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Multiplication through *in vitro* seed germination and pitcher development in *Nepenthes khasiana* Hook. f., a unique insectivorous plant of India

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SUMMARY

In vitro seed germination of *Nepenthes khasiana* Hook. f was accomplished in order to conserve this rare, unique and endemic pitcher plant of India. Seed germination was studied on different nutrient media, *viz.* MS (Murashige and Skoog), ½ MS, ¼ MS, B_5 (Gamborg *et al.*), ½ B_5, ¼ B_5, NN (Nitsch and Nitsch), ½ NN, and ¼ NN to ascertain the nutritional requirements of germinating seeds. Seventy-percent germination of seeds was observed on ¼ MS medium. Pitchers were found to have developed after 150 d of *in vitro* culture in seedlings cultured on ¼ MS medium devoid of growth regulators. Growth regulators in the medium were also found to have a pronounced effect on the development of pitchers on *in vitro*-raised seedlings. Well-developed healthy pitchers were observed after 120 d of culture on ¼ MS medium supplemented with 2.68 µM naphthalene acetic acid.

Carnivorous plants are among the curiosities of nature as they differ from normal plants in their mode of nutrition. They are specialised for trapping insects and are popularly known as insectivorous plants. 'Passive' insectivorous plants have a 'pitfall' mechanism (i.e., a jar or pitcher-like structure) into which the insect slips and falls, eventually to be digested. *Nepenthes khasiana* Hook. f. is the only species of pitcher plant found in India and is indigenous to the state of Meghalaya (northeast India). It is a short, stout, prostrate-to-climbing undershrub, which grows in boggy, acidic, and nitrogen-deficient soils. Due to uncontrolled exploitation for trade, and unplanned human activities, *N. khasiana* has become threatened in its natural habitat (Jain and Shastry, 1980). In nature, seeds of *N. khasiana* take up to 223 d to germinate, and the germination percentage is low (Bordoloi, 1977). *In vitro* multiplication of *N. khasiana* through enhanced axillary branching has been attempted (Rathore *et al.*, 1991; Latha and Seeni, 1994; Tandon and Rathore, 1994). However, micropropagation leads to genetically identical plants which would not preserve the genetic diversity required for the conservation of a rare plant. On the other hand, multiplication through seed would ensure genetic diversity, as seeds are heterozygous. *In vitro* seed germination is a biotechnological approach for the mass propagation and conservation of many rare and endangered plants (Tandon, 2004). The pitchers of *Nepenthes* are modified leaf blades which impart its ornamental value and are of interest to growers, worldwide. Here we report on an efficient protocol for the rapid multiplication and large-scale propagation of *N. khasiana* through *in vitro* seed germination, and the development of pitchers under the combined influence of reduced nutrients and plant growth regulators.

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MATERIALS AND METHODS

Seed source and sterilisation

N. khasiana seeds were collected from its natural habitat at Jarain in the Jaintia Hills of Meghalaya, India. Seeds were surface sterilised with 15% (v/v) sodium hypochlorite [4% (w/v) available chlorine] for 5 min and rinsed thoroughly three-to-four times with sterile distilled water. The seeds were then blot-dried by placing them on sterilised filter paper before culture.

Effect of different media on seed germination

The effects of three different basal media [MS (Murashige and Skoog, 1962), B_5 (Gamborg *et al.*, 1968), and NN (Nitsch and Nitsch, 1969)] on the germination of seeds were assessed. Half and one-fourth strengths of the major and minor salts of each of the three media were also tested. Vitamins and sucrose levels were incorporated in full. Additional additives such as 284 µM ascorbic acid and 47.62 µM citric acid were added to all media (Rathore *et al.*, 1991). Agar [0.8% (w/v)] was added, and the pH of each medium was adjusted to 5.8 prior to autoclaving at 1.06 kg cm^{-2} and 121°C for 15 min. The autoclaved media were then dispensed as 15 ml aliquots into 60 mm-diameter Petri plates (Tarson Products Pvt. Ltd., Kolkata, India). Approx. 30 seeds were cultured in each Petri plate. Ten replicate plates were taken for each treatment and were sealed with a single layer of Parafilm™ (Pechiney Plastic Packaging, Menasha, WI, USA) to avoid dehydration and contamination during culture. The seed cultures were incubated at 25° ± 2°C under white fluorescent light at an intensity of 150 µmol m^{-2} s^{-1} with a 12 h photoperiod. All seeds were observed each day for germination. The percentage of seed germination was recorded every 30 d of culture. The data obtained were subjected to statistical analysis using Student's *t*-test.

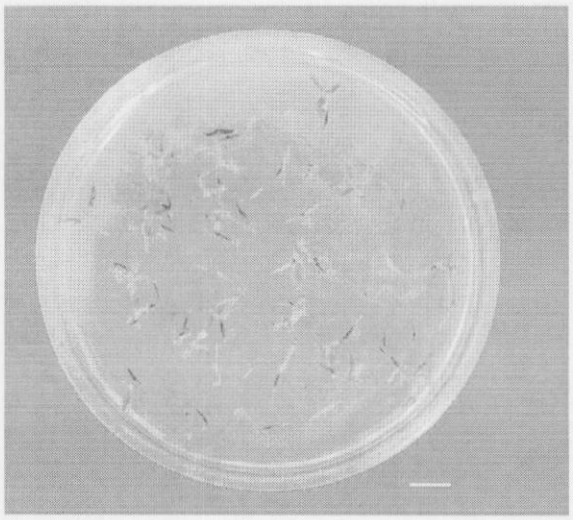

FIG. 1
Germinated seeds of *N. khasiana* after 90 d of culture on ½ MS medium in a 9 cm Petri dish.

Effect of growth regulators on seed germination and pitcher development

To enhance seed germination and to promote pitcher development in cultured seedlings, the effects of auxins [indole-3-acetic acid (IAA), α-naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D)], cytokinins [kinetin (KN), 6-benzylaminopurine (BAP)], and gibberrellic acid (GA_3), each incorporated singly into ½ MS medium over a range of concentrations (2.20 – 57.14 μM), were examined. Ascorbic acid (284 mM) and citric acid (47.62 μM) were also incorporated in ½ MS medium. Agar [0.8% (w/v)] was added to the ½ MS medium and the pH was adjusted to 5.8 before autoclaving, as described earlier. The percentage of seed germination was recorded every 30 d of culture. After 120 d, various growth parameters (e.g., root number, seedling length, and pitcher size) were recorded in the developing seedlings. An average of 50 seedlings were evaluated per treatment, and the data obtained were subjected to statistical analysis using Student's *t*-test.

RESULTS AND DISCUSSION

Seed germination in *N. khasiana* was initiated within 30 d of culture on both MS and B_5 media (Table I),

TABLE I
Effect of different culture media on mean seed germination percentage (± SE) of Nepenthes khasiana *in vitro*

Medium[z]	Mean germination (%)		
	30 d	60 d	90 d
MS	2 ± 1.9	4 ± 0.8	4 ± 0.8a[y]
½ MS	7 ± 0.5	42 ± 1.7	47 ± 2.3b
¼ MS	≠	36 ± 1.6	70 ± 1.8c
B_5	6 ± 1.1	7 ± 1.9	7 ± 1.9a
½ B_5	17 ± 7.4	41 ± 1.1	44 ± 4.4bd
¼ B_5	≠	27 ± 3.6	34 ± 6.1e
NN	≠	20 ± 3.8	20 ± 3.8f
½ NN	≠	23 ± 6.6	29 ± 4.8eg
¼ NN	≠	1 ± 0.8	14 ± 1.8h

≠, no germination.
[y]Mean values (± SE) followed by a different lower-case letter were significantly different at $P = 0.05$ by Student's *t*-test.
[z]See text.

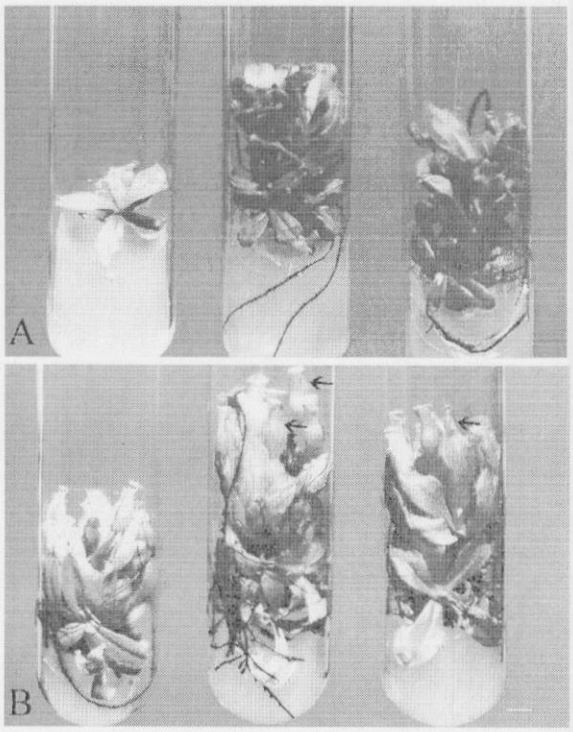

FIG. 2
Panel A, 150 d-old seedlings growing in (left-to-right) MS, ½ MS, or ¼ MS; scale bar = 1.0 cm. Panel B, 120 d-old seedlings with pitchers (arrows) growing in ¼ MS medium containing 2.68 μM NAA (scale bar = 0.5 cm).

suggesting that the composition and concentration of the medium did not affect the initiation of seed germination. However, observations after 60 d and 90 d of culture showed that seed germination ceased after 60 d on the full-strength media. A subsequent increase in the

TABLE II
Mean seed germination percentage of Nepenthes khasiana *in vitro on ¼ MS medium supplemented with different growth regulators*

Growth regulator	Conc. (mM)	Mean germination (%)		
		30 d	60 d	90 d
Control	–	≠	36 ± 2.8	67 ± 2.3a[y]
NAA	2.68	≠	53 ± 8.7	63 ± 5.5a
	13.44	≠	33 ± 8.1	43 ± 5.5b
	26.88	≠	17 ± 4.7	17 ± 4.7b
	53.76	≠	7 ± 3.8	7 ± 3.8b
IAA	2.86	≠	43 ± 4.8	63 ± 6.6a
	14.28	≠	23 ± 7.6	43 ± 5.9b
	28.57	≠	13 ± 2.9	13 ± 2.9b
	57.14	≠	3 ± 3.0	3 ± 3.0b
2,4-D	2.26	≠	34 ± 0.7	34 ± 0.7b
	11.31	≠	8 ± 0.6	8 ± 0.6b
	22.62	≠	8 ± 1.4	8 ± 1.4b
	45.24	≠	4 ± 0.8	4 ± 0.8b
BAP	2.20	53 ± 1.9	63 ± 4.7	67 ± 2.9a
	11.10	50 ± 1.1	63 ± 2.0	63 ± 2.0a
	22.20	50 ± 5.6	63 ± 3.1	63 ± 3.1a
	44.40	40 ± 6.0	60 ± 2.6	60 ± 2.6a
KN	23.20	20 ± 5.5	54 ± 1.1	59 ± 4.0a
	11.60	33 ± 5.2	59 ± 4.0	59 ± 4.0a
	23.20	56 ± 3.6	57 ± 1.9	57 ± 1.9a
	46.60	17 ± 4.7	54 ± 1.1	54 ± 1.1a
GA_3	1.44	3 ± 2.9	67 ± 2.9	68 ± 3.0a
	7.20	17 ± 5.9	68 ± 3.0	69 ± 3.0a
	14.45	7 ± 4.7	60 ± 2.9	67 ± 2.9a
	28.90	≠	46 ± 2.4	50 ± 2.6b

≠, no germination.
[y]Mean values (± SE) followed by a different lower-case letter were significantly different at $P = 0.05$ by Student's *t*-test.

TABLE III
Effect of growth regulators in ¼ MS on in vitro *seedling growth and pitcher development*

Growth regulator	Conc. (mM)	Shoot length (cm)	Root No.	Root length (cm)	No. of pitchers	Length of pitcher (cm)
Control	–	0.55 ± 0.05a[†]	2.00 ± 0.4a	1.1 ± 0.07a	≠	–
NAA	2.68	0.42 ± 0.05a	7.50 ± 0.3b	1.3 ± 0.01a	3.8 ± 0.25a	0.8 ± 0.03a
	13.44	≠	≠	≠	≠	≠
	26.88	≠	≠	≠	≠	≠
	53.76	≠	≠	≠	≠	≠
IAA	2.86	0.34 ± 0.02b	1.75 ± 0.3a	1.1 ± 0.13a	≠	≠
	14.28	0.26 ± 0.02b	1.75 ± 0.3a	2.3 ± 0.80b	≠	≠
	28.57	0.22 ± 0.03b	1.25 ± 0.2a	0.6 ± 0.04b	≠	≠
	57.14	0.18 ± 0.02b	1.00 ± 0.0a	0.4 ± 0.04b	≠	≠
BAP	2.20	0.45 ± 0.03a	1.24 ± 0. 2a	1.75 ± 0.1b	≠	≠
	11.10	0.67 ± 0.03a	3.25 ± 1.6a	2.12 ± 0.8a	≠	≠
	22.20	0.60 ± 0.04a	1.00 ± 0.0a	0.5 ± 0.03b	≠	≠
	44.40	≠	≠	≠	≠	≠
KN	23.20	0.37 ± 0.02a	4.75 ± 0.2b	1.32 ± 0.1a	≠	≠
	11.60	0.40 ± 0.04a	2.00 ± 0.0a	0.8 ± 0.05b	≠	≠
	23.20	0.22± 0.02b	1.00 ± 0.0a	1.2 ± 0.05b	≠	≠
	46.60	0.22 ± 0.02b	1.00 ± 0.0a	0.2 ± 0.02b	≠	≠
GA_3	1.44	0.35 ± 0.02b	2.00 ± 0.0a	0.8 ± 0.03b	≠	≠
	7.20	0.68 ± 0.04a	2.25 ± 0.3a	0.7 ± 0.06b	2.3 ± 0.2b	0.31 ± 0.03b
	14.45	0.55 ± 0.03a	4.75 ± 0.3b	1.8 ± 0.09b	3.5 ± 0.3a	0.32 ± 0.02b
	28.90	0.45 ± 0.03a	2.00 ± 0.0a	1.4 ± 0.07a	3.3 ± 0.2ab	0.26 ± 0.01b

≠, no root, shoot, or pitcher developed. All measurements were made after 120 d in culture.
[†]Mean values (± SE) followed by a different lower-case letter were significantly different at $P = 0.05$ by Student's *t*-test.

percentage of germination was found only on reduced-strength nutrient media, suggesting that *N. khasiana* seeds rely on lower concentrations of nutrients to support enhanced germination. The highest percentage of seed germination (70%) was recorded after 90 d of culture on ¼ MS medium (Figure 1), followed by ½ MS, then ½ B_5 media (Table I). All the media used in the present study contained a nitrogen source, but the form and concentration in which it was presented differed among them. While an inorganic nitrogen source was reduced in the low-nutrient media, vitamins and sucrose were incorporated at full strength. The ammonium content was highest in MS medium, which may have contributed to the highest overall germination rates. It is well known that the rapid assimilation and use of NH_4^+-N in most plants causes enhanced germination, compared to NO_3^--N which can accumulate at toxic levels (Raghavan and Torrey, 1964). The addition of auxins to the media did not have a significant effect on seed germination, with the maximum germination percentage (63%) recorded on media containing 2.68 µM NAA and 2.86 µM IAA (Table II). A decrease in the percentage germination was recorded with an increase in the auxin concentration in the media. The rate of seed germination was facilitated by the addition of cytokinins (BAP or KN) and GA_3 to the media. The initiation of germination was observed within 30 d of culture on media containing different concentrations of cytokinins and GA_3 (except for 28.9 µM GA_3). The use of GA_3 and cytokinins in the culture medium to induce seed germination has been reported by many workers (Blackman *et al.*, 1996; Sharma *et al.*, 1996; Miyoshi and Sato, 1997; Vargas *et al.*, 2004, Stewart and Kane, 2006). Media additives such as ascorbic acid and citric acid help to reduce phenolic exudates and browning of the medium at the time of seed germination (Rathore *et al.*, 1991). The development of pitchers was observed after 150 d of culture on the initial ¼ MS medium, without sub-culture (Figure 2A). This development mimics events in nature, where pitchers develop on *N. khasiana* plants to supplement nitrogen deficiency (Bordoloi, 1977).

Seedlings growing in ¼ MS media containing 13.44 µM, 26.88 µM, or 53.76 µM NAA, 44.4 µM BAP, or any concentration of 2, 4-D, failed to survive. The inhibitory effects of higher concentrations of growth regulators on the growth of many plant species have been widely observed (Sharma and Tandon, 1986; George *et al.*, 1989; Sahoo and Chand, 1998). However, in the present study, the growth and development of pitchers may be enhanced by the incorporation of growth regulators into reduced-strength medium (¼ MS). The optimal size and number of pitchers was obtained on ¼ MS including 2.68 µM NAA (Table III; Figure 2B). In this treatment, root number was also greatly enhanced, which may have facilitated increased nutrient absorption from the medium which, in turn, could have resulted in faster pitcher development in the *in vitro*-grown seedlings.

This is the first study on the seed germination requirements of *N. khasiana*. Data concerning the growth and development of *in vitro*-grown seedlings are also represented. Considering the threatened status of these species in its natural habitat, these data provide critical information which can be used for mass propagation for the ornamental industry, as well as for conservation and recovery of this important indigenous species.

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