

BRIEF COMMUNICATION

Effects of L-arginine and L-cysteine on growth, and chlorophyll and mineral contents of shoots of the apple rootstock EM 26 cultured *in vitro*

T.E. SOTIROPOULOS^{*1}, K.N. DIMASSI^{**} and I.N. THERIOS^{**}

*N.AG.RE.F., Pomology Institute, P.O. Box 122, GR-59200 Naoussa, Greece**

*Department of Horticulture, Laboratory of Pomology, Aristotle University, GR-54124 Thessaloniki, Greece***

Abstract

1, 5, or 10 mM arginine and 25, 50, or 100 μ M cysteine were added in the Murashige and Skoog medium. By increasing arginine concentration the number of shoots per explant increased. Inclusion of 50 μ M cysteine in the medium resulted in maximum number of shoots but it was not significantly different in comparison to 10 mM arginine. The chlorophyll content was significantly increased in explants treated with 10 mM arginine in comparison to the control, 1 mM arginine and 25 μ M cysteine. By increasing arginine and cysteine concentrations of the medium, N, K, and Ca contents of explants increased but no significant changes in P, Mg, Fe, Mn, Zn, and B contents were observed.

Additional key words: amino acids, cell proliferation, micropropagation, nitrogen.

The improvement of micropropagation efficiency of the EM 26 apple rootstock is very important for commercial practices. The different nitrogen forms and their proportion may influence cell division, growth and development of somatic embryos, chlorophyll (Chl) content, ribulose-1,5-bisphosphate carboxylase/oxygenase activity, electron transport rate, photosynthetic rate, fresh mass, soluble protein concentration, and osmotic pressure of the cell sap of various cultures *in vitro* (Mordhorst and Lorz 1993, Jain *et al.* 1999, Mashayekhi-Nezamabadi 2000). Despite the fact that nitrate and ammonium salts have been universally used as N source in tissue culture media, numerous reports specify that reduced nitrogen forms, particularly amides and amino acids, *e.g.* glutamine, glutamic acid, proline, and alanine, can improve cell proliferation as well as regeneration in specific genotypes (Olsen 1987, Neumann 1995, Vasudevan *et al.* 2004). Furthermore, amino acids have a specific role in plant responses to stresses (Rai 2002). However, the influence of reduced organic N on cell division and cell growth is not altogether comprehensive and not always positive. Filner (1966) monitored that L forms of alanine, asparagine, aspartic acid, glutamic acid, proline, valine, histidine, and leucine inhibited cell

growth and repressed nitrate reductase activity in short-term cultivation of tobacco cells in culture media containing one of these amino acids. Proline and serine stimulated somatic embryogenesis in *Daucus* and *Dactylis* (Ronchi *et al.* 1984, Trigiano and Conger 1987). The objectives of the present research were to check the effect of arginine and cysteine on growth, and chlorophyll and mineral contents of shoots of the apple rootstock EM 26 cultured *in vitro*.

The explants employed were apple (*Malus domestica* Borkh.) shoots of the EM 26 rootstock, 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 25 \times 100 mm glass test tube containing 10 cm³ of the MS culture medium (Murashige and Skoog 1962) supplemented with 1 g m⁻³ benzyladenine (BA). This medium was used as a control. Arginine (1, 5, or 10 mM) and cysteine (25, 50, or 100 μ M) were used. The pH of the media was adjusted to 5.8 before autoclaving at 121 $^{\circ}$ C for 15 min. The tubes were maintained in the growth room at 22 \pm 1 $^{\circ}$ C and 16-h photoperiod (cool white fluorescent tubes, irradiance of 45 μ mol m⁻² s⁻¹, 400 - 700 nm). After ten weeks in culture, the number of shoots, length of shoots, and fresh

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Abbreviations: Chl - chlorophyll; MS medium - Murashige and Skoog medium.

¹ Corresponding author; fax: (+30) 2332041178, e-mail: thosotir@alfanet.gr

mass of explants were measured. Leaf Chl content was estimated non-destructively in leaves by using a SPAD meter (*Minolta* model 502, Illinois, USA). For determination of the mineral composition, leaves and stems from each plantlet were harvested and rinsed twice with distilled water. These organs 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. After ashing, the residue was dissolved in 10 cm³ of 6 M HCl and brought to a volume of 50 cm³. These extracts were measured for K, Ca, Mg, Mn, Fe, and Zn by atomic absorption spectrometry (*Perkin-Elmer* model 2380, Wellesley, USA). Nitrogen was determined by the Kjeldahl's procedure, phosphorous by the ammonium phospho-vanado-molybdate method, and boron by the azomethine-H method (Wolf 1974). N, P, K, Ca, and Mg contents were expressed as g kg⁻¹(d.m.), while Fe, Mn, Zn, and B contents were expressed as mg kg⁻¹(d.m.). Each treatment included fifteen replicates (tubes). The experiment was 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 Duncan's multiple range test at $P \leq 0.05$.

By increasing arginine concentration of the culture medium from 1 to 10 mM, the number of shoots per explant increased (Table 1). Inclusion of 50 µM cysteine in the medium resulted in maximum number of shoots but it was not significantly different in comparison to 10 mM arginine. All treatments except 1 mM arginine significantly increased shoot proliferation in comparison to the control. However, arginine and cysteine did not significantly alter shoot length in comparison to the control. Kim *et al.* (1990) reported that L-proline inclusion in culture media enhanced shoot regeneration from excised primary leaf nodes of soybean. According to Shetty *et al.* (1992), partial substitution of certain amino acids for NH₄NO₃ in MS medium enhanced organogenesis of soybean seedling explants. Inclusion of 10 mM methionine in the culture medium significantly increased the number of shoots and length of shoots of MrS 2/5 rootstock in comparison to the control (Dimassi-

Theriou 1995). Sudarsana *et al.* (2001) reported that by supplementing culture medium with proline, glutamine, asparagine, and cysteine, shoot bud development to microshoots was enhanced at least by two fold. Putrescine influences shoot multiplication and *in vitro* flowering in *Cichorium intybus* L. (Bais *et al.* 2000). Furthermore, Montague *et al.* (1979) reported that arginine serves as a precursor in polyamine synthesis. The primary events of cell division, cell size increase, and entry into S-phase of DNA synthesis were improved and the rate of cell mortality decreased when *Asparagus officinalis* mesophyll cells were cultured in MS medium with 30 mM L-glutamine (Jullien *et al.* 1979). Fresh mass of explants grown on media containing 5 mM arginine was significantly lower in comparison to 1 mM arginine and 50 µM cysteine (Table 1). Proline and serine added to culture media during the growth of carrot suspensions stimulated growth (Mashayekhi-Nezamabadi 2000).

The Chl content (SPAD units) was significantly increased in explants treated with 10 mM arginine in comparison to the control, 1 mM arginine, and 25 µM cysteine (Table 1). By increasing arginine and cysteine concentrations of the medium, N content of explants

Table 1. Effect of arginine and cysteine concentrations of the culture medium on the number of shoots per explant, shoot length, fresh mass, and chlorophyll content (SPAD units) of the EM 26 apple rootstock shoots *in vitro*. Means followed by the same letter in the same column are not significantly different (Duncan's multiple range test $P \leq 0.05$).

Amino acid	Conc.	Number of shoots [explant ⁻¹]	Shoot length [mm]	Fresh mass [g]	SPAD units
Control	0	3.6 e	15.35 a	0.28 ab	32 b
Arginine	1 mM	3.5 e	15.59 a	0.33 a	33 b
	5 mM	4.5 c	14.70 a	0.28 ab	35 ab
	10 mM	5.0 ab	15.12 a	0.28 ab	37 a
Cysteine	25 µM	4.0 d	15.30 a	0.29 ab	32 b
	50 µM	5.3 a	15.51 a	0.34 a	34 ab
	100 µM	4.9 b	15.12 a	0.28 ab	35 ab

Table 2. Effect of arginine and cysteine concentrations of the culture medium on N, P, K, Ca, Mg [g kg⁻¹(d.m.)], Fe, Mn, B, and Zn [mg kg⁻¹(d.m.)] contents of the EM 26 apple rootstock shoots *in vitro*. Means followed by the same letter in the same column are not significantly different (Duncan's multiple range test $P \leq 0.05$).

Amino acid	Conc.	N	P	K	Ca	Mg	Fe	Mn	B	Zn
Control		2.98 c	5.8 a	17.5 c	5.2 d	1.5 a	105 a	126 a	84 a	153 a
Arginine	1 mM	3.00 c	5.1 a	19.7 b	6.3 ab	1.9 a	102 a	120 a	82 a	158 a
	5 mM	3.17 ab	5.2 a	22.0 a	5.6 cd	1.6 a	104 a	119 a	85 a	156 a
	10 mM	3.28 a	5.2 a	16.9 c	6.0 bc	1.7 a	107 a	121 a	83 a	151 a
Cysteine	25 µM	2.99 c	5.9 a	21.8 a	6.6 a	1.8 a	104 a	122 a	84 a	154 a
	50 µM	3.07 b	5.9 a	19.5 b	5.9 bc	1.8 a	108 a	125 a	83 a	159 a
	100 µM	3.16 ab	5.1 a	20.0 b	5.1 d	1.5 a	103 a	122 a	84 a	153 a

increased (Table 2). Amino acids can adjust the nitrogen utilization of *in vitro* cultures by regulating primary nitrogen assimilation. Concurrently, many amino acids can be readily transformed into other amino acids and integrated into proteins in the cell culture (Thorpe 1993). Inclusion of arginine and cysteine in the medium significantly increased K content of explants. Calcium

content of explants treated with arginine and cysteine at 25 and 50 μM was significantly higher in comparison to the control. P, Mg, Fe, Mn, Zn, and B contents of cultures grown on media supplied with arginine and cysteine were not significantly altered in comparison to the control (Table 2).

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