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Chemical Stimulation of Seed Germination in *ex situ* Produced Seeds in *Swertia chirayita*, A Critically Endangered Medicinal Herb

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Abstract: Present study on *S. chirayita* was taken to (1) assess the germination potential of seeds procured form *ex situ* source and (2) enhance seed germination in *ex situ* produced seeds, using various pre-sowing chemical treatments. In *Swertia chirayita*, a critically endangered and high value medicinal plant, the seeds procured from 6 *ex situ* set ups, shrubberies, forest-slope, open-slope, tree-canopy; and green-house and net-shade, showing poor germination at initial testing (12 to 31%) were subjected to 11 pre-sowing chemical treatments. Among the pre-sowing treatments, gibberellic acid (50 to 350 μM) most effectively stimulated seed germination (96.7%, maximum; $p<0.001$) and reduced mean germination time ($p<0.05$), followed by potassium nitrate (100 mM) and sodium hypochlorite (5 min) ($p<0.001$). Study confirms *ex situ* produced seeds attained physiological dormancy, which was broken by pre-sowing treatments, as a tool to *ex situ* species conservation.

Key words: Critically endangered, *ex situ*, Himalayan, seed germination, *Swertia chirayita*

INTRODUCTION

One of the richest pools of biological diversity in the world, Indian Himalaya, has been experiencing unreasonable extraction of wild medicinal plants due to market demand, endangering many of its high-value gene stock (Badola and Pal, 2002, 2003; Badola and Aitken, 2003; Butola and Badola, 2008a) including *S. chirayita* (Bhattarai and Acharya, 1997; Badola and Pal, 2002, 2003; Dutta, 2004; Olsen, 2005; Pradhan and Badola, 2008a). *Ex situ* cultivation is considered as a possible solution to the conservation of endangered taxa and at the same time to meet out commercial raw material demand (Badola and Pal, 2002; Badola and Aitken, 2003; Badola and Singh, 2003; Heywood and Iriondo, 2003; Badola and Butola, 2003, 2005). It has been realized that to sustain availability of continuous stock of planting material for commercial cultivation as well as for species recovery, it is crucial to have in hand an appropriate multiplication technique to build a massive reserve of *ex situ* plants (Badola and Butola, 2003, 2005; Butola and Badola, 2007, 2008b). Additionally, the selection of species specific appropriate propagation technology (Fay and Muir, 1990; Butola and Badola, 2004a, b, 2006a, b) and ideal propagation conditions for mass multiplication (Butola and Badola, 2008b) would minimize the waste of propagule resource.

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However, poor seed germination of viable seeds in several Himalayan plant species is experienced as a limiting factor in large scale plant multiplication (Nadeem *et al*., 2000; Butola and Badola, 2004a, b, 2006b, 2007, 2008a, b; Pradhan and Badola, 2008a). Pre-sowing chemical treatments are used to enhance seed germination of wild sources of several Himalayan medicinal plants (Nadeem *et al*., 2000; Pandey *et al*., 2000; Joshi and Dhar, 2003; Manjkhola *et al*., 2003; Butola and Badola, 2004ab, 2006a, 2007; Shivkumar *et al*., 2006) and in plants of other regions (Plummer and Bell, 1995; Yamaguchi and Kamiya, 2001; Ghimire *et al*., 2006; Kulkarni *et al*., 2007; Vandelook *et al*., 2007; Kaur *et al*., 2009). The above studies were mostly confined to test seeds from wild. Seeds of only a few endangered Himalayan medicinal herbs have been evaluated, following establishment of *ex situ* set-ups, for their germination potential assessment, especially using various chemical stimulants and growing conditions (Butola and Badola, 2006a, 2007).

A critically endangered Himalayan herb (Ved *et al*., 2003) and prioritized on top for conservation through *ex situ* cultivation by the international experts (Badola and Pal, 2002), *Swertia chirayita* (Roxb. ex Fleming) H. Karst was targeted for the current study. The genus *Swertia* (Gentianaceae) comprises of over 170 species globally, of which 79, 27 and 40 species are distributed in China, Nepal and India, respectively. Sikkim alone, a North-Eastern state of India, harbours 13-14 species of *Swertia*. Amongst these, *S. chirayita*, which grows mostly in temperate Himalaya (1200-3000 m.a.s.l.) from Kashmir to Bhutan and in Khasia hills of Meghlaya, India (Chadha, 1976) in open pastures, grassy slopes and forests, is of great medicinal importance. Distribution of *S. chirayita* depends upon the altitude and slope and is not uniform. The species prefers to grow on north facing slopes and descends below 1500 m, while on south facing slope the plants are found between 1500 and 3000 m. In general, 2000 m altitude is a suitable range preferred by the species in Nepal (Bhattarai and Shrestha, 1996) and 1800-2300 m in Sikkim Himalaya (present authors; *unpublished*). Over centuries, *S. chirayita* is used ethno-medicinally (Wawrosch *et al*., 2005; Pradhan and Badola, 2008b) for having numerous medicinal properties (Nadkarni, 1976; Biswas and Chopra, 1982; Kirtikar and Basu, 1984). In *S. chirayita*, seed is the only viable solution to the reproduction of the plant. Very limited publications are available on seed germination of *S. chirayita*, all limited to wild seed resources (Raina *et al*., 1994; Pradhan and Badola, 2008a).

The objectives of the present study on *S. chirayita* were, (1) germination assessment of seeds from six *ex situ* produced sources and (2) enhancement of seed germination, in *ex situ* produced seeds, using various pre-sowing chemical treatments. So, far the authors' knowledge is concerned, it is for the first time the present study makes a move beyond wild stock and targets further the descendent seeds as a stock to put on viability of *ex situ* cultivation technology on *S. chirayita* for conservation. The current study makes a break from the ground and attempts to make a crucial step to understand process of domestication of species to attain predictability. The study targets to compare the *ex situ* produced seeds of *S. chirayita* from six sources and make various disclosers on the germination behavior of seeds.

MATERIALS AND METHODS

In March 2006, nursery raised (at, ca 2000 m altitude) 8 month old healthy seedlings of *Swertia chirayita*, initially developed through mass sowing (under biodiversity conservation programme) of wild seeds collected from the forests in East Sikkim, India (2050 m), were transplanted (140 seedlings; 35 plants/condition), maintaining 50 cm distance between plants, under four natural habitats of the species (shrubberies, forest-slope, open-slope and

tree-canopy) and two nursery conditions (70 seedlings; 35 plants/condition), on soil amended beds using garden soil and forest humus (open net-shade and temperature controlled green-house; 25°C±5), located at Pangthang-Gangtok, Sikkim, India (ca 2000 m altitude; N 27°21'51.6"; E 088°34'10.1"). Average minimum and maximum temperature of the area ranged between 6.42°C (January) and 20.1°C (August) and average minimum and maximum relative humidity varied between 70.3% (December) and 98.4% (July). Average monthly rainfall for nearest station, Gangtok, varied between 8.0 mm (minimum: December) and 691.1 mm (maximum: July), which remained high in August (617.8 mm). The percent survival of all transplanted plants was recorded. In December 2007, mature seeds were harvested from above six *ex situ* conditions and mixed well individually for each source. To determine moisture content, 100 seeds/replicate (3 replicates/seed source) were weighed and oven dried (60°C; 48 h). Seeds were counted for 10 healthy fruits per source. Seed size was measured for 30 seeds per sample (10 seeds each of 3 replicates) under a microscope. The remaining seeds were air dried in room temperature for 15 days. The seed viability test could not be performed due to minute seed size (Pradhan and Badola, 2008a). In January 2008, seed germination potential using distilled water was tested (6 replicates; 30 seeds each) in a seed germinator, which yielded poor germination. The remaining seeds were stored in air tight specimen tubes in refrigerator (4°C) till the initiation of the pre-sowing chemical treatment experiment which was performed soon after the initial test was over.

Seed Germination Test

In February 2008, air dried seeds were surface sterilized by dipping in 0.04% aqueous solution of Mercuric chloride ($HgCl_2$) for 10 sec to discourage fungal infection and washed thoroughly with double distilled water in all cases. The disinfected seeds were soaked in beakers containing various freshly prepared test solutions, viz., gibberellic acid (GA_3: 50, 150, 250, 350 μM); potassium nitrate (KNO_3: 50, 150, 250 mM) for 24 h and sodium hypochlorite (NaOCl, 5% available chlorine) for 5, 10 and 15 min. Treated seeds were washed 2-3 times with double distilled water and placed in petri-plates (90 mm) lined with single layer of Whattman No. 1 filter paper moistened with double distilled water. Control sets were maintained using double distilled water. Each treatment had 06 replicates of 30 seeds each, which were incubated for a maximum of 45 days in germination chamber (temperature: 25± 2°C; photo period: 14/10 h light/dark), following a complete randomized design. Seeds were checked daily and moistened with double distilled water as and when required and recorded as germinated on radical emergence. Germinated seedlings were counted and removed.

Statistical Analysis

The data were analyzed for mean and standard deviation (SD). Mean germination time (MGT) was calculated using equation:

$$MGT = \frac{\sum(nd)}{\sum N}$$

where, n is number of newly germinated seeds after each incubation period in days d and N is total number of seeds emerged at the end of the test (Hartmann and Kester, 1989). Analysis of variance was conducted on different parameters. The difference among the means was compared by Least Significant Difference (LSD) test (Snedecor and Cochran, 1967).

RESULTS

Before fruit harvest in December 2007, the final plant survival varied in different growing conditions, *viz*., 71% (Shrubberies), 54% (forest-slope), 22% (open-slope), 71% (tree-canopy), 43% (net-shade) and 88% (green-house). At attaining full maturity of plants, fruits harvested in December 2007 showed significant differences amongst sources. Number of seeds per fruit was significantly higher for shrubberies, which further varied amongst plot sources ($p<0.01$). Seed moisture content ranged from 14 to 22%. Significant differences ($p<0.05$) were obtained for seed weight, seed size and the moisture content amongst sources (Table 1). In initial control test, very low seed germination was recorded for all sources, viz., shrubberies: 31%; forest-slope: 12%; open-slope: 20%; tree-canopy: 21%; net-shade: 23%; green-house: 19%. Seeds produced in shrubberies showed significantly higher germination ($p<0.001$) compared to other sources, except net-shade, which differed at $p<0.01$.

In pre-sowing chemical stimulation experiment, in control, the seeds from forest-slope showed lowest germination (9.44%) comparing to other plots; this followed more or less same trend to that of earlier initial control test. Pre-sowing chemical treatments significantly stimulated seed germination in *S. chirayita* over control, which varied amongst sources (Table 2). In general, GA_3 was most effective in stimulating seed germination over other treatments. Across seed sources, compared to control, GA_3 treatments improved the percent germination significantly ($p<0.001$). Seed germination value ranged between 70% (minimum; net-shade) and 97% (maximum; forest-slope and tree-canopy) using different GA_3 concentrations. Amongst them, GA_3 250 μM followed by GA_3 350 μM effectively stimulated seed germination ($p<0.001$). Seed sources did not show uniformity of germination specifically to a particular concentration of GA_3. For example, seeds from shrubberies responded best with 50 μM GA_3; from open slope best germination was obtained for 250 and 350 μM GA_3,

Table 1: Seed characteristics of *Swertia chirayita* grown under different conditions at ca. 2000 m.a.s.l.

Seed source	Shrubberies	Forest-slope	Open-slope	Tree-canopy	Net-shade	Green-house	p<0.05	F-value
Site conditions	Slope: 20°, Dominant species: *Symploccos theifolia, S. glomerata*	Slope: 40°, Dominant species: *Bucklundia populnea*	Slope: 10°; Dominant species: *Osbeckia* sp.	Slope: 20°, Dominant species: *Castanopsis tribuloides*	Flat beds (soil amended with forest humus)	Flat beds (soil amended with forest humus)		
No. seeds per fruit	316.00	230.00	184.00	146.00	172.00	200.00	60.42	26.04
Seed weight (mg 100^{-1} seeds)	3.20	2.93	2.67	2.73	2.90	3.03	0.34	2.93
Seed length (μm)	362.20	394.07	480.17	379.83	417.77	361.90	1.96	40.60
Seed Width (μm)	315.27	310.40	355.70	306.10	345.77	293.63	2.15	11.28
Moisture content (%)	14.62	14.81	17.47	21.91	17.38	20.89	4.45	4.10

Table 2: Effect of different chemical treatments on seed germination in *Swertia Chirayita*, sourced from six *ex situ* conditions

	Germination (%)									
Treatments	Shrubberies	Forest-slope	Open-slope	Tree-canopy	Net-shade	Green-house	p<0.05	p<0.01	p<0.001	F-value
Control	28.33	9.44	20.56	19.44	21.11	17.78	5.91	7.93	10.46	8.77
GA_3 (50 μM)	91.67	88.89	83.33	85.00	70.00	82.78	10.61	14.23	18.77	4.11
GA_3 (150 μM)	83.33	96.67	83.89	92.78	73.33	82.22	9.75	13.09	17.25	5.95
GA_3 (250 μM)	86.11	95.56	91.67	96.67	89.44	90.56	7.39	9.92	13.07	2.33
GA_3 (350 μM)	85.56	96.11	91.11	91.11	85.56	82.22	6.75	9.05	11.94	4.64
KNO_3 (50 mM)	72.22	23.89	20.00	31.67	48.89	27.22	8.61	11.56	15.24	43.69
KNO_3 (100 mM)	59.44	35.00	42.22	37.78	45.00	37.78	9.30	12.47	16.44	7.51
KNO_3 (150 mM)	45.56	07.22	06.11	05.00	32.78	18.89	6.41	8.60	11.33	56.05
$NaHClO_3$ (5 min)	56.11	53.33	48.33	52.22	48.89	54.44	9.84	13.20	17.41	0.81
$NaHClO_3$ (10 min)	24.44	20.56	31.67	23.89	21.67	42.22	9.61	12.89	16.99	6.05
$NaHClO_3$ (15 min)	12.22	5.00	23.33	13.33	7.78	10.00	7.07	9.48	12.50	6.64
Statistical analysis										
p<0.05	9.50	6.87	9.09	7.98	7.95	8.16				
p<0.01	12.63	9.13	12.08	10.60	10.56	10.84				
p<0.001	16.40	11.86	15.69	13.77	13.72	14.08				
F-value	68.78	255.83	101.21	155.37	94.73	109.64				

Table 3: Effect of different chemical treatments on mean germination time in *Swertia Chirayita*, sourced from six *ex situ* conditions

	Mean germination time							
Treatments	Shrubberies	Forest-slope	Open-slope	Tree-canopy	Net-shade	Green-house	p<0.05	F-value
Control	23.55	27.26	17.53	20.46	22.88	20.20	2.69	12.82
GA_3 (50 μM)	15.51	17.85	16.22	15.97	18.38	16.94	1.87	2.97
GA_3 (150 μM)	14.27	15.66	15.29	13.74	16.41	14.65	1.18	5.63
GA_3 (250 μM)	15.44	17.58	14.92	14.96	15.73	15.33	0.91	9.74
GA_3 (350 μM)	14.16	16.72	14.52	15.21	15.24	15.37	0.95	7.07
KNO_3 (50 mM)	19.17	22.64	20.45	21.08	21.14	20.08	1.61	4.37
KNO_3 (100 mM)	20.24	22.19	19.04	22.81	20.46	22.18	1.53	7.54
KNO_3 (150 mM)	23.86	24.25	23.33	20.00	23.83	20.66	2.60	4.12
$NaHClO_3$ (5 min)	19.77	18.62	18.11	17.79	19.13	18.45	2.67	0.59
$NaHClO_3$ (10 min)	19.46	23.18	19.62	17.76	18.53	17.56	3.07	3.69
$NaHClO_3$ (15 min)	17.93	20.06	17.56	20.89	20.43	18.28	3.35	1.51
Statistical analysis								
p<0.05	2.20	2.27	1.97	2.28	2.23	2.03		
F-value	19.02	20.29	14.44	13.83	12.61	11.70		

whereas seeds from tree-canopy, net-shade and green-house responded best to GA_3 250 μM (Table 2). Seeds from forest-slope showed an exceptional uniformity in germination of about 96% with all GA_3 concentrations except GA_3 50 μM (89%). Comparing to control, KNO_3 treatments proved beneficial in improving seed germination in majority of cases; in which, KNO_3 100 mM proved equally effective to stimulate seed germination in all sources ($p<0.001$). Individually, maximum seed germination (72%; $p<0.001$) was achieved with KNO_3 50 mM for shrubberies. However, KNO_3 150 mM was detrimental in case of three seed sources (forest-slope, open-slope and tree-canopy). Soaking in NaOCl (5 min) had significantly ($p<0.001$) stimulated seed germination (48%: open-slope to 56%: shrubberies). Increased soaking time in NaOCl (15 min) appeared harmful to the delicate seeds of *S. chirayita*.

Significant decline ($p<0.05$) in germination delay (number of days taken for 1st seed germination) was recorded with different concentrations of GA_3 over control, with lowest of 10 days for GA_3 50, 150 and 350 μM, which varied amongst seed sources from experimental plots. The KNO_3 and NaOCl were effective in reducing germination delay only on the seeds collected from shrubberies and forest slope. The half of the maximum seed germination time (days taken to reach 50% of final germination) of 26 days was recorded in control for the forest-slope and a minimum of 13 days in GA_3 150 μM (shrubberies and tree-canopy), GA_3 250 μM (tree-canopy) and GA_3 350 μM (shrubberies and open-slope). Gibberllic acid significantly reduced ($p<0.05$) Mean Germination Time (MGT) over control compared to other treatments (Table 3). After soaking in different concentration of KNO_3 and for different period in NaOCl, significant reduction ($p<0.05$) in MGT was achieved for the seeds collected from shrubberies, forest-slope and net-shade. Individually, significant reduction in MGT ($p<0.05$) was recorded for tree-canopy in NaOCl (5 and 10 min) and for green-house in NaOCl (10 min). In general, among all treatments, GA_3 had greatly improved percent germination (Table 2) and lowered the MGT (Table 3).

DISCUSSION

In the present study, *ex situ* produced seeds of *S. chirayita* harvested from different growing conditions showed very low germination, initially, under control. Likewise in many Himalayan herbs (Pandey *et al*., 2000; Butola and Badola, 2004a, b, 2006a, 2007; Shivkumar *et al*., 2006), pre-sowing chemical treatments significantly improved germination percentage, onset and final germination of *S. chirayita* in present study. Here, gibberellic

acid most effectively stimulated seed germination and minimized the difference in onset and final germination which can be attributed to increased activity of hydrolytic enzymes (Al-Helal, 1996; Joshi and Dhar, 2003; Manjkhola *et al*., 2003) or mobilization of nutrients in dormant seeds (Kumar and Purohit, 1986; Hartmann and Kester, 1989). Further, gibberellic acid is known to promote germination by breaking dormancy in wide range of seeds (Vandelook *et al*., 2007; Perez-Gracia, 2008). In *S. chirayita*, GA_3 in all concentrations was highly stimulatory to seed germination. Further, except for two sources, i.e., tree-canopy and net-shade, the difference amongst GA_3 treatments was insignificant in other four sources. That suggested effectiveness of all used concentrations of GA_3 in stimulating seed germination as well as reducing mean germination time in *S. chirayita* as gibberellic acid is known to affect physiological as well as metabolic activities of seeds resulting in early germination (Tipirdamaz and Ve-Gömürgen, 2000; Chuanren *et al*., 2004). With the increase in GA_3 concentrations, increase in percent germination was observed (Abdel-Hady *et al*., 2008; Keshtkar *et al*., 2008). However, present study indicated non-uniformity in germination with different concentrations of GA_3 amongst the seed sources as reported by Bhatt *et al*. (2005) for *Swertia angustifolia*. However, exogenous application of GA_3 had no effect on the germination of seeds in *Ferula assafoetida* (Otroshy *et al*., 2009).

Sodium hypochlorite overcomes seed dormancy by either increasing permeability of the seed coat to oxygen through the removal of phenolics (Hurley *et al*., 1989) or by scarification or modification of the seed coat (Hsiao, 1979; Butola and Badola, 2004a, b, 2007) which might have resulted in early seedling onset and increased percent germination in seeds treated with NaOCl (5 and 10 min) compared to control for all tested sources in the present experiment. The NaOCl (10 min) could significantly stimulated seed germination for only two sources (forest-slope and green-house) at $p<0.05$. However, the time period for soaking in NaOCl needs to be standardized from species to species. For instance, seed germination was enhanced by soaking for 5 to 60 sec in *Amaranthus powellii* but declined as the soaking period exceeded 60 sec in annual weed (Tommaso and Nurse, 2004). Similarly, seed germination was increased from 2 to 19% in *Zizania palustris* when soaked in NaOCl for 2 h (Oelke and Albrecht, 1985). A range of soaking time in NaOCl (15-45 min) effectively used in many Himalayan herbs (Butola and Badola, 2004ab, 2006b, 2007). Since, the seeds are susceptible to fungal infection during germination, but, treating with NaOCl further reduces the chances of fungal infection to seeds (Butola and Badola, 2007) thereby resulting in improved germination as in the present study.

In present study, KNO_3 50 and 100 mM proved significantly effective for all sources used, lesser stimulatory comparing to GA_3. Whereas, increased concentration of KNO_3 (150 mM) was beneficial significantly only in case of shrubberies and net-shade. Contrarily, in *Angelica glauca* (with large sized seed, compared to minute seeds in *S. chirayita*), KNO_3 (150 mM) was found effective in stimulating seed germination (Butola and Badola, 2004a), may relate to the seed size as an important factor, need to be considered while standardizing level of concentration. Effectiveness of KNO_3 in stimulating seed germination may be possibly due to oxidized form of nitrogen causing a shift in respiratory metabolism to the pentose phosphate pathway (Roberts and Smith, 1977). Nitrogenous compounds in various forms, particularly nitrates (e.g., KNO_3) have been used to stimulate germination (Choudhary *et al*., 1996; McIntyre *et al*., 1996). They play a critical role in increasing the physiological efficiency (Bhargava and Banerjee, 1994) and influence germination through change in water relationship (Nikolaeva, 1977). The responses of seeds to a particular concentration of KNO_3 may vary with species. In Gentianaceae family, in general, physiological dormancy is reported (Nikolaeva, 1977). Contrarily, the researcher's

assessment on 13 wild populations of *S. chirayita* (Gentianaceae) did not show any seed dormancy (Pradhan and Badola, 2008a). However, in the present study, *ex situ* produced seeds were found with high dormancy. The results with improved germination rates in current study under GA_3 and KNO_3 treatments further correspond well with these generalizations, as both these substances are considered best for breaking physiological dormancy (Nikolaeva, 1977). The present study confirms that the seeds of *S. chirayita* from *ex situ* grown sources, initially using wild seed stock, had physiological dormancy, which was broken by pre-sowing chemical treatments.

Present authors had earlier recorded over 70% (minimum) seed germination in thirteen wild populations of *S. chirayita*, which gradually declined over time and further differed amongst sources under storage conditions (Pradhan and Badola, 2008a). Seed germination potential differences in the same species/genus have been reported (Celiklar *et al*., 2006). Such differences may be due to the environmental variations in the light and temperature (Karlsson and Milberg, 2007; Vandelook *et al*., 2007) and climate present in the native habitats of the species (Celiklar *et al*., 2006), but if proper strategies in respect of seed collection time etc., is maintained, such difference can be minimized to some extent. The present results suggest, there might be various environmental factors, the plants grown in or transplanted from green-house to natural habitats had to encounter during acclimatization and adaptation over two more growing seasons, might have induced greater physiological seed dormancy in second generation, which was later broken by chemical treatments. This can be viewed as a part of defence mechanism of a plant's survival tactics in response to a sudden change in its' eco-climatic condition. More detailed investigations are recommended to assess such eco-physiological changes occur from a sudden snap from wild species adapted environmentally over ages. The study further recommends GA_3 (50 to 350 μM) as the best treatment for breaking seed dormancy and greatly improving germination in *ex situ* produced seeds in *S. chirayita*. Good survival in natural conditions following transplantation of plants developed in green-house, using above pre-sowing treatments, has appeared as a technological package for strengthening conservation as well *ex situ* cultivation of targeted endangered taxa, *S. chirayita*, in Himalaya.

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