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ETHANOL INDUCED SEED GERMINATION IN *ACONITUM HETEROPHYLLUM* WALL.: AN ENDANGERED MEDICINAL HERB OF THE NORTH-WEST HIMALAYAS

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SUMMARY

***Aconitum heterophyllum* is an important medicinal herb of high altitudes of western Himalayas (India). Over exploitation from the natural habitats by the drug industries and prolonged seed dormancy made this species endangered. Attempts were made for domestication of this plant species through seeds at comparatively lower altitudes by removing seed dormancy. The seeds of *Aconitum heterophyllum* were germinated by overcoming dormancy with ethanol. This low cost organic solvent improved the per cent germination by enhancing the growth potential of the embryonic axis. In the seed protein profiles a disappearance was observed in the content of 28 and 21 kd proteins in second phase of ethanol induced seed germination. These proteins might have a significant role in enhancing the germination percentage as well as increase in growth potential of the embryonic axis. This study suggests that seed germination and potential for establishment of nurseries of *A. heterophyllum* can be improved with ethanol.**

***Key words: Aconitum heterophyllum,* ethanol, germination protein profiles, radicle growth potential, seed germination**

INTRODUCTION

Aconitum heterophyllum Wall is a herbaceous, rhizomatous species of great medicinal importance that is now endangered in India (Nayar and Sastry 1990). This plant species is distributed in restricted pockets throughout the alpine Himalayan region. Chakravarty and Chakravarti (1954) identified 13 species of *Aconitum* that have drug value. Tuberous roots are commonly known as 'aconites' or 'monkshood' and yields aconitine and alkaloids. The aconites are medicinally important as antipyretic, antiperiodic, aphrodisiac and as an astringent tonic. These are also used for diarrhoea, indigestion, and cough troubles in children. Conservation of medicinal plants is receiving increased attention all over the globe in view of the resurgence of interest in herbal medicines for health care and over exploitation from nature. In India, large quantities of medicinal plants are extracted from the wild species to meet the increasing demand of raw material for domestic consumption and export. As a result, natural habitats are depleting at a fast pace.

Delayed and asynchronous seedling emergence is a major problem in *Aconitum* which results in a reduced crop stand lowering root yield and quality. Under natural conditions, seed germination and seedling establishment in *A. atrox* is rare (Nautiyal *et al.* 1985, Nautiyal 1986, Bhadula *et al*. 2000). Seed size, viability, sowing depth, soil moisture and temperature are all known to contribute to poor and/or sporadic germination and radicle emergence (Bewley and Black 1982, Rajasekaran *et al*. 1992). Pandey *et al*. (2005) attained higher per cent germination in *Aconitum* by breaking seed dormancy using hot water treatment. Beigh *et al*. (2005) reported

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that chilling also improves seed germination in *Aconitum*. Modification of plant architecture with some inducers (Plant growth regulators and chemicals) at a specific stage of development, leads to improved harvest index without reducing total yield. Organic solvents like ethanol, methanol, acetone and peroxides etc. are known as seed germination inducers (Di Nola *et al*. 1990, Amritphale *et al.* 1993, Sreenivasulu and Amritphale 2000). A low concentration of ethanol is non-toxic for the plants (Caddick *et al.* 1998, Ait-ali *et al*. 2003, Vregdenhil *et al.* 2006). In this study, we investigated the effect of ethanol on *Aconitum* seed germination and seedling establishment. These finding will be useful to understand the physiology of germination and to develop strategies for cultivation of the species through seeds and to rehabilitate the degraded habitats and helps in conservation.

MATERIAL AND METHODS

Mature fruits of *Aconitum heterophyllum* were collected from the sub-alpine regions of Lahul-Spiti (4000 msl) located in the upper parts of Western Himalayan region (India) during the month of August 2005. Fresh seeds were separated from fruits and air dried at room temperature. The seeds were kept in a climate controlled room (25 ºC at 60% relative humidity) until a moisture content of 12% (fresh weight basis) was attained after10 days. The seeds (viability 98%) were then stored in air tight plastic containers at 4ºC until the beginning of the experiments. These seeds were used in the present study after washing them thoroughly with deionized water and drying between layers of filter paper.

All germination tests were conducted with 25 seeds per replicate and 4 replicates per treatment. Seeds were placed in 10-cm Petri dishes on Whatman No.1 filter paper circles moistened with 5 ml distilled water. The Petri dishes were kept at 20±1°C for germination in the incubator. The criterion for germination was emergence of radicle through testa. All of the experiments were repeated at least twice and the averages were presented. Germination Index was calculated as germination per cent multiplied with root length after 17 days (GI = Germination per cent X Root length in mm).

Chemical treatment: Seeds were immersed in acetone (Qualigen fine chemicals, Mumbai, India), H_2O_2 (30% (W/V), Qualigen fine chemicals, Mumbai, India), Methanol (Merck India Ltd, Mumbai) and ethanol (99%, Bengal Chemicals and Pharmaceutical Ltd., Calcutta, India), in a glass-stoppered flask placed at 10°C for 30, 60, 180, 300 and 600 sec. Seeds were air dried and tested for viability at 25 ºC by tetrazolium staining as per ISTA (1985) standard procedure.

Measurement of growth potential: Both ethanol treated and untreated seeds (25 seeds per replicate and four replicates) were placed in 10 cm Petri dishes containing filter paper circles moistened with 5.0 ml Polyethylene glycol (PEG) solution (Michel 1983) and with distilled water control. Germination was recorded at 20±1°C after 9 days up to 17 days. The criterion for germination was emergence of radicle through the testa. Root length was determined after 17 days.

Isolation and analysis of proteins: Total proteins were isolated from both ethanol treated and un-treated seeds imbibed for various time periods. One gram of imbibed seeds of each was homogenized separately in 2 ml of 50 mM Tris-HCl (pH6.8) containing 2%SDS, 100 mM DTT in a pre-chilled mortar and pestle. The homogenate was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant proteins were concentrated by acetone precipitation following Hames (1981). Protein contents were estimated following the dye-binding method of Bradford (1976). For electrophoresis, proteins were dissolved in SDS-sample buffer (Hames 1981). One dimensional SDS-PAGE was performed with 3.5% stacking gel and 10% resolving gel according to Laemmli (1970).

Experiments were arranged in Randomised Block Design (RBD) with four replicates. Regression analysis at 95% confidence interval was carried to compare differences in treatment means.

RESULTS

Most of the high altitude plant species show poor seed germination under natural conditions due to their morpho-physiological dormancy (Bhadula *et al.* 2000).

Most of the organic chemicals including ethanol enhance seed germination of high altitude plant species like *Podophyllum, Arnebia, Ephedra, Aconitum* and *Brunicum perscicum* (unpublished results). Among these species, *Aconitum* was selected for further detailed study. Germination data of *Aconitum* seeds in response to various chemicals applied to them in un-imbibed state has been shown in Table 1. Among all the tested chemicals ethanol evoked a high per cent seed germination (95%) whereas, acetone, methanol and H_2O_2 enhanced germination in the range of 81-86%. On the basis of dormancy of *Aconitum* seeds, ethanol was selected to elucidate the germination mechanism.

Table 1. Effect of various chemicals on *Aconitum* seed germination. Seeds were treated with different organic solvents for 30 sec at room temperature and air-dried for 24 h, and allowed to germinate in distilled water for 17 days at 20±1ºC. The values (±) are mean SD.

Chemical	Germination (%)
Control	46 ± 1.0
Acetone	81 ± 1.5
Ethanol	95 ± 2.8
H_2O_2	83 ± 1.1
Methanol	86 ± 2.4

Dose-response curves were prepared by treating the dry seed with ethanol for various time periods. Treated seeds were air dried and kept for germination. Figure 1 depicts *Aconitum* seed germination and GI at 20±1°C after ethanol treatment. Ethanol doubles the germination when compared to that of control. Ethanol treatment time of 30, 60 and 180 sec showed similar response on germination but >60 sec treatments showed a slight decrease in per cent germination with visible adverse effects on subsequent seedling growth (browning of the roots). Hence, 30 sec duration of ethanol treatment was administered to the seeds in the subsequent studies. Germination of a seed depends on the growth potential of its embryonic axis. Growth capacities of the embryonic axis were quantified as Germination Index (GI) during ethanol induced germination. GI was maximum at 30 and 60 sec treatment duration but decreased progressively with further increase in duration from 180-600 sec (Fig. 1).

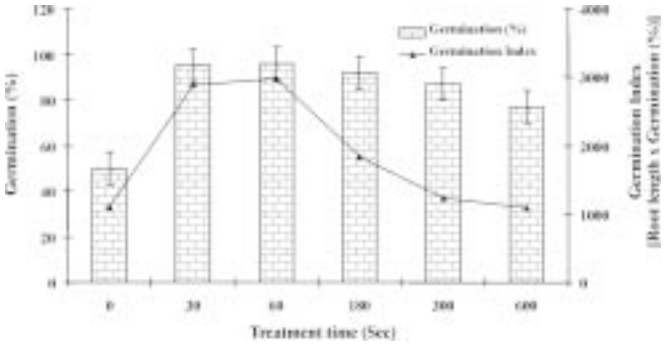

Fig. 1. Seed germination in *A. heterophyllum* seeds. Seeds treated with the ethanol and kept for germination at 20ºC and recorded the germination on each day for 17 days. Error bars are ± SEM values. GI was calculated as per cent seed germination multiplied with the root length.

Regression lines were obtained by plotting the rate of germination against incubation times. The correlation coefficients were highly significant in case of treated seeds (Fig. 2). Ethanol treated seeds showed advancement in germination by 2 days besides exhibiting higher rate of germination to the extent of 90 - 95%, when compared to untreated (Fig. 2).

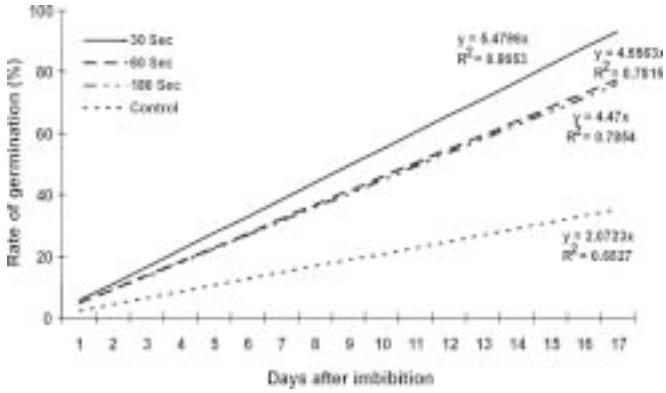

Fig. 2. Rate of germination in *A. heterophyllum* seeds after ethanol treatment. Regression equations of seeds during ethanol induced germination. Increase in per cent germination with ethanol treatment was found to be significant.

Investigation on the germination of ethanol treated and untreated seeds in a solution of different osmotic strength allow quantification on the growth potential of

the embryonic axis. Fig. 3 shows the seed germination and growth of the radicle on different strengths of osmotica. Germination per cent and root length were decreased as increased in osmoticum concentration both in treated and their untreated controls. Ethanol treated seeds showed 47.8, 50, 53, 57 and 67.6 per cent more germination with 2.6, 1.3, 21, 38.6, 38.4 and 47.1% more length in radical growth when compared to their respective untreated controls of DW, -0.5, -1.0, -1.5 and -2.0 MPa osmoticum, respectively (Fig. 3).

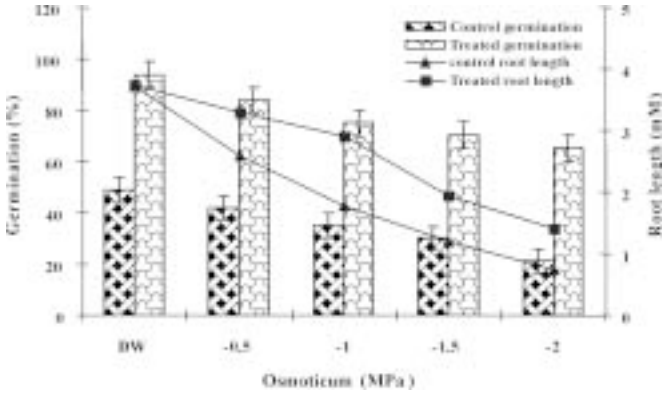

Fig. 3. Germination of *Aconitum* seeds after ethanol treatment as a function of growth potential against different concentrations of osmoticum. Untreated and ethanol treated seeds kept for germination on different concentrations of PEG solutions. Per cent germination and root lengths were measured after 17 days

Studies were conducted to assess changes in proteins during ethanol induced germination. Protein profiles were prepared by isolating proteins at different time intervals of ethanol induced seed germination. Different time intervals were selected on the basis of seed water uptake curve (data not shown) for isolating proteins representing first two phases i.e. 0, 2 (Phase I) and 4, 6, 7 days (Phase II), during the germination. Seed protein profiles were generated on SDS-PAGE for both the untreated and ethanol treated imbibed seeds. No significant changes were observed between the protein profiles of ethanol-treated and untreated seeds in dry condition (Fig. 4). Disappearance of high molecular weight proteins (>70 kd) was noticed in the protein profiles of ethanol treated seeds with two days of incubation on water. During initial stages of the Phase II of the growth curve (i.e. 4 days), no or little changes were observed. It is obvious that this phase of the growth curve is a physical process where the water imbibition takes place. However, significant changes were recorded in the protein profile of 6 and 7 days (II phase of germination) imbibed ethanol treated seeds when compared to its corresponding controls. Disappearance of 28 kd and 21 kd proteins was recorded in the ethanol treated seed protein profiles.

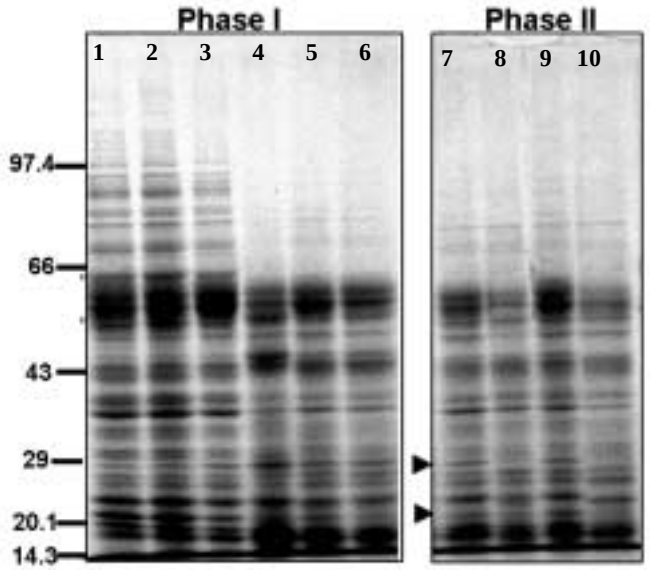

Fig. 4. Change in protein profiles of *A.heterophyllum* seeds at different phases of the germination. Untreated and ethanol treated seeds kept for germination at 20ºC. Total proteins were isolated at various time intervals during germination and analyzed on SDS-PAGE. Lanes 1, 3, 5, 7 and 9 are the proteins from the untreated seeds and 2, 4, 6, 8 and 10 are the proteins from the ethanol-treated seeds after 0, 2, 4, 6 and 7 days respectively, after incubation during the germination. Arrow heads indicate the disappeared proteins

DISCUSSION

In *Aconitum heterophyllum*, an important medicinal herb, regeneration under natural conditions is poor because of long dormancy periods and poor seed germination (Beigh *et al*. 2005, Pandey *et al*. 2005). The present study was intended to provide a method for overcoming seed dormancy in this species. A large number of non-hormonal, organic and inorganic chemicals including alcohols, aldehydes and ketones are known to break seed dormancy in many of the plant species (Taylorson and Hendricks 1979, Cohn *et al*. 1989, Taylorson 1991, Bewley and Black 1982, 1994) and

terminates the developmental arrest (Cohn and Hillhorst 2000). Besides having an applied potential, they serve as molecular probes to explore the mechanisms involved in the transition from developmental arrest to growth (Cohn 1996).

Under natural conditions, seed germination and seedling establishment is rare in *Aconitum* (Nautiyal 1986, Bhadula *et al*. 2000). Seed germination has been reported to be poor in this species (Nautiyal *et al.* 1985). In the present study various chemicals like acetone, ethanol, H_2O_2, and methanol were found to induce the germination in *Aconitum* seeds. Among these ethanol induced highest germination. A number of workers (Adkins *et al.* 1984, Corbineau *et al.* 1991; Footitt and Cohn 1995) proposed a metabolic role for alcohols and related substances in stimulation of germination. Many other workers have suggested that they act at the membrane level (Taylorson 1982, Taylorson 1988, Di Nola *et al.* 1990, Sreenivasulu and Amritphale 2000). Interestingly, dormancy breaking activity of a large number of substances was shown to be correlated with the lipophilicity of the applied chemical (Cohn *et al.* 1989, Cohn 1996, Sreenivasulu and Amritphale 1998).

Seed germination i.e. occurance of radicle protrusion is determined by a balance between the growth potential of the embryo and the mechanical resistance of the endosperm (Nonogaki *et al*. 1992, Nonogaki and Morohashi 1996, Nonogaki *et al*. 2000). Either increase in growth potential of the embryo, decrease in mechanical resistance of the endosperm or both have to occur to change the balance (Nonogaki 2006). In case of *Aconitum* seeds increase in per cent germination and no much difference was observed in radicle length of treated seeds on distilled water, whereas increase in per cent germination as well as more radicle length was recorded in ethanol treated seeds. This increase can be attributed to the increase in growth potential of the embryos on stress. High GI was recorded in treated seeds. Decrease in GI in more than 60 sec treatments was revealed in the seedlings by showing adverse effects in subsequent growth (data not shown). These results confirm that the ethanol treated seeds show better germination performance in stress conditions than the untreated ones.

In the present study a general decrease in the content of high molecular weight proteins (>70 kDa) was noticed with the seed germination. Disappearance in the content of 28 and 21 kDa proteins was also noticed specifically in the protein profiles of ethanol treated seeds on 8th and 9th day during germination. This time point falls in late stage of phase II of the imbibition. In this phase, metabolic processes that are required for embryo growth and completion of germination are activated (Bradford 2004). These protein changes can be attributed to its significant role in germination process. Di Nola *et al*. (1990) identified an increase in the content of a 23 kDa protein prior to visible germination in case of n-propanol treated *E. crus-galli* seeds. A similar study of secondarily dormant cucumber (*Cucumis sativus* L.) seeds identified an increase in the 20 and 36 kDa proteins and decline of a 14 kDa protein in the plasma membranes of the embryonic axes from ethanol treated dormant seeds (Sreenivasulu and Amritphale 2000).

These incubation periods were representing the II phase of the growth curve where new physiological mechanisms prepare cell expansion in the embryonic axes, culminating in the start of cell elongation. These changes might be attributed to the significant role in germination. The change in proteins in later stages can be attributed to post germination events associated with the seedling growth. The present study, showed ethanol induced germination of *Aconitum* seeds by increasing the water uptake, reduced the mean germination time and increased GI. These changes are coinciding with protein changes. The protein changes might be having a significant role in ethanol induced germination in *Aconitum*. On the basis of above results, it can be concluded that seed germination in *Aconitum* can be improved by simple seed treatments with ethanol and this method may be helpful in mass propagation of this high value endangered medicinal plant.

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