DOI: 10.1007/s10535-015-0562-6 BIOLOGIA PLANTARUM 60 (1): 1-12, 2016

Effect of asparagine, cysteine, citrulline, and glutamine on *in vitro* rooting and biochemical constituents in cherry rootstocks

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Abstract

Effects of four amino acids, L-asparagine, L-cysteine, L-citrulline, and L-glutamine in different concentrations (0, 0.5, 1, and 2 mg dm^{-3}) combined with 2 mg dm^{-3} indole-3-butyric acid, on *in vitro* rooting and biochemical constituents of cherry rootstocks CAB-6P (*Prunus cerasus* L.) and Gisela 6 (*P. canescens* × *P. cerasus*) were investigated. In CAB-6P, root number and root fresh mass (FM) were maximum at 0.5 mg dm^{-3} cysteine. All amino acids reduced root length in CAB-6P and root number as well as root FM in Gisela 6. In Gisela 6, 0.5 mg dm^{-3} asparagine or 2 mg dm^{-3} glutamine reduced root length. In CAB-6P, 100 % rooting was achieved in the control and with 1 and 2 mg dm^{-3} cysteine or 1 mg dm^{-3} citrulline. In Gisela 6, the rooting percentage was maximum (76.92 %) with 0.5 mg dm^{-3} asparagine. Callus FM in CAB-6P was the greatest at 1 mg dm^{-3} and in Gisela 6 at 2 mg dm^{-3} citrulline. Callusing was 100 % in the majority of treatments for CAB-6P and 92.31 % for Gisela 6 with 0.5 or 2 mg dm^{-3} citrulline. Cysteine, citrulline, and glutamine diminished chlorophyll content in Gisela 6 whereas in CAB-6P all four amino acids hardly affected it. Carotenoid and porphyrin content in CAB-6P was decreased due to asparagine (0.5 or 1 mg dm^{-3}). Porphyrin content in CAB-6P was also reduced by adding 0.5 or 1 mg dm^{-3} cysteine or 2 mg dm^{-3} citrulline. In Gisela 6, all amino acids decreased carotenoid and porphyrin content. In CAB-6P, all treatments except 0.5 mg dm^{-3} glutamine or 2 mg dm^{-3} asparagine increased leaf sucrose content. In roots, both sucrose and proline content were increased only at 1 mg dm^{-3} cysteine whereas in leaves only 0.5 mg dm^{-3} asparagine caused a 3-fold increase in proline content. A decrease in root proline in CAB-6P was observed due to asparagine, citrulline, or glutamine. In Gisela 6, decreased leaf sucrose and proline content was recorded at 2 mg dm^{-3} cysteine. All amino acids did not alter root sugar content remarkably whereas root proline content was raised by adding 0.5 mg dm^{-3} glutamine or 1 mg dm^{-3} cysteine.

Additional key words: amino acids, carotenoids, chlorophyll, micropropagation, porphyrins, proline, rhizogenesis, sugars.

Introduction

Plants absorb mainly inorganic nitrogen as ammonium or nitrate ions. The ammonium ions are reduced to organic N, mostly amino acids which are transferred to various plant parts through xylem and phloem. Moreover, plants may absorb amino acids directly from soil (Nasholm and Persson 2001). Addition of amino acids provides a readily available nitrogen in tissue cultures, and their uptake can occur more rapidly than from an inorganic nitrogen source in the same medium (George *et al.* 2008).

Amino acids may influence rooting. The observed inhibition of rooting has been related to increased content of endogenous amino acids including glutamine and glutamate (Welander 1978). The variations of the endogenous content of amino acids may regulate the synthesis rate of proteins, *e.g.*, during seed germination (Aguilar and Sanchez de Jimenez 1984) and organogenesis *in vitro* (Zhu *et al.* 1990). Since many amino acids are involved in ethylene biosynthesis (Sudarsana Rao *et al.* 2001), and plant tissues and organs cultured *in vitro* produce ethylene, the accumulated endogenous ethylene in the vessels may influence explant morphogenesis (Sauerbrey *et al.* 1988). Also, some amino acids added to the culture medium affect organogenesis (Orlikowska 1992). Kavi Kishor (1989)

Submitted 4 January 2015, *last revision* 13 June 2015, *accepted* 7 July 2015.

Abbreviations: AS - asparagine synthase; 2,4-D - 2,4-dichlorophenoxy acetic acid; DM - dry mass, FM - fresh mass; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; MS - Murashige and Skoog; NAA - α-naphthalene acetic acid.

Acknowledgements: We would like to express our sincere gratitude to the Fitotechniki Bros Company and especially to Angelos Xylogiannis for kindly providing the CAB-6P and Gisela 6 rootstocks, and to Sofia Kuti and Vasiliki Tsakiridou for technical assistance. The authors gratefully acknowledge the financial support of Aristotle University of Thessaloniki.

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suggested that increased metabolism of amino acids correlates to the induction of root and shoot formation in tobacco callus cultures. In shoot cultures of *Torenia fournieri,* various amino acids induce root formation in the presence of auxin (Kamada and Harada 1979). In apple rootstock (P2) explants, aspartic acid, glutamate, and ornithine significantly increase the number of roots and rooting percentage, but tryptophan and arginine slightly increase only the number of roots, asparagine has no effect whereas proline reduces the number of roots (Orlikowska 1992). In another apple rootstock (P60), arginine, ornithine, glutamate, and glycine increase the number of roots, proline had no effect whereas asparagine, tyrosine, methionine, cysteine, and glutamine reduce it (Orlikowska 1992).

Therefore, the aim of this experiment was to evaluate the effect of addition of four amino acids (L-asparagine, L-cysteine, L-citrulline, L-glutamine) into a medium on *in vitro* rooting, callusing, and vegetative growth of cherry rootstocks CAB-6P and Gisela 6.

Materials and methods

In these experiments, the effect of four amino acids (L-asparagine, L-cysteine, L-citrulline, L-glutamine) in 0, 0.5, 1.0 and 2.0 mg dm^{-3} concentrations on growth, rooting, and callusing of Gisela 6 (*Prunus canescens* L. × *Prunus cerasus* L.) and CAB-6P (*Prunus cerasus* L.) were studied. Shoot tips (approximately 1 to 2.5 cm in length) obtained from previous subcultures were placed onto a Murashige and Skoog (1962; MS) nutrient medium containing all the necessary macro- and micro-elements, including chelate iron (Fe-EDTA), 2 mg dm^{-3} indole-3-butyric acid (IBA), vitamins, and amino acids. Moreover, sucrose 30 g dm^{-3} and agar 6 g dm^{-3} were added and pH was adjusted to 5.8 prior to autoclaving at 121 °C for 20 min. Each treatment included a total of 12 replications (6 in the first + 6 in the second experiment) with one microcutting in each 25 × 100 mm test tube that contained 10 cm^3 of the medium. Afterwards, the cultures were maintained at a temperature of 21 - 23 °C, a 16 h photoperiod, and an irradiance of 90 µmol m^{-2} s^{-1} (*Philips* cool white fluorescent tubes). After seven weeks in culture, measurements of root number, root length, root fresh mass (FM), rooting percentage, callus FM, callusing percentage, shoot length, shoot FM, total leaf chlorophyll, carotenoid, and porphyrin content, as well as sucrose and proline content in both leaves and roots were taken.

For chlorophyll measurement, 0.1 g of a frozen (-18 °C) leaf material was placed in glass test tubes (25 cm^3), 15 cm^3 of 96 % (v/v) ethanol was added, and the tubes were incubated in a water bath at 79.8 °C until a complete sample discoloration and chlorophyll extraction. The absorbances of chlorophylls *a* and *b* were measured at 665 and 649 nm in a spectrophotometer (*Shimadzu UV-1800*, Kyoto, Japan). Chlorophyll content was calculated according to Wintermans and De Mots (1965).

Carotenoid and porphyrin content was determined as decribed Lichenthaler (1987) and Porra *et al.* (1989) and modified by Yang *et al*. (1998). Samples (5 mg) were homogenized with 5 cm^3 of 80 % (v/v) acetone in a cooled mortar and pestle (-4 °C). The homogenate was centrifuged at 1 500 *g* for 5 min, and the supernatant was stored. The pellet was re-extracted with acetone of the same concentration and centrifuged again. This process was continued until the supernatant was colourless, and then the supernatant was pooled. Absorbance was measured at 663.6, 646.6, 440.5, 575, 590, and 628 nm corresponding to the absorption peaks of chlorophyll *a*, chlorophyll *b*, carotenoids, proto-porphyrin, magnesium-protoporphyrin, and proto-chlorophyllide, respectively.

For proline determination, leaf or root frozen tissue (0.1 g), was chopped into small pieces and placed in glass test tubes of 25 cm^3. In each tube, 10 cm^3 of 80 % (v/v) ethanol was added and they were placed in a water bath of 60 °C for 30 min (Khan *et al.* 2000). The extracts were filtered, and 80 % ethanol was added to a total volume of 15 cm^3; then 4 cm^3 of toluene was added and mixed well with a vortex. Two layers were visible in each tube. The supernatant (toluene layer) was removed with a Pasteur pipette and placed in a glass cuvette. Absorbance was measured at 518 nm. The extract was filtered with *Whatman No. 1* filter paper and free proline was measured (Troll and Lindsley 1955) with an acid ninhydrin solution. Proline content was calculated from a standard curve using L-proline (*Sigma*, St. Louis, USA) at 0 - 0.2 mM concentrations.

Sucrose determination in plant tissues was conducted by using the anthrone method (Plummer 1987). For reagent preparation, 1 g of anthrone was diluted in 500 cm^3 of concentrated sulfuric acid. The plant ethanolic extract for sucrose determination was the same as that used for proline with the only difference that it was diluted 10 times with 80 % ethanol. In each test tube, 2 cm^3 of the anthrone reagent was added and maintained in an ice bath. Subsequently, the diluted extract (10 % of the initial) was added dropwise in contact with the test tube walls in order to avoid blackening the samples. After shaking the tubes with a vortex, the samples were incubated in a water bath of 95 °C for 15 min. Afterwards the tubes were placed in a cold water bath for cooling, and absorbance was measured at 625 nm. Endogenous sucrose content was calculated from a standard curve by using 0 - 0.2 mM sucrose concentrations.

The experimental layout was completely randomized and the experiment was repeated twice. Thus, the reported data are the means of the two experiments. The means were subjected to analysis of variance (*ANOVA*) and compared by using Duncan's multiple-range test

(α = 0.05). The main effect of factors (rootstock, amino acid type, and amino acid concentration) and their interactions were determined by the *General Linear Model* (*3-way ANOVA*).

Results

In CAB-6P, root length was highest (21.31 mm) in the control plants (Fig. 1*A*), thus, all four amino acids tested negatively influenced the elongation of roots (Table 1). Root number per rooted explant (14.18) and root FM (0.231 g) were maximum with 0.5 mg dm^{-3} cysteine (Fig. 1*E*). Only this treatment significantly augmented root number (2-fold increase) and root FM (3-fold increase) in comparison to the control plants. The rooting

Fig. 1. Effect of amino acids on *in vitro* rooting of CAB-6P microcuttings: *A* - control; *B* - 0.5 mg dm^{-3} asparagine; *C* - 1 mg dm^{-3} asparagine; *D* - 2 mg dm^{-3} asparagine; E - 0.5 mg dm^{-3} cysteine; *F* - 1 mg dm^{-3} cysteine; *G* - 2 mg dm^{-3} cysteine; *H* - 0.5 mg dm^{-3} citrulline; *I* - 1 mg dm^{-3} citrulline; *J* - 2 mg dm^{-3} citrulline; *K* - 0.5 mg dm^{-3} glutamine; *L* - 1 mg dm^{-3} glutamine; *M* - 2 mg dm^{-3} glutamine.

Fig. 2. Effect of amino acids on *in vitro* rooting of Gisela 6 microcuttings: *A* - Control; *B* - 0.5 mg dm^{-3} aparagine; *C* - 1 mg dm^{-3} aparagine; *D* - 2 mg dm^{-3} aparagine; E - 0.5 mg dm^{-3} cysteine; *F* - 1 mg dm^{-3} cysteine; *G* - 2 mg dm^{-3} cysteine; *H* - 0.5 mg dm^{-3} citrulline; *I* - 1 mg dm^{-3} citrulline; *J* - 2 mg dm^{-3} citrulline; *K* - 0.5 mg dm^{-3} glutamine; *L* - 1 mg dm^{-3} glutamine; *M* - 2 mg dm^{-3} glutamine.

percentage was maximum (100 %) in the control as well as with 1 or 2 mg dm^{-3} cysteine (Fig. 1*F*,*G*) and 1 mg dm^{-3} citrulline (Fig. 1*I*). Both asparagine (Fig. 1*B*,*C*,*D*) and glutamine (Fig. 1*K*,*L*,*M*) decreased rooting percentage in all concentrations tested. However, the percentage of the explants forming roots was lowest (84.62 %) with 0.5 mg dm^{-3} cysteine (Fig. 1*E*) or 0.5 mg dm^{-3} citrulline (Fig. 1*H*).

In Gisela 6, the number of roots per rooted explant (6.57) and root FM (0.159 g) were greatest in the control plants (Table 1, Fig. 2*A*). Except cysteine at 1 mg dm^{-3} which exhibited similar results to the control, all the other treatments decreased root number. Among treatments, asparagine (0.5 or 1 mg dm^{-3}; Fig. 2*B*,*C*), cysteine (1 or 2 mg dm^{-3}; Fig. 2*F*,*G*), citrulline (0.5 or 1 mg dm^{-3}; Fig. 2*H*,*I*) and glutamine in all concentrations applied (Fig. 2*K*,*L*,*M*) decreased root FM. Root length was negatively affected by 0.5 mg dm^{-3} asparagine (Fig. 2*B*) or 2 mg dm^{-3} glutamine (Fig. 2*M*). The percentage of the explants forming roots was augmented with 0.5 or 2 mg dm^{-3} asparagine (Fig. 2*B*,*D*), 0.5 or 1 mg dm^{-3} cysteine (Fig. 2*E*,*F*), and 2 mg dm^{-3} citrulline (Fig. 2*I*,*J*).

Among these treatments, 0.5 mg dm^{-3} asparagine gave the highest rooting percentage (76.92 %).

In CAB-6P, only the addition of 1 mg dm^{-3} citrulline substantially increased (doubled) callus FM in relation to the control (Table 1, Fig. 1*J*). Asparagine or citrulline at 1 mg dm^{-3} as well as 0.5 mg dm^{-3} cysteine decreased callusing from 100 to 92.31 %. In all the other treatments, the frequency of callus formation was 100 % as in the control. In Gisela 6, only 2 mg dm^{-3} citrulline substantially increased callus FM. Callusing percentage was augmented to a greater extent from 61.54 to 92.31 % by incorporating 0.5 or 2 mg dm^{-3} citrulline into the culture medium. Cysteine reduced callus formation percentage in all concentrations tested whereas 0.5 mg dm^{-3} asparagine or 1 mg dm^{-3} glutamine had a positive effect on it. In all amino acid treatments, the Gisela 6 explants formed a smaller but more compact callus than CAB-6P and the callus of Gisela 6 was green-yellow-brown whereas that of CAB-6P was yellowish. In both genotypes, calli formed at the base of the explants were externally compact but internally soft and fragile.

In CAB-6P, no significant changes were observed in terms of both shoot length and shoot FM among the treatments (Table 2). In Gisela 6, however, among the amino acids tested, only cysteine at 1 mg dm^{-3} or glutamine at 2 mg dm^{-3} caused a remarkable decrement in shoot length. Shoot FM of the Gisela 6 microcuttings was substantially decreased only when 2 mg dm^{-3} citrulline was incorporated into the culture medium. In both the CAB-6P and Gisela 6 cherry rootstocks, all the other treatments did not modify meaningfully shoot length and shoot FM. The growth of the Gisela 6 microshoots was better (a higher shoot length and shoot FM) than of CAB-6P.

Table 1. The effect of four amino acids combined with 2 mg dm^{-3} indole-3-butyric acid on rooting, callusing, and *P*-values from *ANOVA* for variables amino acid type (A), amino acid concentration (B), rootstock (C), and interactions. Means followed by the same letters within each column and for each rootstock separately are not significantly different at $P \geq 0.05$ based on Duncan's multiple range test, $n = 12$; ns - $P \geq 0.05$, * - $P \leq 0.5$, ** - $P \leq 0.1$, *** - $P \leq 0.01$.

Rootstock	Amino acid	[mg dm^{-3}]	Root number [explant^{-1}]	Root length [mm]	Root FM [g]	Rooting [%]	Callus FM [g]	Callusing [%]
CAB-6P	control	0	6.31 a	21.31 c	0.080 a	100 c	0.213 a	100 b
	asparagine	0.5	7.75 a	16.10 b	0.125 a	92.31 b	0.304 ab	100 b
		1.0	7.50 a	13.71 ab	0.115 a	92.31 b	0.302 ab	92.31 a
		2.0	6.17 a	11.65 ab	0.087 a	92.31 b	0.380 ab	100 b
	cysteine	0.5	14.18 b	10.81 a	0.231 b	84.62 a	0.339 ab	92.31 a
		1.0	10.69 ab	11.38 a	0.149 ab	100 c	0.406 ab	100 b
		2.0	9.92 ab	12.32 ab	0.126 a	100 c	0.280 a	100 b
	citrulline	0.5	7.91 a	12.10 ab	0.135 ab	84.62 a	0.344 ab	100 b
		1.0	9.62 ab	12.12 ab	0.165 ab	100 c	0.492 b	92.31 a
		2.0	8.92 a	11.39 a	0.126 a	92.31 b	0.372 ab	100 b
	glutamine	0.5	11.08 ab	11.99 ab	0.158 ab	92.31 b	0.358 ab	100 b
		1.0	7.00 a	12.52 ab	0.118 a	92.31 b	0.390 ab	100 b
		2.0	8.17 a	10.82 a	0.108 a	92.31 b	0.303 ab	100 b
Gisela 6	control	0.0	6.57 d	20.59 bc	0.159 d	53.85 b	0.094 ab	61.54 c
	asparagine	0.5	3.10 ab	12.68 a	0.055 ab	76.92 e	0.108 ab	69.23 d
		1.0	4.14 bc	14.69 ab	0.068 ab	53.85 b	0.150 bc	61.54 c
		2.0	4.33 bc	20.29 bc	0.113 bcd	69.23 d	0.149 abc	53.85 b
	cysteine	0.5	4.00 bc	18.31 abc	0.106 bcd	61.54 c	0.122 abc	53.85 b
		1.0	5.22 cd	23.89 c	0.097 bc	69.23 d	0.130 abc	53.85 b
		2.0	3.43 bc	18.67 abc	0.061 ab	53.85 b	0.124 abc	38.46 a
	citrulline	0.5	4.00 bc	17.11 abc	0.086 bc	38.46 a	0.093 a	92.31 e
		1.0	4.00 bc	20.90 bc	0.094 bc	61.54 c	0.098 ab	69.23 d
		2.0	4.63 bc	21.77 c	0.142 cd	61.54 c	0.167 c	92.31 e
	glutamine	0.5	3.00 ab	21.62 bc	0.086 bc	53.85 b	0.129 abc	61.54 c
		1.0	3.71 bc	20.82 bc	0.095 bc	53.85 b	0.123 abc	69.23 d
		2.0	1.60 a	12.60 a	0.020 a	38.46 a	0.112 abc	61.54 c
P-values		A	0.010**	0.804 ns	0.065ns	0.000***	0.604ns	0.000***
		B	0.568 ns	0.000***	0.275ns	0.000***	0.000***	0.000***
		C	0.000***	0.000***	0.030*	0.000***	0.000***	0.000***
		A×B	0.276 ns	0.137 ns	0.068ns	0.000***	0.722ns	0.000***
		A×C	0.057 ns	0.023*	0.378ns	0.000***	0.367ns	0.000***
		B×C	0.000***	0.000***	0.000***	0.000***	0.005**	0.000***
		A×B×C	0.390 ns	0.009**	0.485ns	0.000***	0.629ns	0.000***

Table 2. The effect of four amino acids combined with 2 mg dm^{-3} indole-3-butyric acid on shoot length, shoot FM, leaf chlorophyll, carotenoid and porphyrin content, and *P*-values. For details see Table 1.

Rootstock	Amino acid	[mg dm^{-3}]	Shoot length [mm]	Shoot FM [g]	Chl (*a*+*b*) [mg g^{-1}(FM)]	Chl (*a*+*b*) [mg g^{-1}(DM)]	Carotenoids [mg g^{-1}(FM)]	Porphyrins [mg g^{-1}(FM)]
CAB-6P	control	0	17.31 ab	0.084 abc	3.088 a	20.32 abc	0.416 b	6.786 c
	asparagine	0.5	14.00 a	0.104 abc	2.781 a	17.83 ab	0.212 a	5.872 ab
		1.0	14.23 a	0.087 abc	2.619 a	18.27 abc	0.125 a	5.452 a
		2.0	16.54 ab	0.104 abc	3.144 a	16.65 ab	0.339 ab	6.912 c
	cysteine	0.5	14.62 a	0.074 ab	2.841 a	27.58 c	0.225 ab	5.557 ab
		1.0	16.54 ab	0.093 abc	2.591 a	23.12 bc	0.219 ab	5.433 a
		2.0	19.62 ab	0.120 c	2.951 a	19.01 abc	0.268 ab	6.349 bc
	citrulline	0.5	18.08 ab	0.096 abc	2.906 a	16.18 ab	0.251 ab	6.121 abc
		1.0	12.69 a	0.063 a	3.076 a	20.79 abc	0.376 ab	6.767 c
		2.0	11.92 a	0.071 ab	2.732 a	13.13 a	0.302 ab	5.842 ab
	glutamine	0.5	17.69 ab	0.093 abc	2.870 a	18.98 abc	0.266 ab	6.182 abc
		1.0	22.69 b	0.107 bc	2.771 a	17.44 ab	0.265 ab	6.054 abc
		2.0	16.15 ab	0.088 abc	3.166 a	20.88 abc	0.292 ab	6.854 c
Gisela 6	control	0.0	22.69 bc	0.128 bcd	5.477 d	33.75 c	3.039 c	12.248 d
	asparagine	0.5	23.46 c	0.106 abc	4.954 cd	33.70 c	2.231 ab	10.778 cd
		1.0	18.08 ab	0.103 abc	4.374 bcd	29.90 bc	2.110 ab	9.573 bc
		2.0	17.69 ab	0.130 bcd	3.812 abc	22.09 abc	2.020 ab	8.384 ab
	cysteine	0.5	19.62 abc	0.154 d	3.042 a	14.56 a	1.891 a	7.005 ab
		1.0	16.15 a	0.123 abcd	3.435 ab	19.75 ab	1.662 a	7.572 ab
		2.0	20.38 abc	0.105 abc	3.526 ab	15.02 a	1.801 a	7.814 ab
	citrulline	0.5	20.77 abc	0.087 ab	3.339 ab	12.95 a	1.654 a	7.368 ab
		1.0	17.69 ab	0.086 ab	4.000 abc	20.43 ab	1.831 a	8.501 abc
		2.0	18.46 abc	0.077 a	3.246 ab	16.42 a	1.410 a	7.102 ab
	glutamine	0.5	18.46 abc	0.099 abc	3.446 ab	16.42 a	1.578 a	7.621 ab
		1.0	20.77 abc	0.136 cd	2.828 a	10.42 a	1.401 a	6.244 a
		2.0	17.31 a	0.107 abc	3.075 a	12.19 a	1.421 a	6.728 a
P-values		A	0.418ns	0.003*	0.000***	0.000***	0.057ns	0.000***
		B	0.019*	0.832ns	0.000***	0.000***	0.000***	0.000***
		C	0.001***	0.001***	0.000***	0.003**	0.000***	0.000***
		A×B	0.056ns	0.197ns	0.022ns	0.068ns	0.722ns	0.003**
		A×C	0.213ns	0.629ns	0.000***	0.000***	0.011*	0.000***
		B×C	0.225ns	0.094ns	0.000***	0.000***	0.000***	0.000***
		A×B×C	0.639ns	0.144ns	0.034*	0.031*	0.814ns	0.011*

In CAB-6P, all four amino acids tested did not influence leaf chlorophyll content significantly (Table 2). Leaf carotenoid content was substantially decreased only when 0.5 or 1 mg dm^{-3} asparagine was applied. Porphyrin content diminished at 0.5 or 1 mg dm^{-3} asparagine, 0.5 or 1 mg dm^{-3} cysteine, and 2 mg dm^{-3} citrulline. In Gisela 6, when chlorophyll content was expressed as mg g^{-1}(FM), except asparagine (0.5 or 1 mg dm^{-3}), the other three amino acids tested (cysteine, citrulline, and glutamine) as well as asparagine at the highest applied concentration of 2 mg dm^{-3} resulted in diminished leaf chlorophyll content. When chlorophyll content was expressed as mg g^{-1}(DM), cysteine, citrulline, and glutamine led to decreased leaf chlorophyll content whereas asparagine gave similar results to the control. Content of carotenoids and porphyrins was decreased by the addition of all the four amino acids tested, except asparagine at 0.5 mg dm^{-3} for porphyrin content.

In CAB-6P, all the treatments increased leaf sucrose content except 2 mg dm^{-3} asparagine or 0.5 mg dm^{-3} glutamine which resulted in reduced leaf sugar content (Table 3). Particularly, the leaf content of sucrose was maximum with 0.5 mg dm^{-3} asparagine or 1 mg dm^{-3} cysteine. Among the treatments, only asparagine at 0.5 mg dm^{-3} raised (a 3-fold increase) leaf proline content. In roots, the content of both sucrose and proline was augmented only when 1 mg dm^{-3} cysteine was applied to the culture medium. On the other hand, 2 mg dm^{-3} asparagine, 0.5 mg dm^{-3} cysteine, and 0.5 or 1 mg dm^{-3} glutamine and citrulline in all tested concentrations decreased root sucrose content. Citrulline and glutamine regardless of concentration and asparagine and cysteine both applied either at 0.5 or 2 mg dm^{-3} resulted in decreased proline content. In Gisela 6, all four amino acids did not alter significantly the content of sucrose in roots. Only 0.5 mg dm^{-3} glutamine or 1 mg dm^{-3} cysteine raised root proline content remarkably.

Discussion

From the results of these experiments, it is proved that the amino acid type and amino acid concentration affected rooting, callus formation, and vegetative growth of shoot tip explants differently in two cherry rootstocks CAB-6P and Gisela 6. Among the four amino acids tested, 0.5 mg dm^{-3} cysteine proved to be the best treatment in terms of root number and root FM in the CAB-6P rootstock. In the Gisela 6 explants, on the contrary, all four amino acids except cysteine at the 1 mg dm^{-3} concentration negatively influenced root number. Our findings in Gisela 6 agree with those of Orlikowska (1992), who found that in the dwarf apple rootstock P60, asparagine, cysteine, and glutamine supplied at a concentration of 200 mg dm^{-3} (100× higher concentration) significantly reduce the number of roots. Addition of amino acids into substrate may either induce or inhibit rooting. This depends on the amino acid, explant type, and genotype (Faye *et al.* 1989). The role of amino acids in plant metabolism and their connection with rooting is not very clear. However, accumulation of amino acids at the base of microcuttings has been often referred (Haissig 1986). Interestingly, Welander (1978) attributed the inhibition of rooting in pelargonium explants to an increased concentration of arginine, aspartic acid, alanine, asparagine, glutamine, and glutamate acid. In general, an organic N source has a better effect on rooting, somatic embryogenesis, and shoot formation compared to inorganic N sources due to a higher mobility and a lower energy consumption needed for protein biosynthesis (Kim and Moon 2007). Kavi Kishor (1989) culturing tobacco callus suggested that an increased metabolism of aromatic amino acids is related to the induction of root and shoot formation. According to Kamada and Harada (1979), glutamine induces rooting at the presence of α-naphthalene acetic acid (NAA) in a *Torenia fournieri* shoot culture. Similarly, 4 - 32 mg dm^{-3}

Table 3. The effect of four amino acids combined with 2 mg dm^{-3} indole-3-butyric acid on sucrose and proline content in leaves and roots of CAB-6P and Gisela 6 explants and *P*-values. For details see Table 1.

Rootstock	Amino acid	[mg dm^{-3}]	Sucrose -leaf [μmol g^{-1}(FM)]	Proline - leaf [μmol g^{-1}(FM)]	Sucrose - root [μmol g^{-1}(FM)]	Proline - root [μmol g^{-1}(FM)]
CAB-6P	control	0	40.57 b	3.006 abcd	39.30 d	1.504 e
	asparagine	0.5	63.67 f	10.076 e	41.01 d	1.271 d
		1.0	50.37 e	3.641 bcd	34.31 cd	1.453 e
		2.0	35.56 a	2.839 abcd	26.04 ab	1.125 c
	cysteine	0.5	50.11 e	3.750 bcd	18.66 a	0.797 a
		1.0	60.81 f	3.896 d	51.51 e	1.690 f
		2.0	49.28 de	3.550 bcd	40.70 d	1.107 c
	citrulline	0.5	48.18 de	3.386 bcd	24.80 ab	0.943 b
		1.0	47.20 de	3.367 bcd	21.42 a	1.271 d
		2.0	45.64 cd	2.583 abc	23.24 a	0.888 ab
	glutamine	0.5	34.78 a	1.745 a	18.98 a	0.797 a
		1.0	42.00 bc	2.365 ab	31.50 bc	0.815 ab
		2.0	46.68 de	2.820 abc	35.50 cd	0.906 ab
Gisela 6	control	0.0	66.38 bcd	4.366 b	54.55 abc	1.121 ab
	asparagine	0.5	58.45 abc	3.826 ab	33.88 ab	0.910 ab
		1.0	54.43 ab	4.433 b	62.88 bc	1.022 ab
		2.0	73.82 d	4.094 b	56.27 abc	1.512 bc
	cysteine	0.5	74.05 d	4.032 ab	49.32 abc	1.524 bc
		1.0	72.30 cd	3.523 ab	48.08 abc	1.799 cd
		2.0	45.21 a	2.587 a	32.27 a	0.653 a
	citrulline	0.5	57.46 ab	3.888 ab	50.95 abc	1.148 ab
		1.0	75.96 d	4.274 b	46.77 abc	1.443 bc
		2.0	68.29 bcd	3.168 ab	39.89 abc	0.941 ab
	glutamine	0.5	69.06 bcd	3.888 ab	64.49 c	2.233 d
		1.0	72.93 cd	4.083 b	46.82 abc	1.561 bc
		2.0	80.35 d	3.888 ab	57.82 abc	1.338 bc
P-values		A	0.690ns	0.000***	0.217ns	0.255ns
		B	0.000***	0.000***	0.000***	0.000***
		C	0.000***	0.000***	0.000***	0.000***
		A×B	0.000***	0.003**	0.410ns	0.000***
		A×C	0.000***	0.000***	0.087ns	0.000***
		B×C	0.024*	0.000***	0.855ns	0.022*
		A×B×C	0.000***	0.000***	0.013*	0.011*

glutamine increases root number, root length, and rooting percentage in *Jatropha curcas* microshoots (Liu *et al.* 2015).

In the CAB-6P rootstock, asparagine and glutamine regardless of concentration as well as cysteine and citrulline at the lowest applied concentration of 0.5 mg dm^{-3} reduced the explant ability to form roots. These results agree with those reported by Shibaoka *et al.* (1967) suggesting that 3.6 mg dm^{-3} cysteine almost completely inhibits root formation in *Phaseolus mungo*. Opposite results were reported by Arce and Balboa (1991) who found that 10 mg dm^{-3} cysteine enhances the positive effect of NAA on rooting *Prosopis chilensis*. Our results in CAB-6P regarding the rooting percentage are in line with those of Orlikowska (1992), who found that 200 mg dm^{-3} asparagine, cysteine, or glutamine significantly reduces the rooting percentage in the dwarf apple rootstock P60. The effect of asparagine on rooting apple P2 explants is also inhibitory (Orlikowska 1992). However, the application of glutamine leads to a significant increase in the rooting percentage of *Linum usitatissimum* (Belonogova and Raldugina 2006), pineapple (Hamasaki *et al.* 2005), and *Artemisia vulgaris* (Kumar and Kumari 2010). In Gisela 6, except glutamine that led to decreased rooting percentages, the other three amino acids positively influenced the rooting percentage. In *Vitis labrusca* and *V. vinifera*, asparagine, cysteine, and glutamine when applied at a concentration of 40 mg dm^{-3} have a promoting effect on rooting percentage (Carvalho *et al.* 2013).

In CAB-6P, all four amino acids tested had an inhibitory effect on root length. On the contrary, in fox grape and grapevine, cysteine and glutamine enhance root length (Carvalho *et al.* 2013). In Gisela 6, only asparagine at the lowest (0.5 mg dm^{-3}) and glutamine at the highest (2 mg dm^{-3}) applied concentrations negatively affected root elongation. According to Carvalho *et al.* (2013), asparagine (40 mg dm^{-3}) does not influence substantially root length of fox grape and grapevine microcuttings. The inhibitory action of glutamine on root elongation has also been reported by Pedrotti *et al.* (1994) in *Prunus avium*. Our findings are not in line with those of Kakkar and Rai (1988), who suggested that glutamine and asparagine have a positive effect on root length in *Phaseolus vulgaris*. The negative effect of the amino acids on root length in our study may be attributed to their possible decomposition due to the high temperature during autoclaving and their transformation into inhibitory compounds such as 5-oxoproline (Polaneur *et al.* 1992). Similar results have been reported for lettuce where several products of proline were found (Tsuji *et al.* 1992) when glutamine was added after autoclaving, demonstrating its direct role in root length inhibition, bearing in mind that glutamine is precursor of IAA synthesis (Cohen and Bialek 1984).

Roots frequently emerge through the callus leading to the belief that callus formation is essential for rooting. However, in easy-to-root species, the formation of callus and the formation of roots are independent of each other, even though both involve cell division. Their simultaneous occurrence is due to their dependence upon similar internal and environmental conditions. In some species, callus formation is precursor of adventitious root formation whereas in other species, excess callusing may hinder rooting. The origin of adventitious roots from callus tissue has been found in difficult-to-root species (Macháčková *et al.* 2008). The optimum concentration of amino acid to achieve maximum callus formation depends on the plant species and explant type. Amino acids as sources of organic N are assimilated faster than inorganic N, inducing a faster cell growth (Grimes and Hodges 1990). In detail, glutamine is the main intrinsic amino acid involved in plant metabolism providing necessary N for the composition of proteins, nucleic acids, and other compounds (Coruzzi and Last 2000). Among the applied amino acids, only citrulline at concentrations of 1 mg dm^{-3} in CAB-6P and 2 mg dm^{-3} in Gisela 6 had a stimulatory effect on callus FM. This finding is in agreement with the results of Abou El-Nil (1989) who referred that citrulline increases callus FM in date palm. In general, the beneficial effect of amino acids on callusing may be due to an increase in number, volume, and FM of explant cells. This suggestion was proved for glutamine, asparagine, and cysteine effects on callus cells of a *Catharanthus roseus* leaf explant culture *in vitro* (Taha *et al.* 2009). However, in cucumber, asparagine and glutamine result in a significant decline in callus FM (Locy and Wehner 1982). The inhibition of the growth rate of a callus due to amino acid inclusion may be attributed to cessation of nitrate reductase activity. Similar explanations were given by Filner (1966) and Fukunaga and King (1982) for similar experiments with tobacco and *Datura innoxia*, respectively. In CAB-6P, cysteine at 0.5 mg dm^{-3} as well as asparagine or citrulline at 1 mg dm^{-3} decreased the frequency of callus formation. In contrast, 500 mg dm^{-3} glutamine in olive explants (Pritsa and Voyatzis 2004) and cysteine (0.5 or 1 mg dm^{-3}) in *Artemisia vulgaris* (Kumar and Kumari 2010) improve the induction of callus formation. In Gisela 6, cysteine in all concentrations applied resulted in reduced callogenesis percentages. Our findings are in disagreement with those reported by El-Shiaty *et al.* (2004), where 10 or 50 mg dm^{-3} cysteine, 10 - 100 mg dm^{-3} glutamine and 1 - 100 mg dm^{-3} asparagine increase the callusing percentage of *Phoenix dactylifera* shoot tips. In Gisela 6, 0.5 mg dm^{-3} asparagine, 1 mg dm^{-3} glutamine, or citrulline in all concentrations tested had a stimulatory effect on callus formation percentage, and this response was amino acid type- and amino acid concentration-dependent. Similarly, glutamine improves callus formation in *Acacia auriculiformis* (Ranga Rao and Prasad 1991), *Juniperus excelsa* (Shanhani 2003), and upland rice cv. Lamsam (Shahsavari 2011) explants. Shohael *et al.* (2003) reported that 100 - 200 mg dm^{-3} asparagine substantially augments the callusing percentage in maize.

In the current research, rooting both the CAB-6P and Gisela 6 microshoots was not optimized due to the presence of a callus at their base. In both the cherry rootstocks of our study, although callus formation is not suitable for the rooting process, these results can constitute a valuable source of information in establishing protocols for adventitious shoot regeneration in these genotypes.

Comparing the two studied rootstocks, CAB-6P presented a greater ability of rooting and callusing than Gisela 6. The differences between the two cherry rootstocks may be attributed to the genotype, rooting ability, content of endogenous IAA (James 1983b), or sensitivity of target-cells to auxin (James 1983a).

In CAB-6P, amino acids had no effect on shoot length and shoot FM. Similarly, Carvalho *et al.* (2013) found that in fox grape and grapevine, 40 mg dm^{-3} cysteine does not affect shoot length considerably. Moreover, in *Alstromeria*, the combined supplementation with glutamine and asparagine, both at 30 mg dm^{-3}, does not affect substantially the height of regenerated shoots (Shahriari *et al.* 2012). Our findings in CAB-6P are also in agreement with those found in *Citrus reticulata* cultured *in vitro* where glutamine (25 - 100 mg dm^{-3}) does not have a significant effect on shoot length (Siwach *et al.* 2012). In Gisela 6, on the other hand, shoot length was negatively affected by adding 1 mg dm^{-3} cysteine or 2 mg dm^{-3} glutamine to the culture medium, and shoot FM when the shoot tip explants were treated with 2 mg dm^{-3} citrulline. Our findings in Gisela 6 are in agreement with the results obtained by Haroun *et al.* (2010) who reported than in *Phaseolus vulgaris* cultured *in vitro*, a significant decrease in shoot length was shown by 3 - 5 mM asparagine or glutamine. Furthermore, our results in Gisela 6 are in line with those reported by Zhang *et al.* (1999) who assured that the growth of *Arabidopsis* is inhibited by using concentrations of glutamine higher than 1 mM. According to Groot *et al.* (2003), a decrease in different growth parameters in response to asparagine or glutamine treatments may be mediated by a change in the content of naturally synthesized hormones.

In CAB-6P, no significant changes on leaf chlorophyll content were observed as result of the inclusion of each of the amino acids in the culture medium. In Gisela 6, on the other hand, except asparagine, the other three amino acids (cysteine, citrulline, and glutamine) resulted in decreased chlorophyll content. Our findings are not in line with the results obtained in onion (Lokhande and Gaikwad 2014) and in the apple rootstock EM 26 (Sotiropoulos *et al.* 2005) where cysteine increases total chlorophyll content. Furthermore, 2 or 4 mM glutamine raises total chlorophyll content in *Dianthus caryophyllus* (Kazemi *et al.* 2012). In Gisela 6, all four amino acids decreased the content of carotenoids whereas in CAB-6P, only asparagine (0.5 or 1 mg dm^{-3}) led to diminished leaf carotenoid content. Similarly, in onion, cysteine (5 or 20 mg dm^{-3}) slightly decreases carotenoid content (Lokhande and Gaikwad 2014). According to Haroun *et al.* (2010), on the contrary, 1 mM asparagine or glutamine increases total chlorophyll and carotenoid content in *Phaseolus vulgaris*. Increase in total chlorophyll and carotenoid content due to glutamine application was also reported in maize (Nunes *et al.* 2014) and in onion (Amin *et al.* 2011). A reason for a decrease in chlorophyll content is the biosynthesis of chlorophyllase which may participate in chlorophyll degradation, destruction of chloroplasts, mitochondria, and plasmalema structure, and production of reactive oxygen species (Dolatabadian and Jouneghani 2009).

Regarding leaf porphyrin content, in CAB-6P, asparagine or cysteine both at 0.5 or 1 mg dm^{-3} concentrations, and citrulline (2 mg dm^{-3}) led to decreased porphyrin content whereas glutamine did not have a significant effect on it. In the Gisela 6 microcuttings, all four amino acids (except 0.5 mg dm^{-3} asparagine) led to decreased porphyrin content. The mechanism proposed for the inhibition of the photosynthetic apparatus and the reduction of leaf porphyrin content in both cherry rootstocks, which was more pronounced in Gisela 6, is the replacement of magnesium in the chlorophyll molecule. Consequently, cells accumulate protoporphyrin and chlorophyll synthesis is blocked (De Filippis *et al.* 1981).

According to Ahkami *et al*. (2008), there is a strong correlation between sucrose content and root number as in petunia petioles, sucrose content increased during the root formation phase. Correa e*t al.* (2012) found that in detached *Arabidopsis thaliana* leaves, soluble sugars appear as one of the most distinct biochemical markers of the rooting process. The higher content of sucrose and proline in leaves than in roots in both the cherry rootstocks in the current study might be due to a decline in their translocation rate from leaves to roots. Another explanation was proposed by Agulló-Antón *et al.* (2008) who found that in carnation microcuttings, sucrose continues to be composed during rooting in the dark but it is translocated from leaves to petioles and accumulates there. In CAB-6P, cysteine and citrulline at all concentrations applied as well as asparagine (0.5 or 1 mg dm^{-3}) and glutamine (2 mg dm^{-3}) had a stimulatory effect on leaf sucrose content. In roots, only cysteine (1 mg dm^{-3}) resulted in elevated sucrose content. According to Haroun *et al.* (2010), 1 mM asparagine or glutamine increases sucrose, glucose, total soluble sugars, and total sugars content in *Phaseolus vulgaris* plants under *in vitro* conditions. In Gisela 6, however, cysteine at the highest applied concentration of 2 mg dm^{-3} led to a substantial decrease in leaf sucrose content. In CAB-6P roots, asparagine, glutamine, and citrulline at specific concentrations diminished sucrose content whereas in Gisela 6 roots, no significant changes in their sucrose content were observed. Similarly to CAB-6P roots, in onion, cysteine (5 or 20 mg dm^{-3}) slightly decreases sucrose content (Lokhande and Gaikwad 2014). The observed decrease in sucrose content in Gisela 6 leaves was possibly due to decreased chlorophyll content. A similar explanation was given by Arulanantham *et al.* (1990).

Increase or decrease of proline is an indication of a critical point for growth of plantlet under stress or non-stress conditions, respectively (Watanabe *et al.* 2001). Thus, the depleted content of proline in CAB-6P roots pointed out osmotic stress induced by the incorporation of asparagine, cysteine, or citrulline in specific, and glutamine in all concentrations tested into the culture medium. Similarly, 2 or 4 mM glutamine diminishproline content in carnation (Kazemi *et al.* 2012). In Gisela 6 leaves, among the four applied amino acids, only cysteine at 2 mg dm^{-3} caused a remarkable reduction in proline content. On the other hand, proline accumulation is not just a sign of cellular injury resulting in response to stress, but it is also a marker of stress tolerance having a definite osmoregulatory role in plants subjected to stressful conditions (Jabeen *et al.* 2008). In CAB-6P leaves, only asparagine at 0.5 mg dm^{-3} substantially raised proline content. Proline may be overproduced only when the degree of stress reaches a critical point for plant growth. The synthesis of osmolytes (proline, betaine, and reducing sugars) is biologically very important for the detection, tolerance, and adaptation of plants to specific stresses (Haq *et al.* 2011). In Gisela 6 roots, only cysteine (1 mg dm^{-3}) and glutamine (0.5 mg dm^{-3}) at specific concentrations led to elevated proline content.

In conclusion, all four amino acids had direct effects on *in vitro* rooting the CAB-6P and Gisela 6 explants. Furthermore, it is clear that L-asparagine, L-cysteine, L-citrulline, and L-glutamine influenced leaf chlorophyll, carotenoid or porphyrin content, sugar biosynthesis and metabolism, as well as proline accumulation in both leaves and roots.

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