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EFFECT OF NITROGEN SOURCE ON GROWTH AND MORPHOGENESIS IN THREE MICROPROPAGATED *NEPENTHES* SPP.

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SUMMARY

The effect of the two main nitrogen sources, i.e. $NH_4(NO_3)$ and $Ca(NO_3)_2$ in Woody Plant Medium (WPM) on three micropropagated *Nepenthes* spp. showed differences among three species in percentage nitrogen content in dried leaf, shoot production, shoot length and fresh plant weight. Higher number of shoots and longer shoot length were observed on treatment with a single nitrogen source rather than a combination of the two sources. The three species showed higher uptake of nitrogen from nutrient with single source $Ca(NO_3)_2$ but in general produced lower fresh weights on treatment with higher $Ca(NO_3)_2$. Abnormality in leaf morphology was observed in *N. khasiana* and *N. pervillei* on the treatments with a single nitrogen source. The study concludes that a combination of two nitrogen sources are required rather than a single source for normal growth of the *Nepenthes* species.

***Key words*: Micropropagation, *Nepenthes* spp., nitrogen source.**

INTRODUCTION

Nepenthes species grow in acidic and nitrogen deficient soils, where they experience high rainfall and warm temperatures. Pitchers developed at the tip of the leaf trap a wide group of insects to compensate for the nitrogen deficiency in the soil (Kitching and Schofield 1986). In contrast, *in vitro* cultured plants depend entirely on the nutrient medium for their nitrogen requirement. Thus, nitrogen is an important component of the culture medium, and the nitrogen source used can markedly influence growth and morphogenesis in plant tissue cultures (Gamborg and Shyluk 1970). Furthermore, differences in uptake has been observed for several ions in many plant species (Clark 1983, Jeschke 1983, Rodgers and Barneix 1988). Some micropropagated plant species have been shown to grow better on Murashige and Skooge (MS) (Murashige and Skooge 1962) medium, while others do better on Woody Plant Medium (WPM)(Lloyd and McCown 1981). Plants which grow well in poor, often acid soils have been reported to grow better on low level of nutrients *in vitro*. Notable examples are Rhododendron (Anderson 1984) and Kalmia (Lloyd and McCown 1981) which grow better on WPM, with markedly lower nitrate, ammonium and potassium levels than those of MS. Latha and Seeni (1984) compared induction of shoots from nodal segments of *N. khasiana* with basal nutrient media, i.e. WPM, MS and KC (Knudson C) (Knudson 1946). WPM was superior to the other two media.

The objective of the experiment was therefore, to single out the two main nitrogen sources (i.e. $Ca(NO_3)_2$ & NH_4NO_3) in WPM and study the effect of these

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sources individually on the three micropropagated *Nepenthes* species i.e. *N. khasiana*, *N. pervillei* and *N. vieillardii*. The paper presents for the first time the effect of the two main nitrogen sources in WPM on the three micropropagated *Nepenthes* spp.

MATERIALS AND METHODS

Shoot cuttings (1.0 cm long) with four leaf axes on each cutting were taken from *in vitro* plantlets of the three *Nepenthes* spp. for all experiments. The two nitrogen sources, i.e. $Ca(NO_3)_2$ and NH_4NO_3 were used singly rather than in a combination (Table 1). Two different concentrations of each nitrogen source were used in the culture medium. The treatments are abbreviated to the capital letter 'T' with a subscript number. The subscript number represents the first digit of the nitrogen source concentration in the medium. Each number has two digits separated by a point. The subscript digit to the left of the point stand for NH_4NO_3 and to the right for $Ca(NO_3)_2$. For example, basal Woody Plant Medium (WPM) is abbreviated as $T_{4.2}$ because it contains both the nitrogen sources [i.e. 4.96 µM NH_4NO_3 and 2.35 µM $Ca(NO_3)_2$]. WPM medium ($T_{4.2}$) was used as the standard culture nutrient medium for comparison in the study. The media were supplemented with 8.9µM 6-benzyladenine (BA) in all treatments based on the optimal concentration of growth regulators for shoot regeneration in the three species. The pH of the medium was adjusted to 5.8 before adding 0.7% (w/v) Difco-Bacto agar. Aliquots (20 ml) of medium were dispensed into 20 x 150 mm test tubes and autoclaved at 121^0C and 1.1 $kgcm^{-2}$ for 20 minutes. All cultures were maintained at an irradiance of 160 µmol $m^{-2}s^{-1}$, with a 16 h photoperiod and at a temperature of 24 ± 2^0C.

Table 1. Experimental nitrogen treatments.

Treatments	Nitrogen source	
	NH_4NO_3 mM	$Ca(NO_3)_2$ mM
$T_{4.0}$	4.96	0.0
$T_{9.0}$	9.93	0.0
$T_{0.2}$	0.0	2.35
$T_{0.4}$	0.0	4.70
$T_{4.2}$	4.96	2.35

After 8 weeks, the cultures were scored for (i) number of shoots regenerated (ii) average length of shoots (iii) fresh weight of the plants (iv) fresh weight of leaves and (v) percentage of N content in the leaves on dry weight basis.

For the analysis of nitrogen content in leaves in this experiment fresh leaves were used rather than dry leaves due to limited leaf material produced in culture. Using the dry weight of the leaves would have reduced the material to a very low quantity. In addition some of the nitrogen content would be lost in the drying process. Given these factors fresh leaves were used in preference to the normal procedure of dry matter analysis used for larger samples. Three samples from each treatment were taken randomly from the cultures for the analysis of nitrogen content. The leaves of each sample were cut and placed in a tin capsule, weighed individually. The nitrogen content in the leaves was determined using the Dumas technique for nitrogen determination (Leo Instruments Ltd., UK) At the same time three samples each of fresh leaves from the standard treatment ($T_{4.2}$) were dried in oven at 70^0C for 70 hrs and then the dry weights were recorded. Dry weights of the standard treatment were adopted as standard dry weight for all treatments. Nitrogen content in fresh tissue was then converted into percentage dry weight per sample. In order to analyse the increase in fresh plant weight, the initial weights of the plants (i.e. *N. khasiana* 0.1180g, *N. pervillei* 0.1891g, *N. vieillardii* 0.0868g) were recorded and then the fresh weight after 8 weeks in culture were measured.

The data in all the experiments were tested for normality of the distribution and based on the results, the data were transformed to $\log_{10}$ or square root. The analyses of variance (ANOVA) for the data were carried out using PROC GLM (General Linear model) in SAS package. Treatment means were compared statistically by using the least significant difference (LSD) test.

RESULTS AND DISCUSSION

A comparison of the effect of nitrogen source on the three *Nepenthes* species has shown differences in nutrient uptake. Nutrient uptake differences, however,

have been recognized for a long time (Vose 1963). In the present study percentage of nitrogen content in the leaf on dry weight basis in *N. khasiana* and *N. pervillei* showed significant differences between the treatments. In *N. khasiana* the treatments with $T_{0.2}$ and $T_{4.0}$ produced significantly higher nitrogen content than the other treatments (Table 2). In *N. pervillei*, $T_{0.4,}$ $T_{4.2}$ and $T_{4.0}$ treatments were significantly higher than the other treatments (Table 2).

Table 2. Percentage (on dry weight basis) of nitrogen in leaf after 8 weeks in culture in *Nepenthes* spp.

Treatment	*N. khasiana*	*N. pervillei*
$T_{4.0}$	1.07ab	0.90ab
$T_{9.0}$	0.62c	0.75b
$T_{0.2}$	1.20a	0.81b
$T_{0.4}$	0.92b	1.30a
$T_{4.2}$	0.60c	0.98ab
$LSD_{5\%}$	0.22	0.44
SE's (df=14)	+0.06	+0.13

Means with the same letter in a column are not significantly different (P>0.05).

N. khasiana: Mean fresh weight of leaves 0.4242 mg, Mean dry weight of leaves 0.0565 mg, dry matter = 13.28%.

N. pervilei: Average fresh weight of leaves 0.4168 mg, Average dry weight of leaves 0.0477 mg, dry matter = 11.46%.

Rao *et al*. (1977) reported that the genotypic differences in nitrogen absorption capacity in short-term (6-8h) experiments were not necessarily reflected in the longer term. However, in the present study, the differences in nutrient uptake between treatments were confirmed after 8 weeks in culture by estimating percentage nitrogen content in leaf dry weights, counting the number of shoots produced, shoot length and plant fresh weight. There were differences between the treatments and between species. In *N. khasiana*, $T_{9.0}$ produced significantly higher fresh weight than $T_{0.4}$ (Table 3) but there were no significant differences in the number of shoots and shoot lengths between the treatments (Table 3). In *N. pervillei*, there were significant differences between the treatments for the number of shoots produced and shoot lengths. The lowest

Table 3. Mean number of shoots, shoot lengths and increase in fresh plant weights in *N. khasiana* after 8 weeks (10 replicates of each). (Means of log transformed data).

Treatments	Mean no. of shoots	Mean shoot length (mm)	Mean increase in fresh weight (mg)
$T_{4.0}$	1.58a (3.4)	1.80a (4.8)	8.14ab (3780.6)
$T_{9.0}$	1.69a (4.0)	2.04a (6.8)	8.48a (5406.0)
$T_{0.2}$	1.59a (3.2)	2.03a (6.4)	8.41ab (4942.3)
$T_{0.4}$	1.37a (2.2)	1.63a (4.7)	7.92b (3309.5)
$T_{4.2}$	1.33a (2.1)	1.68a (5.0)	8.04ab (3734.5)
LSD (5%)	0.39	0.57	0.54
SE's (df=44)	+0.14	+0.20	+0.19

Initial average weight of fresh plants 0.1180 g, mean with the same letter in each column are not significantly different (P>0.05), numbers in the brackets are means of the untransformed data.

number of shoot was produced in $T_{4.0}$ whereas $T_{0.4}$ produced significantly shorter shoots, lower in fresh weight than the other treatments (Table 4). In *N. vieillardii*, the highest numbers of shoots was produced in $T_{9.0}$ and was significantly different from the other treatments, whereas, $T_{4.2,}$ $T_{0.2}$ and $T_{9.0}$ produced significantly longer shoots with higher weight than the other treatments (Table 5).

Despite the differences observed between the treatments, the three species in general, performed better on a nutrient medium with a single main nitrogen source rather than a combination of the two main sources of nitrogen, i.e. compared to standard WPM basal medium in terms of shoot regeneration. In all the three species $T_{9.0}$ treatment produced more shoots than other treatments, followed by $T_{0.2}$. However, in terms of shoot length and increase in fresh weight the three species differed. The overall results of the three species showed that the treatments with higher number of shoots did not produce optimal shoot length and fresh weight.

Table 4. Mean number of shoots, shoot lengths and increase in fresh plant weights in *N. pervillei* after 8 weeks (10 replicates of each). (Means of square root for numbers of shoots, shoot lengths and log transformed data for fresh weights).

Treatments	Mean no. of shoots	Mean shoot length (mm)	Mean increase in fresh weight (mg)
$T_{4.0}$	2.10b (4.9)	1.17b (1.0)	7.50bc (2029.4)
$T_{9.0}$	3.20a (10.1)	2.03a (3.8)	8.00ab (3603.1)
$T_{0.2}$	3.05a (9.0)	2.34a (9.0)	7.99ab (3120.4)
$T_{0.4}$	3.09a (9.4)	1.51b (9.4)	7.41c (1983.0)
$T_{4.2}$	2.91a (8.2)	2.61a (4.8)	8.10a (4512.9)
LSD (5%)	0.61	0.35	0.57
SE's (df=44)	+0.21	+0.12	+0.20

Initial average weight of fresh plants 0.1891 g, mean with the same letter in each column are not significantly different (P>0.05), numbers in the brackets are means of the untransformed data.

Table 5. Mean number of shoots, shoot lengths and increase in fresh weight in *N. vieillardii* after 8 weeks (10 replicates of each). (Means of square root for numbers of shoots, shoot lengths and log transformed data for fresh weights).

Treatments	Mean no. of shoots	Mean shoot length (mm)	Mean increase in fresh weight (mg)
$T_{4.0}$	1.60c (2.4)	1.17bc (0.9)	7.04b (1212.1)
$T_{9.0}$	3.21a (10.0)	1.33ab (1.3)	7.40a (1787.1)
$T_{0.2}$	2.47b (5.8)	1.45a (1.7)	7.65a (2192.4)
$T_{0.4}$	2.29b (5.1)	1.07c (0.7)	6.67c (878.1)
$T_{4.2}$	2.42b (5.7)	1.52a (1.9)	7.58a (2062.2)
LSD (5%)	0.48	0.20	0.35
SE's (df=44)	+0.17	+0.07	+0.12

Initial average weight of fresh plants 0.0868 g, mean with the same letter in each column are not significantly different (P>0.05), numbers in the brackets are means of the untransformed data.

Oscarson and Larsson (1986) reported that growth rate can be tightly controlled by nitrate supply. In the present experiments nitrate supply to the plant was not limited but varied in quantity and source, consequently, uptake was not restricted. There were significant differences in nitrogen content in leaves between the treatments in the three *Nepenthes* spp. studied. It seems plausible that the regulatory mechanism responds to changes in the concentration of a specific compound(s) containing reduced N before major changes in total N concentration can be detected, as reported in wheat cultivars (Rodgers and Barneix 1988). The treatments with $Ca(NO_3)_2$ alone, i.e. $T_{0.2}$ in *N. khasiana* and $T_{0.4}$ in *N. pervillei* gave higher nitrogen content in leaves compared to the other treatments. However, in general the treatments with $Ca(NO_3)_2$ produced lower fresh weight than the other treatments. The differences in the estimated percentage of nitrogen content in dry weight of leaves in the present investigation therefore, did not reflect growth rate, but merely showed differences in the uptake of the nitrate from the nutrient medium. The higher nitrogen content in the treatments with $Ca(NO_3)_2$ may be correlated with the reports that the NO_3^- uptake is inhibited by NH_4^+ (Breteler and Siegerist 1984) and by several amino acids (Breteler and Arnozis 1985).

Abnomalities in the morphology of leaves was observed in *N. khasiana* and *N. vieillardii*. In *N. khasiana*, 37.5% of the explants produced fused leaves (i.e. two leaves fused together) in $T_{4.0}$, 50% in $T_{9.0}$ and 12.5% in $T_{0.4}$. *N. vieillardii* showed small and poorly developed leaves. The abnormalities in the leaf morphology of *N. khasiana* and *N. vieillardii* may suggest that the quantity and nature of the nitrogen supply to the plant had affected the leaf morphology, a characteristic known in higher plants (Marschner 1986). Yoshida and Kohno (1982) also reported that media containing high level of NH_4^+ tend to inhibit chlorophyll synthesis and make aggregates in suspension cultures friable. It may be said that the high ions concentrations

in $T_{0.4}$ in the present study was inhibitory for the normal growth of *Nepenthes* spp. High ions concentration are reported to be inhibitory to some species or group of plants. For example, in woody plant tissue culture, it has been frequently found to grow better *in vitro* on media containing reduced concentrations of one or more ions (Lloyd and McCown 1981, George and Sherrington 1984). In *N. pervillei* no abnormalities in the leaves were observed. This may be attributable to its prolific growth and ease of micropropagated nature compared to the other two species.

The earlier report by Latha and Seeni (1994) that WPM was superior to MS or Knudson C basal media for *N. khasiana* tissue culture may, therefore, be due to the lack of $Ca(NO_3)_2$ in MS and NH_4NO_3 in Knudson C nutrient media. The present investigation on the three *Nepenthes* spp. suggests that for normal growth in tissue culture a combination of the two nitrogen sources, i.e. NH_4NO_3 and $Ca(NO_3)_2$ was required rather than one source in the nutrient medium.

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REFERENCES

Anderson, W.C. (1984). A revised medium for shoot proliferation of Rhododendron. *J. Amer. Soc. Hort. Sci.* **109:** 343-347

Breteler, H. and Arnozis, P. (1985). Effect of exogenous and endogenous nitrate utilization by dwarf bean. *Phytochem.* **24:** 653-658.

Breteler, H. and Siegerist, M. (1985). Effect of ammonium on nitrate utilization by roots of dwarf bean. *Plant Physiol.* **75:** 1099-1103.

Clark, R.B. (1983). Plant genotype differences in the uptake, translocation, accumulation, and used of mineral elements required for plant growth. *Plant & Soil* **72:** 175-196.

Gamborg, O.L. and Shyluk, J.P. (1970). The culture of plant cells with ammonoum salt as sole nitrogen source. *Plant Physiol.* **45:** 598-600.

George, E.F. and Sheerington, P.D. (1984). Plant Propagation by Tissue Cuture; Handbook and Dictionary of Commercial Laboratories. Exegetic Ltd., Edington, UK.

Jeschke, W.D. (1983). Cation fluxes in excised and intact roots in relation to specific and varietal differences. *Plant & Soil* **72:** 197-212.

Kitching, R. and Schofield, C. (1986). Every pitcher tells a story. *New Scientist* **23:** 48-51.

Knudson, L. (1946). A new nutrient solution for germination of orchid seed. *Amer. Orch. Soc. Bull.* **15:** 214-217.

Latha, P.G. and Seeni, S. (1994). Multiplication of the endangered Indian pitcher plant (*Nepenthes khasiana*) through enhanced axillary branching *in vitro*. *Plant Cell Tissue Organ Cult.* **38:** 69-71

Lloyd, G. and McCown, B. (1981). Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. *Int. Plant Pro. Soc. Proc.* **30:** 421-427

Marshner, H. (1986). Mineral Nutrition of Higher Plants. Academic Press, London.

Murashige, T. and Skooge, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15:** 473-479.

Oscarson, P. and Larsson, C.M. (1986). Relations between uptake and utilization of NO_3^- in *Pisum* growing exponentially under nitrogen limitation. *Physiol. Plant.* **67:** 109-117.

Rao, P.K., Rains, D.W., Qualset, C.O. and Huffaker, R.C. (1977). Nitrogen nutrition and grain protein in two spring wheat genotype differing in nitrate reductase activity. *Crop Sci.* **17:** 283-286.

Rodger, C.O. and Bareix, A.J. (1988). Cultivar differences in the rate of nitrate uptake by intact wheat plants as related to growth rate. *Physiol. Plant.* **72:** 121-126.

Vose, P.B. (1963). Varietal differences in plant nutrition. *Herbage Abstract* **33:** 1-13.

Yoshida, F. and Kohno, H. (1982). Effect of media containing NH_4 as the sole nitrogen source on cultured cells of tobacco and rice. In: A. Fujiwara (ed.), Proc. Vth International Congress of Plant Tissue and Cell Culture, pp. 231-232. Tokyo, Japan.