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MODE OF ACTION AND METABOLISM

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Duration of yellow nutsedge (*Cyperus esculentus*) competitiveness after treatment with various herbicides.

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ABSTRACT

Experiments were initiated to determine the amount of time required for POST herbicides to render yellow nutsedge physiologically non-competitive. The rate of net CO_2 assimilation (A_N) was chosen as the response variable to describe competitiveness. When A_N of treated plants declined below 50% (A_{N50}) of the untreated control, the plants were no longer considered competitive. The time to reach A_{N50} for halosulfuron, imazapic, glyphosate and MSMA were 2.6, 3.1, 4.2, and 4.3 days, respectively. An A_{N50} value was not calculated for bentazon since A_N rapidly decreased below 50%, but recovered to >50% by 10 DAT. Stomatal conductance (g_s), a measure highly correlated with transpiration, declined similarly with A_N over time for halosulfuron, imazapic, and glyphosate treatments. However, stomatal conductance rates for MSMA treated plants was near 95% of the untreated control while A_N was near 35% 12 DAT. MSMA reduced carbon assimilation one day after treatment and by 5 days after treatment, respiration exceeded carbon assimilation during the photoperiod for MSMA. MSMA rapidly and completely eliminated carbon assimilation, but had almost no effect on plant water use. Halosulfuron and MSMA reduced shoot regrowth to between 0 and 5% of the control. Mesotrione treatment allowed some 58% regrowth. In the field, these three treatments would likely have both quantitatively and qualitatively different effects on the competitive ability of yellow nutsedge.

INTRODUCTION

Yellow nutsedge is a C_4 perennial that infests 21 crops in as many as 30 countries (Holm *et al.*, 1991). It has been shown to be competitively superior to many crop species due to its higher photosynthetic rate, fast sprouting and early growth rate (Holt and Orcutt, 1991).

The highly competitive nature of yellow nutsedge has raised questions concerning the amount of time required for various postemergence herbicides to render the plant non-competitive. Since some enzyme inhibiting herbicides, such as glyphosate and halosulfuron, often do not cause visual injury symptoms for 7-10 days after application (Vencill, 2002), it was questioned whether the yellow nutsedge plants were remaining competitive during that period (Ferrell *et al.*, 2003).

Little work has focused on the physiological response of yellow nutsedge after herbicide treatment. The objective of this study was to compare the time required for several herbicides to render yellow nutsedge physiologically non-competitive. A second objective was to compare MSMA with halosulfuron and mesotrione, for effects on both whole plant carbon assimilation and water use.

MATERIALS AND METHODS

Tubers were planted into 4 litre pots containing a 2:1 sand: sandy loam soil mix and placed in the greenhouse. The tubers sprouted and grew for 5 weeks before treatment. The plants were watered daily and fertilized weekly.

Study 1

Herbicide treatments consisted of halosulfuron (70 g ai ha^{-1}), imazapic (70 kg ai ha^{-1}), glyphosate (840 kg ai ha^{-1}), bentazon (840 kg ai ha^{-1}), and MSMA (2.2 kg ai ha^{-1}), with an untreated control. Non-ionic surfactant was included with each herbicide (0.25% v/v), except bentazon, which received crop oil concentrate (1% v/v). Herbicides were applied in a spray cabinet calibrated to deliver 187 litre/ha with an operating pressure of 270 kPa. Each plant had one mature leaf tagged prior to herbicide application. This leaf was used exclusively throughout the experiment for data collection. Data was collected for 12 days after herbicide application. Measurements on day 1 occurred 4 hours after herbicide application.

Photosynthesis was measured using a portable, open-flow gas exchange system (Ferrell *et al.*, 2003). For each measuring day, rate of CO_2 assimilation (A_N) and stomatal conductance (g_s) data were expressed as a percent of the control, to adjust for daily variations not associated with the herbicide treatment. From these data, the time required for 50% reduction in A_N and g_s for each treatment and replicate was obtained via linear interpolation. Analysis of variance (ANOVA) was employed and means were separated by Fisher's Protected LSD test (P=0.05).

Study 2

Before transfer from the greenhouse, each pot was watered to excess, and then allowed to drain to constant weight in the dark. This was taken as the saturated weight + plant fresh weight (W_{SAT+P}). The saturated weight was also determined for a pot containing the same amount of soil but no plant (W_{SAT}), and the weight of water in the soil at 100% water holding capacity (W_{water}) was calculated as the difference between W_{SAT} and the weight of just the pot and dry soil. Then, the weight of each pot at 80% water holding capacity was calculated as $W_{SAT+P} - 0.2W_{water}$.

Each pot was transferred to a different chamber of an eight-chamber open flow CO_2 exchange measurement system (van Iersel and Bugbee, 2000) at 28 ± 2 °C and approximately 350 µmol m^{-2} s^{-1} during the 16-h photoperiod (20 mol m^{-2} d^{-1}). Net carbon dioxide assimilation was recorded for each chamber once every five minutes during the experiment, and average net assimilation rates were determined for each chamber during each photoperiod and each dark period. Soil and plant respiration rates were assumed to be the same for both the photoperiod and dark period, and so mean gross CO_2 assimilation rates were calculated for each day as the mean daily net assimilation rate, minus the mean respiration rate measured during the following dark period. Temperature and relative humidity of each chamber were also recorded to calculate the average daily vapor pressure deficit for each day of the experiment.

After spending three days in the chambers (one acclimation day, then two normalization days), each plant was removed at the end of the photoperiod and received one of four herbicide treatments: MSMA (2.2 kg ai ha^{-1}), halosulfuron (63 g ai ha^{-1}), mesotrione (110 g ai ha^{-1}), and untreated. MSMA and halosulfuron were applied with non-ionic surfactant at a concentration

of 0.25% v v^{-1} while mesotrione was applied with crop oil concentrate at 1% v v^{-1}. After returning the plants to the chambers, CO_2 assimilation and plant water use were recorded for an additional 11 days.

To determine plant transpiration from daily measurements of pot weights, it was necessary to adjust for the amount of water that evaporated directly from the soil surface. The daily soil water loss had a strong linear relationship to daily mean vapour pressure deficit. This function along with the vapor pressure deficit data from the main experiment was then used to estimate water lost to soil evaporation for each day of the experiment. This was subtracted from individual pot weight loss on each day to estimate the amount of water transpired by the plants.

Net CO_2 assimilation rates and daily water use were normalized for differences in plant size that existed at the beginning of the experiment. This expression of plant water use rate is mathematically equivalent to the "normalized transpiration ratio" (Ray and Sinclair, 1997). Net assimilation and water use data were analyzed separately for each day of the experiment using ANOVA, and means separations were via a protected LSD test ($P = 0.05$).

To determine the effects of the herbicide treatments on regrowth, all plant foliage was removed and pots were returned to the greenhouse, where water and fertilizer routines continued. After two weeks, shoot regrowth from each pot was harvested, dried and weighed. The data were analyzed by ANOVA, and protected LSD test ($P = 0.05$).

Statistical Analysis. Experimental design consisted of a randomized complete block with five replications. The experiment was conducted twice.

RESULTS AND DISCUSSION

Study 1

The most commonly used parameter to measure weed/crop competition is weed biomass accumulation. Biomass accumulation is, in essence, an integrated value of the rate of CO_2 assimilation (A_N) and stomatal conductance (g_s). This experiment measured both of these parameters at the leaf level. It was our assumption that plants possessing high rates of A_N and g_s were the most competitive and would likely have the highest rates of biomass accumulation. Likewise, a reduction in A_N and g_s can be related to a loss in competitive ability.

Figure 1 describes the response of yellow nutsedge to three herbicides that inhibit amino acid biosynthesis. These data demonstrate that halosulfuron and imazapic reduced A_N to 50% after 2.6 and 3.1 d, respectively. The lack of differences between these herbicides was expected, considering their similar mechanism of action as well as their comparably high levels of yellow nutsedge control (Grichar and Nester, 1997). Glyphosate, although displaying a similar response over 12 d to halosulfuron and imazapic, acted more slowly, taking 4.2 d to reach A_{N50}.

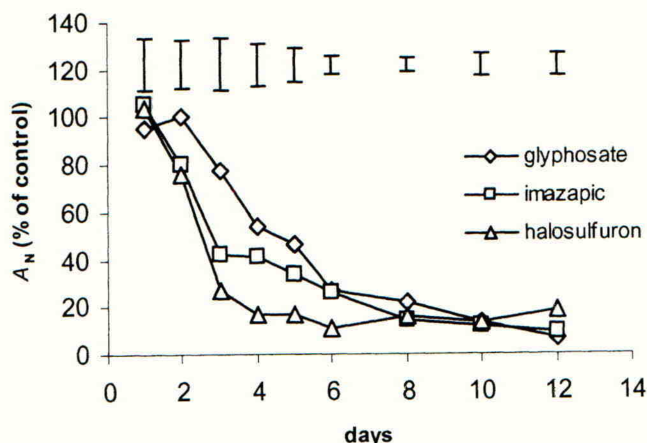

Figure 1. Photosynthetic response of yellow nutsedge to glyphosate, imazapic, and halosulfuron for 12 days after treatment. Bars indicate $LSD_{0.05}$ values.

Reduction of A_N with MSMA (Figure 2) was slower than for the herbicide treatments displayed in Figure 1. Although the pattern of A_N reduction was dissimilar, the time to reach A_{N50} was similar to that of glyphosate at 4.3 d. Conversely, bentazon displayed a trend that was dissimilar to all other treatments observed. The bentazon treatment displayed a reduction in A_N to 60% of the control after only 4 hours of exposure. A_N continued to decline until negative values were recorded on days 2-4. Negative A_N values denote that cellular respiration, on days 2-4, was greater than total CO_2 fixation by the dark reactions. However, recovery began to occur at 5 d and continued until A_N was at 60% of the control by 12 d.

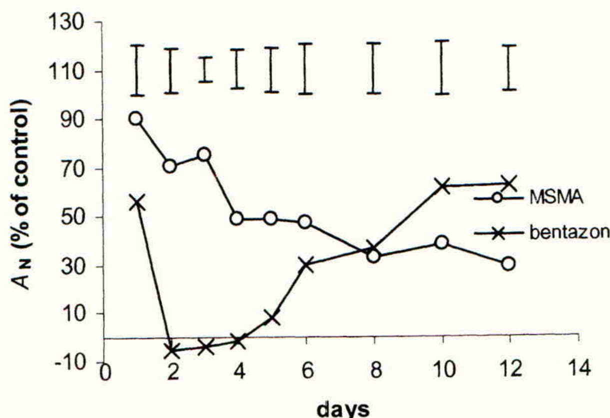

Figure 2. Photosynthetic response of yellow nutsedge to MSMA and bentazon for 12 days after treatment. Bars indicate $LSD_{0.05}$ values.

Competition for water is often considered the most important source of weed/crop competition. Therefore, g_s was monitored for each treatment, relative to the control (Figure 3). For glyphosate, imazapic, and halosulfuron, declines in g_s were highly correlated to reduction in A_N. Reduction in A_N increases leaf internal CO_2 concentrations, which signals stomata to close (Jones, 1992). However, the MSMA treatment displayed a quite different pattern between A_N and g_s. The initial decline in g_s for the MSMA treatment was similar to that of A_N. However, recovery occurred after 12 d and g_s was near 95% of the untreated control while A_N was near 35%. Therefore, MSMA treated sedge plants were transpiring water at near full capacity while A_N was reduced by 70%.

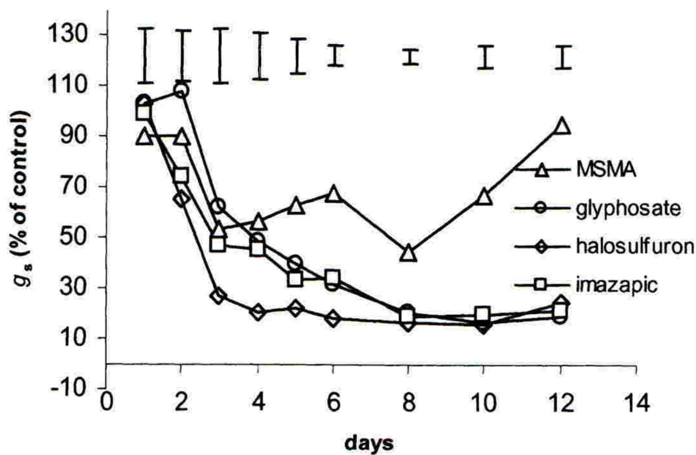

Figure 3. Yellow nutsedge stomatal conductance response to glyphosate, imazapic, halosulfuron, and MSMA treatment. Bars indicate $LSD_{0.05}$ values.

Foliar regrowth was examined after each herbicide treatment. Halosulfuron, imazapic, glyphosate, and MSMA treatments allowed between 0 and 5% regrowth, relative to the control. The bentazon treatment allowed 44% regrowth, relative to the control.

Taken together, these data suggest that application of halosulfuron or imazapic would reduce, or eliminate, yellow nutsedge competition with desirable plants more quickly than glyphosate, MSMA, or bentazon. Application of bentazon was shown to be insufficient to alleviate yellow nutsedge competition due to photosynthetic recovery after 6 d. Moreover, bentazon treatment allowed regrowth to 44% of the untreated control. MSMA did little to reduce competition for water, although reducing A_N by 70% after 12 d.

Study 2

Daytime gross carbon assimilation rates stayed fairly constant for untreated plants over the course of the experiment, dropping from 111% to 90% of the average rate during the

normalization days. All three herbicide treatments significantly reduced the daytime carbon assimilation relative to the control over time, but this reduction was greater and occurred more quickly with MSMA than with mesotrione. Halosulfuron was intermediate in its effect (Figure 4). The effect of MSMA was significant even one day after treatment (DAT), and by 5 DAT respiration exceeded carbon assimilation during the photoperiod (i.e., net carbon assimilation rate was negative) (data not shown). Halosulfuron reduced gross carbon assimilation to 30% of the pre-treatment rate by the end of the experiment, while mesotrione never reduced carbon assimilation below 59% of the pre-treatment rate (Figure 4). Night-time respiration rates were also significantly reduced by MSMA treatment by 6 DAT.

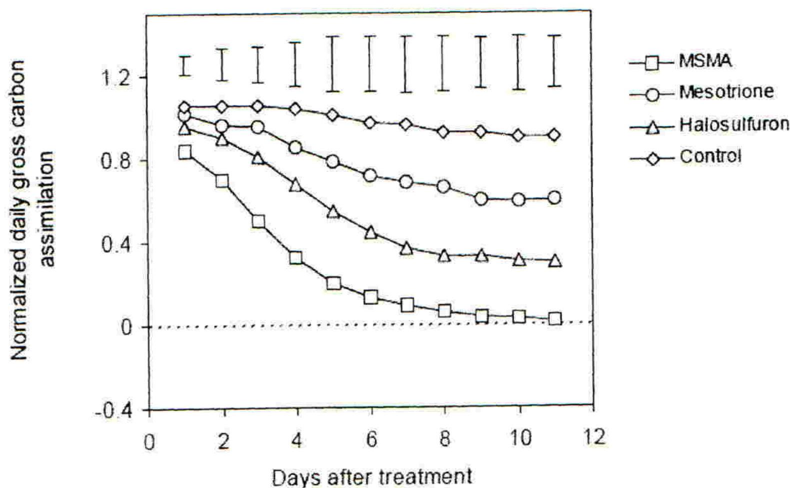

Figure 4. Effects of three herbicide treatments on daytime net carbon assimilation of yellow nutsedge plants.

The effects of the herbicide treatments on whole plant water use contrasted sharply with the effects on photosynthetic carbon assimilation. MSMA treatment resulted in small, but statistically significant, reductions in whole plant water use at 2 and 4 DAT, but otherwise had normalized transpiration rates very close to 1.0 (i.e., no indication of transpiration being reduced relative to control plants). Water use by halosulfuron treated plants declined steadily over the first 7 DAT, and was significantly lower than water use of MSMA treated plants from 6 DAT until the end of the experiment. Similar to MSMA, yellow nutsedge treated with mesotrione showed no discernible reduction in whole plant water use over the course of the experiment.

Halosulfuron and MSMA reduced shoot regrowth to between 0 and 5% of the control (Table 1). Halosulfuron effectively reduces yellow nutsedge shoot number, shoot weight, and root-tuber production (Vencill *et al.,* 1995). Conversely, the mesotrione treatment allowed some 58% regrowth. The ineffectiveness of mesotrione for yellow nutsedge rhizome control was expected, considering that yellow nutsedge does not appear on the product label as a weed that is controlled by postemergence applications.

Table 1. Regrowth of above ground phytomass occurring after treatment with herbicides. Means followed by the same letter do not differ according to a protected LSD test ($P < 0.05$). $n = 4$.

Herbicide	Rate (g/ha)	Dry Weight of Regrowth (g)
Untreated		9.5 a
Mesotrione	110	5.5 b
MSMA	2200	0.5 c
Halosulfuron	63	0.0 c
LSD (0.05)		0.8

In study 1, the application of halosulfuron or imazapic reduced, or eliminated yellow nutsedge competition with desirable plants more quickly than glyphosate, MSMA, or bentazon. However, the MSMA treatment displayed a quite different pattern between A_N and g_s. The initial decline in g_s for the MSMA treatment was similar to that of A_N. However, recovery occurred after 12 d and g_s was near 95% of the untreated control while A_N was near 35%. MSMA treated sedge plants were transpiring water at near full capacity while A_N was reduced by 70%. Study 2 was initiated to examine this phenomenon and confirmed the effect. MSMA rapidly and completely eliminated carbon assimilation, but had almost no effect on plant water use. The mechanism of action for MSMA is not well understood (Vencill 2002). However, there has been evidence to suggest that MSMA inhibits the malic enzyme in C_4 plants (Knowles and Benson 1983). The accumulation of malic acid could then lead to cessation of carbon fixation and photooxidative damage. This hypothesis is supported by the visual symptomology present after MSMA application to susceptible plants. Photooxidative damage generally results in cellular disintegration or leakage. Therefore, we suggest that application of MSMA to yellow nutsedge results in destruction of the guard cells, which would lead to unregulated water loss through the stomata.

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Metamifop: mechanism of herbicidal activity and selectivity in rice and barnyardgrass

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ABSTRACT

Metamifop (coded DBH129, ISO proposed) is a new aryloxyphenoxypropionate (AOPP) post-emergence herbicide developed by Dongbu Hannong Chemical Co Ltd, Korea. One of the most outstanding features of metamifop is that it shows an exclusive whole plant safety to rice with a high control efficacy to annual grass weeds, especially barnyardgrass. To determine the reason for the selectivity of metamifop, we examined ACCase sensitivity, absorption and translocation of [^{14}C] metamifop in both rice (tolerant) and barnyardgrass (susceptible). The I_{50} values for inhibition of ACCase by metamifop was >10 μM in rice and 0.5 μM in barnyardgrass. This differential sensitivity is consistent with whole plant sensitivity under greenhouse conditions. More [^{14}C] metamifop was absorbed through the leaf surface in barnyardgrass than in rice, with about 83% and 56% of the total applied [^{14}C] penetrating 72 hrs after application respectively. Translocation was not significantly different between the two species. These data demonstrated that the selectivity of metamifop between rice and barnyardgrass could be due to both differential foliar absorption rate and differential ACCase sensitivity.

INTRODUCTION

Metamifop [coded DBH-129, (*R*)-2-[4-(6-chloro-1,3-benzoxazol-2-yloxy)phenoxy]-2'-fluoro-*N*-methylpropionanilide] (Figure 1), is a new post-emergence herbicide discovered first by the Korea Research Institute of Chemical Technology (KRICT). For a member of the AOPP class of herbicides, metamifop shows excellent whole plant selectivity between rice and barnyardgrass. Like other AOPP herbicides, metamifop is an inhibitor of ACCase which catalyses the first committed step in fatty acid biosynthesis in plants. Metamifop strongly inhibits plant ACCase, with an I_{50} value of approximately 0.6 μM for the partially purified ACCase from barnyardgrass (Kim *et al.*, 2002 & 2003). This is the first report of the selectivity of metamifop within grass species. In this study, we confirm the primary target site of metamifop and determine the fundamental mechanisms involved in the robust rice safety.

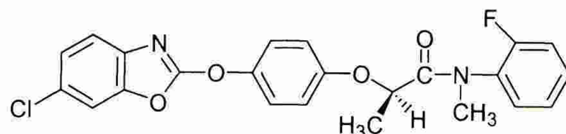

Figure 1. Chemical structure of metamifop

MATERIALS AND METHODS

Chemicals

Two stereoisomers of (R) and (S) metamifop with > 97% purity were prepared by KRICT. The [^{14}C] metamifop (specific activity 1,620 MBq mmol^{-1}) was provided from Korea Radiochemical Center, Suwon, Korea. The (S) metamifop was separated by HPLC using a chiral column.

Preparation of ACCase and assay of activity

ACCase was assayed as described previously (Kim *et al.*, 2000), and all procedures were carried out in a cold chamber at 4°C. Meristematic tissues of the 2-leaf stage grown under greenhouse conditions were harvested and stored in a deep freezer at –70°C until used. The leaf tissues (10 g) were ground with a 30 ml of 100 mM Tris buffer (pH 8.5) containing 1 mM EDTA, 10 % (v/v) glycerol, 2 mM D-isoascorbic acid, 1 mM phenylmethylsulfonyl fluoride, 0.5 % (w/v) polyvinylpolypyrrolidone 40, and 20 mM DL-dithiothreitol. The homogenates were then filtered through a layer of Miracloth (Calbiochem, USA) and centrifuged (J2-21M/E, Beckman) at 27,000 *g* for 10 min. To obtain an adequate reaction in barnyardgrass, the decanted supernatant was adjusted to 20% ammonium sulfate for 30 min. After centrifugation for 30 min at 27,000 *g*, the protein in the supernatant was precipitated by adding ammonium sulfate to 40% saturation. The final pellet was then resuspended in an elution buffer (2.5 ml) of 50 mM Tricine (pH 8.5) containing 2.5 mM $MgCl_2 6H_2O$, 1.0 mM DTT and 50 mM KCl. The enzyme extracts were desalted through a PD-10 (Amersham Pharmarcia Biotech, Sweden) column that had been equilibrated with the elution buffer.

ACCase activity was measured by the incorporation of [^{14}C] into heat-stable products following incubation with $H^{14}CO_3$. A 30 µl of enzyme preparation was pre-incubated for 2 min in a water bath at 32°C in a medium of 20 mM Tris (pH 8.5) containing 10 mM KCl, 5 mM ATP, 2 mM $MgCl_2$, 2.5 mM DTT, 0.15 mM $NaH^{14}CO_3$ (Dupont-NEN, specific activity 251.6 MBq mole^{-1}). A 10 µl aliquot of various concentrations up to 2.5 µM of (R) metamifop dissolved in dimethyl sulfoxide (DMSO) was applied in the reaction. The final volume of reaction mixture was adjusted to 200 µl, and 100 µg protein was used in each reaction. The enzyme activity was initiated by adding 10 µl of 16 mM acetyl-CoA, allowed to proceed for 10 min at 32°C and stopped by adding 20 µl of 12 N HCl. The samples were then dried under a stream of air in a water bath at 90°C for 1 hr and dissolved with 0.5 ml ethanol and 4 ml scintillation cocktail solution. The amount of radioactive products generated during the reaction interval was quantified by a liquid scintillation counting (LS6500, Beckman). Background [^{14}C] fixation was determined by substituting water for acetyl-CoA, and background counts were commonly less than 5% of ^{14}C-fixation in no-herbicide controls. The protein content in enzyme preparations was determined by the method of Bradford using bovine serum albumin as a standard.

All experiments were conducted at least twice, with 3 replicates for each experiment. The data from all experiments were pooled, and the means with standard deviation are presented.

Foliar uptake and translocation

Solutions of [^{14}C] metamifop were prepared in 50% aqueous acetone containing 0.1% Tween 20 so that each 10 μl included approximately 6.4 KBq (440,000 dpm). Using a micropipette, the 10 μl solution was applied as 10 microdroplets to middle of the fully expanded 3rd leaves in rice and barnyardgrass. At various time of intervals, the treated parts were excised, and unabsorbed [^{14}C] metamifop was washed out of the leaf surface by shaking 40 times by hand with 10 ml of 50% aqueous methanol containing 0.1% Tween 20. The amount of [^{14}C] in 1 ml of each wash was determined by liquid scintillation counting.

For the translocation study, the treated leaves were separated from the remainder of shoot 72 hrs after application. The treated leaf was subdivided into the treated area, the upper portion (toward the leaf tip), and the lower portion. The plant tissues were then dried in an oven at 50ºC for a week and combusted in a biological sample oxidizer (Packard 306). The evolved [^{14}C] in 15 ml of carbosob/Permafluor E^+ (5/10, v/v) was determined by liquid scintillation counting. The amount of [^{14}C] metamifop absorbed through the leaf surface was calculated as the total amount of radioactivity recovered in the treated plant and expressed as a percentage of the total applied. The translocation amount was expressed as a percentage of the total radioactivity absorbed by the plants.

RESULTS AND DISCUSSION

Although the chemical structure of metamifop is grouped in the AOPPs, the most prominent feature of this new herbicide is the remarkable whole plant selectivity shown between rice and barnyardgrass (Kim *et al.*, 2003). Under greenhouse conditions, whole plant dose-responses to metamifop were remarkably different in rice and barnyardgrass. Barnyardgrass was completely controlled with a rate of 8 g a.i./ha, while rice survived even at a rate of 2,000 g a.i./ha metamifop (Table 1). It has been reported (Kim *et al.*, 2003) that the primary target site

Table 1. Whole plant dose response of metamifop on rice and barnyardgrass under greenhouse conditions

Treatment	Rate	Rice	Barnyardgrass
	(g a.i./ha)	(% of injury)	(% of control)
(R) metamifop	2,000	21	100
	1,000	0	100
	500	0	100
	250	0	100
	125	0	100
	63	0	100
	32	0	100
	16	0	98
	8	0	99
	4	0	63

of metamifop is ACCase, which is known as the first committed step in fatty acid biosynthesis in plants. In that report, the I_{50} value for metamifop against the partially purified barnyardgrass ACCase was about 0.6 μM. Metamifop showed steroselectivity both in whole plant and *in vitro* ACCase assays, the (S) isomer being much less active than the (R) isomer (data not shown). These results confirmed that the primary target site of metamifop in plants is ACCase.

Selectivity was also seen between rice and barnyardgrass in the sensitivity of ACCase to metamifop. The *in vitro* I_{50} values for ACCase inhibition were >10.0 μM in rice and 0.5 μM in barnyardgrass, demonstrating that rice ACCase is at least 20 times less sensitive to metamifop than that of barnyardgrass (Figure 2). This differential *in vitro* ACCase sensitivity mirrors the whole plant sensitivity shown in Table 1. This result suggests that the binding sites of metamifop on rice and barnyardgrass ACCase are different, and that this could be a major contributor to the whole plant selectivity.

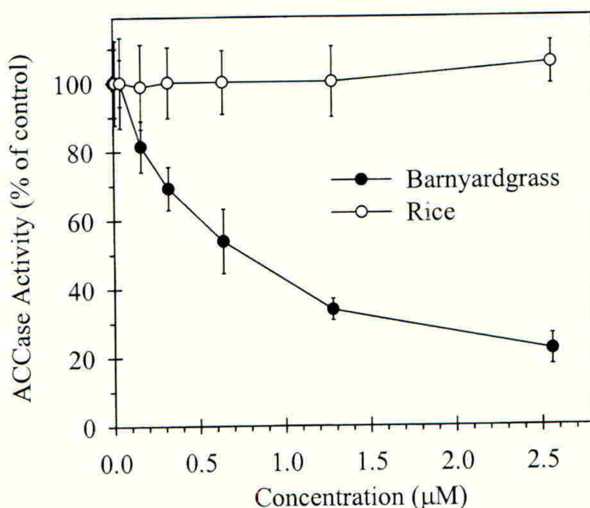

Figure 2. Effect of metamifop on *in vitro* ACCase activity in rice and barnyardgrass.

Foliar absorption of [^{14}C] metamifop through the leaf surface of rice and barnyardgrass was almost complete within 24 hrs after treatment, and was not significantly changed afterward (Figure 3). Rice showed a lower foliar uptake than barnyardgrass 72 hrs after application, values being about 85% and 50% of total applied [^{14}C] metamifop, respectively (Figure 3).

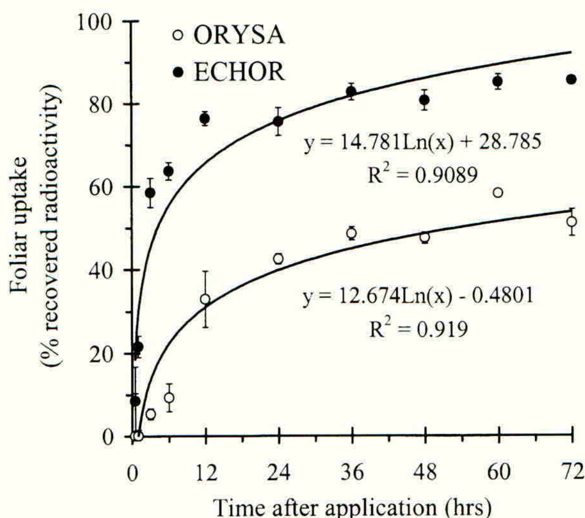

Figure 3. Foliar absorption of [^{14}C] metamifop into rice (ORYSA) and barnyardgrass (ECHOR).

Translocation was very similar between rice and barnyardgrass 72 hrs after application, with about 75% of absorbed [^{14}C] remaining in the treated zone and about 25% exported from the treated zone in both species (Table 2). Of the exported [^{14}C] amounts, around 19% moved to the upper part of treated zone while less than 1% was recovered in the lower part, suggesting that metamifop moves mainly through xylem rather than through phloem. Only small [^{14}C] amounts, 0.8% in rice and 1.0% in barnyardgrass, were recovered from the remaining parts of the plant.

Table 2. Distribution of [^{14}C] in rice and barnyardgrass 72 hrs after treatment

Species	[^{14}C] content (% of absorbed)				Recovery rate
	Treated zone	Upper leaf	Lower leaf	Remainder of plant	
Rice	74.2	18.3	0.7	0.8	0.94
Barnyardgrass	75.4	19.5	1.0	1.0	0.97

In conclusion, the different levels of foliar absorption could contribute to the selectivity between rice and barnyardgrass; however, the differential ACCase sensitivity is fundamental in explaining the robust rice safety of metamifop.

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Herbicide resistance in *Lolium multiflorum* Lam. (Italian rye-grass): involvement of glutathione *S*-transferases

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ABSTRACT

Italian rye-grass (*Lolium multiflorum* Lam.) is a major weed of arable crops in the UK. The extent of herbicide resistance in this species is now second only to that of black-grass and hinders the effective control of this grass weed. Elucidation of the mechanisms underpinning this resistance is vital in the battle to control rye-grass. Glutathione *S*-transferases (GSTs) have been implicated in herbicide resistance in many species, enabling resistant individuals to detoxify herbicides at an enhanced rate. Data is presented from glasshouse trials on a number of rye-grass biotypes that have previously been shown to be poorly controlled by herbicides in the field. GST activity and abundance data is presented for these biotypes, demonstrating that this important enzyme family may be implicated in herbicide resistance in the biotypes studied. These findings will be discussed in relation to both herbicide resistance and GST activity in other UK grasses.

INTRODUCTION

Lolium multiflorum Lam. (Italian Rye-grass) is a major weed of arable crops, especially cereals, in the UK and Northern Europe. Its presence results in significant yield losses, poor quality product and increased lodging of crops.

Herbicide resistance is the inheritable ability of a weed biotype to survive the application of a dose of herbicide that would be lethal to a susceptible population of the weed. Resistance in *Lolium multiflorum* in the UK was first reported in 1993 to the herbicides diclofop-methyl, fenoxaprop-ethyl and fluazifop-P-butyl (Moss *et al.*, 1993). Since then, incidents of resistance have increased and the number of reports of resistance in UK rye-grass populations is now only second to that of *Alopecurus myosuroides* (black-grass). By 2003 over 100 cases of resistance have been reported from 21 UK counties (WRAG, 2003). Herbicide resistant populations of rye-grass result in chemical control being less effective, more expensive and less predictable.

Glutathione *S*-transferases (GSTs) are a family of enzymes that catalyse the conjugation of a variety of xenobiotic chemicals, including some herbicides, to the tripeptide glutathione. This often results in both increased solubility and decreased toxicity of the xenobiotic (Marrs, 1996). GSTs have been implicated as being involved in enhanced metabolism-based resistance in *A. myosuroides* (Reade and Cobb, 1999; 2002). Resistant biotypes of this weed possessed constitutively higher GST activities and GST polypeptide abundance than susceptible biotypes. Investigation of resistance mechanisms in *L. multiflorum* have also

suggested a role for GSTs in this species (Cocker *et al.,* 2001). The role of GSTs in herbicide resistance in grasses has recently been reviewed (Reade and Cobb 2003).

This study presents findings from glasshouse-based herbicide trials and measurements of GST activities and abundance for *L. multiflorum* biotypes that have previously proved to be poorly controlled in the field. Results are discussed in comparison to herbicide resistance in *A. myosuroides*, population plasticity and the possibility of correlations between GSTs and herbicide resistance.

MATERIALS AND METHODS

Materials

L. multiflorum seeds from a susceptible standard and from biotypes demonstrating poor control in the field were provided by Syngenta Crop Protection UK. All herbicides were commercial grade. Propaquizafop was provided by Makhteshim-Agan.

Glasshouse Trial

Plants were grown as previously described for *A. myosuroides* (Reade and Cobb, 1999) using John Innes No 2 compost, under glasshouse conditions. Herbicide treatments were delivered at GS 12/13, as detailed in Table 1, using a patent pot sprayer with 03 F110 nozzles delivering a medium quality spray at a volume of 200 litres ha^{-1}, 45cm above the plants. Fresh weights of individual plants were determined three weeks after herbicide treatment.

Table 1. Rate of application (g a.i./ha) of herbicides in glasshouse trials

Active Ingredient	Fenoxaprop-ethyl	Clodinafop-propargyl	Isoproturon	Propaquizafop	Sethoxydim
Rate Applied	70g/ha	60g/ha	1500g/ha	120g/ha	338g/ha

Protein extraction and GST assay

Proteins were extracted as described by Reade and Cobb (1999). GST activities were assayed against the artificial substrate 1-chloro-2,4-dinitrobenzene (CDNB) using the micro GST assay described by Reade and Cobb (2002). Protein concentration of extracts were determined using a modification of the Bradford dye-binding assay (Bradford, 1976), as previously described (Milner *et al*, 2001). GST activity data is expressed as nmoles CDNB min^{-1} mg^{-1} total protein.

Determination of GST polypeptide abundance

GST polypeptide abundance was determined by enzyme-linked immunosorbent assay (ELISA) using monoclonal antiserum raised against the 30-kD GST subunit purified from *A.myosuroides* biotype Peldon (Reade and Cobb, 2002).

Data analysis

Fresh weight data from herbicide trials were converted to a resistance classification as described by Moss *et al.* (1999). GST activity data are expressed as the ratio of activity to that extracted from susceptible biotype. GST abundance data are expressed as normalised data, taking biotype B1 as 1.0. Three replications were used in all experimentation and analyses.

RESULTS

The results of the glasshouse herbicide trial are shown in Table 2. All biotypes, except the susceptible standard (B1), demonstrated resistance to clodinafop. Cross-resistance to fenoxaprop was detected in 3 biotypes and resistance to propaquizafop in only one biotype (B11). No biotype demonstrated resistance to sethoxydim. Two biotypes, B9 and B12, also showed resistance to isoproturon.

Table 2. Glasshouse-based herbicide trials of five *L. multiflorum* biotypes. Resistance classifications (S, R, RR) were calculated as described by Moss *et al.*, (1999)

Biotype	B1	B9	B10	B11	B12
Isoproturon	S	RR	S	S	RR
Fenoxaprop	S	RR	S	RR	RR
Clodinafop	S	R	R	RR	RR
Propaquizafop	S	S	S	RR	S
Sethoxydim	S	S	S	S	S

GST activities are shown in Figure 1. All biotypes identified as possessing resistance to at least one of the herbicides studied had extractable GST activities greater than that of the susceptible biotype. These ranged from 1.9 to 4.3 times the activity in the susceptible biotype (Table 3). GST abundance was greater in three of the four biotypes displaying resistance to at least one of the herbicides studied, ranging from 2.74 to 5.91. In biotype B11 a relative abundance of 0.37 was obtained.

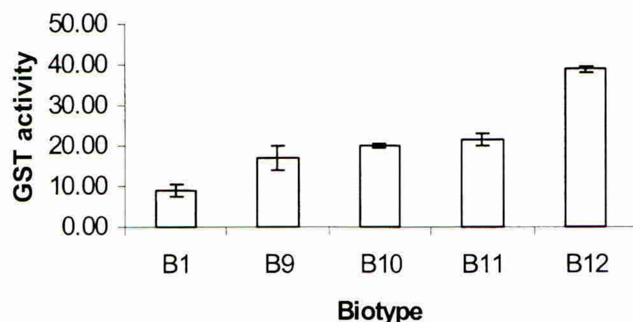

Figure 1. GST activities (nmoles min^{-1} mg^{-1} total protein ± standard error) for five *L. multiflorum* biotypes

Table 3. GST activities and abundance for five *L. multiflorum* biotypes expressed as ratios to those of biotype B1

Biotype	B1	B9	B10	B11	B12
GST activity (ratio to biotype B1)	1.0	1.9	2.3	2.4	4.3
GST Abundance (ratio to biotype B1)	1.0	2.74	5.91	0.37	5.76

DISCUSSION

Herbicides from two mode of action classes were used in the glasshouse trials, namely inhibition of photosynthesis at PS II (IPU) and inhibition of ACCase ('fops' and 'dims'). Biotypes B9 and B12 demonstrated resistance against herbicides from both of these classes, indicating the presence of multiple resistance in these biotypes. The other resistant biotypes (B10 and B11) were susceptible to IPU, but possessed different susceptibilities to the 'fops' used. Absence of resistance to sethoxydim suggests that target site resistance in the form of herbicide-insensitive ACCase is not present in any of the biotypes studied (Moss *et al.*, 2003), indicating that resistance in these biotypes to 'fops' is due to enhanced metabolism of these herbicides.

GST activities were lowest in the susceptible biotype (B1), with resistant biotypes possessing between 1.9 and 4.3 times the extractable GST activity against CDNB (Table 3). GST abundance was greater in three of the four resistant biotypes compared to the susceptible biotype B1. A high degree of correlation between activity and abundance was not observed. However, CDNB is not a substrate for all GSTs and antiserum was raised against an individual GST polypeptide, so correlation between GST activity and abundance may not always be seen. In this respect the *L. multiflorum* biotypes studied show similarities to *A. myosuroides*, where resistant biotype Peldon contains approximately double the GST activity and greater GST polypeptide abundance than susceptible biotype Herbiseed (Reade and Cobb, 1999). Other resistant *A. myosuroides* biotypes also demonstrate enhanced GST activities and abundance (Reade and Cobb, 2002). In addition, the enhanced GST activity in *L. multiflorum* is constitutive, not a result of herbicide application. It is therefore possible that enhanced GST activity in *L. multiflorum* biotypes is responsible for an enhanced rate of herbicide conjugation and therefore herbicide resistance. Alternatively, GSTs may be acting as peroxidases in the detoxification of either herbicides or active oxygen species resulting from herbicide activity, as previously reported in *A. myosuroides* (Cummins *et al.*, 1999).

It is interesting to note that antiserum raised against a GST polypeptide from *A. myosuroides* detected polypeptides in *L. multiflorum* extracts. This indicates that polypeptides with similar epitopes are present and that the monoclonal antiserum will be useful in future studies in *L. multiflorum*.

The complex patterns of cross-resistance shown in the biotypes studied suggests that GSTs are not the only factor imparting resistance in *L. multiflorum*, as if this were the case then more consistency between GST activities/abundance and resistance to individual herbicides might be expected. It is postulated that increased GST activities play a role in resistance, as in *A. myosuroides*, but that they are involved alongside other stress-related enzymes in detoxification of herbicides and protection from herbicide damage.

The large variation in GST activities and abundance between biotypes suggests that there may be a high degree of plasticity in this species with respect to GSTs. This has been observed in field populations of *A. myosuroides* (Reade and Cobb, 2002) and it has been postulated that herbicide application to such populations results in selection of those individuals possessing greater GST activity/abundance. The observed variation in GST activity and abundance in *L. multiflorum* biotypes suggests that the same could be happening in this species.

CONCLUSIONS

The *L. multiflorum* biotypes studied demonstrate individual, complex, cross-resistance patterns to the herbicides studied. They are unlikely to possess target site resistance due to their sensitivity to sethoxydim. All demonstrated enhanced GST activity and three of the four possessed greater abundance of a polypeptide recognised by antiserum raised against a 30kDa GST polypeptide from *A. myosuroides*. It is postulated that GSTs may play an important role in enhanced metabolism-based herbicide resistance in *L. multiflorum*.

ACKNOWLEDGEMENTS

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Structure of dichloromethyl-ketal safeners affects the expression of glutathione S-transferase isoforms

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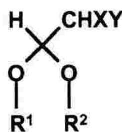
ABSTRACT

The herbicide safener MG-191 and its acetal and ketal analogues as well as mono- and dichloroacetamides were tested for their ability to alleviate toxicity of acetochlor to maize and differentially enhance the glutathione (GSH) content and the expression of glutathione transferase (GST) isoforms in maize. Our results demonstrate that the safener structure affects the specific expression of GSTs mediating the detoxication of acetochlor. No correlation was found between the degree of induction of GSH and GSTs and the safening activity.

INTRODUCTION

Safeners are chemical agents that increase the tolerance of crop plants to herbicides without affecting the weed control efficacy. They appear to induce a set of genes that encode enzymes and the biosynthesis of cofactors involved in herbicide detoxication (Gatz, 1997). Glutathione S-transferase isoenzymes (GSTs) and endogenous glutathione (GSH) play a vital role in chloroacetamide herbicide detoxication by GSH conjugation. Safeners of various chemical classes were found to induce the activity of GSTs and the level of GSH in the protected plants (Davies & Caseley, 1999).

The highly active dichloromethyl ketal MG-191 (2-dichloromethyl-2-methyl-1,3-dioxolane) is used in the safening of maize against thiocarbamate and to a lesser extent chloroacetamide herbicides. MG-191 has been found to induce *Zm*GSTU1-2, a tau (U) class GST isoform of maize (Jablonkai, *et al.*, 2001). In order to further clarify the significance of GST and GSH enhancement in safening maize against the herbicide acetochlor (**Ac,** 2-chloro-N-(ethoxymethyl)-N-(2-ethyl-6-methylphenyl) acetamide) for this safener class, the relationship of structure to safening efficacy, GSH and GST inducibility was examined using halogenated acetals (**1a-l**), ketals (**2a-k**) and acetamides (**3a-d**).



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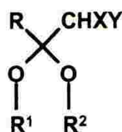
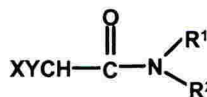


2a-k



3a-d

METHODS AND MATERIALS

Chemicals

Open-chain dichloromethyl acetals and ketals were synthesized from dichloroacetaldehyde, 1,1-dichloroacetone, and 1,1-dichloroacetophenone (Dutka, 1991). Cyclic acetals and ketals were prepared from diethyl acetal and ketal of dichloroacetaldehyde and 1,1-dichloroacetone by transacetalisation. Acetamides were synthesised by haloacetylation of amines using standard Schotten-Baumann conditions. Crude reaction products were purified by either distillation or silica gel column chromatography. Acetochlor was purified by column chromatography from the commercial product. [Carbonyl-^{14}C] acetochlor (sp. act. 37 MBq /mmol) was a sample prepared previously (Jablonkai & Hatzios, 1991). Purity for all compounds was greater than 95 %. All other chemicals were purchased from Aldrich (Sigma-Aldrich Kft., Budapest, Hungary)

Safener activity of experimental molecules

Seeds of maize (Gazda MV) were soaked in water and planted in plastic cups (6 cm diameter, 9 cm deep, 3 seeds/cup) containing air-dried foundry sand (250 g, OH-4 type). Treatment solutions (50 ml) containing safener (50 M) and/or acetochlor (50 M) were applied to each cup. Seeds were placed 2 cm deep. The plants were grown in a growth room (temperature: 23 $\pm$ 1 °C; relative humidity: 60 $\pm$ 5 %; light intensity: 10 klux; light period: 16 h per day). The plants were watered three times a week to bring the weight of cups to 300 g. Plants were harvested two weeks after the treatment and shoot lengths measured. The experiment was carried out twice with four replicates.

Plant material and enzyme isolation

For GST activity analyses seeds (25) of maize were placed in Petri dishes (18.5 cm in diameter) on two layers of filter paper wetted by aqueous solution (20 ml, 50 M) of chemicals studied. The dishes were placed in a germination thermostat. The seedlings were grown in the dark for 5 days at 27 °C. Five-day-old seedlings were thoroughly washed with tap water and separated shoots were homogenized in a mortar and pestle using quartz sand then extracted with 5 volumes of cold Tris-HCl buffer (100 mM, pH 7.5) containing 2 mM EDTA, 1 mM dithiothreitol and 5 % (w/v) polyvinyl polypirrolidone. The homogenates were filtered through two layers of Miracloth and the filtrates were centrifuged at 10,000 x g for 20 min at 4 °C. The supernatants were brought to 80% $(NH_4)_2SO_4$ saturation and centrifuged at 10,000 x g for 20 min at 4 °C. Aliquots of the protein precipitates were resuspended in potassium phosphate buffer (20 mM pH 6.5) and desalted by gel filtration (Sephadex G25, medium) before use for enzymatic studies.

For Western blot experiments shoot and root enzyme extracts of maize (Cecilia) were prepared as reported previously (Jablonkai *et al.*, 2001)

For determination of GSH contents, shoot tissues of etiolated seedlings were grown as described earlier. Tissues were frozen and homogenized in liquid nitrogen and extracted with 4 volumes of 70% ethanol. The homogenates were centrifuged at 10,000 x g for 20 min at 4 °C and the supernatants were collected.

Analysis of GSTs and GSH

Glutathione *S*-transferase activities of desalted enzymes were determined with CDNB (1-chloro-2,4-dinitrobenzene) and [carbonyl-^{14}C]acetochlor (Ac) substrates. GST(CDNB) activities were determined spectrophotometrically (340 nm) and expressed as nmol product formed per second (nkat) per mg protein (Dixon *et al.*, 1998a). GST(Ac) activities of the samples were determined by liquid scintillation counting of the conjugate formed in the reaction of [carbonyl-^{14}C]acetochlor (0.75 mM) and GSH (10.0 mM) mediated by the desalted enzymes at 37 °C in 30 min. The GST(Ac) activity was expressed as pmol conjugate per second (pkat) per mg protein. Protein contents of the extracts were determined spectrophotometrically using a Coomassie Brillant Blue reagent with bovine serum albumin as reference protein.

The polypeptide composition of the GST preparations were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting was carried out using antisera raised to *Zm*GSTF1-2 and *Zm*GSTU1-2 (Dixon *et al.*, 1998a).

Non-protein thiol (GSH) content of alcoholic supernatant was measured spectrophotometrically (412 nm) using DTNB reagent (Jablonkai & Hatzios, 1991).

RESULTS AND DISCUSSION

Safening experiments were carried out in sand at a relatively high pre-emergence acetochlor rate (2.4 kg/ha) and at high moisture content. Under these conditions the herbicide is extremely phytotoxic and no complete protection can be achieved. The bromoacetaldehyde diethyl acetal (**1b**) showed moderate safening effects while the monochloroacetal **1a** and the dichloroacetals (**1c-l**) exhibited poor or no safening activity (Table 1). Among dialkyl ketals having increasing alkyl chain length (**2a-d**) the highest safening activity was observed for the diethyl (**2a**) and dipropyl (**2b**) derivative. In general, cyclic ketals (**2e-k**) were effective safeners. Derivatives having 1,3-dioxolane (**2f**), dioxane (**2g**) and dioxepane (**2i**) ring in their stucture were the most active molecules. Interestingly, dioxacycloalkanes with 8- and 9-membered ring were still active. The safening activity of ketals also exceeded that of acetals against the thiocarbamate EPTC (Dutka, 1991). It seems that the hydrolytic cleavage of the acetals or ketals is likely not involved in their mode of safening action since both the dichloroacetaldehyde and the dichloroacetone, the products of the hydrolysis, were inactive safeners (Dutka, 1991). Among amides the marketed safener dichloroacetyl-diallylamide (dichlormid, **3a**) was highly protective. Decreasing the number of halogens and allyl groups yielded less active molecules. On the other hand, the monochloroacetamide **3c**, which is an alkylating agent with herbicidal activity, was protective against EPTC (Pallos *et al.*, 1975).

The GSH content of shoot tissues of safener-treated plants was significantly increased by treatment with monohalomethyl acetals (**1a-b**) and was not affected by the dihalo derivatives as compared to that of untreated control (Table 1). Among ketals only cyclic derivatives (**2e-k**) induced GSH biosynthesis while open-chain ketals (**2a-d**) were not inducers. Both mono- (**3b-c**) and dichloroacetamide (**3a**) pretreatment elevated the GSH levels. It appears that there is no direct correlation between the elevation of GSH content and the safening efficacy. While twofold increase was found with the moderately safening **2e** cyclic ketal only a low or no inducing effect was observed for the more active molecules **2a-b**. Elevation of GSH content has

been observed for many safeners (Davies & Casely, 1999). However, safener efficacy is not well correlated with elevated GSH levels.

Table 1. Safening activity and inducibility of shoot GSH content and GST activities by acetals, ketals and amides in maize

Code	R	X	Y	R^1	R^2	Protection[a] (%)	GSH[b]	GST(CDNB)[c]	GST (Ac)[d]
							treated/control		
Ac	-	-	-	-	-	-	1.11	1.48	3.74
1a	-	H	Cl	Et	Et	24	1.49	0.69	2.03
1b	-	H	Br	Et	Et	60	1.53	0.94	3.76
1c	-	Cl	Cl	Et	Et	8	0.69	1.42	1.83
1d	-	Cl	Cl	Pr	Pr	0	0.80	0.95	1.38
1e	-	Cl	Cl	Bu	Bu	-6	1.22	0.96	0.91
1f	-	Cl	Cl	i-Bu	i-Bu	-2	1.20	0.90	1.61
1g	-	Cl	Cl	$-(CH_2)_2-$		18	0.93	0.88	1.23
1h	-	Cl	Cl	$-(CH_2)_3-$		14	0.60	0.89	0.90
1i	-	Cl	Cl	$-CH_2C(CH_3)_2CH_2-$		-3	0.91	0.88	1.33
1j	-	Cl	Cl	$-(CH_2)_4-$		11	0.95	1.03	1.33
1k	-	Cl	Cl	$-(CH_2)_5-$		0	0.98	1.24	0.58
1l	-	Cl	Cl	$-(CH_2)_6-$		3	0.82	1.32	0.83
2a	Me	Cl	Cl	Et	Et	62	1.15	1.22	0.65
2b	Me	Cl	Cl	Pr	Pr	63	0.98	1.18	0.85
2c	Me	Cl	Cl	Bu	Bu	38	0.78	0.88	3.93
2d	Me	Cl	Cl	i-Bu	i-Bu	14	0.85	1.07	4.72
2e	Ph	Cl	Cl	$-(CH_2)_2-$		41	2.00	1.94	2.23
2f	Me	Cl	Cl	$-(CH_2)_2-$		64	1.18	1.83	3.93
2g	Me	Cl	Cl	$-(CH_2)_3-$		68	1.31	1.49	1.96
2h	Me	Cl	Cl	$-CH_2C(CH_3)_2CH_2-$		66	1.62	1.77	1.44
2i	Me	Cl	Cl	$-(CH_2)_4-$		70	1.71	1.48	0.92
2j	Me	Cl	Cl	$-(CH_2)_5-$		50	1.24	1.27	1.19
2k	Me	Cl	Cl	$-(CH_2)_6-$		60	1.38	1.39	1.14
3a	-	Cl	Cl	allyl	allyl	81	1.78	1.24	4.69
3b	-	H	Cl	H	allyl	48	2.25	1.25	3.60
3c	-	H	Cl	allyl	allyl	2	1.45	1.16	2.39
3d	-	H	Br	allyl	allyl	22	0.98	0.90	2.98

[a] based on shoot length; protection (%) = 100 x [(herbicide + safener)] / [control - herbicide]; shoot lengths 14 DAT: control, 27.9±5.3 cm, acetochlor, 3.1±0.3 cm

[b] GSH content relative to that of untreated control; $GSH_{contr.}$: 0.55±09 μmol/g fresh weight

[c] GST(CDNB) activity as compared to that of untreated control; $GST_{contr.}$: 3.87±0.33 nkat/mg protein

[d] GST(Ac) activity as compared to that of untreated control; $GST_{contr.}$: 8.26±1.68 pkat/mg protein

GST(CDNB) activity of shoot tissues was only slightly affected by pretreatment with safeners (Table 1). Acetals except for **1c** and open-chain ketals (**2a-d**) did not influence GST(CDNB) activities. Cyclic ketals were all inducers of this GST isoform and derivatives having 1,3-dioxolane backbone (**2e** and **2f**) exerted a twofold increase. Amides were less effective inducers than cyclic ketals. The herbicide acetochlor in itself also increased this isozyme activity by 50%. No correlation exists between GST(CDNB) enhancement and the safening activity of the experimental molecules. GST(CDNB) activity associated with the safener inducible *Zm*GSTF1-2 isozyme was increased by the dichlormid in both roots and shoots of maize (Dixon *et al.*, 1997) and only slightly by the MG-191 (Jablonkai *et al.*, 2001).

GST(Ac) activities were enhanced by both protective and less effective structures as compared to that of untreated control (Table 1). Among acetals pretreatment with the most active safener **1b** induced the highest (3.76-fold) increase in the enzyme activity. Safening activity of the ketals was not correlated with their effects on GST(Ac) activity. While a high increase (4.72-fold) was shown after treatment with the hardly active **2d**, there was no effect of the most active ketal **2i**. For the amides the degree of induction of the isoenzyme activity was parallel with their safening potential. Correlation between GST(Ac) induction and safening activity exists only for the amides. Pretreatment of maize seedlings with acetochlor resulted in a very high degree of induction of the enzyme activity indicating that the induction of GST isoforms by both chloroacetanilides and their safeners is based on a similar mechanism.

The polypeptide compositions of the GSTs in safener-treated and untreated control root and shoot tissues were examined by SDS-page and Western blotting experiments in order to understand which maize GSTs were induced by the safeners. In this study only the more active ketal derivatives were used. The resulting blots were probed using antisera raised to a phi class *Zm*GSTF1-2 and a tau class *Zm*GSTU1-2 (Dixon *et al.*, 1998a). A higher inducibility of these GST isoforms was observed in root tissues (Figure 1). In shoots, when the heterodimer *Zm*GSTF1-2 was used the expression of constitutive *Zm*GSTF1 and inducible *Zm*GSTF2 was enhanced only by **2f** (MG-191) and its analogue **2g** having a 6-membered ring (Figure 1b).

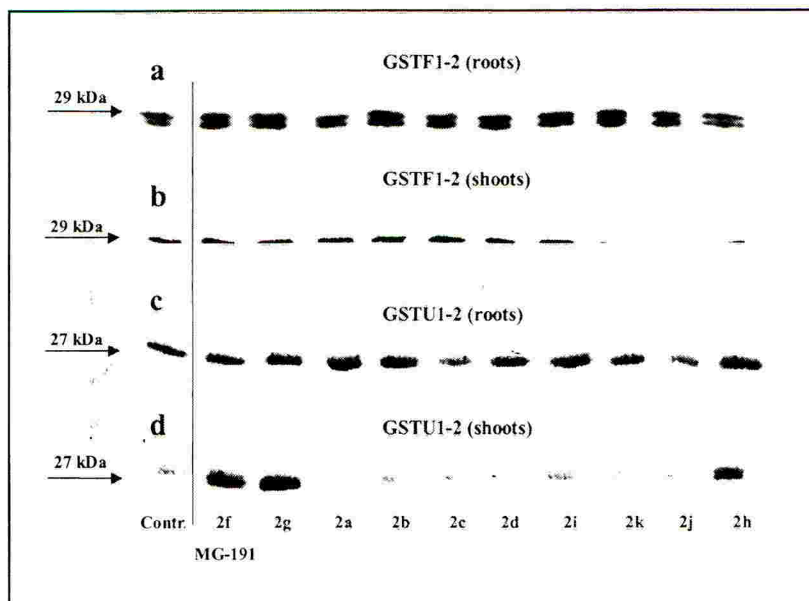


Figure 1. Western blots of crude GST extracts from maize roots and shoots; (a) and (b) analysis of GSTs using the anti-*Zm*GSTF1-2 serum from maize roots and shoots; (c) and (d) analysis of GSTs using the anti-*Zm*GSTU1-2 serum from maize roots and shoots.

These molecules and also **2h** were the most potent inducers of the expression of tau class *Zm*GSTU1 in shoot tissues (Figure 1c). *Zm*GSTU1 has previously been shown to play a key role in metabolism of nitrodiphenyl ether herbicides (Cole *et al.*, 1997). It seems that dichloromethyl ketal type safeners are more specific inducers *Zm*GSTU1-2 than other compounds commonly used to safen thiocarbamate and chloroacetanilide herbicides in maize (Jablonkai *et al.*, 2001).

The exact mechanism of the safener-mediated enhancement of GST activity is not completely understood. GSTs are induced by a diverse range of chemicals and accompanied by the production of active oxygen species. Thus the connection between safener-mediated protection of crops and oxidative stress tolerance has been suggested (Theodoulou *et al.*, 2003). Many GSTs are effective not only in conjugating electrophilic substrates but also function as glutathione peroxidases. Safeners may induce GST expression by mimicking oxidative insult (Dixon *et al.* 1998b). Our results indicate that safener structure plays a decisive role in specific expression of GSTs mediating the detoxication of chloroacetamide herbicides. Since no correlation between the degree of induction of levels of GSH and GST isoforms and the safener activity was found the mode of action of safeners is a more complex process than simply promoting the metabolism of herbicides.

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Mode of action of the rediscovered fumigant - ethyl formate

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ABSTRACT

Ethyl formate is a potential replacement for the ozone-depleting fumigant, methyl bromide. We have investigated the mode of toxic action of ethyl formate as part of an evaluation of its efficacy against insects. The acute toxicity of a range of alkyl esters, ethanol and formic acid were tested in the adult rice weevil, *Sitophilus oryzae* (L.) and adult grain borer, *Rhyzopertha dominica* (F.). All alkyl formates and formic acid were similarly toxic to *S. oryzae* and at least twice as potent as ethyl propionate, methyl acetate or ethanol. The order of potency was similar in *R. dominica* for the tested substances. Alkyl formates were rapidly metabolised *in vitro* to formic acid, when incubated with insect homogenates, presumably through the action of esterases. *S. oryzae* and *R. dominica* fumigated with a lethal dose of ethyl formate had 8 and 17-fold higher concentrations of formic acid, respectively, in their bodies than untreated controls. Cytochrome *c* oxidase activity from isolated insect mitochondria was unaffected by acetate and propionate esters or salts, ethanol or methanol but sodium formate was inhibitory. Toxicity of volatile formate esters to insects is much higher than related alkyl esters, due to hydrolysis of the formate esters to form formic acid and its inhibition of cytochrome *c* oxidase. From our knowledge of the mechanism of action, the development of insect resistance to ethyl formate is predicted to be slow.

INTRODUCTION

Ethyl formate is being re-evaluated as an alternative fumigant for methyl bromide, which is being phased out due to its contribution to depletion of stratospheric ozone (Bell, 2000). Alternatives to methyl bromide should be efficacious against a variety of insect pests and safe to consumer and workers, whilst not damaging either the stored product or the environment. Ethyl formate may satisfy these requirements. It is currently registered in Australia for protection of dried fruit but is being investigated for use as a horticultural and stored grain fumigant. Ethyl formate is similar to methyl bromide in killing all stages of insects within hours.

There is a range of volatile formate, acetate and propionate esters with similar physical and chemical characteristics to ethyl formate that could potentially be used as insecticidal fumigants of fresh and dried commodities, but little is known of their comparative toxicity to insects. Formate, or more probably, formic acid causes toxicity in mammals through its binding to cytochrome a_3 and inhibition of cytochrome *c* oxidase, the terminal oxidase of the electron transport chain (Nicholls, 1975). Therefore, it is reasonable to examine the inhibition of cytochrome *c* oxidase of insects by formate/formic acid in insects as a mechanism of ethyl formate toxicity, as formic acid may be generated in insects from hydrolysis of formate esters.

The relative toxicities of a range of alkyl esters and related compounds toward two pests of stored commodities, the rice weevil *Sitophilus oryzae* (L.) (Coleoptera; Curculionidae) and the lesser grain borer *Rhyzopertha dominica* (F.) (Coleoptera; Bostrichidae) were investigated through mortality bioassays and their inhibition of cytochrome *c* oxidase activity. The intention was to discover the toxic agent of the alkyl esters in insects; that is, to determine whether it is the intact ester or one or more of its components. The capacity of insects to convert ethyl formate to formic acid as a result of exposure was also examined.

MATERIALS AND METHODS

Insect culturing and mortality assessments

S. oryzae (L.) and *R. dominica* (F.) were obtained from laboratory strains cultured at CSIRO Entomology. Insects were cultured on soft wheat, which included some wholemeal flour for *R. dominica*, and were held at 25°C for *S. oryzae* and 30°C for *R. dominica* and 60% relative humidity.

Adult insects (100 per species) were exposed to one of a range of concentrations of volatile alkyl esters or related chemicals, having similar physical and chemical characteristics, in 2.7 litre sealed desiccators. The experiments were repeated at least twice to obtain sufficient data to determine lethal concentrations. The insects were exposed to the fumigants for 3 or 24 h at 25°C, then aired for 1 h, and placed on fresh medium in the culturing rooms for 4 d, after which they were assessed for mortality. LC_{50} and LC_{90} values were determined using a log concentration probit scale program (Finney, 1971). The confidence intervals for LC_{50} and LC_{90} values in *S. oryzae* exposed to methyl acetate could not be calculated, as the slope of the curve was too steep for sufficient points to be obtained.

Formic acid measurement in whole insect homogenate

Whole adult *S. oryzae* or *R. dominica* (3 g) were ground in a mortar and pestle at 4°C with 15 ml of 100 mM phosphate buffer pH 7.4 plus 1 mM disodium ethylene diamine tetraacetic acid (EDTA) and centrifuged at 9,000 *g* for 20 min at 4°C. The supernatant was removed and combined with ethyl formate at 20, 30 or 50 mM (final concentration) in sealed vials, in triplicate samples, and incubated for 10 min at 30°C. Formic acid, was detected and quantified by established method (Lang & Lang, 1972). Protein concentration of the supernatant was determined by the dye binding method (Bradford, 1976).

Insects (3 g each species, three replicates) were fumigated for 2 h with ethyl formate at 270 μmol $litre^{-1}$ containing 10% carbon dioxide and then aired in a fume cupboard. The treated insects and untreated controls from the same culture were processed as described above and the total formic acid content of their bodies was quantified.

Isolation of mitochondria and cytochrome *c* oxidase measurement

Adult *S. oryzae* (5 g) were ground at 4°C in 50 mM Tris buffer pH 7.4 containing 250 mM sucrose, 1 mM EDTA, 5 mM $MgSO_4$ and 0.2% bovine serum albumin, filtered, then centrifuged at 300 *g* for 10 min. The supernatant was removed and centrifuged at 5000 *g* for

10 min. and the resulting mitochondrial pellet was resuspended in isolation medium and again centrifuged at 5000 *g* for 10 min. The mitochondrial pellet was finally resuspended into a volume of 1.5 ml.

Cytochrome *c* oxidase activity was measured by monitoring the oxidation of cytochrome *c* at 550 nm in the presence of detergent-solubilised mitochondria (Storrie & Madden, 1990). Intact mitochondria gave a background rate of cytochrome *c* oxidation of less than 5% of the solubilised rate.

The effect of formate, acetate and propionate esters and related compounds on mitochondrial cytochrome *c* oxidase was determined by addition, with mixing, of pure ester into the assay buffer just prior to addition of mitochondria and cytochrome *c*. Concentrated stocks of cyanide, formate, acetate, and propionate as their sodium salts, were prepared in assay buffer containing lauryl maltoside. The effect on cytochrome *c* oxidase activity due to the inhibitor was determined from the difference in rates between inhibited and control samples and tested in triplicate. The maximum final concentration tested was 100 mM.

RESULTS AND DISCUSSION

To explore the comparative toxicities of volatile esters, mortality bioassays were conducted with a range of alkyl formates, methyl acetate and ethyl propionate against *S. oryzae* and *R. dominica* after 24 h exposure. In Figure 1, the concentration required to kill 50% or 90% of the sample of insects (LC_{50} or LC_{90}) for each volatile ester or related chemical is shown graphically, along with the confidence intervals for those parameters. In both insect species LC_{50} and LC_{90} values for formate esters and formic acid are very close and always lower than those for ethyl propionate, methyl acetate and ethanol. In *S. oryzae* and *R. dominica,* the LC_{50} values for the formate esters show they are approximately 5- and 9-fold more toxic, respectively, than that of methyl acetate (Figure 1 A&C). The higher toxicity of substances able to generate formic acid directly by hydrolysis is especially marked in *R. dominica* (Figure 1 C & D).

To determine the approximate rates of ethyl formate hydrolysis to ethanol and formic acid in insects, homogenates were prepared from the whole bodies of *S. oryzae* and *R. dominica* and incubated with ethyl formate in sealed vials. The production of formic acid was determined in the incubation mixture. The rate of metabolism was fast in both insect species. Mean rate of formate production at 20, 30 and 50 mM ethyl formate was 96, 122, 166 and 42, 72, 111 $nmol.min^{-1}.mg^{-1}$ protein for *R. dominica* and *S. oryzae*, respectively.

When live insects were exposed to ethyl formate at 270 $\mu mol\ L^{-1}$ and 10% carbon dioxide, *S. oryzae* and *R. dominica* were killed within two hours. These conditions were chosen to ensure as little as possible degradation of metabolites in the insect bodies before analysis. The ethyl formate concentration in the desiccator containing insects decreased by a total of 219 µmol compared with an identically dosed but empty desiccator. Therefore, the theoretical maximum uptake of ethyl formate by the insects in the desiccator was 36 $\mu mol.g^{-1}$ insect. Following the airing period, the insects were processed and the formic acid concentrations were compared with batches of unexposed insects from the same culture. The concentrations of formic acid in fumigated *S. oryzae* and *R. dominica* were 23 and 34 $\mu mol\ g^{-1}$ and substantially higher than the unexposed controls (3 and 2 $\mu mol\ g^{-1}$ insect, respectively). The higher concentration of

formic acid in *R. dominica* is consistent with its higher rate of hydrolysis *in vitro* as described previously.

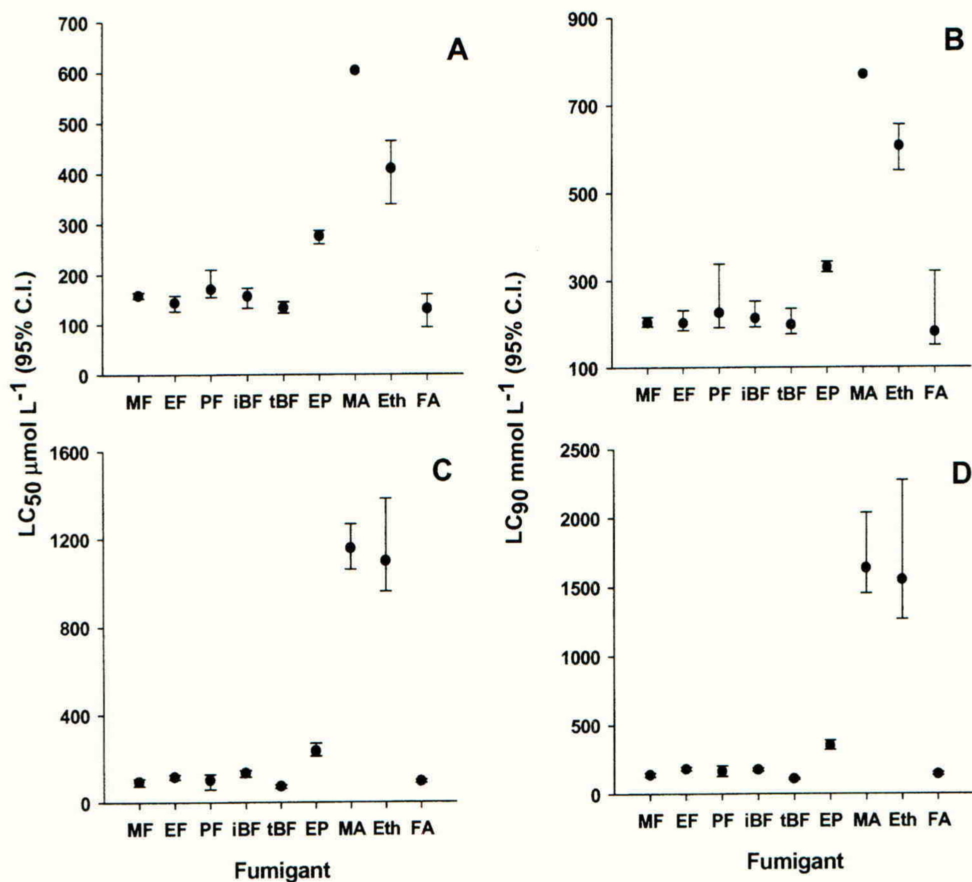

Figure 1. Comparative toxicity of volatile alkyl formate, acetate and propionate esters and related chemicals after a 24 h exposure. (A) LC_{50} with 95% confidence intervals (C.I.) for *S. oryzae* (B) LC_{90} *S. oryzae* (C) LC_{50} *R. dominica* (D) LC_{90} *R. dominica*. Key: Formate ester series: MF – methyl, EF – ethyl, PF – propyl, iBF – *iso*-butyl, tBF – *tert*-butyl; EP – ethyl propionate; MA – methyl acetate; Eth – ethanol; FA – Formic acid.

Cytochrome *c* oxidase activity in detergent solubilized mitochondria isolated from *S. oryzae* was 11 µmol cytochrome *c* oxidised.min^{-1}.mg^{-1}. The inhibitory potential of alkyl esters and related chemicals toward the terminal oxidase of the mitochondrial electron transport chain was determined (Figure 2). Sodium cyanide was used as a standard inhibitor of cytochrome *c* oxidase and to demonstrate the integrity of the system.

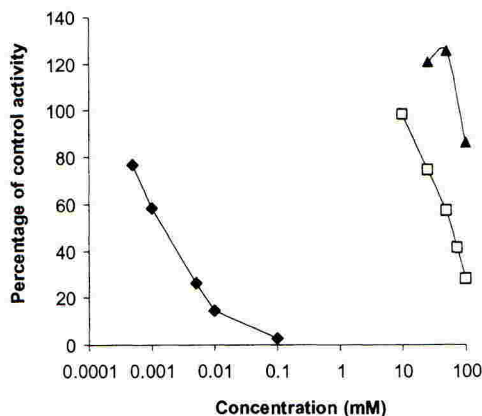

Figure 2. Inhibition of cytochrome *c* oxidase activity in mitochondria isolated from *S. oryzae* by sodium salts of cyanide (solid diamonds), formate (open squares) or acetate (solid triangles).

In comparison to cyanide, formate was a weaker inhibitor of cytochrome *c* oxidase, indicated by a shift in the inhibition curve of almost four magnitudes toward higher concentrations (Figure 2). Sodium acetate stimulated the activity of cytochrome *c* oxidase at 25 and 50 mM but was slightly inhibitory at 100 mM. The concentration to inhibit 50% of activity (IC_{50}) values calculated for cytochrome *c* oxidase inhibitors were 0.0015 and 57 mM for cyanide and formate respectively. Alkyl esters, methanol, ethanol and sodium propionate were tested at both 50 and 100 mM and none were found to be substantially inhibitory (data not shown).

In conclusion, the consistently higher toxicity of formate-containing compounds strongly suggests that formate is the main toxic component of these esters. This conclusion is supported by the >11-fold higher toxicity (based on LC50 values) of formic acid to *R. dominca* compared with ethanol, the two components of ethyl formate, and by the demonstration that formate esters are hydrolysed to produce formic acid in the insect. Thus, our experimental findings show that the toxicity of formate esters toward insects may be fully attributable to formic acid generated by enzyme-catalysed hydrolysis of the parent ester, once it has been absorbed by insects. The fast toxic action of ethyl formate is consistent with a mechanism of toxicity involving inhibition of cytochrome *c* oxidase, leading to a reduction in oxidative phosphorylation and depletion of cellular energy stores.

We argue that development of resistance in insects to the effects of alkyl formates via a loss of esterase activity would be unlikely to occur due to the presence of a plethora of esterases capable of hydrolysing short chain alkyl formates. Similarly, a resistance mechanism involving modification of cytochrome a to prevent binding by formic acid and hence inhibition of cytochrome c oxidase activity is improbable. However, other mechanisms of resistance could develop in insects under selection pressure.

ACKNOWLEDGMENTS

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Two different vacuolar enzymes are responsible for degradation of glutathione-S-conjugates in barley (*Hordeum vulgare* L.)

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ABSTRACT

In plants, glutathione conjugation is a major pathway in the detoxification of organic xenobiotics with electrophilic sites. Glutathione S-conjugates (GS-X) are formed in the cytosol and postulated to be transported across the tonoplast into the vacuole for final storage. In recent studies with barley (*Hordeum vulgare* L.) we could show that GS-X undergo further degradation to a γ-glutamyl-cysteinyl-conjugate (γ-GC-X) catalysed by a vacuolar carboxypeptidase (CP). The high content of cysteine-conjugates (C-X) in vacuolar extracts measured with HPLC suggested a second enzyme catalysed step in the degradation of the conjugate. We were able to demonstrate the presence of two additional peptidolytic vacuolar enzyme activities in different fractions of a purified protein extract.

INTRODUCTION

Plants as sessile organisms are defencelessly exposed to pesticides and other hazardous foreign compounds in the environment. Numerous studies have investigated uptake and metabolic mechanisms of plant strategies in detoxification. The widely accepted "green liver"-concept describes a model derived from animal physiology but demonstrates in this case the co-evolutional development of biochemical detoxification pathways for the detoxification of organic xenobiotics (Sandermann, 1994). The concept consists of three phases. In the first phase the compounds are activated by P450 monooxygenases to yield primary metabolites of higher reactivity for the further reactions. The second phase is the detoxification phase in the strict sense of the word, as it comprises the conjugation of the xenobiotic with biomolecules like sugars or glutathione. These reactions are catalysed by glycosyltransferases and glutathione S-transferases (GST).

The third phase is often described in the literature as storage and sequestration and is usually initiated by export of the conjugate from the cytosol into the large central vacuole of the plant cell. This sequestration reaction might be of high importance, as glutathione conjugates have been shown to be inhibitory to glutathione reductase as well as to glutathione S-transferase (Schroeder *et al.*, 2001). The transport of GS-conjugates into the vacuole is mediated by tonoplast ATPases known as ABC-transporters. This concept of storage excretion is widely accepted in the literature.

However, in the past, metabolic studies with cell cultures have shown that glutathione conjugates generally undergo a rapid metabolism to a large number of intermediary metabolites within few days or weeks after application of the xenobiotic (Lamoureux *et al.*, 1989, 1991). It was the aim of our study to investigate the possible vacuolar degradation of xenobiotic glutathione conjugates in order to demonstrate that the vacuole is an active

compartment in the metabolism of xenobiotics rather than a passive storage site. Our group has previously been able to demonstrate that the GS-conjugate of the chloroacetamide herbicide, Alachlor, (GS-X) undergoes cleavage in the vacuole of barley (Wolf *et al.*, 1996).

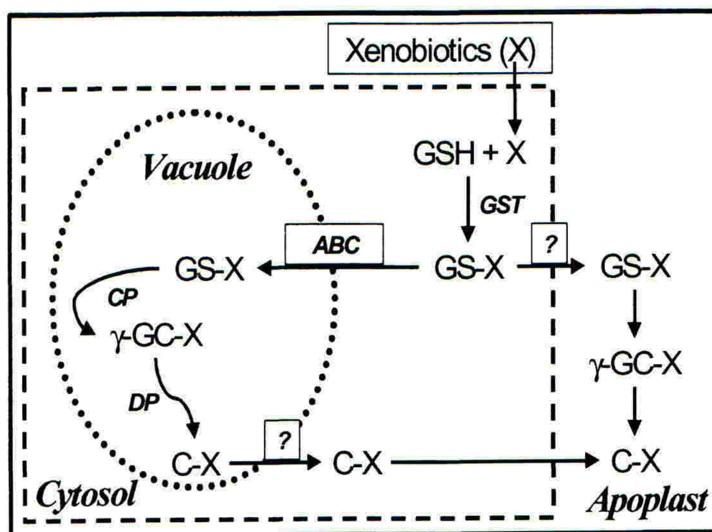

Fig. 1: Schematic overview of the working hypothesis underlying our study. Abbreviations: GSH: glutathione, GS-X: glutathionyl conjugate, γ-GC-X: γ-glutamylcysteinyl conjugate, C-X: cysteinyl conjugate, GST: glutathione S-transferase, CP: carboxypeptidase, DP: dipeptidase.

In a first step, glycine is hydrolysed from the GS-X and a γ-glutamylcysteine-conjugate is formed. This reaction is catalysed by a carboxypeptidase (Wolf *et al.*, 1996). The resulting conjugate is unstable and was assumed to be further metabolised to yield a cysteinyl conjugate or thiol containing metabolites. The degradation in the vacuole is assumed to be part of a complex cascade of reactions finally leading to soluble or bound residues in the cell wall (see Fig. 1).

MATERIALS AND METHODS

All experiments were carried out with 10 day old barley (*Hordeum vulgare* L, var. Cherie) seedlings grown in climate chambers at 17°C with 12 hr artificial light cycle. Plant leaves were frozen in liquid N_2 immediately after harvest.

Vacuoles were isolated from lower epidermal tissue of barley leaves as previously described (Wolf *et al.,* 1996). Protoplasts and vacuoles from the mesophyll of 5-day-old barley leaves were isolated as described by Rentsch and Martinoia (1991).

For the enzymatic studies, frozen barley leaves were ground with a mortar and pestle. The resulting powder was added to five volumes of MES extraction buffer (100 mM, pH 5) containing 5 mM EDTA and 5 mM pepstatin and allowed to stand for 10 min. Crude protein

was precipitated from freshly ground leaves in two steps (35 and 80%) with $(NH_4)_2SO_4$. Pellets were resuspended in extraction buffer, and protein was desalted with PD-10 columns.

Further purification was performed by loading desalted protein on an FPLC cation exchange column (High S, BioRad). Protein was eluted within a linear gradient of NaCl (0-1M, flow rate: 1ml/min) and were collected in 1ml fractions which were concentrated 5-fold by lyophilisation and stored at –80°C for further use.

GST activity was determined utilizing 1-chloro-2,4-dinitrobenzene (CDNB, e= 9.6 $mM^{-1}cm^{-1}$) as a model substrate (Habig *et al.,* 1974). Alachlor conjugation was determined as previously described (Wolf *et al.*, 1996).
Carboxypeptidase and dipeptidase activities were determined as follows: 60µl of the purified protein was incubated (4h, 25°C) with 80µl of 1mM GSH- and g-GC-CDNB conjugates which were synthezised *in vitro*. The reaction was stopped by adding 20% acetonitrile containing 0.1% tri-fluoroacetic acid (TFA). Protein concentration was determined using the dye binding method of Bradford (Bradford, 1976) with bovine serum albumin as standard. All enzyme and protein assays were performed in triplicate.

The samples were analyzed by HPLC on a RP C-18 column (Hypersil, Bischoff) using a linear gradient of acetonitrile from 20 to 100% in 30 min (flow rate 1 ml/min). Peaks were detected in an UV/VIS detector at 280 nm and co-chromatographed with commercially available reference compounds (Sigma).

RESULTS AND DISCUSSION

Crude protein extracts of barley leaves contained GST activity against several xenobiotic substrates, including alachlor and CDNB. In the same extracts, activity of carboxypeptidase (determined with S-dinitrobenzyl-glutathione) and dipeptidase (determined with γ-glutamyl-S-cysteinyl-dinitrobenzene) was observed.

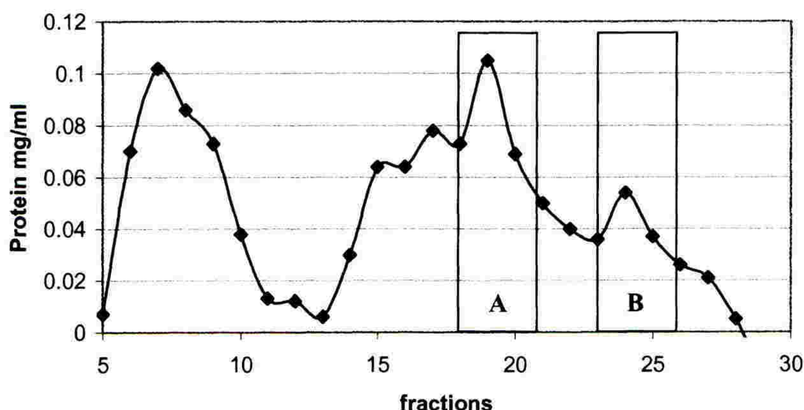

Fig. 2: Protein pattern obtained during cation exchange chromatography of an enzyme extract from barley leaves. Peaks A and B contain peptidolytic enzyme activity as explained in the text.

When the protein extracts were subjected to cation exchange chromatography on an FPLC system, three distinct peaks were resolved (Fig. 2). Of all fractions, activity for the cleavage of the dinitrobenzyl-glutathione was only observed in two peaks: A and B (Fig. 2). Dipeptidase activity for the cleavage of the γ-glutamylcysteinyl-conjugate was only detected in peak B, indicating that this activity represents a separate protein.

The purification factor for the carboxypeptidase after cation exchange chromatography was 160 fold as compared to crude extracts (Table 1). Longer incubation times or incubation of the CP-peak with γ-glutamylcysteinyl-DNB did not yield other reaction products. Hence, it is concluded that this peak does not contain other peptidolytic activtity. In contrast, the dipeptidase was purified 45fold by cation exchange chromatography (Tab. 2). The proposed product of this reaction, the cysteinyl-conjugate, was not further metabolised by any of the fractions.

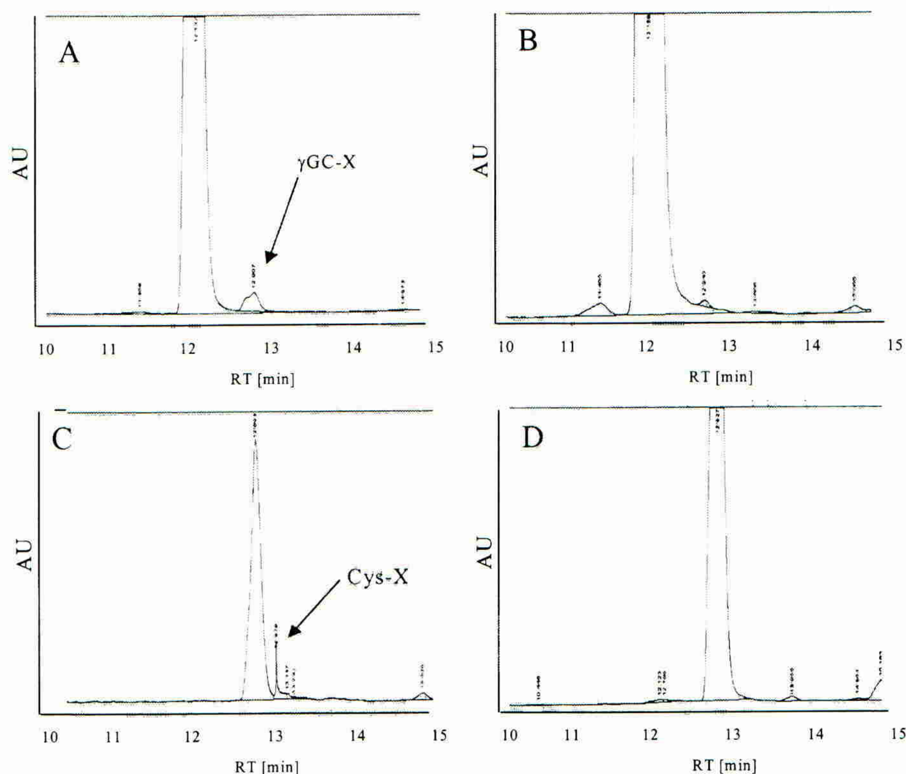

Fig. 3: HPLC chromatogram of the metabolites formed during the catabolism of glutathione conjugates in barley. In the presence of the first fraction (A) GS-X was degraded to γ-GC-X, indicating the presence of the known CP. When γ-GC-X conjugate was incubated with the second fraction (C) the respective cysteine conjugate C-X was formed. Control incubations of GS-X with the second (B) or of γ-GC-X with the first fraction (D) were negative.

Dipeptidase activity also seemed to be quite specific because incubation with the respective glutathionyl- or with the cysteinyl-metabolites did not result in the formation of products. Examples for the respective HPLC chromatograms of substrates and products are shown in Figure 3.

Table 1: Two-step purification of a carboxypeptidase from barley leaves by cation exchange chromatography. Barley leaves were precipitated in a two step ammonium sulfate precipitation and desalted via PD10 columns. The extract was further purified by cation exchange chromatography.

Purification step	Protein (mg/ml)	Total activity (µmol/min)	Specific activity (U/mg)	Purification factor
PD10-extract	2.85	0.016	0.005	1
FPLC-cation exchange peak	0.063	0.04	0.81	162

From the catalytic and biophysical properties and from inhibition studies (to be published elsewhere) it was concluded that the purified carboxypeptidase was of serine type and belongs to the class of endopeptidases previously detected in barley seedlings. Proteolytic activity during germination had been attributed to these CPs (Breddam *et al.*, 1983). Contrary to this, the dipeptidase co-purified from the same barley protein extract has not been described before.

In animal metabolism, degradation of glutathione conjugates starts with the cleavage of the γ-glutamyl-moiety, thus producing a cysteinyl-glycine-conjugate. The latter is subsequently cleaved by a dipeptidase to yield the respective cysteinyl-conjugate (Schröder 2001).

Table 2: Two-step purification of a dipeptidase from barley leaves by cation exchange chromatography. Barley leaves were precipitated in a two step ammonium sulfate precipitation and desalted on PD10 columns. The extract was further purified by cation exchange chromatography.

Purification step	Protein (mg/ml)	Total activity (µmol/min)	Specific activity (U/mg)	Purification factor
PD10-extract	2.85	0.154	0.054	1
FPLC-cation exchange peak	0.02	0.048	2.44	45

Evidence that the identified peptidolytic enzymes were localized in the vacuole was obtained from pilot experiments with isolated barley mesophyll vacuoles. It could be clearly demonstrated that incubation of osmotically shocked vacuoles resulted in the same degradation products of the DNB-glutathione- and γ-glutamylcysteinyl-conjugates as with the isolated enzymes. Further studies will be performed to determine catalytic properties and inducibility of these proteins, as well as to elucidate the fate of the cysteinyl-conjugate formed as a product.

CONCLUSIONS

Glutathione conjugates of xenobiotics are frequently metabolized rather than stored in plants. Upon sequestration in the vacuole, glutathione conjugates are cleaved by carboxy-peptidases (CP). In plants, this CP-activity seems to be specific for glycine removal, and γ-glutamylcysteinyl conjugates are frequently found in the vacuole. Further degradation of the CP-product, γ-glutamyl-cysteinyl-X, is performed by a second peptidolytic enzyme, probably a dipeptidase so far not described. This work shows that in barley leaves these enzyme activities can be separated and are distinct form each other. The fate of the resulting cysteinyl conjugates in the vacuole and the cytosol remains to be elucidated.

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Can picoxystrobin protect winter wheat from environmental stress?

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ABSTRACT

Strobilurin fungicides have been used in cereals in the UK for six years, and have activity against a number of important cereal fungal diseases. They have also been reported to affect crop physiology resulting in enhanced yield by prolonged greening and reduced appearance of stress symptoms. These effects may be a result of increased disease control or a direct interaction between the fungicide and the physiology of the crop plant. We investigated the physiological consequences of picoxystrobin treatment in the presence of environmental stressors. Drought, temperature, light, nitrogen and sulphur availability were investigated in winter wheat cv. Claire under experimental conditions in the glasshouse and in growth cabinets. Initial data suggest that physiological stress symptoms are ameliorated in picoxystrobin-treated plants. In the absence of any obvious crop disease, these observations indicate a direct interaction between picoxystrobin and crop physiology, especially with respect to chlorophyll content.

INTRODUCTION

The strobilurins are a family of fungicides based on naturally occurring compounds, such as strobilurins A and B (Anke *et al.*, 1977). Although structurally diverse they all act at a common site, inhibiting mitochondrial electron transport at the cytochrome bc_1 complex of the respiratory chain. Bartlett *et al.* (2002) have recently reviewed the history, chemistry, mode of action and biology of strobilurins. Picoxystrobin is a new systemic broad-spectrum fungicide with activity against a number of important cereal fungal diseases (Godwin *et al.*, 2000).

Strobilurins have been reported to affect crop physiology, resulting in enhanced or prolonged greening, reduced appearance of stress symptoms, yield enhancement and improved grain quality (Gooding *et al.*, 2000; Mercer & Ruddock, 1998; Ypema & Gold, 1999). These effects have been attributed to decreased ethylene production, reduced degradation of and/or increased endogenous concentrations of cytokinins (Grossmann & Retzlaff, 1997), increases in antioxidant defence systems (Wu & von Tiedemann, 2001) and increased nitrogen assimilation (Glaab & Kaiser, 1999). However, reduced senescence and delayed chlorosis may be a result of the broad spectrum disease control provided by this fungicide family (for example, Bayles, 1999).

We have investigated the effects of picoxystrobin on winter wheat cv. Claire grown under a variety of environmental conditions in the glasshouse or growth cabinet, and both morphological and physiological responses have been examined. Further experiments were performed in which plants were pre-treated with the fungicide quinoxyfen to assess the contribution of existing disease to physiological symptoms observed, thus testing the

hypothesis that the enhanced appearance of picoxystrobin-treated plants was solely due to control of visible symptoms of disease. Water stress was imposed using polyethylene glycol (PEG), an osmotically active macromolecule that sequesters water, therefore making it unavailable to the plant.

MATERIALS AND METHODS

Plant growth

Winter wheat cv. Claire was sown in a Perlite medium (a naturally occurring silicaceous rock) saturated from below with modified Hoagland's growth medium and grown under glasshouse conditions (15°C/14 h day: 8°C/10h night ± 5°C).

Fungicide treatments

Picoxystrobin (250g a.i ha^{-1}; field rate) was applied at growth stage (GS) 21 (one tiller present) with a precision pot sprayer delivering the equivalent of 200 l ha^{-1} through 2 flat fan nozzles at 7 bar pressure at a height of 45cm above the pots. Where indicated, plants were pre-treated with quinoxyfen (Fortress™, 500g a.i l^{-1}; applied as 10 ppm a.i at 200 l ha^{-1}) at GS11/12 (one to two leaves emerged).

Stress conditions

Immediately after application, half the treated and half the untreated plants were grown under the following stress conditions; low light (10µmol m^{-2} sec^{-1}), low nitrogen (1/10th N Hoagland's, 1.025mM nitrogen), low sulphur (1/10th S Hoagland's, 0.16mM sulphur), high temperature (25°C), drought (imposed by 5% and 10% aqueous polyethylene glycol, PEG 8000). Individual experiments examined each environmental stress and monitoring was carried out from picoxystrobin treatment (0 days after treatment, DAT) to the end of the experiment (35 DAT).

Analyses

Non-invasive and invasive analyses were carried out at 0 DAT and at 7-day intervals to 35 DAT. Plant growth stage (GS), root and shoot fresh weight were recorded at each interval and both tip and whole-leaf senescence were assessed. Gas exchange by intact leaves was determined by infrared gas analysis (Cerius II, PP Systems, UK) and values for transpiration, stomatal conductance, carbon dioxide assimilation and sub-stomatal carbon dioxide concentration were calculated. Leaf thickness was measured at the midpoint of the leaf using a micrometer and leaf water potential was determined using a dew-point microvoltmeter (HR-33T, Wescor, UK). All measurements were carried out on the oldest fully expanded non-senesced leaf of each plant. Harvested tissue was frozen in liquid nitrogen and stored at -80°C until needed. Chlorophyll and carotenoid analyses were performed using an adaptation of the methods of Lichtenthaler & Wellburn (1983) and Hendry & Price (1993). Shoot tissue (0.5g) was extracted overnight in 40ml of 80% (v/v) aqueous acetone containing 1mg magnesium carbonate and 0.5mg sodium bisulfite. Extracts were clarified by centrifugation (15000g, 15 mins at 4°C) and their absorbance determined at 480, 645, 663 and 710nm.

RESULTS

Effect of picoxystrobin on stress symptoms

The effects of picoxystrobin on the development of stress symptoms in winter wheat are summarised in Table 1. Picoxystrobin treatment in the absence of stress consistently increased shoot and root fresh weight, increased root to shoot ratios and increased chlorophyll content. In addition, picoxystrobin-treated plants developed less leaf senescence than untreated plants. Similar patterns were noted in picoxystrobin-treated plants in the presence of stresses (Table 1). Treatment caused the amelioration of some stress symptoms in all conditions tested. The most common symptom of picoxystrobin treatment was the retardation of senescence. Under drought stress induced by PEG, treated plants demonstrated similar differences to treated plants in the absence of stress. The effects of picoxystrobin treatment were most pronounced in the drought-stressed plants.

Table 1. Effects of picoxystrobin on the development of stress symptoms in winter wheat.
↑, increased with treatment; ↓, decreased with treatment; X, not affected by treatment; NA , no data currently available. Results presented are from observations over the 35 DAT.

Environmental parameter	Shoot weight (g)	Root weight (g)	Root:Shoot ratio	Senescence	Chlorophyll content
Unstressed	↑	↑	↑	↓	↑
Low Light	X	X	X	X	↑
Low Nitrogen	X	↑	↑	↓	X
Low Sulphur	X	X	X	↓	NA
High Temperature	↑	↑	↑	X	NA
Drought	↑	↑	↑	↓	↑

Effects of drought stress and picoxystrobin on chlorophyll content

The effect of picoxystrobin on leaf chlorophyll content in drought stressed winter wheat plants is shown in Tables 2 and 3. In the presence of drought stress induced by PEG, picoxystrobin increased chlorophyll content by between 32 and 44% between day 0 and day 35. Under similar conditions, but in the absence of picoxystrobin, leaf chlorophyll content fell by between 5 and 34%. Picoxystrobin treatment also resulted in increased chlorophyll a and b content in the absence of drought stress.

Table 2. Total leaf chlorophylls (% change from day 0) for plants grown in the presence and absence of picoxystrobin and drought stress (5% PEG).

Growth conditions/treatment	Days after treatment	Total leaf chlorophylls (% change from day 0)
- PEG – picoxystrobin	28	+53
	35	+37
- PEG + picoxystrobin	28	-51
	35	+154
+ PEG – picoxystrobin	28	-25
	35	-5
+ PEG + picoxystrobin	28	+52
	35	+44

Table 3. Total leaf chlorophylls (% change from day 0) for plants grown in the presence and absence of picoxystrobin and drought stress (10% PEG).

Growth conditions/treatment	Days after treatment	Total leaf chlorophylls (% change from day 0)
- PEG – picoxystrobin	28	+40
	35	0
- PEG + picoxystrobin	28	+16
	35	+26
+ PEG – picoxystrobin	28	-16
	35	-34
+ PEG + picoxystrobin	28	+26
	35	+32

Pre-treatment with quinoxyfen

The effect of picoxystrobin on leaf chlorophyll content in drought stressed winter wheat plants pre-treated with quinoxyfen is shown in Tables 4 and 5.

Table 4. Total leaf chlorophylls (% change from day 0) for plants pre-treated with quinoxyfen and grown in the presence and absence of picoxystrobin and drought stress (5% PEG).

Growth conditions/treatment	Days after treatment	Total leaf chlorophylls (% change from day 0)
- PEG – picoxystrobin	28	-22
	35	-28
- PEG + picoxystrobin	28	-11
	35	-18
+ PEG – picoxystrobin	28	-11
	35	+5
+ PEG + picoxystrobin	28	+9
	35	+1

Table 5. Total leaf chlorophylls (% change from day 0) for plants pre-treated with quinoxyfen and grown in the presence and absence of picoxystrobin and drought stress (10% PEG).

Growth conditions/treatment	Days after treatment	Total leaf chlorophylls (% change from day 0)
- PEG – picoxystrobin	28	0
	35	-6
- PEG + picoxystrobin	28	+6
	35	+57
+ PEG – picoxystrobin	28	-4
	35	+16
+ PEG + picoxystrobin	28	-8
	35	+25

DISCUSSION

These preliminary studies have demonstrated that when winter wheat plants were treated with picoxystrobin less senescence was observed, as previously reported for both azoxystrobin (Bertelsen *et al.*, 2001) and kresoxim-methyl (Grossman & Retzlaff, 1997). This response was enhanced when plants were grown under stress, especially drought induced by PEG. Under these conditions, picoxystrobin further reduced stress development to the extent that stressed treated plants appeared similar to unstressed plants. This may be due to a direct effect of picoxystrobin on the physiology of the plant or may be due to the broad spectrum of disease control provided by this fungicide. In the latter case, the reduction in disease burden may result in the plant being better equipped to combat external stresses, such as drought or reduced nutrient availability. To investigate this possibility, further experiments were carried out utilising plants that had been pre-treated with quinoxyfen to reduce disease interaction with the development of stress symptoms.

Pre-treatment with quinoxyfen resulted in a decrease in the differences between picoxystrobin-treated and untreated plants with respect to total chlorophyll content. However, plants treated with picoxystrobin still had more chlorophylls per g fresh weight than untreated plants grown under similar conditions. Even when all plants were treated with quinoxyfen to control existing disease, picoxystrobin treatment increased chlorophyll content, indicating a possible physiological role in either chlorophyll production or protection of chlorophyll from the damaging effects of active oxygen species.

It should be noted that these observations were carried out on plants grown under controlled conditions. Further study is now necessary to establish the mechanisms that explain picoxystrobin interacts with environmental stressors and with the development of senescence under field conditions.

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