SNP (1 mM) and SNP (10 mM), respectively after 70 d, as compared to 73% germination in control (Fig. 2, 3). A S (5 min.), SNP (1 mM), chilling (1 month) and A S + KNO_3 (0.2%) were not much effective. They caused 70, 77, 77 and 75% germination, respectively (Figure 2).

On the basis of final germination count, the effectiveness assigned to different pretreatments in freshly harvested seeds was in order: GA_3 (1 mM) > A S + GA_3 (1 mM) > A S + SNP (10 mM) > A S + GA_3 (0.1 mM) > $NaHClO_3$ (5 min.) > GA_3 (0.1 mM) > KNO_3 (0.2%) > A S + SNP (1 mM) > SNP (10 mM) > SNP (1 mM) > Chilling (1 month) > A S + KNO_3 (0.2%) > Control (DW) > A S (5 min.).

Storage-dependent alteration in seed germination and responsiveness to different effectors: There was a marked decline in *D. deltoidea* seed germination during 3 years of storage period. A maximum of 50, 23 and 8% germination was observed in 1-, 2-and 3-year stored seeds, respectively (Figure 3 B, C, and D) against a 73% germination in freshly harvested ones. The seed germination due to various physico-chemical and GA_3 pretreatment in differentially stored seeds was also substantially reduced as compared to the freshly harvested seeds. For example, in 3-year stored seeds, GA_3 (1 mM), A S + GA_3 (1 mM), SNP (1 mM), A S + SNP (1 mM) and $NaHClO_3$ (5 min.) treatments caused 17, 13, 12, 12 and 10% seed germination, respectively (Figure 3; Plate 3) in contrast to 92, 90, 77, 82 and 85% seed germination in case of freshly harvested seeds. Similar pattern(s) of reduction with some quantitative variations were also noticed with all other treatments. Although there was a marked reduction in germination percentage under various pretreatments, the responsiveness of seeds towards above effectors appeared to be enhanced as the storage period progressed. For example, in 3-year stored seeds, GA_3 (1 mM), A S + SNP (10 mM), A S + GA_3 (0.1 mM), GA_3 (0.1 mM), SNP (10 mM), A S + GA_3 (1 mM), A S + KNO_3 (0.2%), SNP (1 mM), A S + SNP (1 mM), KNO_3 (0.2%), $NaHClO_3$ (5 min.) and chilling (1 month) promoted the seed germination by 100, 100, 100, 80, 60, 60, 60, 39, 39, 20, 20 and 11%, respectively.

The effectiveness of different treatments in 3-year stored seeds assigned on the basis of final germination count was in the following order: GA_3 (1 mM) > A S + GA_3 (0.1 mM) > A S + SNP (10 mM) > GA_3 (0.1 mM) > A S + GA_3 (1 mM) > A S + KNO_3 (0.2%) > SNP (10 mM) > SNP (1 mM) > A S + SNP (1 mM) > $NaHClO_3$ (5 min.) > KNO_3 (0.2%) > Chilling (1 month) > A S (5 min.) > Control.

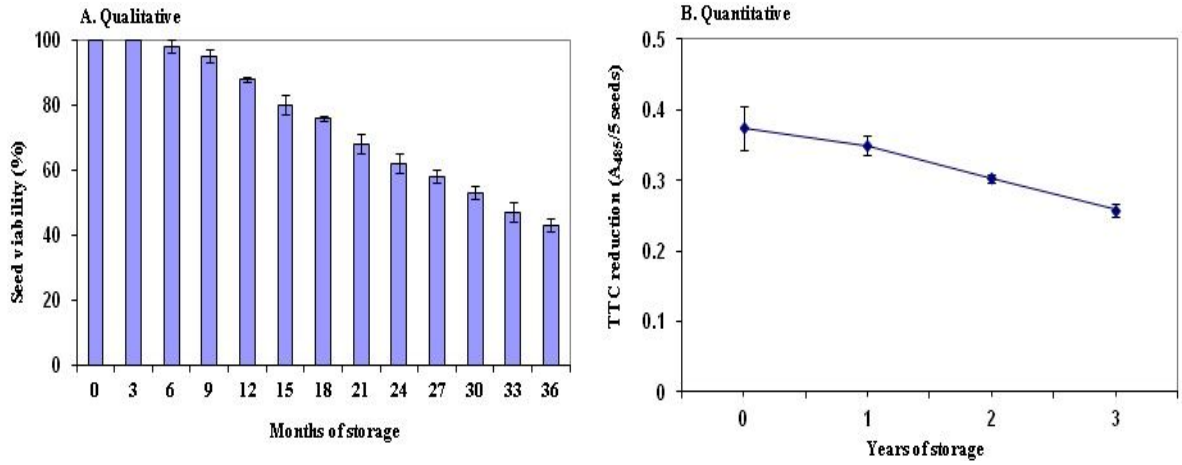

Figure 1: Changes in viability of *D. deltoidea* seeds during 3-year storage: A. Qualitative and B. Quantitative. Values are arithmetic means ± S.D.; n=3.

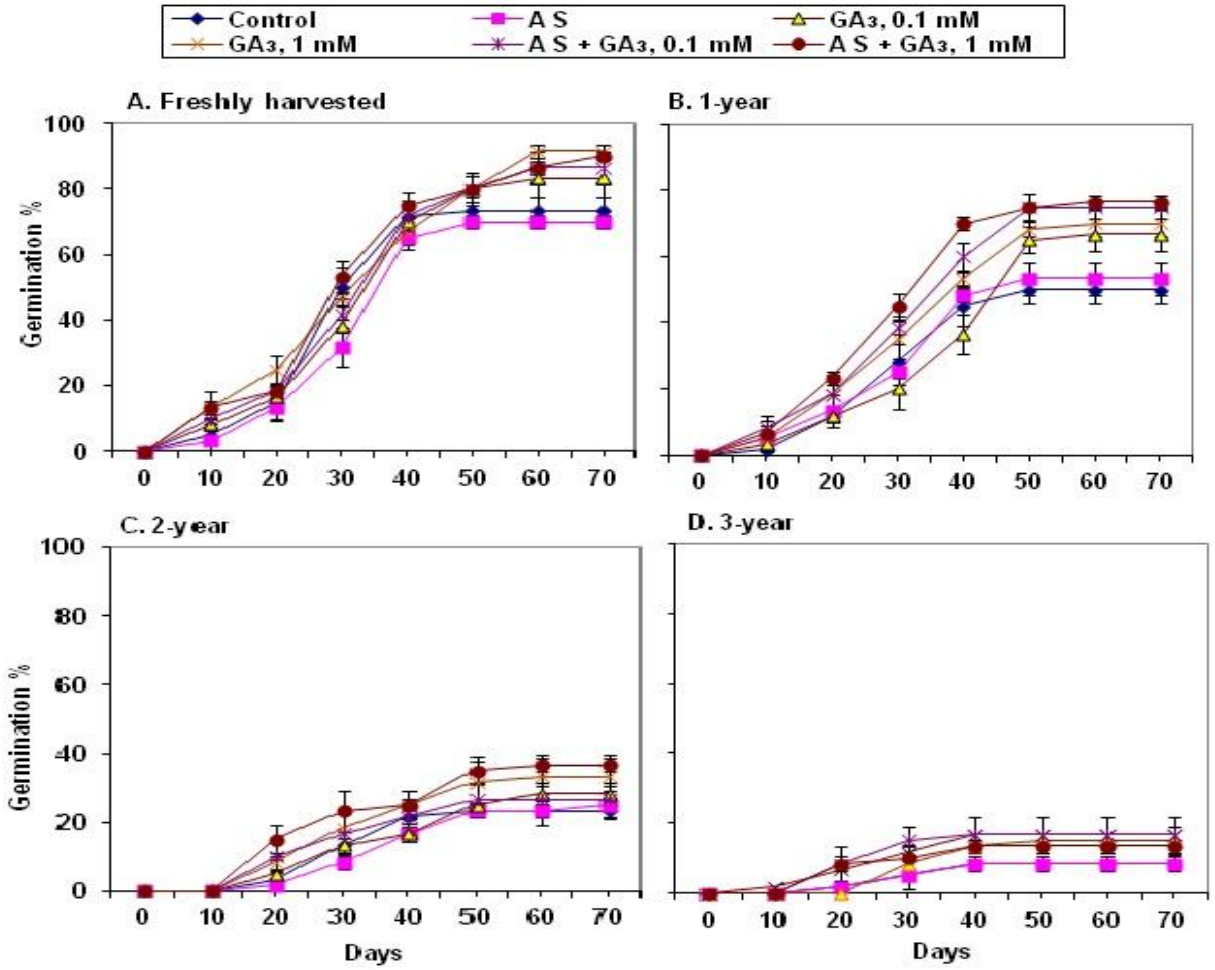

Figure 2: Time-course of seed germination in differentially stored seeds of *D. deltoidea* as affected by GA_3, applied directly or to the acid scarified seeds. Data are arithmetic means of 3 replicates ± S.D. A S-Acid Scarification.

D. deltoidea, commonly known as singli-mingli, has a great demand in pharmaceutical industries. It is categorized as endangered[17]. The populations in the study area are also under threat[11]. The plant is mainly propagated through root division but also by seeds. Its multiplication through shoot tip culture as well as nodal stem cuttings has also been reported[9, 3]. However, there is a dearth of information concerning the physiological aspects of seeds of *D. deltoidea*.

Maintenance of high (100%) seed viability for about half a year observed in the present study is of advantage for seed-based plant propagation. Even after

3 years of storage, > 50% seed viability was evident that could be increased via suitable temperature/RH regimes. The freshly harvested seeds of *D. deltoidea* exhibited reasonably good (73%) germination in control that could be improved through various physico-chemical and GA_3 pretreatments. As in other cases, GA_3 was most effective. Softening of seed coat did not alter the response to GA_3. KNO_3, SNP and $NaHClO_3$ pretreatments promoted the seed germination to varying magnitudes. The nitrates have been widely used to overcome seed dormancy[5]. Also, nitrogen compounds can break seed dormancy by decreasing C_6/C_1 ratio of CO and changing metabolic pathway, so they are usually used as germination accelerators[1]. [6]found that in seeds of *Sisymbrium officinale*, the nitrate content of the seeds from different habitats was directly proportional to their rate of germination.

The promoting effects of different pretreatments on seed germination were accompanied by appropriately altered α-amylase and dehydrogenase activities. Despite a marked reduction in germination percentage, the responsiveness of seeds towards various effectors actually increased as the storage period progressed. However, certain non-enzymatic reactions, such as Amadori and Maillard reactions, could occur even at very low moisture content[18, 16]. These reactions are also known to inhibit the activities of several antioxidative enzymes[10], ribonuclease and certain dehydrogenases[18]. Therefore, the protein or DNA damages by Amadori and Maillard reaction may accumulate during dry storage and eventually contribute to seed death.

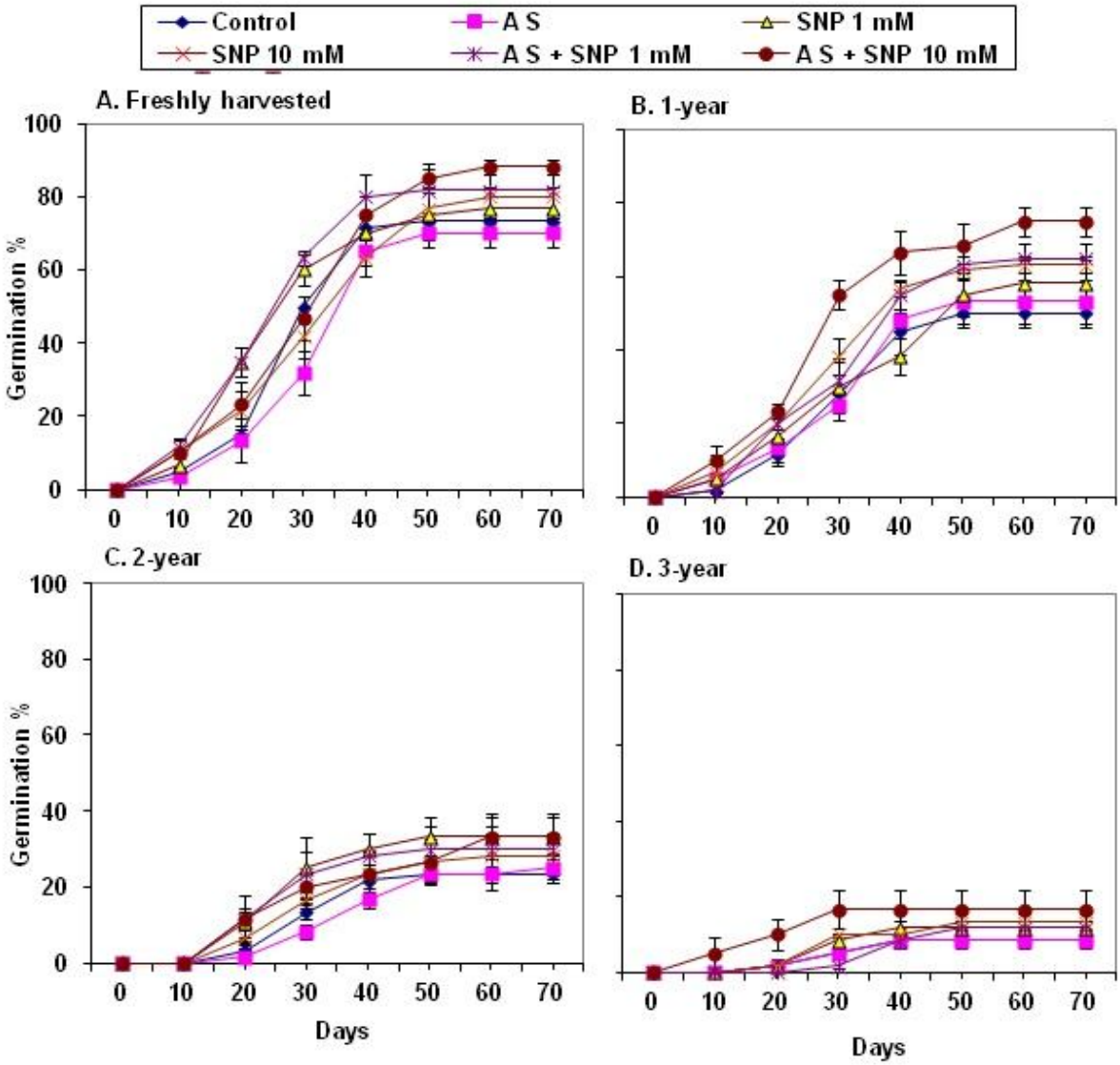

Figure 3: Time-course of seed germination in differentially stored seeds of *D. deltoidea* as affected by sodium nitroprusside (SNP), applied directly or to the acid-scarified seeds. Data are arithmetic means of 3 replicates ± S.D. A S – Acid Scarification.

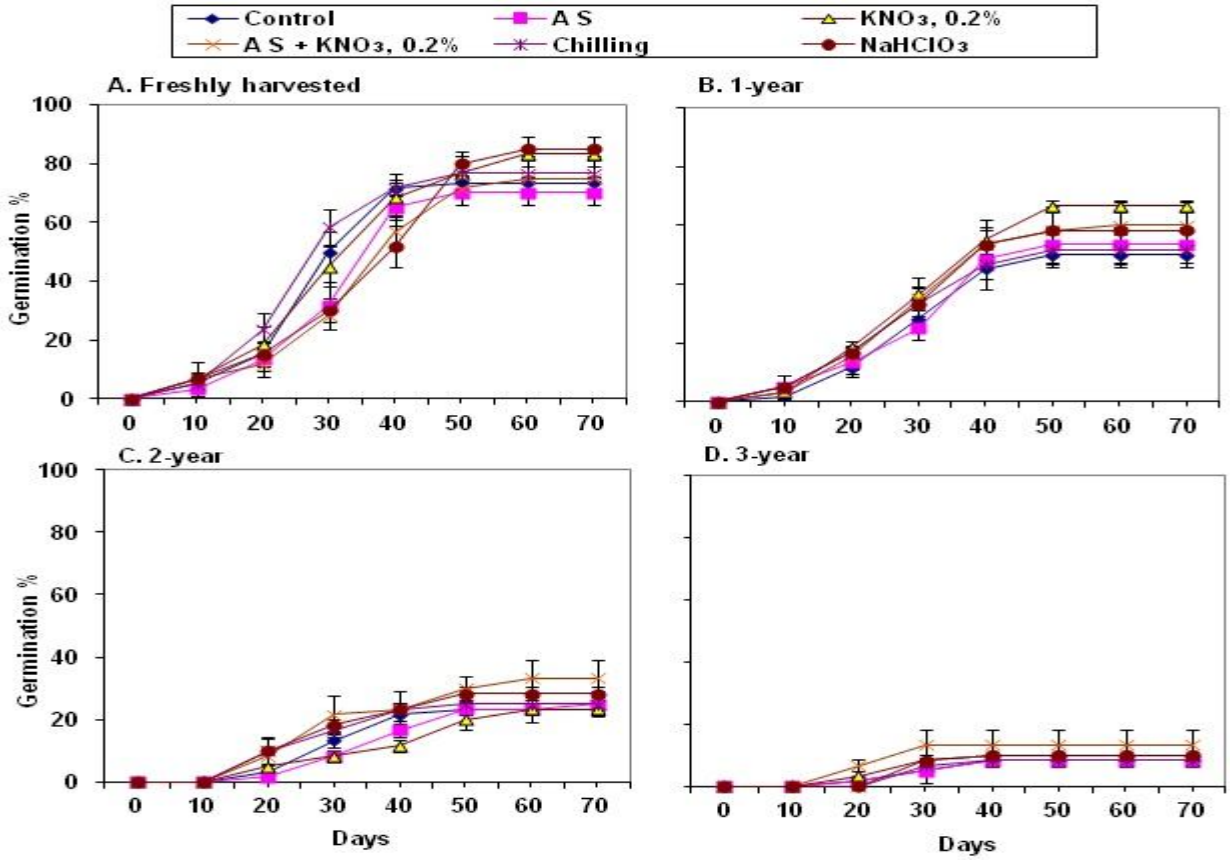

Figure 4: Time-course of seed germination in differentially stored seeds of *D. deltoidea* as affected by some physico-chemical treatments. Data are arithmetic means of 3 replicates ± S.D. A S – Acid Scarification.

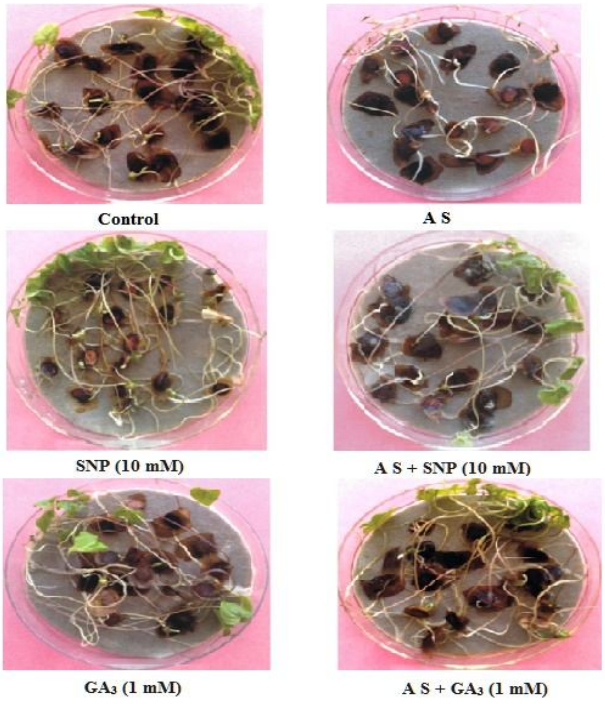

Plate 2: Effect of GA_3 and SNP applied directly or following acid (H_2SO_4) scarification on seed germination of freshly harvested seeds of *D. deltoidea.*

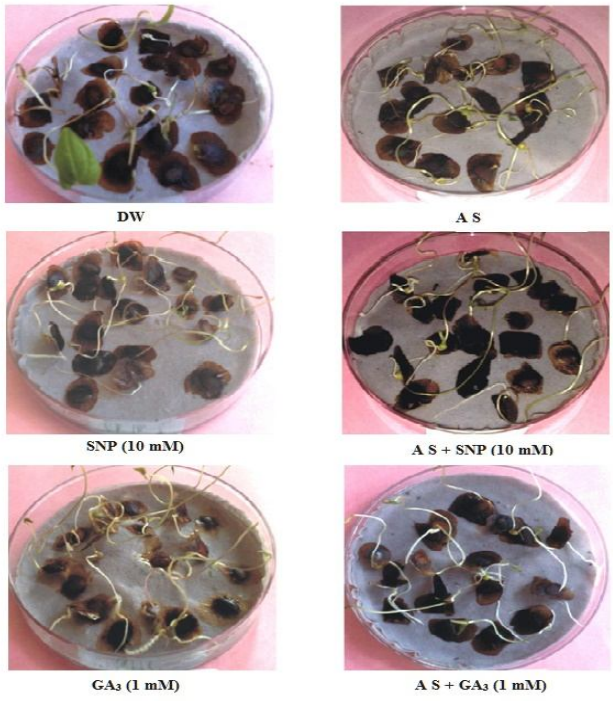

Plate 3: Effect of GA_3 and SNP applied directly or following acid (H_2SO_4) scarification on seed germination of 3-year stored seeds of *D. deltoidea.*

The findings from the present study provide insight into the seed germination and dormancy behaviour important medicinal plant species from Kinnaur (H. P.). Of particular significance are the storage-dependent alterations in the status of seed dormancy/germination and also in the responsiveness of seeds to the tested physico-chemical and GA_3 treatments. Furthermore, the experimental approaches adopted contribute to the understanding of the metabolic basis of storage- as well as- diverse effector-induced changes in the seeds.

CONCLUSION: The findings from present study suggest the suitability of some cost-effective seed pretreatments for achieving a reasonably good seed germination performance in the plant species considered. In conclusion, the present data have implications for conservation and cultivation of the studied important medicinal plant species from Kinnaur (H. P).

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