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Effect of pre-sowing treatments using phytohormones and other dormancy breaking chemicals on seed germination of *Dioscorea deltoidea* Wall. Ex Griseb.: an Endangered Medicinal Plant Species of North Western Himalaya

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ABSTRACTS

The objective of the present study was to develop effective pre-sowing treatments to improve the seed germination, and to reduce mean germination time, so that protocols for commercial production of the plant could be developed and natural populations restored to ensure proper conservation of the species. In the present study phytohormones and other seed dormancy breaking chemicals were used to investigate their effect on seed germination *Dioscorea deltoidea.* An experiment was performed with 15 treatments and 3 replications in a completely randomized design. Germination started in different treatments on different dates. It started on day 4th in chilling treatments Ch_1 (30 days chilled seeds) and Ch_2 (40 days chilled seeds) and was observed till 36th day in all the treatments (Table 1). The highest mean percentage of germination was observed in chilling treatment Ch_1 (92±0.577) followed by treatment $GA_3$1 (82±1.155), Ch_2 (81±3.180), $GA_3$2 (77±4.041) and Ch_3 (70±2.887) respectively. Potassium nitrate treated treatments, K1 (0.2 %) and K2 (0.3 %) showed more percentage germination (62±1.732 and 55±2.887) than naphthalene acid treated treatments NAA1 (25 ppm) (50±2.887) and NAA2 (50 ppm) (47±1.155). In case of combined treatments, Com 1(H_2SO_4/$GA_3$1) and Com. 2 (H_2SO_4/$GA_3$1), mean percentage of germination ± SD (35±2.887 and 30±0.577) was lower as compared to control treatment (36±1.155). Similar was the case with treatments Com 3(H_2SO_4/NAA1) and Com. 4 (H_2SO_4/NAA2) with mean percentage of germination ± SD of 27±1.155 and 26±1.155 respectively. The more effective treatment in breakinh the dormancy and enhancing the seed germination percentage are the pre chilling treatment (3-4°C) for a period of 30 days and treatment of seeds with gibberellic acid.

Key words : *Seed germination, Chilling, Naphthalene acetic acid, Gibberllic acid, Dioscoreadeltoidea*

Introduction

Seed germination is a complex physiological processes that response to environmental signals such as water potential, light and other factors. Poor seed germinationis the major limiting factor of threatened medicinal plants for large scale production and cultivation (Bhardwaj *et al.*, 2016). Germination is a

critical stage in the life cycle of every crop plant and often controls population dynamics, with major practical implications. Seed germination is the critical stage for species survival (Huang *et al.,* 2003 and Yang *et al.,* 2008). Seed germination studies proved to be useful in development of conservation study (Kandari *et al.,* 2007). Seeds may be non-dormant at maturity and thus germinate soon after dispersal if environmental conditions are favourable for them to germinate. However, favourable conditions may not persist long enough for the resulting plant to become established. Seed dormancy prevents seeds from germinating under unfavorable conditions, thus reducing the chances of seedling mortality and there by contributing to the success of population regeneration (Gutterman, 2012). However, poor seed germination of viable seeds in several Himalayan plant species is experienced as a limiting factor in multiplication of plants at a large scale (Baskin and Baskin, 2014 and Nadeem *et al.,* 2002).

D.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 ams l (Chopra *et al*., 1956). *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 (Ozo *et al*., 1984 and Bhandari *et al*., 2003). Diosgenin is the main active principle found in the rhizomes of *D. deltoidea.* The phenolic compounds, widely present in *D. deltoidea* plants are reported to have multiple biological effects, including antioxidant activity, antitumour, antimutagenic and antibacterial properties (Shiu, and Leong, 2002). There are two ways of plant propagation namely, by seeds and by vegetative parts (rhizome cuttings) of the plants. *D.deltoidea* has been much sought after by private agencies and pharmaceutical firms, having been continuously collected in India, except perhaps in the more inaccessible areas of the Himalayas (Satapau, 1970 and Qureshiand Kaul, 1970). The roots yield cortisone, a steroidal hormone, used in treating rheumatic diseases and ophthalmic disorders. The genus has recently gained much repute as a source of steroidal sapogenins like diosgenin. These are promising starting material for synthesis of cortisone, which is useful in treatment of rheumatic arthritis, and in preparation of sex hormones (Jain, 1968). Diosgenin is a precursor for the chemical synthesis of steroidal drugs, and is tremendously important to the pharmaceutical industry (Eibl and Eibl, 2006). *D. deltoidea* advertises an immense potential in the pharmaceutical industry, as well in the traditional medicine. As the species is an endangered because of the fragile nature of its habitat, habitat destruction, illegal harvesting from the wild source, great market demand and destructive harvesting practices, the objective of the present study was to develop effective pre-sowing treatments, to improve the seed germination, so that protocols for commercial production of the plant could develope and natural populations could be restored to ensure proper conservation of the species.

Materials and Methods

Seeds of *D.deltoidea* were collected at maturity from natural populations from Mahadave hills of Kashmir Himalaya (3000–4000 m asl) during August to September 2017. The seeds were air-dried for a fortnight at room temperature (15±2 °C) and then were stored at room temperature (15±2 °C). Seeds were washed with 0.1% mercuric chloride for 5-7 minutes and then with 70% alcohol for 1 minute and thoroughly rinsed with double distilled water and divided into groups of 50 seeds each.

Physical treatment

Stratification/Chilling : The surface sterilized seeds (using mercuric chloride) were soaked in distilled water for 24 hours and then subjected to chilling at low temperature (3-4°C) for different durations [(Chilling treatment (Ch): Ch1 = 30 days, Ch2 = 40 days and Ch3 = 50 days)] using Refrigerator (Make L.G.).

Acid scarification/Sulphuric acid (H_2SO_4) treatment: The seeds were treated with concentrated sulphuric acid for 1 min: S_1 followed by thorough washing in distilled water. Sulphuric acid (Sigma) used was 99.9% pure.

Chemical treatment

Potassium nitrate (KNO_3): Surface-sterilized seeds were moistened with different concentrations of aqueous solution potassium nitrate (K1 = 0.2%, K2= 0.3%) for 24 hours followed by germination on sub-

stratum moistened with different concentrations of aqueous solution potassium nitrate (K1= 0.2%, K2= 0.3%).

Gibbrellic acid and Naphthalene acetic acid treatment: The surface sterilized seeds shall be kept submerged in aqueous solution of GA_3 and NAA with a concentration of 10^{-3} M and 10^{-4} M ($GA_3$1= 10^{-3} M, $GA_3$2= 10^{-4} M, NAA1=25 ppm, NAA2= 50 ppm) for 24 hours.

Combined treatment: Combination of sulphuric acid and hormones. Seeds were treated with H_2SO_4 for 1 min followed by through washing and then soaking in solution of hormone (GA_3 solution and NAA solution) for 24 hours and subsequent germination on substratum moistened with GA_3 s and NAA solution{ (Combined treatment (Com) H_2SO_4/ GA_3: Com 1= $H_2SO_4/GA_3$1, Com 2= $H_2SO_4/GA_3$2) (Com) H_2SO_4/NAA: Com 3= H_2SO_4/NAA1, Com 4= H_2SO_4/NAA2)

One treatment was kept as control (Cont.). There were fifteen treatments; fifty seeds in triplicate were used for each treatment. The experiment was laid in complete randomized design (CRD) with 3 replications each. After the treatment, the seeds were subjected to germination test by allowing them to germinate on the moistened filter paper. The germination of the seeds was monitored over the next one and a half month at an average temperature of 15-20 C^0. Observation on the no. of days taken for the first seed to germinate, total no of days for complete germination and the total no of seeds germinated were noted on regular basis. One way ANOVA was used to find out various statistical terms. The relative effectiveness of different physio-chemical and hormonal treatments in dormancy removal and germination improvement was calculated and the seedlings were transferred to the pots.

Results and Discussion

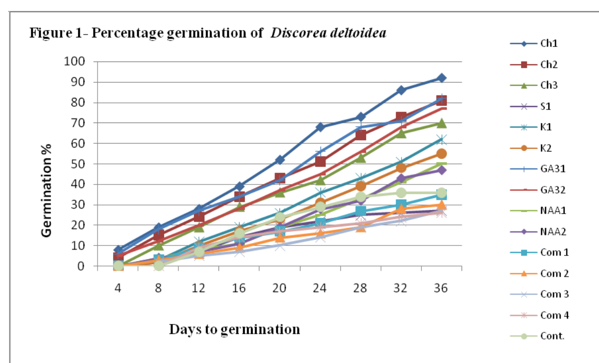
Germination started in different treatments on different dates. It started on day 3rd in chilling treatment Ch1 and completed by 36th day of seed treatment in all the treatments. The highest mean percentage of germination was observed in chilling treatment Ch_1 (30 day chilled seeds) followed by treatment $GA_3$1 in all most all day intervals and least was observed in Com 1, Com 2, Com 3, Com 4 and S_1 treatments respectively.

On 36th day the highest mean percentage of germination ± SE (92±0.57) was observed in chilling treatment Ch_1 followed by 82±1.15 per cent in treatment $GA_3$2 and least of 26±1.15 percent was observed in treatmentCom.4 (Fig. 1, Table 3). Treat-

Table 1. Seed germination studies of *Dioscorea deltoidea*

Treatments	No. of days taken for Ist seed to germinate	No. of days taken for last seed to germinate	Total no. of days taken for complete germination
Ch_1	4	36	33
Ch_2	4	36	33
Ch_3	7	40	34
S_1	7	30	24
K1	7	45	38
K2	7	42	36
$GA_3$1	4	41	35
$GA_3$2	7	40	34
NAA1	7	38	32
NAA2	7	38	32
Com 1	7	37	31
Com 2	7	37	31
Com 3	7	38	32
Com 4	10	41	32
Cont.	11	55	45

{Chilling treatment (Ch),: Ch1= 30 days, Ch2= 40 days, Ch3= 50 days: Potassium nitrate (K) with K1= 0.2 %, K2= 0.3%: Gibbrellic acid (GA_3) with $GA_3$1= 10^{-3} M and $GA_3$2= 10^{-4} M: Naphthalene Acetic Acid with NAA1=25 ppm and NAA2= 50 ppm:Combination of sulphuric acid and GA_3 (Com), Com 1= $H_2SO_4/GA_3$1, Com 2= $H_2SO_4/GA_3$2: Sulphuric acid treatment= S_1: Control =Cont.}



ments Ch_1 and K_1 were statistically different from all other treatments. Treatments $Ch_2$and $GA_3$1 were statistically different from all other treatments, but were at par with each other. Ch_3 and $GA_3$2 were at par with each other, but were statistically different from all other treatments. Treatment S1 is at par with treatments Com 2, Com 3 and Com 4. Treatment K2 is at par with treatment NAA only but is statistically different from all other treatments. Treatments NAA1 and NAA2 are at par with each other but are statistically different from all other treatments. Treatment Com 1 is at par with Com 2 and control treatment but is statistically different from all other treatments. Treatment Com 2 is also at par with S1, Com 3 and Com 4 but statistically different from all other treatments. Control treatment is at par with Com 1, Com 2, Com 3 and Com 4 treatments. Treatment Ch1, with 92±0.57 per cent germination was the best treatment among all other treatments (Table 2, 3).

On 32th day of germination, highest percentage of germination (86±1.73) was observed in chilling treatment Ch_1 followed by Ch_2(73±1.73) and $GA_3$1 (71±1.15) treatments and least percentage of 21±1.15 percent was observed in treatment Com.4. Treatments $Ch_1$was statistically different from all other treatments. Treatment Ch2 was at par with $GA_3$1 and $GA_3$2 but was statistically different from all other treatments. Chilling treatment Ch_3 was at par with were at par with $GA_3$2, but was statistically different from all other treatments. Treatments S1 Com 2, Com 3 and Com 4 were at par with each other, but was statistically different from all other treatments. Treatment K1 and K2 were at par with each other, but were statistically different from all other treatments. Treatments $GA_3$1 and $GA_3$2 were at par with each other, $GA_3$2 is also at par with Ch_3, but is statistically different from all other treatments. Treatments NAA1 and NAA2 were at par with each other, NAA1 was also at par with control treatment, butwas statistically different from all other treatments. Treatments 4Com 1, Com 2 and S1 were at par with each other but statistically different fromtreatments. Treatments Com 3, and Com 4 were at par with each other, but statistically different from other treatments (Table 2, 3).

On 28[th]day, highestmean percentage of germina-

Table 2. Statistical parameters of seed germination of *Dioscorea deltoidea* during different day intervals

Day intervals	Critical difference	Standard error (d)	Coefficient of variation
Day 4	1.145	0.558	44.552
Day 8	2.029	0.989	18.728
Day 12	2.870	1.398	12.975
Day 16	3.488	1.700	10.513
Day 20	3.488	1.700	7.845
Day 24	4.739	2.309	8.352
Day 28	4.939	2.407	7.316
Day 32	5.287	2.576	6.663
Day 36	6.289	3.065	7.067

Table 3. Mean percentage of germination on 32th and 36th day of seed treatment.

Treatments	Mean±S.E.	Mean±S.E.
Ch_1	86±1.732	92±0.577
Ch_2	73±1.732	81±3.180
Ch_3	65±2.309	70±2.887
S_1	26±0.577	27±0.577
K1	51±1.732	62±1.732
K2	48±2.028	55±2.887
$GA_3$1	71±0.577	82±1.155
$GA_3$2	68±1.732	77±4.041
NAA1	41±2.028	50±2.887
NAA2	43±1.155	47±1.155
Com 1	30±2.887	35±2.887
Com 2	28±1.732	30±0.577
Com 3	22±1.732	27±1.155
Com 4	21±1.155	26±1.155
Cont.	35±1.155	36±1.155
C.D.	5.287	6.289
SE(d)	2.576	3.065
C.V.	6.663	7.067

{Chilling treatment (Ch),: Ch1= 30 days, Ch2= 40 days, Ch3= 50 days: Potassium nitrate (K) with K1= 0.2 %, K2= 0.3%: Gibbrellic acid (GA_3) with $GA_3$1= 10^{-3} M and $GA_3$2= 10^{-4} M: Naphthalene Acetic Acid with NAA1=25 ppm and NAA2= 50 ppm:Combination of sulphuric acid and GA_3 (Com), Com 1= $H_2SO_4/GA_3$1, Com 2= $H_2SO_4/GA_3$2: Sulphuric acid treatment= S_1: Control =Cont.}

tion ±SE (73±1.15) was observed in chilling treatment Ch_1 followed by $GA_3$1 (68±0.57) and least percentage (19%) was observed in Com 2, Com 3 and Com 4 treatments. Treatment Ch_1 was the best treatment and was statistically different from all other treatments. Treatments Ch_1 and $GA_3$1 were at par with each other but were statistically different from all other treatments. Chilling treatment Ch_3 was at par with treatment $GA_3$2 but different from all other treatments. Treatment K1 and K2 were at par with each other, but were statistically different from all other treatments. Treatment $GA_3$1 was at par with Ch_2 and $GA_3$2 but was statistically different from all other treatments. Treatments $GA_3$2 was at par with Ch_3 and $GA_3$1 butwas statistically different from all other treatments. Treatments NAA1 and NAA2 were at par with each other, but was statistically different from all other treatments. Treatments Com 2, Com 3, and Com 4 were at par with each other, but statistically different from other treatments (Table 2, 3).

On 24th day, treatments Ch_1, Ch_2, $GA_3$1 and K1 were statistically different from each other as well as from other treatments as well. Chilling treatment Ch_3 was at par with treatment $GA_3$2 but different from all other treatments, $GA_3$2 is itself statistically different from all other treatments. Treatments K2, NAA2 and control were at par with each other but statistically different fron other treatments. Treatments NAA1 and NAA2 were at par with each other. Treatment Ch_1 was the best treatment with highest mean percentage of germination ±SE (68±2.30)(Table 2, 3).

The highest mean percentage of germination in chilling treatment Ch_1 (30 day chilled seeds) in almost all day intervals may be because chilling performs a pivotal role in inducing the stimulus that is needed to surmount dormancy. It is regarded to initiate an increase in the concentration of gibberllic acid (Bretzloff and Pellett, 1979). Chilling is useful to relieve primary inactiveness of many Northern hemisphere species (Baskin, 2001). It has been commonly used as a pre-sowing treatment to overcome dormancy and enhances percentage of germination of dormant seeds of many different species (Fang *et al*., 2006). The pre-chilling treatment conditions may actually be simulating the events that occur during the winter season just before the appearance of summer.

The next higher mean percentage of germination was observed in treatment $GA_3$1 (Fig. 1)(Table 3). It seems that pre chilling requirement in this species is replaced by gibberellin treatment, as it took only 30 days for complete germination. However, seeds were chilled for varying durations (1-month, 40-days and 50 days) before observing their germination over a period of one month. So treatment of seeds with GA3 saves a time period of approximately one month. Similar results have been reported by Sharma *et al.,* (2006) while studying the seed germination behavior of some medicinal plants species. However, our results were contradictory to Shabir *et al.,* (2010) as they did not obtain any germination in gibberellin treated seeds. Gibberellins surmount seed and bud dormancy in many species, thus serving as a substitute for low temperatures, long days or red light (Salisbury and Ross, 1992). In the present study, this response to germination was influenced by proportion of applied GA3. At lower concentrations (GA32= 10-4 M), germination was lower (75%) and at higher concentration (GA31= 10-3 M), it was higher (80%) (Table 3, Fig. 1). Dormant seeds, which demand cold temperature treatment, dry storage following maturation as initiator or stimulator of germination are mostly treated with GA3 to surmount their dormancy (Nadjafi *et al*., 2006). Plant growth hormones are chemicals which in small quantities can regulate various plant processes in addition to seed dormancy. Different plant hormones can control different plant processes including seed dormancy and germination, growth and development of various plant parts (Agraeber *et al*., 2012). Gibberellins are mostly employed to destroy the low temperature requirements of some plant seeds and enhance their germination percentage (El-Dengawy, 2005). It plays a role in inducing enhancement of enzyme synthesis that changes stored nutrients carbohydrates, which are required for quick cell respiration during germination (Bakrim *et al*., 2007). Similar mean percentage of germination ± SD (70±2.3) was observed in treatments $GA_3$2 (10-4 M) and Ch_3 (50 day chilled seeds), respectively, again justifying the positive role of gibberellins in enhancement of germination.

Increase in germination percentage was reported by other workers from studies carried out on other species, such as *Ferula gummosa* (Nadjafi *et al*., 2006), *Sesamum indicum* (Kyauk *et al*., 1995) and *Rumex dentatus* (Ali *et al*., 1996).

The third highest percentage of germination was observed in treatments K_1 and K_2 (KNO_3, 0.1% and 0.2) (Table 3) may be because of the fact that nitrogen compounds can break seed dormancy by de-

creasing C6/C1 ratio of CO and changing metabolic pathway, so they are usually used as germination accelerators. This may also be due to the positive action of KNO_3 on the seed membrane (Asaadi, 2017). Similar results were also reported by Abdollahi *et al.*, (2010) (in Sanguisorba minor Scop., *Pimpinella anisum* L., *Melissa officinalis* L. and *Nigella sativa* L.), Ghobadi *et al*., 2012 (in *Triticum aestivum* L.) Azimi *et al*., 2015 (*Glycine max* L. and *Brassica napus* L.), Yazdanshenas *et al.*, 2015 (*Portulaca oleracea* L.) and Çavuþoðlu, *et al*., 2017 (*Allium cepa* L.). However, Golizadeh *et al.* (2015) reported reductionin germination percentage in *Cannabis sativa* L. seeds when treated with KNO3.

In naphthalene acid treated treatments NAA1 and NAA2, mean percentage of germination ± SD (66±21.6 and 53±16.77) was higher as compared to control treatment (41±14.19). However, it was lower compared to gibberllic acid treated treatments GA31 (79±27.04) and GA32 (70±23.30) treatments. Shinde *et al*., (1994) also reported increased percentage germination as well as seedling vigour with the exogenous application of naphthalene acetic acid (NAA). This is because of the positive effects of hormone naphthalene acetamide, (synthetic auxin that acts as a rooting hormone) on the seed coat as the partial exposure of the cotyledons of the seeds permits the process of hydrolysis whereby hormones such as auxins and ethylene, which could increase nucleic acid metabolism and protein synthesis, are released (Uwaegbute, 1996). Maku *et al.,* (2014) also reported higher percentage of germination in *T. tetraptera* seeds when treated with naphthalene acetic acid (NAA).

Similarly lower percentage of germination was observed in sulphuric acid treated seeds (S_1) may be because of the fact that acids have negative influence on seed germination. Conc. Sulphuric acid breaks the phospho-diester bonds, which serve as a backbone for DNA, resulting in damage to DNA and cells. Thus the embryo gets killed. Similar results were observed by Nasiri and Eisavand (2001) and Shabir *et al.,* (2010) while studying the influence of acidic treatments on germination of *Ceratonia siliqua* and *I. racemosa,* respectively. Contrary to the present findings, Saied *et al*. (2008) on *Ziziphus*, Khaleghi *et al*. (2009), on Tamarind, Nasiri and Eisavand (2001), on *Albiziajulibrissin* and Hojati *et al*. (2007), on *Cycas revolute* Bhardwaj *et al.* (2016) on *Inulara cemosa* had introduced sulfuric acid as the best treatment.

In case of treatments Com. 1($H_2SO_4/GA_3$1) and Com. 2 ($H_2SO_4/GA_3$1), mean percentage of germination ± SD (37±11.84 and 31±10.34) was lower compared to control treatment (41±14.19). It may be because of the negative effect of sulphuric acid on seed germination. Similar was the case with treatments Com 3(H_2SO_4/NAA1) and Com. 4 (H_2SO_4/NAA2). Our results are contradictory to Bhardwaj *et al., (*2016), as they reported higher percentage of germination as compared to control treatment. As treatment of seeds with conc. sulphuric acid may be injurious to the seeds as acid may rapture vital parts of the embryo. According to Levitt (1974) and Nikoleave, (1977) immersion of seed in concentrated sulphuric acid disrupts the seed coat. However, Shabir *et al.,* (2010) reported higher percentage of germination in *Inularacemosa* seeds that were scarified and then treated with gibberellin.

Conclusion

Present experimental findings reveal that the prechilling treatment (3-4ºC) for a period of 30 days and treatment with GA_3 are that the most practical and useful pre-treatments for propagation of *Dioscorea deltoidea* seeds. Low temperature treatment can also be replaced by GA_3treatment. Further, application of GA_3 has also resulted in the reduction of germination time period, which in turn will help to produce large quantity of the plant material. Present findings will ensure production of large quantities of plant material which has immense potential in pharmaceutical companies and can serve as an axle to generate new sources of income for resource poor farming community.

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