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Effect of Some Herbicides as Chemical Pollutants on the Activity of Some Enzymes, Pigments Content and Nucleic Acids Level of *Lupinus termis* Cotyledons

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Abstract: Two herbicides clomazone and metazachlor were non-competitively inhibited the activities of chorismate mutase (CM, EC 5.4.99.5), prephenate dehydratase (PD, EC 5.2.1.51), acetohydroxyacid synthase (AHAS, 4.1.3.18) and phenylalanine ammonia-lyase (PAL, 4.3.1.5) in cotyledons of *Lupinus termis*. Inhibition was dependent on the type and concentration of each individual herbicide. L-tryptophan and its analog 5-methyltryptophan activated CM and PD while L-tyrosine, *m*-fluorotyrosine, *p*-fluorophenylalanine and L-phenylalanine inhibited both enzymes particularly in herbicide-treated seedlings. PAL was inhibited by phenylpropanoid intermediates involved in flavonol and anthocyanin biosynthetic pathway particularly *p*-coumaric acid, naringenin and quercetin. PAL inhibition by these intermediates was more pronounced in cotyledons of herbicide-treated seedlings. The levels of chlorophyll a, chlorophyll b, carotenoids, anthocyanin and nucleic acids were decreased by the two tested herbicides.

Key words: Clomazone, metazachlor, *Lupinus termis*, enzyme inhibition, pigments, nucleic acids.

Introduction

Environmental pollution has revealed crises proportions over the past several decades and is now having a significant impact on the health of forests and agricultural production (Hopkins, 1995).

Modern agricultural practices depend heavily on protection of the crop species against competition from weeds. Herbicides and the pesticides are most likely known to affect the enzymes and metabolism of plant including secondary metabolism (Lydon and Duke, 1989; El-Shora, 1999).

The common aromatic pathway in plants results in the biosynthesis of chorismate, the common precursor of many important aromatic amino acids such as tryptophan, tyrosine and phenylalanine (Bender, 1984). Chorismate mutase (EC 5.4.99.5) and prephenate dehydratase (EC 4.2.1.51) are important enzymes in synthesis of the aromatic amino acids (Strack, 1997; Taiz and Zeiger, 1998).

The interface between phenylalanine and secondary phenylpropanoid metabolism is controlled by the enzyme phenylalanine ammonia- lyase (EC 4.3.1.5). This enzyme catalyzes a non-oxidative deamination of phenylalanine to yield *trans*-cinnamic acid (Lea, 1990; D'Cunha *et al.*,1996; Sarma and Sharma, 1999)and regulate biosynthetic routes leading to the biosynthesis of phenylpropanoid-derived secondary products in plants such as lignins, flavonoid pigments, UV protectants, plant hormones and phytoalexins (Hao *et al.*, 1996; Gatehouse, 1997). Thus, PAL is considered as a switch for secondary metabolism (Cosio *et al.*, 1985; Molin *et al.*, 1986; Gottstein *et al.*, 1991).

Acetohydroxyacid synthase (EC 4.1.3.18) is the first common enzyme in the pathways leading to the biosynthesis of valine, leucine and isoleucine in different organisms (Barak *et al.*, 1987; Singh *et al.*, 1988).

A vital role of carotenoids in photosynthetic tissues is photoprotection by quenching the triplet state of chlorophyll and scavenging for singlet oxygen. This function is associated with the ability of the carotenoid molecules to participate in photochemical reactions such as singlet-singlet energy, triplet-triplet energy, oxidation, reduction and isomerization (Bramley, 1997).

So, to investigate the effect of clomazone and metazachlor on the activities of CM, PD, AHAS, PAL and to see whether these compounds inhibit these enzymes in *Lupinus* cotyledons as a C_3 plant. Also, it was investigated whether these compounds reduce the pigment level and nucleic acids content in the C_3 cotyledons.

Materials and Methods

Seeds of *Lupinus termis* were surface sterilized with 0.1 % $HgCl_2$, and washed with distilled water then germinated on 3 layers of filter paper in Petri dishes. The filter papers were moistened with H_2O for 2 days in the light at 30 °C. The seedlings were grown in growth chamber at 30°C for 5 days in one–third strength Hoagland's nutrient salt solution with gentle aeration under continuous white light from fluorescent lamps. The photosynthetic photon flux density, at the top of the plants, was 100 μmol m^{-2} s^{-1}.The cotyledons from uniformly grown seedlings were collected and used for preparing the extracts of the different enzymes.

Effect of herbicides: Clomazone and metazachlor were applied at either 10 or 100 μM with the nutrient solution and samples were taken daily for enzyme assays as well as determination of pigments and nucleic acids throughout 5 days. Control seedlings were treated with nutrient solution without herbicide.

Extraction and assay of enzymes:

Chorismate mutase (CM):

Extraction of CM: The enzyme was extracted according to the method of Goers and Jensen (1984a). Fifty gm of *Lupinus* cotyledons were homogenized in 10 ml of 50 mM Tris buffer (pH 7.5) contained 1 mM EDTA, 1 mM cysteine and 1 mM $MnCl_2$. The homogenate was centrifuged at 5000 rpm for 20 min. and the resulting supernatant was used as the crude enzyme extract.

Assay of CM: CM was assayed according to the method of Nelms *et al.* (1992). The reaction medium contained 1 mM chorismate, 100 mM Tris-HCl buffer (pH 7.5), 0.5 mM EDTA, 0.1 % bovine serum albumin, and enzyme extract to start the reaction. The reaction mixture was incubated for 5 min at 37°C and then terminated by adding 0.1 ml of 4.5 M HCl. The reaction mixture was incubated for an additional 10 min. at 37°C to convert all of the prephenate to phenylpyruvate, and then 0.1 ml of 12 M NaOH was added and the absorbance was measured at 320 nm. One unit (U) of CM activity was defined as the quantity of enzyme, which catalyzed the conversion of 1 μmol of chorismate to prephenate in 1 min

under assay conditions.

Prephenate dehydratase (PD)

Extraction of PD: The enzyme was extracted according to the method of Goers and Jensen (1984a). Fifty gm of *Lupinus* cotyledons are homogenized in 10 ml of extraction medium contained 100 mM Na phosphate buffer (pH 7.5), 1 mM EDTA, 5 % cysteine, 5 mM Na pyruvate, 0.2 mM FAD and 1 mM $MgCl_2$. The resulting homogenate was centrifuged at 5000 rpm for 20 min and the supernatant was used as the crude enzyme extract.

Assay of PD: PD was assayed according to the method adopted by Nelms *et al.* (1992). The reaction mixture contained 27mM Tris-HCl buffer (pH 8), 1 mM K-prephenate, and enzyme extract to start the reaction. The preparation was incubated at 37°C for 10 min, then 0.25 ml sample was removed and mixed with 0.75 ml of NaOH. The absorbance was measured at 320 nm. One unit (U) of PD activity was defined as the quantity of the enzyme that catalyzed the conversion of 1 μmol of prephenate to phenylpyruvate in 1 min under assay conditions.

Phenylalanine ammonia-lyase (PAL)

Extraction of PAL: The enzyme was extracted according to the method of Sadasivam and Manickam (1996). Fifty gm of Lupinus cotyledons were homogenized in 10ml of cold 25 mM borate-HCl buffer (pH 7.5) containing 5 mM mercaptoethanol (0.4 ml/L). The homogenate was centrifuged at 5000 rpm for 20 min. The resulting supernatant was used as the crude enzyme extract.

Assay of PAL: The enzyme was assayed using the modified method of Hao *et al.* (1996). The reaction mixture contained 100 mM tris buffer (pH 8.6), 75 mM L-phenlalanine, 1 mM, 2-mercaptoethanol and enzyme extract to start the reaction. The incubations were allowed to proceed for 1h at 37°C. The inactivation of the enzyme with concentrated HCl terminated the reaction. A_{290} of the clear sdution was measured. Reaction mixture in which the substrate was added after termination of the amount of the reaction served as control. One unit (U) of the enzyme was defined as the amount of enzyme required for conversion 1 μmol of L-phenylalanine to product per min at 37°C.

Acetohydroxyacid synthase (AHAS):

Extraction AHAS: The enzyme was extracted by the modified method of Rhodes *et al.* (1987). Fifty gm of Lupinus cotyledons were homogenized in 10ml of extraction medium contained 100 mM Na phosphate buffer (pH 7.5), 1 mM EDTA, 5% cysteine, 5 mM Na pyruvate, 0.2 mM FAD and 1 mM $MgCl_2$. The resulting homogenate was centrifuged at 5000 rpm for 20 min and the supernatant was used as the crude enzyme extract.

Assay of AHAS: The enzyme was assayed according to the modified method of Singh *et al.* (1988). The reaction mixture contained 100 mM Na phosphate buffer (pH, 7.5), 1 mM thiamine pyrophosphate, 0.1 mM FAD, 2 mM $MgCl_2$, 5 mM sodium pyruvate and enzyme extract. The reaction mixture was incubated at 37°C for one hour. Adding 4.5 M Hcl stopped the reaction and the reaction product was left to decarboxylate at 50 Oc. for 20 min. The acetoin formed was determined by incubating with 0.2% creatine and α-naphthol (1.7 % in 4 N NaOH) for 20 min at 50 Oc.. The reaction mixture was cooled and centrifuged at 5000 rpm for 20 min. The absorption of the resulting color was measured at 520 nm. One unit (U) of AHAS activity is defined as the quantity of the enzyme that produces 1 μmol of acetoin per min under assay conditions.

Kinetic studies of enzymes: Enzymes were partially purified from the hydroxylapatite step of fractionation described by Goers and Jensen (1984a).

Determination of chlorophyll a, b and carotenoids: Chlorophyll a, b and carotenoids contents were determined (Chauhan and Senboku, 1996). Chlorophyll is extracted in 80 % acetone and the absorption (A) at 663, 645 and 470nm in a spectrophotometer was noted. Chlorophyll a, b and carotenoids were calculated as:

Chl. a = $12.7 \times A_{663} - 2.69 \times A_{645}$

Chl. b = $22.9 \times A_{645} - 4.68 \times A_{663}$

Total carotenoids = $4.2\ A_{453} - (0.03$ Chl.a + 0.43 Chl. b).

Determination of anthocyanin: The anthocyanin was extracted (Sarma and Sharma ,1999) in 2 ml of acidified (1 % v/v HCl) methanol for 24 h at 4 °C with occasional shaking. The anthocyanin was quantitatively estimated by measuring the A_{535}.

Determination of nucleic acids: RNA and DNA were extracted and determined by the method of Sadasivam and Manickam (1996).

Effect of phenylpropanoid metabolites: The phenolic compounds were dissolved in 10-50 % (v/v) ethanol to make stock solutions (10 mM). The final concentration of metabolites in the PAL assay mixture was 1 mM. In the control assay mixture an equivalent amount of ethanol was added. The PAL activity is expressed as U min^{-1} and the inhibition percentage was calculated with respect to control enzyme sample without compounds.

Results and Discussion

Treatment of *Lupinus termis* with clomazone or metazachlor at either 10 μM or 100 μM reduced the activity of CM (Fig.1A) below the control level. The magnitude of reduction was most pronounced with the higher concentration of each herbicide but metazachlor was the better effective inhibitor. PD activity (Fig.1B) was also influenced by the tested herbicides. During the first 2 days of treatment, the activity was not remarkably affected by 10 μM of clomazone. In subsequent days, the enzyme activity was decreased continuously by 100 μM clomazone or in presence of metazachlor. The decreased level of both CM and PD by the tested herbicides might be due to binding of the herbicide to the enzyme- substrate complex *in vivo* and thus alter catalysis (Holt *et al.*, 1993). A second possibility is that the enzyme becomes very unstable when bound with the herbicide and is rapidly broken down or degraded either in the plant or during the extraction procedures (Moreland, 1980).

The sensitivity of CM and PD, extracted from cotyledons of treated- and untreated-seedlings with 100 μM clomazone or metazachlor, to the aromatic amino acids and their analogs was tested. The aromatic amino acids or their analogs were examined at 1mM in the extraction medium. The results obtained for CM and PD are shown in Figs. 2A and 2B, respectively. These results reveal that L-tryptophan is a potent activator for both enzymes. L-tyrosine was a strong inhibitor whereas L-phenylalanine seemed to be less effective. Fluorotryptophan was a good activator analog of L-tryptophan; methyltyrosine was a good inhibitor analog of L-tyrosine and *m*-fluorophenylalanine was a good inhibitor analog of L-

Table 1: Effect of phenylpropanoid compounds on PAL activity extracted from cotyledons of treated and untreated-seedlings with herbicides.

PAL activity						
Treated seedlings with				Untreated seedlings		
Metazachlor (100 μM)		Clomazone (100 μM)		% Inhibition	(U mg^{-1} protein)	
% Inhibition	(U mg^{-1} protein)	% Inhibition	(U mg^{-1} protein)			Compounds (1 mM)
---	20.6 ± 0.9	---	25.5 ± 0.6	---	40.0 ± 0.9	None
28.4 ± 0.4	14.7 ± 0.3	17.2 ± 0.3	21.1 ± 0.3	9.0	36.4 ± 0.4	Ferulic acid
54.4 ± 0.7	9.4 ± 0.7	46.3 ± 0.6	13.7 ± 0.5	37.2	25.1 ± 0.7	Naringenin
31.3 ± 0.6	14.2 ± 0.5	20.4 ± 0.9	20.3 ± 0.9	14.0	34.4 ± 0.9	Cinnamic acid
24.5 ± 0.9	15.6 ± 0.5	13.3 ± 0.7	22.1 ± 0.3	8.3	36.7 ± 0.2	Caffeic acid
72.5 ± 0.5	5.7 ± 0.8	63.5 ± 0.8	9.3 ± 0.4	55.0	18.0 ± 0.5	*p*-Coumaric acid
46.3 ± 0.3	11.1 ± 0.7	34.4 ± 0.2	16.7 ± 0.2	29.2	28.3 ± 0.8	Quercetin

The data represents mean ± S. E. of three replications.

phenylalanine. Generally, the sensitivity of CM and PD to aromatic amino acids was increased in cotyledons of herbicide-treated seedlings. The inhibition of the CM and PD by amino acids is in accordance with the results of Cole *et al.* (1980). The relatively greater ability of L-tryptophan to activate both enzymes could enhance the output of aromatic amino acid biosynthesis as reported by Goers and Jensen (1984b).

The activity of AHAS (Fig. 3A) was not remarkably affected with the lower concentration of clomazone throughout the experimental period. However, the higher concentration of clomazone or the two concentrations of metazachlor inhibited the enzyme activity during the entire experiment. The inhibition of AHAS by the tested herbicides is in agreement with the results of Gerwick *et al.* (1990) and Hart *et al.* (1992).

PAL activity (Fig.3B) was increased over the control values during the first 2 days of treatment with 10 μM clomazone. This effect may be caused by enzyme activation, increase of its synthesis or decrease of its degradation (Johnson and Smith, 1978). However, PAL activity of the differently treated-seedlings declined rapidly after the third day of treatment. In support, other herbicides completely inhibit the activity of PAL, such as diuron (Amrhein and Zenk, 1971), atrazine (Hoagland, 1989) and metribuzin (Hoagland and Duke, 1981). This inhibition of either AHAS or PAL may result from depletion of the end products; depletion of intermediates of the pathway for some critical process, or a build up of toxic substrate (Singh *et al.*, 1995). It was suggested that inhibition of AHAS by herbicides causes accumulation of 2-ketobutyrate and 2-aminobutyrate and this build-up is what kills the plant (Rhodes *et al.*, 1987).

It was thought to study the regulation of PAL activity extracted from cotyledons of untreated- and herbicide-treated seedlings by feedback inhibition mechanism utilizing phenylpropanoid intermediates. Thus, PAL was incubated and assayed in presence and absence of several phenolic compounds. The results in Table 1 showed that the tested compounds inhibited the enzyme activity with *p*-coumaric acid, naringenin and quercetin being the most potent inhibitors. Furthermore, the inhibition of PAL by these compounds was more pronounced in case of herbicide-treated seedlings. These results are in harmony with the results of Sarma and Sharma (1999). Since PAL is the first enzyme for the general phenylpropanoid pathway, the strict feedback control of PAL provides a selective way for its regulation of overall flux into the pathway. It is therefore likely that the accumulation of these compounds results in a feedback inhibition of the enzyme to regulate the flux of the pathway. In addition, the inhibition was increased for PAL extracted from herbicide-treated seedlings.

Since both clomazone and metazachlor reduced the extractable activity of the four tested enzymes. Clomazone or metazachlor was included in the assay mixture of each individual enzyme at 100 μM using various substrate concentrations. There was a distinct response from each enzyme towards its substrate (Figs. 4 & 5). While the K_m remained unchanged, V_{max} values for the substrate changed with the herbicide. These results suggest that both clomazone and metazachlor inhibit noncompetitively the four-tested enzymes.

The content of chlorophyll a (Fig. 6A) or chlorophyll b (Fig. 6B) was slightly reduced by the lower concentration of each examined herbicide up to the end of the experiment. However, the higher concentration resulted in a remarkable reduction in the content of each type of chlorophyll below the control values. These results are in accordance with those of other investigators (Schnelle and Honsley, 1990; Marco and Orus 1993; Wilkinson, 1993). The effect of the herbicides on chlorophyll content could be explained by their ability to cause abnormal accumulation of tetrapyrroles in addition to the blockage of the porphyrin pathway (Yu and Masiunas, 1992). Consequently, this would inhibit the chlorophyll synthesis inducing lethal photo oxidation reactions. Another possibility is the degradation of the chlorophyll in presence of the tested herbicides (Kitchen *et al.*, 1981; Matile *et al.*, 1996).

Carotenoids content (Fig. 7A) was, in general, remarkably decreased in presence of herbicides. The magnitude of response was more pronounced with metazachlor. These results are consistent with those of Norman *et al.* (1990). Both carotenoids and phytol moiety of chlorophyll molecules are synthesized *via* the terpenoid pathway predominantly in the chloroplast (Kleinig, 1989; Frank and Cogdell, 1996). Carotenoids protect chlorophyll from photo oxidation (Moreland, 1980; Bramley, 1997). Therefore, the inhibition of carotenoid synthesis leads to chlorophyll degradation in the light as a result of the loss of the photoprotection that carotenoids are considered to provide.

The anthocyanin pigment (Fig. 7B) was increased at the first two days of treatment over the control values in presence of 10 μM clomazone or metazachlor. After the third day of treatment the pigment content was remarkably decreased by the two concentrations of the herbicides. Thus, the anthocyanin content was correlated positively with PAL activity; both of which were simultaneously increased or reported that induction of flavonoid formation was

accompanied by an increase in the activity of the enzymes involved in their biosynthesis (Kombrink and Somssich, 1995; Taiz and Zeiger, 1998). The synthesis of anthocyanin requires CoA (Taiz and Zeiger, 1998) and α-chloroacetanilides are known to interact chemically with CoA (Lydon and Duke, 1989). Therefore, the effect of metazachlor may be due to a reduction of CoA availability.

The content of RNA (Fig. 8A) or DNA (Fig. 8B) was decreased below the control level particularly in presence of metazachlor, which was the effective inhibitor at the two concentrations. Also, DNA content was reduced remarkably after the 4th day of treatment. Nucleic acids biosynthesis has been reported as potential target sites affected by α-chloroacetanilides and other herbicides (Foley *et al.*, 1983). Correlation between inhibition of nucleic acids and reduced tissue ATP concentrations was reported (Duke *et al.*, 1975). This correlation suggests that interference with the production of energy, required to drive the biosynthetic reactions, could be the mechanism through which the inhibitory herbicides expressed their effects. However, direct interference with a specific step in the biosynthetic pathway remains a distinct possibility. The direct effect of herbicides on DNA and /or RNA synthesis could occur by altering chromatin integrity or by binding to chromatin in such a way that transcription was restricted (Moreland, 1980).

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