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EFFECTS OF Cu^{2+}, CYTOKININS AND JASMONATE ON CONTENT OF TWO FLAVONOLS IDENTIFIED IN ZUCCHINI COTYLEDONS

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This study examined the effects of cytokinins and methyl jasmonate (JAMe) alone and in combination on the growth and flavonol content of zucchini cotyledons cultured in the presence or absence of Cu^{2+}. During greening of cotyledons in intensive diurnal illumination, rutin was identified as the main flavonol compound. Its accumulation was greatly stimulated by phenylurea cytokinin (4PU-30) but reduced by Cu^{2+}. Stimulation by benzylaminopurine (BA) was less. JAMe showed an inhibitory effect, alone and with simultaneous addition of Cu^{2+} or cytokinins. In moderate excess (100 μM and 250 μM $CuSO_4$), Cu^{2+} enhanced stimulation by 4PU-30 of rutin accumulation; at a higher concentration or with other substances it decreased the rutin level. The other flavonol detected, kaempferol-3-rhamnoside, increased after JAMe or 4PU-30 were added; in the other cases it decreased. The data obtained indicate that cytokinins can increase rutin content in developing *Cucurbita* cotyledons. Rutin content usually decreased under stress induced by excess Cu^{2+}, but Cu^{2+} in moderate excess had a stimulating effect in the presence of higher phenylurea cytokinin levels.

Key words: Copper, *Cucurbita pepo* cotyledons, cytokinins, flavonoids, growth, heavy metals, methyl jasmonate, rutin.

INTRODUCTION

Over 4000 flavonoids based on the C6-C3-C6 skeleton have been found in plants. In the genus *Cucurbita*, the occurrence of three flavonol glycosides has been reported in stigmata of four *Cucurbita pepo* species (Imperato, 1979). Flavonoid levels in the skin of apples showed a high correlation with light intensity (Awad et al., 2001). The antiherbivory effect induced by solar UV-B radiation may be mediated at least in part by accumulation of phenolic derivatives similar to those induced in plants in response to insect herbivory (Izaguirre et al., 2007).

Some literature data indicate that plant growth regulators (cytokinins, jasmonates) and heavy metals can be important factors in induction of changes in plant secondary metabolism. Flavonoid metabolism appears extremely flexible, capable of adjusting quickly to environmental changes (Cooper-Driver and Bhattacharya, 1998; Yoshitama, 2000). An increase of flavone content is usually regarded as a sign of an increase in the antioxidant capacity of plant tissues. The flavonoids rutin and quercetin are known to decompose H_2O_2 to substantially prevent Cu^{2+} and H_2O_2-caused damage to collagen and hyaluronic acid. Flavonoids have scavenging action against oxygen radicals and chelating action against Cu^{2+} (Park et al., 1991). Their level increased in runner bean plants under Cd and Cu stress, particularly in young plants (Skórzyńska-Polit et al., 2004). However, data on the effects of heavy metal stress on flavonoid synthesis in plant experimental systems is still scarce.

Jasmonates are ubiquitously occurring lipid-derived compounds with signalling functions in plant responses to abiotic and biotic stresses, as well as in plant growth and development (Wasternack, 2007). Accumulation of secondary metabolites is one of the final results of metabolic changes induced by jasmonates. Recent studies indicate that externally applied jasmonic acid or JAMe

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Abbreviations: BA – N6-benzylaminopurine; 4PU-30 – N1-(2-chloro-4-pyridyl)-N2-phenylurea; methyl jasmonate – JAMe

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usually increased the levels of some flavonoids in different plant species, depending on their organ type, for example in leaves of barley (Ishihara et al., 2002) and *Arabidopsis thaliana* (Hendrawati et al., 2006), peach shoots (Saniewski et al., 1998) and in cell suspension of *Medicago truncatula* (Farag et al., 2008), *Glycyrrhiza inflata* (Yang et al., 2008) and grapevine (Belhadj et al., 2008). In *Fragopyrum esculentum*, on the other hand, JAMe inhibited anthocyanin accumulation (Horbowicz et al., 2008).

In addition to JAMe, synthesis of some classes of flavonoids such as isoflavonoids and anthocyanins was reported to be aided by exogenous phenylurea, 6-benzylaminopurine, and N-6-benzyladenine (Del Rho et al., 1998; Dedio and Clark, 2006; Goossens and Vendrig, 1982; Tamari et al., 1995). Relatively few studies have discussed the effects of cytokinins on accumulation of flavonol glycosides (Angelova et al., 2001; Ali and Abbas, 2003). The level of phenols in tobacco callus tissue was higher under the influence of kinetin than under the influence of 4PU-30 (Angelova et al., 2001). Saline stress in barley seedlings causes an increase in total phenolic compounds and flavonoids, associated with enhancement of peroxidase and indoleacetic acid oxidase activity and a growth rate decrease. The detrimental effects of salt stress on germination, antioxidant enzymes, phenolic compounds and flavonoids were partially rectified by phenylurea (Ali and Abbas, 2003). Flavonoid accumulation correlated with the formation of adventitious roots was differentially affected by purine cytokinin treatment (Curir et al., 1990). There is no available information about effects of cytokinins and jasmonates on flavonol glycoside metabolism. Here we examine the possible role of different kinds of growth factors (4PU-30, BA, jasmonate and Cu^{2+}) on flavonoid accumulation in the cotyledons of *Cucurbita pepo*, and relate that to their effects on cotyledon growth.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWTH CONDITIONS

Seeds of one fruit of *Cucurbita pepo* L., cv. Cocozelle var. Tripolis (zucchini) were stripped of their seed coats, and cotyledons isolated from the embryonic axes of the seed were placed in covered Petri dishes on filter paper soaked with distilled water (control) or with water supplemented as indicated with either $CuSO_4$ (100, 250 or 500 μM), 10 μM BA (N6-benzylaminopurine), 10 μM 4PU-30; JAMe (100 μM) and combinations thereof. We used the high concentration of Cu to obtain evident inhibition of growth. The cotyledons were allowed to develop under diurnal intensive light (150 μmol m^{-2} s^{-1}) at 28°C for 10 days. Seeds of several different batches (originating from different zucchini fruits) were tested.

GROWTH ANALYSIS

The growth of the cotyledons was characterized by an increase in the area of at least 15 cotyledons in each of three independent experiments. For area measurement the cotyledons were carefully flattened and scanned (Epson Perfection 2480 Photo scanner, Matsumoto-shi City, Japan). Cotyledon area was measured using 3DDoctor software (Able Software Corp., Lexington, MA, USA).

The following purine cytokinins were tested: purine cytokinin BA (N6-benzylaminopurine) from Sigma (St. Louis, U.S.A.), phenylurea cytokinin 4PU-30 (N-(2-chloro-4pyridyl)-N´-phenylurea) kindly provided by Prof. Koichi Shudo (University of Tokyo, Japan), and methyl jasmonate from Serva (Heidelberg, Germany).

ISOLATION OF TOTAL FLAVONOID FRACTIONS

Powdered plant material (0.04 g from each sample) was individually extracted in MeOH:H_2O (80:20, v/v). The extracts obtained were filtered, and concentrated mixtures were extracted with dichloromethane to eliminate lipophilic compounds. Water solutions were extracted three times with EtOAc. The combined EtOAc layers were evaporated in a vacuum. The dry extracts were dissolved in methanol to check for the presence of flavonoids.

TLC ANALYSIS

TLC used the following flavonoid fractions for analysis: ethyl acetate:formic acid:acetic acid:water (100:11:11:27). Flavonoid fractions were spotted on Merck Kiselgel 60 F254 aluminum sheets (0.2 mm thin layer, 10 × 20 cm). Chromatograms were viewed under 336 nm UV before and after spraying with Naturstoffreagenz A, 1% solution of diphenylboric acid-ethanolamine complex in methanol. Flavonoid glycoside was identified by direct TLC comparison with markers.

QUANTITATIVE FLAVONOID ANALYSIS

Rutin (5, 2.5, 1.3 μg/spots) was applied, together with 15 μl from extracts of each sample with unknown concentrations, on Merck Kiselgel 60 F254 aluminum sheets. Ethyl acetate:formic acid:acetic acid:water (100:11:11:27, v/v) solution

TABLE 1. Changes in the amount of rutin and kaempferol (mg/g DW) in cotyledons after treatment of zucchini plants with cytokinins, methyl jasmonate or excess copper. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

	Treatment	Rutin	Kaempferol-3-rutinoside
A	Control	1.60±0.3	0.40±0.09
B	BA (10 µM)	2.20±0.5	0.53±0.15
C	4PU-30(10 µM)	4.60±0.4***	0.63±0.21
D	JAMe (100 µM)	1.30±0.3	0.62±0.21
E-1,2	$CuSO_4$ (100µM or 250 µM)	Trace	Trace
E-3	$CuSO_4$ (500 µM)	not detected	Trace
F-1	BA+$CuSO_4$ (100µM)	1.10±0.3	Trace
F-2	BA+$CuSO_4$ (250 µM)	0.80±0.7	Trace
F-3	BA+$CuSO_4$ (500µM)	0.53±0.5	Trace
G	BA+JAMe	0.90±0.15	not detected
H	BA+JAMe+$CuSO_4$(100µM)	1.00±0.4	not detected
I-1	4PU-30+$CuSO_4$ (100µM)	6.20±0.5***	0.52±0.25
I-2	4PU-30+$CuSO_4$ (250 µM)	6.00±0.3***	0.41±0.28
I-3	4PU-30+$CuSO_4$ (500 µM)	0.70±0.4	0.20±1.9
J	4PU-30+JAMe	0.50±0.5	not detected
K	4PU-30+$CuSO_4$ (100µM)+JAMe	0.50±0.4	Trace
L-1	JAMe+$CuSO_4$ (100µM)	Trace	not detected
L-2	JAMe+$CuSO_4$ (250 µM)	Trace	not detected
L-3	JAMe+$CuSO_4$ (500 µM)	not detected	not detected

was used for plate development. The compounds were visualized after spraying with FlCL3 reagent. The TLC plates were scanned and the images were analyzed with QuantiScan 2.1® Biosoft software. The content of compounds per sample was calculated from the densitogram peak areas by comparison with the three standards analyzed on the same plate (Nikolova et al., 2004).

STATISTICAL ANALYSIS

The statistical analyses were applied to at least 15 cotyledons from each of the three independent experiments, which used seeds originating from different zucchini fruits. One-way ANOVA was followed by the Dunnett post t-test. The levels of significance were taken as $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$.

RESULTS AND DISCUSSION

Two flavonoids were identified by co-chromatography with authentic markers. The compounds were identified as rutin (quercetin-3-rutinoside) and kaempferol-3-rutinoside. Rutin is the major flavonol, while kaempferol-3-rutinoside occurs in trace amounts in the cotyledons. Rutin is a flavonol glycoside composed of flavonol quercetin and disaccharide rutinose (Fig. 1).

The changes in the amount of rutin in the cotyledons after treatments were compared with the amount of rutin in the control cotyledons (Tab. 1). Rutin synthesis was more strongly suppressed by $CuSO_4$ applied alone and in combination with the cotyledon growth regulators. This result stands in contrast to data from *Phaseolus coccineus* leaves (Skórzyńska-Polit et al., 2004), indicating that the flavonol response to heavy metal stress is species-specific. Because of its low content, rutin might not be a part of the defense against excess Cu stress in zucchini cotyledons at the basal level of cytokinin. Rutin accumulation was highest under the influence of phenylurea cytokinin (4PU-30), attenuated the inhibitory effect of Cu ions, and was lowest in cotyledons developed in medium with purine cytokinin (BA). Methyl jasmonate weakly inhibited rutin accumulation and nonsignificantly stimulated accumulation of kaempferol-3-ruti-

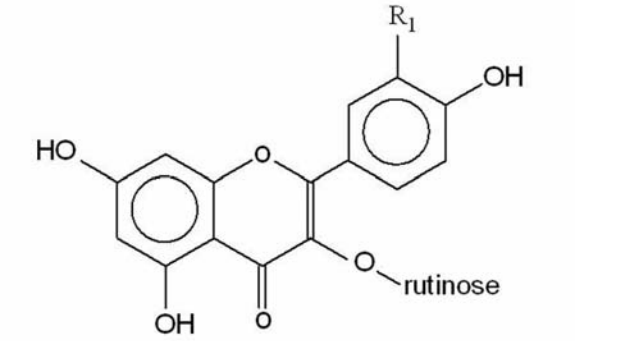

Fig. 1. Structure of the flavonols identified in zucchini. R1=OH Rutin (quercetin-3-rutinoside); R1=H Kaempferol-3-rutinoside; rutinose (6-O-L-rhamnosyl-D-glucose).

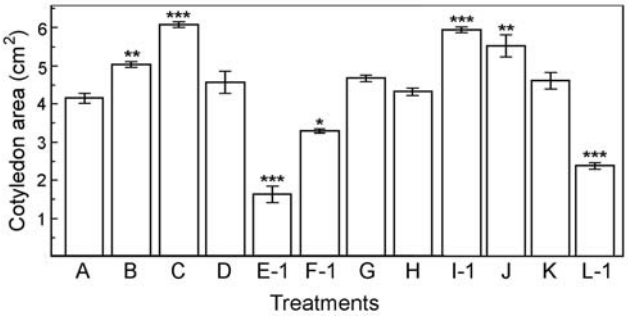

Fig. 2. Area of cotyledons of control zucchini plants and plants exposed for 10 days to cytokinins, methyl jasmonate or copper as described in Table 1. Data are means ± SE. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

noside. Interestingly, moderate excess of Cu^{2+} (100 and 250 μM $CuSO_4$) enhanced the stimulating effect of 4PU-30 on rutin accumulation.

Some studies have implicated factors other than environmental stress in changes in phenolic compounds. Changes in metabolites could be related to ontogenetic transformation of plant metabolism. Jeoman et al. (1980) were among the first to show that secondary metabolism has not merely ecological functions but is rather multifunctional. The synthesis of some secondary metabolites was shown to begin at the transition of cell cultures to subcellular differentiation of organelles. According to Abreu et al., (2004), quercetin and rutin, identified in analysis of *Hypericum brasiliense* shoots, reached the highest concentration during flowering (together with other phenolic compounds, 1,5-dihydroxyxanthone and isouliginosin B).

Phenolic compounds are usually regarded as growth inhibitors in many plant tissues, but there are opposing results. Flavonoid accumulation is correlated with adventitious root formation in *Eucalyptus gunnii* (Curir et al., 1990). Trans-cinnamic acid, gallic acid, ferulic acid, tannin, coumarin, chalcone, rutin, morin, quercetin and naringenin accumulation was also shown to decrease or even abolish the inhibitory effect of ABA on *Amaranthus caudatus* hypocotyl growth (Ray et al., 1980). The significant increase of rutin content provoked by phenylurea cytokinin we found in cotyledons is not consistent with the results of Angelova et al. (2001) in tobacco callus tissue. They reported the opposite correlation between callus growth induced by different types of cytokinins and accumulation of total phenols, and the amount of phenols was higher under the influence of kinetin than with 4PU-30. No differences in rutin content were found during the whole culture period (Angelova et al., 2001). This difference in results may be due to differences in the studied species and their endogenous hormonal status. In our work, more pronounced cotyledon enlargement (Figs. 2, 3) coincides with a higher amount of rutin, but no close correlation between growth and flavonoid enrichment could be established, as, for example, jasmonate-treated cotyledons had larger cotyledons but lower rutin content. The accumulation of rutin and quercetin provoked by cotyledon growth regulators is more closely related to their ability to stimulate cell proliferation. Recently we found that jasmonate inhibits palisade cell division (Stoynova-Bakalova et al., 2008), while cytokinins (especially 4PU-30) are strong stimulators of cell growth and cell divisions in zucchini (Stoynova-Bakalova et al., 2007) and in *Arabidopsis* cotyledons (Stoynova-Bakalova et al., 2004). The effect of jasmonate in decreasing rutin is similar, although lower than that of excess copper. This similarity could be based on some similarities in the transduction chains of their signals. In support of this is the finding that jasmonic acid activates the same suite of genes as copper and cadmium do (Xiang and Oliver, 1998; see also Maksymiec, 2007), and these heavy metals can induce jasmonate accumulation (Maksymiec et al., 2005).

CONCLUSIONS

We identified two flavonol glycosides, rutin and kaempferol-3 rhamnoside, in the cotyledons of *Cucurbita pepo*. Copper ions in excess suppressed rutin and kaempferol-3 rhamnoside accumulation, while the examined cytokinins increased it. Phenylurea cytokinin 4PU-30 generally increased flavonol accumulation to the greatest extent, and reduced Cu^{2+}-induced inhibition of both cotyledon growth and flavonol glycoside accumulation. JAMe stimulated cotyledon growth and increased kaempferol rhamnoside content, but reduced rutin accumulation.

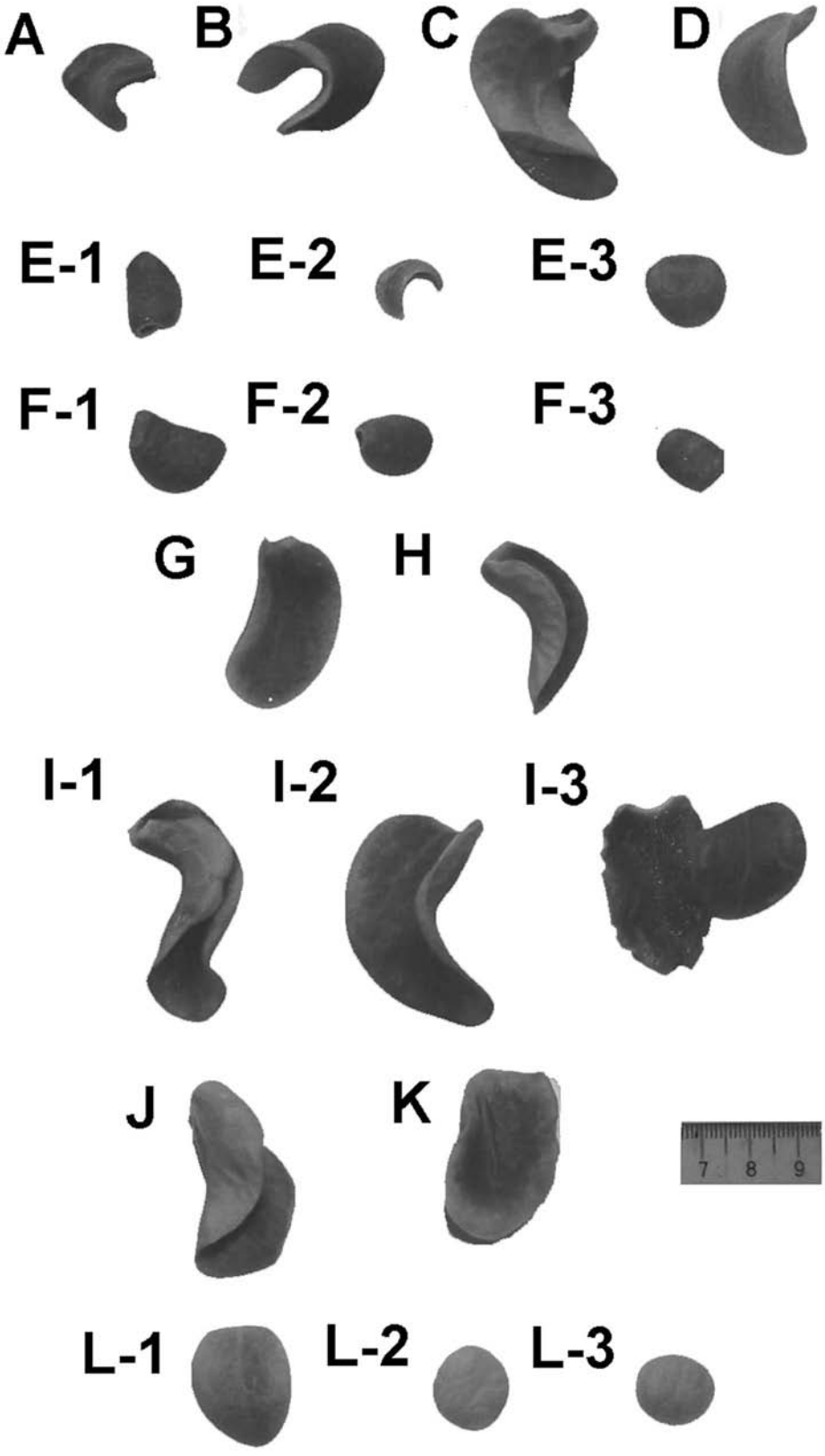

Fig. 3. Cotyledons of control zucchini plants or plants exposed for 10 days to cytokinins, methyl jasmonate or copper as described in Table 1.

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