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OPTIMIZATION OF *IN VITRO* SEED GERMINATION PROTOCOL OF *ARNEBIA BENTHAMII* : A THREATENED MEDICINAL HERB OF HIMALAYA

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ABSTRACT

Supplementing tissue culture medium with plant growth regulators is known to influence and regulate germination effectively. This approach can be utilized as a strategy to maximize the germination outcome in many plant species. Present study aims to optimize a method to improve seed germination of *Arnebia benthamii* through *in vitro* seed culture. $HgCl_2$ (0.1% and 0.2%) at different incubation times was used. Seeds treated for 3 min. in 0.1% $HgCl_2$ solution showed least contamination (10±5.78%) while keeping the germination percent at 86.6±15.27. Gibberellic acid (GA_3), Benzylaminopurine (BA), and Sodium nitroprusside (SNP) were supplied with the half-strength Murashige and Skoog (MS) basal medium. The results indicated that half-strength MS medium supported germination fairly well (83.33±4.4%). Application of plant growth regulators *viz*., GA_3, BA and SNP considerably improved germination and significantly ($p<0.05$) reduced the mean germination time compared to the control. MS medium supplied with 5µM GA_3 showed maximum germination (98.33±1.67%) followed by 20µM-GA_3 (93.33±6.67%) and 2µM BA (93.33±1.67%). The minimum mean germination was observed in the medium supplied with 2 µM-BA (4.26±0.13 days) followed by medium with 5µM GA_3 (4.65±0.27 days). The findings demonstrate the potential of using these growth regulators to improve the germination and shorten the mean germination time with ensuring uniform population and high survivability of *A. benthamii* seedlings, offering valuable insights for conservation and *in vitro* cultivation practices of this medicinally important species.
***Keywords*:** Germination regulation, Medicinal Plant, Seed surface sterilization, Western Himalaya.

Introduction

Arnebia benthamii (Wall. ex G. Don) I.M. Johnston (Boraginaceae), is a perennial herb found in the alpines of Western and Central Himalya at 3000-3900 m asl (Manjkhola and Dhar, 2002). The leaves, flowers, shoots and roots of the plant are collected from the wild and used by several local communities for treating liver problems (Ahmad *et al*., 2018), cough, fever, urine burning etc (Mahmood *et al*., 2011). Rhizome is also used to extract red-purple dye, that is used as a hair tonic, textile colouring and for other cosmetic purposes. Flowering shoots are reported to be used in the preparation of jam and syrup (Manjkhola and Dhar, 2002). Besides, several studies report the use of plant extract to exhibit antioxidant, anti-inflammatory and wound healing properties (Ganie *et al*., 2014; Shameem *et al*., 2015; Pradhan *et al*., 2024).

Seed germination and embryo growth are critical stages in a plant's life cycle, as they directly influence population success. Propagation of *A. benthamii* occurs by rhizome buds or seeds (Guna, 2019). Natural regeneration of the plant is quite low which could be due to overexploitation and collection of plant material much before flowering (Kandari *et al*., 2011). In protected areas also, the population density of this plant has been reported to be very low (Kala, 2005). Consequently, the plant has been categorized as endangered to critically endangered for different states (Kala *et al*., 2004).

Plant tissue culture based biotechnological approaches have been effectively used as a sustainable

technique for the conservation of endangered medicinal plant species, reducing pressure on the wild populations of medicinal plants (Haldar *et al*., 2021).

By optimizing factors such as medium composition, nutrient strength, growth regulators, temperature, pH and photoperiod, it is possible to achieve high germination rates and superior seedlings production with enhanced survivability (Hesami *et al*., 2021). *In vitro* produced seedlings can serve as explants, either whole or in parts, for initiating mass propagation, significantly reducing the time required for the large-scale production of the plant material (Khan *et al*., 2013). However, contamination by microorganisms is often a serious problem encountered in plant tissue cultures. Therefore, sterilization of explants and seeds forms a crucial step in initiating a successful culture practice (Babaei *et al*., 2013). Chemicals like sodium hypochlorite and mercuric chloride are commonly used in plant tissue culture for this purpose. However, it is essential to standardize the concentrations and exposure times of these sterilizing agents for different plant species to establish an effective disinfection strategy while minimizing phytotoxicity and avoiding damage to the explants (Agogbua and Okoli, 2022).

The use of plant growth regulators (PGRs) to break dormancy and achieve synchronized germination in seeds seems to be another measure in establishing successful plant culture (Pandey *et al*., 2022). In the previous attempts to understand the *in vitro* seed germination of *A. benthamii*, pre-sowing treatments with PGRs were found to be very effective in improving seed germination percentage. Gibberellic acid (GA_3) and cytokinins like 6 benzylaminopurine (BAP) (Kandari et at., 2011; Manjkhola *et al*., 2003) and Kinetin (Ganai *et al*., 2011) improved the germination with reducing the mean germination time. Manjkhola *et al*. (2003) used thiourea, a nitric oxide (NO) donor to increase the seed germination in *A. benthamii*. Pre-treatments with sodium nitroprusside (SNP), another NO donor also increased the germination significantly in *A. euchroma* (Sharma and Sharma, 2020). These studies establish that application of PGRs has a significant impact on seed germination of *Arnebia* species and suggest that supplementing PGRs with growth medium could hold a potential in improving germination parameters while establishing *in vitro* seed cultures.

The threatened status of *A. benthamii*, coupled with low germination rates, underscores the importance of employing all potential measures to ensure a high germination success rate. The present study was designed to establish a reliable method for successful seed germination and seed culture establishment. The approach first involved ensuring seed sterility for culture initiation by testing the effect of $HgCl_2$ at different concentrations and incubation times followed by an evaluation of the effect of the plant growth regulators (GA_3, BA, and SNP) supplied in the growth medium at varying concentrations, to assess their impact on germination parameters.

Materials and Methods

Seed collection

The seeds of *A. benthamii* were collected in September 2023 from Balpata bugyal (3317.65±31m, 30.316374º N, 79.551684º E) Chamoli, Uttarakhand.

Seed sterilization

The seeds were gently stirred in water containing Tween 20 (Himedia, India) for 10 min., washed properly, followed by treatment with Bavistin (0.5%, w/v) for 20 min., and then thoroughly rinsed with distilled water. Following this, the seeds were dipped in the 0.1% and 0.2% (w/v) $HgCl_2$ (Himedia) solution for 1-4 min. Finally, the $HgCl_2$-treated seeds were washed properly (7-8 times) with autoclaved distilled water and were used for inoculation. The efficacy of sterilization was assessed by monitoring the inoculated seed for 10 days to observe seed germination and contamination, if any. The results were expressed as germination percent (GP) and contamination percent (CP).

Medium preparation and culture conditions

Basal half-strength Murashige and Skoog medium (MS,) supplemented with sucrose (15g/L) and agar powder (7.5 g/L) (Himedia, Mumbai) with pH set at 5.8 was used throughout the study. The medium was supplemented with plant growth regulators (PGRs) (Himedia)- GA_3 (5-30µM), BA (2-12µM) and SNP (2-12µM) for germination experiments, medium without PGRs served as control. Glass jars (250 mL) were each filled with 25 mL of half-MS medium and sealed with caps. The media was autoclaved at 15 lbs/inch^2 121º C for 20 min. The prepared medium was kept under observation for one week to ensure sterility. The culture conditions were maintained at 25±1°C temperature and 16/8 hours photoperiod using white fluorescent tube lights (40 µmol $m^{-2}s^{-1}$). Germination was observed from the first day after inoculation until the last recorded germination in any treatment, day 16.

Data analyses

For seed-sterilization experiments, 30 seeds in total were used for every treatment (10 for each

replication, 3 replicates), whereas, to see the effect of PGRs, 60 seeds per treatment and control (all contamination free) were used (20 for each replication, 3 replicates). All the values were expressed as mean and standard error. ANOVA (Analysis of Variance) followed by Tukey's HSD test was performed for all the experiments. The width of cotyledonary leaves was measured using ImageJ software and statistical analyses were done using Origin Pro 2024 software. The following formulae were used for calculating the parameters used in the study:

Contamination Percent (CP) = (Total contaminated seeds/ Total inoculated seeds) × 100

Germination Percent (GP) = (Total germinated seeds/ Total inoculated seeds) × 100

Mean Germination Time (MGT) = Total number of days to germination/ Total number of seeds germinated. Where, total number of days to germination is the sum of number of seeds germinated × number of days, for each day (Hartmann and Kester, 1989).

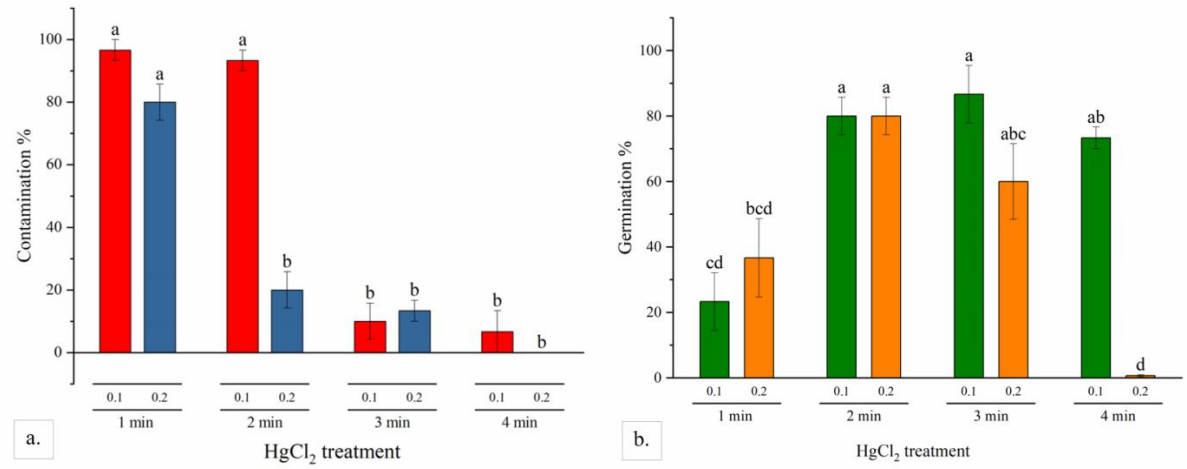

Fig. 1 : Effect of $HgCl_2$ treatment during *in vitro* seed germination of *Arnebia benthamii*. Bars represent-(a.) Contamination % and (b.) Germination % at 0.1% and 0.2% (w/v) concentrations of $HgCl_2$ across different incubation times (1-4 min.). Values are expressed as Mean ± SE. Different letters on the top indicate significant differences ($P<0.05$), while the identical letters denote the lack of significance, as determined by two-way ANOVA with Tukey's post-hoc test.

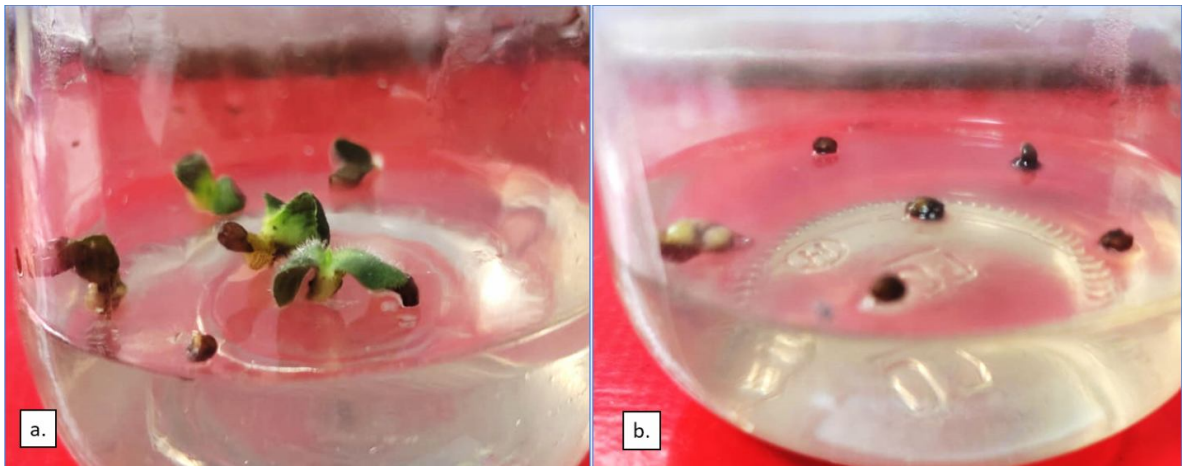

Fig. 2 : a) Germinated seeds with ruptured seed coat with $HgCl_2$ (0.1%) treatment for 3 min., and (b.) seeds treated with 0.2% $HgCl_2$ for 4 min

Fig. 3 : Germinated seeds of *A. benthamii* on the 9th day after inoculation under the influence of PGR supplemented growth medium at different concentrations. (Bar=1cm)

Table 1 : Results of ANOVA showing the individual and interaction effects of incubation period and concentration of $HgCl_2$ on the contamination and germination (%) of *A. benthamii* seeds.

	Source	DF	SS	MS	F value	P value
CP	$HgCl_2$-incubation period	3	28566.67	9522.22	142.83	<0.0001
	$HgCl_2$ Concentration	1	3266.67	3266.67	49	<0.0001
	Concentration× Incubation period	3	5300	1766.67	26.5	<0.0001
	Error	16	1066.67	66.67		
	Total	23	38200			
GP	$HgCl_2$-incubation period	3	11460.5	3820.17	19.92697	<0.0001
	$HgCl_2$ Concentration	1	2773.5	2773.5	14.46729	0.0015
	Concentration× Incubation period	3	6480.5	2160.17	11.26799	0.0003
	Error	16	3067.33	191.71		
	Total	23	23781.833			

Where, DF= degree of freedom, SS= sum of squares, MS= Mean square, CP= contamination %, GP= germination (%)

Table 2 : Germination percent (GP), mean germination time (MGT) and cotyledonary leaf width in *A. benthamii* seeds under different treatments of plant growth regulators

Treatment	GP (%)	MGT (days)	Cotyledonary leaf width (cm)
Control	83.33 ± 4.40^{ab}	8.5 ± 0.45^{ab}	0.32 ± 0.007^{f}
GA_3-5µM	98.33 ± 1.67^{a}	4.65 ± 0.27^{ef}	1.22 ± 0.004^{ab}
GA_3-15µM	90 ± 2.89^{ab}	5.44 ± 0.07^{def}	0.79 ± 0.006^{de}
GA_3-20µM	93.33 ± 6.67^{ab}	5.02 ± 0.49^{ef}	0.46 ± 0.195^{ef}
GA_3-30µM	80 ± 5.7^{abc}	5.62 ± 0.11^{def}	0.51 ± 0.008^{ef}
BA-2µM	93.33 ± 1.67^{ab}	4.26 ± 0.13^{f}	1.43 ± 0.009^{a}
BA-4µM	88.33 ± 6.09^{ab}	4.86 ± 0.56^{ef}	1.09 ± 0.07^{bc}
BA-8µM	75 ± 2.88^{abc}	6.15 ± 0.14^{de}	0.89 ± 0.012^{cd}
BA-12µM	58.33 ± 7.26^{c}	9.24 ± 0.95^{a}	0.43 ± 0.05^{ef}
SNP-2µM	73.33 ± 6.66^{bc}	8.47 ± 1.33^{ab}	0.47 ± 0.017^{ef}
SNP-4µM	86.67 ± 1.67^{ab}	7.97 ± 0.31^{abc}	0.42 ± 0.016^{ef}
SNP-8µM	88.33 ± 4.40^{ab}	7.11 ± 0.36^{bcd}	0.48 ± 0.02^{ef}
SNP-12µM	91.67 ± 3.33^{ab}	6.33 ± 0.15^{cde}	0.65 ± 0.003^{de}

Values are expressed as Mean ± SE. Different letters on the top indicate significant differences ($P<0.05$), while the identical letters denote the lack of significance, as determined by one-way ANOVA with Tukey's post-hoc test.

Results and Discussion

Seed sterilization

The results revealed that $HgCl_2$ had a significant impact on eliminating contamination during *in vitro* germination of *A. benthamii*. $HgCl_2$ is widely used in plant tissue culture for sterilizing explants and seeds due to its effectiveness in eliminating microbial contamination while maintaining the viability of plant tissues. Fig. 1 illustrates the effects of surface seed sterilization on *A. benthamii* seeds using 0.1% and 0.2% $HgCl_2$, with incubation periods ranging from 1 to 4 min., based on germination (GP) and contamination percentages (CP). The highest germination (88±8.81%) along with lowest contamination (10±5.7%) was observed in the 0.1% $HgCl_2$ treatment for 3 min., followed by 0.2% $HgCl_2$ treatment for 2 min (GP, 80±10% and CP, 20±5.7%). Incubation period for 1 min. (for both 0.1% and 0.2% $HgCl_2$) resulted in high contamination rates (96.6±3.3 and 80±5.7% respectively), which adversely affected the germination (GP, 23.67±8.81 and 36.66±12.0% respectively). Notably, 0.2% $HgCl_2$ for 4 min. completely eliminated contamination (CP, 0%) but was lethal to nearly all the seeds (GP: 0.67±0.57%). In a previous report on the seed culture of *A. benthamii*, 0.1% $HgCl_2$ treatment was used for 5 minutes (Parray *et al*., 2018). In the current study, application of 0.1% $HgCl_2$ for 4 min. reduced contamination significantly (CP, 6.67±6.67%) but affected germination (GP, 73.33±3.33%) as well.

The concentration and exposure time of $HgCl_2$ are critical factors that influence the success of sterilization protocols. Depending on the plant species, concentrations between 0.1% and 1% are usually tested, with specific exposure times ranging from 1 to 30 min (Şekerli, 2024). Previous studies have reported similar trends. Parray *et al*. (2018) used 0.1% $HgCl_2$ for 5 minutes in *A. benthamii*, while Bisht *et al*. (2022) reported 0.1% $HgCl_2$ for 10 minutes as the most effective treatment for *Berberis asiatica*. However, overexposure has been linked to reduced germination, as seen in *Althaea officinalis* where 0.3% $HgCl_2$ for 7 minutes minimized contamination but dropped GP to 44% (Younesikelaki *et al*., 2016). Kaur *et al*. (2014) also reported hindered seed germination and slower seedling growth rates when eggplants seeds were surface sterilized with higher concentrations of different disinfectants. In the current study, statistical analysis confirmed that $HgCl_2$ concentration, incubation time, and their interaction significantly influenced both CP and GP ($P<0.05$, Table 1).

Softer plant tissues respond differently to $HgCl_2$ treatment than the harder plant tissues. Upon exposure to $HgCl_2$, studies reveal that softer plant tissues exhibit reduced viability compared to harder tissues. (Ahmad *et al*., 2016; Kaya & Özatay, 2024). The seeds of *A. benthamii* are soft, with a papery-thin seed coat through which water can be easily absorbed (Ganaie *et al*., 2011). This could potentially make them more susceptible to water-based $HgCl_2$ treatments, particularly if the incubation time and concentration exceed the required levels. The significant interaction effects suggest that determining the optimal combination of $HgCl_2$ concentration and incubation time for each plant species is crucial for effectively controlling contamination while ensuring successful aseptic germination in the growth medium.

Seed germination in basal medium

The seeds of *A. benthamii*, once inoculated in the half strength MS medium, swell-up, and ruptured the seed coat, initiating germination. Eventually, the cotyledons turned green, expanded and following that shoot budding takes place (Fig. 3). Seeds of *A. benthamii* with a radicle length of 1–2 mm and a ruptured seed coat were considered germinated. The results of the study have been reported in terms of germination percent (GP) and mean germination time (days) taken for germination for each treatment (Table 2). In the current study, the inoculation of sterilized seeds on the half-strength MS basal medium (Control) resulted in considerable germination (88.33±4.40%) with the mean germination time (MGT) of 8.5±0.45 days. MS medium is a rich source of nutrients that balances supplementing macro and micro elements to the plants (Abbaszadeh *et al*., 2018). There are several reports that suggest that nutrients in the growth medium possibly help with enhanced germination (Paul et. al, 2012; Pradhan *et al*., 2013; Hunhoff *et al*., 2018). Previous studies on the seed germination of *A. benthamii* reported significantly lower germination percentages (5–40%) in the control group; where moistened filter paper was used as a substrate for germination (Ganaie & Nawchoo, 2002; Manjkhola *et al*., 2003; Ganaie *et al*., 2011; Kandari *et al*., 2011). Pre-treatments with KNO_3, which is a major component of MS medium, have been reported to enhance the germination percentage of *A. benthamii* seeds, where short term (one hour) KNO_3 pre-treatments at lower concentrations (50, 100 and 150 mg/L) significantly enhanced seed germination compared to control seeds (Ganaie *et al*., 2011). However, prolonged exposure (24 hours) to higher concentrations (50-100 mM) of KNO_3 either increased

the GP only marginally, or decreased it (Manjkhola *et al*., 2003).

Contrary to our findings, Parray *et al.* (2018) reported no germination response when *A. benthamii* seeds were inoculated in basal half-strength MS medium. Several factors may account for this discrepancy. Notably, seed viability of *A. benthamii* declines over time. Kandari *et al*. (2011) observed that freshly collected seeds exhibited 84% viability, which dropped to 50% after just one year of storage at 4°C. In the current study, for germination experiments, seeds stored for six months at 4°C were used. The study of Parray *et al*. (2018) specified the seed collection period (July–September 2013), it did not provide details on when their experiment was initiated, making it difficult to conclude whether the aging of seeds was the factor responsible for no germination response in the growth medium. Additionally, interpopulation variation in viability percentage has been reported in *A. benthamii* (Manjkhola *et al*., 2003; Ganaie *et al*., 2011). Manjkhola *et al*. (2003) reported varied germination percentage and seeds' responses to different pre-treatments in seeds collected from subalpine and alpine regions. Ganaie and Nawchoo (2002) in their study of germination reported that the seeds of *A. benthamii* with 8 mg average weight showed 30% more GP than the seeds with 6 mg average weight.

Seed Germination in Medium Supplemented with Plant Growth Regulators- Among all the PGR based treatments, the highest germination rate (GP) was recorded in 5µM GA_3 (98.33±1.67%), followed by 20µM GA_3 and 2µM BA (93.33±6.67 and 93.33±1.67% respectively). SNP treatment at higher concentration (12µM) also resulted in good germination (91.67±3.33%) (Table 2). Although these treatments improved GP compared to the control (half MS medium; 83.33±4.4%) but the differences were only partially significant. All the other treatments showed insignificant differences in GP from the control.

The mean germination time (MGT) was however, significantly reduced by using PGR supplemented media. BA-2µM yielded the fastest germination (4.26±0.13 days) among all the treatments, closely followed by GA_3-5µM (4.65±0.27 days) both of which were significantly lower than the MGT of the control (8.5±0.45 days). The high BA concentration (12µM) on the other hand, not only reduced GP (58.33±7.26%) but also delayed the germination (MGT, 9.24±0.95 days). These findings indicate that PGRs accelerated the germination speed and their effect was concentration dependant.

The morphology of cotyledonary leaves developed from the seeds of *A. benthamii* (under the influence of different PGRs, on day nine from the inoculation) is depicted in Fig. 4. The cotyledonary leaves with larger width (Table 2) are a direct outcome of the speed of germination. The treatments with low MGT values had the largest cotyledonary leaves, as early germination was followed by early seedling development.

The exogenous application of GA_3 increases the endogenous levels of GA while decreasing abscisic acid (ABA) levels. In *Panax notoginseng,* exogenous application of GA_3 stimulated GA biosynthesis and repressed the expression of genes related to ABA signal-transduction. This action promotes early seed coat rupturing and embryo development (Ge *et al*., 2023). Additionally, GA_3 enhances amylase activity, mobilizing starch reserves, and boosts antioxidant enzymes such as superoxide dismutase, catalase, and peroxidase, which mitigate oxidative stress during germination as observed in *Malania oleifera* (Su *et al*., 2025). In the present study, GA_3 supplementation improved both GP and MGT, though higher concentrations (30 µM) were inhibitory, which is consistent with a previous report in *Berberis asiatica* (Bisht *et al*., 2022). Exogenous application of GA_3 in form of pre-sowing treatments or supplementation with growth medium improved germination percent in *A. benthamii* (Manjkhola *et al*., 2002; Kandari *et al*.,2008; Parray *et al*. 2018). Parray *et al*., (2018) reported only 35% GP on half strength MS media supplemented with 5µM GA_3 in *A. bethamii* and no germination response in basal medium devoid of GA_3 or with lower concentrations of GA_3 (1.5 and 2.5) µM in *A. benthamii.* The present study reported 98.33±1.67% GP on 5µM GA_3 which is 3 times more than Parray *et al*. (2018).

BA also promoted germination at lower concentrations. It is known to play role in seed germination due to antagonistic effects on ABA (Ferreira *et al.*, 2016) and their capacity to alleviate stress (Nikolic *et al*., 2006). Nikolic *et al*. (2006) explored the effect of different cytokinins (zeatin, isopentenyl adenine, thidiazuron, and BA) supplied in MS medium on *in vitro* seed germination of *Lotus corniculatus* and found that all the cytokinins enhanced GP, with BA being the most effective in promoting seed germination among the used cytokinins. In our study, 2 µM BA significantly reduced MGT (4.26±0.13 days) while maintaining high GP (93.33±1.67) which is in accordance with the study of Manjkhola *et al*. (2003) where fastest MGT (9.2 days)

was noticed in *A. benthamii* seeds with BA pre-treatment (200µM) compared to other growth regulators or control (MGT, >15 days). Conversely, higher BA levels (12 µM) were inhibitory, consistent with the findings in *Digitalis purpurea* where 10 µM BA yielded the maximum GP (65.5%), while further increases in concentrations reduced GP (Patil *et al*., 2012).

The application of SNP, which is a nitric oxide (NO) donor, also contributed to improved germination in *A. benthamii*, though only at higher concentration (12 µM) with less noticeable effect on MGT. NO actually alleviates ABA-induced inhibition of germination and promotes cotyledon development (Sarath *et al*., 2006; Wang *et al*., 2015). SNP (1mM) pretreatment significantly increased GP in *A. euchroma* (70%) although, the treatment was able to reduce MGT by merely one day (Sharma and Sharma, 2020). Hameed *et al*. (2015) also reported high GP and reduced MGT along with improved stress tolerance in *Triticum aestivum* seeds primed with SNP.

Conclusion

From the study it can be concluded that selecting the right combination of concentration of the sterilizing agent $HgCl_2$ and its exposure time is essential, as it minimizes contamination while preserving germination potential which is critical for successful initiation of *in vitro* propagation of the plant. The seeds of *A. benthamii* show good germination response in MS basal medium and supplementing the medium with GA_3 (5 µM) considerably improved all the germination parameters. Optimizing GA_3 and BA levels in the growth medium can significantly ensure rapid seedling establishment. However, the excess concentrations of these regulators reduce germination efficiency. The implications of the study can play a crucial role in improving the propagation techniques for *A. benthamii*, which is essential for its conservation as well as establishing its commercial cultivation. By understanding the specific responses of *A. benthamii* to different growth regulators, targeted strategies can be adopted that maximize germination rates, paving the way for effective restoration projects and sustainable propagation practices.

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