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INVOLVEMENT OF REACTIVE OXYGEN SPECIES GENERATED FROM MELANIN SYNTHESIS PATHWAY IN PHYTOTOXICTY OF L-DOPA

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Abstract—L-DOPA is an active allelochemical that inhibits plant growth. To determine whether the phytotoxicity is due to the reactive oxygen species generated during its oxidation to melanin, oxidative damage, melanin accumulation, and the effect of antioxidants on its phytotoxicity were examined in L-DOPA-tolerant (barnyard grass) and -susceptible (lettuce) plants, and in suspension-cultured carrot cells. L-DOPA suppressed root elongation in lettuce compared to barnyard grass. Levels of melanin and thiobarbituric acid reactive substances (TBARS) increased remarkably in L-DOPA-treated lettuce roots, but not in barnyard grass. L-DOPA also suppressed carrot cell growth to 60% of the control at 1 mM. Melanin content in 1 mM L-DOPA-treated carrot cells increased continuously; however, ascorbic acid and α-tocopherol suppressed accumulation. When melanin formation was inhibited by ascorbic acid and α-tocopherol, growth of L-DOPA-treated cells was restored. TBARS levels were higher in 1 mM L-DOPA-treated carrot cells than in untreated control cells 2 d after treatment, but not at 4 or 6 d. Ascorbic acid and α-tocopherol suppressed the production of lipid peroxide during the initial 2 d. These results suggest that the phytotoxicity of L-DOPA is due to oxidative stress caused by reactive oxygen species from the melanin synthesis pathway.

Key Words—L-DOPA, inhibition of root elongation, melanin synthesis pathway, lipid peroxidation, reactive oxygen species.

INTRODUCTION

L-DOPA (L-3, 4-dihydroxyphenylalanine), a precursor for alkaloids, phenylpropanoids, flavonoids, lignin, and melanin (Halbrock and Scheel, 1989) is an active allelochemical. Velvet bean (*Mucuna pruriens*), a leguminous species, exudes a

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large amount of L-DOPA from its root and inhibits the growth of nearby species (Fujii et al., 1991). Previous studies have revealed that L-DOPA suppresses the growth of roots more significantly than shoots, and the inhibitory effect is selective among plant species (Fujii, 1994; Fujii et al., 1991; Nakajima et al., 1999; Hachinohe et al., 2004).

In the past few decades, natural toxins, including allelochemicals that suppress or eliminate competing plant species near the source plant, have received special attention due to their agricultural potential as herbicides (Dayan et al., 2000). Although hundreds of allelochemicals have been identified, the mode of action of many of these is not yet clear. This study focuses on the phytotoxic mechanism of L-DOPA, which is synthesized via oxidation of tyrosine in the presence of the copper-containing enzyme tyrosinase and molecular O_2. In plant cells, L-DOPA is metabolized to several chatecholamines, phenylpropanoids, and melanin (Pattison et al., 2002). Nakajima et al. (1999) reported that cucumber plants detoxify L-DOPA to nontoxic amino acids such as phenylalanine or tyrosine. We previously compared the metabolic activity of L-DOPA in barnyard grass (*Echinochloa crus-galli* L.: tolerant) and lettuce (*Lactuca sativa* L. cv. Great lakes 366: susceptible) (Hachinohe et al., 2004). There was no significant difference in the composition of metabolites between the species; however, L-DOPA accumulated solely in lettuce suggesting that it is an active principle of the phytotoxic action.

There are numerous studies on the effect of L-DOPA in animal cells. It is a potential therapeutic agent for Parkinson's disease; however, there is considerable concern about the long-term effects caused by L-DOPA-induced cell apotosis and cytotoxicity (Basma et al., 1995; Lai and Yu, 1997; Melamed et al., 1998; Haque et al., 2003). L-DOPA can be oxidized non-enzymatically to form melanin (Riley, 1997); a process that generates reactive oxygen species (Kruk et al., 1999). The cytotoxicity of L-DOPA in nerve cells is due to oxidative damage from reactive oxygen species and can be suppressed with antioxidative enzymes or antioxidants in many cases.

This study was conducted to determine whether the phytotoxicity of L-DOPA is due to reactive oxygen species formed during oxidation to melanin. Oxidative damage and the effect of antioxidants on phytotoxicity were examined in L-DOPA-tolerant (barnyard grass) and -susceptible (lettuce) plants, and in suspension-cultured carrot cells.

METHODS AND MATERIALS

Plant Materials. Seeds of barnyard grass and lettuce were sown on aluminum mesh trays covered with cheesecloth and set on plastic boxes ($310 \times 220 \times 5$ mm^3) containing distilled water. They were germinated in a growth chamber

under 25°C/20°C (day/night, 12 hr each, 80–100 μmol m^{-2} s^{-1}). The uniformly germinated seeds, 1-d-old lettuce and 4-d-old barnyard grass were used for the experiments.

Effect on Root Elongation. Ten germinated seeds of barnyardgrass and lettuce were placed in a plastic box (60 × 60 × 100 mm^3) containing 200 ml of a 0.5% agar culture medium (pH 6.0) with L-DOPA (0.1 mM). They were kept in the chamber, and their root lengths were measured at 1, 2, 3, and 5 d. Each experiment was conducted using five boxes, and the experiment was repeated three times.

Cell Culture and Effect on Plant Growth. Suspension cultures of carrot (*Daucus carota* L. cv. Harumakigosun) cells were grown in Murashige and Skoog medium (MS medium), pH 6.0, containing 30 g L^{-1} of sucrose and 2 mg L^{-1} of 2,4-dichlorophenoxyacetic acid (Matusmoto et al., 2002). Cultures were maintained on a gyratory shaker (NR-20; TATITEC Corporation, Saitama, Japan) agitated at 100 rpm under dim light at 25°C, and subcultured at 2-wk intervals by transferring 20 ml of cell culture into 100 ml of fresh medium. Cell cultures were grown with medium containing L-DOPA with/without ascorbic acid or α-tocopherol for 6 or 8 d. The effect on growth was determined by measuring packed cell volume (Warabi et al., 2001) at 2, 4, and 6 d after treatment.

Spectrophotometric Assay of Melanin. Total melanin was determined according to Wakamatsu and Ito (2002). Roots of lettuce and barnyard grass (30 mg FW) were homogenized in 3 ml of a 90% (v/v) Soluene-350 solution, and solubilized completely by incubation for 30 min at 100°C. After cooling, absorbance at 500 nm was determined by using synthetic melanin as a standard. In carrot cells, the yellow pigments were removed with methanol before the cells, 0.2 ml, were homogenated in 3 ml of Soluene-350.

Determination of Lipid Peroxidation. Lipid peroxidation was determined with the thiobarbituric acid (TBA) test (Velikova et al., 2000). Roots of lettuce and barnyard grass (500 mg) were homogenized in a 0.1% (w/v) TCA solution (5 ml). The homogenate was centrifuged at 10,000 *g* for 20 min, and 0.5 ml of the supernatant were added to 1 ml of 0.5% (w/v) TBA in a 20% TCA solution. The mixture was incubated for 30 min at 98°C, and the reaction was stopped by cooling in an ice bath. The samples were centrifuged at 10,000 *g* for 5 min, and the absorbance of supernatants was analyzed at 532 nm. The value for non-specific absorbance at 600 nm was subtracted. The amount of thiobarbituric acid reactive substances (TBARS) was calculated with an extinction coefficient of 155 mM^{-1} cm^{-1}.

Statistical Analysis. Data were analyzed with Statcel (OMS, Saitama, Japan), and tested for normality and homogeneity of variances (F-test or Bartlett test) followed by ANOVA or simple linear regression analysis. Differences between treatments were determined by Fisher's PLSD test, Student's t-test, or Welch's t-test.

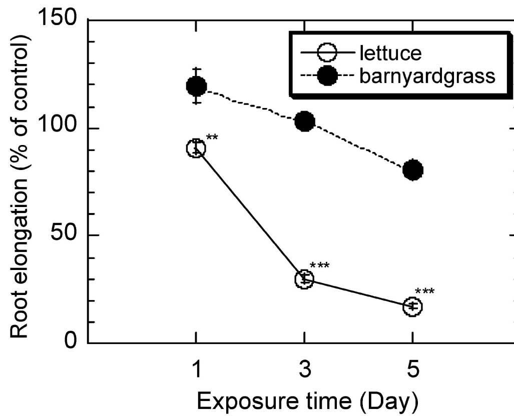

FIG. 1. Effect of 0.1 mM L-DOPA on growth of barnyard grass and lettuce roots. Bars indicate ±SE of the mean ($N = 5$). Asterisks indicate a significant preference between control and L-DOPA treatment ($^{**}P < 0.01$; $^{***}P < 0.001$).

RESULTS

L-DOPA caused selective suppression of root elongation in barnyardgrass and lettuce (Figure 1). The root lengths of both L-DOPA-treated species were approximately 80% and 20% of untreated control root lengths, respectively.

Although L-DOPA induced accumulation of melanin in the roots of both species, the increase was more remarkable in lettuce (Figure 2). Melanin content was positively correlated with growth inhibition of L-DOPA-treated roots

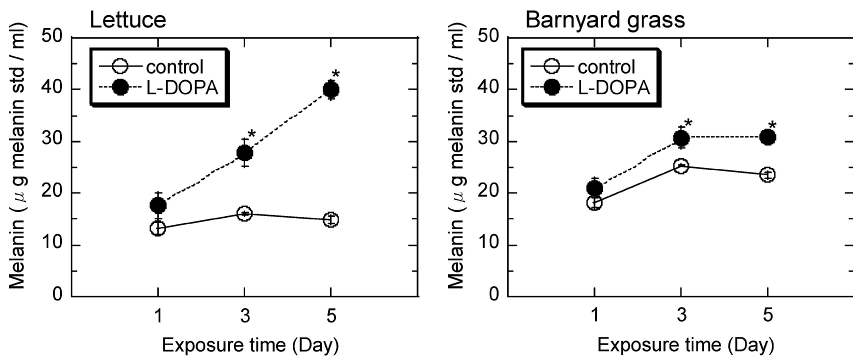

FIG. 2. Melanin levels in roots of barnyard grass and lettuce treated with L-DOPA. Bars indicate ±SE of the mean ($N = 3$). Asterisks indicate a significant preference between control and L-DOPA treatment ($^{*}P < 0.05$; $^{**}P < 0.01$).

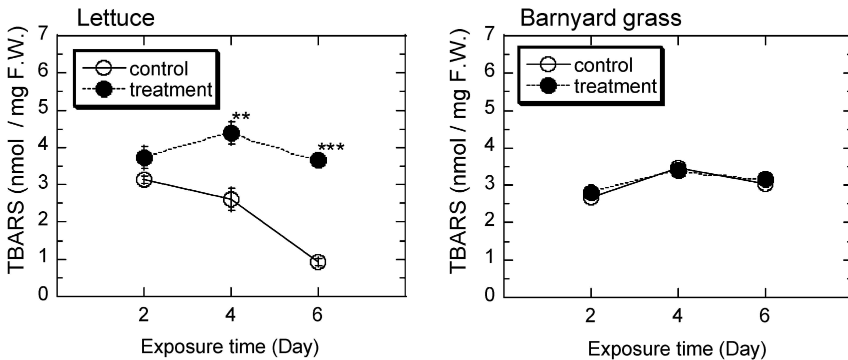

FIG. 3. Effects of L-DOPA on TBARS levels in roots of barnyard grass and lettuce. Bars indicate ±SE of the mean ($N = 3$). Asterisks indicate a significant preference between control and L-DOPA treatment ($^{**}P < 0.01$; $^{***}P < 0.001$).

($r^2 = 0.85$, $F_{1,28} = 152.8$, $P < 0.001$). Levels of TBARS were higher in L-DOPA-treated lettuce roots, but not in barnyard grass (Figure 3). Lipid peroxidation also correlated with growth inhibition ($r^2 = 0.67$, $F_{1,10} = 20.4$, $P < 0.05$) in the regression analysis.

Toxicity of L-DOPA in nerve cells is caused by the reactive oxygen species, H_2O_2, $^1O_2$, O_2^-, and $OH^\bullet$, generated from its oxidative metabolism to melanin (Graham et al., 1978; Rosenberg, 1988; Kruk et al., 1999; Pattison et al., 2002). Cytotoxicity was restored with the addition of antioxidative enzymes or antioxidants (Parsons, 1985; Basma et al., 1995; Lai and Yu, 1997; Haque et al., 2003). To investigate the involvement of this oxidative process in L-DOPA's action, melanin content and TBARS content were determined in carrot cells cultured with MS medium. Suspension-cultured carrot cells were used for the experiments because it is easy to regulate concentrations of chemicals in the medium.

At 6 d after treatment, L-DOPA suppressed cell growth to 85% and 60% of the control level at 0.1 mM and 1 mM, respectively (Figure 4). The melanin content increased continuously in 1 mM L-DOPA-treated cells for 6 d (Figure 4). The amount of melanin accumulated in cells depended on the concentration of L-DOPA (data not shown). Ascorbic acid and α-tocopherol suppressed melanin formation in L-DOPA-treated carrot cells (Figure 5). When melanin production was inhibited by ascorbic acid and α-tocopherol, growth of L-DOPA-treated cells was restored (Figure 6). TBARS levels were higher in 1 mM L-DOPA-treated carrot cells than untreated control cells 2 d after treatment, but not at 4 and 6 d (Figure 7). Ascorbic acid and α-tocopherol suppressed this initial increase in the formation of the lipid peroxide.

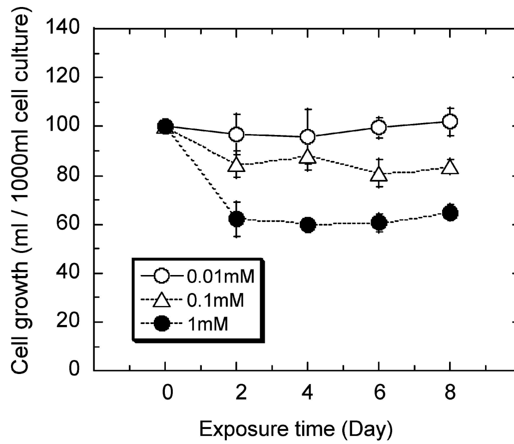

FIG. 4. Effect of L-DOPA on growth of carrot cells. Bars indicate ±SE of the mean ($N = 3$). A one-way ANOVA was performed ($F_{11,24} = 7.22$, $P < 0.001$).

DISCUSSION

L-DOPA is a strong neurotoxic agent owing to its ability to induce apoptosis (Melamed et al., 1998; Haque et al., 2003) and adverse effects on neurological degenerative diseases in animals (Enochs et al., 1994; Bowling and Beal, 1995;

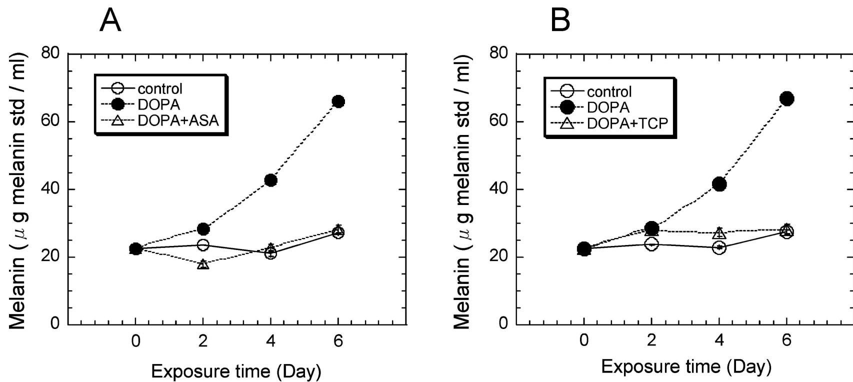

FIG. 5. Effect of ascorbic acid (A) and α-tocopherol (B) on melanin levels in carrot cells treated with L-DOPA. Concentrations of L-DOPA and antioxidants are 1 mM. Bars indicate ±SE of the mean ($N = 3$). A one-way ANOVA was performed: ascorbic acid at 4 d ($F_{2,6} = 2476.7$, $P < 0.001$); ascorbic acid at 6 d ($F_{2,6} = 980.2$, $P < 0.001$); α-tocopherol at 4 d ($F_{2,6} = 1221.2$, $P < 0.001$); α-tocopherol at 6 d ($F_{2,6} = 1223.3$, $P < 0.001$).

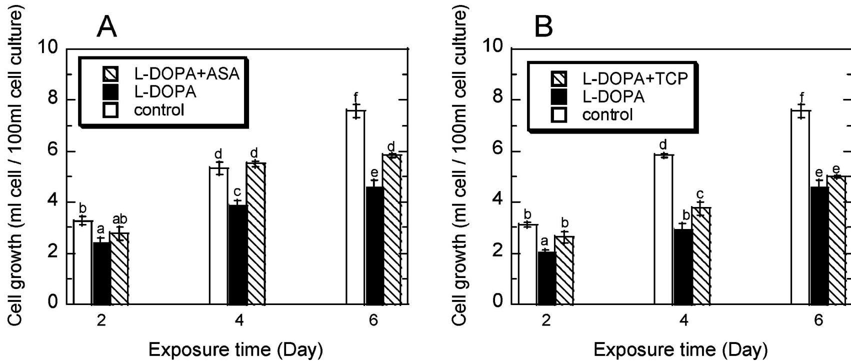

FIG. 6. Effect of ascorbic acid (A) and α-tocopherol (B) on growth of carrot cells treated with L-DOPA. Concentrations of L-DOPA and antioxidants are 1 mM. Bars indicate ±SE of the mean ($N = 3$). A one-way ANOVA was performed: ascorbic acid ($F_{8,18} = 67.72$, $P < 0.001$); α-tocopherol ($F_{8,18} = 86.56$, $P < 0.001$). Bars labeled with same letters are not significantly different ($P = 0.05$).

Smythies, 1997). Several studies have proposed that the cytotoxicity is attributable to reactive oxygen species generated during its oxidation to melanin. The purpose of this study was to examine whether L-DOPA acts on plant cells as it does on animal cells.

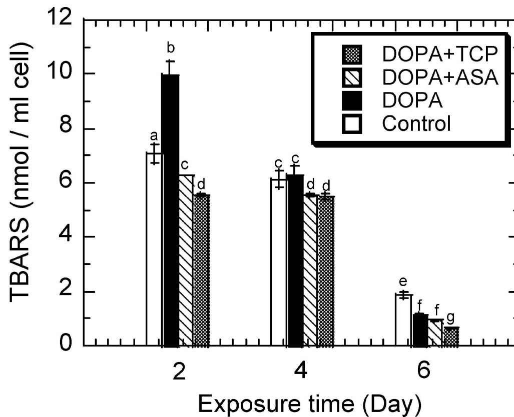

FIG. 7. Effect of antioxidants on TBARS levels in carrot cells treated with L-DOPA. Bars indicate ±SE of the mean ($N = 3$). A one-way ANOVA was performed: ($F_{11,24} = 8376.87$, $P < 0.001$). Bars labeled with same letters are not significantly different ($P = 0.05$).

Based on our previous study, barnyard grass and lettuce were selected as L-DOPA-tolerant and -susceptible species, respectively (Hachinohe et al., 2004). First, we confirmed the difference in sensitivity between them (Figure 1). A greater melanin accumulation was observed in L-DOPA-treated lettuce (Figure 2). Our results indicate that the melanin synthesis pathway in plants metabolizes exogenously applied L-DOPA. From a regression analysis, the amount of accumulated melanin correlated with the growth inhibitory activity of L-DOPA. Therefore, the melanin synthesis pathway might be closely linked to the L-DOPA phytotoxic mechanism. We checked the effect of exogenously applied melanin on lettuce root. Elongation was not inhibited (92.9% of the untreated control) when plants were grown with 60 μg ml^{-1} melanin containing agar medium formed by polyphenol oxidase-catalyzed oxidation of medium containing 0.1 mM L-DOPA. Moreover, melanin itself has not been reported to be a cytotoxic substance, but is a free radical sink that protects cells from oxidative damage (Peters and Schraermeyer, 2001). Some intermediate(s) or by-product(s) of the melanin synthesis pathway may be involved in the phytotoxicity.

In neuroblastoma SH-SY5Y cells, Lai and Yu (1997) showed a correlation between L-DOPA cytotoxicity and formation of melanin. The cells suffered oxidative damage from reactive oxygen species that disrupted cell membrane integrity. This ultimately resulted in a reduction in growth or cell death. To estimate the oxidative damage due to L-DOPA in plants, amounts of lipid peroxides were determined. Lipid peroxides levels were much greater compared with the untreated lettuce control, but not in barnyard grass (Figure 3). This suggests the involvement of reactive oxygen species and a mechanism similar to cytotoxicity in animal cells.

Cytotoxic effects in animal cells can be completely prevented by antioxidants or antioxidative enzymes, particularly catalase, superoxide dismutase (SOD), ascorbic acid, and α-tocopherol (Parsons, 1985; Basma et al., 1995; Haque et al., 2003). We confirmed that the phytotoxicity of L-DOPA in carrot cells was stopped with ascorbic acid and α-tocopherol. However, the antioxidative enzymes catalase and SOD had less of a protective effect (data not shown). Macromolecules such as catalase and SOD may not easily penetrate cell membranes (Buckley et al., 1987; Beckman et al., 1988; Clement et al., 2002).

Growth of carrot cells was suppressed by L-DOPA in a concentration-dependent manner (Figure 4), although the cells were more tolerant than lettuce. A significant accumulation of melanin was observed in carrot cells (Figure 5), indicating that the melanin pathway functions in the metabolism of L-DOPA. Ascorbic acid and α-tocopherol suppressed the formation of melanin (Figure 5). Levels of TBARS in carrot cells decreased at 4 and 6 d in the untreated control (Figure 7). This is probably due to increased cell division in the new medium. In L-DOPA-treated cells, great peroxidation was observed at 2 d, but not at 4 or 6 d. When the synthesis of melanin was inhibited by the antioxidant chemicals, the levels of TBARS decreased at 2 d. These results suggest that the melanin

pathway is involved in oxidative damage, and that lipid peroxidation occurs in the early phase of the culture. Rapid recovery of carrot cells from lipid peroxidation might be due to greater levels of carotenoids, anitioxidants, and/or the rapid metabolism of L-DOPA to nontoxic metabolites other than melanin. Although melanin formation and lipid peroxidation were suppressed during the exposure period, the effect of α-tocopherol on cell growth was not remarkable compared to ascorbic acid. The reason for this difference is not clear, but, both antioxidants effectively reduced the phytotoxicity of L-DOPA in the early stages of exposure (Figure 6).

This study suggests that the phytotoxicity of L-DOPA is due to oxidative stress, and that reactive oxygen species generated by the synthesis of melanin are involved. This is the first report suggesting such involvement in the allelopathic activity of L-DOPA. Our future goals are to clarify the mechanisms behind the production of reactive oxygen species in the melanin synthesis pathway, and the selectivity difference between barnyard grass and lettuce.

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