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DELIGNIFICATION OF SEEDS AND CULTURE AS A TOOL FOR EARLY ASYMBIOTIC SEED GERMINATION IN *Habenaria panchganiensis*, A CRITICALLY ENDANGERED TERRESTRIAL ORCHID OF WESTERN GHATS (INDIA)

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ABSTRACT

Terrestrial orchids are very difficult to germinate and cultivate *in vitro*. Orchid seeds are hydrophobic in nature which limits the interaction of seed with culture medium, as a result very low or no germination occurs *in vitro*. In present study, a protocol was established for early asymbiotic seed germination of *Habenaria panchganiensis*. Delignification of seeds was achieved by using variable concentrations of sodium hypochlorite (NaOCl) for a variable time duration. The nutritional requirement of seeds was quantified by subjecting seeds to six different media for asymbiotic seed germination experiments in solid and suspension cultures along with variable concentrations of cytokinins (6-benzylaminopurine & kinetin). BM1 medium proved superior for asymbiotic seed germination in suspension culture (along with 4mgL^{-1} Kin). Different concentrations of sucrose were used to increase the tuber size because tuber reintroduction is easy and effective for the survival of *in vitro* developed plants. BM1 supplemented with 4 mg L^{-1} Kin and 40 g sucrose showed higher tuber length (5.15 ± 0.37 cm), breadth (0.96 ± 0.17 cm) and fresh weight (2.42 ± 0.23 g). This study may help in mass propagation of the species which, in turn, may prove helpful in its conservation.

Keywords: Asymbiotic seed germination, cytokinin, endangered orchid, suspension culture, Western Ghats, *Habenaria panchganiensis.*

INTRODUCTION

Habenaria panchganiensis Santapau & Kapadia is a terrestrial orchid that usually grows in a specific habitat of lateritic plateaus of Western Ghats in India. other terrestrial orchids, *H. panchganiensis* population has shown decline because of the internal factors of the species (Merritt *et al.*, 2014). Habitat destruction of lateritic plateaus due to overgrazing, trampling, horse riding and permissible tourism has added to the disruption of the niche of the species. Due to the low quality of habitat, the taxon is listed as critically endangered in the IUCN Red List Criteria 1994, 2000 and is included in the Appendix- II of CITES (Molur and Walker, 2001). All these internal and external factors are responsible for decline in the species, so there is utmost need to develop an effective conservational strategy for the Red data listed species. *Ex situ* conservation is best option over *in situ* conservation due to topographic limitation of *in situ* conservations (Batty *et al.*, 2001). In present study, we focused

more on *ex situ* conservation of the species which is achieved by multiplying the plant number *in vitro* and reintroducing them in natural habitat. *In vitro* multiplication of *H. panchganiensis* was affected by seed dormancy and requirement of most suitable culture medium.

Seed dormancy is a common problem in many orchid species which affects their germination. Mature orchid seeds have adopted seed dormancy which impart brown colour to the seeds on maturation; however, immature orchid seeds are not dormant and are usually white in colour (Yamazaki and Miyoshi, 2006; Barsberg *et al.*, 2013; Rao *et al.*, 2014). Final stage of seed maturation leads to the lignin synthesis and its deposition on integument (Barsberg *et al.*, 2013; Rao *et al.*, 2014; Yang and Lee, 2014; Pierce *et al.*, 2019). The outer integument of seeds is lignified which helps the seeds to remain viable for longer-time(Rasmussen, 1992; Baskin and Baskin, 1998). Lignins are hydrophobic, extremely hard to degrade and also exclude water imbibition, lead to 'testa imposed dormancy' (Baskin, 2003; Vasudevan and van Staden, 2010; Pierce *et al.*, 2019). In nature, degradation of lignins are catalyzed by ligninase enzymes released by soil fungi and bacteria and reportedly ligninase scarification of seeds facilitate water uptake and removal of seed dormancy (Batista *et al.*, 2008; Pierce *et al.*, 2019). Natural enzymatic scarification promotes seed germination. Similarly chemical scarification agents help in the removal of lignin by oxygen delignification method but excessive exposure may leads to embryo damage(Wilson, 1915; Pierce *et al.*, 2019). Oxygen delignification/oxygen bleaching with the help sodium hypochlorite (NaOCl) has been used to remove lignin deposition and promote faster seed germination (Hubbell and Ragauskas, 2010; Li *et al.*, 2017). The present study was based on two hypothesis, (1) the exposure of mature seeds of *H. panchganiensis* to variable concentrations of NaOCl for variable time protects embryo from damage, and (2) the early and enhanced seed germination in a suitable medium promotes water uptake and provides suitable nutrients for early seed germination.

MATERIAL AND METHODS

In present study five districts of Maharashtra state (India) *viz*., Satara, Sindhudurg, Kolhapur, Nasik, and Raigad were selected for recording the population of *Habenaria panchganiensis*.

Seed collection

The seed of *H. panchganiensis* were collected for seed germination and multiplication of the species, from the selected districts during monsoon season. The seeds were collected in clean filter paper and stored at 4°C in a refrigerator (Magrini *et al.*, 2019).

Standardization of NaOCl treatment and viability test

Seed dormancy is very common in terrestrial orchids due to lignified carapace (Yamazaki and Miyoshi, 2006); and to break the seed dormancy, NaOCl (Sigma Aldrich) was used (Custódio *et al.*, 2016). Different concentrations of NaOCl (5, 10, 15 and 20%) were used for variable time intervals (5, 10 15, and 20 min) for breaking the seed dormancy. The treated seeds were washed with distilled water twice to remove all the traces of NaOCl and the seeds soaked in filtered triphenyl tetrazolium chloride (TTC; Hi-media) solution (1 g 100 mL^{-1} distilled water, pH 6.5-7.0) for 48 h in dark. Seeds were washed 5 times with distilled water. Seeds were agitated between cover slides to remove testa and viewed under a binocular microscope (Carl Zeiss Axiostar). The embryos that stained pink to red were considered viable, while seeds with partially coloured, white or brown embryos were assumed to be non-viable (Van Waes and Debergh, 1986a; Magrini *et al.*, 2019).

Surface sterilization for asymbiotic germination

Harvested seeds were surface disinfected with 0.1% mercuric chloride for 3 min, followed by 70% ethanol for 30 sec and then with 5% NaOCl solution for 15 min and finally rinsed 3 times with

distilled water. Seeds were collected with the help of a sterilized spatula and a small mass of aggregated seeds were sown on different culture media for *in vitro* asymbiotic seed germination.

Standardization of media and plant growth regulators

The nutritional requirement of each plant species is different; therefore, it is necessary to assess the need of plant for better development and growth. In present study, six media *viz*., VC (Vacin and Went, 1949), MM (Malmgren, 1996), KC (Knudson, 1946), ½ MS (Murashige and Skoog, 1962), LM (Llndemann *et al.,* 1970) andBM1(Van Waes and Debergh, 1986b) media, were evaluated for asymbiotic seed germination of *H. panchganiensis* (Thakur and Dongarwar, 2017). All the six media were used in solid and suspension culture to check which medium was the best and in which state (solid or suspension). The best medium was continued with different concentrations of cytokinins *viz*., 6-Benzylaminopurine (BA) and kinetin (Kin) (Sigma Aldrich) to get a developmental profile of seedling. Seedlings were later transferred to the variable concentrations of sucrose in order to increase the size of tubers because healthy tissue cultured tubers are more tolerant in nature than that of tissue cultured plant.

Data analysis

Asymbiotic seed germination percentage was calculated by dividing the number of seeds germinated with total number of seeds inoculated in each medium. The experiments were conducted and 5 replicates were used for each parameter. Data analysis was achieved by performing one-way analysis of variance and the means were compared by Duncan's multiple comparison test ($\alpha = 0.05$) using DSAASTAT version 1.1.

RESULTS AND DISCUSSION

Seed delignification and viability testing

Terrestrial orchids are slow growers and mass multiplication of such orchids for *ex-situ* conservation needs a protocol for early and better germination. In nature terrestrial orchids survive for a long term because of lignin depositions on seeds which leads to seed dormancy (Yamazaki and Miyoshi, 2006). However, seed dormancy of terrestrial orchids can be removed by using bleaching agents like NaOCl (Miyoshi and Mii, 1998; Batty *et al.,* 2001; Yeh *et al.,* 2021). In present study, the NaOCl treated seeds were soaked in TTC solution to check the viability of seeds (Fig. 1B). The 5% NaOCl treatment proved most effective in yielding highest number of viable seeds than other treatments (Table 1). The best exposure time duration was 15 min in which 80% seeds were viable; however, 5, 10, and 20 min exposure showed 52, 75 and 70% viable seeds. Higher doses of NaOCl proved lethal to the seeds and showed less or negligible seed viability. Stewart and Zettler (2002) have reported that 5.25% NaOCl pre-treatment helped in the seed germination of *Habenaria repens*, *H. quinquiseta*, *H. macroceratitis*. Giri *et al.* (2012) worked on *Habenaria edgeworthii* and found 2% NaOCl treatment for 5 min before inoculating the seeds for seed germination effective.

Table 1: Effect of NaOCl treatment on the seed viability of *Habenaria panchganiensis*

NaOCl (%)	Treatment time (min)	Seed viability (%)
5	5	52
	10	75
	15	80
	20	70
10	5	53
	10	30
	15	0
	20	0

Asymbiotic seed germination

In present study, asymbiotic seed germination was observed in all the media tested in both solid and suspension culture (Table 2). On solid medium more time was taken (8-10 weeks) for seed germination, while in suspension culture it took 2 to 6 week's time. Thus, suspension culture proved

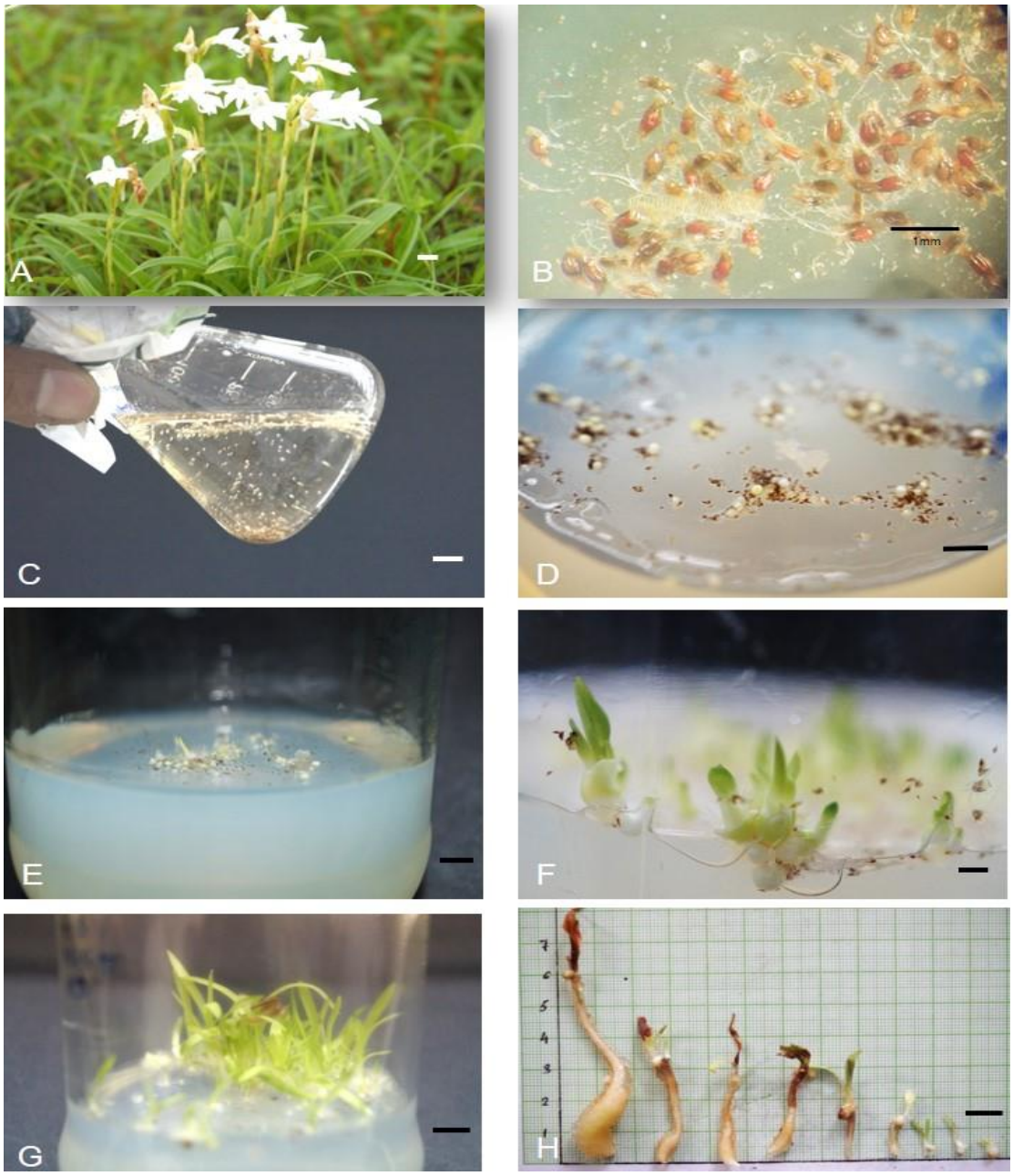

Fig. 1: *Habenaria panchganiensis*. A) *Habenaria panchganiensis* inflorescence in natural habitat. B) Seed viability test in TTC solution, C) Seed germination after 2 weeks in suspension culture of BM1; D) Germinated seeds transferred to solid medium; E-H) Seedling developmental stages. *Scale Bar is 1 cm except in B where scale bar is 1 mm.

superior over solid medium for seed germination. Of all the seed germination media evaluated, BM1, MM and ½ MS gave 55.6, 57.6, and 45.2% seed germination in solid media and 79.0, 62.5, and 74.7% in suspension culture, respectively (Table 1). The seed germination on suspension culture was earlier but the seedling development was comparatively better on solid medium. The seeds after germination were transferred to solid culture (Fig. 1D-G).

In present study asymbiotic seed germination of *H. panchganiensis* is reported for the first time, though earlier Stewart and Kane (2006) have reported asymbiotic seed germination in *H.*

Table 2: Comparative study of seed germination on solid and liquid media of *Habenaria panchganiensis*

Medium	Seen germination (%)	
	Solid media	Liquid media
VW	10.8 ± 0.73[d]	35.5 ± 2.66[d]
KC	11.3 ± 3.00[d]	33.2 ± 1.75[d]
BM 1	55.6 ± 2.53[a]	79.0 ± 1.47[a]
LM	32.4 ± 1.71[c]	52.7 ± 2.95[c]
MM	57.3 ± 1.48[a]	62.5 ± 2.39[b]
½ MS	45.2 ± 1.93[b]	74.7 ± 1.25[a]

*Means ± standard deviation mentioned in the column followed by the same letter do not significantly different from each other according to the Duncan's multiple range test at α = 0.05.

macroceratitis. They observed 89.1% asymbiotic seed germination on the 7th week and elongation of first leaf on protocorm was observed on the 16th week. Stewart and Zettler (2002) worked on the symbiotic seed germination of *Habenaria repens, H. quinquiseta, H. macroceratitis* and reported that the seeds germinated within 3 weeks of inoculation. In comparison to Stewart and Kane (2006) and Stewart and Zettler (2002), it can be concluded that *Habenaria* species seeds took six weeks for asymbiotic germination and 3 weeks for symbiotic seed germination i.e. in nature the seeds grow within 3 weeks. In present study, a protocol for early asymbiotic seed germination has been developed by using suspension culture along with cytokinins which resulted in germination within 2 weeks. Early asymbiotic seed germination is the resultant of removal of hydrobhobicity of seeds by NaOCl and secondly suspension culture helps the seeds to imbibe and fast uptake of nutrients from suspension culture.

All the six media have minor differences in their composition but showed significant differences in the growth and development of the species (Kauth *et al.,* 2008). Nitrogen is available either organic or inorganic form in all the media; but organic form nitrogen is readily available to the seeds. Comparing all the six media, glycine and glutamine are the two amino acids present in BM1 medium may be the reason for good results in that medium (Thakur and Dongarwar, 2017). Other media like LM, MM, and MS are also supplemented with glycine but in very little quantity. Many researchers have reported that terrestrial orchids show early and higher germination on the media supplemented with amino acids (Van Waes and Debergh, 1986b; Stewart and Kane, 2006). In present study all the test media exhibited seed germination and supported seed development but varied in germination percentage and the time taken for seed germination (Table 2 & 3).

Table 3: Effect of plant growth regulators (BA and Kin) on asymbiotic seed germination of *Habenaria panchganiensis* on BM 1 medium

Cytokinin	Conc. (mg L^{-1})	Week 1	Week 2	Week3	Week 4
	0 (Control)	0	2.7 ± 1.15[e]	33.3 ± 1.20[d]	58.5 ± 1.19[e]
Kin	1	3.0 ± 0.57[c]	28.3 ± 0.82[d]	50.7 ± 1.45[c]	62.4 ± 0.71[d]
	2	2.3 ± 0.33[c]	54.1 ± 1.73[b]	64.8 ± 2.04[d]	67.4 ± 1.28[c]
	3	17.0 ± 2.50[b]	76.7 ± 1.20[a]	78.0 ± 0.56[a]	78.7 ± 0.47[b]
	4	27.0 ± 3.20[a]	76.0 ± 1.15[a]	78.0 ± 0.72[a]	80.7 ± 0.58[a]
	5	11.0 ± 2.10[b]	40.7 ± 1.45[c]	61.9 ± 1.18[b]	79.2 ± 0.38[a]
BA	1	22.0 ± 0.57[a]	63.7 ± 0.86[a]	73.0 ± 0.54[a]	77.5 ± 0.75[a]
	2	13.0 ± 0.89[b]	32.0 ± 1.73[b]	52.3 ± 1.45[b]	65.7 ± 0.55[b]
	3	7.3 ± 1.76[c]	27.3 ± 2.51[b]	48.3 ± 0.88[c]	61.6 ± 0.48[c]
	4	11.3 ± 0.88[b]	31.7 ± 1.45[b]	42.5 ± 0.37[d]	59.5 ± 0.59[d]
	5	7.7 ± 0.90[c]	20.1 ± 0.57[c]	40.9 ± 0.88[d]	58.4 ± 0.64[d]

*Means ± standard deviation mentioned in the column followed by the same letter are not significantly different according to Duncan's multiple range test at α = 0.05.

Orchid seeds in nature grow in association with fungi that provide nutrients and growth promoting chemicals which proliferate germination and development. In this experiment the best medium (BM1) was supplemented with variable concentrations of cytokinins (BA and kinetin). BA and kinetin

Table 4: Effect of cytokinins on length, breadth and fresh weight of the tubers of *Habenaria panchganiensis* raised *in vitro*

Kin (mg L^{-1})	Tuber length (cm)	Tuber breadth (cm)	Tuber fresh weight (g)
1	1.86 ± 0.16^{cd}	0.7 ± 0.05^{a}	0.38 ± 0.01^{c}
2	2.44 ± 0.30^{bc}	0.8 ± 0.06^{a}	0.84 ± 0.03^{b}
3	2.54 ± 0.36^{b}	0.73 ± 0.03^{a}	0.89 ± 0.02^{b}
4	3.94 ± 0.12^{a}	0.8 ± 0.06^{a}	1.29 ± 0.21^{a}
5	2.48 ± 0.12^{d}	0.4 ± 0.03^{b}	0.21 ± 0.03^{c}
BA (mg L^{-1})			
1	2.23 ± 0.08^{a}	0.73 ± 0.02^{a}	0.79 ± 0.03^{a}
2	1.60 ± 0.11^{b}	0.33 ± 0.03^{b}	0.17 ± 0.01^{b}
3	1.34 ± 0.24^{b}	0.40 ± 0.05^{b}	0.17 ± 0.01^{b}
4	1.82 ± 0.21^{b}	0.33 ± 0.10^{b}	0.17 ± 0.01^{b}
5	1.52 ± 0.20^{b}	0.36 ± 0.08^{b}	0.17 ± 0.01^{b}

*Means ± standard deviation mentioned in the column followed by the same letter are not significantly different according to Duncan's multiple range test at α=0.05.

supplementation in the medium resulted in higher seed germination especially with1mg L^{-1} BA (77.5%) and 3, 4, 5 mg L^{-1} Kin (78.7, 80.7, and 79.2%, respectively) (Table 3, Fig. 1c). However, seedling development was better with 4 mg L^{-1} kin which showed the tuber length of 3.94 cm (Table 4).

The completely developed plants were shifted to the variable concentrations of sucrose along with best medium and best suited kinetin concentration (BM1 @ 4 mg L^{-1} Kin). The 40 g sucrose yielded healthy tubers with tuber length of 3.9 cm. Later, with the same combination the seedlings were kept for 16 weeks and observed tuber length was 5.15 ± 0.37 cm, tuber breadth 0.96 ± 0.17 cm and tuber fresh weight 2.42±0.23 g (Fig. 1H, Table 4). The tissue cultured tubers were shifted to greenhouse in the first season and after completing the first season in greenhouse tubers were harvested and shifted to their natural habitat.

Table 5: Effect of sucrose on length, breadth and fresh weight of tubers of *H. panchganiensis*

Sucrose (g L^{-1})	Tuber length (cm)	Tuber breadth (cm)	Tuber fresh weight (g)
20	2.25 ± 0.20^{bcd}	0.63 ± 0.02^{c}	0.74 ± 0.03^{b}
30	2.53 ± 0.21^{bc}	0.62 ± 0.01^{c}	0.62 ± 0.01^{b}
40	3.90 ± 0.09^{a}	0.90 ± 0.01^{a}	1.13 ± 0.18^{a}
50	2.30 ± 0.15^{bcd}	0.75 ± 0.03^{b}	0.81 ± 0.01^{b}
60	2.08 ± 0.08^{cd}	0.65 ± 0.03^{c}	0.62 ± 0.01^{b}
70	2.65 ± 0.20^{b}	0.65 ± 0.03^{c}	0.63 ± 0.03^{b}

*Means ± standard deviation mentioned in the column followed by the same letter do not significantly differ from each other according to the Duncan's multiple range test at α = 0.05.

Conclusion: *Habenaria panchganiensis*is a critically endangered and endemic species of Western Ghats hotspot of India. Since species is declining at a faster rate, the present study would help in early and efficient seed germination and seedling development. The protocol developed resulted in enhanced tuber growth so may help in better reintroduction of the species in nature.

Author contributions: Dr. Nitin Dongarwar (ND) made the research plan and Ms. Uma Thakur (UT) completed the tissue culture/lab work. ND and UT executed the seed sample collection, survey and reintroduction work. UT completed the statistical analysis work. UT and ND contributed the manuscript write-up and both finalized the manuscript.

Conflict of interests: The authors declare that they have no conflicts of interest.

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