Metolachlor in Corn (*Zea mays*) and Soybean (*Glycine max*): Persistence and Biochemical Signs of Stress during Its Detoxification†

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Metolachlor translocation and persistence were investigated in corn (*Zea mays* L.) and soybean (*Glycine max* L.) during a 12-day period after treatment. Herbicide uptake was rapid, and its detoxification resulted in small residues of the parent molecule in soybean and negligible residues in corn by the 12th day. The accumulation of metolachlor in the roots and in the shoots was found to be greater in soybean than in corn seedlings. This seems to be due to a faster detoxification in corn than in soybean tissues because of the higher herbicide-induced activity of glutathione *S*-transferase (GST, EC 2.5.1.18) in the corn than in the soybean. A reduction of dry matter, protein, and chlorophyll content in the treated seedlings was observed. A decrease in chlorophyll appeared to be a consequence of reduced activity of 3-aminolevulinate dehydratase (ALA-D, EC 4.2.1.24). The increased activities in the treated seedlings of phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), and of tyrosine ammonia-lyase (TAL, EC 4.3.1), key enzymes in phytoalexin biogenesis, confirmed the metolachlor-induced stress status.

INTRODUCTION

Metolachlor [2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl)acetamide] is a chloroacetanilide herbicide that provides excellent control of many annual grasses and broad-leafed weeds. Therefore, it is widely and successfully used in several major crops, including corn and soybean. Its primary biochemical mechanism of action is unknown; nevertheless, many investigators have observed interferences with growth, protein synthesis, membrane permeability ion transport, endogenous phytohormone levels, enzyme activities, etc. in several plant species (Fedtke, 1982a,b; Mellis et al., 1982; Egli et al., 1985). It has been observed that the tolerant plants, including corn and soybean, are able to detoxify the chloroacetanilide herbicides at sufficient rates to prevent their accumulation and persistence at phytotoxic levels. It has also been recognized that the conjugation of these herbicides with glutathione (GSH) or homoglutathione (h-GSH) by action of GST is major detoxification pathway in plants (Le Baron et al., 1988).

This research was undertaken to ascertain the accumulation and persistence of metolachlor in corn and soybean seedlings; the interference of the metolachlor residues on some biochemical parameters was also investigated to evaluate possible stress signs during detoxification of the herbicide. Hence, the GST activity as well as dry matter, protein, and chlorophyll contents and the PAL and TAL activities were checked.

MATERIALS AND METHODS

Chemicals. Analytical grade metolachlor was supplied by Ciba-Geigy; reduced glutathione and Brilliant Blue G were obtained from Aldrich Chemie (Steinheim, Germany). Porphobilinogen (PBG) was from Calbiochem-Behring Corp. (La Jolla, CA), and 3-aminolevulinic acid (ALA) chlorohydrate, *trans*-cinnamic acid, and *p*-coumaric acid were from Fluka Chemie AG (Buchs, Switzerland). Acetonitrile (HPLC grade) and water (HPLC grade) were purchased from BDH (Poole, England). All other reagents employed were of ACS grade.

Apparatus. A Perkin-Elmer Model 8310 gas chromatograph equipped with a 2-m column packed with 3% OV 17 on 80–200 mesh Gas-chrom Q, and a nitrogen/phosphorus selective detector was used. The HPLC instrument was assembled from the following modular components: two Perkin-Elmer Series 410 LC pumps, a Rheodyne Model 7125-075 injector, a Perkin-Elmer Model LC 235 diode array detector, and an LC column, 25 cm × 4.6 mm i.d., C_{18} reversed phase with a Supelguard LC_{18} guard column (Supelco Inc., Bellefonte, PA). A Varian Model Cary 210 double-beam grating spectrophotometer was used.

Plant Material and Growth Conditions. Corn (*Zea mays* L., hybrid 204) and soybean (*Glycine max* L., Cutler 71) seeds obtained from Agricultural Central Research Ministry of Agriculture Cairo (Egypt) were used. The seeds (100) were placed on filter papers in growth dishes (656 cm^2) moistened with water in a growth chamber in the dark at 25 ± 0.5 °C and a relative humidity of 75 ± 3%. After 4 days, a treatment with metolachlor at the field dose (3.24 kg/ha) was performed. To apply the required concentration, the suggested rate per hectare was calculated according to the surface area per dish, and then the herbicide was mixed in a suitable amount of water, enough to spray the surface area twice, in one direction and crosswise. At this stage (zero time), the seedlings were submitted to day–night conditions (12 h of light, 5000 lux, and 12 h of darkness) at 25 ± 0.5 °C. Samples were collected at zero time and at 4-day intervals during the following 12 days.

Determination of Metolachlor Residues. The metolachlor residues in plant tissues and in growth dishes were determined according to the method of Marucchini et al. (1988). Tissues were extracted with methanol, and the methanolic extract was purified by use of acidic alumina and Florisil. Recovery varied from 88 to 94%, and the limit of sensitivity was 0.01 mg/kg.

Protein Extraction and Determination. The samples of treated and untreated shoots and roots (15 g) were cut into pieces and homogenized by sonication with chilled acetone (−20 °C) (75 mL) for 1 min. The crude homogenates were filtered through a Büchner funnel and washed with chilled acetone. The residues were spread on filter paper and allowed to dry at room temperature to obtain the acetone powders (Harborne, 1988). For the complete protein extraction, aliquots of powders (0.5 g) were covered with buffer solution (25 mL; Tris-HCl, 0.05 M, pH 9.0) and allowed to stand for 10 min at 4 °C; the mixtures were then centrifuged at 48200*g* for 15 min at 4 °C. The total protein content of the supernatant was determined spectrophotometrically by reaction with Brilliant Blue G according to the Bradford (1976) procedure and expressed according to fresh weight. The su-

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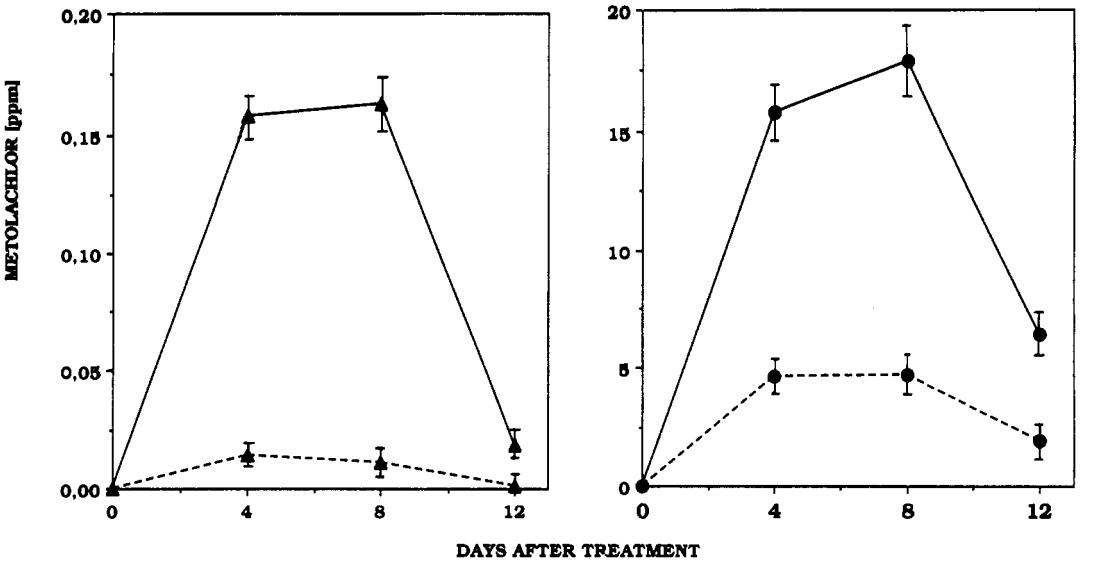

Figure 1. Concentrations of metolachlor in corn (▲) and soybean (●) roots (—) and shoots (- - -) after a treatment equivalent to 3.24 kg/ha. Each value is the mean ± SD of three determinations.

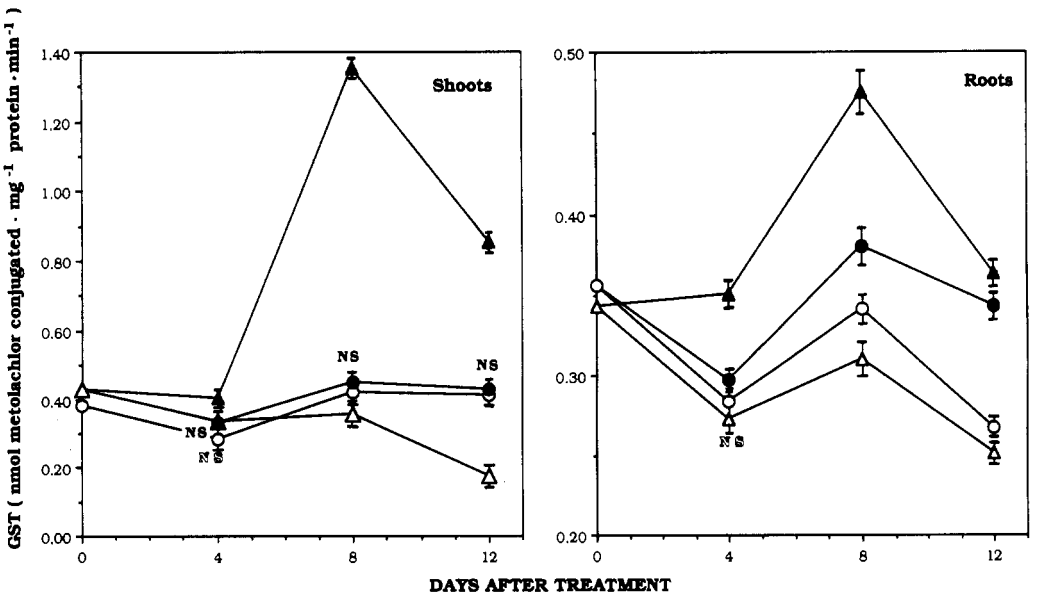

Figure 2. Activity of glutathione *S*-transferase in treated and untreated corn (▲ and △) and soybean (● and ○) seedlings. Each value is the mean ± SD of three determinations. **NS**, not significantly different from the respective untreated control; NS, no significative difference between untreated corn and soybean ($P < 0.05$, on the basis of LSD test).

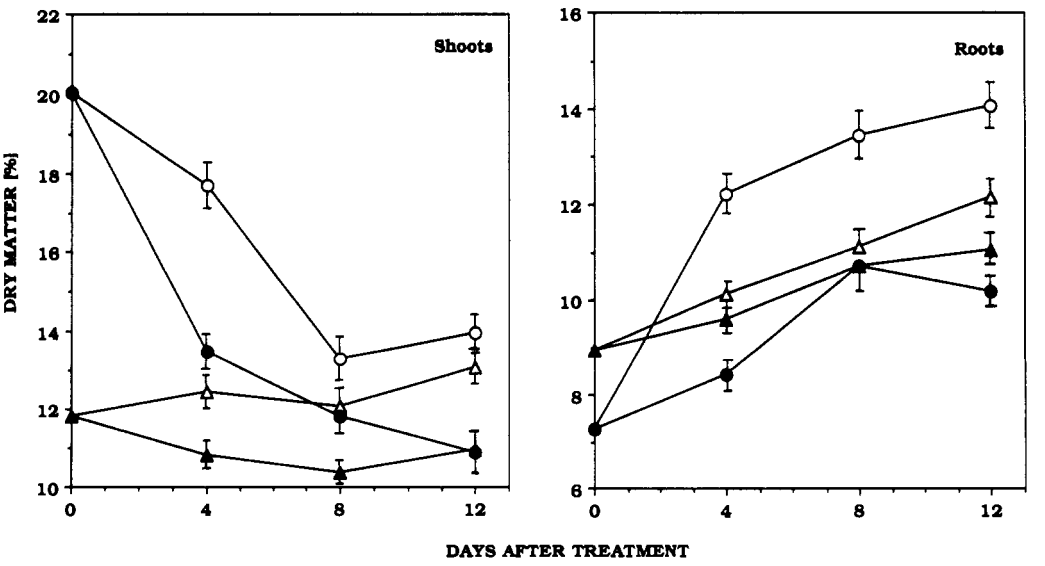

Figure 3. Dry matter contents in treated and untreated corn (▲ and △) and soybean (● and ○) seedlings. Each value is the mean ± SD of three determinations.

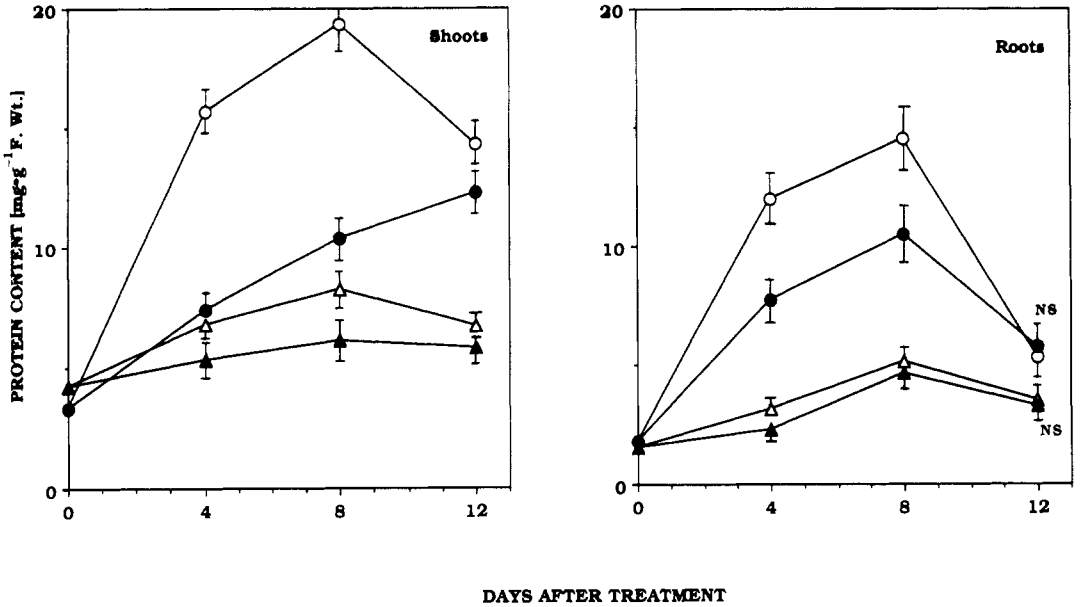

Figure 4. Protein content in treated and untreated corn (▲ and △) and soybean (● and ○) seedlings. Each value is the mean ± SD of three determinations. **NS**, not significantly different from the respective untreated control ($P < 0.05$, on the basis of LSD test).

Table I. Correlation Coefficients between Metolachlor Residues and Some of the Investigated Biochemical Parameters

	corn		soybean	
	roots	shoots	roots	shoots
dry matter decrease	0.633*	0.959**	0.803**	0.105
protein decrease	0.820**	0.682*	0.974**	0.998**
ALA-D decrease		0.765**		0.873**
PAL increase	0.955**	0.889**	0.970**	0.989**
TAL increase	0.864**	0.495	0.871**	0.820**

[a] The values followed by * or ** are significant at $P < 0.05$ or $P < 0.01$, respectively.

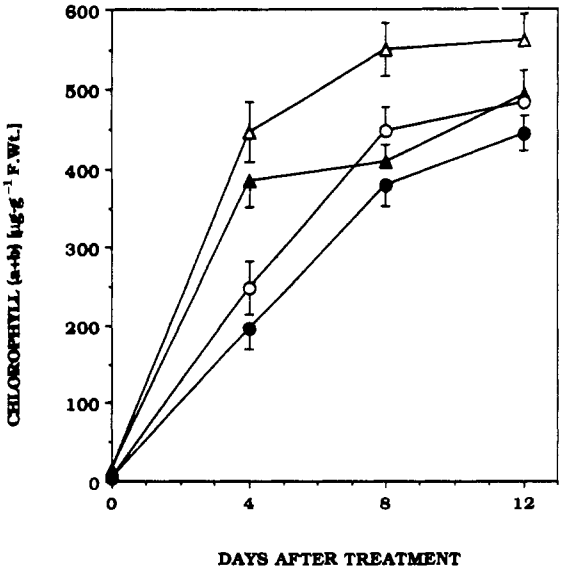

Figure 5. Contents of chlorophyll ($a + b$) in treated and untreated corn (▲ and △) and soybean (● and ○) seedlings. Each value is the mean ± SD of three determinations.

pernatants were also used as crude enzyme extracts for the determination of GST and ALA-D activities.

Assay of GST Activity. The activity of GST was evaluated from the rate of metolachlor–GSH conjugation in reaction mixtures prepared according to the method of Gronwald et al. (1987) and analyzed by an HPLC procedure (Scarponi et al., 1991a,b). To 3.5 mL of phosphate buffer (0.1 M, pH 7.4), which had been deareated by bubbling N_2 through it, were added in sequence a suitable volume of enzyme extract so as to have 2.5

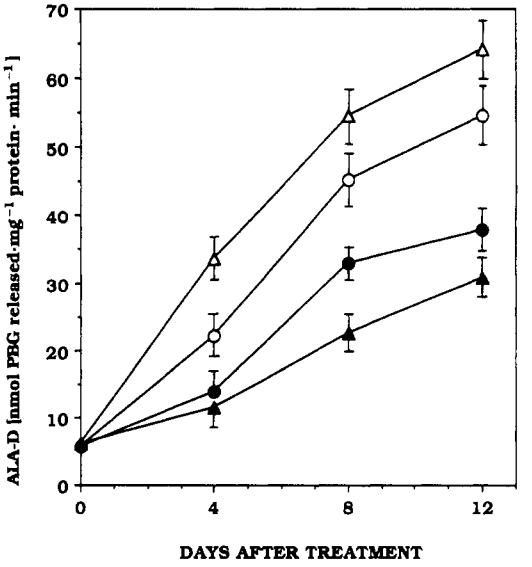

Figure 6. Activity of 3-aminolevulinate dehydratase in treated and untreated corn (▲ and △) and soybean (● and ○) seedlings. Each value is the mean ± SD of three determinations.

mg of protein in the reaction mixture, 10 μmol of GSH in 0.5 mL of the phosphate buffer, and 160 nmol of metolachlor dissolved in the phosphate buffer. The final volume of the mixture was adjusted at 6.0 mL with the phosphate buffer. After 2 h of incubation at 35 °C, the reaction was stopped by freezing in a dry ice–acetone bath and the reaction mixtures were then lyophilized. To check eventual herbicide losses by nonconjugating reactions, control tests were carried out substituting GSH solution with 0.5 mL of the phosphate buffer. The lyophilized material was extracted with 5 mL of methanol and centrifuged at 34800g for 10 min at 4 °C. The supernatant was utilized to determine the residual nonconjugated herbicide. The following isocratic system was employed for the HPLC determination: mobile phase, water–acetonitrile (1:9); flow rate, 1 mL/min; detection, 220 nm.

Assay of ALA-D Activity. ALA-D activity was measured by the amount (milligrams) of PBG formed per hour in 4 mL of incubation mixture containing the enzyme extract (3.6 mL) and a solution (0.4 mL) of ALA (5 mg/mL) in Tris-HCl buffer (0.05 M, pH 7.0). The mixtures were allowed to react at 34 °C for 1 h and then stopped by addition of a solution of 0.1 M $HgCl_2$ in 10% CCl_3COOH (2 mL). The quantity of PBG formed was determined spectrophotometrically by reaction with dimethylaminobenzaldehyde at 555 nm (Mauzerall and Granick, 1956).

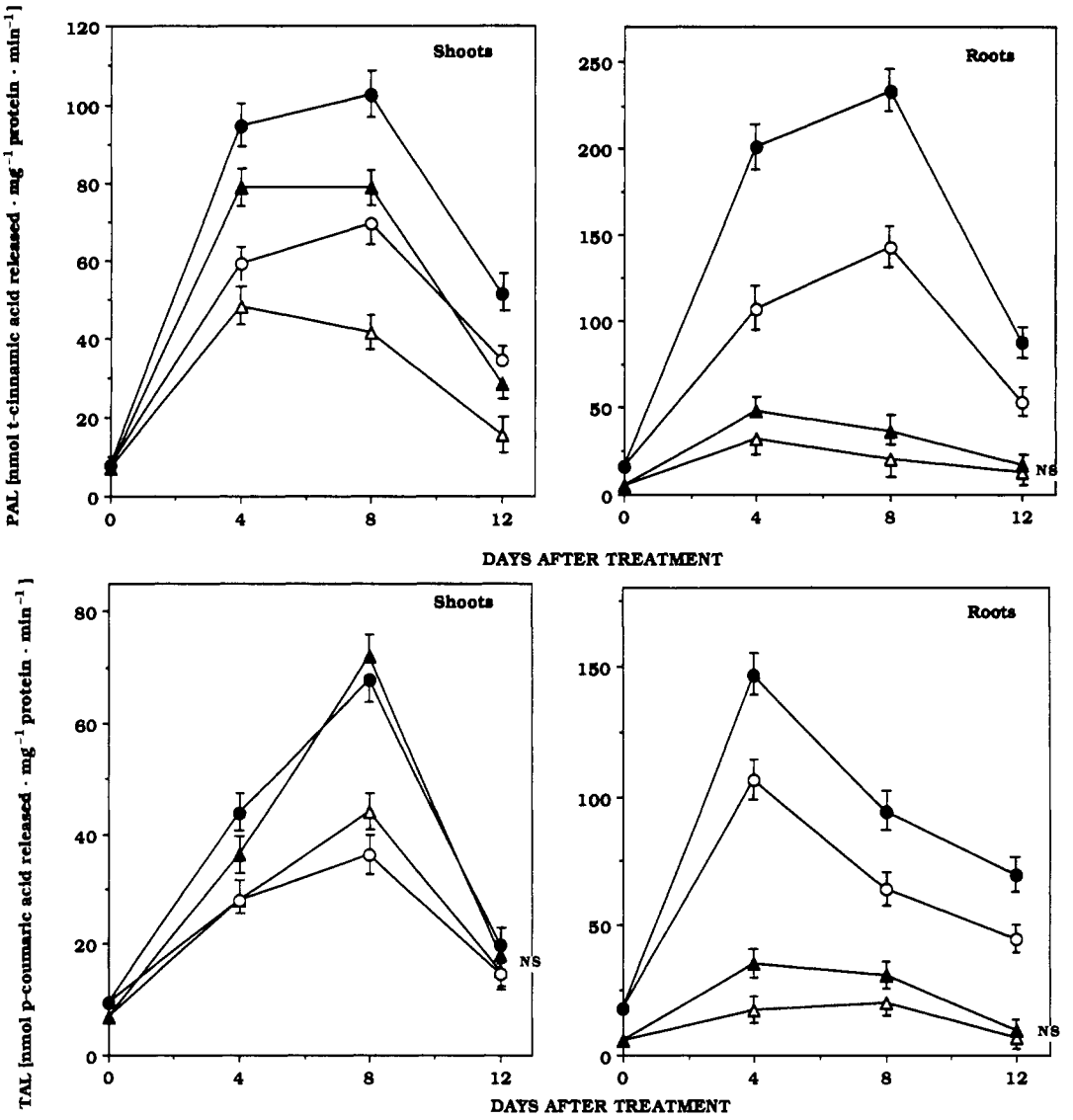

Figure 7. Activities of phenylalanine ammonia-lyase and tyrosine ammonia-lyase in treated and untreated corn (▲ and △) and soybean (● and ○) seedlings. Each value is the mean ± SD of three determinations. **NS**, not significantly different from the respective untreated control ($P < 0.05$, on the basis of LSD test).

Determination of Chlorophyll. Chlorophylls *a* and *b* were determined in the fresh tissues after extraction with 85% acetone according to the spectrophotometric method described by Metzner et al. (1965).

Assay of PAL and TAL Activities. Samples of treated and untreated shoots and roots were cut into pieces and homogenized by sonication with Tris-HCl buffer (0.05 M, pH 8.4) containing 15 mM 2-mercaptoethanol. The homogenates were centrifuged at 40000*g*, and the supernatants were used as enzyme preparations. All steps of the enzyme extraction were carried out at 0–4 °C. The extracts were assayed in PAL and TAL activities according to the methods of Beaudoin-Egan and Thorpe (1985). The reaction mixture in a final volume of 1 mL contained 500 μmol of Tris-HCl buffer (pH 8), 100 μL of enzyme preparation, and either 6 μmol of L-phenylalanine for PAL or 5.5 μmol of L-tyrosine for TAL. The reactions were stopped after 70 min of incubation by the addition of 50 μL of 5 N HCl. The amount of *trans*-cinnamic and *p*-coumaric acids formed was determined by measuring the absorbance at 290 and 333 nm, respectively.

Each plant extraction procedure and subsequent determination of metolachlor residue, enzyme activities, and other assays were replicated three times. All data were statistically analyzed using the least significant differences (LSD) method (Snedecor and Cochran, 1980).

RESULTS AND DISCUSSION

The metolachlor uptake was fast: 4 days after treatment only 2.7 ± 0.3 and 1.8 ± 0.2% of the applied herbicide was found in the growth dishes of soybean and corn, respectively. No detectable residues were found in the growth dishes after 8 and 12 days. Figure 1 shows that the persistence and translocation of metolachlor followed a similar trend in corn and soybean seedlings, but the amounts of parent molecule accumulated in the two plants were quite different: at 8 days 17.90 ppm of herbicide was present in the roots of soybean and only 0.16 ppm in corn. An efficacious detoxification of the parent molecule occurred, so that herbicide residues were greatly reduced in soybean and almost negligible in corn by the 12th day. Figure 1 also shows that a greater portion of metolachlor accumulated in the shoots of soybean than in corn. Four days after treatment, the amounts of herbicide in soybean and corn shoots were 29.5 and 9.2% of the respective levels in the roots. This agrees with the findings of Le Baron et al. (1988), who reported that 2-chloroacetanilides are easily taken up by the roots of dicotyledonous plants and that their translocation is mostly by acropetal movement.

Considering the rapid disappearance of metolachlor in the growth dishes and the amount of its residues in the two plants, a faster detoxification in corn than in soybean tissues can be hypothesized.

The major detoxification pathway of metolachlor in plants is its conjugation with GSH or h-GSH by the action

of GST (Breaux et al., 1987; Le Baron et al., 1988); therefore, the specific GST activity with metolachlor as substrate was investigated in roots and shoots of both untreated and treated samples (Figure 2). In general, the GST activity of the untreated samples was significantly greater in soybean than in corn seedlings, except at the fourth day when no significant differences were found. In the treated samples, higher GST activity in shoots of corn and in roots of both plants was found in comparison to the respective untreated samples; furthermore, the increases in roots were greater in corn than in soybean. Consequently, the GST activity in treated seedlings was always greater in corn than in soybean. This suggests that metolachlor residues have a stimulating effect on the activity of corn and soybean GST. A similar effect has been observed in sorghum (Dean et al., 1990) and with alachlor treatments in corn and soybean (Scarponi et al., 1991a,b). The occurrence of GST induction by residues of other pesticides has also been shown by Feng and Patanella (1989) in the liver cytosol of male rats and by Shivanandappa and Rajendran (1987) in *Trogoderma granarium*. Why this GST metolachlor activation was more efficient in corn than in soybean seedlings is difficult to explain and needs further investigation. Nevertheless, it could confirm the hypothesis that the lower accumulation of metolachlor in corn as compared to that in soybean can derive from its more efficient detoxification by conjugation.

Even though detoxification maintained the metolachlor residues at sublethal levels for both seedlings, the responses of some biochemical parameters were checked to identify possible injury and stress symptoms. Reduction of seedling growth and inhibition of protein synthesis have often been referred to as the first signs of metolachlor injury (Pillai et al., 1979; Deal et al., 1980; Ellis et al., 1983); therefore, the dry matter, protein, and chlorophyll contents as well as the ALA-D activity were checked.

Dry matter and protein were always significantly less in the treated than in the untreated seedlings (Figures 3 and 4). However, the roots of both corn and soybean were able to bring about a complete recovery from the herbicide action with respect to protein synthesis 12 days after treatment. The decreases in dry matter and protein were greater in soybean than in corn seedlings compared to those of the respective untreated controls. This seems to be consistent with the greater metolachlor accumulation in soybean than in corn tissues, because of the generally significant correlations found between herbicide residues and decreases in dry matter and protein (Table I).

As shown in Figure 5, the chlorophyll contents were less in the treated than in the respective untreated shoots. Among the physiological processes supposed to be directly affected by metolachlor treatment, reduction of pigmentation is not clearly referred to in the literature. Nevertheless, the behavior of ALA-D was investigated because it is an enzyme involved in the biosynthesis of porphyrins, which are precursors of chlorophyll. Significant decreases in ALA-D activity were found in the treated with respect to the untreated corn and soybean shoots. In a previous work (Scarponi et al., 1989) it was found that metolachlor affects the V_{max} values of ALA-D in corn but does not modify its K_M values. Hence, it was concluded that metolachlor induces a reduction in ALA-D synthesis rather than affects the catalytic efficiency by acting on the structural factors related to K_M values. These findings support the hypothesis that the decreases in chlorophyll in the treated corn and soybean shoots directly or indirectly might be related to a reduced ALA-D biosynthesis. This, in turn, appears to be an indirect consequence of a stress status determined by metolachlor treatment, as shown by decreases in dry matter and protein.

Tentative confirmation and evaluation of metolachlor-induced stress status were undertaken by investigating PAL and TAL activities. PAL and TAL deaminate phenylalanine and tyrosine to yield *trans*-cinnamic and *p*-coumaric acids, respectively. They are considered key enzymes in the biogenesis of the phytoalexins (Camm and Tower, 1977; Hoagland and Duke, 1981), which are natural defense compounds produced in plant tissues in response to biotic and abiotic infections (Baily, 1982; Komives and Casida, 1983).

The data reported in Figure 7 show significant increases in PAL and TAL activities in the treated with respect to the untreated seedlings. Twelve days after treatment these values were no longer significant in corn roots for PAL and TAL and in corn shoots for TAL, corresponding to the time when most of the metolachlor detoxification occurred. In addition, increases in PAL and TAL were generally correlated with metolachlor residues (Table I). Similar responses to some other herbicides have also been shown for PAL in soybean (Hoagland and Duke, 1983) and for PAL and TAL in corn and soybean (Scarponi et al., 1991a,b). These findings and the generally significant correlations found between metolachlor residues and decreases in dry matter, protein, and ALA-D activity (Table I) confirm the stress status deriving from metolachlor residues, although the metolachlor was maintained at sublethal concentrations. These results also suggest the feasibility of considering increasing PAL and TAL activities as general stress indexes for diagnostic purposes, following metolachlor treatment.

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Registry No. ALA-D, 9036-37-7; PAL, 9024-28-6; GST, 50812-37-8; metolachlor, 51218-45-2.