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ANTIOXIDANT PROFILES AND SELECTED PARAMETERS OF PRIMARY METABOLISM IN *PHYSALIS IXOCARPA* HAIRY ROOTS TRANSFORMED BY TWO *AGROBACTERIUM RHIZOGENES* STRAINS

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We compared the biochemical profiles of *Physalis ixocarpa* hairy roots transformed with *Agrobacterium rhizogenes* ATCC and A4 strains with non-transformed root cultures. The studied clones of A4- and ATCC-induced hairy roots differed significantly; the latter showed greater growth potential and greater ability to produce secondary metabolites (tropane alkaloids) and to biotransform hydroquinone to arbutin. We compared glucose content, alanine and aspartate aminotransferase activity, and L-phenylalanine ammonia-lyase activity. We analyzed markers of prooxidant/antioxidant homeostasis: catalase, ascorbate peroxidase, oxidase, glutathione peroxidase and transferase activity, and the levels of ascorbate, glutathione, tocopherol and lipid peroxidation. We found that transformation induced strain-specific regulation, including regulation based on redox signals, determining the rate of allocation of carbon and nitrogen resources to secondary metabolism pathways. Our results provide evidence that *A. rhizogenes* strain-specific modification of primary metabolites contributed to regulation of secondary metabolism and could determine the ability of *P. ixocarpa* hairy root clones to produce tropane alkaloids and to convert exogenously applied hydroquinone to pharmaceutically valuable arbutin. Of the studied parameters, glucose content, L-phenylalanine ammonia-lyase activity and alanine aminotransferases activity may be indicators of the secondary metabolite-producing potential of different *P. ixocarpa* hairy root clones.

Key words: *Agrobacterium rhizogenes*, arbutin, hairy roots, *Physalis*, primary and secondary metabolism, tropane alkaloids.

INTRODUCTION

Hairy root cultures obtained by infecting plant explants with the soil phytopathogenic bacterium *Agrobacterium rhizogenes* offer an attractive source of high-value secondary metabolites, including pharmaceuticals and other biologically active substances of commercial importance (Ramachandra Rao and Ravishankar, 2002; Guillon et al., 2006). They grow rapidly on hormone-free medium, show genetic stability, and can produce these metabolites over a long period of time. The major drawback of an *Agrobacterium*-mediated transformation system is considerable variation in the stability, integration and expression of the introduced transgenes. Each hairy root clone obtained by *A. rhizogenes*-mediated transformation is genetically different due to random integration of different T-DNA copies into the plant genome (Tzfira and Citovsky, 2006; Veena and Taylor, 2007). They also differ with respect to phenotype, growth rate, metabolite content and productivity (Woo et al., 2004).

Infection with *A. rhizogenes* modulates the host cell metabolic state by, for example, altering the expression of plant defense genes and secondary metabolite synthesis (Stojakowski and Malarz, 2000; Strycharz and Shetty, 2002). Many plant defense pathways interfere with reactive oxygen species (ROS) and the cellular prooxidant/antioxidant equilibrium (Foyer and Noctor, 2009; Kuźniak

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et al., 2009). The involvement of ROS, especially H_2O_2, and redox regulation in mediating secondary metabolite biosynthesis in plant culture in vitro is well documented, although the mechanism is still not clear (Sachan et al., 2010). A similar function has been attributed to low-molecular carbohydrates, especially glucose and sucrose (Gibson, 2005), playing a central role in plant primary metabolism.

The chemical defense related to secondary metabolite biosynthesis largely depends on extensive reprogramming of primary metabolism, especially that related to redistribution of carbon and nitrogen resources, which generates precursors fed into secondary metabolism pathways (Simon et al., 2010).

To gain further insight into *Agrobacterium*-induced reprogramming of plant primary metabolism possibly affecting the production of secondary metabolites we used two *Physalis ixocarpa* hairy root cultures transformed by ATCC and A4 *A. rhizogenes* strains, which differed in their ability to produce tropane alkaloids and in the ability to biotransform hydroquinone (HQ) into its monoglucoside, arbutin, which is not synthesized as a natural secondary product in the genus *Physalis* (Bergier et al., 2008).

Physalis ixocarpa (Solanaceae) is an edible and medicinal plant reported to show great potential for producing biologically active secondary metabolites, among them tropane alkaloids (Su et al., 2002; Pérez-Castorena et al., 2004). The tropane alkaloids hyoscamine, atropine and scopolamine, occurring predominantly in Solanaceae plants, act as a chemical defense in these plants (Alves et al., 2007). They are important medicinal agents in demand commercially (Grynkiewicz and Gadzikowska, 2008).

We compared non-transformed root cultures and hairy roots transformed with *A. rhizogenes* ATCC and A4 strains with respect to glucose (Glc) content as marker of the primary carbon supply, and the activity of alanine aminotransferase (AlaAT, EC 2.6.1.2) and aspartate aminotransferase (AspAT, EC 2.6.1.1), involved in both carbon and nitrogen metabolism. We also examined the activity of L-phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), a key enzyme in phenylpropanoid metabolism, linking primary and secondary metabolism in plant cells. We analyzed selected markers of prooxidant/antioxidant homeostasis: the activity of catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.1), ascorbate oxidase (AO, EC 1.10.3.3), glutathione peroxidase (GSH-Px, EC 1.11.1.9) and glutathione transferase (GST, EC 2.5.1.18), as well as the levels of ascorbate, glutathione, tocopherol (TOC) and lipid peroxidation.

MATERIAL AND METHODS

PHYSALIS IXOCARPA HAIRY ROOT CULTURES

Hairy root cultures of *P. ixocarpa* were obtained after transformation with two agropine wild-type strains of *A. rhizogenes*, ATCC 15834 (pRi 15834) and A4 (pRi A4). Stems of aseptically grown plants were used for induction of hairy roots. After 3 weeks, newly formed roots were excised and the isolated hairy root clones were transferred to B5 liquid medium containing 500 mg l^{-1} ampicillin and 30 g l^{-1} sucrose, without growth regulators and subcultured at 7-day intervals. The concentration of ampicillin was halved each week from 500 to 62.5 mg l^{-1}, and finally cultures free of *A. rhizogenes* were transferred to B5 medium lacking ampicillin. Cultures were maintained by transferring 0.1 g root tissue at 4-week intervals to 100 cm^3 Erlenmeyer flasks containing 25 cm^3 medium and cultivated in the dark at 120 rpm on a rotary shaker at 23°C. The transgenic character of the hairy root cultures was determined by polymerase chain reactions (PCR) as described previously (Bergier et al., 2008, Fig. 1). The clones clearly differed in biomass production (data not shown). The differences in growth parameters were stable in each subculture. One hairy root clone exhibiting the highest growth potential was selected for further studies from the ATCC-induced and from the A4-induced hairy root cultures.

ROOT CULTURES FROM NON-TRANSFORMED PLANTS

The non-transformed controls (NT) were roots excised from 4-week-old *P. ixocarpa* seedlings grown in vitro were cultured on B5 medium supplemented with 30 g l^{-1} sucrose and 0.5 mg l^{-1} indole-3-acetic acid, subcultured every 4 weeks. Root cultures from non-transformed plants were grown in the dark at 120 rpm on a rotary shaker at 23°C.

DETERMINATION OF TROPANE ALKALOIDS

Tropane alkaloids were extracted from three-week-old cultures as described by Kamada et al. (1986) and their content was determined by HPLC using a Dionex system (Sunnyvale, U.S.A) equipped with a photodiode-array detector. Separation of tropane alkaloids was achieved on a reverse-phase column (aQ Hypersil GOLD, 150 mm × 4.6 mm, 5 μm, Polygen, Gliwice, Poland) coupled with a guard column (GOLD aQ Drop-In guards, 10 mm × 4 mm, 5 μm, Polygen, Gliwice, Poland) at 25°C. The mobile phase was made up from 15% acetonitrile and 85% H_2O containing 50 mM K_3PO_4 (pH 3.5). The flow rate was 0.8 cm^3 min^{-1}, and absorbance was meas-

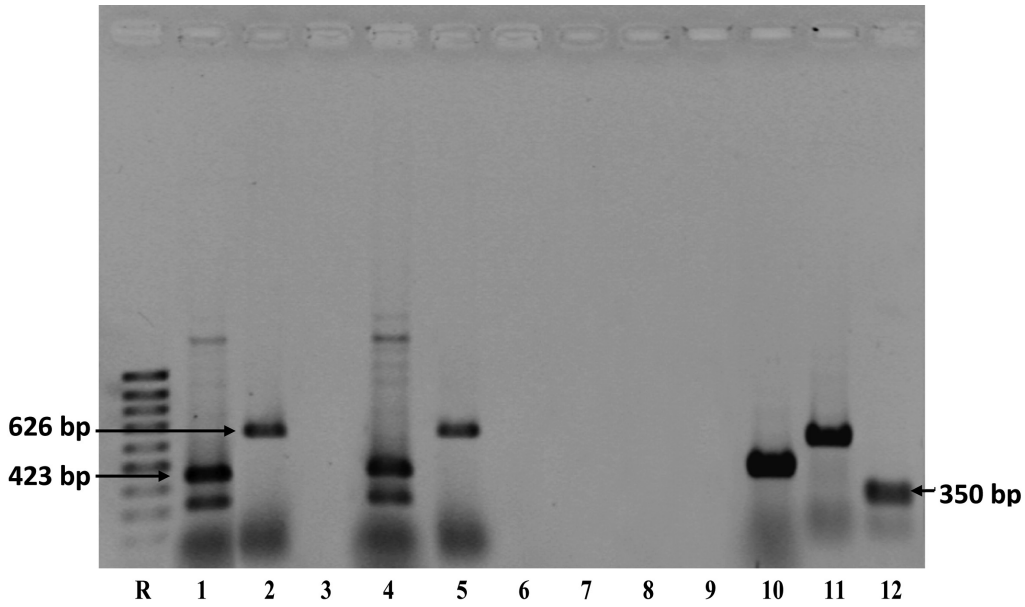

Fig. 1. PCR analysis of *Physalis ixocarpa* roots transformed by *Agrobacterium rhizogenes* ATCC 15834 (lanes 1, 2, 3) and A4 (lanes 4, 5, 6), non-transformed roots (lanes 7, 8, 9) and *Agrobacterium rhizogenes* ATCC 15834 as positive control (lanes 10, 11, 12). GeneRulerTM 100bp DNA ladder (lane R). Arrows show amplified fragments of rolB (423 bp), rolC (626 bp) and virG (350 bp) genes.

ured at 204 nm. Chromatogram peaks were identified by comparing the retention time of authentic standards (atropine, (-)-hyoscyamine-N-oxide hydrochloride, scopolamine) and by on-line UV absorption spectra of samples; quantification was based on the calibration curves for standards.

HYDROQUINONE TREATMENT AND DETERMINATION OF ARBUTIN

Hydroquinone (HQ, 2 mM) dissolved in the medium was added three times at 2-day intervals to a final concentration of 6 mM. It was applied to cultures aseptically through a Millipore filter (0.22 μm) starting from the 16th day of growth. Arbutin was extracted from 3-week-old cultures according to Skrzypczak-Pietraszek et al. (2005). Quantitative determination of arbutin was as described by Bergier et al. (2008).

BIOCHEMICAL ANALYSES

All the examined parameters were determined in one-week-old NT root and hairy root cultures. TOC, TBARS and Glc content was measured and PAL, AlaAT and AspAT activity was assayed in 2- and 3-week-old cultures also.

The samples (0.5 g FW) were immediately homogenized in a mortar in 5 cm^3 ice-cold 0.05 M sodium phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 mM sodium ascorbate and 1% polyvinylpyrrolidone. For TOC determination the homogenate was taken and stored at -20°C. Immediately after centrifugation (20,000 × μg, 20 min, 4°C) the supernatant was used for determination of enzyme activity and glutathione content. For lipid peroxidation determination the supernatant was stored at -20°C. Separate homogenization was performed to determine PAL activity and ascorbate content.

DETERMINATION OF GLUCOSE CONTENT

Glucose content was determined by the colorimetric method with a glucose/sucrose assay kit (Boehringer Mannheim/R-Biopharm, Germany) according to the manufacturer's protocol and is reported as μg mg^{-1} protein.

DETERMINATION OF ASCORBATE AND GLUTATHIONE CONTENT

For determination of ascorbate content, hairy and NT roots (0.5 g FW) were homogenized in 5 cm^3 ice-cold 5% trichloroacetic acid. Ascorbate was determined spectrophotometrically as described by Kampfenkel et al. (1995). Total ascorbate was estimated after reduction of dehydroascorbate (DHA) to reduced ascorbate (AA) with dithiothreitol. The concentration of ascorbate (μmol g^{-1} FW) was determined using a calibration curve for AA as standard. Glutathione content was determined colorimetrically using 5,5'-dithiobis-2-nitrobenzoic acid as described by Brehe and Burch (1976), and its con-

centration is given in nmol mg^{-1} protein. For the specific assay of oxidized glutathione (GSSG) the reduced glutathione (GSH) was masked by derivatization with 2-vinylpyridine. Redox ratios for ascorbate and glutathione were calculated as AA/[AA+DHA] and GSH/[GSH+GSSG], respectively.

DETERMINATION OF TOCOPHEROL CONTENT

Tocopherol content was assayed according to the method of Taylor et al. (1976). After saponification of the sample with KOH in the presence of AA, TOC was extracted to n-hexane. The fluorescence of the organic layer was measured at 280 nm (excitation) and 310 nm (emission). The TOC concentration is expressed in μg mg^{-1} protein.

DETERMINATION OF LIPID PEROXIDATION

Lipid peroxidation, estimated in terms of the concentration of thiobarbituric acid-reactive substances (TBARS), was analyzed fluorometrically according to Yagi (1976). The supernatant, prepared as given for determination of enzymes, was mixed with 29 mM 2-thiobarbituric acid (TBA) in 8.75 M acetic acid and heated at 95°C for 1 h. After cooling, TBARS were extracted to n-butanol and the fluorescence of the organic layer was measured at 531 nm (excitation) and 553 nm (emission). The concentration of TBARS was estimated by referring to the standard 1,1,3,3-tetraetoxypropane and expressed in nmol mg^{-1} protein.

ENZYME ASSAYS

Total GST activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) by the method of Habig et al. (1974). The reaction mixture contained 100 mM potassium phosphate buffer (pH 6.25), 0.75 mM CDNB, 30 mM GSH and the enzyme extract. The product of CDNB conjugation with GSH absorbs at 340 nm (ε = 9.6 mM^{-1} cm^{-1}). One GST activity unit represents the formation of 1 nmol S-conjugate min^{-1} mg^{-1} protein. GSH-Px activity was determined according to the method of Hopkins and Tudhope (1973) with t-butyl hydroperoxide as substrate. The reaction solution contained 0.05 M potassium phosphate buffer (pH 7.0), 0.002 M EDTA, 0.28 μM NADPH, 0.16 U glutathione reductase, 0.073 μM *t*-butyl hydroperoxide and the enzyme extract. Enzyme activity is expressed in units representing 1 nmol NADPH (ε = 6.22 mM^{-1} cm^{-1}) oxidized per minute per mg protein. APX activity was assayed following oxidation of ascorbate to dehydroascorbate (ε = 13.7 mM^{-1} cm^{-1}) at 265 nm by the method of Nakano and Asada (1981), with modifications. The assay mixture consisted of 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA, 0.25 mM sodium ascorbate, 25 μM H_2O_2 and the enzyme extract. Addition of H_2O_2 started the reaction. Enzyme activity is expressed in units representing 1 μmol ascorbate oxidized per minute per mg protein. CAT activity was measured according to Dhindsa et al. (1981). The assay mixture contained 50 mM sodium phosphate buffer (pH 7.0), 15 mM H_2O_2 and the enzyme extract. Decomposition of H_2O_2 (ε = 36 M^{-1} cm^{-1}) was measured at 240 nm. Enzyme activity is expressed in units representing 1 mmol H_2O_2 decomposed per minute per mg protein. AO activity was determined from the decrease in A_{265} at 25°C in a reaction mixture containing 50 mM sodium phosphate (pH 7.0) 1 mM EDTA and 100 μM AA. One unit of AO activity is defined as the oxidation of 1 μmol AA min^{-1} mg^{-1} protein. An extinction coefficient for AA of 14 mM^{-1} cm^{-1} at 265 nm was used in calculations (Nakano and Asada, 1981). AlaAT activity was assayed in the alanine → pyruvate direction by coupling the reaction with NADH oxidation by lactate dehydrogenase. AspAT activity was assayed in the aspartate → oxaloacetate direction by coupling the reaction with NADH oxidation by malate dehydrogenase. The activity of AspAT and AlaAT was measured according to Gajewska and Skłodowska (2009), and is reported as μmol NADPH min^{-1} mg^{-1} protein. PAL activity was assayed according to Zucker (1965). The samples were homogenized in a mortar in ice-cold 0.5 M Tris-HCl buffer (pH 8.8) containing 0.8 mM β-mercaptoethanol and polyvinylpolypyrrolidone (25 mg cm^{-3}). After centrifugation (20,000 × μg, 20 min, 4°C) the supernatant was used for measurement of PAL activity. The reaction mixture consisted of 0.21 M Tris-HCl buffer (pH 8.8), the enzyme extract and 10 mM L-phenylalanine (added after 10 min preincubation at 37°C). After 1 h of incubation at 37°C the reaction was stopped by the addition of HCl (32 mM) and absorbance was read at 290 nm. Enzyme activity is expressed in units representing the amount of enzyme catalyzing the formation of 1 μmol trans-cinnamic acid (ε = 9000 M^{-1} cm^{-1}) per minute per mg protein.

TABLE 1. Growth parameters of *Physalis ixocarpa* hairy root clones and non-transformed (NT) roots. Over a four-week growth cycle the cultures were sampled every three days and the growth index (final FW – initial FW)/initial FW) and biomass doubling time were estimated. The experiments were repeated three times. Values are means of three independent experiments ± SD

Culture	Inoculum (g)	Growth index (fold)	Biomass doubling time (days)
NT	0.1	1.9	10.18
A4	0.1	19.0	5.21
ATCC	0.1	30.1	3.52

TABLE 2. Production of tropane alkaloids and arbutin in hairy root clones of *Physalis ixocarpa*. The metabolite concentrations were determined in 3-week-old cultures. Values are means of three independent experiments ± SD

Culture	Tropane alkaloids (μg g^{-1}FW)				Arbutin (mg g^{-1}FW)
	Hyoscyamine-N-oxide	Scopolamine	Atropine	Total	
NT	35.069±4.208	1.719±0.275	3.972±0.436	40.760±5.830	-
A4	9.870±1.835	29.230±5.261	1.690±0.241	40.790±7.309	0.685±0.201
ATCC	13.672±0.697	46.390±4.244	3.780±0.423	63.840±6.294	2.457±0.622

TABLE 3. Enzymatic activity of *Physalis ixocarpa* cultures. Activity of catalase (CAT), ascorbate peroxidase (APX), ascorbate oxidase (AO), glutathione peroxidase (GSH-Px) and glutathione transferase (GST) were determined in non-transformed roots (NT) and in A4 and ATCC hairy root clones one week after subculture. Values are means of three independent experiments ± SD. Statistically significant differences ($P<0.05$) between the A4 and ATCC clones and non-transformed roots (NT) are asterisked

Culture	CAT	APX	AO	GSH-Px	GST
NT	0.045±0.011	0.041±0.007	0.039±0.003	62.71±2.04	110.56±29.16
A4	0.041±0.009	0.045±0.011	0.051±0.007*	49.55±1.77*	98.79±10.35
ATCC	0.038±0.006	0.032±0.009	0.038±0.005	53.87±2.47*	117.96±11.57

Data in mmol mg^{-1} protein

PROTEIN CONTENT DETERMINATION

Protein content was determined according to Bradford (1976) with a standard curve prepared using bovine serum albumin.

STATISTICAL ANALYSIS

The results presented are the means of three (n=6) independent experiments. Sample variability is given as the standard deviation of the mean. The significance of differences between mean values was determined by the non-parametric Mann-Whitney Rank Sum Test. Statistically significant differences between the non-transformed roots and the A4 and ATCC clones are shown. Differences were considered significant at $P<0.05$.

RESULTS

CHARACTERISTICS OF HAIRY ROOT CULTURES

Agrobacterium rhizogenes ATCC and A4 strains showed similar root-forming efficiency, as 100% of the inoculated stem explants developed roots. Hairy root clones showing the most favorable growth characteristics, one induced by ATCC and one induced by A4, were used for these comparative analyses. They showed similar morphology – highly branched and plagiotropic – and these root phenotypes remained stable after several subcultures (data not shown). In both cases the biomass increase was greatest from days 15 to 27 of culture (data not shown), but the clones differed significantly in growth capacity. *Agrobacterium* ATCC strain-induced hairy roots grew more vigorously, as shown by a higher growth index [growth index = (final FW – initial FW)/initial FW] and shorter biomass-doubling time (Tab. 1). The non-transformed roots did not branch and grew very slowly, with a growth index of 1.8 (Tab. 1).

Agrobacterium ATCC strain-induced hairy roots showed higher tropane alkaloid yield and hydroquinone-to-arbutin biotransformation capacity than the A4 strain-induced cultures. In the ATCC clone the total tropane alkaloid content was ~1.6 times higher than in the cultures transformed with the A4 strain (Tab. 2). *P. ixocarpa* hairy root cultures were able to convert HQ to arbutin, but much less efficiently. Calculated on the basis of arbutin content (mg g^{-1} FW), the clone transformed with the ATCC strain was ~3.5 times more efficient. The NT cultures were unable to produce arbutin (Tab. 2).

ANTIOXIDANT PROFILE OF ONE-WEEK-OLD CULTURES

The activity of the main H_2O_2-scavenging antioxidant enzymes (CAT, APX, GST) did not differ significantly between the two transformed hairy root clones and the NT root cultures. GSH-Px activity significantly declined in both hairy root cultures, and AO activity increased in the A4 clone (Tab. 3). The content of the main non-enzymatic antioxidants (AA, DHA) increased only in the ATCC clone only, by 72% and 120% respectively (Tab. 3). In consequence, the

TABLE 4. Ascorbate and glutathione pools of *Physalis ixocarpa* cultures. Reduced (AA) and oxidized (DHA) ascorbate content, the AA/total ascorbate redox ratio, reduced (GSH) and oxidized (GSSG) glutathione content and the GSH/total glutathione redox ratio were determined in non-transformed roots (NT) and in hairy root clones A4 and ATCC one week after subculture. Values are means of three independent experiments ± SD. Statistically significant differences ($P<0.05$) between the A4 and ATCC clones and non-transformed roots (NT) are asterisked

Culture	AA (µmol g^{-1} FW)	DHA (µmol g^{-1} FW)	Redox ratio	GSH (nmol mg^{-1} protein)	GSSG (nmol mg^{-1} protein)	Redox ratio
NT	0.114±0.017	0.115±0.011	0.50	1.196±0.259	0.302±0.010	0.79
A4	0.115±0.009	0.114±0.010	0.50	0.449±0.091*	0.103±0.027*	0.81
ATCC	0.196±0.014*	0.254±0.021*	0.43	0.233±0.034*	0.289±0.013	0.42

ascorbate redox ratio (AA/total ascorbate) in the ATCC clone was significantly lower than in NT roots. *Agrobacterium*-mediated transformation induced substantial changes in the glutathione pool. Hairy roots initiated using the *A. rhizogenes* A4 strain showed ~3 times lower content of GSH and GSSG than the control cultures, and the ATCC-induced strain showed decreased GSH and a glutathione redox ratio (GSH/total glutathione) roughly half of that in control roots (Tab. 4).

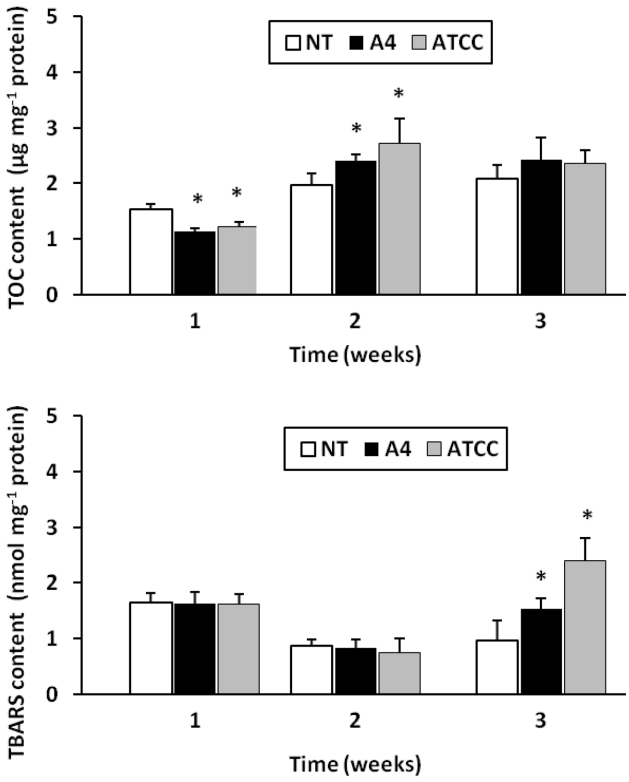

Fig. 2. Tocopherol (TOC) and thiobarbituric acid-reactive substance (TBARS) content in hairy root clones of *Physalis ixocarpa*. Values are means of three independent experiments ± SD. Statistically significant differences ($P<0.05$) between the A4 and ATCC clones and non-transformed roots (NT) are asterisked.

BIOCHEMICAL CHARACTERISTICS OF *PHYSALIS IXOCARPA* HAIRY ROOTS

There were several significant differences between NT root cultures and hairy roots: in sugar content, AlaAt and PAL activity, and TOC and TBARS levels. The timing, profile and intensity of changes induced in the TOC pool were independent of the *Agrobacterium* strain used for transformation. One week after subculture, TOC content was lower in the transformed hairy roots than in the NT roots; in two-week-old cultures of A4 and ATCC it was 22% and 38% higher than the control, respectively (Fig. 2). Transformation had a negligible effect on TBARS level two weeks after subculture (Fig. 2) but in three-week-old A4 and ATCC clones their content was 59% and 149% higher than in the non-transformed roots, respectively (Fig. 2). As to PAL activity, a marked decrease was observed in the A4 clone throughout the experiment and in the ATCC clone 2 and 3 weeks after subculture (Fig. 3). The lowest PAL activity in the A4 and ATCC clones, approximately 25% and 50% of the control level, was found in three-week-old hairy roots.

The glucose pool was significantly altered in hairy roots (Fig. 4). Glc content declined in both transformed clones at all culture time points; the effect was strongest in the two- and three-week-old cultures. For example, 3 weeks after subculture the Glc concentration in the A4 clone was half that of NT roots, and was 39% of the control level in the ATCC clone (Fig. 4).

Agrobacterium rhizogenes-mediated transformation differentially affected the activity of aminotransferases (Fig. 5). AlaAT activity was significantly lower after 2 and 3 weeks of culture in both transformed clones, but transformation had no effect on AspAT activity (Fig. 5).

DISCUSSION

In these experiments the ATCC hairy root clone accumulated more tropane alkaloids than the NT root cultures and the A4 clone. Its other biochemical parameters differed from those of non-transformed

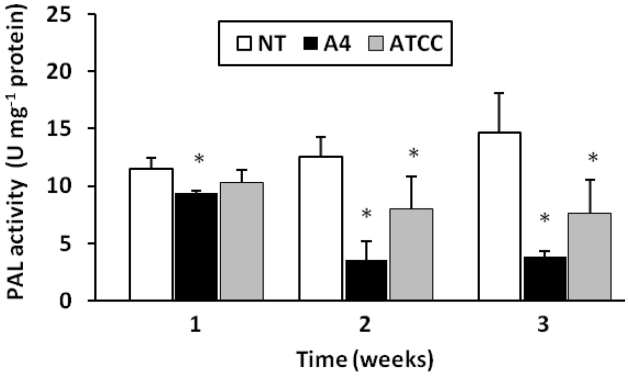

Fig. 3. Activity of L-phenylalanine ammonia-lyase (PAL) in hairy root clones of *Physalis ixocarpa*. Values are means of three independent experiments ± SD. Statistically significant differences ($P<0.05$) between the A4 and ATCC clones and non-transformed roots (NT) are asterisked.

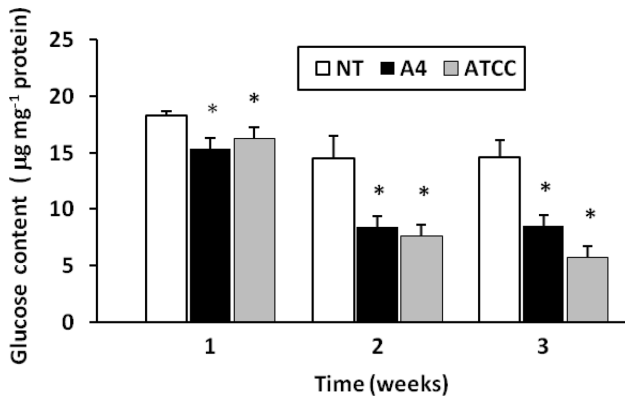

Fig. 4. Glucose content in hairy root clones of *Physalis ixocarpa*. Values are means of three independent experiments ± SD. Statistically significant differences ($P<0.05$) between the A4 and ATCC clones and non-transformed roots (NT) are asterisked.

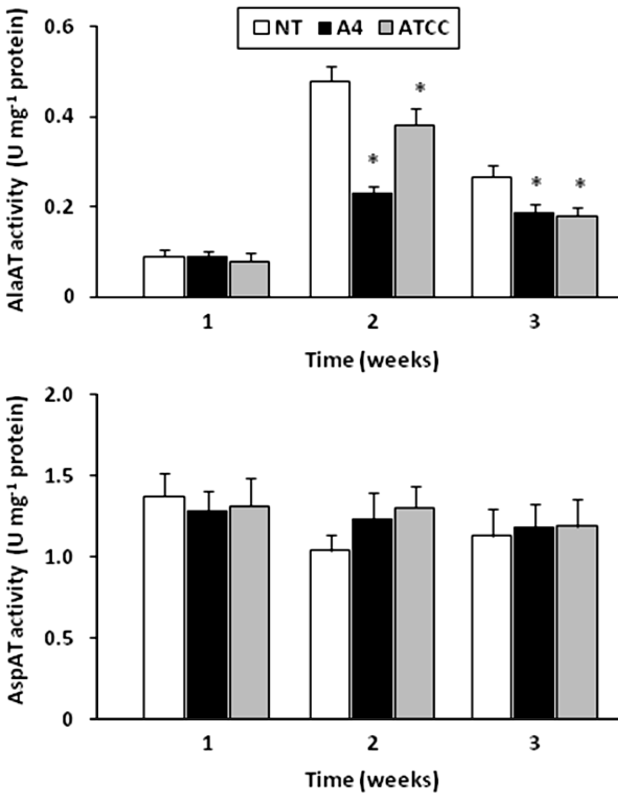

Fig. 5. Activity of alanine aminotransferase (AlaAT) and aspartate aminotransferase (AspAT) in hairy root clones of *Physalis ixocarpa*. Values are means of three independent experiments ± SD. Statistically significant differences ($P<0.05$) between the A4 and ATCC clones and non-transformed roots (NT) are asterisked.

roots more than the A4 clone did. Changes in the prooxidant/antioxidant balance in plant-pathogen interactions resulting from higher ROS generation (Foyer and Noctor, 2009) can trigger the expression of genes encoding secondary metabolite biosynthetic enzymes such as PAL (Desikan et al., 1998). In tobacco callus cultures, however, accumulation of H_2O_2 downregulated nicotinic alkaloid biosynthesis by altering the expression of putrescine N-methyltransferase, an enzyme functioning in biosynthesis of nicotinic and tropane alkaloids (Sachan et al., 2010).

Agrobacterium rhizogenes-mediated transformation did not induce oxidative stress one week after subculture, as there was no change in CAT- and APX-related H_2O_2 scavenging capacity and GSH-Px activity decreased. Simultaneously, however, the AA/total ascorbate and GSH/total glutathione ratios fell in the ATCC clone, suggesting that reduced forms of ascorbate and glutathione as nonenzymatic antioxidants were being utilized. In both examined hairy root clones the reduced and oxidized pools of ascorbate and glutathione were lower than in NT roots, but in A4 the AA/total ascorbate and GSH/total glutathione ratios were similar to those for NT roots. The lowered GSH level may have caused the decline in GSH-PX activity in both cultures. The increase in TOC levels (in two-week-old hairy root cultures) may indicate intensification of prooxidative processes (Munné-Bosch et al., 2007), but in the three-week-old cultures this antioxidant protection mechanism appeared to be ineffective, as the content of TBARS, a marker of oxidative processes, increased significantly, especially in the ATCC clone. This could enhance its ability to produce tropane alkaloids. Non-enzymatic lipid peroxidation under decreased TOC content, as found in the ATCC clone, has been reported to correspond to

increased expression of chemical defense-related genes and phytoalexin biosynthesis (Sattler et al., 2006). However, the increased TBARS content, detected only in three-week-old cultures, might also be growth phase-dependent and related to senescence (Vanacker et al., 2006). The altered cellular redox equilibrium mediated by decreased ascorbate and glutathione redox ratios in one-week-old ATCC cultures seems to enhance the production of tropane alkaloids. Accumulating evidence suggests that GSH/GSSG and AA/DHA redox couples are important in controlling enzyme activities and in regulating the expression of defense genes via redox-sensitive mediators (Foyer and Noctor, 2009; Meyer, 2008). Our results point to the possible role of glutathione- and ascorbate-mediated redox signaling in tropane alkaloid biosynthesis. The shift of the glutathione- and ascorbate-related redox balance towards the oxidative state may constitute a predictor of the biosynthetic capability of hairy root cultures.

Ascorbate, the main hydrophilic non-enzymatic antioxidant in plants, is also an important factor regulating cell growth via apoplastic AO (Pignocchi et al., 2006). Elevated AO, which oxidatively decarboxylates auxins, is correlated with a decreased auxin response in roots (Kerk et al., 2000). Thus, the increased AO activity we found in the A4 clone might also affect its growth pattern and be at least partly responsible for its lower biomass production. In our study the increased AO activity did not produce any changes in the ascorbate pool in the A4 clone, in accord with Pignocchi and Foyer's (2003) finding that AO had a negligible effect on the whole-cell ascorbate pool, as apoplastic ascorbate constitutes ~10% of its total cell content.

In most heterotrophic in vitro cultures, sucrose serves as a carbon and energy source. The use of it relies on its cleavage into hexoses by sucrose synthase (EC 2.4.1.19) and cytosolic alkaline or vacuolar acid invertases (EC 3.2.1.26) (Wang et al., 2000). It has been suggested that in plant tissues the invertase pathway may be generally directed toward growth and expansion (Sturm, 1999), so the greater decrease of Glc content in the ATCC clone than in the A4 clone may reflect its greater demand for carbohydrates to sustain its higher growth potential. Glucose may be also redirected toward increased production of glycosylated secondary metabolites, as shown in our study with respect to arbutin. It can also feed NADPH-producing metabolic pathways and influence mitochondrial metabolism and ascorbate biosynthesis (Smirnoff and Pallanca, 1996).

Interrelations between secondary metabolite production and primary carbon and nitrogen metabolism have been documented. Yu et al. (2005) found that taxol production in cell suspension cultures of *Taxus chinensis* was favored by high activity of glucose-6-phosphate dehydrogenase (G-6-PDH), and that the addition of glutamate increased G-6-PDH activity. G-6-PDH is a key regulatory enzyme in the pentose phosphate pathway that produces NADPH needed for reductive biosynthesis and maintenance of the cellular redox state (Kletzien et al., 1994). In our study, changes in nitrogen metabolism were manifested in decreased PAL and AlaAT activity in the hairy root cultures. The amino acid phenylalanine participates in the biosynthesis of tropane alkaloids delivering the tropic acid moiety (Eich, 2008). It can be also diverted into the phenylpropanoid pathway via PAL. Thus, the suppression of PAL activity in *P. ixocarpa* hairy roots could favor tropane alkaloid production. Similar results were obtained in *Catharanthus roseus* cultures (Godoy-Hernández and Loyola-Vargas, 1991) but not for tropane alkaloid-producing hairy roots of *Brugmansia candida* (Marconi et al., 2007).

In our study the glutamate-producing activities of aminotransferases were differentially regulated in hairy root cultures: AlaAT declined but AspAT remained the same as in NT roots. Glutamate provides amino groups for biosynthesis of major N-containing compounds including amino acids, nucleotides, alkaloids and polyamines, the precursors of tropane alkaloids (Forde and Lea, 2007).

Secondary metabolites are often synthesized at the cost of growth, and growth and alkaloid biosynthesis compete for nitrogen (Liu et al., 1998). Thus the significantly decreased AlaAT activity in the A4 clone in the phase of rapid growth (two-week-old cultures) could contribute to the lower production of tropane alkaloids measured in three-week-old hairy roots by limiting synthesis of their precursors. In the ATCC clone, the action of AspAT and possibly the glutamate synthase/glutamine synthetase system, co-regulating the glutamate pool size (van den Heuvel et al., 2004), may have compensated for the suppressed AlaAT activity. In these cultures the nitrogen content of the medium appeared to be enough to support both biomass production and alkaloid synthesis. The *A. rhizogenes* A4 strain-mediated transformation seems to have induced different regulatory mechanisms, as the growth potential of the A4 clone as well as its ability to produce tropane alkaloids and to biotransform hydroquinone (HQ) to arbutin were considerably lower. These results highlight the complexity of the regulation of carbon/nitrogen interactions that underlie secondary metabolite production in vitro.

CONCLUSIONS

The data from this study suggest the following three points: (1) genetic transformation can induce *A. rhizogenes* strain-dependent regulatory mechanisms,

including those based on redox signals, determining the rate of allocation of carbon and nitrogen resources to secondary metabolite production pathways; (2) these specific modes of coregulation of primary and secondary metabolism are at least in part responsible for the differences in the ability of the two *P. ixocarpa* hairy root clones to produce tropane alkaloids and to convert exogenously applied hydroquinone into pharmaceutically valuable arbutin; and (3) the selected markers of primary metabolism (ascorbate and glutathione redox ratios, Glc content, PAL and AlaAT activity) might be used as indicators of the secondary metabolite-producing potential of different *P. ixocarpa* hairy root clones. Thus, early biochemical analysis may provide a means of predicting secondary metabolite synthetic ability and facilitate preliminary selection of highly productive clones.

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