J Plant Growth Regul (2001) 20:95–100
DOI: 10.1007/s003440010005

J P G R
Journal of Plant Growth Regulation

Cytokinins Increase Epicatechin Content and Fungal Decay Resistance in Avocado Fruits

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ABSTRACT

Treatment of avocado callus originating from avocado mesocarp (MC-2) or pericarp (PC-1) with compounds mimicking cytokinin activity enhanced the level of epicatechin. Increasing thidiazuron (TDZ) in the medium (0.9 µM to 1.8 µM) tripled the epicatechin level of the mesocarp in cytokinin-dependent (MC-2) callus. TDZ also induced the level of epicatechin in the cytokinin-independent clone mesocarp (MC-1), but the overall level of epicatechin in this tissue was 12-fold lower than in the cytokinin-dependent callus. Preharvest spray of avocado cv. Fuerte fruits with TDZ (10 µM) or benzylaminopurine (BAP) (40 µM) increased activities of flavanone-3-hydroxylase (F3H) and dihydroflavanone reductase (DFR) at 2 weeks as well as levels of epicatechin and resistance to *Colletotrichum gloeosporioides.* Preharvest treatments of fruits of avocado cv. Ettinger with benzyl adenine delayed fruit softening and decay by 20%. It is suggested that compounds with cytokinin-like activity stimulate the flavonoid biosynthetic pathway in avocado tissue. This can lead to enhanced fruit resistance as shown by the delay in decay development due to *C. gloeosporioides.*

Key words: *Persea americana*; Lauraceae; Avocado; Pre-formed compounds; Flavonoid biosynthesis; Induced resistance

INTRODUCTION

Resistance of unripe avocado fruits to attack by *Colletotrichum gloeosporioides* is correlated with the presence of fungitoxic concentrations of the pre-formed antifungal compound 1-acetoxy-2-hydroxy-4-oxoheneicosa-12, 15 diene (Prusky and Keen 1993). Decreasing diene levels in ripening fruit and the consequent increase in susceptibility have been attributed to the oxidation of this antifungal compound by lipoxygenase. During avocado ripening, lipoxygenase activity increases as a result of decreased inhibition by the natural antioxidant flavan-3-ol, epicatechin, present in the fruit pericarp (Karni and others 1989). Increased diene levels by different elicitors occur in part via epicatechin inhibition of lipoxygenase-diene-mediated catabolism of this antifungal compound (Prusky and others 1990; Karni and others 1989; Leikin and Prusky 1998). The increase in epicatechin levels is regulated by transcriptional activation of several synthetic genes, including *pal, chs,* and *f3h* (Ardi and others 1998).

Cytokinins promote cell division, delay senescence, stimulate chloroplast development (Horgan 1984) and affect secondary metabolism. Miller (1969) found that callus tissues of *Glycine max*

Received 7 September 2000; accepted 30 January 2001; Online publication 7 June 2001
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quickly responded to cytokinins by synthesizing the flavonoids deoxyisoflavone, daidzein. Clarke and others (1998, 1999) showed that exogenous application of cytokinins (dihydrozeatin and dihydrozeatin riboside) to beans (*Phaseolus vulgaris*) led to inhibition of virus replication. Reports on the effect of cytokinins as activators of the phenylpropanoid pathway in fruits, however, were not found.

In the present work, we demonstrate that epicatechin content in avocado tissue is inducible and the level reached depends on sensitivity of the tissue to cytokinins. This effect offers a new option for modulating resistance to postharvest pathogens in avocado fruits.

Materials and Methods

Source of Material and Experimental Treatments

Experiments were carried out with avocado fruits [*Persea americana* Miller var. drymifolia (Schldl. and Cham.) S.F. Blake] cv. Fuerte, from an orchard at Kibbutz Givat Brenner, Israel. Primary callus from mesocarp or pericarp of 10-week-old avocado fruits (5 cm length) was initiated on basal Murashige and Skoog salt mixture (MS) solid growth medium, with different combinations of indole acetic acid (IAA) (0.1–2 mg l^{-1}), 2,4-dichlorophenoxyacetic acid (2,4-D) (0.01–0.4 mg l^{-1}), or kinetin (0.1–2 mg l^{-1}) at 25°. To prevent surface cell oxidation, the explants were incubated with polyvinyl pyrrolidone (PVP) (av. Mol. wt. 40,000) (250 mg l^{-1}) and vitamin C (10 mg l^{-1}). Subcultivation of acquired primary callus was carried out on MS medium with different combinations of picloram (0.01–0.1 mg l^{-1}) and kinetin (0.1–0.4 mg l^{-1}).

Cytokinin Treatment

Developing callus (approximately 0.3 g) was transferred to plates containing increasing concentrations of N-phenyl-N'- 1,2,3-thidiazol-5-ylurea (TDZ). Whole avocado cv. Fuerte fruits were sprayed 14 days before harvest with 10 µM TDZ or 40 µM benzylaminopurine (BAP) dissolved in 0.01% dimethyl sulfoxide (DMSO) and 0.02% Triton X-100. Control fruits were sprayed with 0.01% DMSO and 0.02% Triton X-100 only. Fruits were left uncovered until harvested and transferred to the lab for extraction or inoculation. Fruits of fairly uniform size and weight (250–270 g) were harvested, washed in water, and used immediately after harvest for experiments.

In a second set of experiments, whole avocado cv. Ettinger trees were sprayed 90, 75, and 60 days before harvest with 50 µg ml^{-1} benzyl adenine (BA, Safe Pack Co. Israel) with 0.1% paclobutrazol [(2RS,3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl)-pentan-3-ol]. Fruits from trees sprayed on the same dates with the commercially used paclobutrazol (PTZ) or from unsprayed trees were used as controls. Each treatment was composed of five different trees from which 100 fruits were stored. Fruits were harvested at the commercial mature stage, transferred to approximately 90% RH and 20°C to simulate ripening on the retail shelf. Firmness, a parameter of fruit ripening, was determined by recording the force in Newtons (10 soft to 90 firm) required to penetrate the fruit skin and flesh. Fruits of similar size and weight (250–270 g) were used and punctured at the central diameter of the fruit.

Extraction of Epicatechin

Epicatechin was extracted from 1-mm thick avocado pericarp slices or from callus tissue, as previously described (Ardi and others 1998). Tissue (3 g fr. wt) was ground in 95% ethanol (1:10 w/v). The ethanol extract was dried in a rotary evaporator, and the residue was redissolved in 10 ml of 1 mM NaCl and extracted twice with 90-ml ethyl acetate. Epicatechin was quantitated by RP-HPLC. Calculation of epicatechin concentration was based on a comparison of the HPLC peak areas with those of authentic standards.

Enzymes Extraction and Activity

Activities of flavanone-3-hydroxylase (F3H) and dihydroflavanone reductase (DFR) were measured in avocado tissue culture and pericarp. The enzymes extracted from avocado pericarp (5 g) were blended with 50 ml cold acetone (–20°), the homogenate filtered through Whatman no.1 filter paper and blended three more times under the same conditions. The pericarp powder was dried at room temperature overnight and stored at –20° until use. Enzymes were extracted from acetone powder of pericarp (100 mg) by stirring for 2 h at 4° in the presence of 4 ml of 0.1 M Tris-HCl pH 7.4, and 0.2 g polyclar *AT* [polyvinyl pyrrolidone insoluable (BDH)]. Fresh tissue culture (100 mg) was homogenized with 1 ml of the same buffer in a mini-bead beater (Biospec) with one-third the volume of glass beads (0.5 mm). The extract was centrifuged at 10,000 g for 20 min at 4° and the supernatant was used as an enzyme source. F3H activity was tested in a reaction mixture consisting of 0.6 ml of 50 mM Tris-HCl pH 7.4, 0.015 mM naringenin (Roth, HPLC grade), 5 mM NADPH,

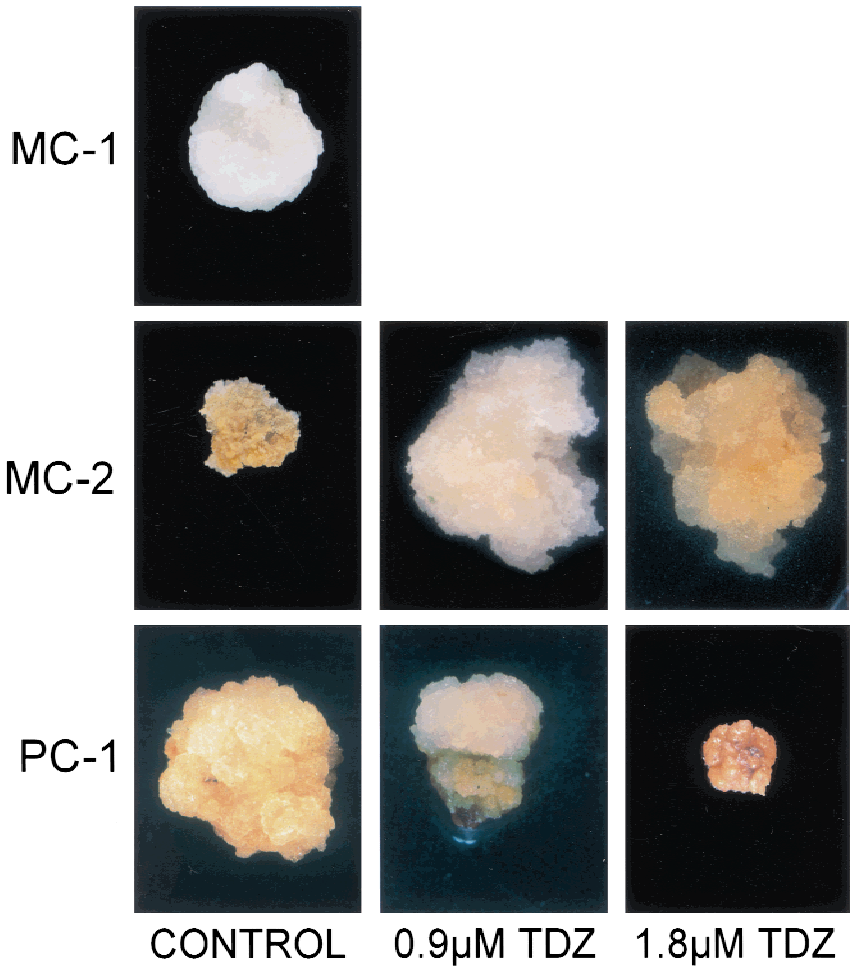

Figure 1. Effect of TDZ on avocado mesocarp (MC-1, MC-2) or pericarp (PC-1) callus. MC-1: a cytokinin-independent clone; MC-2 and PC-1: cytokinin-dependent clones (0.1 mg l^{-1} kinetin in the medium).

and 100 µg enzyme extract. The reaction was incubated for 1 or 2 h at 37°. The reaction was terminated with 50 µl of 5 N HCl and the enzyme substrate-naringenin was extracted three times with 1 ml ethyl acetate. The upper layer was collected and evaporated under N_2. The dry extracts were dissolved in 0.2 ml of a mixture of methanol:doubled distilled water:acetic acid at 55:44:1(v/v/v). Naringenin was analyzed by RP HPLC (RP-18, EcoPack, Merck), and quantified by comparison with authentic standards. The activity is given as the disappearance of naringenin in µmol mg protein $^{-1}$ h $^{-1}$.

DFR activity was assayed as a double-step reductase sequence starting with dihydroquercetin (DHQ) converted to catechin, according to Stafford and Lester (1984). The reaction mixture contained 50 µg DHQ, 5 mM NADPH, and 200 µg of protein from the crude extract in 50 mM Tris-HCl pH 7.4, in a total volume of 0.6 ml. The reaction was stopped with 50 µl 5 N HCl, and the metabolites extracted as the F3H assay. Enzyme activity is presented as disappearance of DHQ in pmol mg protein^{-1} h^{-1}.

Fruit Inoculation

A single-spore-isolate of *C. gloeosporioides,* isolate Cg-14, obtained from decayed avocado fruits, was used for inoculation experiments (Prusky and others 1994). The fungus was maintained on Mathur's medium (M_3S media) at 20°. Inoculation of avocado cv. Fuerte fruits was carried out by placing a 10 µl spore suspension (2×10^6 spores ml^{-1}) on the pericarp at six inoculation spots on the longitudinal axis of the fruit, three on each side. Following inoculation, fruits were maintained at approximately 90% RH for 24 h and then transferred to normal humidity conditions (70–80% RH) at 20° until fruit ripening and symptom development. Symptoms of decay on the pericarp were expressed as the diameter (mm) of brown pericarp tissue. Ten fruits were inoculated per treatment (for a total of 60 inoculated spots per treatment) and experiments were repeated three times.

Decay development of naturally infected cv. Ettinger fruits was evaluated after fruit ripening at

Table 1. Levels of Epicatechin in Selected Callus Clones from Mesocarp (MC-1, MC-2) and Pericarp (PC-1) of Avocado Fruits Exposed to TDZ

Callus Tissue*	Epicatechin (μg · g fr wt^{-1})		
	Control	TDZ (0.9 μM)	TDZ (1.8 μM)
MC-1	**0.237 ± 0.05**	**0.398 ± 0.07**	**0.338 ± 0.07**
MC-2	2.918 ± 0.32	4.783 ± 0.81	9.283 ± 1.66
PC-1	4.727 ± 0.86	14.795 ± 2.33	little growth

Table 2. Dihydroflavanone Reductase (DFR) in Selected Callus Clones from Mesocarp MC-1, MC-2 from Avocado Mesocarp Exposed to TDZ

Callus Tissue	DFR Activity (pmol · mg $protein^{-1}$ · h^{-1})	
	Control	TDZ (0.9 μM)
MC-1	**20.5 ± 2.4**	**25.2 ± 1.2**
MC-2	20.3 ± 3.7	80.5 ± 1.4

20°C. Fruits were considered decayed when the brown pericarp areas exceeded 5 mm.

Results

Effect of Cytokinins on Growth and Epicatechin Level in Selected Callus Clones

Acquired primary callus was subcultured on MS medium with increasing concentrations of picloram (0.01–0.1 mg l^{-1}) or kinetin (0.1–0.4 mg l^{-1}). After the fifth subcultivation, two clones of mesocarp (MC-1 and MC-2) and one of pericarp (PC-1) origin were isolated. MC-1 was cytokinin-independent and grew on medium without kinetin, forming a hard, round, white callus (Figure 1). MC-2 was dependent on 0.1 mg l^{-1} kinetin for growth, forming a soft, irregular callus, which was gray with yellow-brown inclusions (Figure 1). The PC-1 clone was hard, light pink in color, flat, regular, and round in shape (Figure 1), and dependent on 0.1 mg l^{-1} kinetin for growth. PC-1 was sensitive to 1.8 μM TDZ, exhibiting growth suppression and browning of the callus (Figure 1).

The presence of 0.9 μM TDZ in the growth media of the cytokinin-dependent calluses, MC-2 and PC-1, stimulated epicatechin levels by 64% and 212%, respectively (Table 1). Treatment with 1.8 μM TDZ increased epicatechin content in the MC-2 callus by 218% relative to controls, but suppressed growth in the pericarp clone. Growth of MC-1 was not affected by increases in TDZ in the medium but epicatechin levels were lower (12 times) than in MC-2. Addition of 0.9 μM TDZ increased epicatechin levels by 67% in MC-1, but the overall level of this flavonoid in the tissue was still very low.

Activity of DFR, the last enzyme in epicatechin synthesis, increased almost fourfold in the cytokinin-dependent callus as a result of the presence of 0.9 μM TDZ, whereas DFR activity in the cytokinin-independent MC-1 callus was nearly unchanged (25.5 ± 1.2 pmol DHQ mg $protein^{-1}$ h^{-1}) (Table 2).

Table 3. Effect of TDZ and BAP on Epicatechin Concentration in the Pericarp of Avocado cv. Fuerte Fruits

Time after Treatment Days	Epicatechin (μg · g fr · wt^{-1})		
	Control	TDZ (10 μM)	BAP (40 μM)
7	475 ± 161	968 ± 202	1349 ± 326
14	543 ± 192	1055 ± 188	1040 ± 176

Effect of Preharvest Cytokinin Treatments on F3H and DFR Activities, Epicatechin Content, and *C. Gloeosporioides* Development in Avocado Fruit Pericarp

Spraying avocado fruits with 10 μM TDZ or 40 μM BAP 14 days before harvest increased epicatechin content in the pericarp tissue (Table 3). Seven days after treatment, epicatechin levels increased by 103% and 184% in response to 10 μM TDZ and 40 μM BAP, respectively. Fourteen days after cytokinin treatment, epicatechin concentrations were still higher than in untreated fruits. Cytokinin-induced epicatechin accumulation correlated with the increase of F3H activity (Table 4). Cytokinin treatments also had a significant effect on DFR activity, which increased from 24.5 in controls to 44.3 and 65.5 pmol DHQ mg $protein^{-1}$ h^{-1} in TDZ- and BAP-treated fruits, respectively (Table 4).

When pre-harvest cytokinin-treated fruit were inoculated after harvest with spores of *C. gloeosporioides,* decay development was significantly inhibited (Figure 2). *In vitro C. gloeosporioides* germination or germ tube elongation showed no direct effect of either TDZ or BAP (results not shown). Furthermore, preharvest treatments of avocado cv. Ettinger fruits with benzyl adenine delayed fruit softening and reduced the natural percent of decayed fruits by 17% 14 days after harvest (Table 5).

Table 4. Effect of TDZ and BAP Treatment on Flavanone-3-Hydroxylase (F3H) and Dihydroflavanone Reductase (DFR) Activity in the Pericarp of Avocado cv. Fuerte Fruit 14 Days after Treatment

	Activity (Units)		
Enzyme Activity	Control	TDZ (10 μM)	BAP (40 μM)
F3H (μmol · mg protein^{-1} · h^{-1})	6.50 ± 1.7	12.00 ± 1.8	11.50 ± 0.5
DFR (pmol · mg protein^{-1} · h^{-1})	24.5 ± 5.5	44.3 ± 6.1	65.5 ± 13.6

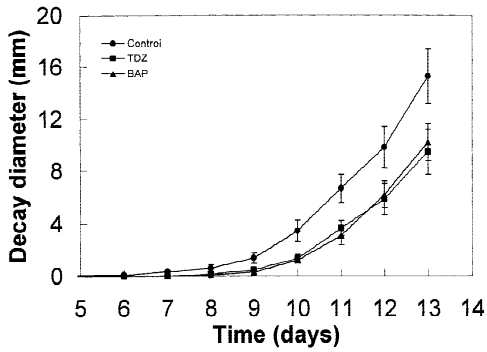

Figure 2. Effect of preharvest cytokinin treatments on decay development on avocado cv. Fuerte fruits caused by *C. gloeosporioides*. Fruits were sprayed with a solution containing 0.01% DMSO, 0.02% Triton X-100, and either 10 μM TDZ or 40 μM BAP 14 days before harvest. Control fruits were sprayed with DMSO and Triton X-100 only. Inoculation with *C. gloeosporioides* (2×10^6 spores ml^{-1}) was carried out after harvesting.

Discussion

Preharvest treatment of avocado fruits with compounds exhibiting cytokinin-like activity of avocado fruits reduced decay development from *C. gloeosporioides* after harvest. Resistance of unripe avocados to attack by *C. gloeosporioides* is mediated by the presence of fungitoxic concentrations of the preformed antifungal compound, 1-acetoxy-2-hydroxy-4-oxoheneicosa-12, 15 diene (diene) in the pericarp of unripe fruits (Prusky and Keen 1993). Increased diene concentrations were accompanied by an increase in the transcriptional activation of genes involved in the biosynthesis of the flavonoid, epicatechin, a natural antioxidant present in the pericarp that regulates the antifungal diene decline during ripening (Ardi and others 1998; Prusky and others 1994). Preharvest treatment with TDZ or BAP also increased F3H and DFR activities and epicatechin levels, confirming early reports that cytokinins activate the phenylpropanoid pathway (Hino and others 1982; Kuboi and Yamada 1976; Thomas and others 1997).

The effects of cytokinins on activation of the phenlypropanoid pathway and epicatechin levels were also evaluated on avocado pericarp and mesocarp cultures. Hino and others (1982) reported that cytokinins enhanced flavonoid biosynthesis in callus tissue. In the avocado system the cytokinin-independent clone, MC-1, contained low levels of epicatechin but in the presence of cytokinin, epicatechin levels were enhanced in both the cytokinin-dependent and cytokinin-independent clones. This may imply that dependence on cytokinin for growth can affect the basic level of epicatechin but not the capability of the callus to increase epicatechin concentrations. Kinetin increased phenolic acid content and the activity of O-methyltransferase activity in tobacco cell culture (Kuboi and Yamada 1976), and synthesis of the deoxyisoflavone diadzein in callus tissue of *G. max* (Miller 1969). However, the mechanism for induction of epicatechin increases in calluses and avocado tissue by cytokinins is not known (Hahlbrock and Scheel 1989).

Cytokinins might have a secondary effect on avocado by delaying senescence and affecting resistance in this way. Musgrave (1994) and Lesham (1987) suggested that cytokinins play a role as oxygen-free-radical scavengers, and maintained high scavenger activity that results in senescence inhibition. Chaitanya and Naithani (1998) claimed that kinetin inhibits and/or reduces membrane damage by suppressing oxidative stress via the enhancement of superoxide dismutase, which could reduce phospholipid peroxidation. This inhibition of senescence resulted in a delay of fruit ripening and natural decay development, suggesting that cytokinin treatment may be a means for improving the quality of stored avocado fruits.

Acknowledgments

This research was supported by research grants No. IS-2435-94C from BARD, The United States—Israel Binational Agricultural Research and Development Fund, under Grant No. C11-246 US-Israel Cooperative Development Research Program, Economic Growth, U.S. Agency for International Development

Table 5. Effect of Preharvest Cytokinin Treatments on Fruit Firmness and Natural Decay Development on Avocado cv. Ettinger Fruits Caused by *C. gloeosporioides*

	Firmness (Newtons)			Decayed fruits (%)		
Days after harvest	Control	PTZ	PTZ ± BA	Control	PTZ	PTZ ± BA
1	94 ± 10	94 ± 10	94 ± 10			
4	71 ± 8	74 ± 5	82 ± 8			
7	35 ± 2	36 ± 2	43 ± 8			
8	26 ± 2	33 ± 5	36 ± 9			
9	23 ± 4	21 ± 7	30 ± 4			
10				20 ± 3	21 ± 4	10 ± 4
14				53 ± 2	61 ± 1	36 ± 2

Fruits were sprayed three times with the commercially used paclobutrazol (PTZ) and a combination of PTZ and 50 mg · l^{-1} benzyl adenine (BA).

and German-Israel Agricultural Research Agreement for the Benefit of the Third World (GIARA).

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