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Ferulic acid mediated changes in oxidative enzymes of maize seedlings: implications in growth

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Abstract

Maize (*Zea mays* L. cv. Ganga-5) seedlings were grown in the presence of ferulic acid (0.5 - 3.0 mM) for 8 d. Treatment with ferulic acid considerably decreased shoot and root length, increased the activity of peroxidase, catalase and indole-3-acetic acid (IAA) oxidase and decreased the activity of polyphenol oxidase. The increased activity of peroxidase correlated with pronounced increase in content of lignin and phenolic compounds.

Additional key words: allelochemicals, catalase, IAA oxidase, peroxidase, polyphenol oxidase, root and shoot length.

Introduction

Phenolic compounds constitute a greater portion of plant secondary metabolites. Ferulic acid (FA), a derivative of cinnamic acid apart from its allelopathic nature (Blum and Rebbeck 1989, Holappa and Blum 1991) exists as a structural component in the cell walls of various monocots and dicots and is a precursor of lignin (Kamisaka *et al.* 1990, Tan *et al.* 1991, 1992, Locher *et al.* 1994). Modulation of several physiological and biochemical processes have been observed in plants grown in the presence of FA (Blum and Rebbeck 1989, Bergmark *et al.* 1992).

Phenolic compounds, including FA, are known to modify a variety of oxidative reactions *in vitro* either acting as substrates, activators or inhibitors depending on the concentration (Lee *et al.* 1982, Pang *et al.* 1989). The objective of this work is to examine the influence of exogenous FA on oxidative enzymes, synthesis of lignin and phenolic compounds and to relate these changes to growth of maize seedlings.

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Material and methods

Plant material: Maize (*Zea mays* L. cv. Ganga-5) seeds were surface sterilized with 5 % sodium hypochlorite solution for 15 min, washed with distilled water and soaked in water for 12 h. Seedlings were raised hydroponically with Hoagland's solution under irradiance of 89 μmol m^{-2} s^{-1}, temperature 25 to 30 °C and treated with 30 cm^3 of 0 - 3 mM FA (increment of 0.5 mM) for 8 d. After this period shoot and root length was determined.

Enzymes preparation: Leaves and roots of 8-d-old seedlings were cut and 1 g of tissue was extracted with 5 cm^3 of 100 mM sodium phosphate buffer, pH 7, for peroxidase, catalase, polyphenol oxidase and potassium phosphate buffer, pH 6, for IAA oxidase. The extract was centrifuged at 18 400 *g* for 20 min at 4 °C and 0.4 cm^3 of the supernatant was desalted on *Sephadex G-25* (20 × 5 mm). For peroxidases, desalted supernatant was used to assay soluble peroxidase and to isolate covalent and ionic peroxidases, the pellets were resuspended in Triton X-100 and centrifuged at 1 000 *g* for 5 min and then washed with water until no activity was detected in the supernatants. The pellets were then washed three times with 1 cm^3 of 1 M NaCl. The washes were pooled and used to assay ionically bound peroxidases. The NaCl washed pellets were resuspended in 2 cm^3 of 0.05 M sodium phosphate buffer (pH 7) containing 10 mg cellulase and incubated at 25 °C overnight. After a 10 min spin at 1 000 *g*, the supernatant was used to determine the activity of covalently bound peroxidase.

Peroxidase (E.C. 1.11.1.7) activity was measured according to Shinshi and Noguchi (1975). Reaction mixture (3 cm^3) contained 50 mM sodium phosphate buffer, pH 7, 0.2 mM guaiacol, 2 mM hydrogen peroxide and 50 μg of protein. The absorbance was read at 470 nm in *Shimadzu UV-160A* spectrophotometer (Japan). Enzyme activity was calculated using an extinction coefficient of 25.5 mM cm^{-1} for tetraguaiacol. One enzyme unit is the amount of protein required to produce 1 mM of tetraguaiacol per min.

Catalase (E.C.1.11.1.6) was assayed according to Abei (1975) in 3 cm^3 of reaction mixture containing 10 mM H_2O_2, 100 mM phosphate buffer and 25 μg protein. The change in absorbance was measured spectrophotometrically at 240 nm. One enzyme unit is the amount of protein required to bring about the change in absorbance of 1 (corresponds to 25 μmoles of H_2O_2 decomposed per 1 cm^3 per min).

IAA oxidase activity was assayed following the method of Talwar *et al.* (1985). Reaction mixture contained 0.2 mM 2,4-dichlorophenol (DCP), 0.2 mM $MnCl_2$, 20 μg of IAA and 50 μg of protein in a total volume of 1 cm^3. The reaction mixture was incubated for 15 min in dark and the reaction was stopped by the addition of 2 cm^3 of Salkowski's reagent and incubated in dark for 30 min. The destruction of IAA was determined by recording the absorbance at 530 nm. One unit of enzyme is the amount of protein required to oxidize 1 μg of IAA.

Polyphenol oxidase (E.C.1.14.18.1) activity was measured according to Tremolieres and Bieth (1984). Reaction mixture (1 cm^3) contained 100 mM sodium phosphate buffer, pH 7, 10 mM catechol and 100 μg of protein. One unit of enzyme

is the amount of protein required to bring about the change in the absorbance of 0.001 per min.

Extraction and estimation of phenolic compounds: Phenolic compounds were extracted with 75 % ethanol at 4 °C. The homogenate was centrifuged at 20 000 *g* for 20 min. Aliquots of the supernatant were used to determine the phenolics colorimetrically according to Seigler *et al.* (1986). After colour development (2 h), the absorbance at 725 nm was measured and phenolic concentration was determined using tannic acid as standard and expressed as mg phenolics per g of fresh mass.

Extraction and estimation of lignin: Samples (leaves and roots) were freeze dried and ground to pass through a 60 μm mesh sieve. Samples were pre-extracted with water at 100 °C for 30 min, with 3 % SDS (120 °C) for 60 min, 85 % alcohol (75 °C) for 30 min and acetone (56 °C) for 30 min. After the final extraction, the material was filtered and extracted free material was left to dry over night at 30 °C. Extracted free samples (10 mg) were weighed into 50 cm^3 volumetric flasks and digested with 2.5 cm^3 of acetyl bromide for 30 min at 70 °C dry heat. After cooling on ice, 15 cm^3 of 2 M NaOH and glacial acetic acid mixture (1:5) was added to the digest. Before bringing the mixture to volume with acetic acid, 0.25 cm^3 of 7.5 M hydroxylamine hydrochloride was added to each flask. The absorbance at 280 nm, A_{280}, was measured after 2 h. The amount of lignin was expressed as percentage of final dry mass following the formula: $(A_{280} \times 100)/(\varepsilon \times \text{final dry mass [g]})$, with an extinction coefficient of lignin standard $\varepsilon = 24$. All the experiments were repeated four times and the results were analyzed for significance following the Student's *t*-test.

Chemicals: Ferulic acid was purchased from *Fluka Chemie AG*, (Switzerland) IAA, guaiacol and catechol were from *Sigma Chemical Co* (USA). All chemicals were of analytical grade.

Results

Application of FA (1 - 3 mM) caused considerable decrease in the growth of maize seedlings, both of shoots and roots (Fig. 1).

The activity of peroxidase (soluble, ionic and covalent) increased over control in leaves and roots (Table 1, 2). Catalase activity of leaves and roots responded differently to FA treatment. In leaves, FA up to 1.5 mM increased the activity up to 19 % while 2.5 - 3 mM FA decreased the activity up to 31 %. Roots, in contrast to leaves, exhibited a significant concentration dependent increase (12 - 296 %) in catalase activity (Table 3). IAA oxidase activity also increased to greater extent in leaves and roots with FA application (Table 4). Compared to the other oxidative enzymes studied, FA exerted lesser influence on polyphenol oxidase activity of leaves as well as roots. FA concentrations lower than 3 mM did not alter the activity of polyphenol oxidase (Table 5). However, 3 mM FA decreased the activity in leaves and in roots (Table 5).

In addition to changes in the activity of oxidative enzymes, an increase in phenolic and lignin contents were observed in FA treated seedlings. In leaves and roots phenolic contents increased (Table 6). Lignin content of treated seedlings increased in roots while decreased in leaves (Table 7).

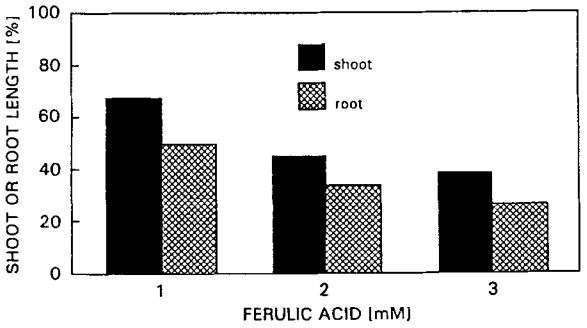

Fig. 1. Effect of ferulic acid on shoot and root length (in % of control) of 8-d-old maize seedlings.

Table 1. Effect of ferulic acid (FA) on peroxidase activity [pkat g^{-1}(f.m.) s^{-1}] in leaves of maize seedlings.

FA [mM]	Peroxidase soluble	ionic	covalent
control	36.1 ± 3.1	6.0 ± 0.23	6.1 ± 0.32
0.5	42.1 ± 5.5 (+16.6)	6.4 ± 0.27 (+ 6.6)	9.3 ± 0.41 (+ 52.4)**
1.0	45.3 ± 9.2 (+25.4)	7.1 ± 0.13 (+18.3)	9.5 ± 0.50 (+ 55.7)**
1.5	45.1 ± 6.9 (+24.9)	7.5 ± 0.23 (+25.0)	9.9 ± 0.98 (+ 62.2)**
2.0	48.6 ± 6.4 (+34.6)*	8.3 ± 0.32 (+38.3*	10.6 ± 0.27 (+ 73.7)**
2.5	50.0 ± 5.5 (+38.5)*	8.3 ± 0.37 (+38.3)*	13.5 ± 0.32 (+121.3)**
3.0	52.7 ± 6.4 (+45.9)**	10.7 ± 0.41 (+78.3)**	13.6 ± 0.55 (+122.9)**

Table 2. Effect of ferulic acid (FA) on peroxidase activity [pkat g^{-1}(f.m.) s^{-1}] in roots of maize seedlings.

FA [mM]	Peroxidase soluble	ionic	covalent
control	123.6 ± 10.1	91.2 ± 7.4	108.3 ± 8.7
0.5	157.9 ± 12.5 (+27.7)	93.0 ± 6.4 (+1.9)	131.0 ± 12.0 (+20.9)
1.0	143.5 ± 8.7 (+16.1)	122.2 ± 11.5 (+33.9)*	120.3 ± 7.4 (+11.1)
1.5	164.8 ± 13.0 (+33.3)*	133.3 ± 15.7 (+46.1)**	138.8 ± 7.4 (+28.2)
2.0	171.2 ± 11.1 (+38.5)*	149.5 ± 9.2 (+63.9)**	143.5 ± 12.5 (+32.5)*
2.5	178.7 ± 14.3 (+44.5)**	172.2 ± 9.7 (+88.8)**	153.2 ± 8.3 (+41.4)**
3.0	181.0 ± 13.4 (+46.4)**	170.4 ± 14.8 (+86.8)**	156.5 ± 12.0 (+44.5)**

Table 3. Effect of ferulic acid (FA) on catalase activity [pkat mg^{-1}(protein) s^{-1}] in leaves and roots of maize seedlings.

FA [mM]	Catalase leaves	roots
control	55.5 ± 10.6	12.7 ± 2.0
0.5	49.7 ± 8.6 (-10.4)	14.3 ± 1.8 (+ 12.5)
1.0	57.7 ± 11.6 (+ 3.9)	14.4 ± 2.7 (+ 13.3)
1.5	66.1 ± 12.6 (+19.0)	19.7 ± 4.5 (+ 55.1)**
2.0	49.8 ± 7.6 (-10.2)	22.5 ± 2.4 (+ 77.1)**
2.5	37 9 ± 5.0 (-31.7)*	27.5 ± 5.1 (+116.5)**
3 0	38.3 ± 4.6 (-30.9)*	50.6 ± 9.7 (+298.4)**

Figures in parentheses are % of increase (+) or decrease (-) over control; * - $P < 0.005$, ** - $P < 0.001$.

Table 4 Effect of ferulic acid (FA) on the activity of IAA oxidase [pkat mg^{-1}(protein) s^{-1}] in leaves and roots of maize seedlings.

FA [mM]	IAA oxidase leaves	roots
control	280 ± 40	400 ± 50
0.5	340 ± 50 (+ 21.4)	470 ± 20 (+ 17.5)
1.0	430 ± 30 (+ 53.5)**	500 ± 60 (+ 25.0)
1.5	510 ± 40 (+ 82.1)**	3008 ± 70 (+652.0)**
2.0	550 ± 50 (+ 96.4)**	3140 ± 70 (+685.0)**
2.5	600 ± 30 (+114.2)**	3180 ± 60 (+695.0)**
3.0	680 ± 10 (+142.8)**	3180 ± 60 (+695.0)**

Table 5. Effect of ferulic acid (FA) on the activity of polyphenol oxidase [pkat mg^{-1}(protein) s^{-1}] in leaves and roots of maize seedlings.

FA [mM]	Polyphenol oxidase leaves	roots
control	8.50 ± 0.85	19.81 ± 3.33
0.5	8.46 ± 0.78 (- 0.4)	22.45 ± 3.16 (+13.3)
1.0	7.96 ± 1.28 (- 6.3)	19.44 ± 3.67 (- 1.8)
1.5	8.18 ± 0.47 (- 3.7)	20.71 ± 2.67 (+ 4.5)
2.0	7.35 ± 0.94 (+13.5)	18.52 ± 2.21 (- 6.5)
2.5	7.52 ± 0.84 (-11.5)	16.56 ± 2.79 (-16.4)
3.0	6.09 ± 1.27 (-28.3)	13.62 ± 3.49 (-31.2)*

Figures in parentheses are % of increase (+) or decrease (-) over control; * - $P < 0.005$, ** - $P < 0.001$.

Table 6. Effect of ferulic acid (FA) on content of phenolic compounds [mg g^{-1}(f.m.)] in leaves and roots of maize seedlings.

FA [mM]	Phenolic compounds leaves	roots
control	2.43 ± 0 17	1.33 ± 0.11
0.5	2.79 ± 0.09 (+ 14.8)	1.49 ± 0.09 (+ 12.0)
1.0	3.04 ± 0.11 (+ 25.1)	1.89 ± 0.23 (+ 42.1)**
2.0	3.58 ± 0.27 (+ 47.3)**	2.69 ± 0.18 (+102.2)**
2.5	4.24 ± 0.28 (+ 74.4)**	3.27 ± 0.22 (+145.8)**
3.0	5.04 ± 0.11 (+107.4)**	3.96 ± 0.35 (+197.7)**

Table 7. Effect of ferulic acid (FA) on lignin content [%(d.m.)] in leaves and roots of maize seedlings.

FA [mM]	Lignin content leaves	roots
control	37.63 ± 4.65	42.52 ± 5.26
0.5	37.69 ± 3.29 (+ 0.1)	44.49 ± 3.77 (+ 4.6)
1.0	34.77 ± 3.96 (- 7.6)	49.55 ± 4.67 (+16.5)
1.5	33.13 ± 4.42 (-11.9)	54.25 ± 4.00 (+27.5)
2.0	33.94 ± 5.36 (- 9.8)	57.60 ± 6.97 (+35.4)**
2.5	31.05 ± 4.13 (-17.4)	60.33 ± 5.84 (+41.8)**
3.0	28.66 ± 3.46 (-23.8)	66.55 ± 6.65 (+56.5)**

Figures in parentheses are % of increase (+) or decrease (-) over control; ** - $P < 0.001$.

Discussion

Application of FA considerably decreased the growth of maize seedlings (Fig. 1) as observed earlier (Blum and Rebbeck 1989, Tan *et al.* 1991, 1992). The activities of oxidative enzymes increased significantly as a result of FA treatment. Significant increase in peroxidase activity (soluble, ionic and covalent) has been observed in leaves as well as roots (Table 1 and 2). In plants the localization of peroxidases depends on their function (Grison and Pilet 1984). Soluble peroxidases associated with cytoplasm are known to catalyze most of the peroxidative reactions in the cell (Ridge and Osborne 1970) while bound peroxidases catalyze the oxidative polymerization of lignin precursors during lignin synthesis (Van Huystee and Zheng 1993, McDougall *et al.* 1994). Accordingly, FA treated seedlings showed increased peroxidase activity accompanied by increased levels of phenolic acids and lignins (Tables 6 and 7). The increase in activity of peroxidase observed in the present study could possibly to facilitate the polymerization of lignin precursors (FA) during lignin synthesis. Similar increase in peroxidase activity associated with binding of FA to cell walls has been reported earlier (Whitemore 1976, Van Huystee and Zheng 1993). Furthermore, Shann and Blum (1987) demonstrated a substantial increase in

peroxidase activity associated with an increase in lignin in barley with exogenous FA application. FA being the precursor of lignin (Shann and Blum 1987), the increase of lignin and phenolic compounds in FA treated seedlings suggest that the exogenously supplied FA might have been diverted to the synthesis of lignin and various phenolic intermediates.

In plants accumulation of phenolic compounds in the cell walls is known to decrease their extensibility and lignification causes cell walls mechanically rigid (Kamisaka *et al.* 1990, Tan *et al.* 1991, 1992). Thus, the inhibition in growth of maize could be related to an increase in phenolic and lignin contents which make the cell wall mechanically rigid. In addition to their role in lignin synthesis, peroxidases are known to catalyze the oxidation of phenolic compounds both *in vitro* and *in vivo* at the expense of H_2O_2 and free radicals are produced during the course of action (Spiker *et al.* 1992, Takahama and Oniki 1992). The increased peroxidase (Table 1, 2) which coincided with increased level of phenolic compounds (Table 6) suggests its role in phenolic oxidation as proposed by Hrubcová *et al.* (1992) since, many of the phenolic compounds act as natural substrates for peroxidases.

In addition to peroxidase, catalase acts on H_2O_2 and other free radicals thus protects the cells from toxic effects of peroxide radicals (Havir and McHale 1990). Stimulation of catalase in FA treated roots could possibly facilitate the removal of H_2O_2 and other peroxides in the face of increased oxidative reactions (H_2O_2-oxidation, IAA oxidation and phenol oxidation; Tables 1, 2, 3, 4) due to FA. In contrast to roots, a decrease in the catalase activity was observed in leaves suggesting existence of tissue specific regulation of the catalase activity by FA.

Apart from peroxidase and catalase, FA treatment resulted in significant increase in the activity of IAA oxidase in leaves as well as roots (Table 4). It is evident from the earlier studies that many of phenolic compounds, including FA, are known to induce a lag in the IAA oxidation *in vitro* acting as protectants of IAA oxidation (Gelinas 1973). However, *in vivo* regulation of IAA oxidase by the phenolic compounds is under debate. The increased activity of IAA oxidase in FA treated seedlings suggests that different mechanism might operate *in vivo* regulating IAA level. It has been shown earlier, that peroxidases in addition to their role in lignin synthesis also catalyze the oxidation of IAA (Gebhardt 1982, Zheng and Van Huystee 1992). Further Talwar *et al.* (1985) reported the association of peroxidase and IAA oxidase with the same protein. The increased activity of peroxidase (Table 1, 2) and IAA oxidase (Table 4) in FA treated leaves and roots described in the present study suggested that peroxidase might have been involved in the oxidation of IAA to a certain extent in addition to a general increase in IAA oxidase. In view of the inverse correlation of IAA oxidase to growth, the increase in IAA oxidase activity may influence the rate of IAA destruction and there by decrease the growth of plants.

Since the phenolic metabolism includes high turnover of enzymes involved, modifications in the activities of enzymes involved in synthesis are expected after exogenous additional supply of phenolic compounds (FA). In plants polyphenol oxidase catalyzes the conversion of monophenols to diphenols (Tremolieres and Bieth 1984). Though a general increase has been observed in the levels of phenolic compounds in FA treated seedlings (Table 6), polyphenol oxidase exhibited a lower

sensitivity to FA (Table 5) as only 3 mM FA could decrease the activity up to 31 %. In plants the polyphenol oxidase activity is regulated since its activity would modulate the levels of *o*-diphenols and quinones which are toxic to plants and inhibitory for IAA oxidase activity. This further suggests the relation between IAA oxidase and polyphenol oxidase.

These results suggest that the oxidative enzymes are acting in a co-ordinated manner in the regulation of lignin, phenolic compounds and IAA. Hence, the observed changes in the enzymes and metabolic products induced by FA could bring about considerable changes in growth of FA treated plants. In conclusion, the inhibitory effect of FA on growth of maize seedlings might be explained by accumulation of growth inhibitory compounds such as phenolic compounds and lignin and by lowering the growth promotory compounds such as IAA via modulating the enzymes involved in their regulation which might have acted together in bringing out the reduction in growth of the seedlings.

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