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Allelopathic effects of (+)-usnic acid on some phytohormone concentrations in tomato plants

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ABSTRACT

Usnic acid (UA), the main phenolic secondary metabolite in some lichens, affected the phytohormone concentrations in tomato organs. Long-term cultivation of tomato plants in Hoagland media supplemented with 30 μM (+)-UA reduced the phytohormone concentrations than control levels in leaves and roots. The reduction in indole-3-acetic acid (IAA), in leaves and roots was 61% and 66%; 62% and 36% for cytokinins (CKs), and 40% and 71% for abscisic acid (ABA) respectively. This may due to blocking or changing the direction of metabolic transformation of precursor(s) and/or increased activity of enzyme(s) engaged in catabolic pathways. UA also reduced the transport of phytohormones between the main site of their synthesis and other organs. This is evident from the changes in the root/leaf ratios of phytohormone molecular concentrations, which for IAA decreased from 3.0 in the control to 2.6 in plants grown in media with (+)-UA and for ABA from 0.35 to 0.16. The root /leaf ratio of cytokinins molar concentrations increased from 1.5 in control to 2.5 in plants cultivated in media with additional 30 μM (+)-UA supplement.

Key words: Abscisic acid, cytokinins, indole-3-acetic acid, lichen metabolites, phytohormones, tomato, usnic acid.

INTRODUCTION

Lichens produce a diverse range of secondary chemicals, which are secreted by the mycobiont and deposited mainly extracellularly on cell walls of fungal hyphae (12). Usnic acid (UA) is the most abundant lichen metabolite (4,10,14) and is located in the upper cortex and comprises up to 6% of dry weight (DW) of thallus (34). The intensity of UA synthesis and accumulation strongly depends on the stressful climatic factors [temperature, humidity, ultraviolet and photosynthetically active radiation (PAR) and oxidative stress (2,5,9,41)]. UA is the main phenolic compound in several lichen genera (*Cetraria, Cladonia, Evernia, Ramalina* and *Usnea*) occurring on substrates such as soil or else as epiphytes on trees and shrubs (14,26). Because lichens and higher plants are in close proximity, ecological interactions *via* their metabolites are possible. Usnic acid can be translocated from lichens into plants in several ways. Lichen hyphae can deeply penetrate plant tissues (13) and secrete secondary chemicals. If the hyphae reaches xylem vessels, lichen metabolites are translocated with the stream of xylem sap within whole plants (26). UA leaches from the lichen thallus by rainwater or morning dew, and accumulates on the plant's surface and in soil profile (11,29).

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UA can pass through the plasmalemma and may be distributed within plant cells (42). On the other hand, ecological investigations (19,40) have questioned whether significant quantities of UA are leached from the lichen layer. Chemical data on UA demonstrates that its solubility is enhanced in solution at high pH, and that its relative toxicity depends on pH (16). UA is very poorly dissolved in pH of rainwater and is broken down in soil through microbial and chemical action (17,18). Hence, root uptake of UA in some ecosystems is negligible and does not exert phytotoxic effects (19).

The physiological activity of UA differs considerably depending on the type of plant treatment (42) and may be modified by cell compartment pH (1). Several studies have confirmed the phytotoxic action of UA in certain conditions (e.g., 8,20,36). Long-term hydroponic culture with addition of a micromolar concentration of (+)-UA (pH 6.9) affects the plant growth parameters, accumulation of biomass and some elements, water uptake, hydraulic conductivity in roots, gas exchange, membrane leakage and other physiological processes (22,23,25). The survival duration of tomato plant depends on the (+)-UA concentration dose > 40 µM kills the plants in few days (25). The mechanism of toxic effects of UA has not yet been fully elucidated.

Plant growth, development and other physiological processes are controlled by phytohormones. There is little information on the influence of UA on the synthesis and metabolism of phytohormones in plant tissues. This aimed to provide data on the effects of long-term feeding of (+)-UA on the concentration of certain phytohormones [indole-3-acetic acid (IAA), abscisic acid (ABA) and cytokinins (CKs)] in plant organs. The tomato plants were used, because they grow easily in hydroponic culture. During plant cultivation, pH was kept at 6.9 and the (+)-UA concentration was kept constant.

MATERIALS AND METHODS

Plant material

We used tomato plants (*Lycopersicon esculentum* Mill. cv. Perkoz F_1) in this study. Within 10 days after seed germination (DAG), the plants were transplanted to a chemostatic system for hydroponic cultivation as previously described (flow rate, aeration and temperature medium) by Bialczyk *et al.* (3). Plants were cultivated in Hoagland media (pH 6.9 ± 0.5) supplemented with 30 µM (+)-UA (Aldrich) or without it (control). Medium pH was monitored, kept constant and corrected when necessary during cultivation. To maintain UA at a constant concentration, the medium in the growth chamber (20 L, 25 plants) was changed every second day; during plant growth it was also assayed by HPLC as described by Caviglia *et al.* (9). Plants were cultivated for 21 days (10 to 31 DAG) in a greenhouse under a natural photoperiod, with maximum PAR at 1000 µmol m^{-2} s^{-1} and 70-75% relative humidity. Plant growth conditions and the method of preparing media with UA were as described elsewhere (25). Blades of secondary leaves and root tips (0.5 cm) were collected for analysis at midpoints of the diurnal cycle. The fresh weight (FW) of one part of the samples was recorded and then those samples were lyophilized to DW.

Determination of IAA and ABA

Upon harvesting, the weighed samples of plant material (100 mg) were preserved in 1 mL methanol and kept at -70°C until analyzed. When batch of samples was accumulated, the methanol from each one was drawn out and kept for further processing, while the sample was homogenized in 500 µL methanol with 1% acetic acid and extracted for 1 h with frequent vortexing. Then the samples were centrifuged, the supernatant collected and the extraction repeated. The supernatants and methanol in which the sample was stored were combined and 10 ng $^{13}C_6$-IAA or 100 ng $^2H_4$-ABA were added as internal standards and the solution was evaporated to dryness in a vacuum in a centrifuging evaporator (5301 concentrator, Eppendorf). The dry residue was dissolved in 150 µL methanol, 1 mL ether solution of diazomethane was added and after 10 min the sample was evaporated to constant weight under nitrogen. The methylated sample was dissolved in 30 µL ether acetate and 1 µL aliquots were analyzed in a gas chromatograph (Agilent 5975B insert XL MSD). The mass spectrometer was set to monitor ions at m/z 130, 136, 189, 195 (mass and molecular ions of methyl ester of IAA and its ^{13}C-labeled standard, respectively) and at m/z 134, 138, 190, 194 (mass and molecular ions of methyl ester of ABA and its deuterated standard, respectively). The content of the compounds in the analyzed samples was calculated as under:

$$\text{IAA}=10\cdot A_{130}/A_{136},\ \text{ng ABA}=100\cdot A_{134}/A_{138}$$

Where, A_x : Relative abundance of given ions.

Determination of cytokinins

The early steps of CK extraction were similar to those described above for IAA and ABA. The samples were extracted in 1 mL 80% methanol with the addition of molybdate to stop phosphatase activity and a known amount of ^3H-[9R]Z (^3H-zeatin riboside) as a marker for estimation of the recovery of sample purification. After extraction and evaporation the dry residue was dissolved in 100 µL mobile phase and was filtered through a 0.2 µm membrane before HPLC separation. CKs were separated by reverse-phase HPLC (Kontron, Garching, Germany) on a 5 µm mesh ODS Hypersil column using the mobile phase of 5 mM triethanolammonium bicarbonate (pH 6.0) combined with a gradient of acetonitrile (10-28%). Separation was done after UV absorbance measurement at 250 nm. The fractions were collected and dried in a vacuum and redissolved in 10% DMSO (dimethyl sulfoxide) in 0.15 M phosphate buffer saline (pH 7.0) with 2.6 mM NaN_3 and then used for ELISA.

The CK-*O*-glucosides were determined after treating the samples with α-glucosidase and separated by HPLC. CK nucleotides were assayed in samples using an ELISA test after alkaline-phosphatase pretreatment and HPLC separation. Recovery was estimated from radioactivity found in the fraction corresponding to [9R]Z and averaged at 85-95%. Conjugates of [9R]iP (isopentenyl-adenosine) to bovine serum albumin were prepared using a technique described by McDonald *et al.* (30). Unconjugated CKs were removed on a Sephadex G-10 column eluted with degassed distilled water. The protein concentration was determined by Bradford's method (6) and adjusted to 10 mg mL^{-1} after concentration in an Amicon B15 microconcentrator. The immunization schedule of rabbits and CKs assay are described elsewhere (24). Both the riboside forms and, to a minor extent, the free base of CKs inhibited cross-reaction with the corresponding antibody. Weak immunoreaction was measured with CKs from other groups. The differences in cross-reactivities of each antibody with a particular CKs were appreciable and could contribute to an estimate of the total amount of Z-types (zeatin-type) and iPA-types (isopentyl-adenosine-type). The procedures for the

ELISA test were as described by Cahill *et al.* (7). The results are presented as the sum of both types of CKs.

Statistical Analyses: Results are expressed as nmol of IAA, ABA or CKs per DW unit. The reported data are means ±SE of five replicates.

RESULTS AND DISCUSSION

The balance between the phytohormone biosynthesis, metabolism and transport from the main sites of synthesis to other organs determines their homeostasis. These are dynamic processes which respond to developmental and environmental signals (31). UA has toxic potential, which may affect the plant development and morphogenesis (13). The long-term effects of (+)-UA on phytohormone concentrations in tomato organs are shown in Figures 1-3. The IAA level in the control plants was about 3-times higher in root tissues than in leaves (Fig. 1). Other reports describe a similar trend (e.g., 15,27,28). The site of IAA synthesis is the apical meristem of stem and roots. IAA formed in the stem is translocated downward and accumulates in roots (15). After 21 days of cultivation of tomato plants in media with 30 µM (+)-UA, the IAA concentration decreased by 61% in leaves and 66% in roots. The root/leaf ratio of IAA molar concentrations decreased from 3.0 in the control to 2.6 in plants grown in media with (+)-UA. UA may interfere with the phytohormone biosynthesis pathway. In this case, it probably blocks or changes the direction of metabolic transformation of L-tryptophane, IAA precursor, resulting in inhibition of IAA biosynthesis. On the other hand, UA enhances the activity of IAA oxidase, which breaks down the structure of IAA molecules. The biological activity of phenolics in IAA catabolism depends on their chemical structure (15). For activity, the number and length of alkyl groups bound to the benzene rings and to phenolic oxygen (33) are important. The fall in the root/leaf ratio of IAA concentrations in plants treated with (+)-UA may be owing to: a) stronger inhibition of biosynthesis in the root meristem due to higher UA concentration there, as documented by Latkowska (21) in plants hydroponically grown with 30 µM (+)-UA supplementation, which contributed UA to roots, stems and leaves in a 12:4:1 ratio; b) UA interferes with both short and long-distance transport in plants (22,23). Polar transport of IAA requires energy for priming uptake *via* the influx carrier, which is driven by the proton electrochemical gradient between the apoplast and symplast (35,38). A long-term effect of (+)-UA was damage to the plasma membrane by lipid peroxidation and reduction of H^+-ATPase activity (22). IAA is also translocated by the xylem sap stream. UA drastically reduced this kind of transport by affecting root hydraulic conductance. In plants cultivated in media with 30 µM (+)-UA for 21 days, root hydraulic conductance fell from the control level by a factor of 12.3 (23).

The CKs concentration in control plants in roots, the main site of biosynthesis, was about 1.5 times the level in leaves (Fig. 2). Cultivation of plants in media with UA reduced the CKs concentration by 36% in roots and 62% in leaves. The difference in the reduction of CKs concentrations between those two organs was reflected in the increase of the root/leaf ratio to 2.5. This result suggests that UA inhibits translocation of CKs from roots to leaves, a suggestion also supported by the ratio of CKs/IAA concentrations, which in UA-treated plants was lower in leaves and higher in roots than in control. Degradation of CKs plays an

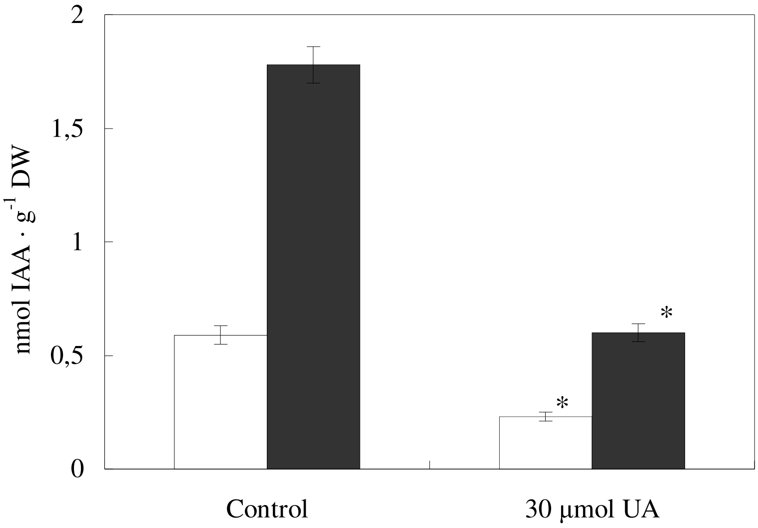

Figure 1. Concentration of IAA in leaves (white column) and roots (black column) of tomato plants cultivated for 21 days in Hoagland solution alone (control) or with 30 µM UA. Values are means of five replicates. *Significant difference from control at $p<0.05$.

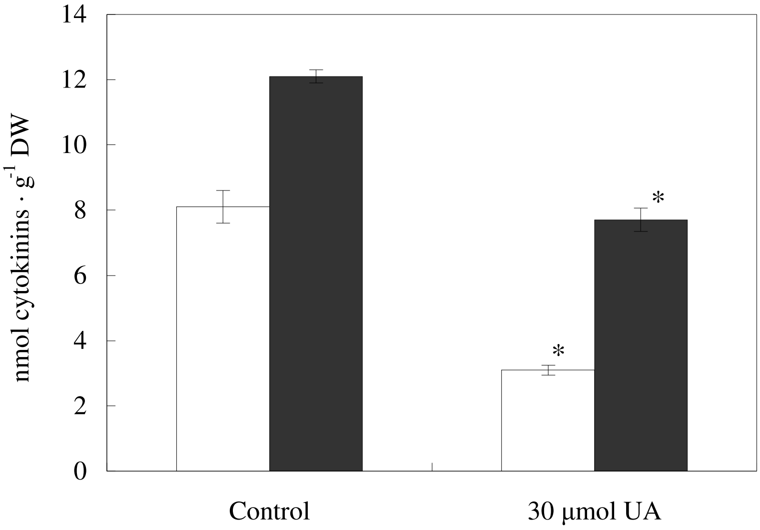

Figure 2. Concentration of cytokinins in leaves (white column) and roots (black column) of tomato plants cultivated for 21 days in Hoagland solution alone (control) or with 30 µM UA. Values are means of five replicates. *Significant difference from control at $p<0.05$.

important homeostatic role in control of their concentration and distribution in plants. The reaction is catalyzed by cytokinin oxidase/dehydrogenase, which irreversibly inactivates CKs by clearing side chain. It is known that phenolic compounds activate cytokinin oxidase/dehydrogenase. These compounds may control cytokinin degradation and also gene expression responsible for cytokinin production (39).

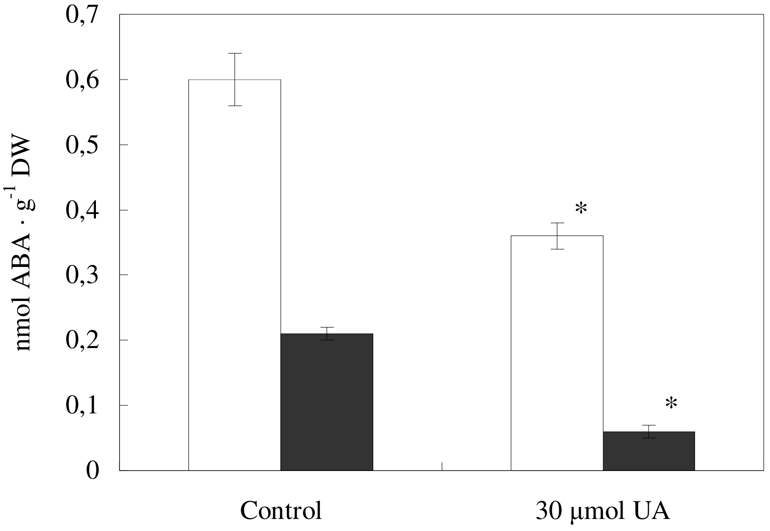

Figure 3. Concentration of ABA in leaves (white column) and roots (black column) of tomato plants cultivated for 21 days in Hoagland solution alone (control) or with 30 µM UA. Values are means of five replicates. *Significant difference from control at $p<0.05$.

The ABA concentration in control plants was 2.8-folds higher in leaves, the main site of its biosynthesis, than in roots. In the UA treatment, ABA content fell by 40% in leaves and 71% in roots. In those conditions the root/leaf ratio of ABA molar concentrations decreased from 0.35 in control to 0.16 in plants grown in media with (+)-UA. This may be due to limitation of xylem translocation between the two organs (23). Besides, it is related to the pathway of ABA synthesis from C_{40} xanthophyll precursor *via* C_{15} intermediates (43). The drop in the ABA concentration in UA-treated tomato organs may be related to decreased synthesis of mevalonic acid, a precursor common to ABA and carotenoids, or switch of mevalonic acid metabolism to the carotenoid C_{40} pathway. As presented earlier, UA had a strong depressive effect on carotenoid accumulation (36). In experimental conditions similar to our present work, carotenoid accumulation dropped by 35% versus the control (23). UA is a strong inhibitor of enzymes of carotenoid biosynthesis pathway: phytoene desaturase, which converts phytoene to carotenoids (37), and/or 4-hydroxyphenylpyruvate dioxygenase (36), catalyzing synthesis of plastoquinone which activates phytoene desaturase (32). Further experiments should clarify the role of UA in regulation of phytohormone biosynthesis.

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