Journal of Tropical Forest Science 11(3):631–638 (1999)

IN VITRO PROPAGATION OF *NEPENTHES MACFARLANEI*

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Received October 1997

CHUA, L. S. L. & HENSHAW, G. 1999. *In vitro* propagation of *Nepenthes macfarlanei*. Half-strength Murashige and Skoog medium supplemented with at least 5×10^{-6} M 6-benzylamino purine was required for shoot multiplication from cotyledonary seedlings of *Nepenthes macfarlanei*. Cotyledonary seedlings produced higher numbers of shoot buds compared to apical shoots and nodal segments. Half-strength Murashige and Skoog medium supplemented with various concentrations of α-naphthalene acetic acid was favourable for rooting of shoot buds.

Key words : Tropical pitcher plants - endemic - conservation strategy

CHUA, L. S. L. & HENSHAW, G. 1999. Pembiakan *in vitro Nepenthes macfarlanei*. Anak benih *Nepenthes macfarlanei* di peringkat kotiledon memerlukan separuh kepekatan media Murashige dan Skoog yang disokong dengan 6-benzylamino purina pada kepekatan sekurang-kurangnya 5×10^{-6} M untuk percambahan tunas melalui teknik pendaraban *in vitro*. Anak benih di peringkat ini menghasilkan lebih banyak tunas berbanding dengan pucuk di pangkal batang dan keratan batang. Media Murashige dan Skoog pada separuh kepekatan disokong dengan pelbagai kepekatan asid asitik α-naftalena dapat mempertingkatkan pertumbuhan akar pada pucuk.

Introduction

Nepenthes spp., commonly known as tropical pitcher plants, belong to the family Nepenthaceae. The genus, which has about 82 species, has its centre of diversity in the Malay Archipelago of the Far East, where 73 species occur (Danser 1928, Jebb & Cheek 1997). The genus is well known in horticulture and is sought for its unique pitchers which come in different shapes, sizes and colours. Prices are high, particularly for species that are rare or difficult to find. Despite its horticultural importance and the high degree of endemism within the genus, little published

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information is available on the techniques of *in vivo* and *in vitro* propagation for *Nepenthes* spp. Although artificial propagation is used to produce a large proportion of the plants sold (Anonymous 1991), such propagation is undertaken by horticultural companies and the techniques are not generally available because of commercial considerations. It is important that such information is more readily available so that these techniques can be incorporated where appropriate into the overall conservation strategy for *Nepenthes* species, especially the rarer endemics.

Micropropagation has been attempted on *N. khasiana* (Rathore *et al.* 1991, Latha & Seeni 1994), *N. carunculata* and *N. madagascariensis* (Redwood & Bowling 1990, Fay 1994). *Nepenthes khasiana*, an endemic to Assam Hills, India, has been successfully established in culture from nodal segments of seedlings. Between ten and twelve shoots were produced from the segments cultured on a modified MS medium containing combinations of plant growth regulators; 80% of these shoots rooted on MS medium provided with NAA (α-napthalene acetic acid) and kinetin (Rathore *et al.* 1991). Latha and Seeni (1994) reported that shoots of *N. khasiana* may proliferate from nodal segments cultured in Woody Plant Medium (Lloyd & McCown 1981) supplemented with BA (N^6-benzyladenine) as the sole plant growth regulator. *In vitro* plantlets of *N. carunculata* and *N. madagascariensis*, which were produced from seedlings, have flowered in culture (Redwood & Bowling 1990).

This paper aims to provide information pertaining to *in vitro* shoot multiplication and plantlet regeneration of *N. macfarlanei* Hemsl., a species which is dioecious, endemic to Peninsular Malaysia and confined to the upper montane forest habitat (Chua 1995).

Materials and methods

Mature seeds were used as parent materials to initiate cultures. A large proportion of the seeds was obtained from Genting Highlands, Peninsular Malaysia while a smaller proportion was obtained from Cameron Highlands. Seed batches were collected at different times of the year and bulked across different mother plants from the same locality. Where necessary, seed batches were stored at 4 °C for up to six months. Seeds were germinated to establish the stock explants for *in vitro* shoot multiplication and plantlet regeneration.

The basal media tested were MS (Murashige & Skoog 1962), GB5 (Gamborg's B5 Basal Medium with Minimal Organics 1968), NN (Nitsch & Nitsch 1969), SH (Schenk & Hildebrant 1972), VW (Vacin & Went 1949) and AS (8 g l^{-1} agar + 30 g l^{-1} sucrose). All media prepared from either stock solutions or powdered mixtures were supplemented with 8 g l^{-1} agar bacteriological technical (Difco Laboratories) and 30 g l^{-1} sucrose; the pH was adjusted to 5.7 prior to the addition of agar. Autoclaving was carried out at 121 °C and 1.1 kg cm^{-2} for 15 min.

For surface sterilisation, the seeds were shaken vigorously for 20 min in a 3% (v/v) solution of commercial bleach (Clorox) containing a drop of Tween 20 detergent, and rinsed three times with sterile distilled water before culture. For the shoot multiplication experiments, intact cotyledonary seedlings aged

approximately two months and apical shoot and nodal parts of seedlings greater than six months old were used.

All cultures were maintained at 25 ± 1 °C with a 12 h photoperiod, a PAR range of 12 to 15 μmol $m^{-2}s^{-1}$ and relative humidity 67 to 75 %. Cultures were incubated in the dark by wrapping them with aluminum foil and placing them in the same environment. Subcultures were undertaken monthly. A completely randomised design was employed for all experiments. Data were analysed using analysis of variance (single classification) and *t*-method for unplanned comparison among treatment means. Arc-sine and square-root transformations for percentages and mean numbers respectively were used where necessary (Sokal & Rohlf 1981).

The rooted plantlets were removed from various auxin treatments, rinsed and planted into a prepared mixture of commercial compost. No fertiliser was added. The plantlets were placed in a 24 ± 2 °C glasshouse and watered daily. After one month, each plantlet was scored for survival and the number of newly-developed leaves.

For shoot multiplication, cotyledonary seedlings, apical shoots and nodal segments were cultured on MS, 1/2MS, GB5, 1/2GB5, NN, SH, VW basal media and AS supplemented with 0, 10^{-6}, 5×10^{-6} and 10^{-5} M BAP (6-benzylamino purine). The number of cotyledonary explants per treatment was twenty, divided into four replicates while the number of apical shoots and nodal segments per treatment was ten, divided into two replicates. The experiment was repeated once (Table 1). Following this, cotyledonary seedlings were cultured on 1/2MS basal media supplemented with either BAP, 2-iP (N^6-[2-isopentenyl] adenine) or kinetin. The concentrations used were 10^{-6}, 5×10^{-6}, 10^{-5}, 2.5×10^{-5} and 5×10^{-5} M. The number of explants per treatment was twenty, divided into four replicates (Table 2). The cultures were maintained for two months.

In order to investigate the potential for the induction of somatic embryogenesis, immature and mature embryos, leaf segments and cotyledonary seedlings were cultured on MS, 1/2MS, GB5, NN, SH and VW basal media supplemented with either 2,4-D (2, 4-dichlorophenoxyacetic acid) or 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) at 10^{-6}, 5×10^{-6} and 10^{-5} M. The cultures were incubated either in the light or the dark for 30 days.

Axillary shoots derived from the shoot apex of cotyledonary seedlings and apical shoots grown on various basal media supplemented with different concentrations of cytokinins were maintained on 1/2MS for one month before being transferred to 1/2MS supplemented with NAA, IBA (indole-3-butyric acid) and IAA (indole-3-acetic acid). The concentrations used were 10^{-6}, 5×10^{-6} and 10^{-5} M. The cultures were maintained for two months.

Results and discussion

Shoot multiplication

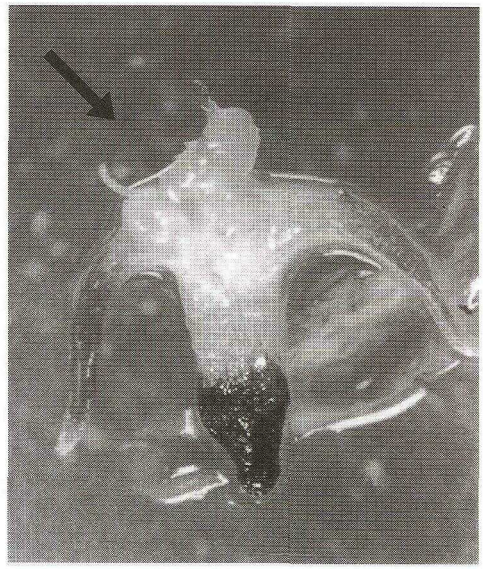
In general, more shoots were produced from cotyledonary seedlings than from apical shoots (Table 1) and all of the nodal segments died without producing

axillary shoots. For cotyledonary seedlings, the differences between the mean numbers were not significantly different for any of the treatments (at $p<0.01$, n=4, Table 1). The fairly high variability within treatments is likely due to the genetic heterogeneity of the species and the relatively small number of replicates used. In media supplemented with BAP, small outgrowths proliferated around the macroscopically invisible shoot apex region within thirty days (Figure 1a). These outgrowths differentiated into shoots after three months of subculture in 1/2MS (Figures 1b & 1c).

Table 1. Number of buds developing from *N. macfarlanei* cotyledonary seedlings and apical shoots cultured on various basal media supplemented with BAP

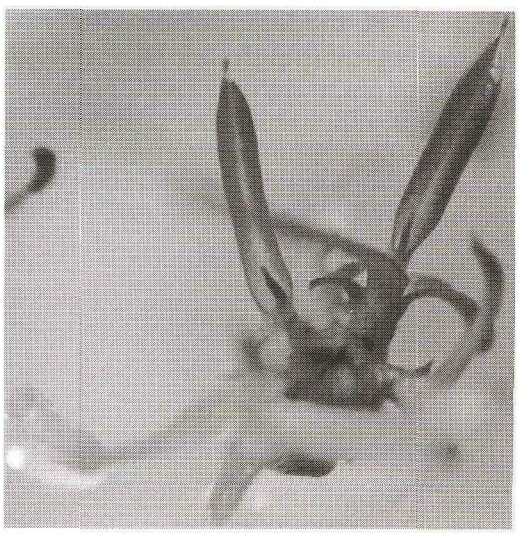
Basal media	BAP (M)	Mean number of shoots ± s.d.	
		Cotyledonary seedlings (n=4)[1]	Apical shoots (n=2)[2]
MS	0	0	0
	10^{-6}	0	1 ± 1.4
	5×10^{-6}	0	0.5 ± 0.7
	10^{-5}	0	1 ± 1.4
1/2MS	0	1 ± 0	0
	10^{-6}	1 ± 0	1.5 ± 1.4
	5×10^{-6}	1.8 ± 0.2	2.0 ± 0
	10^{-5}	3 ± 1.4	2.5 ± 0.7
GB5	0	1 ± 0	0
	10^{-6}	0.5 ± 0.7	0
	5×10^{-6}	1 ± 0	1 ± 1.4
	10^{-5}	0.5 ± 0.7	0.5 ± 1.1
1/2GB5	0	1 ± 0	0
	10^{-6}	1.5 ± 0.7	2.2 ± 0.4
	5×10^{-6}	1 ± 0	0
	10^{-5}	2.5 ± 3.5	0
NN	0	1 ± 0	0
	10^{-6}	1 ±0	0
	5×10^{-6}	0.5 ± 0.7	0
	10^{-5}	1.5 ± 0.7	0
SH	0	1 ± 0	0
	10^{-6}	1 ± 0	0
	5×10^{-6}	1 ± 0	0.5 ± 1.1
	10^{-5}	0.5 ± 0.7	0
VW	0	0.5 ± 0.7	0
	10^{-6}	0.5 ± 0.7	0
	5×10^{-6}	1 ± 0	0.5 ± 0.7
	10^{-5}	0.5 ± 0.7	0
AS	0	0.5 ± 0.7	0
	10^{-6}	1 ± 1.4	1.2 ± 0.4
	5×10^{-6}	1.5 ± 1.4	0.5 ± 0.7
	10^{-5}	2.5 ± 0.7	0

[1]Number of explants per treatment = twenty, divided into four replicates,
[2]Number of explants per treatment = ten, divided into two replicates,
Duration of observations = 3 months,
Experiment was repeated once.



(a)

(b)



(c)

Figure 1. Multiplication of shoot buds from cotyledonary seedlings of *N. macfarlanei* cultured on 1/2 basal MS supplemented with 10^{-5} M BAP

(a) Outgrowths (arrow) from the shoot apex
(b) Morphogenic development of outgrowths into shoot buds
(c) Development of shoot buds

The mean number of shoots produced from apical shoots varied significantly between treatments (at $p< 0.01$, n=2). Greatest number of shoots was produced from 1/2MS supplemented with either 5×10^{-6} M or 10^{-5} M BAP and 1/2GB5 supplemented with 10^{-6}M BAP (Table 1). In these treatments, the shoot continued to produce new leaves during the first two months, after which small outgrowths could be seen close to the apical region in cultures on media supplemented with BAP. These outgrowths developed into shoots after three subcultures.

The poor response of the nodal segments could be due to the persistent effects of apical dominance resulting in the presence of rudimentary axillary meristematic regions. Casual field observations showed that *N. macfarlanei* does not often produce axillary shoots and so this species possibly requires the use of a more complex combination of plant growth hormones than those investigated here to promote the development of the axillary meristems. This result is in contrast to those reported by Rathore *et al.* (1991) and Latha and Seeni (1994) for *N. khasiana.*

The mean number of shoots derived from cotyledonary seedlings varied significantly between 1/2MS medium supplemented with different concentrations of BAP, kinetin and 2-iP (at $p< 0.01$, n=4). Only BAP was able to maintain viability and induce multiple shoot formation while there were hardly any buds developing on seedlings cultured in media supplemented with 2-iP or kinetin (Table 2). The rate of shoot multiplication was enhanced with the increase in BAP concentrations up to 5×10^{-5} M, the highest concentration tested. Again, bud outgrowths, which subsequently grew into shoots, were restricted to the seedling shoot apex.

Table 2. Survival and bud development from cotyledonary seedlings cultured on 1/2MS supplemented with various concentrations of cytokinins

Cytokinin	Concentration (M)	Mean survival ± s.d. (%)	Mean number of shoots ± s.d.
BAP	10^{-6}	50 ± 11.6	1.3 ± 0.5
	5×10^{-6}	60 ± 28.3	2.9 ± 0.8
	10^{-5}	60 ± 16.3	3.6 ± 1.4
	2.5×10^{-5}	60 ± 43.2	4.2 ± 1.2
	5×10^{-5}	75 ± 19.2	6.7 ± 1.3
2-iP	10^{-6}	5 ± 10.0	0.3 ± 0.5
	5×10^{-6}	0	0
	10^{-5}	6.6 ± 11.6	0.3 ± 0.6
	2.5×10^{-5}	5 ± 10.0	1 ± 2.0
	5×10^{-5}	5 ± 10.0	0.2 ± 0.5
Kinetin	10^{-6}	0	0
	5×10^{-6}	5 ± 10.0	1 ± 2.0
	10^{-5}	5 ± 10.0	1 ± 2.0
	2.5×10^{-5}	5 ± 10.0	0.3 ± 0.5
	5×10^{-5}	10 ± 11.6	1 ± 1.4

Number of explants per treatment = twenty, divided into four replicates.

Almost no callus nor embryonic tissues could be induced from any of the treatments. With the exception of cotyledonary seedlings, none of the explants survived the treatments involving either 2,4-D or 2,4,5-T. Cotyledonary seedlings placed in the light had low levels of survival in 10^{-6} M 2,4-D and 10^{-6} M 2,4,5-T.

Rooting of excised shoots

Roots were produced in all media, and although survival was somewhat lower in media containing NAA, these media were the most effective both in terms of the proportion of shoots producing roots (100%) and the number of roots produced (Table 3).

Table 3. *In vitro* rooting of *N. macfarlanei* axillary shoots cultured on 1/2MS supplemented with various concentrations of auxins

Auxin	Concentration (M)	% shoots surviving	% shoots producing roots	% shoots producing mean No. of roots			
				0	1-10	11-20	>20
IAA	10^{-6}	55	73	27.3	27.3	27.3	18.1
	5×10^{-6}	40	75	25	37.5	37.5	0
	10^{-5}	30	50	50	33.3	16.7	0
IBA	10^{-6}	50	30	70	20	0	10
	5×10^{-6}	75	67	26.7	33.3	33.3	6.7
	10^{-5}	45	44	55.6	11.1	11.1	22.2
NAA	10^{-6}	35	100	0	28.6	42.8	28.6
	5×10^{-6}	25	100	0	0	40	60
	10^{-5}	40	100	0	12.5	12.5	75

- Number of explants = twenty replicates, divided into five Petri dishes,
- Duration of observation = 3 months.

Establishment of plantlets in soil

All rooted plantlets, produced as a result of the various auxin treatments, survived when transferred to the compost. By the end of the first month, all of them had developed a healthy young shoot with at least one new leaf while the old leaves withered and died.

Conclusion

Half-strength MS, supplemented with concentrations of BAP ranging from 5×10^{-6} to 5×10^{-5} M, is beneficial for shoot multiplication of *Nepenthes macfarlanei*, while the same basal medium supplemented with NAA was favourable for rooting of excised shoots.

This multiplication protocol, although marginally successful, may be used as part of a conservation strategy, to rescue and recover a wider range of *Nepenthes* spp. It will, however, require modification, particularly in the concentrations of cytokinins and auxins. For conservation purposes, the use of seedling explants is recommended as they are heterogeneous and have a broader genetic base, although in many species, the supply of seeds may be restricted. Even though the use of cotyledonary seedlings as explants is advantageous because it reduces the time required to initiate shoot multiplication and all seedlings are of unique genotypes, the study into the propagation of other types of explant should also be pursued, particularly so that cultures could be initiated when necessary from established plants.

Acknowledgements

Thanks are due to the Director General, Forest Research Institute Malaysia for granting the permission to undertake this study, K. Baskaran, Marzalina Mansor and the staff of the Seed Technology Laboratory, FRIM, for the use of facilities and the anonymous reviewers for their valuable comments and criticisms.

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