Effect of Temperature and Safeners on Glutathione Levels and Glutathione S-Transferase Activity in Maize*

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Studies were conducted to determine the biochemical aspects of chloroacetamide injury to maize and the mechanism by which safeners maintain herbicide tolerance, even at reduced temperatures. The objectives of these studies were threefold: one, determine whether glutathione (GSH) content varies in maize plants grown at three different temperatures in safener-treated and non-treated plants; two, determine whether glutathione S-transferase (GST) activity varies in plants grown at different temperatures; and three, determine if GSH activity is sensitive to low temperatures *in vitro*. The herbicide safeners CGA-154281 [4-(dichloroacetyl)-3,4-dihydro-3-methyl-2 H-1,4-benzoxazine] and dichlormid [2,2-dichloro-*N,N*-di-2-propenylacetamide] were used with metolachlor [2-chloro-*N*-(2-ethyl-6-methylphenyl)-*n*-(2-methoxy-1-methyl)acetamide] or acetochlor [2-chloro-*N*-(ethoxymethyl)-*N*-2-ethyl-6-methylphenyl)-acetamide], respectively, to determine the mechanisms of maize tolerance. CGA-154281 significantly increased GSH levels in maize seedlings grown at 27 °C compared to non-safened seedlings, however significant differences were not seen at 17 or 37 °C. Dichlormid increased GSH levels by 1.6-fold at all growth temperatures. Both CGA-154281 and dichlormid increased GST activity significantly at all growth temperatures. The safener-induced GST activity was maintained at *in vitro* incubation temperatures of 5 and 15 °C for acetochlor and metolachlor, respectively. In contrast, GST activity from non-safened tissue was essentially absent at these temperatures. Therefore, greater GST activity following safener treatment may result in higher levels of herbicide metabolism, even at low temperatures.

Introduction

Chloroacetanilide (= chloroacetamide) herbicides are used extensively for grass control in crop production. However, these herbicides sometimes cause injury to crops. Several environmental factors such as temperature and soil moisture exacerbate chloroacetanilide injury [1–6]. Grass crops, such as maize and sorghum, are especially sensitive to these herbicides since the shoot apices remain at or near the soil surface (site of herbicide treatment) for several weeks during early growth. The development of herbicide safeners has alleviated much of the injury caused by these herbicides. Greenhouse research has shown that the safeners, CGA-154281 and dichlormid**, maintain a high level of maize tolerance, particularly at low growing temperatures, to the herbicides metolachlor and acetochlor, respectively (unpublished research). However, the specific role that temperature plays in herbicide metabolism and injury has not been fully elucidated. Since glutathione and glutathione S-transferase play a major role in chloroacetanilide detoxification, studies were conducted to determine the biochemical aspects of chloroacetanilide injury in maize and the mechanism by which safeners maintain herbicide tolerance, even at reduced temperatures. The objectives of these studies were threefold: one, determine if glutathione content varied in maize plants grown at different temperatures in safener-treated and non-treated plants; two, determine if glutathione S-transferase (GST) activity varied in plants grown at different temperatures in safener-treated and non-treated plants; and three, determine if GST activity is sensitive to low temperatures *in vitro*.

* Based on a paper presented at the International Conference on Herbicide Safeners, August 12–15, 1990 in Budapest, Hungary.

** Dichlormid is the common name adopted by the Weed Science Society of America for 2,2-dichloro-*N,N*-di-2-propenylacetamide.

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Materials and Methods

Plant material

Maize (*Zea mays* L., *var* Pioneer 3320) seeds were soaked for 2 h and then 28 seeds were placed

on 750 grams of soil (Elmwood sandy-loam) in styrofoam trays (30 × 10 × 5 cm). The seeds were then covered with another 750 grams of treated soil. The soil was premoistened with 150 ml of water and an additional 150 ml was added after treatments. The styrofoam trays were wrapped with plastic wrap (to prevent excess moisture loss from the surface) and placed in a dark growth chamber set at 17, 27, or 37 °C. Maize shoots were harvested when 2 cm long.

Glutathione content

Eight 2 cm maize shoots were assayed for glutathione (GSH) content using fluorescence high-performance liquid chromatography (HPLC) according to Newton *et al.* [7] with minor modifications. Shoots were frozen in liquid nitrogen and stored at −80 °C until assayed for GSH. Tissues were homogenized in 0.1 N NaOH (5 ml/g tissue) using a ground glass tissue grinder. Following a 4 min centrifugation (12,000 × *g*), 0.25 ml of supernatant was reduced with 25 µl of sodium borohydride in 1 M NaOH (20 mg/ml) for 15 min. Excess sodium borohydride was decomposed with 25 µl of 3.6 N HCl. Samples were cold centrifuged for another 10 min and 0.25 ml of the supernatant was then reacted with 4 µl of 0.1 M monobromobimane and 25 µl of 26% 4-ethylmorpholine. The reaction mixture was incubated for 30 min then stopped with 90 µl of 1 M NaH_2PO_4 and 5 µl of 3.6 N HCl. Samples were centrifuged for another 4 min, and supernatant was analyzed for thiols by HPLC. Analyses were carried out on a Beckman 126 gradient HPLC equipped with a Beckman 157 fluorescence detector, operating at 1 ml/min in a gradient of 10–55% MeOH in 40 mM Na_2PO_4 (pH 3.0).

Glutathione S-transferase activity

Fifteen 2 cm maize shoots were excised and prepared according to Gronwald *et al.* [8]. The reaction volume of 0.5 ml contained 50 µl of 0.1 M GSH, 0.3 ml of 0.1 M K-phosphate buffer (pH 7.4), 0.1 ml of extract (0.1 M K-phosphate buffer pH 6.8, approx. 0.2 mg of protein) and 50 µl of 0.5 mM [^{14}C]metolachlor or [^{14}C]acetochlor (4.1 nCi). The reaction was initiated by the addition of metolachlor. Radioactivity of the aqueous phase was determined by liquid scintillation spectrometry.

Protein assay

The protein content of the seedlings was determined by the Bradford method [9] using Coomassie blue G-250 dye binding with bovine serum albumin as a standard.

Statistical design

A completely randomized design was used, with three replications for metolachlor and two replications for acetochlor. All data was subjected to analysis of variance and significant means (at $\alpha = 0.05$) were separated using LSD.

Results and Discussion

Effect of CGA-154281 on glutathione content of maize seedlings grown at three different temperatures

Glutathione (GSH) content increased 1.6-fold in plants grown in CGA-154281-treated soil at 27 °C compared to non-treated plants (Table I). Other authors working with CGA-15428 have not measured a significant increase in GSH content [10]. Although the GSH content in plants treated with CGA-154281 grown at 17 and 37 °C was higher when compared to their corresponding non-treated controls, these differences were not significant ($\alpha = 0.05$). Metolachlor treatments did not influence GSH content when compared to non-treated maize plants. There were no differences in GSH contents when comparing each treatment across temperatures, with one exception. This occurred at 27 °C, where GSH content of CGA-154281-treated seedlings was nearly 1.5-times higher than that of plants grown at 17 °C. Therefore, GSH response to

Table I. Effect of temperature on glutathione content of CGA-154281-treated and non-treated maize seedlings.

Treatment	nmol GSH/mg protein 17 °C	27 °C	37 °C
Control	46.8 cd*	43.0 d	48.1 b c d
Metolachlor (2 kg/ha)	41.1 d	47.7 b c d	49.7 b c d
CGA-154281 (0.25 kg/ha)	47.8 b c d	70.2 a	61.0 a b c
Metolachlor + CGA-154281	56.8 a–d	70.6 a	65.2 a b

* Means followed by the same letter are not significantly different at $\alpha = 0.05$.

CGA-154281 was greatest at 27 °C, the temperature at which maize seedling growth is maximal.

Effect of dichlormid on glutathione content of maize seedlings grown at three different temperatures

GSH content increased significantly (1.5- to 2-fold) at all temperatures evaluated in seedlings grown in dichlormid-treated soil compared to seedlings grown in non-treated soil (Table II). Similar increases in GSH content in dichlormid-treated maize seedlings have been reported by a number of other researchers [11, 12]. Acetochlor treatments alone caused an increase in GSH content in seedlings grown at 17 °C. However, significant increases were not observed in seedlings grown at higher temperatures. Increased levels of GSH may be of greater significance in protecting maize seedlings treated with acetochlor, since acetochlor appears to conjugate non-enzymatically more readily with GSH than does metolachlor (Table III). In addition, these data suggest that dichlormid acts more strongly on the GSH system than does CGA-154281.

Effect of CGA-154281 and dichlormid on glutathione S-transferase activity in response to three different growth temperatures

Glutathione S-transferase (GST) activity increased 2-fold in all maize seedlings treated with CGA-154281 grown at both 17 and 27 °C (Table IV). Growth temperature did not have a significant effect on GST activity in plants grown at 17 and 27 °C. Viger *et al.* [10] have reported similar results with CGA-154281. Seedlings grown at 37 °C responded to the CGA-154281 treatment with a 1.4-fold increase in GST activity. However, the safener-induced GST activity in plants grown at 17 and 27 °C was significantly higher than in plants grown at 37 °C.

Glutathione S-transferase activity in dichlormid-treated maize seedlings was approximately 1.7-fold higher than that of non-treated and acetochlor-treated plants at all growth temperatures (Table V). Induction of GST activity, in dichlor-

Table II. Effect of temperature on glutathione content of dichlormid-treated and non-treated maize seedlings.

Treatment	nmol GSH/mg protein 17 °C	27 °C	37 °C
Control	46.1 g*	56.9 f g	60.7 e f
Acetochlor (2 kg/ha)	61.8 e f	56.4 f g	73.3 d e
Dichlormid (0.25 kg/ha)	90.3 b c	99.6 a b	81.3 c d
Acetochlor + Dichlormid	81.4 c d	95.3 a b c	105.5 a

* Means followed by the same letter are not significantly different at $\alpha = 0.05$.

Table III. Rate of non-enzymatic conjugation of metolachlor and acetochlor to glutathione.

Herbicide	nmol Herbicide conjugated × h
Acetochlor	1.5
Metolachlor	0.3

Table IV. Glutathione S-transferase activity*: effect of growth temperature on CGA-154281-treated and non-treated maize seedlings.

Treatment	nmol Metolachlor conjugated/mg protein × h 17 °C	27 °C	37 °C
Control	4.6 e f**	4.6 e f	5.2 e f
Metolachlor (2 kg/ha)	4.3 e f	3.7 f	5.5 d e
CGA-154281 (0.25 kg/ha)	8.6 a b	10.0 a	7.0 c d
Metolachlor + CGA-154281	9.8 a	9.5 a	7.5 b c

* Reactions for these experiments were incubated at room temperature.

** Means followed by the same letter are not significantly different at $\alpha = 0.05$.

Table V. Glutathione S-transferase activity*: effect of growth temperature on dichlormid-treated and non-treated maize seedlings.

Treatment	nmol Acetochlor conjugated/mg protein × h 17 °C	27 °C	37 °C
Control	8.8 c d e**	8.3 d e	9.7 b–e
Acetochlor (2 kg/ha)	6.2 e	5.7 e	7.5 e
Dichlormid (0.25 kg/ha)	14.9 a	11.9 a–d	14.8 a
Acetochlor + Dichlormid	13.1 a b c	14.1 a	13.8 a b

* Reactions for these experiments were incubated at room temperature.

** Means followed by the same letter are not significantly different at $\pi = 0.05$.

mid-treated plants, did not differ significantly with growth temperature.

Determination of GST sensitivity to in vitro temperatures

Experiments were conducted to ascertain enzyme activity at various *in vitro* incubation temperatures in order to determine if the GST enzymes induced by the safeners would respond to temperature in the same manner as GST enzymes in maize seedlings which were not treated with safeners. In seedlings treated with metolachlor or metolachlor plus CGA-154281 at 5 °C (incubation temperature), there was no significant GST activity measured, regardless of treatment (Fig. 1). However, at 15 °C, maize seedlings grown at all three temperatures and treated with metolachlor plus CGA-154281, had significantly higher levels of GST activity when compared to seedlings treated only with metolachlor (Fig. 2). GST activity at this incubation temperature generally decreased as seedling growth temperatures increased. The GST activity continued to increase as the incubation temperatures increased from 15 to 35 °C. Glutathione S-transferase activity in CGA-154281-treated tissue remained significantly higher at incubation temperatures of 25 and 35 °C. The rate of enzymatic metolachlor conjugation at 35 °C from non-safened tissue was considerably higher than the rate of herbicide conjugated at lower temperatures. This rapid conjugation at higher temperatures may account for the decreased metolachlor injury observed at higher temperatures (unpublished data).

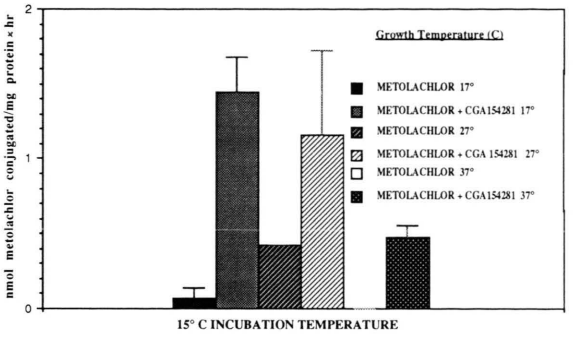

Fig. 2. *In vitro* enzymatic conjugation of metolachlor at 15 °C. Vertical bars represent the standard errors of means of three experiments.

Maize seedlings treated with dichlormid plus acetochlor and grown at 17 and 27 °C more rapidly conjugated acetochlor when incubated at 5 °C compared to plants treated only with acetochlor or grown at 37 °C (Fig. 3). At the 15 °C incubation temperature, dichlormid-treated seedlings from all three growth temperatures had higher levels of GST activity compared to seedlings not treated with the safener. This higher level of GST activity in safener-treated plants remained higher as incubation temperatures increased to 35 °C.

Conclusions

Results from these experiments indicate that treatment of maize seedlings with CGA-154281 caused a significant increase in GSH content when grown at 27 °C. However, large differences were not detected in seedlings grown at 17 or 37 °C. It is

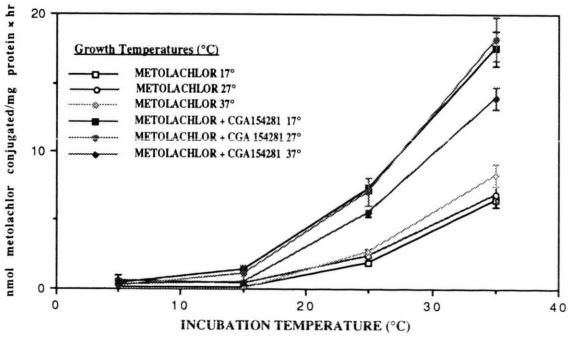

Fig. 1. Effect of temperature on *in vitro* glutathione S-transferase-catalyzed metolachlor conjugation. Vertical bars represent the standard errors of means of three experiments.

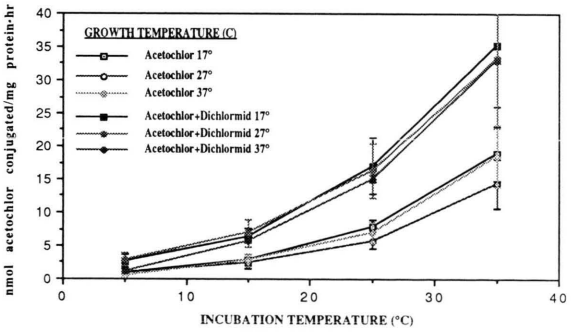

Fig. 3. Effect of temperature *in vitro* glutathione S-transferase-catalyzed acetochlor conjugation. Vertical bars indicate range of the means of two experiments.

unlikely that GSH content probably plays a major role in alleviating metolachlor injury at low temperatures. Dichlormid treatments, however, significantly increased the levels of GSH at all growth temperatures. Therefore, since non-enzymatic conjugation to GSH seems to occur more rapidly with acetochlor than with metolachlor this higher accumulation of GSH may significantly contribute to the safening of maize to acetochlor, even at low temperatures. The high level of GST activity at high incubation temperatures may explain the increased tolerance to these herbicides which is observed at higher growing temperatures. The safener-induced GST activity was maintained at incubation temperatures of 5 and 15 °C for acetochlor and metolachlor, respectively. By contrast, GST activity from non-safened tissue was essentially absent at these temperatures. Consequently, higher GST activity following safener treatment may result in higher levels of herbicide metabolism at lower temperatures.

Acknowledgements

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