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Germination Studies of Critically Endangered Medicinal Angiosperm Plant Species *Meconopsis aculeta* Royle Endemic to Kashmir Himalaya, India: A multipurpose Species

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Abstract

The critically endangered and perennial alpine endemic angiosperm, *Meconopsis aculeta* Royle inhabit such habitats in the Kashmir Himalaya that are characterized by short growing season and heavy snow cover for about 3-4 months during winter season. The seeds of this species under natural conditions experience a long period of pre-chilling during winter prior to their germination in following spring season. Taking cue from such a requirement, present study investigated the effect of exogenous application of growth hormones (BAP, NAA Zeatin, Kinetin and TDZ) on germination percentage of deep-dormant seeds of *Meconopsis aculeta,* under alternate light/dark regimes. Treatment of seeds with different doses of hormonal combinations had a pronounced stimulatory effect on the total germination percentage. In fact, highest germination percentage (78%) was recorded on MS basal supplemented with combinations of Zeatin (4 mg/l) and NAA (0.5 mg/l) within 30 days. The seeds cultured on MS basal medium has no significant influence on germination percentage in *Meconopsis aculeta.* Among various treatments highest *in vitro* shoot regeneration was achieved on MS medium supplemented with combination of Zeatin (2 mg/l) and IAA (0.1 mg/l) with 3.41 ± 1.00 cm mean length of shoots with a culture response of 40% within 28 days. *In vitro* raised shoots regenerated roots on MS medium supplemented with combination of Zeatin (2 mg/l) and IAA (0.1 mg/l) with 14.65 ± 1.23 mean number of roots and 2.76 ± 1.21 cm mean length of roots within 42 days.

Keywords: Endangered; Medicinal plant; *In vitro* seed germination; Seedling development; *Meconopsis aculeta*

Introduction

North West Himalayas, one of the richest pools of biological diversity in the World, has been experiencing ruthless extraction of wild medicinal plants due to ever increasing global inclination towards use of herbal medicine and thus endangering many of its high value gene stock. The continued commercial over-exploitation of these valuable herbal gems has resulted in depleting the population of many such species in their natural habitats. In India, with an estimated 8000 species of medicinal plants [1], more than 90% of medicinal plants for herbal drug industries are drawn from natural habitats, thereby putting them to severe exploitation [2,3]. In addition to the over exploitation, destructive harvesting practices, habitat degradation and agricultural encroachment to wild habitats have also been recognized as contributing factors in the loss of our herbal gems. Owing to increasing market demand, Himalaya has been undergoing unreasonable extraction of wild medicinal plants endangering many of its high-value gene stocks [4].

The genus Meconopsis Vig., known as the "Blue poppy", belongs to the Papaveraceae family. There are a total of 49 species of the Meconopsis genus worldwide, and about 38 species distributed in the west of China, including Qinghai, Sichuan, Yunnan provinces and the Tibet region [5]. *Meconopsis aculeta* is an endemic perennial medicinal herb commonly known as Achatsarmum. A slender rather delicate perennial herb, stem reaching up to 60-80 cm with bristly hairs. Leaves deeply and irregularly pinnately lobed, bristly haired. Flowers usually blue, borne on long stalks. Flowering and fruiting occurs in Mid-June to August. Due to attractive flowers the plant is having aesthetic value and is a potential ornamental plant. The entire plant is used against ulcers, disorders of lungs, liver and inflammation, pharyngitis, backache and disorders of spinal cord. The plant bears a single, unbranched, erect, hard and prickly stem. Single raceme bears many flowers. Flower is showy, actinomorphic, hermaphrodite, complete and hypogynous. Flower has a thin, cylindrical, bristly and erect pedicel. Petals are four, obtuse, obovate, delicate, thin, soft and with wavy margins. Fruit is a many seeded capsules. The whole herb is used as analgesic, chronic, renal pain, tonic, narcotic and febrifuge.

Seed germination studies are vital tools in conservation programs because they can be used for management programs and species reintroduction [6]. Seed germination is a critical stage in the life history of plants [7]. Seeds may be non-dormant at maturity and thus germinate soon after dispersal if environmental conditions are favorable for them germinate. However, favorable 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 [8]. 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 [9,10].

The dormancy characteristics and optimum conditions for seed germination of this species have not yet been described. However, little information is available about standardization methods of *in vitro* seed germination in *Meconopsis aculeta*. It is therefore necessary to develop a reliable *in vitro* propagation method of traditional Himalayan herb. Keeping in view the *in vitro* studies on the germination of seeds has been undertaken with a conservation prospective for the first time.

Materials and Methods

Seeds of Meconopsis aculeta were collected from Sinthontop (Anantnag) during the years 2013 and 2014 from July to September. The health and density of seeds were tested by dipping them in water. Seeds that float were discarded and healthy seeds were selected for sterilization. The viability of seeds was tested by the 2, 3, 5-triphenyltetrazolium chloride (TTC) test [11]. Seeds were washed thoroughly with running tap water for 5 to 10 min to remove surface dirty particles and then sterilized by immersing in 70% ethanol for 1 min with vigorous shaking followed by 20 min in 3% sodium hypochlorite containing one drop of Tween-20. The seeds were then rinsed three times with sterile distilled water in a laminar air flow cabinet to remove minor amounts of disinfection liquid. The surface-sterilized seeds were used for the treatments of *in vitro* germination trials. For germination, the surface-sterilized seeds were cultured in cultural viols containing Murashige and Skoog medium containing 3% sucrose and 0.8% agar alone and along with different of auxins and cytokinins. The pH of the medium was adjusted to 5.8 before the addition of 0.8 (w/v) agar. All the cultures were incubated under controlled conditions 22 ± 20°C temperature and 16 h photoperiod provided by cool fluorescent lamps (2×40w Philips India).

The cultures were observed daily and the data on daily seed germination was collected until the completion of the germination. The final germination percentage (GP) was calculated from the total seeds that germinated on the day of completion. Different growth parameters such as mean number of days taken for germination, mean number of days taken for shoot and root regeneration, mean length of shoots and number of roots of the seedling were recorded. Plantlets with well-developed shoots and roots was transferred to plastic pots containing autoclaved soil and maintained for three weeks in culture rooms. The plantlets were then transferred in a shade net house.

The experiments were designed in Completely Randomized Design (CRD). In each treatment 10 seeds were inoculated in each cultural vial and each treatment were replicated 3 times. The statistical analysis was done by employing ANOVA.

Results and Discussion

Seed viability

In the present investigation, viability of the seeds of *Meconopsis aculeta* Royle was checked using TTC test. The seeds were soaked in TTC solution for 24 h in the dark. All the embryos were stained red. These results showed that the TTC dye which was in contact with embryo got reduced and stained the embryo into red color. Seed viability indicates the capability of seeds to germinate and produce normal seedlings under suitable germination conditions [12]. It has been known that three factors; temperature, seed moisture content and oxygen pressure are most important for viability and longevity of seeds in storage. Total germination depends largely on the viability and vigor of the seeds [13,14]. Viability tests in conjunction with germination experiments explains better to define the reproductive potential of seeds over time [15,16]. It indicated that the 100% seeds were viable. Similar studies were carried on viability testing of the seeds using TTC test in *Uraria picta* showed 100% viability [17], seeds of *Bunium persicum* showed 93% viability [18]. The seed viability of *Indigofera glandulosa* using TTC test showed 100% viability [19].

In vitro plantlet regeneration from seed explants of *Meconopsis aculeta*

Seed germination: Seed germination and seedling growth are known to be regulated by exogenous hormones. Growth regulators used in pre-sowing seed treatment with growth regulator play an important role in regulating germination and vigour. Seed germination and seedling growth are known to be regulated by plant growth regulators. Growth regulators play an important role in regulating germination and vigour [20]. Auxins, Gibberellins and Cytokinins are known to stimulate seed germination in a wide range of plant species [21].

Treatment combinations	Label	Mean no. of days taken for germination	% culture response
MS Basal	A	No response	-
MS Basal+Zeatin (4 mg/l)+NAA (0.5 mg/l)	B	30	78%
MS Basal+Kn (6 mg/l)+BAP (1 mg/l)	C	33	60%
MS Basal+Zeatin (1 mg/l)+NAA (0.1 mg/l)	D	45	50%
MS Basal+BAP (2 mg/l)+NAA (1 mg/l)	E	50	45%

Table 1: Effect of PGR's on *in vitro* seed germination in *Meconopsis aculeta*. Data presented as mean of three replicates.

It is evident from the data presented in Table 1 that the seeds of *Meconopsis aculeta* showed the significantly highest germination percentage on MS medium supplemented with Zeatin at a concentration of (4 mg/l) in combination with NAA (0.5 mg/l) within a period of 30 days with culture response of 78%. Seeds inoculated on MS medium fortified with Kinetin (6 mg/l) in combination with BAP (1 mg/l) germinated within 33 days with 60% culture response. In addition, seeds also germinated on MS basal medium fortified with Zeatin (1 mg/l) in combination with NAA (0.1 mg/l) within 45 days with 50% culture response. Seed germination was also observed on MS medium supplemented with BAP (2 mg/l) in combination with NAA (1 mg/l) with 45% culture response (Figures 1 and 2).

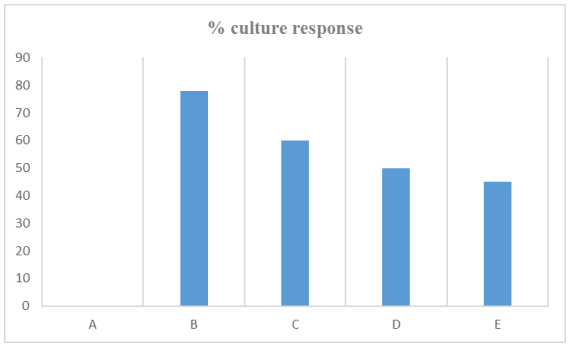

Figure 1: Effect of PGR's on *in vitro* seed germination in *Meconopsis aculeta*.

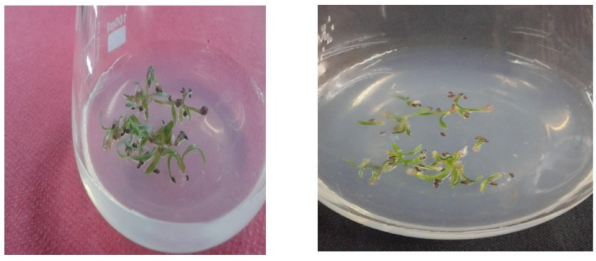

Figure 2: Seed germination in *Meconopsis aculeta.*

Minimum percentage culture response was achieved on MS basal supplemented with combination of BAP (2 mg/l)+NAA (1 mg/l) with 45% culture response. Similar effect of BAP on seed germination in *Phaseolus vulgaris*, has been reported by Malik and Saxena [22] under *in vitro* conditions.

The present investigation showed that percentage culture response increases with the increase in Zeatin along with increase in NAA. Such results are in conformity with recent studies which have suggested that Zeatin in combination with NAA resulted in increased culture response [23]. A little hard seed coat alone is not the only factor to delay seed germination in *Meconopsis aculeta;* so differential germination responses have been observed for the treatments to promote fast germination with uniform seedling emergence. Notably, the best concentrations of Zeatin, BAP and IAA would be effective for various physiological aspects of seeds germination and survival percentage with respect to dormancy alleviating mechanism. Physiological dormancy is reported to be the main cause of delay in seed germination process of several plants [24] that can be overcome by treating seeds in various germination regulating compounds (GRCs) and known as germination alleviating agents; such as Gibberalic acid, Zeatin, BAP and kinetin [25-29]. The evaluation of seed germination and the identification of ideal seed, lots of high performance and dormancy pattern are also an important initiative towards successful seeds germination.

Treatment of seeds with kinetin (6-furfuryl-aminopurine) had a significant influence on germination percentage in *Meconopsis latifolia*. Thus, prolonged chilling of seeds followed by their treatment with GA3 under alternate light/dark conditions are the requirements necessary for seed germination in *Meconopsis aculeta* [30].

Shoot generation

Treatments combinations	Label	Mean number of days taken for shoot regeneration	Mean length of shoots (cm)	Culture response
MS Basal	A	No response	-	-
MS Basal+Zeatin (2 mg/l)+IAA (0.1 mg/l)	B	28	3.41 ± 1.00	40%
MS Basal+ Kinetin (0.5 mg/l)+NAA (0.1 mg/l)	C	45	1.50 ± 1.00	37%
MS Basal+ Kinetin (1mg/l)+NAA (0.1 mg/l)	D	40	1.75 ± 1.00	25%
MS Basal+ Zeatin (4 mg/l)+IAA (1 mg/l)	E	29	1.20 ± 1.00	35%
MS Basal+ BAP (2 mg/l)+NAA (1 mg/l)	F	35	1.10 ± 1.00	32%

Table 2: *In vitro* regeneration of shoots from the germinated seeds of *Meconopsis aculeta.* Data presented as mean ± SD of three replicates.

The data presented in Table 2 showed that MS medium supplemented with Zeatin (2 mg/l)+NAA (0.1 mg/l) proved best medium for shoot regeneration with (3.41 ± 1.00 cm) mean shoot within 28 days with culture response of 40%. MS medium supplemented with Kinetin (0.5 mg/l)+NAA (0.1 mg/l) induced shoot regeneration within 45 days in 37% cultures with mean shoot length of (1.50 ± 1.00). On MS basal medium fortified with Kn (1 mg/l)+NAA (0.1 mg/l) and Zeatin (4 mg/l)+IAA (1 mg/l), shoots were produced within 40 and 29 days with culture response of 25% and 35% with shoot length of (1.75 ± 1.00 cm) and (1.20 ± 1.00 cm) respectively. On MS Medium supplemented with BAP (2 mg/l)+NAA (1 mg/l) shoots were produced within 35 days with 32% culture response with mean length of shoots (1.10 ± 1.00 cm) (Figures 3 and 4).

However, some studies figured out that transferring shoots into MS medium in combination with PGRs or free PGR medium is the successful way for shoot elongation [31]. Shoot multiplication during repeated transfer may be due to inhibition of apical dominance which stimulates basal dormant meristematic cells to produce young shoots [32]. This approach for shoot multiplication has been used in several plant species [33].

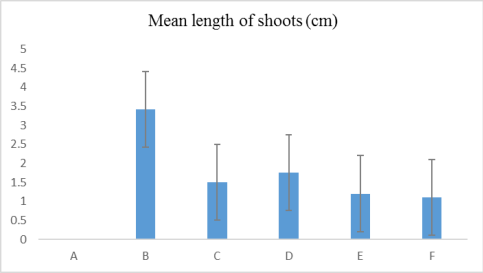

Figure 3: *In vitro* regeneration of shoots from the germinated seeds of *Meconopsis aculeta.*

Figure 4: Shoot regeneration in *Meconopsis aculeta.*

Our results are in agreement with yong et al. who achieved germination of immature seeds of *Meconopsis befonicifolia* on MS medium with 2 or 5 mg/l benzyl adenine, kinetin or isopentenyladenine combined with 0.2 mg/l 2, 4-dichlorophenoxyacetic acid leading to the formation of multiple shoots [34]. Similar result was observed by Tolera et al. in *Saccharum officinarum* where application of Kn showed significant higher value for shoot length as compared to control [35]. This might be due to the fact that increase in Kn concentrations triggers the cell elongation and faster multiplication of the cells that results in rapid growth and development of the seedlings as compared to the treatments with lower hormonal concentrations. Explants originally cultured on benzyl adenine or kinetin containing medium produced more shoots than those originally cultured on isopentenyladenine containing media. Dar et al. [30] also reported seed germination in *Meconopsis latifolia*, when treated with GA3. Our results are in confirmation with Kuris et al. [36] who reported that increased percentage of shoots was achieved in *Origanum vulgare* L. when cultured on MS medium supplemented with IAA and IBA treatments and showed that the IAA and IBA has higher potential of *in vitro* root development.

MS medium augmented with Zeatin (2 mg/l)+IAA (0.1 mg/l) produced 14.65 ± 1.23 roots with mean length of (2.76 ± 1.21 cm) within 42 days and was found to be best medium in terms of number of roots and number of days taken for root regeneration (Table 3).

Treatment combinations	Label	Mean no. of roots	Mean length of roots (cm)	Number of days taken
MS+Zeatin (3 mg/l)	A	3.10 ± 0.45	1.45 ± 0.75	49
MS+Zeatin (2 mg/l)+IAA (0.1 mg/l)	B	14.65 ± 1.23	2.76 ± 1.21	42
MS+Zeatin (2 mg/l)+IAA (3 mg/l)	C	11.22 ± 1.35	2.01 ± 0.90	50
MS+Kinetin (2 mg/l)+NAA (3 mg/l)	D	7.32 ± 0.79	2.06 ± 0.92	55
MS+BAP (3 mg/l)	E	3.0 ± 0.51	2.04 ± 0.91	59

Table 3: Regeneration of roots from *in vitro* raised shoots on MS medium supplemented with different concentrations and combinations of PGR's in *Meconopsis aculeta.* Data presented as mean ± SD of three replicates.

MS medium supplemented with Zeatin (2 mg/l)+IAA (3 mg/l) and Kinetin (2 mg/l)+NAA (3 mg/l) induced production of 11.22 ± 1.35 and 7.32 ± 0.79 roots with mean length of (2.01 ± 0.90 cm) and (2.06 ± 0.92 cm) within 50 days and 55 days respectively. MS medium supplemented with Zeatin at a concentration of (3 mg/l) produced 3.10 ± 0.45 mean number of roots with mean root length of (1.45 ± 0.75 cm) within 49 days while as on MS basal medium supplemented with BAP (3 mg/l), 3.0 ± 0.51 mean number of roots with mean root length of (2.04 ± 0.91 cm) were produced within 59 days (Figures 5 and 6).

Auxins play an important role in induction of roots from the cut ends of *in vitro* raised shoots [37]. Auxins are mainly used in root induction and their effect varies with type and concentration used in different plant species [38]. However, a very low concentration of exogenous auxin is required for better root induction. Our results are in agreement with [34], they studied the formation of multiple roots from immature seeds of *Meconopsis befonicifolia* was achieved on MS basal medium supplemented with combination of kinetin (2.5 mg/l) and 2, 4-dichlorophenoxyacetic acid (0.2 mg/1). Several researchers reported that IBA had positive effects on the rooting of various medicinal and aromatic plants, such as *Origanum vulgare* L., *Mentha piperita* L. and *Melissa officinalis* L. [36] and *Origanum vulgare* var. *hirtum* [39].

Acclimatization/Hardening

Thirty days old plantlets raised from the seed explant of *Meconopsis aculeta* were taken out from the culture vials. The media adhering to the basal portion of plantlets was washed off with double distilled water. After washing they were transferred to pots containing vermi-compost mixed with soil in the ratio of 1:2 ratios (1-part vermi-compost and 2 parts soil) and covered with polythene bags to retain moisture. Later these plantlets were transferred to green house and were maintained under control conditions of temperature (22 ± 4°C)

and a relative humidity of 60%. The hardening of the plants was achieved with 15 days with survival rate of 60% (Figure 7).

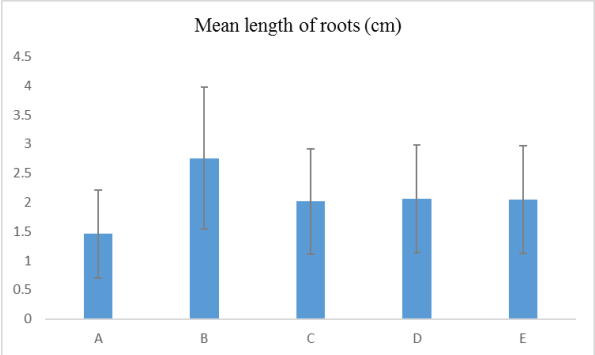

Figure 5: Regeneration of roots from *in vitro* raised shoots on MS medium supplemented with different concentrations and combinations of PGR's in *Meconopsis aculeta.*

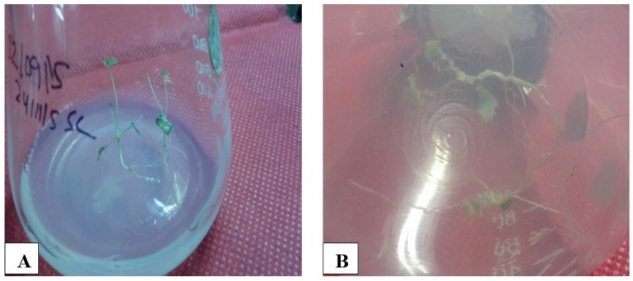

Figure 6: Root regeneration in *Meconopsis aculeta* on MS Zeatin (2 mg/l)+IAA (0.1 mg/l).

Figure 7: Hardening of *in vitro* raised plant.

Conclusion

From the present investigation, it may be concluded that growth hormones gave significantly better response in seed germination and seedling development in *Meconopsis aculeta* Royle. MS medium supplemented with Zeatin (4 mg/l) in combination with NAA (0.5 mg/l) was found more effective in terms of germination percentage culture response (78%) and minimum day required for germination was 30 days. Whereas highest mean length of shoots (3.41 ± 1.00) was achieved within 28 days with 40% culture response on MS medium fortified with Zeatin (2 mg/l)+IAA (0.1 mg/l). Roots regenerated on medium containing Zeatin (2 mg/l)+IAA (0.1 mg/l) with mean length of roots (2.76 ± 1.21) and mean number of roots (14.65 ± 1.23) within 42 days.

So, the present protocol clearly describes that for *in vitro* seed germination of *Meconopsis aculeta* MS medium supplemented with Zeatin (4 mg/l)+NAA (0.5 mg/l) is recorded best. While sub culturing germinated seedlings on MS medium supplemented with Zeatin (2 mg/l)+IAA (0.1 mg/l) is recorded best for *in vitro* shoot regeneration and MS medium supplemented with Zeatin (2 mg/l)+IAA (0.1 mg/l) is recorded best for *in vitro* root regeneration. Since the germination percentage of *Meconopsis aculeta* seeds in natural environment is very poor, the present protocol will be helpful to produce quality seedlings in large quantities and hence, conserve the rare species from natural collection.

Conflict of Interests

The authors did not declare any conflict of interest.

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