SESSION 6C

APPLICATION AND FORMULATION PARAMETERS: THEIR INFLUENCE ON PRODUCT PERFORMANCE AND SAFETY

Session Organiser MR J H TOBUTT
Zeneca Agrochemicals, Yalding, UK

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EFFECTS OF ADDITIVES ON GLYPHOSATE ACTIVITY IN PURPLE NUTSEDGE

R SUWANKETNIKOM
Dept. of Agronomy, Kasetsart University, Chatuchak, Bangkok 10903, Thailand

ABSTRACT

Effects of additives on ^{14}C-glyphosate penetration into purple nutsedge leaves and efficacy of glyphosate for purple nutsedge control were examined in 2 week old plants. It was found that the addition of $(NH_4)_2SO_4$ at 1.0 % (v/v) + diesel oil at 1.0 % (v/v) + Tendal at 1.0 % (v/v) increased ^{14}C-glyphosate penetration into nutsedge leaves more than the addition of either one alone. $(NH_4)_2SO_4$ at 1.0 % + diesel oil at 1.0 % + Tendal at 0.12 or 0.25 % increased phytotoxicity of glyphosate at 0.5 and 0.75 kg ae/ha on nutsedge plants, in the greenhouse. Additives did not enhance glyphosate activity by reducing number of nutsedge tubers.

INTRODUCTION

Purple nutsedge (*Cyperus rotundus* L.) was ranked as one of the most serious weeds in the world (Holm *et al.*, 1979). It is an important weed in corn, sorghum, soybean, mungbean, peanut, cotton, upland rice, and vegetables. Mechanical control of this weed is not successful because they can sprout new shoots from tubers. Various selective pre-emergence herbicides can not control this weed.

Glyphosate is a nonselective, translocated, foliar absorbed herbicide (Weed Science Society of America, 1989). Glyphosate has been reported to control purple nutsedge (Suwunnamek & Parker, 1975). However, application of glyphosate for weed control is restricted by cost. Appropriate adjuvants or additives might be used in combination with glyphosate to maintain its optimum activity but at reduced rate.

$(NH_4)_2SO_4$ has been reported to increase activity of glyphosate for purple nutsedge control (Suwunnamek & Parker, 1975). Furthermore, calcium antagonism of glyphosate has been overcome with $(NH_4)_2SO_4$ (Nalewaja & Matysiak, 1991; Thelen *et al.*, 1995a), citric acid and organosilicone adjuvants (Thelen *et al.*, 1995b).

Various additives including nonionic surfactants (Coret & Chamel, 1993), the organosilicone Silgard 309 (Reddy & Singh, 1992), and both petroleum and seed oils (Gauvrit & Cabanne, 1993) were reported to increase glyphosate activity. Organosilicone Silwet 77 enhanced ^{14}C-glyphosate uptake into bean (*Vicia faba* L.) leaf (Zabkiewicz *et al.*, 1993). Furthermore, oils also increased glyphosate penetration (Gauvrit & Cabanne, 1993).

The objectives of these experiments were to determine the effects of the various additives,

$(NH_4)_2SO_4$, Tendal (surfactant), and diesel oil at appropriate concentrations on ^{14}C-glyphosate penetration into purple nutsedge leaves, and to determine the effects of additives on glyphosate efficacy for purple nutsedge control.

MATERIALS AND METHODS

Laboratory experiment

^{14}C-glyphosate and glyphosate at 1.5 kg ae/ha with several additives in a spray volume of 200 liters/ha were applied when the purple nutsedge plants were 5-6 leaves (approximately 2 weeks after planting). Eight drops of 0.5 μCi ^{14}C-glyphosate were applied on the same leaf of each plant. Plants were harvested at 2 or 24 hours after application. Cellulose acetate (6.0 %) in 9:1 acetone/water was painted on the ^{14}C-glyphosate treated leaves. After 2 minutes the dried cellulose acetate was removed and mixed with 2 ml glacial acetic acid. 200μ liters solution of cellulose acetate in glacial acetic acid was mixed with scintillation cocktail to determine the amount of ^{14}C-glyphosate by liquid scintillation spectometry.

The additive, Sunlite ® is a local detergent. The surfactant, Tendal ® is the blend of 60 % alkyl aryl polyethoxylate and sodium salt of dialkyl sulfosuccinate plus 40 % solubilizer and couplers. Triton X-100 ® is dioctyl sulfonosuccinate, sodium salt. Herbicide, glyphosate is 36 % ae Roundup ®.

Experiments were carried out in a Randomized Complete Block Design with 6 replications. The temperature and relative humidity during application of first, second, and third experiments were 32°C, 65 %; 30°C, 70 % and 28°C 80 % respectively.

Geenhouse experiment

Three purple nutsedge tubers were planted in polyethylene pots containing clay soil. The pot size was 14 cm diameter and 15 cm height. Plants were watered from the surface every day. At 2 weeks after germination, glyphosate was applied alone and in combination with additives. Herbicide was applied by laboratory sprayer at the spray volume of 200 liters/ha and the pressure was 85kPa. The nozzle was a Teejet, flat fan 8001. During application the temperature was 28°C with 75 % relative humidity.

The number of nutsedge shoots and tubers in each pot were recorded at 21 and 60 days after application. A Randomized Complete Block Design with 4 replications was used.

RESULTS AND DISCUSSION

Laboratory experiment

At 2 hours after application there was increased penetration of ^{14}C-glyphosate with the treatments which included Tendal + diesel oil at rates of 0.5% and above or Triton X-100 +

diesel oil at 1% (Table 1). The other treatments gave smaller increases in penetration.

Table 1 Effects of various additives on ^{14}C-glyphosate penetration into purple nutsedge leaves at 2 hours after application

Treatment	% absorption
Glyphosate 1.5 kg ae/ha $(NH_4)_2SO_4$ 1.0 %	6.2 d[1]
Glyphosate 1.5 kg ae/ha $(NH_4)_2SO_4$ 1.0 %+Tendal 0.25 %	24.8 bcd
Glyphosate 1.5 kg ae/ha $(NH_4)_2SO_4$ 1.0 %+Tendal 0.5 %	35.2 abc
Glyphosate 1.5 kg ae/ha $(NH_4)_2SO_4$ 1.0 %+Tendal 1.0 %	26.5 bcd
Glyphosate 1.5 kg ae/ha $(NH_4)_2SO_4$ 1.0 %+Tendal 2.0 %	26.7 bcd
Glyphosate 1.5 kg ae/ha $(NH_4)_2SO_4$ 1.0 %+Tendal 4.0 %	29.9 bcd
Glyphosate 1.5 kg ae/ha $(NH_4)_2SO_4$ 1.0 %+Tendal 0.25 %+diesel oil 0.25 %	15.5 cd
Glyphosate 1.5 kg ae/ha $(NH_4)_2SO_4$ 1.0 %+Tendal 0.5 %+diesel oil 0.5 %	38.6 abc
Glyphosate 1.5 kg ae/ha $(NH_4)_2SO_4$ 1.0 %+Tendal 1.0 %+diesel oil 1.0 %	58.0 a
Glyphosate 1.5 kg ae/ha $(NH_4)_2SO_4$ 1.0 %+Tendal 2.0 %+diesel oil 2.0 %	39.6 abc
Glyphosate 1.5 kg ae/ha $(NH_4)_2SO_4$ 1.0 %+Tendal 4.0 %+diesel oil 4.0 %	46.9 ab
Glyphosate 1.5 kg ae/ha $(NH_4)_2SO_4$ 1.0 %+Triton X-100 1.0 %+diesel oil 1.0 %	47.4 ab

[1] Means in the same column followed by the same letters are not significantly different at 5 % level by DMRT.

Table 2 Effects of $(NH_4)_2SO_4$, Tendal, and diesel oil on ^{14}C-glyphosate penetration into purple nutsedge leaves at 2 hours after application

Treatment	% absorption
Glyphosate 1.5 kg ae/ha + $(NH_4)_2SO_4$ 1.0 %+Tendal 0.5 %	21.6 b[1]
Glyphosate 1.5 kg ae/ha + $(NH_4)_2SO_4$ 1.0 %+Tendal 1.0 %	29.8 ab
Glyphosate 1.5 kg ae/ha + $(NH_4)_2SO_4$ 1.0 %+Tendal 1.0 %+diesel oil 0.25 %	19.5 b
Glyphosate 1.5 kg ae/ha + $(NH_4)_2SO_4$ 1.0 %+Tendal 1.0 %+diesel oil 0.5 %	25.7 b
Glyphosate 1.5 kg ae/ha + $(NH_4)_2SO_4$ 1.0 %+Tendal 1.0 %+diesel oil 1.0 %	42.4 a

[1] Means in the same column followed by the same letters are not significantly different at 5 % level by DMRT.

The treatment which included Glyphosate 1.5 kg ae/ha $(NH_4)_2SO_4$ 1.0 %+Tendal 1.0 %+diesel oil 1.0 % gave the greatest penetration of ^{14}C-glyphosate. A further experiment studied the effect of lower levels of diesel oil (Table 2) which showed that diesel oil at 0.25% and 0.5% reduced the penetration compared to diesel oil at 1% and was no better than the treatment without oil.

At 24 hours after application, $(NH_4)_2SO_4$ 1% + Triton X-100 2% increased ^{14}C-glyphosate penetration into nutsedge leaves(Table 2). The addition of diesel oil at 1% did not give any further improvement. The treatments with $(NH_4)_2SO_4$ alone, Triton X-100 with or without oil and Sunlite with $(NH_4)_2SO_4$ and/or diesel oil did not improve penetration into nutsedge leaves compared to the control.

Table 3 Effects of various additives on ^{14}C-glyphosate penetration into purple nutsedge leaves at 24 hours after application

Treatment	% absorption
Glyphosate 1.5 kg ae/ha	63.8 bc[1]
Glyphosate 1.5 kg ae/ha + $(NH_4)_2SO_4$ 1.0 %	68.2 ab
Glyphosate 1.5 kg ae/ha + Triton X-100 2.0 %	66.2 b
Glyphosate 1.5 kg ae/ha + $(NH_4)_2SO_4$ 1.0 %+Triton X-100 2.0 %	78.2 a
Glyphosate 1.5 kg ae/ha + Triton X-100 2.0 % + diesel oil 1.0 %	66.3 b
Glyphosate 1.5 kg ae/ha + $(NH_4)_2SO_4$ 1.0 %+Triton X-100 2.0 %+diesel oil 1.0 %	72.7 ab
Glyphosate 1.5 kg ae/ha + $(NH_4)_2SO_4$ 1.0 %+Sunlite 4.0 %	63.7 bc
Glyphosate 1.5 kg ae/ha + Sunlite 4.0 %+diesel oil 1.0 %	54.5 c
Glyphosate 1.5 kg ae/ha $(NH_4)_2SO_4$ 1.0 %+Sunlite 4.0 %+diesel oil 1.0 %	65.9 b

[1] Means in the same column followed by the same letters are not significantly different at 5 % level by DMRT

The increased penetration of ^{14}C-glyphosate into nutsedge leaves when using $(NH_4)_2SO_4$ might be due to a change of the glyphosate molecule to a more readily absorbed form. Using NMR spectoscopy Thelen *et al.* (1995a) showed that NH_4^+ from $(NH_4)_2SO_4$ complexed directly with the glyphosate molecule through the phosphonate and carboxylate groups and resulted in a more readily absorbed form of glyphosate. They also found that a nonionic organosilicone adjuvant increased ^{14}C-glyphosate absorption into sunflower leaves (Thelen *et al.*, 1995b). However, the organosilicone adjuvant did not directly interact with glyphosate (Thelen *et al.*, 1995b). The organosilicone adjvants might alter the physical properties of the spray solution or the leaf cuticle to the point where ^{14}C-glyphosate could directly penetrate the leaf.

Oils have seldom been tested with water soluble herbicides, although glyphosate efficacy against wheat was increased by both petroleum and seed oil (Gauvrit & Cabanne, 1993). The

main action of adjuvant oils was increasing herbicide penetration but the mechanisms involved are poorly understood (Gauvrit & Cabanne).

Geenhouse experiment

At 7 days after application, $(NH_4)_2SO_4$ at 1.0 % + oil +Tendal at 0.12 % or 0.25 % increased nutsedge control of glyphosate at 0.75 kg ae/ha (Table 4). However, additives did not increase activity of glyphosate at higher rates. At 14 days after application, $(NH_4)_2SO_4$ at 1.0 % + oil at 1.0 % + Tendal at 0.12 % or 0.25 % increased activity of glyphosate at 0.5 and 0.75 kg ae/ha. However, at 21 days after application, the additives increased activity of glyphosate only at 0.5 kg ae/ha .

Table 4 Purple nutsedge control with glyphosate in combination with various additives under greenhouse condition

Glyphosate	$(NH_4)_2SO_4$	Diesel oil	Tendal	Days after application[1]			
(kg ae/ha)	(% v/v)	(% v/v)	(% v/v)	4	7	14	21
0.5	-	-	-	18 d[2]	45 e	59 c	81 b
0.75	-	-	-	23 bcd	43 e	69 bc	83 a
1.5	-	-	-	28 a-d	65 abc	98 a	100 a
0.5	1	1	0.12	20 cd	45 e	89 a	93 ab
0.75	1	1	0.12	28 a-d	63 a-d	90 a	95 ab
1.5	1	1	0.12	35 ab	75 a	100 a	100 a
0.5	1	1	0.25	25 a-d	53 cde	83 ab	90 ab
0.75	1	1	0.25	30 abc	68 abc	95 a	100 a
1.5	1	1	0.25	33 abc	73 ab	99 a	100 a
0.5	1	1	1	23 bcd	58 b-c	79 ab	93 ab
0.75	1	1	1	25 a-d	48 d-e	82 ab	90 ab
1.5	1	1	1	38 a	73 ab	91 a	91 ab
Untreated	-	-	-	0e	0f	0d	0c

[1] % Weed Control; 0 = no control, 100 = complete control.

[2] Means in the same column followed by the same letters are not significantly different at 5 % level by DM

The additives increased glyphosate phytotoxicity on nutsedge plants, but dry weight and number of tubers were not affected (results not included).

CONCLUSION

The addition of $(NH_4)_2SO_4$ at 1.0 % + oil at 1.0 % + Tendal at 1.0 % increased ^{14}C-glyphosate penetration into nutsedge leaves more than the addition of either one alone.

The addition of $(NH_4)_2SO_4$ at at 1.0 % + oil at 1.0 % + Tendal at 0.12 or 0.25 or 1.0 % increased phytotoxicity of glyphosate at 0.5 and 0.75 kg ae/ha 7 and 14 days after application

in the greenhouse. Additives in combination with glyphosate did not reduce number of nutsedge tubers compared with glyphosate alone.

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ALGINATE-BASED FORMULATIONS OF VOLATILE HERBICIDES: FACTORS AFFECTING RELEASE RATES

R M WILKINS, J ZHAO
Department of Agricultural and Environmental Science, Newcastle University, Newcastle upon Tyne, NE1 7RU, UK

ABSTRACT

Various lignin-filled alginate membranes were prepared by gelification of the slurry of sodium alginate and lignin with calcium chloride. The permeation of a volatile model herbicide through anhydrous and hydrated membranes was evaluated by using a flow cell system. The data obtained from the permeation study suggested that the lignin-alginate matrix may provide effective control of the volatility losses of trifluralin. The release profiles of the lignin-alginate matrix could be moderated by changing the lignin:alginate ratio in the matrix or by selecting a specific type of lignin. The most effective control of release of trifluralin in vapour phase might be found in an organosolv lignin-alginate matrix with a lignin:alginate ratio of 3.3:1.

INTRODUCTION

Trifluralin (α,α,α-trifluoro-2,6-dinitro-N,N-dipropyl-*p*-toluidine) is a selective soil-herbicide, that is used on numerous crops for the pre-emergence control of many annual grasses and broad-leaved weeds. It has been confirmed that the rapid loss of trifluralin by evaporation and photodegradation from the soil surface is the major problem associated with its application

Controlled release technology provides the potential for improving the efficiency and reducing undesirable side effects of herbicides. To establish the effectiveness of controlled release formulations (CRFs), an *in vitro* study of the release profiles of CRFs is essential to understand the release behaviour of the active ingredient in the environment. *In vitro* study of release profiles of trifluralin CRFs is often conducted in an organic solvent, such as acetone or mixed solvent (ethanol: water=1:1), due to its very low water solubility. As trifluralin acts as a root disrupter by diffusion through the vapour phase in the soil from formulations, the accuracy of evaluation of the trifluralin release from CRFs could be low by using the data obtained from the release study in solvent. However, few works have been reported on the gas-phase release of bioactive ingredient under reproducible conditions. Recently, the release profiles of trifluralin by volatilisation from several matrices have been evaluated by using a microbalance (Chen *et al.*, 1992). The release mechanisms of trifluralin into the air phase from formulations has been studied (Garratt & Wilkins, 1997).

Alginate has been used as a component of a matrix in the preparation of CRFs of a number of herbicides to optimise the herbicidal activity. The release of active ingredients with moderate water solubility could be altered by the incorporation of adsorbents, such as vegetable oils, charcoal, clay, or lignin (Pepperman *et al.*, 1995). In this study, trifluralin was selected as a volatile model herbicide to understand the release characteristics of alginate-based CRFs by

investigating the trifluralin permeation through lignin-filled membranes using a flow cell system. Four types of lignin were incorporated into alginate-based membranes to determine the effect on the trifluralin permeation through membranes. In addition, the trifluralin permeation was also evaluated under a wet condition which could simulate the other extreme of moisture content of soils.

MATERIALS AND METHODS

Membrane preparation

Four types of lignin were used as adsorbents in filled membranes. The lignins L_1 and L_6 were commercially available Indulin AT (Westvaco Corp., Charleston. SC, USA) and Alcell (Repap Technologies Inc, Valley Forge, PA, USA). The lignins L_3 and L_5 were kindly provided by Faenquil, Lorena-SP, Brazil. Sodium alginate (BDH, UK) was produced from *Laminaria hyperborea*. Calcium chloride (BDH, UK) was used as a crosslinking agent. Technical grade trifluralin (98%, DowElanco) was used as a model herbicide in the experiments. Various lignin-filled alginate-based membranes were prepared by the previously published method (Zhao & Wilkins, 1997). A code for membranes was used, XL_yZ_r, where X represents the status of membranes (anhydrous or hydrated), L_y is the lignin incorporated in the membrane, Z is the lignin:alginate ratio, r is the replicate of the same composite membranes.

Air permeability

To assess the air permeability of membranes due to its porous structure, air permeability was determined using an air permeation cell system. The cell of PTFE consisted of two horizontal compartments in which a membrane sample was mounted in the middle. Air was supplied to the bottom compartment by a compressed air cylinder with a pressure of 0.5 bar. The air flow (ml/min) through membranes was used as a rough indicator of the air permeability.

Water permeability

Water permeability of membranes was presented by the water loss from the hydrated membranes in a period of time. As in the wet permeation cell used in our previous study (Zhao & Wilkins, 1997), a reservoir was used to maintain the membranes wet. The water loss from the reservoir was monitored at regular time intervals.

Permeation cell systems

Dry and wet permeation cells and the analysis of trifluralin permeated were the same as described in our previous study (Zhao & Wilkins, 1997).

RESULTS AND DISCUSSION

The properties of membranes examined

Twelve alginate-based membranes were prepared: one Ca alginate-only membrane (AA); four with Indulin AT lignin (L_1); two with eucalyptus kraft lignin (L_3); two with bagasse alkali lignin (L_5) (Ferraz *et al.*, 1997); two with Alcell lignin (L_6) (Lora *et al.*, 1989). The composition and physical properties of membranes examined in the dry and wet flow cell systems are summarised in Table 1.

Table 1 The composition and physical properties of membranes examined

Code	Lignin	Thickness (mm)	Density (g/cm^3)	Dv (g/cm^3)	Air permeability (ml/min)	Water loss (g/day)
AL_12_a	pine kraft	0.141	0.551	0.802	23.5	----
AL_12_b	pine kraft	0.121	0.562	0.791	----	----
HL_12	pine kraft	0.104	0.672	0.681	----	7.83
AL_13	pine kraft	0.118	0.612	0.743	----	----
AL_33	eucalyptus kraft	0.125	0.694	0.692	25.7	----
HL_33	eucalyptus kraft	0.117	0.619	0.767	----	----
AL_53_a	bagasse alkali	0.121	0.541	0.838	28.9	----
AL_53_b	bagasse alkali	0.122	0.610	0.768	19.7	----
AL_63_a	hardwood organosolv	0.128	0.551	0.766	17.1	----
AL_63_b	hardwood organosolv	0.129	0.533	0.784	----	----
HL_63	hardwood organosolv	0.130	0.557	0.760	----	8.57
AA	None	0.035	1.293	----	182.2	----

The densities of the four lignins incorporated in membranes, Indulin AT, eucalyptus kraft lignin, bagasse alkali lignin, and Alcell lignin, were 1.361, 1.401, 1.391, 1.311, respectively (Cotterill, 1994). All lignin-filled membranes have a lower density (Table 1) than either the alginate-only membrane or the lignin incorporated. The reduction in density probably indicates that the filled membranes have a porous structure. The void space due to the porosity in the membranes could be roughly estimated by the decrease in density obtained from the following equation: $D_V=(P\times D_L+(1-P)\times D_A)-D_M$, where D_V is the decrease in density due to porosity, P is the proportion of lignin in the membrane expressed as a fraction, D_L, D_A and D_M are the densities of lignin, Ca alginate (assuming no void space), and membrane respectively.

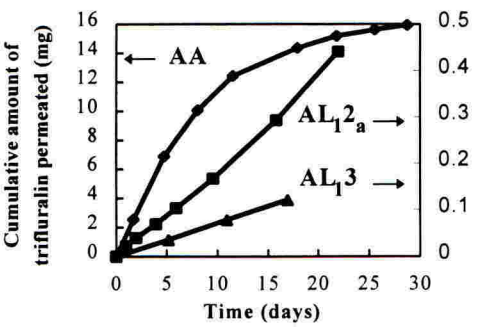

Fig. 1 Permeation of trifluralin through alginate-based membranes with various lignin contents.

Effect of lignin added

The permeation of trifluralin through three types of alginate-based membranes in which the amount of lignin was different is shown in Fig.1. In absence of lignin in the membrane, trifluralin permeated through a dry Ca alginate only membrane (AA) very quickly, about 14 mg within 17 days. The permeation of trifluralin was slightly reduced in comparison with the evaporation of trifluralin from filter paper probably due to the macroporous structure in the membrane (Zhao & Wilkins, 1997). The permeation was dramatically reduced through membranes in which Indulin AT lignin was incorporated. With a higher ratio of lignin to Ca alginate, a reduction of permeation was observed. The amount of trifluralin permeated through AL_13 was 0.12 mg at 17 days, which was 3 times less than through AL_12_a, with ca. 0.32 mg of trifluralin permeated. The decreasing permeation rates with the increasing lignin content in the membrane is possibly due to: ① the decrease of macroporous structure after the incorporation of lignin. The air permeability for AA and AL_12_a were 182.2 and 23.5 ml/min at 0.5 bar air pressure difference. The higher air permeability roughly indicated a more porous structure in the membrane. ② the increase of significant interaction of trifluralin with membranes resulted from the more hydrophobic component, lignin, replacing hydrophilic polysaccharide. The results indicated that the lignin-filled membranes could effectively reduce the permeation of trifluralin vapour.

Effect of varying lignin type

The permeation of trifluralin in the filled membranes varied significantly with the lignin. As can be seen from Fig. 2, the permeation through bagasse kraft lignin-filled membrane (AL_53_b) was 2 times faster than through Alcell lignin-filled membrane (AL_63_a), although these two membranes were almost identical in composition, thickness, air permeability, and porosity (Table 1). This is most likely due to the difference in the chemical structure of lignins. Alcell lignin has a lower content (10.21 %) of total hydroxyl groups than bagasse alkali lignin (12.7 %) (Ferraz *et al.*, 1997), indicating that the hydrophobicity of the Alcell lignin-filled membrane (AL_6) may be increased due to this. Trifluralin is highly hydrophobic, as indicated by its strong adsorbance to soil organic matter (Grover, 1979). Trifluralin may have a greater partitioning into a filled membrane containing less total hydroxyl groups, leading to more pronounced decrease in permeation. Therefore, the slower permeation through AL_6 compared to AL_5 suggests that a much stronger interaction of the trifluralin with lignin probably existed in the membrane AL_6 than AL_5. In addition, the permeation through more porous membranes (AL_53_a & AL_63_b), as indicated by their Dv value (Table 1), was slightly

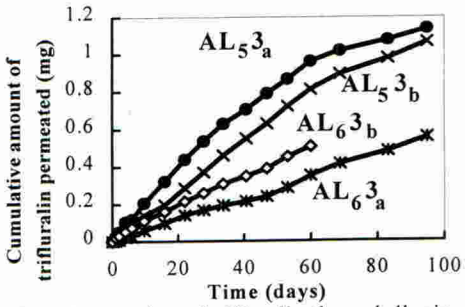

Fig. 2 Permeation of trifluralin through lignin-filled membranes with a lignin: alginate ratio of 3.3:1.

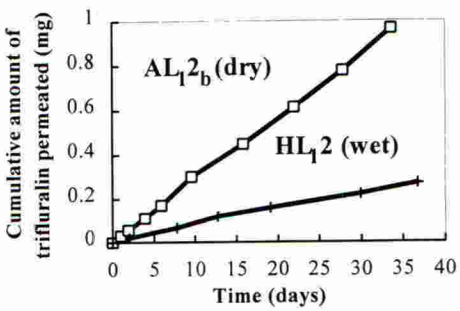

Fig. 3 Permeation of trifluralin through anhydrous and hydrated pine kraft lignin-filled membranes with a lignin:alginate ratio of 2:1.

faster than the counterparts AL_53_b & AL_63_a with lower Dv values, respectively. The results are in good agreement with the data discussed in the previous section, indicating that the permeation rate could be altered by incorporating certain type of lignin into membranes.

Comparison of permeation through anhydrous & hydrated membranes

The permeation of trifluralin through various anhydrous & hydrated membranes is shown in Fig. 3, 4 & 5. As can be seen from these figures, the hydration of lignin-filled membranes resulted in a drop in permeation rate for all membranes to various extents. For the Indulin AT lignin-filled membrane with a lignin:alginate ratio of 2:1, the permeation through the hydrated membrane was about 4 times slower than through the anhydrous one and only 0.22 mg of trifluralin was permeated in 30 days (Fig. 3). The permeation rate of HL_33 was slightly reduced compared to that of AL_33 (Fig. 4). Very little difference in permeation rate could be found for Alcell lignin-filled membranes, AL_63_a & HL_63 (Fig. 5). The results were consistent with our previous study in which anhydrous & hydrated alginate-only membranes had much greater difference in permeation rate than anhydrous & hydrated membranes with a cellulose:alginate ratio of 3.3:1 (Zhao & Wilkins, 1997).

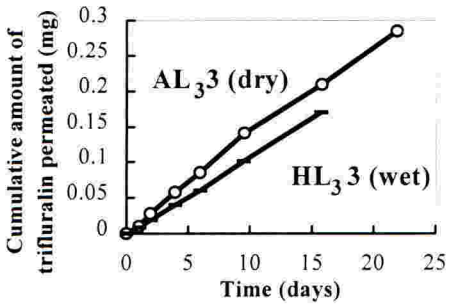

Fig. 4 Permeation of trifluralin through anhydrous and hydrated encalyptus kraft lignin-filled membranes with a lignin:alginate ratio of 3.3:1.

In comparison with anhydrous membranes, the drop in trifluralin permeation through hydrated membranes probably occurred due to the different release mechanism involved. For anhydrous membranes, the permeation was mainly governed by two processes: ① the mass flows through porous membranes; ② the partition of trifluralin vapour with hydrophobic component (lignin) in membranes. For hydrated membranes, however, the permeation was only accomplished by trifluralin dissolved in the water in the membranes. Trifluralin has a very low solubility in water (0.12μg/ml, 25°C). Its movement through hydrated membranes was further governed by ① dissolution of trifluralin; ② partition between water in the pores and membranes; ③ diffusion through hydrated membranes; ④ water permeation through membranes & evaporation from the hydrated membrane. The reduction in permeation of trifluralin for the three pairs of dry & wet membranes suggested that the drop in permeation rate could be reduced by incorporating lignin at a proper ratio. The increased porosity and the decreased hydrophobicity produced by reducing the lignin content or by adding a lignin with a high content of total hydroxyl groups contributed to an increase in permeation through

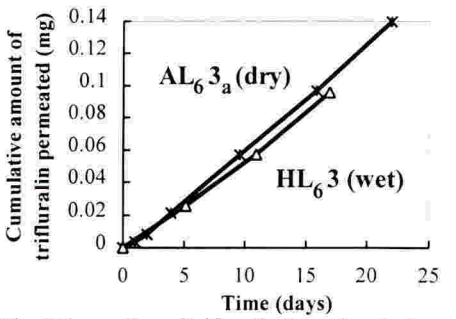

Fig. 5 Permeation of trifluralin through anhydrous and hydrated hardwood organosolv lignin-fille membranes with a lignin:alginate ratio of 3.3:1.

anhydrous membranes. The increase in hydrophilicity by increasing alginate content or by adding the lignin with high content of total hydroxyl groups, resulting in the decrease in water permeation, lead to a reduction in permeation of trifluralin through hydrated membranes.

CONCLUSION

The permeation of highly volatile trifluralin through various lignin-filled alginate membranes could be sufficiently studied by using the flow cell system. This system could be used as a model to understand the release characteristics of alginate-based granules in dry and wet soil. The data obtained from the permeation study suggested that lignin alginate formulations may effectively protect trifluralin from volatility losses. The release profiles could be altered by the appropriate amount or certain type of lignin incorporated in the alginate matrix. The permeation study under two extreme conditions (dry & wet) also suggested that soil moisture varying with irrigation and rainfall could influence the release profiles to various extents for different lignin-alginate matrices. The release of lignin-alginate granules with a lignin : alginate ratio of 2:1 may be significantly subjected to the change in soil moisture, whilst the release from lignin-alginate granules with a lignin : alginate ratio of 3.3:1 and with added lignin of low content of total hydroxyl groups could be kept more stable in various soil moisture regimes. Therefore, a promising lignin-alginate controlled release formulation of volatile trifluralin might be achieved for the effective weed control by optimising lignin-alginate matrix with taking the change of soil moisture into account.

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INFLUENCE OF EMULSIFIABLE OILS AND EMULSIFIER ON THE PERFORMANCE OF PHENMEDIPHAM, METOXURON, SETHOXYDIM AND QUIZALOFOP

H DE RUITER, A J M UFFING, E MEINEN
DLO-Research Institute for Agrobiology and Soil Fertility (AB-DLO), PO Box 14, 6700 AA Wageningen, The Netherlands

ABSTRACT

Field and growth chamber experiments were conducted to investigate the influence of emulsifiable mineral oil, emulsifiable rapeseed oil, and the emulsifier (polyoxyethylene C_{13}/C_{15} oxo alcohol) on the efficacy of four herbicides. Both in the field and in the growth chamber, the emulsifier was at least as effective as the oil adjuvants, when combined with all herbicides except sethoxydim. The herbicide doses giving 50% response (ED_{50}) were determined for the growth chamber experiments. The adjuvants reduced the ED_{50} 2.7-fold with phenmedipham, 51-fold with metoxuron, 2.7-fold with sethoxydim, and 6.8-fold with quizalofop when averaged over the three adjuvants. It was concluded that rapeseed oil (all tested herbicides except quizalofop) or the emulsifier (all tested herbicides except sethoxydim) can serve as a substitute for mineral oil.

INTRODUCTION

Few studies (Manthey *et al.* 1989) have compared the effects of mineral and vegetable oils under the same experimental conditions with regard to type and concentration of emulsifier. In this study we compared the adjuvant effects of a mineral oil with those of a vegetable oil, both containing the same, biodegradable emulsifier.

Despite the extensive use of emulsifiable oil adjuvants little information is available on the specific functions fulfilled by the emulsifier and by the oil in enhancing herbicide performance. The primary reason for adding an emulsifier is to ensure distribution of oil in the water carrier. It is evident that the emulsifier, being a surfactant, may have more functions. Experiments with sethoxydim (Nalewaja and Skrzypczak 1986) and quizalofop (Manthey, Szelezniak *et al.* 1992) demonstrated that emulsifiers in oil adjuvants are involved in enhancing the foliar absorption of these herbicides. That the emulsifier itself may effectively promote herbicide efficacy was demonstrated with the herbicide [(±)-(*EZ*)-2-[1-allyloxyimino)propyl]-3-hydroxy-6-(4-cyanophenyl)cyclohex-2-ene-1-one on grasses (Grayson *et al.* 1993). In this study, we compared the adjuvant effects of the emulsifier itself with those of the emulsifiable mineral and vegetable oils.

MATERIALS AND METHODS

Field experiments

Fat hen was grown in the greenhouse in 6-cm diam. paper pots filled with a mixture of sand and humic potting soil (1:2 by volume). When the plants were in the 4- to 6-leaf stage, the pots were transferred from the greenhouse to 3 m by 2 m field plots on the Droevendaal experimental farm (sandy soil). The paper pots containing plants were placed in the soil at 15-cm intervals in rows, with a row separation of 25 cm. This resulted in a uniform population of fat hen over all field plots. The plants were treated at the 12- to 14-leaf stage.

Winter wheat (cv. 'Arminda') was sown and grown on 4 m by 2 m field plots on the Droevendaal experimental farm. The seeding rate of wheat was 75 kg/ha and the rows were spaced 25 cm apart. The plants were treated at the 3-leaf stage.

Emulsifiable oils were prepared by dissolving the emulsifier 'Lutensol AO 5'(polyoxyethylene (5) C_{13}/C_{15} oxo alcohol) in mineral oil and rapeseed oil at 100 g/L. Commercially formulated herbicides were applied as follows: phenmedipham at 0.24 and at 0.48 kg ai/ha (recommended rate 0.94 kg ai/ha), and metoxuron at 0.6 and at 1.2 kg ai/ha (recommended rate 2.4 kg ai/ha) to fat hen. Sethoxydim was applied at 0.05 and at 0.1 kg ai/ha (recommended rate 0.4 kg ai/ha), and quizalofop(-ethyl) at 0.025 and at 0.05 kg ai/ha (recommended rate 0.2 kg ai/ha) to wheat. The herbicide solutions were applied with an air-pressured sprayer (Birchmeier Helico Sapphire 1.2 mm nozzles fitted with a perforated (0.6 mm) whirling pin 2F) delivering 250 L/ha (phenmedipham) and 400 L/ha (other herbicides) at 202 kPa. Different application volumes were achieved by changing forward speed.

The percent control of fat hen and wheat was estimated visually 1 and 2 wk after treatment. The control was quantified by harvesting the aerial parts from 1-m^2 subplots in each plot.

Growth chamber experiments

Fat hen and winter wheat were grown in a growth chamber under 14 h of light, at 18/12 (±0.5)°C (day/night) temperature, and in 70/80 (±5)% (day/night) relative humidity. Light was provided by high pressure sodium lamps and fluorescent tubes to give 80-120 W/m^2 (PAR) at leaf level. Fat hen was treated at the 6-leaf stage and wheat at the 3-leaf stage.

Each herbicide was applied at ten rates for measuring the dose response relation. The herbicide solutions were applied with a laboratory track sprayer fitted with the same nozzles as used for the field experiments except that 250 L/ha and 400 L/ha were given under a higher pressure of 303 kPa.

Herbicide efficacy was measured by harvesting the aerial parts of the plants. Fat hen was harvested 7 DAT with phenmedipham and 11 DAT with metoxuron. Wheat was harvested 21 DAT with sethoxydim and 21 DAT with quizalofop.

Experimental design and data analysis

Two separate field experiments, one with fat hen and one with wheat, were conducted in 1993 and repeated in 1994. Treatments were arranged in a randomized complete block design with four replications. Data were subjected to analysis of variance using the Genstat 5 statistical package. The means of treatments were compared according to Fisher's LSD (0.05) test.

Two separate experiments with each herbicide were conducted in the growth chamber as a randomized complete block with four replications. Each block contained two untreated controls. To determine the dose response relationship for each adjuvant-herbicide combination, the data of each adjuvant-herbicide combination (ten herbicide rates) were subjected per replication to nonlinear regression (Genstat 5) using the equation:

$$y = a + \frac{c}{1+e^{-b\,(x-m)}}$$

In this equation y is the fresh weight, x is ln(herbicide dose), a is the lower limit at large doses, a+c is the upper limit at zero dose, m is the $\ln(ED_{50})$; ED_{50} is the dose required to reduce the fresh weight to halfway between the upper and the lower limits, and b is the slope-parameter which determines the slope around the ED_{50}. There was no significant block-effect on the variation in fresh weights of the untreated plants within experiments. These fresh weights were therefore pooled in the calculation of the upper limit a+c of the dose response curve. The lower limit a was calculated by pooling the weights of completely dead plants. The $\ln(ED_{50})$ values determined for the different herbicide-adjuvant combinations were subjected to analysis of variance. The geometric means were compared using Fisher's LSD (0.05) test and the arithmetric means are presented.

RESULTS AND DISCUSSION

Phenmedipham

The field experiments demonstrated that mineral oil, rapeseed oil and the emulsifier were generally equally effective in enhancing the efficacy of phenmedipham against fat hen (Table 1). Determination of the ED_{50} revealed that the rapeseed oil and the emulsifier similarly influenced the performance of phenmedipham (Table 2). The ED_{50} was reduced approximately 4-fold. Mineral oil was less effective and reduced the ED_{50} less than 2-fold. Calculation of the average ED_{50} across the three adjuvants and the two experiments revealed that the adjuvants reduced the ED_{50} 2.7-fold versus the no-adjuvant treatment.

The beneficial effect of the emulsifier is notable because previous studies indicated that nonionic surfactants (Miller and Nalewaja 1973; de Ruiter et el. 1991) and cationic, polyoxyethylene alkylamine surfactants (Miller and Nalewaja 1973; Zandvoort 1988) were less effective than oil adjuvants in enhancing phenmedipham performance.

Metoxuron

Mineral oil, rapeseed oil and the emulsifier strongly enhanced the field performance of metoxuron against fat hen (Table 1). The results indicate the great potency of the adjuvant-metoxuron combination for controlling this weed. The three adjuvants, on average, reduced the ED_{50} for metoxuron 51-fold versus the no-adjuvant treatment (Table 2). This agreed with the results of the field experiments.
Our study indicates that the emulsifier alone is sufficient for optimum performance of metoxuron and that oils are not needed. West *et al.* (1988) also found that a nonionic

Table 1. Influence of the adjuvants on herbicide efficacy in the field. Emulsifiable mineral oil and emulsifiable rapeseed oil (emulsifier content 100 g/L) were included in the spray mix at 1 % (v/v). When tested alone, the emulsifier was included at 1 g/L.

			Adjuvants						
			1993			1994			
Herbicide	Rate	Target species	No adjuvant	Mineral oil	Rapeseed oil	No adjuvant	Emulsifier	Mineral oil	Rapeseed oil
	kg ai/ha		% fresh wt reduction [1, 2, 3]						
Phenmedipham	0.24	Fat hen	63	81	81	41	62	65	71
	0.48		77	83	87	55	73	80	84
Metoxuron	0.6	Fat hen	59	90	89	57	90	93	93
	1.2		27	90	91	74	93	93	94
LSD (0.05) within a year and for fat hen			24			10			
Sethoxydim	0.05	Wheat	0	63	58	3	20	64	55
	0.1		7	94	91	10	68	94	89
Quizalofop	0.025	Wheat	0	96	16	23	91	96	21
	0.05		24	97	82	12	96	97	71
LSD (0.05) within a year and for wheat			19			18			

[1] Percent fresh weight reduction relative to non-treated control.
[2] Mean fresh weight of fat hen from untreated plots was 0.65 kg/m^2 in 1993 and 1.32 kg/m^2 in 1994.
[3] Mean fresh weight of wheat from untreated plots was 0.58 kg/m^2 in 1993 and 0.60 kg/m^2 in 1994.

Table 2. Influence of the adjuvants on herbicide efficacy (ED_{50}) under controlled conditions. Emulsifiable mineral oil and emulsifiable rapeseed oil (emusifier content 100 g/L) were included in the spray mix at 1 % (v/v). When tested alone, the emulsifier was included at 1 g/L.

Herbicide	Target species	Experiment	Upper limit a+c [1]	Lower limit a [1]	Adjuvants			
					No adjuvant	Emulsifier	Mineral oil	Rapeseed oil
			g/pot	g/pot	ED_{50} (g ai/ha)[2]			
Phenmedipham	Fat hen	1	8.6	1.0	342 a	76 c	200 b	75 c
		2	10.0	1.1	214 a	72 b	134 a	55 b
Metoxuron	Fat hen	1	4.4	0.1	1546 a	20 b	22 b	24 b
		2	15.3	0.5	1532 a	34 c	37 bc	45 b
Sethoxydim	Wheat	1	52.2	4.3	122 a	57 c	42 d	36 b
		2	49.5	2.9	98 a	48 b	33 c	32 c
Quizalofop	Wheat	1	43.4	2.7	46 a	7 b	6 c	9 d
		2	48.8	3.1	49 a	8 b	6 c	6 c

[1] For explanation, see section on Materials and Methods.
[2] Means within one experiment followed by the same letter do not differ at the 5 % probability level (LSD).

surfactant (polyoxyethylene nonylphenol) can be as effective as emulsifiable oils. These results indicate that adding appropriate adjuvants provide new possibilities for efficient (low dose) post-emergence use of metoxuron. Potential loss of selectivity caused by adjuvants requires attention with each new post-emergence application.

Sethoxydim

Mineral oil and rapeseed oil were equal in enhancing the performance of sethoxydim against field-grown winter wheat (Table 1). Ranking of the adjuvant effects acording to the ED_{50} determined under controlled conditions parallelled the results in the field (Table 2). The oil adjuvants reduced the ED_{50} approximately 3-fold whereas the emulsifier reduced the ED_{50} 2-fold. The adjuvants, on average, reduced the ED_{50} 2.7-fold versus the no-adjuvant treatment.

The similarity of the effects of a vegetable and a mineral oil confirms an earlier study on sethoxydim which also used oil adjuvants containing the same type and amount of emulsifier (Manthey *et al.* 1989). Foliar absorption experiments with sethoxydim demonstrated that a mineral oil, seed oils (both oil types used without emulsifier), and two emulsifiers approximately tripled the uptake of sethoxydim (Nalewaja and Skrzypczak 1986). The foliar uptake study cited and previous efficacy studies with nonionic surfactants (Hartzler and Foy 1983; Manthey *et al.* 1989; de Ruiter *et al.* 1991) and cationic, polyoxyethylene alkylamine surfactants (Kudsk *et al.* 1987; de Ruiter *et al.* 1991; Harker 1992), indicate that surfactants can be at least as effective as emulsifiable oils in enhancing sethoxydim efficacy.

Quizalofop

The mineral oil and the emulsifier were both very effective adjuvants for quizalofop against winter wheat (Table 1). Rapeseed oil had no adjuvant effect at all at the lower application rate (0.025 kg/ha). The poor performance of the rapeseed oil confirms previous data (Manthey *et al.* 1989). Their study demonstrated that addition of once-refined seed oils (sunflower, soybean, and linseed) reduced the activity of quizalofop in the field against three grass species. In contrast, the dose response curves revealed that all adjuvants, including the rapeseed oil, strongly reduced the ED_{50} for quizalofop (Table 2). There were significant differences between the ED_{50} values of the adjuvants but their values were in the same range (6-9 g/ha) which differed greatly from the ED_{50} (46-49 g/ha) determined for quizalofop without adjuvant. The adjuvants, on average, reduced the ED_{50} 6.8-fold.
The good performance of the emulsifier, being an alcohol ethoxylate, agrees with a recent study (Green 1997) reporting that alcohol ethoxylates strongly enhance the efficacy of quizalofop-P.

Rapeseed oil versus mineral oil

The field and the greenhouse experiments indicate that mineral oil can be replaced by rapeseed oil as an adjuvant for the herbicides phenmedipham, metoxuron and sethoxydim. Rapeseed oil was less effective when combined with quizalofop in the field but methylated vegetable oil may serve as an appropriate substitute for mineral oil with this herbicide (Manthey et al 1989). Therefore, it can be concluded that mineral oil is certainly not indispensable as an adjuvant for the herbicides tested. Seed oils, either as triglycerides or as methylated fatty acids, may serve as substitutes.

Emulsifiable oil versus emulsifier alone

A possible difference between the emulsifiable oil and the emulsifier alone regarding their influence on retention of spray drops on the foliage may have contributed to the results reported here. Emulsifiable oils (Nalewaja 1986; de Ruiter *et al.* 1987; Schott *et al.* 1991; Moerkerk and Cobellack 1992) and surfactants (de Ruiter *et al.* 1990) can enhance spray retention. The influence on retention of leaving out oil and application of the emulsifier alone is not known and needs more attention.

The emulsifier used in this study is relatively lipophilic (EO content of 5; HLB=10). Previous studies suggest that the absorption of lipophilic compounds requires relatively lipophilic surfactants (Stevens and Bukovac 1987; Stock *et al.* 1993). This may explain why the emulsifier used in this study is effective when combined with the four relatively lipophilic herbicides. Two factors may contribute to the influence of an adjuvant on foliar uptake: solubilization of the herbicide in the drop deposit, thereby increasing the fraction of active ingredient available for uptake; and facilitated permeation of the leaf cuticle. Both oils and emulsifiers, depending on their lipophilicity (Briggs and Bromilow 1994), can serve as solvents for relatively lipophilic herbicides and both adjuvant types may strongly interact with the lipophilic leaf cuticle. From this point of view it seems logical that emulsifier and oil can enhance foliar absorption of herbicides (Nalewaja and Skrzypczak 1986; Manthey, Szelezniak *et al.* 1992) and increase their efficacy, as observed in this study.

We assume that the physical-chemical properties of the deposit, after evaporation of water and volatilization of solvents of the commercial formulation, determine the uptake of an active ingredient. It is then understandable why a few studies (Manthey, Matsyak *et al.* 1992; McMullan 1993) have mentioned that emulsion stability of the spray solution is not particularly relevant to the efficacy of a herbicide.

Emulsifiable oils and and emulsifiable esterified oils are extensively used as adjuvants for herbicides and other biocides. Our study indicates that these adjuvants can be replaced by relatively low concentrations of an appropriate surfactant.

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EFFECT OF DRIFT CONTROL ADJUVANTS AND A SURFACTANT ON A HERBICIDE APPLIED AT CONVENTIONAL AND ULTRALOW VOLUMES

G D WILLS
Delta Research and Extension Center, PO Box 197, Stoneville, MS 38776 USA

J E HANKS
Application Production Technology Research Unit, U S Department of Agriculture, PO Box 36, Stoneville, MS 38776 USA

E J JONES
Delta Research and Extension Center, PO Box 197, Stoneville, MS 38776 USA

R E MACK
Helena Chemical Company, One Hy Crop Row, Memphis, TN 38120 USA

ABSTRACT

A field experiment evaluated the effect of a surfactant and three drift control adjuvants on the efficacy and drift of a herbicide applied at a conventional and an ultralow spray volume. Weed control was enhanced by the addition of the surfactant. Each drift control adjuvant reduced the amount of herbicide drift with no adverse effect on efficacy. Applications at the ultralow spray volume were often as effective as at the conventional spray volume.

INTRODUCTION

The reduction of off-target movement (drift) of pesticide spray solutions is a concern of many countries (Hall, 1995). Many researchers are working to develop the use of drift control adjuvants to reduce the volume of fine (<150 μm) driftable spray droplets. Hazen and Olsen (1995) were able to reduce the formation of fine droplets by adding a dry polymeric adjuvant to a spray solution. Downer *et al.* (1995) found that polyvinyl polymer adjuvants reduced the spray widths of flat fan spray nozzles by 10 to 15% but did not affect the efficacy of the herbicide being applied. Wills *et al.* found similar effects using drift retardants with glyphosate herbicide.

Herbicides are normally applied at rates of 50 to 200 litres/ha. With conventional hydraulic spray nozzles, lower volume applications require smaller nozzle orifices which frequently become clogged. McWhorter *et al.* (1988) developed a spraying system that was modified by Hanks and McWhorter (1991) which delivers from 2 to 150 litres/ha and does not rely on nozzle orifice size and hydraulic pressure to develop a spray pattern. This system uses a positive displacement pump to meter the spray volume to an air-assist nozzle with orifice sizes up to 2.5 mm in diameter. The spray pattern and droplet size are formed by a low-pressure airflow which is introduced at the nozzle tip (Hanks and McWhorter, 1993).

This study was conducted to determine the effect of three drift control adjuvants with and without a surfactant on the efficacy and drift of paraquat applied at conventional and ultralow spray volumes.

MATERIALS AND METHODS

Applications were made to plots with four rows of soybeans 85 to 90 cm tall, spaced 1 m apart by 12 m long and with *Echinochloa crus-galli* 55 to 60 cm tall, seeded broadcast in the inter-row spacings. Chemicals were applied as shown in Table 1. Treatments were replicated three times in a randomized complete block design. Paraquat was applied at 250 g a.i./ha alone or with the surfactant, HM 8802A, a proprietary blend of methylated seed oil and organosilicone surfactant (0.5%, v/v). Each of these was applied with or without the drift control adjuvant, HM9236, a proprietary blend of polymeric viscosity modifiers and inert ingredients (0.45 g/litre), HM9621, a proprietary blend of nonionic soluble organic polymers, dispersion additives and formulation agents (1.25 ml/litre) or HM9206, a proprietary blend of acrylamide polymers, water and surfactants (1.25 ml/litre).

Treatments were applied broadcast to three inter-row spacings in each plot. Application was made under solid-state, fiberglass hoods which were 70 cm wide, 50 cm high and open at the front and back. The hoods were mounted 10 cm above the ground on metal skids. Treatments at the conventional spray volume of 50 litre/ha were applied with a hydraulic system using 9501 Tee Jet brass spray tips at 390 kPa. Ultralow volume applications were made with an air-assist spray system as described by Hanks and McWhorter (1991). The solution was atomized with air at 30 kPa as it exited the nozzle.

Evaluations were made at five days after treatment. Control of *E. crus-galli* was visually rated over each treatment plot where 0 = no control and 100 = complete kill of shoots. Drift was randomly measured at four locations in each treatment plot as the height in cm above the ground of herbicide injury on adjacent soybeans.

Data were subject to analysis of variance. Means were separated using Fisher's Protected LSD procedure with a significance level of 0.05. Arcsine square root transformations were computed on the percent control data and were separated based on mean separations of the transformed data. The data are displayed in their original units.

RESULTS

The effects of surfactant and drift control adjuvants on the efficacy and drift of paraquat are shown in Table 1. At the conventional spray volume of 50 litres/ha, drift height of paraquat injury on bordering soybeans was 52 and 49 cm with and without added surfactant, respectively. The addition of each drift control adjuvant equally reduced the drift height to 34 to 37 cm both with and without surfactant.

At the ultralow spray volume of 5 litres/ha, drift height was 60 and 62 cm when applied with and without surfactant, respectively. When applied without surfactant, the drift control

adjuvants HM9236 and HM9621 reduced the drift height to 51 and 52 cm, whereas HM9206 reduced the drift to only 56 cm. When applied with surfactant, all three drift control adjuvants reduced the drift to 43 to 48 cm on the bordering soybeans.

Control of *E. crus-galli* was 78 and 75% without surfactant and 88 and 87% with surfactant when paraquat was applied at the conventional and at the ultralow spray volume, respectively. The addition of the drift control adjuvants either increased or had no significant effect on the efficacy of the herbicide.

Applications at the ultralow spray volume were often as effective as at the conventional spray volume. However, at this time, ultralow volume application with paraquat has not been approved for use in the United States of America or elsewhere in the world.

Table 1. Effect of drift control adjuvants and a surfactant on paraquat control of *Echinochloa crus-galli* and the height of drift injury on bordering soybean rows as applied under hoods to 100-cm-wide inter-row spacings at conventional and ultralow spray volumes[1,2/]

Herbicide	Drift control adjuvants			Surfactant	Drift height on bordering	Control of
paraquat	HM9236	HM9621	HM9206	HM8802A	soybeans	*E. crus-galli*
(250 g/ha)	(0.45 g/litre)	(1.25 ml/litre)	(1.25 ml/litre)	(5 ml/litre)	(cm)	(%)
			Applied at 50 litres/ha			
X	–	–	–	–	49	78
X	X	–	–	–	34	83
X	–	X	–	–	36	82
X	–	–	X	–	36	85
X	–	–	–	X	52	88
X	X	–	–	X	37	90
X	–	X	–	X	36	85
X	–	–	X	X	35	88
			Applied at 5 litres/ha			
X	–	–	–	–	62	75
X	X	–	–	–	51	82
X	–	X	–	–	52	73
X	–	–	X	–	56	83
X	–	–	–	X	60	87
X	X	–	–	X	45	87
X	–	X	–	X	43	85
X	–	–	X	X	48	87
Untreated control					0	0
LSD (0.05)					3.39	3.42

[1/]Application was made under solid-state, fiberglass hoods which were 70 cm wide, 50 cm high and open at both ends. The hoods were mounted 10 cm above the ground on metal skids. Applications at 50 litres/ha were made using 9501 Tee Jet brass spray tips. Application at 5 litres/ha were made using Beta T Mizer spray tips with 30 kPa air-assist pressure.

[2/]Application was made in the inter-row of 100-cm spaced rows of soybeans 85 to 90 cm tall and to *Echinochloa crus-galli* 55 to 60 cm tall in the inter-row. Visual evaluation of percent control of *Echinochloa crus-galli* and height of drift of paraquat on soybeans were made at five days after treatment.

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MODIFIED SPRAY NOZZLE DESIGN REDUCES DRIFT WHILST MAINTAINING EFFECTIVE CHEMICAL COVERAGE

A R G CECIL
Billericay Farm Services, Downham, Billericay, Essex, CM11 1QU, UK

ABSTRACT

A major consideration during the design and manufacture of spray nozzles intended for pesticide application concerns accurate application with minimal chemical spray drift. Billericay Farm Services have designed, manufactured and evaluated a simple nozzle design which uses an integral venturi to draw air into the pumped liquid stream before being sprayed through a conventional fan jet. This reduces the proportion of very small drops in the jet stream and incorporates air bubbles in the larger drops. The characteristics of the spray produced is typical of a Medium to Coarse fan jet. Evaluation of the design under laboratory and field trial conditions has demonstrated that a significant reduction in drift is achieved combined with good chemical response.

INTRODUCTION

Pesticides which work through leaf contact require the maximum possible number of contact points between leaf and chemical. It is recognised that Fine sprays achieve better leaf contact but drift potential is markedly increased relative to Medium or Coarse sprays. The current UK legislation requires the pesticide to be confined to the target area. These restrictions demand that operators take all reasonable precautions to minimise spray drift which might inadvertently cause damage to crops or watercourses on adjacent areas.

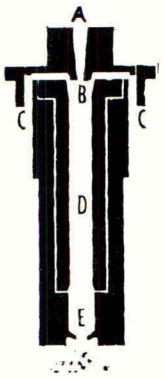
The potential for drift is inversely proportional to the time each drop remains airborne, which functionally is inversely proportional to it's terminal velocity. Stokes law states that terminal velocity is proportional to the square of the diameter of the drop. For a given volume of sprayed liquid the number of drops is inversely proportional to the cube of the volume median diameter (VMD). Thus the drift potential is inversely proportional to the square of the diameter of a drop. The relationship therefore between good chemical coverage ($\alpha\ d^{-3}$) and drift ($\alpha\ d^{-2}$) is proportional to 1/d.

This paper reports a novel spray jet developed by Billericay Farm Services which utilises the inclusion of air into the liquid stream to break the normal relationship between chemical coverage and drift by including air bubbles in the larger drops. It is hypothesised that the individual larger drops collapse on impact and act in a manner similar to a number of smaller drops.

MATERIALS AND METHODS

Nozzle design

The air bubble jet is designed to use the energy of the pumped liquid passing through a venturi to create a vacuum which sucks air into the liquid stream.



The liquid being sprayed passes through a tapered nozzle **A** which accelerates the liquid and projects the flow into the tapered mouth of the venturi **B**. This creates a vacuum which causes air to be sucked in through the slots marked **C**. The mixture of air and liquid is compressed as it passes through the mixing chamber **D** and is then sprayed through a fan nozzle **E**.

EVALUATION OF THE OPERATION OF AIR BUBBLE JET

Laboratory Tests

The VMD of drops produced by the 03 Air Bubble Jet (ABJ) has been evaluated by The International Pesticide Application Research Centre (IPARC) [Craig (1992) - personal communication] using the Malvern droplet sizing tests.

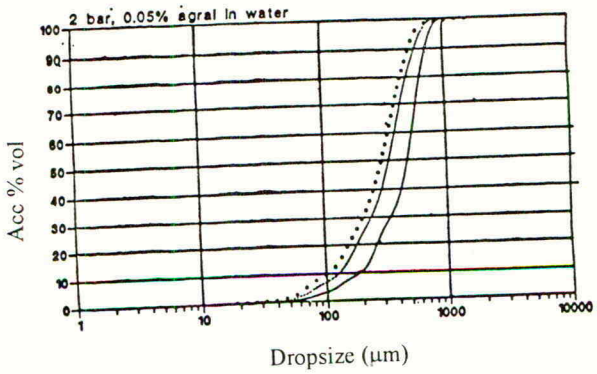


Figure 1. Volume median diameter of droplets produced by ABJ with: ——— Air vent open,····· Air vent closed,••••• Pre-orifice plate removed

These results demonstrate that the presence of an air-vent consistently decreases the percentage of spray particles with a volume of less than 105 micron by about 50% in each case. Evaluation of droplet characteristics, undertaken at Silsoe Research Institute using micro photographic techniques have demonstrated that the individual droplets produced during fluid passage through the air bubble jet nozzle contain small bubbles of air.

Evaluation of spray drift

The spray drift produced by the ABJ has been evaluated by the Long Ashton Research Station [Hislop (1994) - personel communication].

The comparative drift performance of an ABJ 03 nozzle with the air vents blocked (ABJ 03-, flow 1.5 l/min) and ABJ 03 nozzle with the air vents open (ABJ 03+, flow 1.5 l/min) were evaluated against a standard 03 fan nozzle (flow 1.2 l/min).

Six equally spaced horizontal sampling strings (bottom string 70 mm and top string 645 mm above the cereal crop) were positioned 5m down wind in a wind tunnel creating a nominal average wind speed measuring 2.5 m/sec at 800 mm above the crop. A fluorescent dye (fluorescin) was added to the liquid spray solution and drift was determined by calculating the total deposit of dye harvested from the sampling strings, expressed as a percentage of the total dye released, following correction for differences in flow rate.

These results demonstrated that 03 ABJ with open air vents achieved a reduction in drift of 58% compared with a standard 03 fan nozzle.

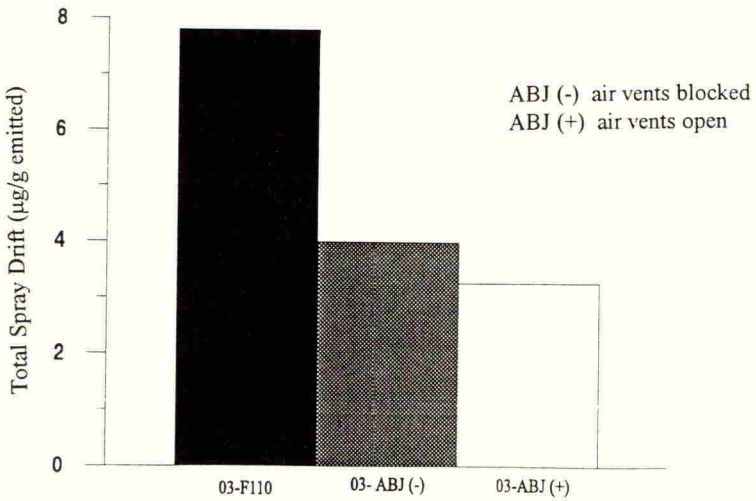


Figure 2. Total spray drift (µg/g dye emitted) harvested at 5m downwind with measured wind speed of 2.5m/sec

RESULTS

Crop trials

The spray characteristics of the ABJ nozzle have been evaluated in collaberation with Hardi International and Harper Adams Agricultural College using two plant species, barley and radish.

which are representative of crops that are targeted for much agrochemical usage [Taylor (1996) and Cooper (1996) - personel communication].

Experimental conditions established for the crop trials involved a Hardi LX tractor mounted machine with a 12 metre boom. Ground speed was 2 m/sec and the spray solution was water with a non-ionic surfactant (Agral, Zeneca) at 0.1% vv. A fluorometric water soluble dye (fluorescin) was added to facilitate quantification of the sprayed deposits. Concentration of fluorescin in harvested samples was measured on a Perkin Elmer LS2 filter fluorimeter against known concentrations of sprayed liquid. Samples were rapidly taken and covered from UV light to stabilise the tracer. The range of treatments applied include four sizes of Air Bubble Jet nozzles and three conventional fan jet nozzles (Hardi 411014, 411020, and 411030) as BCPC reference nozzles for fine, medium and course spray categories to establish differences in total retention due to the nozzle type. During subsequent applications all plants were washed individually to assess the variability of data within each treatment. The values are expressed as the quantity of sprayed solution recovered from the foliar parts of the plants normalised to permit true comparisons between water volume rates (µl dye/plant at100 l/ha spray volume).

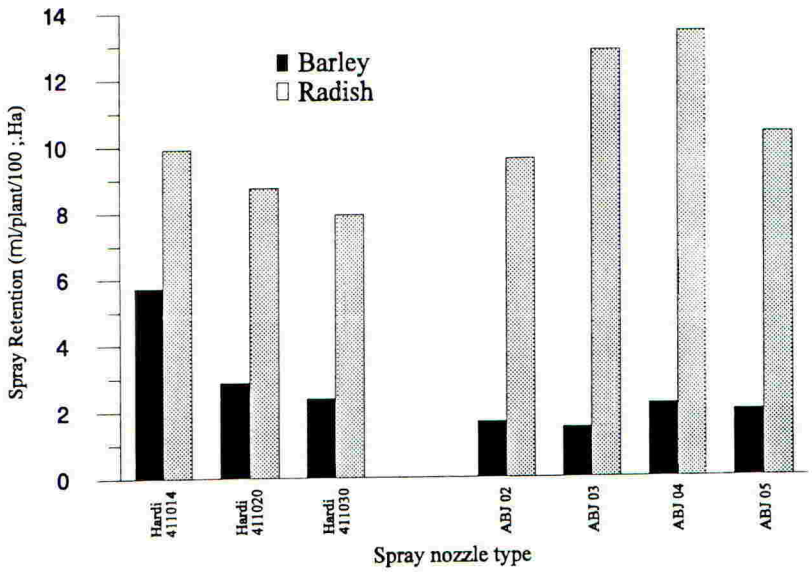


Figure 3. Comparison of the mean spray retention on the foliar parts of barley and radish using the range of Fine, Medium and Course Hardi fan jet nozzles compared to the ABJ jet nozzle range (02,03,04,05). Results are expressed as µl dye/plant at 100 l/ha spray volume.

Field Trials

Field trial assessment of agrochemical application using the novel ABJ spray nozzles (02) compared with standard fan nozzles has been undertaken by Novartis [Robinson (1997) - personel communication]. Agrochemicals were applied using a tractor trailed model 2000 Knight sprayer travelling at 3.3m/sec operating at a standard pressure of 3 bar with an application rate of 200 l/Ha.

Trinex-apat-ethyl growth regulator (Moddus, Novartis) was applied to winter wheat at 0.2l/ha diluted in two differing volumes of water to ensure a final application volume of 80l/ha and

200l/ha using standard fan jets and Air Bubble Jets. All spray applications were performed in triplicate.

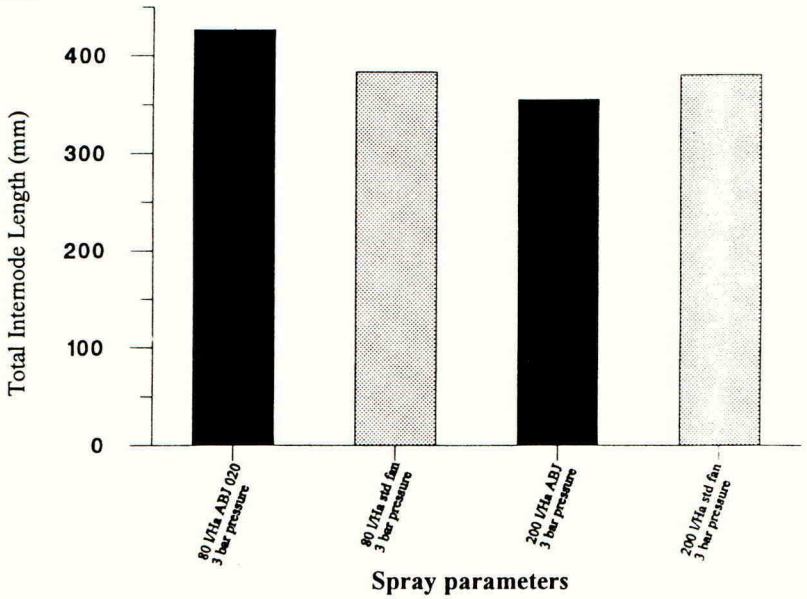


Figure 4. Total internode length of winter wheat measured at 6 weeks and 16 weeks post-application of Trinex-apat-ethyl growth regulator (Moddus, Novartis)

Clodinafop-propargyl (Topic, Novartis) a contact acting herbicide for annual grass weed growth in cereals was applied at 0.125 l/Ha diluted in two differing volumes of water to ensure a final application volume of 80l/ha and 200l/ha using standard fan jets and Air Bubble Jets.

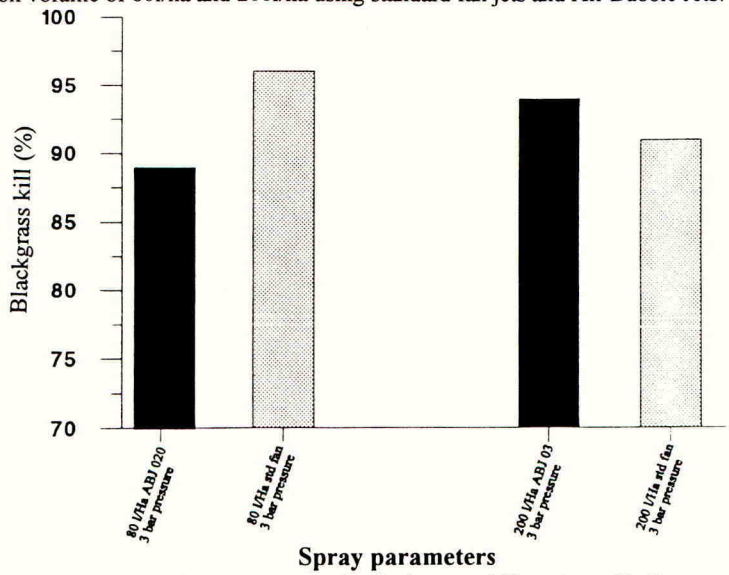


Figure 5 Assessment of percentage of Blackgrass kill using Clodinafop-propargyl (Topic, Novatis)16 weeks after application.

DISCUSSION

The results of laboratory and field trials indicate that Air Bubble Jet nozzles have spray characteristics comparable to Medium and Coarse standard conventional fan spray jets. Wind tunnel evaluation demonstrated conclusively that significant reductions of drift by up to 58% can be achieved using the ABJ nozzles in preference to standard fan nozzles where Coarse or Medium spray agrochemical applications are recommended. The results of the evaluation of foliar retention of agrochemical indicate that broad leaf targets have excellent retention of the larger, air containing, drop produced by the ABJ nozzles. Targets with an upright waxy leaf have better retention of agrochemical, under ideal windless conditions, when the chemical is sprayed from conventional Fine fan nozzles. Environmental and legislative considerations have focussed attention on the requirement to reduce agrochemical drift. The time frame of pesticide effectiveness frequently requires the application within a defined period which may involve spraying under non ideal wind conditions. Trials to assess foliar retention of agrochemicals under non ideal wind conditions sprayed with Fine nozzle jets compared with ABJ nozzle jets are currently in development phase. Air Bubble Jets have been shown to improve agrochemical application, by reducing drift loss, where Medium and Coarse nozzle application is recommended by the manufacturers.

Acknowledgements

We are most grateful for the work undertaken by Long Ashton Research Station, Silsoe Research Centre, IPARC, Novartis and Hardi International

EFFECTS OF ADJUVANT OIL EMULSIONS ON FOLIAR RETENTION AND SPRAY QUALITY

K J HALL, N M WESTERN, P J HOLLOWAY
IACR-Long Ashton Research Station, Department of Agricultural Sciences, University of Bristol, Long Ashton, Bristol BS18 9AF, UK

D STOCK
AgrEvo UK Limited, Chesterford Park, Saffron Walden, Essex CB10 1XL, UK

ABSTRACT

Retention effects of tank-mixing three model ECs containing mineral, vegetable or fatty acid methyl ester oils, were compared in track-sprayer experiments using the water-soluble tracer, fluorescein. Deposition of aqueous solutions of fluorescein onto pea and barley foliage was enhanced by addition of these ECs at 5-20 g/litre. On young foliage, there was little difference in retention efficiency between the three emulsions or between the emulsions and the emulsifier alone, if applied at the concentrations present in the diluted ECs However, on older foliage, the emulsions performed significantly better than the emulsifier. In-flight measurements revealed that droplet-sizes in the spray cloud at the position of impact with the target plants were increased both in the presence of emulsions and emulsifier, when compared with water. These effects on spray quality probably explain why retention enhancement using oil ECs is often inferior to that obtained using other adjuvants, especially surfactants.

INTRODUCTION

Emulsified mineral oils are commonly used as adjuvants for tank-mixing with pesticides, especially herbicides, in order to improve efficacy. Because of increasing concerns about the biodegradability of mineral oils, alternatives with more environmentally benign vegetable oils or derivatives thereof, are increasing in importance. Whilst it is generally agreed that the main mode of action of these oils is by enhancement of pesticide uptake (Gauvrit & Cabanne, 1993), their effects on the efficiency of spray delivery to foliage are still poorly documented.

In the present work, we compare the effects of three chemical types of oils formulated as ECs having a common emulsification system, on the spray retention of fluorescein by two water-repellent species, each at two different growth stages. Retention results are examined in relation to spray quality data determined by in-flight droplet measurements.

MATERIALS AND METHODS

Target species

Pea (*Pisium sativum* cv. Meteor, three per pot) and barley (*Hordeum vulgare* cv. Triumph, ten per pot) plants were raised from seed under controlled environment conditions (20°C light / 15°C dark with a 16h photoperiod). Peas were sprayed 18d or 22d after sowing, barley 10d or 15d after sowing.

Oils

Light Liquid Paraffin BP (LLP) (Thornton Ross, UK) was the mineral oil. The vegetable-based products were soya oil (S) (Seatons, UK) and methyl soyate (MS) (Henkel, Germany), a mixture of soya fatty acid methyl esters.

ECs

ECs of the three oils were prepared using 10% *wt / wt* of a 1:1 mixture of Ethylans D253 (a 3 mole ethoxylated (EO) primary alcohol) and C12AH (a 35% EO castor oil) (Akcros, UK).

Bench-mark deposition agents

Surfactant- and solvent-based adjuvants were used. The surfactant was Agral (NP10EO) (Zeneca, UK), a 10 mole EO nonylphenol. Aqueous acetone (1:1 *v/v*) was the solvent.

Spray application

A gear and tooth-driven track-sprayer fitted with an 80015E even-spray nozzle (Spraying Systems Company, USA; BCPC nozzle code FE80/0.59/3), placed 400 mm above target plants was used for all applications. Spray rates were *ca* 200 litres/ha (flow rate 0.43 litres/min; air pressure 200 kPa; track speed 0.45 m/s). Recirculation by a pneumatically operated diaphragm pump provided continuous agitation of the reservoir.

Spray retention

Spray solutions contained sodium fluorescein in distilled water (0.05 g/litre) and tracer recoveries were quantified using the spectrofluorimetric method described by van Toor *et al.* (1994). Retention data expressed as deposits per unit emission (DUE), *viz.*, ng fluorescein / g foliage dry wt / g fluorescein applied / ha, were subjected to analysis of variance, with $\log_n$ transformations where necessary.

Spray quality

In-flight droplet sizes and velocities were determined by phase-Doppler analysis (PDA) (Aerometrics Inc., USA) at a vertical distance of 400 mm below the nozzle, from three consecutive 300 mm short axis scans through the centre-line of the spray fan and 80 mm either side of it. Spray parameters are expressed as mean values from three separate measurements.

RESULTS

Spray retention on pea

On 18-d-old foliage, addition of either the oil ECs at 10 and 20 g/litre or the equivalent concentrations of emulsifier alone (1 and 2 g/litre) all enhanced fluorescein retention (Table 1), when compared with water (DUE 108). However, the increases produced were generally less than those achieved using NP10EO at 2 g/litre (DUE 439) or aqueous acetone (DUE 738). There were no significant differences in deposition between the emulsions and the emulsifier alone (DUE 253-412), except for the LLP EC at 20 g/litre, which gave less retention (DUE 150) than the other formulations.

Table 1. Effects of emulsions and emulsifier on fluorescein retention by 18-d-old peas

Spray liquid	Mean DUE values *			
	1 g/litre	2 g/litre	10 g/litre	20 g/litre
Emulsifier	5.72 (305)	5.92 (373)	-	-
Soya EC	-	-	6.02 (412)	5.94 (380)
Methyl soyate EC	-	-	5.53 (253)	5.58 (266)
LLP EC	-	-	5.58 (266)	5.01 (150)

Water 4.68 (108); NP10EO at 2 g/litre 6.08 (439); aqueous acetone 6.60 (738).
* $\log_n$-transformed values, LSD (p=0.05) 0.34; back-transformed values in parentheses.

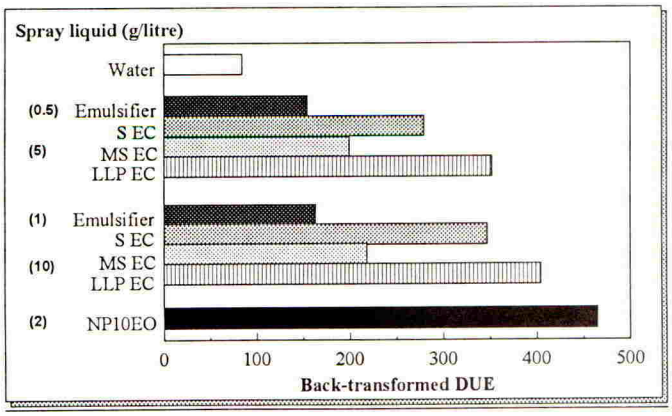


Fig. 1. Effects of emulsions and emulsifier on fluorescein retention by 22-d-old peas.

On more mature 22-d-old plants, foliar deposition was also enhanced by tank-mixing with the oil ECs at 5 and 10 g/litre, as well as by the emulsifier alone (0.5 and 1 g/litre) (Fig. 1). However, retention enhancement by emulsions was significantly greater than that from emulsifier at both concentrations tested; differences were also observed according to oil composition. For example, for ECs at 5 g/litre, DUE values for soya, methyl soyate and LLP were 348, 200 and 353, respectively, compared with 155 for the emulsifier itself. The performance of methyl soyate emulsions was consistently inferior to those containing the

other two oils (Fig. 1). Although at 5 g/litre EC LLP gave better retention than soya, at 10 g/litre, the two emulsions behaved similarly.

Spray retention on barley

Differences between the relative retention-enhancing efficiencies of emulsions and emulsifier with plant age were not as marked on barley as on peas. Again, on younger 10-d-old foliage, there were no significant differences in fluorescein deposition between the three oil ECs or between the emulsions and emulsifier, either at 10 or 20 g/litres (DUE 152-295) (Table 2); the DUE value for water was 122. Both bench-mark adjuvants produced much better enhancement (NP10EO, DUE 502; aqueous acetone, DUE 926).

Table 2. Effects of emulsions and emulsifier on fluorescein retention by 10-d-old barley

Spray liquid	Mean DUE values *			
	1 g/litre	2 g/litre	10 g/litre	20 g/litre
Emulsifier	5.02 (152)	5.45 (233)	-	-
Soya EC	-	-	5.30 (201)	5.46 (235)
Methyl soyate EC	-	-	4.89 (133)	5.32 (204)
LLP EC	-	-	5.06 (158)	5.69 (295)

Water 4.80 (122); NP10EO 6.22 (502); aqueous acetone 6.83 (926).
* $\log_n$-transformed values, LSD (p=0.05) 0.55; back-transformed values in parentheses.

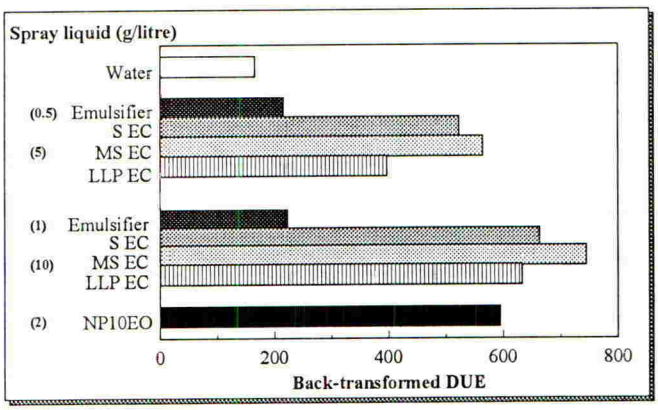


Fig. 2. Effects of emulsions and emulsifier on fluorescein retention by 15-d-old barley.

On 15-d-old plants, the three ECs at 5 and 10 g/litre also enhanced retention considerably more than the equivalent concentrations of emulsifier alone (0.5 and 1 g/litre) (Fig. 2). At 5 g/litre, the DUE values for soya, methyl soyate and LLP were 524, 566 and 398, respectively, the corresponding value for the emulsifier being 217. The performance of the ECs was similar at 10 g/litre but, at 5 g/litre, methyl soyate was superior to the other two oils. In addition, there was significantly better deposition with the LLP EC at 10 g/litre (DUE 634) than at 5 g/litre (DUE 398). When used at the highest rate, the retention-

enhancing performance of the three oil emulsions was equivalent to that of NP10EO at 2 g/litre (DUE 596).

Spray quality

Addition of either emulsions (10g/litre) or emulsifier (1g/litre) affected atomisation of the spray liquid through the hydraulic nozzle (Table 3). In all cases, droplet volume median diameters (VMDs) were increased substantially (243-259μm) in comparison with water (217μm). These changes produced corresponding decreases in the % volume in droplets < 100μm diameter, from 6.1% for water to 2.0 - 2.9%, reflected in increases in mean droplet velocities. Similar results were obtained using the oil ECs at 5-20 g/litre. On the other hand, NP10EO at 2 g/litre produced the opposite effect, droplet VMDs decreasing (195 μm) and the small droplet component increasing (10.6%), in comparison with water.

Table 3. Spray parameters for emulsions and emulsifier

Spray liquid (g/litre)	VMD* (μm)	SDC† (%)	Mean droplet velocity (m/s)
Water	217	6.1	4.1
Emulsifier (1)	254	2.0	6.4
Soya EC (10)	243	2.9	6.0
Methyl soyate EC (10)	248	2.3	5.9
LLP EC (10)	259	2.1	6.3
NP10EO (2)	195	10.6	3.4

* Volume median diameter. † Small droplet component (% volume in droplets < 100μm diameter).

DISCUSSION

The model emulsions containing either hydrocarbon (LLP), triacylglycerol (soya) or fatty acid methyl ester (methyl soyate) oils at *ca* 5-20 g/litre increased fluorescein deposition on the two difficult-to-wet species examined (Tables 1 and 2; Figs 1 and 2), confirming our earlier work on commercial oil ECs (Hall *et al.*, 1997). However, in the present investigation, the behaviour of the emulsifier was found to vary according to the size of the target. On small targets, retention efficiencies of emulsions and emulsifier were similar (*cf.* Hall *et al.*, 1997), whereas on larger plants emulsions gave superior performance to the formulant alone. Although the reasons for this difference are unclear, it should be noted that emulsifiers have been reported to be as effective as the corresponding emulsified oil in promoting the activity of several pesticides (Grayson *et al.*, 1993; de Ruiter *et al.*, 1997).

Similar effects on atomisation and resultant spray quality, namely, increased VMDs and decreased proportions of droplets with diameters <100μm in comparison with water (Table 3), to those found previously for emulsions (Quinn *et al.*. 1986; Merritt & Morrison, 1988; Butler Ellis *et al.*, 1997) and lipophilic surfactants (Holloway, 1994), i.e. the emulsifier, were recorded. Such parameters would appear to be typical of spray fluids containing finely dispersed matter, suggesting that substantial shortening of the liquid sheet occurs during atomisation (Butler Ellis *et al.*, 1997); retention efficiency would be predicted (Holloway, 1994) to be inferior to other adjuvants which decrease droplet size, such as NP10EO.

There was good agreement between spray cloud characteristics and retention efficiency on small pea and barley targets but not on bigger ones, suggesting the existence of additional physicochemical interactions between the oil, the emulsifier and the target surface, other than droplet-size. Oil composition appears to have only a minor influence on retention performance and spray quality.

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EFFECT OF LIQUID PROPERTIES ON THE POTENTIAL FOR SPRAY DRIFT FROM FLAT-FAN HYDRAULIC NOZZLES.

K U SARKER, C S PARKIN, B J WILLIAMS
Silsoe College, Cranfield University, Silsoe, Bedford, MK45 4DT

ABSTRACT

The potential for spray drift from flat-fan hydraulic nozzles was assessed using a 2 m x 1 m x 7 m wind tunnel operated at 2.63 m s^{-1} wind speed. The proportion of the spray displaced 2 m downwind was measured using line drift collectors and a fluorescence technique. Spray liquid viscosity was varied from 1.2 mPa s to 4.8 mPa s using 0 -40% v/v aqueous solutions of glycerol. Viscosity was determined using a Contraves Low Shear 30 Rheometer. Surface tension was varied from 73 mN m^{-1} to 29 mN m^{-1} using 0 - 1% v/v aqueous solutions of Agral. Dynamic surface tension was determined using the Maximum Bubble Pressure method. A Lurmark 02-F110 nozzle was operated at 300 kPa pressure and located at 0.5 m above the tunnel floor. Spray drift was found to increase from 11% to 18% as dynamic surface tension decreased. Increasing viscosity marginally increased drift. Similar trends were obtained by a study of droplet spectra.

INTRODUCTION

The measurement of spray Drift Potential using wind tunnels is now an accepted technique for assessing nozzle performance (Miller et al 1993). Test liquids are usually restricted to either to tap water or a 0.1% v/v solution of the non-ionic surfactant Agral. If these liquids are to be considered as representative of typical pesticide spray liquids, the influence on spray drift potential of a range of typical liquid properties is required. The work reported here covered these aspects. It did not seek to find ways of reducing drift by modifying liquid properties (e.g. Huddleston et al 1995)

It is well known that surface tension and viscosity influence the size of drops produced by hydraulic spray nozzles (Lefebvre 1993) and thereby influence spray drift. When solutions are sprayed the surface age of the liquid sheet influences the surface tension and atomisation characteristics. With solutions of long-chain molecules, such as surfactants, static measurements of surface tension do not reflect the characteristics of atomisation. To determine the effect of varying surface tension a series of Agral solutions were prepared, and their dynamic surface tension measured. In order to determine the effects of viscosity a series of aqueous solutions of glycerol were prepared.

Using a typical nozzle and pressure, the drift potential of these solutions was measured in a wind tunnel using a suitable wind speed. The trends observed in the wind tunnel data were confirmed by measurements of drop spectra.

METHOD AND MATERIALS

Spray Liquids

The test liquids for assessing the influence of dynamic surface tension on spray drift were 0, 0.1, 0.5 and 1 % v/v aqueous solutions of Agral. To asses the influence of viscosity 0, 10, 20, 30, and 40% v/v aqueous solutions of glycerol were used.

Surface Tension Measurement

The surface tension of the Agral solutions was measured using the Maximum Bubble Pressure method (Holloway 1994). Details of how this method was implemented were described by Williams (1992). The surface age of the solutions obtained using this method cannot approach that of atomisation but the method can represent the broad effects of surface age especially if the solute molecules are relatively mobile. A standard surface age of 500 ms was used to characterise these effects. To check for interaction, the influence of glycerol on surface tension was also determined.

Viscosity Measurement

The rheological properties of the glycerol solutions was measured using a Contraves Low Shear 30 Rheometer. Because the viscosity of glycerol solutions changes with temperature, and the drift measurements were made at ambient temperature, the influence of temperature was required. A series of relationships between temperature and viscosity were established by measurement at different temperatures. The value of viscosity at the time of spraying was calculated by interpolation. To check for interaction, the influence of Agral on viscosity was measured.

Wind Tunnel

The Silsoe College low-speed suction wind tunnel was used in the experiments (Sarker and Parkin 1995). The tunnel has a width of 2 m, is 1 m high, and 7 m long. A wind speed of 2.63 m s^{-1} was used for the tests. A single Lurmark 02-F110 spray nozzle (BCPC Code F110/0.8/3.0) was mounted on a boom 2 m downwind of the tunnel entrance and at 500 mm above the tunnel floor. The floor was covered with artificial turf to prevent drop re-entrainment and the section directly under the nozzle was fitted with a small sump to collect the majority of the spray liquid leaving the nozzle. The spray system consisted of a 10 l pressurised container delivering liquid to the boom through a solenoid valve. The solenoid valve was operated by an electronic timer which was set to provide spray periods of 10 s.

Drift Measurement

The spray was traced using a standard fluorescence technique (Merritt 1989). Spray solutions contained of 0.1% (w/v) sodium fluorescein. Airborne spray was sampled downwind of the spray nozzle using 2 mm o.d. polyethylene tubing stretched across the tunnel at 100 mm height intervals. The fraction of the spray airborne downwind of the nozzle, or Drift Potential (*Dp*),

was calculated from the dimensions of the collectors, the height intervals between collectors, the duration of the spray, the nozzle discharge, and the tracer collected on the sample.

Drop Measurement

Drop spectra were measured using a Particle Measuring Systems OAP-260X 1D laser probe. (Parkin et al 1980). The probe was fitted with a Bulk Area Rejection Circuit to remove coincidence errors. Data from the probe was stored in a Holtech 5000 Interface and analysed by software developed by Silsoe College. The nozzle was operated at the same pressure as in the wind tunnel. The nozzle was moved back and forth at 600 mm above the probe using a computer controlled x-y transporter (Sarker 1997).

RESULTS

Liquid Properties

The influence of Agral concentration on dynamic surface tension is shown on Table 1. Over a wide range of surface ages, and concentrations the influence on glycerol on dynamic surface tension was found to be negligible (70-73 mN m^{-1}) (Butler-Ellis personal communication).

Concentration of Agral % v/v	Surface Tension mN m^{-1}
0	73
0.1	50
0.5	33
1	29

Table 1. Surface Tension of Agral Solutions at 500 ms Surface Age

The influence of concentration and temperature on the viscosity of glycerol solutions is shown on Table 2. The glycerol solutions exhibited Newtonian behavior. Because Agral concentrations were limited to 1%, the solutions of Agral exhibited negligible influence on viscosity (Williams 1992).

	Viscosity mPa s				
Glycerol Concentration % v/v	Temperature °C				
	10	15	20	25	30
0	1.12	1.01	0.95	0.80	0.75
10	1.58	1.37	1.33	1.13	1.01
20	2.35	2.10	1.85	1.62	1.41
30	3.78	3.24	2.81	2.52	2.13
40	6.34	5.24	4.46	3.98	3.14

Table 2. Viscosity of solutions of Glycerol and Tap Water at varying Temperatures

Drift Measurements

Drift Potential significantly decreased with increasing dynamic surface tension as can be seen from Figure 1a. Increasing viscosity showed only a marginal increase in Drift Potential (Figure 1b). The effect was however greater than could be explained by the small decrease in dynamic surface tension that resulted from increasing glycerol concentrations.

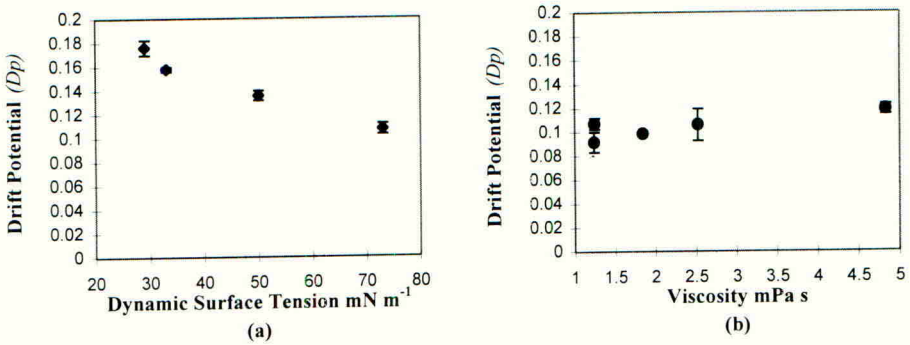

Figure 1 (a) Influence of Dynamic Surface Tension {500 ms surface age} and (b) Viscosity on Drift Potential 2m downwind from a Lurmark 02-110 flat-fan nozzle operated at 300 kPa. Error bars represent 95% Confidence Interval.

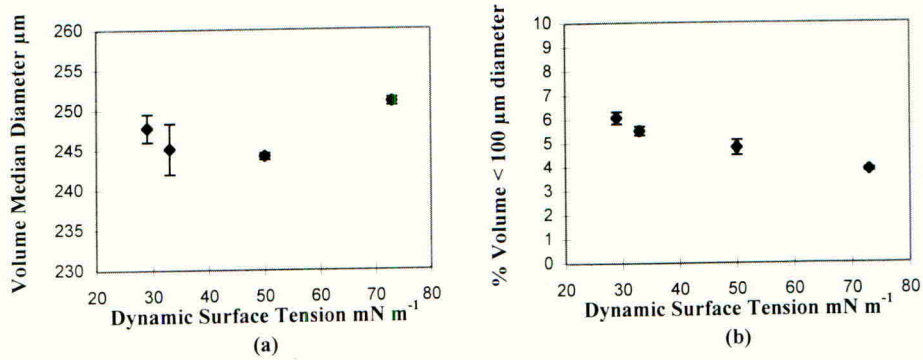

Figure 2 Influence of Dynamic Surface Tension {500 ms surface age} on (a) VMD and (b) % Volume < 100 µm diameter for a Lurmark 02-110 flat-fan nozzle operated at 300 kPa. Error bars represent 95% Confidence Interval.

Drop Spectra Measurements

The measured drop spectra confirmed the Drift Potential results. Although increasing dynamic surface tension generally increased VMD (Figure 2a) and increasing viscosity generally decreased VMD (Figure 3a), the % Volume < 100 µm Diameter followed closely the trends

exhibited by the Drift Potential results (Figures 2b and 3b). This is important since % Volume < 100 μm Diameter is often used as an indicator of potential drift (Parkin 1993).

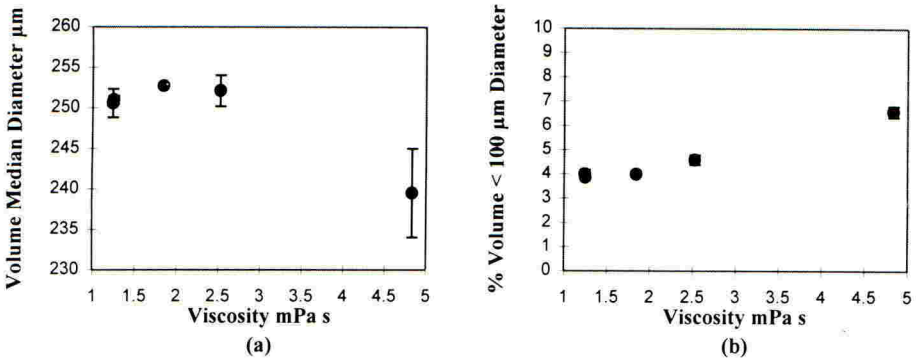

Figure 3 Influence of Viscosity on (a) VMD and (b) % Volume < 100 μm diameter for a Lurmark 02-110 flat-fan nozzle operated at 300 kPa. Error bars represent 95% Confidence Interval.

DISCUSSION

The wind tunnel results appear to be confirmed by the drop spectra results. Although some interaction was evident, with Agral solutions influencing viscosity and the glycerol solutions influencing surface tension, these effects were negligible. It appears that with low viscosity aqueous solutions, increasing surface tension decreases spray drift. Within the range of values tested, the effects of viscosity were weak but increasing viscosity increased Drift Potential. This is contrary to the evidence obtained with drift control agents (Goering & Butler 1975, Bode et al 1976, Ozkan et al 1993). It should be noted that drift control agents have a more profound effect on viscosity than glycerol and are predominantly non-Newtonian in their rheological behaviour (Yates et al 1976).

CONCLUSIONS AND FURTHER WORK

Although dynamic surface tension can significantly alter the Drift Potential from hydraulic nozzles, the use of simple test liquids to simulate the spray drift performance of nozzles applying conventional formulations appears to be valid. In this work only one nozzle at one pressure was investigated. Although conditions were typical of field operations, it is possible that complex nozzle designs which include such features as secondary chambers (Castell 1993) and air-entrainment might produce effects which could invalidate the use of simple test liquids such as water.

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EMULSIONS AND THEIR EFFECT ON SPRAY FORMATION AND DROPLET SIZE WITH AGRICULTURAL FLAT FAN NOZZLES

M C BUTLER ELLIS, C R TUCK, P C H MILLER
Silsoe Research Institute, Wrest Park, Silsoe, Beds, MK45 4HS, UK

ABSTRACT

Measurements were made of the spray droplet size produced when spraying dilute emulsions through a flat fan nozzle at a range of concentrations and photographs of spray formation were taken. It was found that all the emulsions used caused break-up through perforation of the liquid sheet resulting in larger spray droplets than when spraying water alone. Although the number of perforations increased with increasing concentration, and consequently changed the length of the liquid sheet, there was no effect on spray droplet size.

INTRODUCTION

In any pesticide application, spray droplet size and velocity are crucial factors in ensuring maximum efficiency through their influence on off-target contamination and efficacy of the active ingredient. Work carried out by many authors, reviewed by Miller and Butler Ellis (1997) has shown that liquid properties can have a significant effect on droplet size in sprays generated by hydraulic pressure nozzles. Thus the addition of pesticide formulations and adjuvants will alter the droplet size spectrum and can therefore have consequences for efficacy and for spray drift.

Spray liquids have been shown to influence spray formation by flat fan nozzles (Dombrowski and Fraser, 1954, Butler Ellis et al, 1997) by affecting the break-up of the liquid sheet immediately downstream of the nozzle. In particular, spray liquids which are emulsions have been shown to cause perforations in the liquid sheet, leading to earlier sheet break-up and larger spray droplets with higher velocities. Although the occurrence of perforations with a wide range of spray liquids is well established, the mechanism which causes them is not well understood.

This paper investigates in more detail the phenomenon of break-up by perforation by studying the extent to which the concentration of the emulsifiable liquid affects sheet break-up and spray droplet size. Measurements of spray droplet sizes were made and photographs of liquid sheet break-up were taken with a spray liquid containing different concentrations of a blank emulsifiable concentrate or one of three oil-based adjuvants. Previous measurements (Butler Ellis and Tuck, 1997) over a limited concentration range with a PMS imaging probe suggested that there was no effect of concentration on droplet size. The use of Phase Doppler analysis for measurement of droplet size improves the resolution between liquids. Further experiments were therefore made using both PMS and PDA with sprays containing emulsifiable liquids at a wider range of concentrations than previously used. Measurements were also made of size distribution of emulsion droplets of the spray liquids.

MATERIALS AND METHODS

Earlier work has shown that similar effects of liquids properties on spray formation were obtained with a range of flat fan nozzles (Butler Ellis et al,1997) and therefore only one nozzle was used for these investigations, designated F110/0.8/3 (Lurmark) at pressures of 1 bar and 2.5 bar. In the work reported by Butler Ellis and Tuck (1997), concentrations of blank e.c of 0.0625%, 0.125% and 0.25% were used. In this work, additional measurements of 1% e.c. were made with both instruments to enhance any effect of concentration. Three emulsion-forming adjuvants were also used at two or three concentrations.

Two different instruments were used to measure spray droplet size, a PMS imaging probe (Particle Measuring Systems, Boulder, Colorado) and a phase Doppler analyser, or PDA (Dantec, Bristol, UK). Measurements were made 350mm below the nozzle by carrying out a full two-dimensional scan, as described in Tuck et al (1997). Two photographs were taken of the liquid sheet at each pressure for each liquid as described in Butler Ellis et al (1997) and estimates of sheet length were made for each photograph.

A Malvern Particle Sizer 2600 series was used with a flow-through cell and dispersion unit (Malvern Instruments, Malvern, Worcs., UK) to measure the size distribution of emulsion droplets in the spray liquid. This system pumps the emulsion through the flow cell and has a built in stirrer. The 63mm lens was used to measure droplets between 1.2 and 118μm. The maximum concentration which could be measured by the instrument depended on the emulsion droplet size, but was of the order of 0.05%. This was significantly less than the 1% at which pesticides and oil adjuvants are commonly used and so at these concentrations, further dilutions were necessary in order to make the measurements.

RESULTS AND DISCUSSION

Volume Median Diameters (VMD) of spray droplets, measured with both PMS and PDA instruments, are shown in Tables 1 and 2. The PDA was unable to measure the blank e.c. or Codacide at a concentration of 1% and so only PMS data has been included at this concentration. Concentration had no effect on spray droplet size.

All the photographs confirm that emulsions are associated with perforation of the liquid sheet. The number and distance of the perforations from the nozzle varies with liquid. Clear differences in the appearance of the liquid sheet can be seen between some of the spray liquids, in terms of the number, size and position of the perforations. Examples are shown for two concentrations of two of the liquids at 1.0 bar in Figs 1-4. There was a clear effect of concentration upon the length of the liquid sheet , also shown in Fig. 5, although it is difficult to be conclusive without taking large numbers of photographs. A large change in concentration is necessary to produce a visible change in sheet length. For example, a twenty-fold increase in concentration only causes a 10-30% reduction in sheet length.

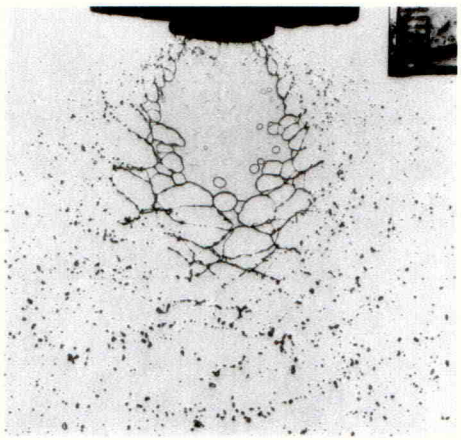

Fig 1. 0.05% Codacide

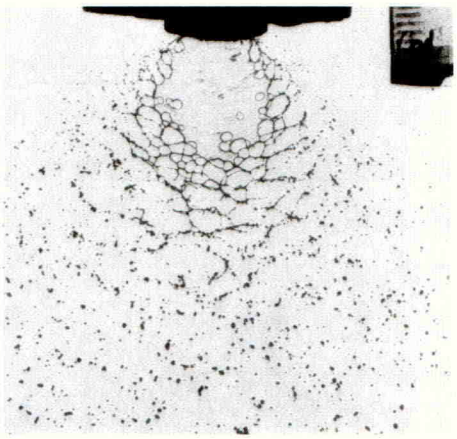

Fig 2. 1% Codacide.

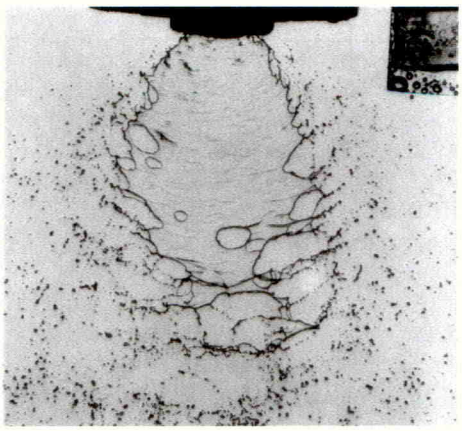

Fig 3. 0.05% Actipron.

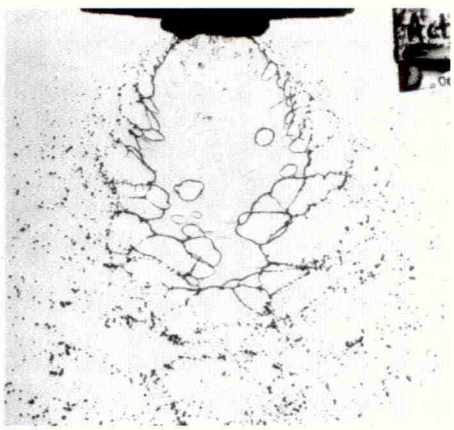

Fig 4. 1% Actipron.

Table 1. Variation of VMD with concentration of blank e.c. F110/0.8/3 nozzle at 2.5 bar

	PDA		PMS	
Liquid	VMD	standard deviation	VMD	standard deviation
water	216	1.9	266	1.7
0.0625% e.c.	234	3.4	278	1.1
0.125% e.c.	233	2.5	277	0.9
0.25% e.c.	237	8.2	273	0.8
1% e.c.	-	-	278	1.4

Table 2. Variation of VMD with concentration for 3 adjuvants. F110/0.8/3 nozzle at 2.5 bar

	PDA		PMS	
Liquid	VMD	Standard deviation	VMD	Standard deviation
water	217	1.8	245	1.3
0.03% Codacide	233	3.8	255	1.5
1% Codacide	-	-	255	1.1
0.05% Actipron	225	2.1	250	0.7
1% Actipron	226	2.0	251	1.3
0.05% LI-700	242	4.2	253	1.7
0.1% LI-700	243	7.1	251	2.8
0.5% LI-700	238	5.7	251	3.2

The length of the liquid sheet is virtually independent of pressure (Fig 5), despite the difference in sheet velocity and liquid flow rate. This means that the age of the sheet at break-up and the thickness of the sheet at break-up will vary as pressure varies. The hypothesis originally put forward by Dombrowski and Fraser (1954) and more recently by Butler Ellis and Tuck (1997), that perforations occur when the diameter of the emulsion droplets and the thickness of the liquid sheet are the same, seems largely unfounded.

There is some indication, when comparing different liquids, that a reduction in the length of the liquid sheet is associated with an increase in spray VMD. For example, there is a correlation coefficient of -0.97 between sheet length and spray VMD for water and each of the four spray liquids at their lowest concentration. However, with a single liquid, reducing the length of the liquid sheet by increasing the concentration is not associated with any change in spray VMD (Tables 2 and 3).

The measurements of emulsion size showed some day-to-day variability, but typical values of emulsion droplet VMD suggest that the larger the emulsion droplet, the longer the liquid sheet (Table 3), contrary to the hypothesis suggested in Butler Ellis and Tuck (1997). It is possible that emulsion droplet size does not directly influence sheet break-up, but some other factor (such as low interfacial tension) is responsible for both early sheet perforation and small emulsion droplets. Dombrowski and Fraser (1954) suggested that wettability of particles determined their ability to cause perforations and that perforations were caused by non-wettable particles. High wettability is associated with low interfacial tension, and a liquid

which spontaneously emulsifies has a low interfacial tension and is therefore wettable. All the emulsion particles in this study would therefore be considered to be wettable, but there may be some differences in their interfacial tension.

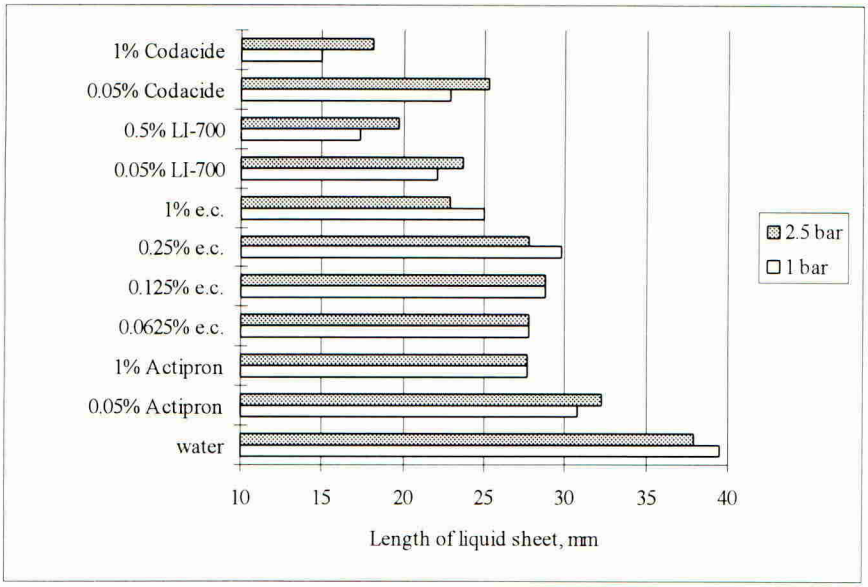

Fig. 5 The variation of liquid sheet length with liquid, concentration and pressure.

Table 3. Emulsion droplet VMD and liquid sheet length for the F110/0.8/3.0 nozzle at two pressures for the four spray liquids

Liquid	emulsion droplet VMD, μm	Sheet length, mm	
		1 bar	2.5 bar
1% Codacide	4.9	15.01	18.17
1% Actipron	11.1	27.65	27.65
0.5% LI-700	2.9	17.38	19.75
1% blank e.c.	21.6	25.02	22.94

There appear to be at least two mechanisms affecting the length of the liquid sheet: one which is associated with different liquids, which might be related to the size of the emulsion droplets, and one which is associated with increasing concentration of emulsifiable liquid. The former results in a change in spray VMD, whereas the latter does not. Increasing the concentration of emulsifiable liquid will also affect bulk liquid properties, such as surface tension, viscosity etc. It is possible that, although reducing the liquid sheet would normally result in larger spray droplets, the change in other liquid properties might counteract this increase.

It seems more probable that the properties of an individual emulsion droplet are responsible for determining its ability to cause perforations. These may be droplet viscosity, or interfacial properties such as interfacial tension, viscosity or elasticity.

CONCLUSIONS

The formation of spray by a flat fan nozzle, when spraying the emulsions used in this work, was through perforation of the liquid sheet. This increased the droplet size above that for water alone. The number and position of perforations was dependent both on the liquid and its concentration. Spray droplet size also varied with spray liquid but was independent of the concentration.

There is an indication, however, for the four liquids tested in this work, that the different emulsion droplet sizes may be associated with different sheet lengths and consequently different spray droplet sizes. There is no link between sheet length and spray droplet size for different concentrations of the same liquid. It is hypothesised that the important physical properties which determine break-up and spray droplet size are most likely to be those related to individual emulsion droplets rather than bulk liquid properties.

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ASSESSMENT OF ACTUAL PESTICIDE HAZARD TO THE APPLICATORS

A I POTAPOV, V N RAKITSKY, A V ILNITSKAYA, L I LIPKINA, T V YUDINA
FF Erisman Moscow Research Institute of Hygiene Moscow Region Semashko, 2, Mytishchy, 141000, RF

ABSTRACT

The paper discloses basic approaches to evaluation of hazardous effects of pesticides on agricultural workers. The authors introduce an original method of actual assessment of hazard from pesticides for workers and formula of cumulative toxicity levels evaluation.

Screening of over 50 pesticides of different chemical groups and types (like herbicides, insecticides, fungicides, etc.) proves that the biggest hazard results from direct dermal exposure.

INTRODUCTION

In the present of the art of toxicology of plant protection chemicals, the development of methods of assessing actual hazard (risk) of pesticides to the applicators happens to be a vital issue. Among these are filling attendants, tractor drivers, pilots, sowers, etc. The importance of the problem resides in the fact that the effectiveness and feasibility of prevention to mitigate negative impact of biologically hyperactive compounds on the human body must depend on the appropriate assessment of the actual hazard of pesticides to the applicators.

The most common currently used models of health risk assessment of applicators exposed to pesticides are those developed by the German and British researchers (Westphal *et al.*, 1993, Martin, 1990)

These models make it possible to define the relationship between the predictive cumulative value of inhalation, dermal and oral exposure and the safe level determined as a result of subacute toxicity experiment in test animals.

DEVELOPMENT OF MODEL

This presentation deals with the original model of actual assessment of hazard (risk) of pesticides to the applicators on the basis of long-standing experience gained by the Russian toxicologists and hygienists.

The following scientific approaches were used for the development of the Russian model of assessing actual pesticide hazard to the applicators.

Firstly, it is the concept of different assessment of actual hazard of pesticides to humans (Rakitskiy, 1981) which is used to determine the relationship between the maximum actual amount of xenobiotic substances that might be ingested by the human body in real life situations, and the degree of the observed disruption of the body's compensatory capacity, including the environmental effects and the violation of health-based regulations (standards).

Secondly, it is the experience gained in the course of many years of health-based regulation of xenobiotic substances, including pesticides, detected in the occupational air and as a result of human dermal exposure (Ygorov, 1978, Sanotsky *et al.*, 1975, Kondrashov, 1993). Maximum allowable concentrations (MAC) for occupational air and dermal exposure were developed for hundreds of xenobiotic substances. This, in its turn, made it possible to develop reliable models to compute preliminary safe exposure levels of xenobiotics in the occupational air (PSEL o.a., mg/m^3) and preliminary allowable levels of dermal exposure (PAL d.e., mg/m^3) on the basis of toxic parameters found in acute and subacute toxicity experiments in test animals. In recent decades, a high degree of reliability was proved both by epidemiological studies and prolonged chronic tests in long-term exposure studies.

The basis of the proposed model of assessment of actual pesticide hazard (risk) to the applicators centres on the following. Actual inhalation hazard is determined by the relation between the observed pesticides concentration in the occupational air (I o.a., mg/m^3) and MAC or PSEL in the occupational air (mg/m^3). In order to evaluate the actual hazard of the dermal route of pesticide exposure, it has been proposed that a tentative permissible level of skin contamination (TPL sc), expressed in mg/cm^2, should be calculated. This level is determined for the entire skin surface. The above approach is based both on the data that has been published in literature and on our own data, which suggests that not only open skin areas, but also skin areas covered (protected) with clothing may be affected by pesticides. The penetration of an active substance into the human organism may occur both through a direct contact with the skin(e.g. when droplets or solid particles get onto it or when there is a contact with contaminated equipment, protective clothing, respirator, etc.) and as a result of the absorption by the skin of gases, vapours and aerosols from the air.

Vapours and aerosols may get under clothing relatively easily and be absorbed by the skin, this being reflected in the documents for the prompt justification of maximum permissible level of skin contamination with harmful substances, developed in Russia.

Therefore, the TPL^{SC} represents a kind of hygenic regulation which reflects not only the norm of permissible contact skin contamination but also that of permissible pesticide absorption, i.e. it effectively approaches the exposure tests used in practice when a new substance is massively applied. The TPL^{SC} calculation is performed using a calculated tentative quantity LIMcCH (mg/kg) for the index of acute skin toxicity DL50C (mg/kg) as follows:

$$TPL^{SC}\ (mg/cm^2) = \frac{Lim^{C}_{CH}\ mg/kg \times M \times K_{RES} \times K_{REL\ PENET}}{S \times Kr}$$

Where: Lim^{c}_{ch} (mg/kg) is the threshold dose, which is either found experimentally (in a chronic experiment) or calculated using $LD_{50}{}^{c}$ value;

M - the human body mass, assumed, on average, to equal> 70 kg;

Kres - the residual factor which expresses the exposure ratio of the amount of the substance that penetrated the skin to that of the substance that remained on skin, this ratio equalling, on average, 0.25;

S - the human skin total area which, on average, is equal to 16120 cm^2;

Krel.penet - the coefficient of relative penetration (or relative penetrability), found experimentally or estimated tentatively as equal to 2;

Kr - the reserve coefficient, assumed to be between 20 and 10 for substances of the 1st and 2nd classes of hazardness (with respect to skin exposure), between 10 and 3 for substances of classes 3 and 4, and over 20 for substances showing delayed and sensitizing effects.

Lim^{C}_{CH} (mg/kg) for 1st and 2nd class of hazard substances is calculated using the following formula:

$$Lim^{C}_{CH}\ mg/kg = 0.0002 \times LD_{50}{}^{C},\ mg/kg$$

For 3rd and 4th class of hazard substances, it is given by

$$Lim^{C}_{CH}\ mg/kg = 0.001 \times LD_{50}{}^{C}\ mg/kg$$

The actual skin exposure, (Da mg/cm) is found from the average dermal exposure, (Dav mg/cm^2) based on wash-off from covered and open skin areas, taking account of the operator's actual performance (treated area) and the daily norm of substance application. The actual skin exposure (Da) is given by:

$$Da\ mg/cm^2 = \frac{Dav\ mg/cm^2 \times F}{F_1}$$

Where:

F - the daily norm (rate) of application (ha) or the daily norm of seed dressing, (tones);

F_1 - the actual area of application (ha) or the actual amount of seeds that have been dressed.

MODEL EXAMPLES

As an example, we demonstrate the calculation of actual hazard of pesticides to the applicators under application of different types of pesticides.

1. Application of fungicide on wheat:

   the rate of application - 0.33 kg/ha (or 0.1124 kg a.i./ha);
   the treated area - 2 ha;
   the daily norm (rate) of application - 50 ha;
   the average content of active ingredient on skin (an average dermal exposure), Dav - 0.00000096 mg/cm^2;
   the actual skin exposure (with taking into account the operator's actual performance and daily
   norm of fungicide application), Da - 0.000024 mg/cm^2.

   Therefore, the tenative permissible level of skin contamination (TPLsc) (under LD_{50}^{C} = 4000 mg/kg make up:

$$TPLsc\ (mg/cm^2) = \frac{0.001 \times 4000 \times 70 \times 0.25 \times 2 = 0.00043\ mg/cm^2}{16120}$$

   The hazard of influence up on skin is determined by the ratio between Da and TPLsc, which is equal 0.0558 (the permissible value < 1)

2. Application of herbicide on maize:

   the rate of application - 2.0 l/ha (or 1.8 kg a.i./ha);
   the treated area - 25 ha;
   the average dermal exposure, Dav - 0.000095 mg/cm^2;
   the actual skin exposure, Da - 0.00019 mg/cm^2
   TPLsc - 0.00009 mg/cm^2

   The operator's dermal hazard - 2.1 (the permissible value <1).

   The average concentration of active ingredient in the occupational air (Io.a) -0.008 mg/m^3.

   The inhalation hazard determined as a ratio between Io.a. and MAC o.a. (0.5 mg/cm^3) make up: 0.016 (the permissible value <1).

   Application of insecticide on barley:
   the application rate - 1.4 l/ha (or 0.57 kg a.i./ha);
   the treated area - 5 ha;
   the daily norm (rate) of application - 50 ha;
   the average dermal exposure, Dav - 0.0000007 mg/cm^2
   the actual skin exposure, Da - 0.000007 mg/cm^2;

TPLsc - 0.0000092 mg/cm^2

The operator's dermal hazard - 0.76 (the permissible value <1).

The average concentration of active ingredient in the occupational air, Io.a. - 0.025 mg/m^3;

The inhalation hazard determined as a ratio between Io.a. and MACo.a. (0.5 mg/m^3) - 0.05 (the permissible value <1).

CONCLUSION

As you can see demonstrated by the examples the distinguishing feature of suggested risk assessment model is a calculation of corresponding safety factors (SF) which are determined by a ratio of the actual levels of active ingredient exposure and MAC or PSEL. The method of risk assessment (real danger) provides for obtaining the information which is intended for developing preventive and toughening control measures for pesticides usage, as well as for establishing the main routes of dangerous substances entering in the human body. Safety factors in this case should be below 1. The given relationships are used at all stages of pesticide application technology (including such procedures as filling-up, spraying, etc.) as well as under extreme conditions (maximum application rate and so on).

Total pesticide inhalation and dermal exposure for the applicators is determined according to the formula of cumulative toxicity given below:

$$\frac{Io.a.}{MAC_{O.A.}} + \frac{Dr}{MAC_{D.E.}} < 1$$

As far as the applicators are concerned, many years of field and pilot studies have demonstrated that the relative contribution of oral ingestion of pesticides is rather insignificant and happens to be the consequence of non-compliance with safety rules. In this connection the model in question disregards a potential hazard of oral exposure.

The assessment of actual hazard associated with over 50 pesticides of different uses and chemical classes (herbicides, insecticides, fungicides, disinfectants, etc.) conducted by means of the given model has shown that for the majority of such products, the most serious health hazard for the applicators is associated with dermal exposure, being consistent with the results of using the German or British model. At the same time a comparative analysis of assessing actual pesticide hazard to the applicators made by running the Russian, German and British models demonstrated contradictory findings related to the allowable risk.

So further improvement of methods of assessing actual pesticide hazard (risk) to the applicators stemming from the Russian, German and British experience will contribute to the harmonization of international requirements in this area and soundly substantiate a complex of preventive measures to mitigate unfavorable pesticide effects produced on health of the applicators.

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SESSION 7A

NEW CHALLENGES FOR HERBICIDE DISCOVERY AND SELECTIVITY

Chairman and Session Organiser	DR K E PALLETT *Rhône-Poulenc Agriculture Limited, Ongar, UK*
Papers	7A-1 to 7A-5

HERBICIDE TARGET SITES, RECENT TRENDS AND NEW CHALLENGES

K E PALLETT
Rhône-Poulenc Agriculture Limited, Fyfield Road, Ongar, Essex CM5 0HW, UK

ABSTRACT

One of the key challenges ahead for research scientists involved in herbicide discovery is the search for new molecules possessing novel modes of action. Recent trends in new molecule announcements show two target sites, namely acetolactate synthase and protoporphyrinogen oxidase accounting for two-thirds of 51 new molecules announced since 1991. Only two new modes of action have been definedfor commercialised herbicides since 1992, namely, hydroxyphenylpyruvate dioxygenase and auxin transport inhibition.

The incorporation of *in vitro* high throughput screening (HTS) provides a further challenge in identifying the target sites for such tests. The choice between established and novel unproven target sites is briefly discussed.

INTRODUCTION

The objective of this session at the 1997 BCPC - Weeds conference is to identify the challenges ahead for research scientists involved in herbicide discovery.

These challenges fall into two key areas:

i to discover new molecules with commercial potential as herbicides, which possess novel modes of action;

ii to discover new molecules with an agronomically effective crop selectivity.

This short paper will briefly review recent trends in new herbicide mode of action and summarise some of the present challenges. Others in the session will cover recent trends and the challenges ahead with respect to herbicide selectivity and our ability to move towards "design" of crop selective herbicides (Barrett, 1997; Hallahan *et al*, 1997; Tecle *et al*, 1997). One further paper will address the role of natural products as sources of leads for new herbicides and as useful tools to identify lethal target sites for future herbicides (Duke *et al*, 1997).

WHY NEW HERBICIDE MODES OF ACTION?

Herbicide mode of action has taken on a new interest in recent years due to the incorporation of high throughput screening (HTS) strategies in herbicide discovery. Furthermore, the development of resistance in weeds due to altered target site has led to management recommendations to use herbicides of differing modes of action in rotation (Shaner, 1995).

New modes of action for future molecules will greatly assist the management of weed resistance and maintain diversity and complementarity for product mixtures. Identification of the target site also enables modification of crop tolerance to herbicides through recombinant techniques.

HTS procedures in agrochemical discovery has been the subject of a number of symposia in recent years. A major issue for *in vitro* screening is the choice of target sites for HTS.

There is a choice between two alternatives:

i established target site for existing herbicides, or
ii novel targets

ESTABLISHED HERBICIDE TARGET SITES

The benefit of choosing a proven target is that its inhibition is known to be associated with lethality. Should any *in vitro* inhibitor possess all the necessary features to be transported to the target site after application to foliage or soil, then it will be herbicidal.

Table 1 lists the target sites of herbicides based on Pillmoor *et al* (1995). Since this review, which was based on the Ninth Edition of the Pesticide Manual (Worthing and Hance, 1991) new molecule announcements have led to only one further defined target site (Table 2). Therefore, if one considers the desire for lower and lower application rate herbicides preferably acting on plant-specific processes, the actual number of known targets is less than those identified in Tables 1 and 2. Furthermore, the significant development of resistance at the level of the target site may reduce the choice of target for HTS further (Table 1).

Table 1 The defined herbicide targets.

Target	Plant specific	Target site resistance reported
acetolactate synthase (ALS)	Yes	Yes
glutamine synthetase	No	No
enoylpyruvyl shikimate phosphate synthase	Yes	No
acetyl CoA carboxylase	Yes	Yes
non-specific chloroacetanilide target	?	?
protoporphyrinogen oxidase (protox)	No	No
phytoene desaturase	Yes	No
lycopene cyclase	Yes	No
hydroxyphenylpyruvate dioxygenase	No	No
photosystem II	Yes	Yes
photosystem I	Yes	No
oxidative phosphorylation	No	No
cellulose biosynthesis	Yes	No
folate biosynthesis	Yes	No
auxin mimics	Yes	No
microtubule assembly and function	No	Yes

No attempt has been made to assign any target site to a herbicide defined as having unknown modes of action.

However, such techniques require significant investments of time and resources to identify a single potential target (Akers, 1996).

The data presented in Table 2 reflect a recent trend in herbicide discovery, namely that two target sites are dominating the modes of action of new herbicides. The exploitation of the sulphonylurea family is the major factor contributing to the numbers of new ALS inhibitors. Whereas, the susceptibility of protox to a variety of chemical classes of inhibitors has led to 15 new molecule announcements in the last five years.

Table 2. The target site of 51 compounds announced since the publication of the Ninth Edition of Pesticide Manual (Worthing and Hance, 1991).

Target Site	Numbers
Acetolactate synthase	19
protoporphyrinogen oxidase	15
non-specific chloroacetanilide target	4
acetyl-CoA carboxylase	2
hydroxyphenylpyruvate dioxygenase	2
cell division (not defined)	2
photosystem II	2
phytoene desaturase	1
microtubule assembly and function	1
auxin transport inhibitor	1
unknown or not stated	2
Total	**51**

NOVEL HERBICIDE TARGETS

Empirical screening of tens of thousands of molecules over the years has selected the 17 known target sites identified above. Current genome analysis of *Arabidopsis thaliana* indicates the presence of over 10,000 genes, which in theory are potential herbicide targets. The challenge is to identify if any of these genes possesses the characteristics necessary to be an effective herbicide target, i.e. inhibition of which causes:

cessation of plant growth and/or
rate limiting metabolic step, and/or
accumulation of toxic intermediates.

Several attempts have been made to select novel targets and to either design or screen for herbicides (see Lead Inspiration in Pillmoor *et* al, 1995).

Problems with this approach are exemplified with acetohydroxy acid isomeroreductase, the enzyme which follows ALS in the branched chain amino acid pathway. Design and screening approaches have led to the discovery of two novel inhibitors (IpOHA and HOE704) but neither have been commercialised as herbicides. Although mechanistic reasons have been proposed for the lack of herbicidal activity (Dumas, *et al*, 1995).

Molecular biology can provide tools to search for new targets. Reverse genetics or antisense techniques can reduce gene expression and 'mimic' herbicidal inhibition (Foster and Höfgen, 1993). However, this does necessitate the availability of the gene or cDNA of the target. Identification and characterisation of mutants showing lethal phenotypes also offers potential for selectivity novel targets.

Alternatively, active searching for the targets of *in vivo* herbicide compounds or natural products (see Duke *et al*, 1997) will also be a valuable approach in the search for novel targets.

CONCLUSION

In recent years traditional herbicide discovery approaches have resulted in the domination of herbicide mode of action by ALS and protox. The incorporation of HTS procedures which will involve *in vitro* screening has led to interest in new targets. These will arise from a combination of conventional biochemical approaches to *in vivo* active molecules (herbicidal or natural products), molecular biology and 'lead inspiration'.

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NATURAL PRODUCTS AS LEADS FOR NEW HERBICIDE MODES OF ACTION

S O DUKE, F E DAYAN, A HERNANDEZ
United States Department of Agriculture, Agricultural Research Service, Natural Products Utilization Research Unit, P. O. Box 8048, University, Mississippi 38677, USA

M V DUKE, H K ABBAS
United States Department of Agriculture, Agricultural Research Service, Southern Weed Science Laboratory, P. O. Box 350, Stoneville, Mississippi 38776 USA

ABSTRACT

A major objective of the discovery effort of the herbicide industry is to find new molecular target sites. One strategy for discovery of new molecular targets is to determine those for naturally-occurring phytotoxins. The relatively few molecular targets of natural products that are known are almost all different from those of commercial herbicides. We describe the novel sites of action and novel mechanisms of action of some of the compounds with which we have worked. These include AAL-toxin, artemisinin, australifungin, cornexistin, 1,8-cineole, cyperin, and hydantocidin. Each of these compounds appears to have a mechanism of action that is very different from that of any commercial herbicide.

RATIONALE

New herbicide modes of action are sought by the pesticide industry for several reasons. Novel modes of action usually offer additional tools to combat the evolution of resistance to currently available herbicides and alternatives to herbicides to which resistance has already evolved. Furthermore, new modes of action often translate into different properties and attributes in the field, potentially creating an addtional market niche. Theoretically, proprietary rights could be centered around a new molecular site of action. Sometimes molecular target sites that are exclusive to plants can support claims of toxicological safety, although this is often a disingenuous argument.

Biorational discovery programs can be oriented around newly discovered molecular target sites. Considerable effort is now going into discovery of new molecular target sites, in some cases independently of a herbicide screen. Such strategies can involve antisense molecular biology and site-directed mutagenesis. Another approach is to explore the mechanisms of action of natural phytotoxins.

Naturally occurring compounds offer not only leads for new herbicide chemical classes, but have also been useful in identifying new potential sites of action for herbicides. There is very little overlap between the known molecular target sites of natural phytotoxins and those of commercial or soon to be commercialized synthetic herbicides (Table 1). Of the fifteen or so molecular target sites of commercial herbicides, only that of glufosinate (the synthetic

Table 1. Summary of the known molecular modes of action of herbicides and natural phytotoxins (Adapted in part from Duke *et al.*, 1996a.)

Physiological site	Molecular site	Synthetic or natural phytotoxin[a]
Amino acid synthesis		
aromatic amino acids	EPSP synthase	glyphosate
branched chain amino acids	acetolactate synthase	imidazolinones, sulfonylureas, others
glutamine synthesis	glutamine synthetase	*many, including phosphinothricin, oxetin*
glutamate synthesis	aspartate amino transferase	*gostatin*
general amino acid synthesis	many transaminases	*gabaculin*
ornithine synthesis	ornithine carbamoyl transferase	*phaseolotoxin*
methionine synthesis	β-cystathionase	*rhizobitoxin*
Photosynthesis		
electron transport	D-1, quinone-binding protein	many, including triazines, substituted ureas.; *sorgoleone, cyanobacterin*
photophosphorylation	CF_1 ATPase	*tentoxin*
electron transport diverters	photosystem I	bipyridiliums (*e.g.*, paraquat)
plastoquinone synthesis	4-hydroxyphenylpyruvate dioxygenase	isoxazoles, pyrazoles, and triketones
Pigment synthesis		
porphyrins	protoporphyrinogen oxidase	diphenyl ethers, oxadiazoles, *etc.*
	ALA synthase	*gabaculin*
carotenoids	phytoene desaturase	many, including pyridazinones, *etc.*
	a prenyl transferase	isoxazolidinones
Cell division		
mitotic disruptors	β-tubulin	dinitroanilines, phosphoric amides; V*inca alkaloids, colchicine, etc.*
Vitamin synthesis		
folate synthesis	dihydropteroate synthase	asulam
Lipid synthesis		
	acetyl-CoA carboxylase	aryloxyphenoxypropanoates, cyclohexanediones
	acetyl-CoA transacylase	*thiolactomycin*
	3-oxoacyl-ACP synthase	*cerulenin*
	ceramide synthase	*AAL-toxin and analogues*
Nucleic acid synthesis		
plastid nucleic acid synthesis	RNA polymerase	*tagetitoxin*
	adenylosuccinate synthase	*hydantocidin*
	AMP deaminase	*carbocyclic coformycin*
Plasma membrane function		
	H^+-ATPase	*syringomycin*
Cell wall synthesis		
	cellulose synthesis?	dichlobenil
	unknown site	isoxaben

[a] compounds in italics are natural products

version of the microbial product phosphinothricin) is the result of natural product research(Lydon & Duke, In press). We know relatively few molecular sites of action of natural phytotoxins, yet few of them have sites of action common with commercial herbicides. This may be partly due to some of them having toxicologically undesirable target sites. Several examples of this are given below. However, most natural products appear to have environmentally desirable properties due to their relatively short half-life, compared to synthetic pesticides.

The topic of natural products as new molecular target site leads has been partially discussed in several previous reviews (*e.g.*, Duke, 1986a, b, Duke *et al.*, 1996a), although only one previous review has dealt with the topic in detail (Duke *et al.*, 1996b). Natural products as herbicides have been twice before discussed at this conference (Poole & Chrystal, 1985, Duke *et al.,* 1991). This short review will update the topic and provide some perspective. We also mention several natural products that appear to have novel mechanisms of action, although their exact molecular site is not yet established.

EXAMPLES

Microbial products

Microbially-generated compounds have been the most lucrative source of novel phytotoxins with new sites of action. Most of these have been from non-pathogenic soil or saprophytic microbes. The most successful of these has been phosphinothricin, which is now produced as the synthetic herbicide glufosinate (Lydon & Duke, In press). Clearly, plant pathogens should be a good source of such compounds (Strobel *et al.*, 1991), but these organisms are much more difficult to isolate and culture and have received relatively little attention. We have worked with compounds from both types of microbes.

AAL-toxin and australifungin

Until recently, AAL-toxin (Figure 1) was considered to be a host-specific phytotoxin produced by *Alternaria alternata* pathovars that infect tomato. Its mode of action was reported to be inhibition of RNA synthesis. Actually, this analogue of fumonisin is not host-specific at all (Abbas *et al.*, 1993, 1995b). It is active at submicromolar levels (Abbas *et al.*, 1995b). The molecular site of action is the plant equivalent of ceramide synthase (Abbas, *et al.*, 1994; Abbas *et al.*, 1995a), the same site of action of fumonisin in animals and plants. AAL-toxin is an analogue of the sphingoid base substrates (sphinganine and phytosphinogosine) of this enzyme and is presumably a competitive inhibitor. Inhibition causes rapid accumulation of phytotoxic levels of these substrates. The symptoms caused by application of the substrates, which are almost undetectable in untreated tissues, are the same as those caused by AAL-toxin or fumonisin (Tanaka *et al.*, 1993). AAL-toxin is more toxic to plants than animals, whereas fumonisin is less active as a plant phytotoxin, but more active against animals. Australifungin (Figure 1) is a structurally unrelated ceramide synthase inhibitor. It is active against both plants and animals (Abbas *et al.*, 1997). Clearly, this work demonstrates that ceramide synthase is an excellent herbicide target site, provided a plant-specific inhibitor could be found. Work with a series of AAL-toxin analogues (Abbas *et al.*, 1995c) has not yielded such a compound.

Cornexistin

Cornexistin (Figure 1) is a nonadride phytotoxin from the coprophyllic basidiomycete *Paecilomyces variotii.* It has good herbicidal activity against monocotyledonous and dicotyledonous weeds, but maize is resistant to it (Nakajima *et al.*, 1989). Its mode of action appears to be that of a proherbicide, being metabolized to an inhibitor of a least one isozyme of aspartate amino transferase (AAT) (Amagasa *et al.*, 1994). This view is supported by the finding that the protective effects obtained by certain chemical treatments also reverse the effects of aminooxyacetic acid, a well known AAT inhibitor. Cornexistin has no *in vitro* effect on ATT, however, upon incubation in a cell-free plant extract, an AAT inhibitor is generated. A hypothesized metabolite of cornexistin is an analogue of gostatin, a known ATT inhibitor from a microbial source (Nishima & Murao, 1983).

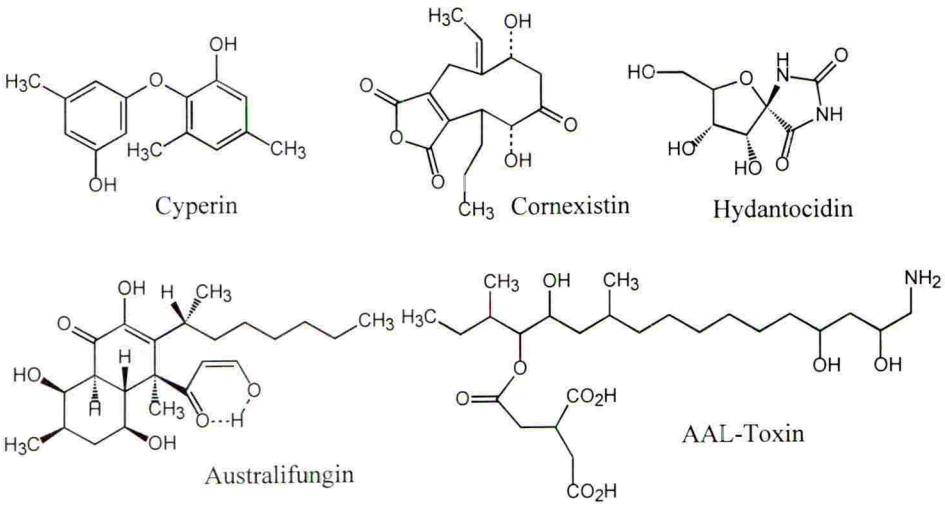


Figure 1. Structures of microbial compounds mentioned in the text

Cyperin

Cyperin (Figure 1) is a naturally-occurring diphenyl ether produced by *Ascochyta cypericola*, a fungal pathogen of purple nutsedge (*Cyperus rotundus*) (Stierle *et al.*, 1991), by *Preussia fleischhakii*, a coprophilous fungus (Weber & Gloer, 1988), and by *Phoma sorghina,* a pathogen of pokeweed (*Phytolacca americana*) (Venkatasubbaiah *et al.*, 1992). Cyperin is a moderately active growth inhibitor and membrane disrupter (Harrington *et al.*, 1995). It causes loss of chlorophyll in the light and the dark (Duke *et al.*, unpublished). However, it is a weak inhibitor of protoporphyrinogen oxidase, with an I_{50} of 60 µM (Duke *et al.*, unpublished). Our preliminary data strongly suggests that this compound induces rapid enzymatic degradation of chlorophyll by a novel mechanism of action.

Hydantocidin

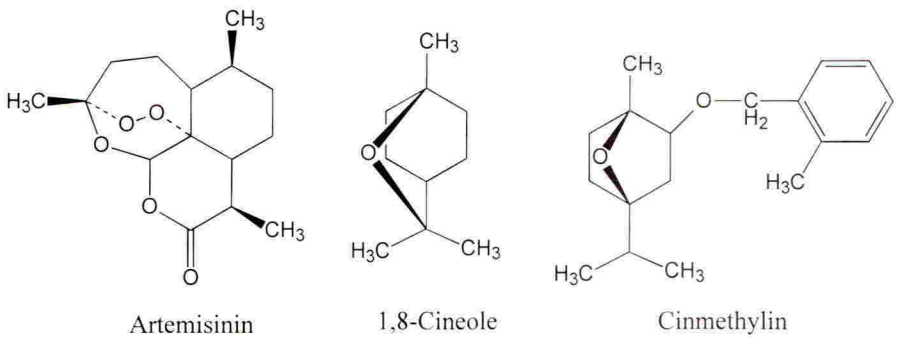
Hydantocidin (Figure 1) is a product of *Streptomyces hygroscopicus* that has significant herbicidal activity (Nakajima *et al.*, 1991). Its growth-inhibiting activity can be largely

reversed by growing plants on media containing adenine, AMP, or adenylosuccinate, but IMP has no such effect (Duke *et al.*, unpublished). It blocks conversion of IMP to AMP (Heim *et al.*, 1995, Siehl *et al.*, 1996). These data suggest that hydantocidin is a strong inhibitor of adenylosuccinate synthetase. While hydantocidin has no *in vitro* effect (Siehl *et al.*, 1996), the 5'-phospho derivative does inhibit this enzyme strongly (Fonné-Pfister *et al.*, 1996, Siehl *et al.*, 1996) by competing with IMP, forming a dead-end complex that is about 1000-fold tighter than that with IMP (Walters *et al.*, 1997). Two other microbial products, hadacidin and alanosine, are also inhibitors of this enzyme. Many analogues of hydantocidin and have been synthesized without much success in producing a more potent and safer herbicide than hydantocidin.

Plant products

Artemisinin

Artemisinin (Figure 2) is a sesquiterpene lactone from *Artemisia annua* that is an effective antimalarial drug. It is also a potent plant growth inhibitor (Duke *et al.*, 1987, 1988, DiTomaso & Duke, 1991). It is autotoxic to the producing plant, but the plant protects itself from harm by sequestering it in the space between the cell wall and cuticle of the surface of the glandular trichomes that cover the surface of its foliage and flowers (Duke *et al.*, 1994). Its mode of action as an antimalarial drug is unknown, but its ability to cross-link with heme has been demonstrated. Preliminary data from our laboratory indicates that, as a phytotoxin, artemisinin may also interact with porphyrins to inactivate them.



Artemisinin 1,8-Cineole Cinmethylin

Figure 2. Structures of plant-derived compounds mentioned in the text

1,8-Cineole and cinmethylin

For almost 40 years, 1,8-cineole (Figure 2), a monoterpene constituent of many aromatic plants, has been known to be highly phytotoxic. A synthesized analogue, cinmethylin (Figure 2), is sold as a herbicide in Europe. The molecular site(s) of action of these compounds are unknown. Cineole inhibits respiration (Lorber & Muller, 1980). Cinmethylin is a growth inhibitor with good activity against most grassy weeds and some dicotyledonous weeds. All that is published of its mode of action is that it prevents entry of meristematic cells into mitosis (El Deek & Hess, 1986). Cineole has a similar effect (Muller,

1965). Inhibition of any one of many metabolic processes could cause such an effect. Cinmethylin has been examined for effects on polyamine synthesis, but no dramatic effects were noted (DiTomaso & Duke, 1991). What little we know suggests that these compounds have a novel mechanism of action.

SUMMARY AND CAVEATS

Our limited experience clearly indicates that natural products offer relatively rapid clues for the discovery of new molecular target sites for herbicides. However, there are a few caveats about this approach. First, just as bioassay-directed isolation and identification of naturally-occurring phytotoxins can result in much time wasted in rediscovery of known compounds, there is no guarantee that the mode of action of natural phytotoxins will be novel. For example, there are well documented natural PS II inhibitors such as sorgoleone (Nimbal *et al.*, 1996) and cyanobacterin (Gleason *et al.*, 1986). The incidence of proherbicides among natural phytotoxins may be higher than previously realized (*e.g.*, bialaphos, cornexistin, and hydantocidin). This complicates discovery of the mechanism of action. A significant problem has been the difficulty in finding more cost-effectve synthetic analogues of natural phytotoxins. In some cases, nature appears to have optimized the activity in compounds that would be too expensive for development as a commercial herbicide. Furthermore, as mentioned above, there is no assurance that natural products are less toxicologically benign than synthetic pesticides (*e.g.*, AAL-toxin, fumonisins). As this area of research matures, diminishing returns will occur. However, the literature would suggest that this is presently not a significant problem.

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HERBICIDE SELECTIVITY MECHANISMS IN MAIZE: USING WHAT WE KNOW FOR THE FUTURE

M BARRETT
Department of Agronomy, University of Kentucky, Lexington, KY, 40546-0091, USA

ABSTRACT

Selectivity based upon differential herbicide metabolism by tolerant crops compared to susceptible weeds is the basis for the widespread use of these chemicals in crop production today. Although crop tolerance to herbicides achieved through tissue selection or genetic engineering receives much of the attention today, differential metabolism will remain important to new herbicide discovery. A review of the selectivity mechanisms for maize, and indeed for most crops, herbicides shows that glutathione transferases and cytochrome P450 monooxygenases are by far the most important plant enzyme systems for herbicide detoxification. These enzymes are a logical starting point to expand our knowledge of herbicide selectivity mechanisms. The current understanding of these enzymes in maize is reviewed, including our studies to characterize the numbers and substrate specificity of P450s involved in herbicide detoxification. The paper ends with a discussion of the possibilities for using our expanding knowledge of these enzymes in herbicide discovery.

INTRODUCTION

The miracle of selective weed control in crops begun with the discovery of 2,4-D control of broadleaf weeds in cereal crops currently continues as the basis for the extensive use of these chemicals in crop production. Although perhaps not often appreciated, metabolic detoxification of herbicides has been as crucial to the development of herbicides as the discovery of new modes of action. Also, whereas the enzymology of the interactions between herbicides and their site of action has been increasingly well characterized at the molecular level, this level of understanding has not been achieved for the detoxification enzymes.

Maize is arguably the single most important crop market for herbicides. Using maize as an example, a review of the selectivity mechanisms for herbicides currently used and some of those under development shows that metabolism by glutathione transferases (GSTs) and cytochrome P450 monooxygenases (P450s) accounts for the selectivity of the majority of herbicide groups (Table 1). Although the development, through tissue culture selection or genetic engineering, of herbicide resistant crop varieties receives much attention today, only four maize herbicide groups (imidazolinone, glufosinate, glyphosate, cyclohexanedione) have achieved selectivity through this route (Shaner *et al.*, 1996, Vasil, 1996, Padgette *et al.*, 1996, Slack *et al.*, 1997, Somers, 1996). The four herbicide groups for which resistant maize varieties have been developed share the characteristics that they were discovered and developed for other uses plus their mode of action was well understood before the maize varieties were developed. The potential commercial importance of these groups will not be

argued but the discovery of new maize herbicides is more likely to come from metabolic.

Table 1. Selectivity mechanisms for selected herbicides used in maize

Herbicide Family	Example	Selectivity Mechanism[1]	Reference
Acetamide	Acetochlor	GST, P450?	Hatton *et al.*, 1996
Thiocarbamate	EPTC	GST, Peroxygenase	Shimabukuro, 1985
Triazine	Atrazine	GST, P450?	Hatton *et al.*, 1996
Sulfonylurea	Nicosulfuron	P450, GTase?	Barrett, 1995
Triazolopyrimidine	Flumetsulam	P450	Frear *et al.*, 1993
Benzoic Acid	Dicamba	P450?	Ashton & Crafts, 1981
Phenoxy Acid	2,4-D	P450?	Montgomery *et al.*, 1971
Nitrile	Bromoxynil	P450?	Buckland *et al.*, 1973
Aryl Triazolinone	FMC 8426	P450?	Anon., 1995
Benzoyl Isoxaflutole	RPA 201772	P450?	Pallett *et al.*, 1997
Unclassified	Bentazon	P450	McFadden *et al.*, 1990
Phenylpyridazine	Pyridate	GTase?	Zohner, 1987
Phthalimide	Flumiclorac-pentyl	Not Reported	
Pyridine	Clopyralid	Not Reported	
Imidazolinone	Imazethapyr	Altered ALS, P450?, GTase?	Shaner et al., 1996 Baerg, 1994, Shaner & Mallipudi, 1991
Unclassified	Glufosinate	Bacterial Acetylase	Vasil, 1996
Cyclohexanedione	Sethoxydim	Altered ACCase	Somers, 1996
Unclassified	Glyphosate	Altered EPSPS	Padgette *et al.*, 1996, Slack *et al.*, 1997

[1]Abbreviations: GST, glutathione transferase, P450, cytochrome P450 monooxygenase, GTase, glucosyltransferase, ALS, acetolactate synthase (also known as acetohydoxyacid synthase), ACCase, acetyl-CoA carboxylase, EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase

selectivity than from genetic engineering. For this reason, this paper focuses on the mechanisms of selective herbicide metabolism by maize with particular attention given to GSTs and P450s in maize. In addition to metabolic detoxification based selectivity, positional selectivity (avoidance of herbicide contact with the plant) is a primary means of selective use of such herbicides as linuron, pendimethalin and paraquat in maize. Although P450 metabolism of linuron was demonstrated in other species (Frear *et al.*, 1991) and may contribute to maize tolerance to this herbicide, it would not the major factor in tolerance. These groups will not be discussed further.

HERBICIDE METABOLISM IN MAIZE

Briefly reviewing the information in Table 1, the activity of GSTs has been shown in several ways (including in specific cases genetic studies and safener experiments) to be critical for the tolerance of maize to acetamides, thiocarbamates, and triazines. Metabolism of acetamides by P450 in maize microsomes has also been documented but its contribution to overall tolerance is not known (Jablonkai & Hatzios, 1994). Thiocarbamates are oxidized first by Phase I reactions to the sulfoxides and sulfones and then conjugated in a Phase II reaction catalyzed by a GST

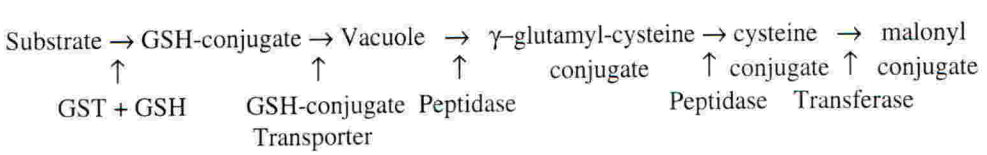
with glutathione. The initial step was thought to be catalyzed by a P450 but two studies (Blee, 1991, Jablonkai & Hatzios, 1994) suggest that a peroxygenase, which utilizes hydroperoxides as an O_2 donor and which does not use NADPH as an electron donor, is the actual catalytic enzyme. Demonstration of P450 metabolism of simazine in *Lolium rigidum* suggests that this could also contribute to maize tolerance of this herbicide (and perhaps other triazines) but GST metabolism is the governing detoxification route (Preston *et al.*, 1996). The involvement of maize P450s in the metabolism of the acetolactate synthase (ALS) inhibitor herbicide families sulfonylureas and triazolopyrimidine has been conclusively shown (Moreland *et al.*, 1993, Frear *et al.*, 1993). The importance of the P450 to tolerance was emphasized by findings that inhibition of the activity by organophosphate insecticides resulted in crop injury (Kreuz & Pfister, 1992, Diehl *et al.*, 1995). The P450 metabolism of sulfonylureas can produce only partially inactive products that require glucose conjugation for complete detoxification (Sweetser, 1985). Commercially acceptable imazethapyr, a member of the imidazolinone ALS inhibitor family, tolerance required the selection of maize lines through tissue culture with an insensitive ALS form. Imazethapyr is metabolized by P450 in maize but the hydroxylated product represents only a partial detoxification (Baerg, 1994, Shaner & Mallipudi, 1991). Lack of glucose conjugation of the hydroxylated imazethapyr in maize, in contrast to tolerant soybean, prevents complete imazethapyr detoxification by metabolism (Shaner & Mallipudi, 1991). Bentazon is another herbicide for which P450 metabolism accounts for maize selectivity (McFadden, 1990, Moreland *et al.*, 1993). However, for herbicides such as dicamba, 2,4-D, bromoxynil, and FMC 8426 (proposed name carfentrazone-ethyl, ethyl *a*, 2-dichloro-5-[4-(difluoromethyl)-4,5-dihydro-3-methyl-5-oxo-1 *H*-1,2,4-triazol-1-yl]-4-fluorobenzenepropanoate) it is only the presence of hydroxylated metabolites in the maize and other tolerant plant metabolic pathways which suggest the involvement of P450 reactions (Anon., 1995, Buckland *et al.*, 1973, Montgomery *et al.*, 1971, Ray & Wilcox, 1967). Rapid metabolic detoxification in maize, perhaps through P450, has been suggested for the benzoyl isoxazole herbicide RPA 201772 (proposed common name isoxaflutole, 5-cyclopropyl-4-[2-methylsulphonyl-4-trifluoromethylbenzoyl]isoxazole) (Pallett *et al.*, 1997). Pyridate is an unusual maize herbicide in that its selectivity is based on the formation of *N* and *O*-glycosides (Zohner, 1987). Of course, for some herbicides used in maize, the herbicide metabolism pathway and its contribution to selectivity have not been established.

Given the importance of GSTs and P450s in the metabolism and selectivity of herbicides in maize, it is apparent that a greater knowledge of these enzyme systems will increase our understanding of how the selective metabolism is achieved and may help develop new maize selective herbicides.

THE GSTs OF MAIZE

Several recent reviews concerning GSTs in plants have appeared and the reader is referred to those for further detailed information (Dixon *et al.*, 1997, Droog, 1997, Kreuz *et al.*, 1996, Marrs, 1996). GSTs catalyze the nucleophilic attack of the electrophilic center of the substrate by the sulfur of glutathione (GST). Plant GSTs are cytosolic enzymes and most frequently are homo- or heterodimers with subunits in the range of 23-39 kD. A subunit will have both a specific GSH binding site and a substrate binding site which may have broad specificity. Like P450s, GSTs belong to a gene superfamily but, in agreement with the wide variety of substrates utilized, can share little sequence homology. However, there are conserved residues associated

with GSH binding, thiolate ion formation, and maintenance of enzyme structure. It should be remembered that the plant GSTs perform many natural functions in biosynthesis, localization and movement of secondary metabolites, prevention of oxidative stress, and in auxin transport and storage. Also, although the focus is on formation of glutathione conjugates as a primary detoxification reaction for herbicides, further processing and vacuolization of the conjugates occurs. This is likely important to the overall health of the plant as GSH-conjugates are inhibitors of GSTs, GSH-reductases, and possibly other plant processes. The overall fate of GSH-conjugates is the subject of active research but some of the current understanding can be summarized in the following scheme:



Many studies utilize 1-chloro-2,4-dinitrobenzene (CDNB) as a convenient model substrate to measure GST activity. However, there can be major discrepancies between activities measured with CDNB and herbicide, or other, substrates.

Unlike the P450s, a comprehensive scheme for naming the GSTs has not been developed. This will likely come as more genes, as opposed to activities, are described. Mammalian GST subunits are classified into five groups; alpha, mu, pi, theta, and sigma, based on N-terminal sequences and reactivity to human GST antibodies. The theta class is considered the most ancient and, although somewhat a default grouping, also contains GSTs from bacteria, insects, plants, and fish. Plant GSTs have been further subdivided into types I, II, and III based upon amino acid sequence and intron/exon arrangement. However, it was recently proposed that type III plant GSTs evolutionarily represent a new subunit group, designated tau, the type I GSTs remain in the theta group, and the type II GSTs are not related to either tau or theta (Droog, 1997). Although, the number of type II sequences available (3) is too small to determine if they represent a third group. Plant theta (type I) GSTs appear to be involved in plant protection (including herbicide detoxification), type II GSTs are floral ethylene and senescence related, and tau (type III) GSTs are involved in stress responses.

Identified maize GSTs belong to both the theta (type I) and tau (type III) groups (Table 2). Herbicide metabolizing GSTs dominate the known GSTs from maize, likely due to the focus of the conducted research. Herbicide metabolizing GSTs have been named in the order of their discovery and presently number six. The five herbicide metabolizing maize theta GSTs (GST I-IV) are composed of different combinations of the same three subunits, I, II, and III and it was proposed that they be renamed based on their subunit composition (Table 2). The sixth herbicide metabolizing GST from maize, GST V, has low (approximately 15%) amino acid sequence identity to the previously described subunits I-III but has higher sequence identity with two auxin-inducible GSTs from tobacco, 33 and 54%, respectively (Irzyk & Fuerst, 1996). It may thus belong to the tau group of GSTs. In a direct comparison of relative activities towards herbicide substrates of GST I/I, GST I/II, and GST I/III, all three metabolized atrazine with approximately equal (0.07-0.11 nkat mg^{-1} protein) efficiency (Dixon *et al.*, 1997). Alachlor metabolism was more than 10x that of atrazine and twice as high in GST I/II as the other two forms. Little metolachlor metabolism by GST I/I was detected, equivalent activity for alachlor as with GST I/III, and half that of alachlor with GST I/II. Similar results as with

metolachlor occurred with fluorodifen but fluorodifen activities were approximately 10% of those with metolachlor. Expressed as a fraction of the total recovered activity following column purification of maize root extracts, the majority of atrazine metabolism would be performed by GST I/I and that of alachlor, metolachlor and fluorodifen

Table 2. Identified maize GSTs

Name	Type	Subunits (kD)	Subunit Class[1]	Substrates	Induction	Comments
GST I	Theta (I)	29 Homo-dimer	I/I	alachlor, atrazine, CDNB	flurazole, dichlormid	Major constitutive GST in roots and shoots
GST II	Theta (I)	29,27	I/II	alachlor, metolachlor, fluorodifen, atrazine, CDNB	flurazole, dichlormid, R-29148	Constitutive in roots, not induced by wounding, ethylene, salicyclic acid, subunit II may have peroxidase activity
GST IIIa	Theta (I)	26 Homo-dimer	III/III	alachlor, metolachlor, CDNB	dichlormid	
GST IIIb	Theta (I)	29/26	I/III	alachlor, metolachlor, fluorodifen, atrazine, CDNB	dichlormid	
GST IV	Theta (I)	27 Homo-dimer	II/II	alachlor, metolachlor, triazines	benoxacor	CDNB not a substrate
GST V	Tau (III)?	27 Homo-dimer		metolachlor, CDNB	benoxacor, metolachlor	Activity inhibited by ethacrynic acid, kaempferol, quercitin but not induced by these or CDNB
Bronze-2	Tau (III)	26		CDNB, cyanadin-3-glucoside	Cd, ABA, arsenite	Anthocyanin synthesis, not induced by 2,4-D
CT24						41% identity and 61% similarity to Bronze-2

[1]Subunit classification according to Dixon *et al.*, 1997

by GST I/II. However, GST I/III would contribute to metabolism of all the herbicides as would GST I/II to atrazine metabolism. GST II/II, although metabolizing triazines and chloroacetamides, is distinguished by its inability to metabolize CDNB. GST V, as an apparent member of the tau group, is unique among the herbicide metabolizing GSTs. It serves to demonstrate that herbicide metabolism is not confined to one group of GSTs but will result from the fortuitous interaction of a GST with a particular herbicide structure. Reported substrates for GST V are metolachlor and CDNB (Irzyk & Fuerst, 1996).

In addition to the herbicide metabolizing GSTs, two other GSTs, bronze-2 and CT24 (related to bronze-2), have been identified. Bronze-2 catalyzes the conjugation of cyanidin-3-glucoside, the anthocyanin precursor, to GSH and the conjugate is transferred to the vacuole where the anthocyanin color develops. A mutation in the bronze-2 gene causes accumulation of the cyanidin-3-glucoside in the cytoplasm resulting in necrosis, poor growth, or even death.

There are also several reports of a GST in maize that will conjugate GSH to *trans*- or *para*-cinnamic acid. However, the enzyme was unusual as a GST in that it functioned as a monomer, could use both cysteine and GSH as S-donors, and activity towards trans-cinnamic acid was enhanced by *para*-cinnamic acid or hydroxycoumarin. However, subsequent work suggested the GSH conjugation of cinnamic acid was mediated by a peroxidase ,rather than a GST (Dean & Devarenne, 1997). Peroxidases have been shown to conjugate GSH to alkyl double bonds and can have a broad substrate specificity. They represent another enzyme class that could contribute to herbicide metabolism.

A characteristic of GSTs is there inducibility by various agents and environmental conditions. All the herbicide metabolizing GSTs are induced by specific safeners (Table 2) and GST V is induced by a substrate, metolachlor, but not as strongly as the safener benoxacor (Irzyk & Fuerst, 1996). However, there are enough differences in tissue constitutive expression, response to safeners between tissues and maize lines, and the specificity of the enzymes induced to demonstrate complex regulation of GST expression and substrate specificity (Dixon *et al.*, 1997).

Past research on GSTs in maize identified several isozymes by focusing on purification of specific activities. More isozymes may wait to be resolved as, depending on the specific interpretation of activities eluting from a column, maize homogenates had as many as 11 activities. However, the number of GST genes identified may rapidly increase as techniques of molecular biology (particularly PCR and library screening using conserved sequences) are applied to the search. The challenge, as seen with P450s discussed below, is to link these genes with known reactions.

THE P450s OF MAIZE

Plant P450s have been the subject of two recent reviews and their involvement in xenobiotic metabolism, both generally and in maize in particular, have also been covered (Barrett, 1995, Bolwell, *et al.*, 1994, Kreuz *et al.*, 1996, Schuler, 1996). P450s are heme-thiolate containing oxidases using NADPH and/or NADH plus O_2 to produce an oxidized organic substrate and water. Although a wide variety of reactions can be catalyzed by P450s, reactions with herbicides most often involve aromatic or alkyl hydroxylations and *O,N*-dealkylations. Although often a complete detoxification of the herbicide, the hydroxylated products must be further metabolized to glycosyl conjugates for complete loss of toxicity in some cases as discussed above. The fate of the conjugates is not well understood but they may be transported to the vacuole or apoplast. A general reaction scheme can be summarized as:

$$\underset{\text{P450 + P450 reductase}}{\text{X-H} + O_2 + \text{NADPH} \rightarrow \text{X-OH} + H_2O} + \underset{\text{Transferase}}{\text{NADP}^+ \rightarrow \text{X-O-Glucose}}$$

P450s are membrane (ER) bound enzymes of 45-62 kD. The proteins can be widely divergent in sequence and mutation of a single amino acid can alter substrate specificity. Across a wide range of P450s, only a few (5) residues associated with covalent attachment of the heme are highly conserved. Although P450s are involved in biosynthesis of numerous secondary metabolites, only one physiological activity, lauric acid hydroxylation, has been associated with

herbicide, diclofop hydroxylation, metabolism. Although, the P450 cinnamic acid hydroxylase expressed at high levels in yeast can metabolise chlorotoluron at low rates.

P450s are members of a gene super family (over 500 known gene sequences, designated CYP) and are classified by sequence homology. Sequences >40% identical at the amino acid level belong to the same family (CYP#), >55% the same sub-family (letter after family and number for gene), >97% are allelic variants, <3% difference in a species are designated v1, v2, etc. As of January 1997, there were 32 families of plant P450 genes numbered CYP51, CYP71-99, and CYP710 and 702 (these sequences can be accessed through the home page of David Nelson at http://drnelson,utmem.edu/nelsonhomepage.html). Maize has 21 P450s identified in the data base in 8 families. In addition, there are numerous P450 ESTs in the maize cDNA program plus other, unsubmitted, maize P450 sequences. A summary of the P450 genes in maize is found in Table 3. Using the P450s identified in the *Arabidopsis* ESTs, there are at least 51 P450s in this species.

Table 3. Identified maize P450 genes

Gene Family	Members	Comments
CYP51		EST Fragment, wheat obtusifoliol 14a-demethylase
CYP71	C1, C2, C2v2, C3, C3v2, C4, C5	Found as gene cluster on chromosome 4
CYP73	A6, A7, A8	CYP73A1 is cinnamic acid hydroxylase
CYP78	A1	Found in developing inflorescence
CYP81	A1, A2, A3, A4	Induced by ethanol
CYP88	A1	Associated with gibberellin biosynthesis
CYP92	A1	Tobacco CYP92 associated with the hypersensitive response
CYP95	A1	

Unlike the GSTs, P450s have been identified through molecular biology as genes for which only few functions have been assigned or as activities in crude homogenates. The membrane bound nature of the P450s, combined with their low activity, has made purification of individual isozymes difficult. This can be particularly true in monocot species where the P450 allene oxide synthase can be the predominant P450 form in the tissue. This has left open questions concerning how many P450s in maize metabolize herbicides, their substrate specificity, and their regulation. However, using a combination of genetic, induction, and inhibitor studies, we have shown that their are two activities for bentazon hydroxylation in coleoptiles. One is inducible by safeners and one is not. The safener inducible activity is also associated with metabolism of sulfonylureas, imidazolinones, and malathion plus inhibition by terbufos-sulfone. Similarly, flumetsulam hydroxylation at two different positions was suggested to involve two P450s (Frear *et al.*, 1993). Primisulfuron hydroxylation at two positions may also be catalyzed by separate P450s.

In the past ten years, we have moved from uncertainty over the involvement of P450 in herbicide metabolism to demonstration of multiple herbicides detoxified by P450 in maize and multiple maize P450 genes. Clearly, the P450s are a complex system that represent a challenge to understand individual contributions to herbicide metabolism and regulatory control by safeners and other factors.

USING WHAT WE KNOW

We are approaching an era where the tools could be at hand to characterize and model the reactions of novel herbicide chemistries with GST and P450 enzymes. A first step will be a more complete inventory of the GSTs and P450s in maize. This can be a daunting task given the estimates of P450s in a given species (50-100?) and the likelihood that molecular biology approaches could reveal a plethora of new GSTs in maize. The EST data base can help in this search but caution should be exercised from the example of ten named *Arabidopsis* P450 genes that are not represented in the ESTs.

Obtaining sequences will likely be less problematic than assessing function of the gene products. Plant GSTs and P450s have both been expressed in bacteria and yeast. The requirement for both membrane insertion and coupling with reductase makes P450 expression more problematic than cytosolic GST expression. We have experienced difficulty in expressing maize P450s in both bacterial and yeast expression systems. Expression will need to be more routine before a heterologously expressed library of maize GSTs and P450s is possible. This would have utility in studying herbicide metabolism in maize and could serve to generate metabolites from unlabelled precursors. Transformation of *Arabidopsis*, where it is sensitive to the herbicide of interest, could be used as an alternative to heterologous expression. A series of *Arabidopsis* lines expressing single maize detoxifying genes could be developed.

Alternatively, the "Trait Utility System for Corn" developed by Pioneer Hi-Bred International, Inc. which uses a collection of maize lines containing multiple copies of the transposable element family, *Mutator* (*Mu*), to disrupt gene function could be employed to determine potential contributions of a GST or P450 gene to herbicide tolerance. As several GSTs or P450s could be involved in detoxification of a single herbicide, loss of one enzyme may not produce complete sensitivity to a herbicide. Careful dose response studies could identify shifts in sensitivity due to loss of a detoxifying enzyme. In addition, determining whether multiple GSTs or P450s are involved in tolerance could help predict the occurrence of sensitive inbred and hybrid lines. For example, one gene appears to control sulfonylurea sensitivity in maize, sensitive hybrids are relatively frequent, but as two genes control bentazon sensitivity, sensitive hybrids are very rare. Further, identification of herbicides detoxified by P450s sensitive to organophosphate insecticide inhibition, or other deleterious interactions, could help prevent these types of interactions. Given the similarity between monocots, it may be possible to use the maize systems to identify homologues in other grass crops and weeds. Information on which enzymes detoxify a herbicide in maize may lead to predictions of which other species will also tolerate the chemical.

Finally, it is a dream to be able to model herbicide structure and selectivity. In many cases, it is possible to predict from experience which structures will better serve as substrates for detoxifying enzymes. However, there are also examples where only in hindsight was it possible to understand how molecular changes in a herbicide structure resulted in profound alterations in selectivity. Unlike the search for new sites of action where new interactions between a potential herbicide and a protein target must be described, there are a finite number, albeit large, of GSTs and P450s in maize. Once they are characterized and, hopefully, expressed they will serve as a continuing resource.

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MOLECULAR ENZYMOLOGY OF PLANT CYTOCHROMES P450

J A MAUGHAN, J H A NUGENT
Biology Department, University College London, Gower Street, London WC1E 6BT, UK

I M CLARK, A C G WESTLAKE, D L HALLAHAN
Biochemistry & Physiology Department, IACR-Rothamsted, Harpenden, Herts. AL5 2JQ, UK

ABSTRACT

Isozymes of the cytochrome P450 superfamily in plants are known to play key roles in a variety of metabolic pathways, biosynthetic as well as degradative. Studies of these plant enzymes have until recently been hampered by the difficulties involved in their isolation. Molecular biological approaches have shown that far more isozymes are present in plants than previously appreciated. Despite the advances in molecular biology, cloning of a specific cyt P450 isozyme remains a challenge. Molecular approaches have also allowed detailed characterisation of several of these isozymes following heterologous expression of cloned cDNAs. However, it is now becoming clear that expressed cyt P450s may not couple efficiently with endogenous host cyt P450 reductase (where present), and that in these cases alternative strategies for co-expression of the cyt P450 and an appropriate reductase must be adopted. Thus although the early 1990s have seen tremendous progress in the field of plant cyt P450 research, significant challenges remain, particularly in the assignment of catalytic activity to 'orphan' plant cyt P450 cDNAs.

INTRODUCTION

Since the initial discovery that plants possessed cytochrome P450 enzymes in the late 1960s, an increasing number of activities have been attributed to this class of enzyme (Schuler, 1996). It is now well established that cyt P450s are key enzymes in the metabolism of a large number of xenobiotics, including herbicides, in plants (Schuler, 1996, Bolwell *et al.*, 1994). Rapid cyt P450-mediated metabolism of herbicides in cereal crops, for example, provides the basis for the selectivity of important classes of herbicides such as the phenylureas and sulfonylureas (Barrett, 1995). The importance of these enzymes has spurred attempts to purify them and analyse their activity, but in the main these attempts have been successful only with specific isozymes expressed to relatively high levels in tissues which may be obtained in bulk, eg, CYP71A1 from avocado fruit, or berbamunine synthase from cell cultures (O'Keefe & Leto, 1988, Stadler & Zenk, 1993). Many of the more interesting isozymes are present at much lower levels, and although their activity may be analysed, their purification (and subsequent molecular cloning) has not been possible. In the case of cereals such as wheat and maize, one isozyme (allene oxide synthase) constitutes the majority of the detectable cyt P450 (Lau *et al.*, 1993), thus purification of any one of the remaining isozymes represents a real challenge to the current state of the art.

Latterly, with the application of molecular biological techniques, it is becoming clear that the cyt P450 isozymes identified biochemically represent only a fraction of the cyt P450 genes present in plants. For example, 33 plant families have been identified thus far, and examination of the *Arabidopsis thaliana* expressed sequence tag (EST) database has shown that 15 different families of cyt P450 can be identified, and in one subfamily (CYP71B) at least 12 genes are present (data analysed and presented by D R Nelson, which can be accessed on the world wide web at the url http://drnelson.utmem.edu /nelsonhomepage.html). Overall, it is estimated that a minimum of 51 cyt P450 genes exist and are expressed in *A. thaliana*. Such numbers are supported by data from other species. For example, amplification of cyt P450 cDNAs using the polymerase chain reaction (PCR) with a series of degenerate primers has shown that at least 70 different genes are present in petunia (Holton & Lester, 1996).

It is clear, then, that the next major advances in the study of plant cyt P450 will be made initially through molecular biological approaches. However, the 'orphan' cDNAs or genes identified through shotgun cloning or mutant analysis will require assignment or confirmation of function of the corresponding proteins. In this paper, we briefly review the methodologies in use for the isolation of cyt P450 cDNAs from plants, and for biochemical analysis of the corresponding proteins following heterologous expression.

MOLECULAR CLONING OF PLANT CYT P450

The random sequence analysis of large numbers of cloned cDNAs (ESTs) has yielded considerable information on plant cyt P450s, as described above. However, in the main researchers are interested in identifying genes corresponding to specific cyt P450 isozymes, which may not be expressed in plants for which EST programmes are currently underway (*A. thaliana*, maize, rice). A variety of approaches have been successfully used for the isolation of specific cyt P450 cDNAs. The classical approach, requiring purification of a specific isozyme followed by library screening using antibodies or oligonucleotide probes (or PCR primers) derived from peptide sequencing, has proved successful in a number of cases (eg., Teutsch *et al.*, 1993; Matsui *et al.*, 1996; Bak *et al.*, 1997).

The inducibility of many cyt P450-associated enzyme activities in plants is well documented, and it has been assumed that this reflects gene expression. Differential cDNA hybridization was used to isolate the first plant cyt P450 cloned, the ripening-related CYP71A1 from avocado fruit (Bozak *et al.*, 1990). Similar approaches have been used in the cloning of CYP75A2, whose gene transcripts are inducible by UV illumination, thus suggesting a role for the gene product in anthocyanin biosynthesis (Toguri *et al.*, 1993). Differential display was used to isolate a cyt P450, CYP93, induced by methyl-jasmonate in soybean cell suspension cultures (Suzuki *et al.*, 1996), and differential screening led to the identification of a number of cyt P450 genes of the CYP71 family in maize (Frey *et al.*, 1995). It is likely that these or similar techniques such as subtractive hybridization will prove particularly useful in the isolation of cyt P450 cDNAs induced in response to xenobiotic treatments, such as herbicide safeners.

With the isolation of the first plant cyt P450 cDNAs, it became apparent that their sequences contained a number of conserved motifs, often in common with sequences from

other organisms. This has facilitated the design of degenerate oligonucleotide primers for amplification of partial cDNAs using PCR. Combinations of specific and degenerate primers have been employed to isolate 16 different cyt P450 cDNAs from the periwinkle *Catharanthus roseus* (Meijer *et al.*, 1993), 2 from the catmint *Nepeta racemosa* (Clark et al., 1997), and 70 from petunia (Holton & Lester, 1997). The latter work showed the value of repeated rounds of primer design, amplification and library screening in the isolation of large numbers of novel sequences. However, given the diversity of the plant cyt P450 families, single rounds of PCR with primers designed against existing sequence data are unlikely to amplify highly novel cDNAs.

Library screening at low stringency using existing cDNAs has been used to isolate cyt P450 cDNAs belonging to different families within the same species (Umemoto *et al.*, 1993), from the same family in a different species (Toguri *et al.*, 1993), and even from a different family in a different species (Cabello-Hurtada *et al.*, 1997).

Transposon or T-DNA insertional mutants of plants have also been useful in the isolation of plant cyt P450s. Analysis of an *A. thaliana* T-DNA mutant exhibiting constitutive photomorphogenesis and dwarfism revealed that the T-DNA was inserted within a novel cyt P450, CYP90, involved in brassinosteroid biosynthesis (Szekeres *et al.*, 1996). Similarly, ferulate 5-hydroxylase was identified by analysis of the T-DNA tagged *A. thaliana* mutant *fah1* (Meyer *et al.*, 1996), and *dwarf* genes (believed to encode gibberellin hydroxylases) isolated from maize (Winkler & Helentjaris, 1995) and tomato (Bishop *et al.*, 1996). The recent identification of the roles of three cyt P450s in hydroxamic acid biosynthesis in maize confirms the power of such approaches (Frey *et al.*, 1997)

HETEROLOGOUS EXPRESSION OF PLANT CYT P450

A number of methods are available for the expression of plant cyt P450, each with their own advantages and drawbacks (Gonzalez & Korzekwa, 1995). The most widely used have been the bacterium *Escherichia coli*, the yeast *Saccaromyces cerevisiae*, insect cell culture, and plant expression systems. The choice between these is dictated by the information required; for instance, plant expression is most useful for phenotypic complementation studies or the introduction of new characteristics, but is unsuitable for enzyme recovery and *in vitro* mechanistic and functional experiments (O'Keefe, 1995). A number of cyt P450s have been expressed in plant systems; for instance, the genes encoding flavonoid 3',5'-hydroxylase and cathasterone hydroxylase have been identified using complementation in mutant plants (Holton *et al.*, 1993; Szekeres *et al*, 1996). Bacterial cyt P450s which activate pro-herbicides such as sulphonylureas have also been expressed in plant systems (O'Keefe *et al.*, 1994).

Functional studies on cyt P450s *in vitro* require the presence of a suitable NADPH-cytochrome P450 reductase (CPR). This may be either native to the host organism, or introduced *via* genetic manipulation. Alternatively, P450 activity may be driven by oxygen donors such as cumene hydroperoxide, which in many cases give increased activity relative to NADPH-driven reactions. Such donors may react directly with substrates or cause production of different enzymatic reaction products, and are also likely to inactivate cyt P450.

Plant cyt P450 enzymes may be expressed in *E. coli*, although this is complicated by the fact that this organism does not produce any detectable level of CPR. Activity studies therefore require either co-expression with a suitable CPR (Dong & Porter, 1996), or purification of the cyt P450 and reconstitution with the CPR in an artificial membrane environment (Halkier *et al.*, 1995). The N-terminal membrane anchor region must also be modified for efficient expression in *E. coli* (Barnes, 1996). Enzymes which have been successfully expressed in *E. coli* include cyt P450tyr (Halkier *et al.*, 1995) and obtusifoliol 14-α-demethylase (Bak *et al.*, 1997). One strategy which may particularly be used to advantage in *E. coli* is the generation of genetically constructed translational fusions between the cyt P450 and the CPR, and this has been successfully applied to the expression of cinnamate 4-hydroxylase from periwinkle (Hotze *et al.*, 1995).

Another system widely employed for the expression of cyt P450 enzymes is the use of baculovirus-infected insect cell cultures. This method has the advantage of often providing high specific activities of cyt P450 per cell; however, the growth period is much longer than for *E. coli* or *S. cerevisiae*. The host cells contain an endogenous CPR, which has been found to donate electrons to berbamumine synthase from *Berberis stolonifera* (Kraus & Kutchan, 1995). The cinnamate 4-hydroxylase from *A. thaliana* has also been successfully expressed using this system (Mizutani *et al.*, 1997).

Possibly the simplest and most widely-used system is expression in *S. cerevisiae*. Strains have been developed which express a low level of endogenous P450, and allow transformation by vectors carrying common selectable markers (Fang *et al.*, 1994). Yeast has been used to express a variety of plant cyt P450 enzymes, including cinnamate 4-hydroxylase (Urban *et al.*, 1994), obtusifoliol 14-α-demethylase (Cabello-Hurtado *et al.*, 1997) as well as CYP71A1 from avocado and CYP71B7 from *A. thaliana*, which have no established function (Bozak *et al.*, 1992; Maughan *et al.*, 1997). Yeast contains an endogenous CPR which often serves to support NADPH-driven activity, and further recombinant strains have been developed which over-express either the yeast reductase, or one of two *A. thaliana* reductases (Pompon *et al.*, 1996). Optimum expression is often obtained when the 5'- and 3'- untranslated regions are removed from the cyt P450 cDNA prior to insertion into the expression vector, although the levels of expression in yeast are low by comparison with those obtained in other hosts.

HETEROLOGOUS EXPRESSION OF CYP71B7 FROM *A. THALIANA*

In order to determine whether catalytic activity might be attributed to an orphan plant cyt P450 cDNA, we expressed CYP71B7 from *A. thaliana* in yeast (Maughan *et al.*, 1997). The CYP71B7 cDNA had been isolated originally as an EST from *A. thaliana*, with no information as to its function *in vivo*. To screen for potential substrates, we decided to examine a range of compounds for their ability to inhibit enzymatic activity of the expressed protein towards a synthetic model substrate. Compounds exhibiting competitive inhibition in such an assay can be assumed to bind to the catalytic site of the enzyme, and thus may well be substrates of the enzyme (Hallahan *et al.*, 1992). Membranes prepared from transgenic yeast were found to catalyse ethoxycoumarin-*O*-deethylase (ECOD) activity, which is conveniently measured by monitoring production of the fluorescent

product umbelliferone (Werck-Reichhart *et al.*, 1990). Such activity was not detected in untransformed yeast or in yeast transformed with an empty plasmid. ECOD activity in yeast expressing CYP71B7 was not supported by NADPH, implying that the endogenous yeast CPR was incapable of transferring electrons to the *A. thaliana* cyt P450. However, activity was supported by the organic hydroperoxide, cumene hydroperoxide.

A variety of compounds, either known or potential plant cyt P450 substrates, were added to the reaction mixture, to a concentration of 250 μM (Maughan, 1997). The amino acids L-tryptophan, L-phenylalanine, L-methionine, and mono- di- and tri-homo-methionine, potential glucosinolate precursors, were not inhibitory. Other compounds from different chemical classes known to be substrates of plant cyt P450 were not inhibitory at the concentration employed, including the fatty acid lauric acid, the diterpenoid kaurene, the flavonoid kaempferol, and the xenobiotic herbicide chlorotoluron. However, several compounds from two chemical classes (the phenylpropanoids and monoterpenoids) proved inhibitory to the activity of this enzyme (Table 1). The monoterpenoids *S*-limonene, *S*-citronellol, terpinolene, linalool, nerol and geraniol exhibited significant inhibition, as did the phenylpropanoids ferulic and *p*-coumaric acid. Not all phenylpropanoids or monoterpenoids tested were inhibitory; *t*-cinnamic, benzoic and salicylic acids, as well as the monoterpenoids cineole and abscissic acid, were ineffective at the concentrations tested.

Table 1. Screening of various phenylpropanoids and monoterpenoids for inhibition of ethoxycoumarin *O*-deethylation catalysed by yeast-expressed CYP71B7 from *A. thaliana*

Class	Compound	Inhibition
Phenylpropanoids		
	p-coumaric acid	+
	ferulic acid	+
	t-cinnamic acid	-
	benzoic acid	-
	salicylic acid	-
Monoterpenoids	*S*-limonene	+
	terpinolene	+
	p-menth-1-ene	+
	S-citronelleol	+
	nerol	+
	geraniol	+
	linalool	+
	cineole	-
	abscissic acid	-

This study illustrates that screening heterologously-expressed orphan cyt P450 cDNAs against a panel of potential substrates in this manner can be informative, at least indicating a subset of compounds for more detailed analysis. In the case of CYP71B7 of *A. thaliana*, it would appear that the most likely substrates of this enzyme are monoterpenoids, although the possibility that these compounds merely act as inhibitors cannot be ruled out. For example, it was interesting to find inhibition by the phenylpropanoid ferulic acid, since

a quite different cDNA encoding ferulate 5-hydroxylase has been isolated from *A. thaliana* (Meyer *et al.*, 1996). This enzyme was classified in a different family (CYP 84) on the basis of its sequence.

This study also highlights a potential stumbling block with yeast systems, as the endogenous CPR did not support NADPH-driven catalytic activity. Thus future work on the analysis of orphan plant cyt P450 cDNAs will most likely necessitate the development of more robust yeast expression systems, in which NADPH-supported activity is reconstituted. This may be achieved by co-expression of an appropriate CPR, and/or additional components such as cyt b_5, as described above. The levels of expression in yeast are often quite low, so although questions regarding substrate specificity can be answered through expression in yeast, more detailed structural and spectroscopic studies will have to be carried out in hosts yielding greater amounts of enzyme, for example bacteria such as *E. coli*. Through such approaches, the challenge of assigning catalytic activity (and physiological roles) to orphan plant cyt P450 DNAs should be met.

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COMPARATIVE METABOLISM OF IMIDAZOLINONE HERBICIDES

B TECLE, D L SHANER, A D CUNHA, P J DEVINE, M R VAN ELLIS
American Cyanamid Company, Agricultural Research Center, P.O.Box 400, Princeton, NJ 08543

ABSTRACT

AC 263222, imazethapyr and imazamox are low use rate herbicides from the imidazolinone class of herbicides discovered and developed by American Cyanamid Company. The selectivity of this class of herbicides is based on the rate and route of metabolism. Although these three imidazolinones are closely related analogs, the pathways of metabolism of AC 263222, imazethapyr and imazamox varies unpredictably in soybean, maize and peanuts due to the presence of different metabolic pathways in the different species. There are two competing pathways for imidazolinones in soybeans. AC 263222 is metabolized via one of these pathways, while imazethapyr and imazamox follow the other. On the other hand, in peanuts there is only one pathway of metabolism by which imidazolinones are readily detoxified via hydroxylation and glycosylation. In maize, imazethapyr and AC 263222 are rapidly hydroxylated, but imazamox is not.

INTRODUCTION

Imazethapyr and imazamox are selective herbicides for use in soybean. AC 263222, (2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-5-methyl-3-pyridinecarboxylic acid), on the other hand, is only selective to peanuts and some varieties of soybeans.

Imidazolinone herbicides exhibit structural commonalties as well as diversity (Figure 1). They all share the substituted imidazolinone ring with the methyl-isopropyl side chain attached to an aromatic ring with a carboxylic acid function. They differ in the type of aromatic ring and the substituents on the aromatic ring. These three imidazolinones , imazethapyr, AC 263222 and imazamox, all have substituents at the 5'-position on the pyridine ring. These three herbicides also differ in their crop selectivity that is due to the differences in the substituents at the 5'-position.

Imidazolinone herbicides kill plants by interfering with the activity of acetohydroxyacid synthase (AHAS), the first enzyme in the pathway leading to biosynthesis of valine, leucine and isoleucine (Stidham and Singh, 1991).

The objective of our study was to determine the rates and routes of metabolism of these imidazolinone herbicide analogs in tolerant and susceptible plants and relate the differences to herbicide selectivity to the differences in the substituent at the 5'-position.

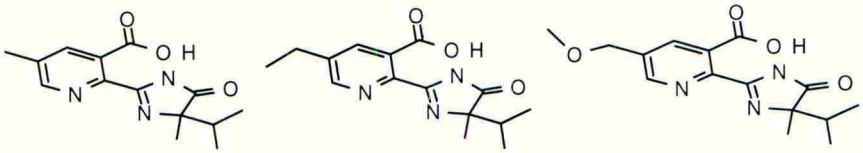

Figure 1: Imidazolinone Structures

MATERIALS AND METHODS

Chemicals

[Ring-^{14}C]AC 263222, imazethapyr and imazamox were used at specific activity of 100.5, 21.4 and 180 μCi/mg, respectively. The radiochemical purity was 98% as determined by HPLC on reverse phase column. The respective [^{13}C] labeled analogs were utilized as a mass marker for positive identification of the purified metabolites by mass spectrometry. For the isolation and identification of major metabolites, hydroponically-grown seedlings were exposed to a herbicide solution which contained the isotopes [^{12}C:^{13}C (1:1), ^{14}C]. At least 20-30 seedlings were harvested at 48 and 72 hours after treatment.

Tissue Extraction

The roots and shoots of hydroponically grown seedlings at each time point were cut into pieces and homogenized three times in acetone:methanol:water (1:1:1; 10 ml/gm tissue). The extracts from plants at 48 and 72 hours after treatment were combined and the solvent removed on the rotary evaporator. The residue was dissolved in water (4 mL) and centrifuged. The metabolites were then enriched on ODS SPE columns. Chromatographically pure metabolites were obtained after repurification on analytical reverse phase HPLC columns.

Chromatography

HPLC separations were performed with a Zorbax ODS 4.6 mm x 25 cm column on a Beckman model 345 Liquid Chromatograph equipped with a variable UV detector and a 171 Beckman radioisotope detector. The mobile phase used for purification and analysis was 27% methanol:70% water:2% tetrahydrofuran (THF):1% acetic acid (v/v) at a flow rate of 1 ml/min.

Mass Spectroscopy

Mass spectral analysis were performed on a Finnigan-MAT TSQ-70 triple stage mass spectrometer equipped with either the direct chemical ionization probe (DCI) or the thermospray (TSP) liquid chromatography interface.

RESULTS AND DISCUSSION

Crop Selectivity

The sensitivity of soybean, peanut and maize to these three imidazolinone herbicides varies. Soybeans and peanuts are very tolerant of imazethapyr and imazamox, while AC 263222 is only selective on peanuts (Table 1). Although none of these herbicides can be used selectively in maize, there are differences in maize's sensitivity to the three imidazolinones (Table 1).

Table 1 : Imidazolinone herbicides Safe Rate (g/ha)[a]

Herbicide	Soybean	Peanut	Maize
Imazethapyr	>500	>500	24
AC 263222	20	>200	10
Imazamox	>250	--	6

[a]A safe rate causes less than 15% injury to the crop.

Mechanisms of Herbicide Selectivity

In general, herbicide crop tolerance can result from such factors as reduced absorption and translocation by the crop, differential sensitivity at the site of action or rapid metabolic inactivation of the herbicide by the tolerant crop.

Studies on the comparative herbicides uptake and translocation in both tolerant and sensitive plant species have shown that these imidazolinones are readily absorbed and translocated in both tolerant and sensitive species and this factor can not be the major basis for the imidazolinone herbicides crop selectivity (Little and Shaner, 1991).
These three imidazolinone herbicides are potent inhibitors of acetohydroxyacid synthase. There are minor differences in the I_{50} s of these three imidazolinone on AHAS from different species (Stidham and Singh, 1991) but these differences would not explain their selectivity.

Herbicide Metabolism

Herbicide metabolism plays a major role on the crop selectivity of a number of herbicides. When we began research to determine the mechanisms of selectivity these three imidazolinones, we already knew the metabolic pathway of imazethapyr in soybeans, maize and peanuts. Based on this information , we predicted how AC 263222 and imazamox would be metabolized in these three crops. However, the different 5' -substituent had unpredictable effects on the metabolism of these herbicides in plants and it is these differences which determine the crop selectivity of these three herbicides

Metabolism in soybean

Our studies showed that imazethapyr and imazamox follow similar metabolic pathway in soybeans. (Figure 2). In both cases, the 5'-substituent is hydroxylated and then conjugated to

glucose. In the plant there is little accumulation of the hydroxylated metabolite. Instead, there is rapid accumulation of the glucose conjugate. This conjugate is completely inactive.

AC 263222 is also metabolized in soybeans, but at a very much reduced rate. Before starting this work, we had assumed that AC 263222 would be metabolized via a similar route as imazethapyr. That is, the methyl substituent would be hydroxylated followed by conjugation. We were puzzled that AC 263222 was not more selective on soybeans than it was. Much to our surprise we found that AC 263222 did not follow the same path way as imazethapyr. Instead it was metabolized via the same route as imazaquin (Shaner and Mallipudi, 1991) (Figure 3). That is, the first metabolite was more hydrophobic than the parent herbicide due to a cyclization between the carboxylic acid function and one of the nitrogens in the imidazolinone ring. This was followed by a cleavage of the imidazolinone ring and eventually the production of two products, with imidazolinone ring completely opened. This is the same path by which soybeans metabolize imazaquin. However, soybeans metabolize imazaquin much more rapidly and extensively than it metabolizes AC 263222. Thus, AC 263222 is not selective in soybeans.

Metabolism of AC 263222 and imazethapyr in peanuts:

Unlike soybeans, AC 263222, imazamox, and imazethapyr are metabolized in a similar manner in peanuts (Figure 4). All three herbicides undergo hydroxylation followed by conjugation to glucose. The glucose conjugate is completely inactive as a herbicide at both the enzyme and whole plant level. We saw no trace of the hydrophobic metabolites of AC 263222 in peanuts and indeed, imazaquin is not selective on peanuts because it is not metabolized.

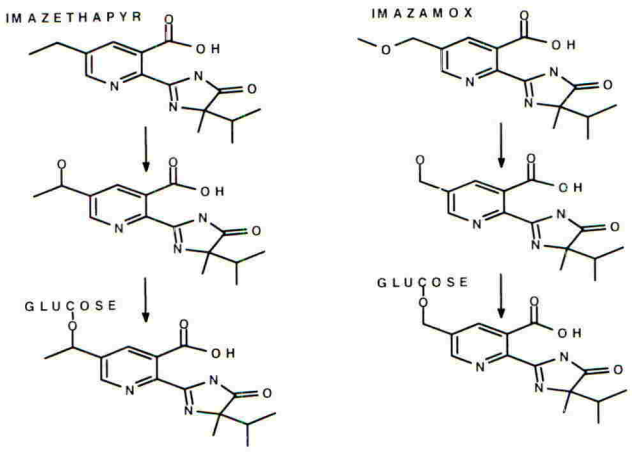

Figure 2: Proposed major metabolic pathways of imazethapyr and imazamox in soybean

Pathways of metabolism of imidazolinone herbicides in maize

In maize, these herbicides are hydroxylated, presumably via a mixed function oxidases. However, unlike soybeans, there is no subsequent rapid conjugation of these herbicides to

glucose. Instead the hydroxylated products accumulate. In addition, the mixed function oxidases do not have the same selectivity as seen in soybeans and peanuts. That is, while imazamox is rapidly hydroxylated in soybeans ($T^{1/2}$=24 h) it is only slowly hydroxylated in maize($T^{1/2}$>72h). Imazethapyr, is rapidly hydroxylated in all three crops ($T^{1/2}\leq$18h). AC 263222 is hydroxylated at a rate intermediate between imazethapyr and imazamox ($T^{1/2}$=48h). These differences appear to be due to differences to the substrate specificity's of the mixed function oxidases in the different species.

Hydroxylating, the parent imidazolinone herbicides reduces the herbicidal activity of these compounds, but it does not eliminate it. The two metabolites found in maize after treatment with either imazethapyr and AC 263222 are still potent inhibitors of AHAS (I_{50} 2.5 uM). These compounds, however, are not as mobile within the plant as their parent herbicides and , hence they are less active at the whole plant level.

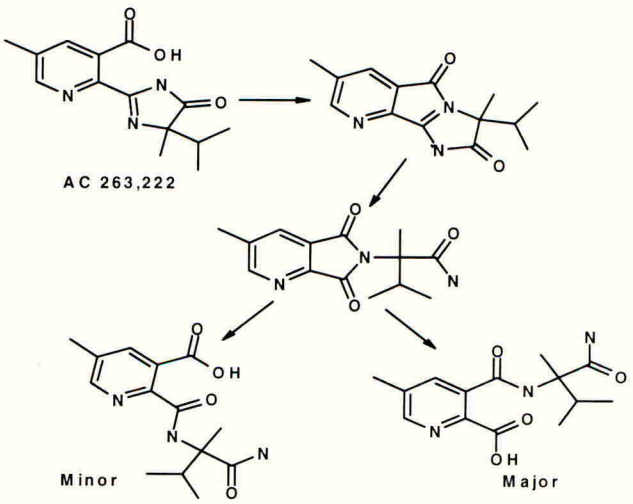

Figure 3: Proposed metabolic pathway of AC 263222 in soybean

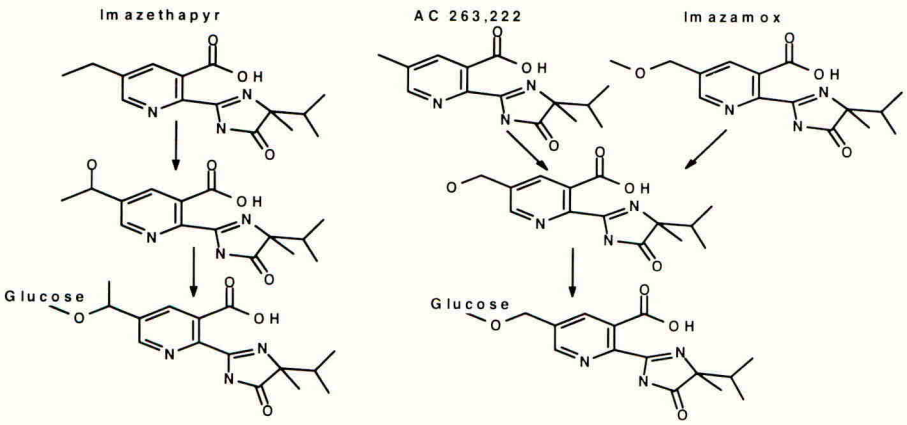

Figure 4: Proposed metabolic pathway of AC 263222 imazamox and imazethapyr in peanut

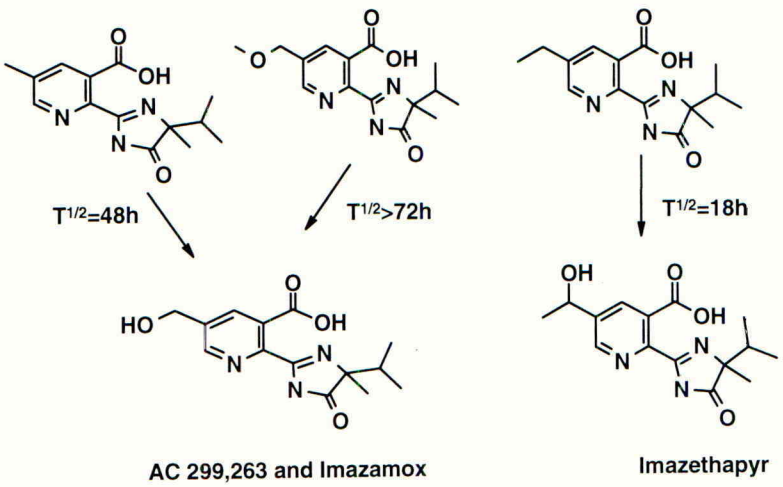

Figure 5: Proposed metabolic pathway of AC 263222 and imazethapyr in maize

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