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BRIEF COMMUNICATION

Effects of different N-sources on growth, nutritional status, chlorophyll content, and photosynthetic parameters of shoots of the apple rootstock MM 106 cultured *in vitro*

T.E. SOTIROPOULOS*[1], G.N. MOUHTARIDOU*, T. THOMIDIS*, V. TSIRAKOGLOU**, K.N. DIMASSI** and I.N. THERIOS**

*N.AG.RE.F., Pomology Institute, P.O. Box 122, GR-59200 Naoussa, Greece**
*Laboratory of Pomology, School of Agriculture, Aristotle University of Thessaloniki, GR-54124 Thessaloniki, Greece***

Abstract

The effects of five different N-sources (KNO_3 + NH_4NO_3 = control, KNO_3, NH_4NO_3, $NH_4H_2PO_4$, L-alanine) on growth, nutritional status, chlorophyll (Chl) content, and photosynthetic parameters of the apple rootstock MM 106 shoots cultured *in vitro* were investigated. In comparison to all the other treatments, control explants grown on a MS medium containing KNO_3 + NH_4NO_3 had the highest fresh mass, Chl content, net photosynthetic rate, transpiration rate, and stomatal conductance.

Additional key words: cell proliferation, ion contents, micropropagation, net photosynthetic rate, nitrogen, stomatal conductance, transpiration rate.

The MM 106 is an apple rootstock used extensively in many countries to produce semi-dwarf trees. Therefore, its micropropagation *in vitro* is very important for commercial practices. Nitrate and ammonium ions are the most common inorganic nitrogen sources used as mineral salts in tissue culture media for *in vitro* propagation (Murashige and Skoog 1962, Niedz 1994). The different forms of nitrogen in the culture media alter the endogenous levels of cell metabolites as well as of proteins, organic acids, and plant hormones (Preece 1995). Moreover, N concentration, its forms and their proportion may influence cell division, differentiation, growth, and development of somatic embryos *in vitro* (Mordhorst and Lorz 1993). The N nutrition does not only affect somatic embryogenesis, but also chlorophyll (Chl) content, Rubisco activity, electron transport rate, photosynthetic rate, anthocyanin production, fresh mass, soluble protein concentration, and osmotic pressure of the cell sap of various cultures *in vitro* such as carrot, tomato and wheat (Guidi *et al.* 1998, Jain *et al.* 1999, Mashayekhi-Nezamabadi 2000). The objective of this research was to study the effects of different N-sources on growth, nutritional status, Chl content, and photosynthetic parameters of the apple rootstock cultured *in vitro*.

The explants employed were shoots of the apple (*Malus domestica* Borkh.) rootstock MM 106 of about 25 mm in length, preserved from previous *in vitro* cultures and maintained in the growth room. Each explant was transferred and grown in a 15 × 100 mm glass test tube containing 3 cm^3 of the Murashige and Skoog (1962; MS) nutrient medium. The nutrient medium was supplemented with 30 kg m^{-3} sucrose, 1.5 g m^{-3} benzyl-adenine, and 1 g m^{-3} gibberellic acid. The following treatments with MS medium containing different N-sources (KNO_3 + NH_4NO_3 = control, KNO_3, NH_4NO_3, $NH_4H_2PO_4$, L-alanine) were used. The N concentration in all treatments was 4 mM. The pH of the media was adjusted to 5.8 before autoclaving at 121 °C for 15 min. The tubes were closed with aluminum foil and

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Abbreviations: Chl - chlorophyll; MS - Murashige and Skoog; Rubisco - ribulose-1,5-bisphosphate carboxylase/oxygenase.
[1] Author for correspondence; fax: (+30) 2332041178, e-mail: thosotir@alfanet.gr

maintained in the growth room at 22 ± 1 °C and 16-h photoperiod with irradiance 45 μmol m^{-2} s^{-1} (400 - 700 nm) provided by cool white fluorescent lamps, supplied by *TLD 36W/84* lamps.

After five weeks in culture, the fresh mass (FM) of explants was measured. Leaf Chl content was estimated in leaves by using *a SPAD* meter (*Minolta 502*, Osaka, Japan). For determination of the mineral composition, leaves and stems of each plantlet were harvested and rinsed twice with distilled water. These were then dried at 68 °C for 48 h, ground to pass a 30-mesh screen and dry ashed at 530 °C for 16 h. Ca, K, Mg, Zn, Fe, and Mn contents in ash were determined by atomic absorption spectroscopy (*Perkin-Elmer 2380*, Wellesley, USA). Phosphorus was determined colorimetrically by the ammonium phosphovanadomolybdate method, nitrogen by the Kjeldahl's procedure, and boron by the azomethine-H method (Wolf 1974). Each treatment included fifteen replicates (tubes). The experiment was conducted and repeated twice, and the reported data are the means of the two experiments. The statistical design employed was the randomized complete block one. Differences between means were evaluated by using the Duncan's multiple range test at $P < 0.05$.

Explants treated with KNO_3 + NH_4NO_3 (control) produced the highest fresh mass, while those treated with $NH_4H_2PO_4$ the lowest (Table 1). Many higher plant species show toxicity symptoms, when ammonium ion is the exclusive N-source. The toxicity of NH_4^+ causing low pH is particularly severe in maize and bean (Yan *et al.* 1992). A very important feature of the N-forms used, is their effect on the pH of the culture medium. Due to the release of H^+ in the $NH_4H_2PO_4$ treatment, the pH of the culture medium was reduced, while in the KNO_3 treatment, the pH of the medium was increased, due to OH^- release (Mashayekhi-Nezamabadi 2000). Previous experiments with tobacco cell cultures showed that cells proliferate better in a medium containing nitrate as the sole nitrogen source, than other N forms (Neumann 1995). Furthermore, in strawberry and in carrot cultures, the low NH_4^+ concentration in medium favoured dry mass accumulation (Hidder *et al.* 1994). In potato microtubers, the low NH_4^+ concentration improved dry matter accumulation (Chen and Liao 1993). Avilla *et al.* (1998) working with *in vitro* cultures of potato concluded that the ratio NH_4^+/NO_3^- was important in determining C and N use, because by decreasing the N concentration in the medium and the proportion of NH_4^+ C use is increased, as reflected in dry matter accumulation.

The Chl content (*SPAD* units) was significantly increased in explants treated with KNO_3 + NH_4NO_3 in comparison to the other treatments (Table 1). The lowest Chl content was measured when $NH_4H_2PO_4$ was added in the culture medium. In the same treatment, leaf chlorosis was observed. Mashayekhi-Nezamabadi (2000) have pointed out that NH_4^+ treatments of carrot cultures *in vitro* produced a very low amount of Chl because enzyme activities and the growth of cells were reduced

Table 1. Effect of N-sources in the culture media on fresh mass per plantlet, chlorophyll content, photosynthetic rate, transpiration rate, and stomatal conductance of the apple rootstock MM *106 in vitro*. N concentration at all treatments was 4 mM. Means in the same column followed by the same letter are not significantly different (Duncan's multiple range test, $P = 0.05$).

N-source	Fresh mass [g $plant^{-1}$]	Chl content [*SPAD* units]	Net photosynthetic rate [μmol m^{-2} s^{-1}]	Transpiration rate [mmol m^{-2} s^{-1}]	Stomatal conductance [mol m^{-2} s^{-1}]
KNO_3 + NH_4NO_3	0.72a	35.10a	1.48a	1.52a	0.22a
KNO_3	0.65bc	31.16b	1.26b	1.04c	0.20ab
$NH_4H_2PO_4$	0.58d	20.77c	0.88c	0.83d	0.15c
NH_4NO_3	0.69ab	29.35b	1.40ab	1.44b	0.17bc
L-alanine	0.63c	29.08b	0.57d	0.54e	0.13c

Table 2. Effect of N-sources in the culture media on N, P, K, Ca, Mg, Fe, Mn, Zn, and B contents of the apple rootstock MM 106 *in vitro*. *N* concentration at all treatments 4 mM. Means in the same column followed by the same letter are not significantly different (Duncan's multiple range test, $P = 0.05$).

N-source	N [%(d.m.)]	P [%(d.m.)]	K [%(d.m.)]	Ca [%(d.m.)]	Mg [%(d.m.)]	Fe [μg g^{-1}(d.m.)]	Mn [μg g^{-1}(d.m.)]	Zn [μg g^{-1}(d.m.)]	B [μg g^{-1}(d.m.)]
KNO_3 + NH_4NO_3	2.96a	0.21b	2.40b	0.32b	0.12a	73a	87a	36a	43a
KNO_3	2.62b	0.23b	3.86a	0.34b	0.14a	75a	85a	32a	46a
$NH_4H_2PO_4$	2.68b	0.40a	1.62c	0.31b	0.13a	76a	89a	36a	44a
NH_4NO_3	2.80ab	0.22b	1.72c	0.32b	0.12a	70a	92a	31a	45a
L-alanine	2.07c	0.24b	1.67c	0.45a	0.11a	72a	85a	34a	43a

due to low pH of the medium. Hsu *et al.* (2003) confirmed that NH_4^+ accumulation in leaves increased leaf sensitivity to ethylene, which in turn resulted in an enhancement of Chl loss. Photosynthetic and transpiration rates and stomatal conductance exhibited the highest values in the control treatment and the lowest in the L-alanine one (Table 1). This effect could be attributed to the lower N concentrations of explants in the L-alanine treatment in comparison to the other N sources. Despite the fact that nitrate and ammonium salts have been universally used as nutrients in tissue culture media, certain reports specify that several amino acids such as asparagine, proline, and alanine could improve cell proliferation, as well as regeneration in *Medicago sativa* (Stuart and Strickland 1984, Olsen 1987). However, the function of these compounds in the induction and expression of morphogenesis is explicit. The impact of reduced organic N in cell cultures on cell division and cell growth is not always positive (Filner 1966). In the present experiment, L-alanine reduced fresh mass per explant in comparison to the control.

Nitrogen content of cultures was highest when KNO_3 + NH_4NO_3 were added in the culture medium (Table 2). Mengel (1991) proposed that the effective N uptake *in vitro* depends on a balance between both nitrate and ammonium ions. The lowest N content of cultures, was measured in the presence of L-alanine. Phosphorus content of cultures was highest when $NH_4H_2PO_4$ was added in the culture medium, due to the presence of the $H_2PO_4^-$ ion, and did not differ significantly among the other treatments (Table 2). Potassium content of cultures was decreased in the following order: KNO_3 > control > $NH_4H_2PO_4$ = NH_4NO_3 = L-alanine. The highest K content of cultures in the KNO_3-treatment could be ascribed to the presence of K^+. The highest Ca content of cultures was measured in the presence of L-alanine in the culture medium, and did not differ significantly among the other treatments. Finally, Mg, Fe, Mn, Zn, and B contents of cultures did not differ significantly for the various N-sources employed (Table 2).

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