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Eugenol Reduces Growth and Increases Activity of S-adenosylmethionine Decarboxylase in the Phytopathogenic Fungus *Botrytis fabae*

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This study was undertaken to examine the possibility that eugenol-induced reductions in growth of *Botrytis fabae* are associated with alterations in polyamine metabolism. *B. fabae* was grown in liquid medium amended with different concentrations of eugenol. Changes in fungal biomass, and activities of enzymes of polyamine biosynthesis and catabolism were studied. An examination was also made of the incorporation of radioactivity from ornithine into polyamines. Activity of the polyamine biosynthetic enzyme S-adenosylmethionine decarboxylase (AdoMetDC) and flux of label from ornithine into the polyamine spermine were greatly increased in *B. fabae* grown in the presence of eugenol. However, no significant changes were observed in polyamine catabolism or in the concentrations of free polyamines in treated fungal tissue.

KEY WORDS: *Botrytis fabae;* eugenol; polyamines; spermine; polyamine oxidation.

INTRODUCTION

Two classes of compounds, phenylpropenes and terpenoids, make up the bulk of plant volatile oils. The phenylpropene eugenol is a major constituent of cloves, accounting for between 70% and 90% of its volatile oil content and up to 15% of the dry weight of clove buds (4). Eugenol is also found in other plant species and together with other phenylpropenes can account for up to 90% of the volatile oil composition of basil (*Ocimum basilicum)* (5). The adaptive value of the toxic properties of phenylpropenes like eugenol are probably responsible for their widespread distribution among plants. The toxic properties of these compounds have also attracted the attention of people wishing to protect plants and their products. Thus, eugenol exhibits antibacterial activity (2), is an effective antifungal agent (1), kills nematodes at low doses (13) and has a marked insect antiherbivory effect (15).

Most of the reports of antifungal effects of eugenol relate to food spoilage fungi (12) and little work has been targeted specifically at plant pathogenic fungi. Moreover, although there are numerous reports that plant volatile oils and / or their constituents exhibit activity against plant pathogenic fungi (10,11,14), nothing is known about their mode of action. In their study of the antiproliferative effects of the monoterpene geraniol against human colon cancer cells, Carnesecchi *et al.* (3) found that polyamine catabolism was activated. This led them to suggest that geraniol may exert its antiproliferative effects *via* a perturbation of

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polyamine metabolism. In view of these results, and since previous work in this laboratory has suggested a link between polyamine catabolism and plant resistance to fungal infection, it was decided to examine the effects of eugenol on polyamine biosynthesis, accumulation and catabolism in the phytopathogenic fungus *Botrytis fabae.*

MATERIALS AND METHODS

Materials L-[1-^{14}C] ornithine hydrochloride (specific activity 57 mCi mmol^{-1}), S-adenosyl-L-[carboxyl-^{14}C] methionine (specific activity 55 mCi mmol^{-1}), L-[U-^{14}C] ornithine hydrochloride (specific activity 242 mCi mmol^{-1}) and [^{14}C] spermine were purchased from Amersham Pharmacia Biotech UK Ltd. Eugenol was obtained from Sigma (Poole, Dorset, UK).

Effects of basil oil and its individual constituents on fungal growth in liquid culture In an initial experiment, a growth curve for *B. fabae* was constructed in order to determine the timing of its exponential growth phase. This information was then used in all subsequent experiments conducted using liquid culture. Conical flasks (250 ml) containing 100 ml of sterile liquid medium (9) were inoculated with 7-mm-diam plugs of *B. fabae* taken from stock plates of the fungus growing on potato dextrose agar. Flasks were placed in an orbital incubator set at 90 rpm and 19°C. For determination of the growth curve, three flasks were harvested every 12 h for 108 h. Fungal material was harvested using a fine mesh sieve, centrifuged and weighed. Based upon the growth curve obtained, in all subsequent experiments flasks were harvested after 3 days. To examine the effect on growth of *B. fabae,* eugenol was added to flasks containing liquid medium to obtain concentrations between 10 and 30 ppm. Essential oils are considered to be sterile (17) and so flasks containing oils were not autoclaved but used immediately in experiments.

Determination of enzyme activities and polyamine concentrations Enzyme extract was prepared by grinding 0.5 g of fungal tissue in a pre-chilled mortar and pestle with 1.75 ml of potassium phosphate buffer containing 2 mM dithiothreitol, 1 mM $MgCl_2$, 0.1 mM EDTA and 0.1 mM pyridoxal phosphate, adjusted to pH 7.6. For the ornithine decarboxylase (ODC) assay, extracts were placed in glass test tubes and sonicated using a Soniprep 150 for 10 cycles of 10 s on / 20 s off. Test tubes were kept on ice during sonication. Suspensions were then centrifuged for 15 min at 24,000 *g* at 0°C, after which the supernatant was dialyzed in 30 volumes of buffer overnight at 4°C. ODC activity was assayed by measuring the $^{14}CO_2$ released following incubation with radio-labeled ornithine. The reaction mixture contained in a final volume of 0.3 ml: 50 mM Tris-HCl (pH 8.0), 0.05 mM L-ornithine monohydrochloride, 0.031 mM pyridoxal-5-phosphate, 0.1 ml enzyme extract and 2.5 μl L-[1-^{14}C] ornithine hydrochloride. The test tubes were fitted with silicone rubber stoppers and 35-mm-long, 22-gauge needles. A 25 mm^2 filter paper impregnated with 10 μl of 2 M KOH was fitted to each needle to trap $^{14}CO_2$ released during the reaction. The test tubes were placed in a water bath at 37°C for 30 min, after which 0.2 ml of 6% perchloric acid was added to each tube to stop the reaction, before incubation for a further 30 min. The filter paper was then removed and placed in 12 ml of Emulsifier Safe scintillant (Packard Bioscience) and left overnight before counting the radioactivity using a Packard Tri-Carb 1900 TR liquid scintillation counter.

For the S-adenosylmethionine decarboxylase (AdoMetDC) assay, enzyme extract was prepared as described above, after which ammonium sulfate (754 mg) was added to the supernatant (cytosolic fraction) and the suspension centrifuged at 24,000 *g* for 20 min at 0°C. The pellet obtained was resuspended in 1.75 ml of buffer and dialyzed as described above. AdoMetDC activity was assayed by measuring $^{14}CO_2$ released after incubation with S-adenosyl-L-[carboxyl-^{14}C] methionine. The reaction mixture contained in a total volume of 0.3 ml: 0.1 M sodium phosphate buffer (pH 7.4), 0.2 mM S-adenosyl-L-methionine, 1.0 mM putrescine, 0.1 ml of enzyme extract and 10 μl of S-adenosyl-L-[carboxyl-^{14}C] methionine. The remainder of the reaction was carried out as described for ODC.

To determine the flux of radio-labeled ornithine into polyamines, an ODC assay was carried out as described above, except that 0.125 μCi of L-[U-^{14}C] ornithine hydrochloride was used in the reaction mixture. An aliquot (100 μl) of the reaction mixture was removed, 200 μl of saturated sodium carbonate solution and 400 μl of dansyl chloride in acetone (30 mg per ml of acetone) were added, and the reaction mixture was incubated in the dark at 60°C for 25 min. Proline (100 μl of a 100 mg per ml solution in distilled water) was added and the samples was incubated in darkness for a further 10 min at room temperature to convert excess dansyl chloride to dansyl proline. Toluene (500 μl) was added and the reaction mixture shaken for 20 s to extract the dansylated polyamines. An aliquot (25 μl) was then spotted onto silica gel TLC plates which had been incubated in an oven at 110°C for 90 min. The plates were developed in chloroform:triethylamine (12:1; v/v) and the polyamines visualized under UV light. Spots were scraped off the plate into 10 ml of Emulsifier Safe scintillant (Packard Bioscience) and radioactivity was counted as described above.

For the polyamine oxidase (PAO) assay, fungus (0.6 g) was ground using a mortar and pestle with 4 ml of a buffer containing 100 mM potassium phosphate and 2 mM dithiothreitol, adjusted to a pH of 8.0. The enzyme extract was centrifuged at 20,000 *g* for 20 min at 4°C, after which 0.5 ml of the supernatant and 0.15 μCi of [^{14}C] spermidine were added to 0.5 ml of a buffer containing 100 mM potassium phosphate, 1 mM spermidine and 30 μg catalase, adjusted to pH 8.0. The assay was carried out in 100 mm glass test tubes fitted with silicone rubber stoppers. Test tubes were incubated in a water bath at 37°C for 30 min before the reaction was stopped by adding 4 M sodium hydroxide (1 ml) and shaking. Enzymic products were extracted into toluene (2 ml), vortexed for 10 s and then left to stand for 30 min. An aliquot (1 ml) was taken from the toluene layer, transferred to a vial containing 10 ml of scintillation cocktail (Emulsifier Safe, Packard Bioscience) and radioactivity counted as described above.

Statistical analyses All values presented are the means of four replicates. All experiments were repeated with similar results and statistical significance was assessed using Student's *t*-test.

RESULTS AND DISCUSSION

Eugenol added to the liquid medium reduced growth of *B. fabae* at concentrations of 10 ppm and greater, with 30 ppm eugenol reducing fungal growth by 73% (Fig. 1). These growth reductions were accompanied by alterations on polyamine metabolism. Thus, growth of *B. fabae* in the presence of eugenol led to a fivefold increase in activity of the enzyme responsible for synthesis of the polyamines spermidine and spermine, AdoMetDC, although activity of the putrescine biosynthetic enzyme ODC was not altered significantly (Table 1). The increased AdoMetDC activity was confirmed by the sixfold increase in the flux of label from ornithine through to spermine (Table 2). Although the flux of label into spermidine was also increased, this difference was not significant. There was, however, a significant decrease in movement of label into the diamine putrescine (Table 2), which is not surprising, since putrescine is a substrate for AdoMetDC. These findings suggest that the concentration of free spermine should increase in eugenol-treated tissue, but there was no significant change in spermine level in *B. fabae* (Table 3). This could reflect enhanced spermine oxidation, although PAO activity was not significantly changed in fungus grown in eugenol (Table 1), nor was there enhanced excretion of spermine from fungal cells (data not shown). So, given the evidence for increased spermine formation in *B. fabae* treated with eugenol, what was the fate of the spermine? Polyamines can be acetylated in fungi (8) and it is possible therefore that the spermine could have been converted to acetylspermine. Although acetylspermine can be oxidized by PAO (8), it may have accumulated in eugenol-treated fungal tissue. At present this must remain as speculation, since levels of acetylated polyamines were not measured in *B. fabae* grown in the presence of eugenol. It would seem prudent to examine not just the formation of acetylated polyamines in eugenol-treated *B. fabae,* but also to conduct a more detailed examination of polyamine catabolism. Such studies would be worthwhile, since the toxic products of polyamine oxidation, including hydrogen peroxide, have been implicated in cell death induced by free polyamines (7), polyamine analogs (6) and plant resistance to fungal infection (16).

TABLE 1. Effect of eugenol on activities of enzymes of polyamine biosynthesis and catabolism in *Botrytis fabae*

Enzyme	Control	Eugenol
Ornithine decarboxylase (ODC) (pmol CO_2 [mg protein]$^{-1}$ h^{-1})	47±8.4	54±9.9
S-adenosylmethionine decarboxylase (AdoMetDC) (pmol CO_2 [mg protein]$^{-1}$ h^{-1})	14±0.9	73±5.0***
Diamine oxidase (DAO) (pmol product [mg protein]$^{-1}$ h^{-1})	348±65.6	481±127.8
Polyamine oxidase (PAO) (pmol product [mg protein]$^{-1}$ h^{-1})	92±3.4	105±6.2

Values are means of four replicates ±S.E. Significant difference from the control is shown as ***$P \leq 0.001$.

TABLE 2. Effect of eugenol on incorporation of radio-labeled ornithine into polyamines in *Botrytis fabae*

Treatment	Radioactivity in polyamines (dpm [mg protein]$^{-1}$)		
	Spermine	Spermidine	Putrescine
Control	48±5.7	24±8.5	52±10.4
Eugenol	314±36.3 ***	186 ±93.2	17±6.5 *

Values are means of four replicates ±S.E. Significant differences from the control are shown as *$P \leq 0.5$; ***$P \leq 0.001$.

TABLE 3. Effect of eugenol on concentrations of free polyamines in *Botrytis fabae*

Treatment	Free polyamine concentration (μmol g^{-1} fresh wt.)		
	Spermine	Spermidine	Putrescine
Control	1193±47.0	2300±131.1	629±92.7
Eugenol	1602±126.9	1858±65.6	747±279.5

Values are means of four replicates ±S.E.

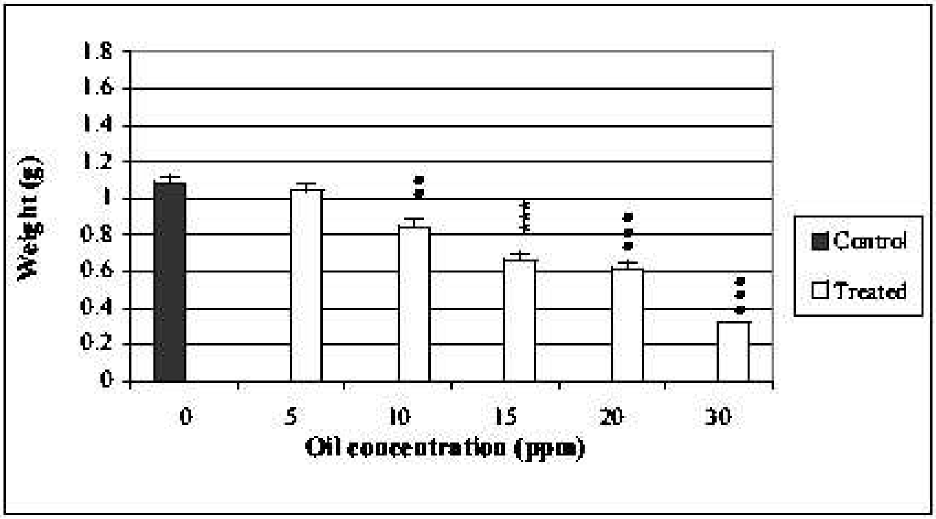

Fig. 1. Effects of various concentrations of eugenol on mycelial growth of *Botrytis fabae* in liquid culture. Values are the means of four replicates ±S.E. Significant differences from the control calculated using Student's *t*-test are shown as ** $P<0.01$; *** $P<0.001$.

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