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Reducing herbicide input to reduce costs and to prevent and overcome resistance

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Executive Summary

We have used two approaches to find ways to lower herbicide use while also endeavoring to preclude and/or overcome herbicide resistance use of herbicide mixtures whereby one component exerts negative cross resistance, fully understand the modes of resistance in a few cases, as well as to design strategies to prevent or delay resistance

Molecular analysis of chloroplast *psbA* gene of susceptible and resistant to atrazine forms was continued for Polish biotypes of *C canadensis* and *E crus-galli* which confirmed the site mutation in resistant forms Weak additional band *"susceptible"* was found in resistant forms Farther increase in the number of perennial species was noted An increase of in the ratio of resistant to susceptible biotypes in populations was observed whenever atrazine was applied even with lowest rate

The work on finding synergists did not lead to a sufficiently active compound to lead to field testing, so only the basic physico biochemical aspects of that research has been followed up Great effort has been put into better understanding the modes of oxidant tolerance in the paraquat We report below how a series of enzymes, higher levels of the polyamine putrescine, as well as elevated transcript levels of the mRNAs are all correlated with resistance

Modelling resistance management strategies has continued, both for cases where herbicides are seed treated to control parasitic weeds and for where low herbicide rates are used

The results of this project has led to a series of publications by both groups, including a joint publication

Section I

A Research Objectives

The control of resistant weeds requires additional herbicides, which erodes agricultural effectiveness and aggravates environmental problems Nowadays, the populace demands that modern agriculture maintain the quantity and quality of final products while using a lowered input of chemicals to the agroecosystem The appearance in agriculture of resistant weeds makes this goal even more difficult to attain Studies on agroecology and biology of resistant weed biotypes is useful in practice as well as theoretically interesting Even if we start to change our conventional agriculture with its high energy input to more sustainable low energy input, a transition period is necessary, characterized by the rational use of some herbicides Our objective is to enter this period of transition almost immediately by finding ways to decrease amounts of applied chemicals utilizing the phenomena of negative cross resistance and/or use synergistically active compounds Herbicides exerting negative cross resistance in resistant weed species should have better environmental and agronomic impact and decreased probability of evolving additional resistances We have performed both laboratory and field experiments with available herbicides and have synthesized new synergists that considerably reduce the herbicide levels in a coalitive manner, where the levels herbicide and synergist are so low that when used alone each has no phytotoxicity To perform this, we have had to better understand a mechanism of resistance Our results are beginning to meet the objectives of both lowering herbicide use and preventing or overcoming resistance

B Research Accomplishments

a Molecular analysis of chloroplast *psbA* gene of Polish resistant and susceptible biotypes of *C canadensis* and of *E crus-galli* (Poland)

Molecular analyses of chloroplast *psbA* gene of susceptible and resistant to atrazine forms were continued for the Polish biotypes of *C canadensis* and *E crus-galli* Procedures of CTAB DNA extraction, PCR amplification, BstXI enzyme digestion and electrophoresis were as described in Annual Report 1995 Both in previous and this year's studies results of electrophoresis of the DNA incubated with the restriction enzyme BstXI confirmed the site mutation in codon 264 and

clearly distinguished the resistant and susceptible biotypes of both species based on the cleavage of DNA at the site mutation However, when a large abundance of PCR product from resistant biotypes, was fractionated from an additional weak band appeared in the position of susceptible band (not cut by BstXI) At the same time DNA samples of the susceptible biotypes of both species were not cut by the enzyme and never showed "resistant" bands

A detailed analysis was undertaken to explain the appearance of the additional *"susceptible"* band in the resistant biotypes Assuming that not all DNA was cut, the incubation was performed with higher quantity of the BstXI enzyme and was extended for longer period of time This modification in the procedure did not change the result and the additional *"susceptible"* band was still present There are several restriction enzymes that are sensitive to various base modifications occurring in the DNA sequence what may reduce the rate of cleavage of the modified sites or even completely block it This however, is not the case with BstXI This enzyme is sensitive to methylation but this type of modification can be excluded when PCR products are used with Taq polymerase because there are no evidence that Taq polymerase modifies nucleotides Therefore, we may assume that the additional band is not a methodology artifact

DNA fragments containing this band were recovered from the agarose gel with PCR products purification kit (Fermentas, Lithuania) to explain the reason of appearance of the additional *"susceptible"* band The excised band was melted and mixed with NaI solution and then bound to silica powder, and eluted into water and divided into two parts The first was used for incubation with BstXI and other for cycle sequence analysis Even though the recovered DNA was from resistant biotypes, little DN was digested suggesting an absence of the site recognized by the enzyme

There was a difference in the *E crus-galli* sequence patterns Although only resistant individuals were analyzed, the nucleotide sequence of this additional fragment (recovered from the gel) was the same as for susceptible biotype, 264 codon mutation Other nucleotides matched the species-specific sequence of nucleotides for susceptible *E crus-galli* reported previously (Annual Report IV) It is believed that *psbA* gene is one of the most conservative gene and confirmation of the presence of this species-specific sequence allowed us to exclude impurities from other sources in our samples In case of *C canadiensis* we also obtained the additional band, but the analysis is still in progress

It is difficult to explain the appearance of "susceptible" band in the resistant plants But some assumption can be made There are many copies of the chloroplast genome in the cell If not all of them are in the mutated version they will not be cut by the enzyme leading to the additional "susceptible" band appearance if much enough DNA material was used for the analysis It also can be taken into a count that the process of reversion of resistant individuals to susceptible takes place especially when having in mind that the studies were conducted under conditions free of the atrazine selection

b Changes in the expression of resistance to atrazine in progeny of resistant biotypes of *C canadensis* and *E crus-galli* (Poland)

Greenhouse tests

These results are summarized in our joint manuscript (Appendix A)

c The expression of negative cross resistance in the field conditions

The expression of negative cross-resistance by *E crus-galli* and by *C canadensis* was studied in field condition in separate experiments for each species During the whole five year experimental period the soil on the plots was not cultivated and the herbicides were applied according to agronomic recommendations The effect of five years of herbicides treatments on weed infestation, community structure and changes in the number of plants per unit area were evaluated on 1 m^{-2} plots in August 1997 and the results are presented in Tables 1 and 2

In the last project year, the number of weed species noted on the plots was slightly higher than in last year 17 vs 13 in *E crus-galli* and 18 vs 17 in *C canadensis* in the number of weed species in *E crus-galli* and in *C canadensis* plots treated with two herbicides decreased to a much

Table 1 Species structure of weed community after four years of treatment for *Echinochloa crus-galli*

Species	Herbicides [kg*ha ^{1}]										
	control	atrazine 2 0	glyphosate 1 6	metolachlor 1 6	fluazifop butyl 0 25	atrazine 1 0 plus glyphosate 0 8	metolachlor 0 8	fluazifop butyl 0 25	atrazine 0 5 plus glyphosate 0 4	metolachlor 0 4	fluazifop butyl 0 06
	plants*m ^{2}										
Achillea millefolium				1 0	0 3						
Amaranthus retroflexus				0 3							
Anthemis arvensis				0 3							
Agropyron repens				3 3	1 3				0 3	1 0	7 0
Capsella bursa pastoris			0 3								
Chenopodium album	2 0			0 5	1 8						
Cirsium arvense	3 5		0 3	1 0	0 3		7 8	0 3	0 3	2 3	0 8
Conyza canadensis		3 8				0 3	1 0	5 0		1 0	
Echinochloa cruss galli	79 0	305 0	348 0	13 8	52 5	273 3	24 5	177 0	330 0	41 5	238 0
Geum urbanum					1 3						
Lamium sp	1 3		0 8	0 3	4 5					0 3	
Poa annua	12 0	1 3	0 8	11 8	23 5	1 5	3 5	1 8	0 8	2 5	
Polygonum aviculare					0 3						
Senecio vulgaris			0 5	4 3	4 8						
Sonchus oleraceus	0 3										
Stelaria media					0 3						
Taraxacum officinale	17 0			9 0	16 8	0 3	1 5	1 3	11 0	0 8	1 0
Total [a]	**115 0ab**	**310 0d**	**350 5d**	**45 3a**	**107 3ab**	**275 3cd**	**38 3a**	**185 3bc**	**342 3d**	**49 3a**	**246 8cd**
Percent of control	**100 0**	**269 6**	**304 8**	**39 4**	**93 3**	**239 4**	**33 3**	**161 1**	**297 7**	**42 9**	**214 6**

[a] Means followed by the same letter are not significantly different at the 10% level according to Duncan s multiple Range Test

Table 2 Species structure of weed community (number of plants) after fourth year of herbicide treatment for *Conyza canadensis* control

Species	Herbicides [kg*ha^{-1}]											
	control	trazine	glyphosate	2 4 D	pyridate	atrazine 1 0 plus			atrazine 0 5 plus			
		2 0	1 6	1 8	1 0	glyphosate 0 8	2 4 D 0 9	pyridate 0 5	glyphosate 0 4	2 4 D 0 45	pyridate 0 25	
	plants*m^{-2}											
Achillea millefolium	3 3				0 3							
Amaranthus retroflexus	0 3		1 3	0 3	0 3				9 8	0 3	1 5	
Anthemis arvensis	1 0				0 5							
Artemisia vulgaris					0 3							
Capsella bursa pastoris	0 8		1 8									
Cerastium viscosum	5 0		0 5						1 0			
Chenopodium album	5 3		9 3	7 0	1 0				0 8			
Cirsium arvense		1 8		0 5	1 8		0 3	3 5	1 3		0 5	
Conyza canadensis	2 5	8 3	4 0	2 8		0 3	0 5	0 3	0 3	0 3		
Daucus carota						0 3						
Echinochloa cruss galli			3 3					2 3				
Epilobium adenocaulon	1 8	14 8	1 0		0 8	12 3	0 8		0 3			
Equisetum arvense							1 5	0 8	4 5			
Lamium sp	2 3		3 3	8 5	25 3						0 3	
Plantago maior	1 0				6 3							
Poa annua		5 8		23 8	2 0	1 0	16 3	5 8		21 5	5 3	
Polygonum sp	0 8			2 1	0 8					0 8	0 8	
Rorippa silvestris			3 5		12 0							
Rumex acetosella	1 3									3 0	3 5	
Senecio vulgaris	1 0		10 3	5 8						0 5	2 0	
Sonchus arvensis					0 5							
Sonchus oleraceus	14 0						0 3		0 3	0 3		
Stellaria media	13 0		3 8	0 3	8 0							
Taraxacum officinale	18 5	0 3	43 3	33 8	39 5	0 3		0 8	39 5	15 5	23 0	
Veronica persica	1 0				0 3							
Viola arvensis	0 3					0 3						
Total [a]	**72 8cde**	**30 8ab**	**85 0de**	**84 8de**	**99 3e**	**14 3a**	**19.3ab**	**13 3a**	**57 5bcd**	**42 0abc**	**36 8abc**	
Percent of control	**100 0**	**42 3**	**116 8**	**116 5**	**136 4**	**19 6**	**26 5**	**18 3**	**79 0**	**57 7**	**50 5**	

[a] Means followed by the same letter are not significantly different at the 10% level according to Duncan s multiple Range Test

higher degree than when treated with single compound This was true even when the herbicides were reduced to a quarter the recommended rates

The *E crus-galli* plots were infested with annual *Poa annua* and *Senecio vulagaris* and perennials *Taraxacum officinale, Cirsium arvense* and *Agropyron repens* (Table 1) Negative cross resistance was exerted on *E crus-galli* in the field plots that were treated with metolachlor, fluazifop-butyl and in combination with atrazine (1 0 kg•ha^{-1}) plus metolachlor (0 8 kg•ha^{-1}) and atrazine (0 5 kg•ha ^{1}) plus metolachlor (0 4 kg•ha ^{1}) Metalachlor exerted results shows very strong negative cross resistance, at the lowest rate (0 4 kg•ha^{-1}) applied together with atrazine Superior control of resistant plants of resistant *C canadensis* was obtained with pyridate alone and together with atrazine (Table 2) These results well correspond to data obtained in the greenhouse studies (Appendix A)

d Synthesis and characterization of synergists that prevent loss of toxicity of oxidant generating herbicides (Israel)

Oxidant-generating herbicides cause the generation of oxygen radicals that rapidly cause cell and plant death We have reported how enzymes of the Halliwell-Asada pathway can detoxify these radicals as they form, protecting a plant from damage In our previous reports we discussed how chelators that remove copper from the first enzymes of the pathway can prevent oxidant detoxification The results of the synthesis program, based on dithiocarbamate derivatives, are summarized in Appendix B As none of these compounds proved to have a sufficient level of synergy to consider for use in the field, the applicable side of such studies was terminated, and the graduate student working on this has been working only on the physical-biochemical aspects of the interaction of these chelators with pure superoxide dismutase, which is beyond the aegis of this project (Rogachev et al ,1998b)

More effort has been exerted in the other aspects of this project

e Inducibility of enzymes (and their mRNAs) that detoxify active oxygen species (Israel) (in conjunction with a sister project funded by GIARA)

We have shown that both the magnitude of resistance in a strain of the weed *Conyza bonariensis* that has evolved resistance to the oxidant generator paraquat changes throughout development (see previous reports) Only at the time of maximum resistance (8-11 weeks) was it possible to measure differences in the enzymes that detoxify active oxygen

Others have shown that oxidant stresses can enhance tolerance to oxidant generators while elevating of the oxidant-tolerating enzymes We also performed a series of experiments to ascertain whether this was the case in *Conyza* of different ages and resistance to paraquat

<u>Enzymes</u>

Pre-stressing the plants with an oxidative stress (the herbicide paraquat) enhances the resistance of the resistant *Conyza* strain This treatment also increased the levels of the oxidant-protecting enzymes, including two enzymes that we had not studied previously in *Conyza* monodehydroascorbate reductase, and dehydroascorbate reductase (Ye and Gressel 1998)

We followed this up by assaying another enzyme, glutathione peroxidase implicated by others to be related to stress tolerance in annual and plant systems In plants this can only be considered to be a tenuous implication by gene transcript similarity to an animal enzyme and by an indirect enzyme assay fraught with possible artifacts Months of research led to a direct assay for glutathione peroxidase and we indeed found that there were constitutively elevated levels of this enzyme in the paraquat-resistant biotype of *Conyza bonariensis* Furthermore, a 24 h prestressing of the plants with 1μM paraquat induced even higher levels of this enzyme (Ye et al, 1998a) Glutathione peroxidase can directly accept electrons from peroxide, augmenting the previously reported effects of ascorbate peroxidase and mono and hydroascorbate reductases

Glutathione reductase is key to regenerating the scavenging power of all these enzymes We therefore wished to see whether a specific isozyme of this enzyme is elevated, and found the gel assays used by others as severely wanting We developed new method that allows the selective staining and quantification of glutathione reductases in cell extracts following acrylamide gel

electrophoresis The method is based on modifications of two previous procedures, it uses DTNB [5,5'-dithio-*bis*-(2-nitrobenzoic acid)] to develop a yellow color on reaction with GSH formed from the NADPH-dependent reduction of oxidized glutathione This is followed by specific counter-staining of glutathione reductase with dichlorphenolindophenol / nitroblue tetrazolium The use of DTNB in the initial staining step inhibits enzymes other than glutathione reductase, that could be stained with the dichlorphenolindophenol /nitroblue tetrazolium counterstain Enzymes such as thioredoxin reductase, which can directly reduce DTNB with NADPH may be non-selectively stained by this new procedure Plant ferredoxin-thioredoxin reductase is not reduced by NADPH and therefore does not appear Glutathione reductase stains much quicker with DTNB in the presence of GSSG than thioredoxin reductase, allowing them to be distinguished, if parallel gels are run without GSSG, where the two enzymes react at the same rate The sequential use of two staining procedures results in distinct, sharp permanent bands that can be used to quantify the activity of glutathione reductase while precluding artifacts generated by the previous methods The new method is also far more sensitive than the older methods (Ye et al , 1997a)

We have DNA probes for the genes coding for some of these enzymes In general, whenever there was an increase in enzyme activity, we found an increase in mRNA by northern blotting analyses These results are summarized in Ye et al (1998b) It is clear that the transcript levels of the mRNAs are elevated more or less in parallel with the activity levels, both constitutive and induced, in the oxidant resistant *Conyza* Additionally, we were recently able to obtain a probe for the putative glutathione peroxidase of *Citrus,* which we used to challenge Conyza *mRNA* It is clear from the blots that the resistant biotype can be induced by low level oxidative stress to produce more mRNA correlating with the glutathione peroxidase activity data

Polyamines

Polyamines have also been noted as scavengers of radicals, and there was even a report that they are elevated in a paraquat-resistant *Conyza canadensis* from Hungary (Szigeti et al J Environ Sci Health B31 599-604, 1996) We validated and extended their findings by measuring polyamine levels at two weeks (when the paraquat resistant *Conyza bonariensis* is mildly resistant) and at 8-10 weeks when it is highly resistant Only a change in putrescine was observed The putrescine level was >2 times higher in the resistant biotype at two weeks and >5 times higher at ten weeks We are developing a hypothesis that we will study, that the elevated putrescine level is responsible for the mild resistance (as we and others have not been able to measure differences in the oxidant detoxifying enzymes at two weeks), whereas the detoxifying enzymes give rise to the high level resistance at ten weeks (Ye et al 1997b)

f Strategies for resistance management

A key part of this project has been to design and test strategies for resistance management (see above and Appendix A) We have been following up and verifying a previous strategy we designed - using the negative cross resistance exerted by some herbicides We have been designing novel strategies to deal with newer types of resistance, especially those brought about by using low rates of herbicides, which specifically select for polygenic resistances (Gressel et al 1996, Gardner et al , 1998) Indeed, the newest and most frightening case of resistance, to the herbicide glyphosate (Gressel 1997b) may be due to this mechanism, and the strategies outlined may be helpful in assuaging resistance, without using megadoses of herbicides, i e keeping in tune with the lowering herbicide use, as an integral part of this project

Additionally, we have delineated new strategies to deal with evolution of resistance to herbicides where a specific case dose rates were lowered by using seed treatments of herbicide (Gressel, Segel and Ransom, 1996) As these strategies have been published or are in press, they will not be summarized at length herein

B Publications based on this research

Abayo, G O , J K Ransom, J Gressel, and G D Odhiambo, (1996) *Striga hermonthica* control with acetolactate synthase inhibiting herbicides seed-dressed to maize with target site resistance Advances in Parasitic Weed Research, Junta de Andalucia, pp 762-768

Ciarka D and S W Gawronski 1996 Secondary effects following acquisition of triazine resistance in weeds Proceedings of the International Symposium on Weed and Crop Resistance to Herbicides Univ of Cordoba (R De Prado, J Jorrin and G Marshall, eds) p 149 -151

Gadamski G , Ciarka D , Gawronski S W 1996 Molecular survey of Polish resistant biotypes of weeds Proceedings of the Second International Weed Control Congress, Copenhagen, Denmark p 547- 550

Gadamski, G , D Ciarka, J Gressel, S W Gawronski (1998) Negative cross-resistance in trazine resistant biotypes of barnyardgrass (*Echinochloa crus-galli*) and horseweed (*Conyza canadensis*) Weed Science (submitted)

Gardner, S N , J Gressel, M Mangel (1998) A revolving dose strategy to delay the evolution of both polygenic and monogenic pesticide and drug resistances Intl J Pest Management (in press)

Gawronski S W and G Gadamski 1996 Negative cross resistance in the weeds with different resistance mechanism Proceedings of the International Symposium on Weed and Crop Resistance to Herbicides Cordoba (R De Prado, J Jorrin and G Marshall, eds) p 80-81

Gressel, J (1995) Creeping resistances The outcome of using marginally effective or reduced rates of herbicides Brighton Crop Protection Conf - Weeds pp 587-590

Gressel, J (1996) Fewer constraints than proclaimed to the evolution of glyphosate-resistant weeds Resistant Pest Management 8 (1) 2-5

Gressel, J (1997) Burgeoning resistance requires new strategies *in* Weed and Crop Resistance to Herbicides (R De Prado, ed), Kluwer, Amsterdam, pp 1-14

Gressel, J , J K Ransom and E A Hassan (1996) Biotech-derived herbicide-resistant crops for third world needs Ann New-York Acad Sci 792 140-153

Gressel, J , S Gardner, and M Mangel (1996) Prevention vs remediation in resistance management *in* Molecular Genetics and Evolution of Pesticide Resistance, (T M Brown, ed), Amer Chem Soc , Washington, D C , pp 169-186

Gressel, J , L E Segel and J K Ransom (1996) Managing the delay of evolution of herbicide resistance in parasitic weeds Intl J Pest Management 42 113-129

Gressel, J (1996) $ynergizing herbicides Proc 2nd Intl Weed Control Cong - Copenhagen pp 1211-1221

Rogachev, I , V Kampel, V Gusis, N Cohen, J Gressel and A Warshawsky (1998a) Synthesis, properties and use of copper-chelating amphiphilic dithiocarbamates as synergists of oxidant-generating herbicides Pestic Biochem Physiol (in press)

Rogachev, I , L Weiner, J Gressel, and A Warshawsky (1998b) Inhibition of Cu/Zn superoxide dismutase activities by amphiphilic dithiocarbamates (in manuscript)

Ye, B and J Gressel (1998) Transient oxidant inducibility of antioxidant enzymes and oxidant resistance to higher levels in paraquat-resistant *Conyza bonariensis* (submitted)

Ye, B , C Gitler and J Gressel (1997a) A high sensitivity, single gel, PAGE method for quantification of glutathione reductases Anal Biochem 246 159-165

Ye, B , H Muller, J Ziang and J Gressel (1997b) Elevated putrescine levels and putrecine generating enzymes constitutively correlated with oxidant stress resistance in *Conyza bonariensis* and wheat Plant Physiol 115 1443-1451

Ye, B , H Tzivon, Z Faltin, G Ben-Hayyim, Y Eshdat, and J Gressel (1998a) Involvement of glutathione peroxidase in oxidative stress resistance in *Conyza* determination by a direct fluorometric assay (submitted)

Ye, B , H Tzivon, , and J Gressel (1998b) Coordinately-induced elevated levels of mRNAs encoding antioxidant enzymes correlate with induced enzyme levels and oxidative stress tolerance in paraquat-resistant *Conyza bonariensis* (in manuscript)

C Scientific Impact of Collaboration

The senior investigators met at meetings in Poland in 1995 and most recently in the U S in Jan 1996 and later at the International Weed Control Congress in Copenhagen, where both presented findings emanating from this project Additionally, S Gawronski went to Israel in 1996 under the auspices of the project Below is his report

The publications list from this project and its sister projects, including our joint publication (Appendix A) summarize as best one can, the scientific impact of the project

D Project Impact

Developing countries have generated some of the worst problems of resistance due to overuse of single herbicides This has become evident especially in Hungary, Poland, India and Australia (which some consider somewhat backward in herbicide-use practices, if not "developing") Dr Gawronski's work is a step in the right direction towards rationale herbicide use, and his findings are ready for limited field testing by farmers The impact from the collaboration so far is from the theoretical framework promulgated by the Rehovot group that the Warsaw group is reducing to practice The Rehovot group has continued their theoretical work modelling methods of delaying resistance, as an integral part of this project

Section II

A Managerial Issues

No issues beyond those discussed in earlier reports

B Budget

No major changes were made

C Special Concerns

None

D Collaboration, Travel, Training and Publications

See Sections I B, IC There was also considerable collaboration by phone, letter and fax and email

E Request for A I D / BOSTID Actions

None

Appendix A

Submitted to Weed Science

Negative cross-resistance in trazine resistant biotypes of barnyardgrass (*Echinochloa crus-galli*) and horseweed (*Conyza canadensis*).

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Abstract Negative cross resistance at the whole plant level was studied in *C canadensis* and *E crus-galli* two very important global weeds as it can be a most useful tool in cost and evolutionary effective resistance management Seeds of triazine susceptible as well as resistant biotypes collected in orchards treated continuously with atrazine, were treated in a greenhouse, with herbicides from different chemical groups triazines, benzothiadazole, phenyl-pyridazine, arylophenoxy-propionates, cyclohexanodiones, phenoxycarboxylic acids, pyridate carboxylic acids, phosphinic acids, glyphosate, chloracetamides, sulfonoureas, bipyridyliums Eleven of the 18 herbicides tested exerted significant negative cross resistance where the resistant biotype was affected at 0 03 to 0 67 the concentrations that affected the triazine sensitive type Bentazon and fluoroxypyr, which separately exerted negative cross resistance were tested in mixture No synergism was found between these compounds and a resistance index did not indicate better control of resistant biotypes Still, using this mixture allows a reduction in environmental impact, and could assist in controlling a broader spectrum of other weeds

Key words negative cross resistance, atriazine resistance, herbicide mixture, mechanisms of negative cross resistance

1 INTRODUCTION

Monoculture is all too common in conventional agriculture It can contribute to soil erosion, as well as water and air pollution, while enhancing the rate of evolution of resistance when there is also no rotation of pesticides It is necessary to decrease the input of chemicals to agroecosystems to resolve some of these problems and to prevent new ones from emerging The evolution of resistant weed biotypes makes these goals even more difficult to attain, especially if yields and crop quality have to be maintained

More than 60 different weed species have evolved triazine resistance creating a very long and still growing list since the first reported case in 1970 (Heap, 1997, Powles and Holtum, 1994, Ryan, 1970) These weeds are estimated to cover 6M ha, where alternative herbicides are added to the triazines, to attain broad spectrum and resistant weed control, with added costs to farmers Triazine-resistant biotypes have evolved first in monocultures continuously treated with atrazine continuously for 6-10 years (Gressel and Segel, 1990) The mechanism of atrazine resistance in weeds is almost invariably based on the point, mutation in codon 264 of the chloroplast *psbA* gene leading to an amino acids replacement of serine by glycine (Hirschberg and McIntosh, 1983, Gronwald, 1994, Gawronski *et al* , 1992) There is only one exception found in the field where a serine 264 to threonine change evolved, in *Portulaca oleracea* treated with linuron

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continuously for 25 years (Masabni and Zandstra, 1996) This biotype was highly cross resistant for atrazine Resistant biotypes of *Conyza canadensis* and *Echinochloa crus-galli* both with the 264 serine to mutation survive >32 times more atrazine than their susceptible biotypes, precluding control with atrazine (Gadamski *et al*, 1996) Fortunately, the mutant herbicide binding domain can allow other herbicides to bind better (Kless et al, 1994, Sobolev and Edelman, 1995), leading to phenomenon of negative cross-resistance (collateral sensitivity) where individual resistant to one group of pesticides have a higher sensitivity to other chemical groups The unfitness of resistant biotypes also renders them more susceptible to some herbicides than the susceptible biotype, also leading to negative cross-resistance Our interests focussed on negative cross resistance in plants resistant to herbicides as a weed management strategy and resistance management tool as proposed, on theoretical grounds by Gressel and Segel (1990) Negative cross resistance for herbicide-resistant biotypes was first seen at the level of isolated plastids, some PSII inhibitors were more effective on plastids from resistant plants (Fuerst *et al*, 1986, Oettmeier *et al*, 1982) Studies of negative cross resistance at the whole plant level are of great importance if this phenomenon is going to be utilized in practice So far, not many data are available in the literature on the negative cross resistance for the whole plants (Arlt and Juttersonke, 1992, De Prado *et al* , 1992, Parks *et al*, 1996) Many studies on the control of atrazine-resistant weeds did not test the same chemicals on the susceptible biotype and thus much potentially useful information has been lost, that could be valuable in developing resistance management strategies

Another is using mixtures of herbicides powerful method of weed control This technique has important advantages as herbicides can often be applied in lower rates, especially when synergistic interactions appear (Streibig, 1987, Gressel, 1990) Mixtures can be environmentally safer and less expensive than continuous application of one compound, and can often control a broader spectrum of weed species Moreover resistant individuals are effectively controlled and resistant populations rarely evolve when herbicides with different mechanisms are used simultaneously

The goal of this study was to search for herbicides that exert negative cross-resistance atthe whole plant level for control atrazine-resistant biotypes of a grass *E crus*-galli and a broad leaf species *C canadensis* Additionally, a mixture of bentazon and fluoroxypyr was examined to ascertain if these herbicides are synergistic in exerting negative cross resistance Together these herbicides have wide spectrum of activity and well control resistant biotypes

The results of this study can also be useful as a paradigm for selecting herbicides that are effective in controlling of atrazine-resistant weeds, but also provides information about the possible mechanisms

2 MATERIALS AND METHODS

Plants of resistant and susceptible biotypes of *E crus-galli* and *C canadensis* were used in these studies Seeds of resistant biotypes were collected from plants grown in orchards that were continuously treated with atrazine for more than ten years and from susceptible plants from neighboring places free of such treatment As pollen transmission of this maternally-inherited resistance is rare, there is little likelihood of gene flow into these neighboring populations Plants were grown in the greenhouse in 12-cm diameter pots containing a soil mixture of peat-moss and sand (2 1 v/v) The herbicides used, and their modes of action, are shown in Table 1 Based on results of our previous work (Gawronski *et al*, 1992) in selecting herbicides for this study, we omitted those that are already known as not exerting negative cross resistance Herbicides were

applied either by mixing with soil just before seedlings were transplanted to the pots or sprayed at the 4-5 leaf growth stage Depending on the compound, 5-6 geometrically progressing rates were used The median rate was near the recommended one in practice, and a pesticide free treatment was used as the control All experiments were repeated at least two times The plants were harvested, and biomass recorded three to four weeks after application of herbicides The level of resistance to atrazine and the effectiveness of the herbicides were estimated on the basis of 50 % reduction of added plant growth (GR_{50}) (Gawronski *et al*, 1995) Eight replications with three plants per replication were used per treatment, i e 24 plants per treatment The data are presented using probit analysis (Finney 1952) The resistance index (RI) was calculated as the ratio of the GR_{50} for the resistant to the GR_{50} for the susceptible A value of RI below 1 0 denotes a higher sensitivity of the atrazine resistant biotype than the susceptible one, i e negative cross resistance The lower the RI the greater the magnitude of negative cross resistance

Herbicide mixture

In the experiment where a mixture of herbicides was tested, plants of *C canadiensis* were treated with bentazon and fluoroxypyr Together, these compounds control a wide spectrum of weed species, are stable and effective, and exerted moderate negative cross resistance Plants were grown and treated as described earlier with the exception that herbicides in the mixture were used in 1 1 (w/w) proportion, and the rates from 45g ha^{-1} to 750 g ha^{-1} were increased geometrically

3 RESULTS

Eleven of 18 tested herbicides exerted negative cross resistance with values of RI ranging from 0 03 to 0 67 for *E crus-galli* (Table 1) and *C canadensis* (Table 2) The RI for the remaining herbicides was either 1 0 or higher for three and six other compounds, respectively showing appearance of other phenomenon that is cross resistance The highest RI (GR_{50}>32 kg ha^{1} for resistant biotypes of both species studied was noted for atrazine, as expected

The greatest negative cross resistances were found for fluazifop-butyl (RI=0 03) in *E crus-galli* and MCPA (RI=0 12) in *C canadensis* Both of these herbicides were the most effective ones A low rate of 10 g ha^{1} reduced growth of both resistant biotypes by 50% Among other chemical families, low resistance indices were found for the chloracetamide metolachlor, (RI=0 49) and the phenylpyridazine, pyridate (RI=0 14) The benzothiadazole, bentazon and the bipiridylium, diquat controlled resistant biotypes at half of the rate used for susceptible ones Glyphosate exerted strong negative cross resistance on *C canadensis* but a rather weak one on *E crus-galli* Glufosinate was only weakly effective and only on *E crus-galli* A wide variation of RI was noted not only for different chemical families but even for herbicides that belong to the same group No significant negative cross resistance was observed for 2,4-D (RI=0 95) while it was very high for the analogous MCPA Similar situations were observed for graminicides used to control *E crus-galli* where four out of seven used herbicides were less effective for resistant than for susceptible biotypes that led to a cross-resistance The highest RI=1 81 in this group of herbicides was noted for fenoxaprop-ethyl which is rather hard to understand

An analysis of negative cross resistance was used as the basis for selection of the herbicides to be used in mixture The resistance index for both bentazon and fluoroxypyr were around 0 5 Therefore, their combination in mixture should sufficiently control biotype of an atrazine resistant *C canadiensis* resistant to atrazine The mixture was at first evaluated in relation to the herbicide input to the environment The mixture was more than twice as active

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(GR_{50}=0 60/0 29 kg ha^{-1} for susceptible / resistant biotype) than fluoroxypyr alone (GR_{50}=1 38/0 72 kg ha^{-1}) but still three times less effective than bentazon used alone (GR50=0 19/0 10 kg•ha^{-1}) (Table 2) This effect was observed both for susceptible and resistant to atrazine biotypes of *C canadensis* The mixture was also analyzed according to changes of RI The level of negative cross resistance for the mixture (RI=0 48) was almost the same as for herbicides used separately (the RI for bentazon and fluoroxypyr were both near 0 5) There was clearly no interaction between these compounds that further lowered the RI, i e enhanced the negative cross resistance

The GR_{50} values were lower than expected, probably due to the near optimal growing conditions in the greenhouse Therefore, plants were delicate and more sensitive to herbicides The temperature and humidity in the greenhouse was slightly higher than is usual in the field, and herbicide uptake and metabolism could have been altered as well

4 DISCUSSION

The phenomenon of negative cross resistance was more common than expected among the herbicides used, those including herbicides that do not affect PSII This is probably due to the fact that in atrazine- resistant biotypes, are typically less fit, than the susceptibles, and thus may be more susceptible to secondary stresses Several processes on different levels of plant organization are altered Changes in biology of growth and reproduction of resistant biotypes are well known (Gronwald, 1994 Ciarka *et al*, 1995, Ciarka, in preparation) Reduction of the fundamental life process i e photosynthesis has been found when resistant biotypes have lower ATP and NADPH production and thus lower CO_2 fixation (15-25%) because of the slower rate of electron transfer in PSII (Holt et al , 1981, Ahrens and Stoller, 1983) Changes in chloroplast membrane structure connected with higher level of unsaturated acids have been measured in resistant plants (Pillai and St John, 1981, Burke *et al*, 1982) These changes are pleiotropic results of a single target-site mutation in the *psbA* gene (Gronwald 1994) The same mutation has been found for both species used in this study (Gawronski *et al*, 1992, Gadamski *et al*, 1996) The reduced fitness of the triazine-resistant biotypes cannot completely explain the phenomenon, as there then should be negative cross resistance to all herbicides, irrespective of site of action

Aryloxyphenoxypropionates and cyclohexanediones graminicides inhibit lipid biosynthesis by blocking acetyl-coenzyme A carboxylase (ACCase) mediated acetate conversion into fatty acids via herbicide binding to ACCase (Devine *et al* 1993) It is not clear why there are RI differences among these herbicides The only common denominator is ACCase inhibition, leading to decrease to membrane synthesis and the thylakoid membrane activity of PSII inhibitors There is no evidence that de novo fatty acid synthesis occurs outside of plastids Therefore, the phenomenon of negative cross resistance might result from the changes of membrane composition Plants resistant to triazines have altered thylakoids and with increased proportion of unsaturated to saturated fatty acids (Pillai and St John, 1981, Burke *et al*, 1982) Lipophillic herbicides may penetrate more easily to resistant plants, as proposed by Chapman *et al* (1985) Thus, higher herbicide solubility in organic solvents might be responsible for lower RI Acetone solubility of arylophenoxypropionates showed strong negative correlation (R^2=0 98%) with the level of negative cross resistance (Fig 1) One must test many compounds from each chemical family as it was done for arylophenoxypropionates to be able to extrapolate this hypothesis to other herbicide families The chemical structure of herbicides could be considered in the respect with the exception of fenoxaprop ethyl Arylophenoxypropionates are

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used as esters to facilitate absorption into plant tissue The resistance indices seem to relate to the length of alcohol group building these esters (Fig 1) Peculiarly hydroxylation to the active ACCase inhibitor takes place soon after the herbicide enters plant cell tissue, before reaching the chloroplast site of action Therefore, ACCase-related phytotoxicity is associated with free acids and negative cross resistance is correlated with their ester solubility This correlation cannot yet be extrapolated to the cyclocyclohexanediones, which also inhibit ACCase at overlapping binding sites

Glyphosate reliably exerted negative cross resistance to a much higher degree on *C canadensis* than on *E crus-galli* (Tables 1 and 2) Glyphosate inhibits the EPSP synthase both before and especially after glyphosate it enters the chloroplast (Devine *et al*, 1993) as little is known about chloroplasts

Strong negative cross resistance was found for bentazon and pyridate (Table 1, 2 and De Prado *et al*, 1992, Parks *et al*, 1996) Moreover, pyridate is used in practice for resistance management in the field Bentazon and pyridate both inhibit photosynthesis be considered as good candidates for negative cross resistance because they seem to act like phenolic type of PSII inhibitors, and bind more tightly to the mutated *psbA* gene product, and thus can exert greater negative cross resistance (Gressel and Evron, 1992)

The graminocide metolachlor, was excellent for controlling resistant *E crus-galli* (Table 1) (just as the broad leaf pyridate had been for *C canadensis*) Metolachlor exerted good negative cross resistance in greenhouse and even better in the field conditions (Gadamski *et al* in preparation) However, the mechanisms involved in negative cross resistance exerted by metolachlor has no logical explanation at present

Mixture of bentazon and fluoroxypyr exerted the same negative cross resistance but not synergistically with each other as hoped Lack of synergy is due to mechanisms of negative cross resistance are involved in these two compounds

There is no universal negative cross resistance mixture that can be recommended for use when biotypes resistant to atrazine appear Still, this mixture controls wider spectrum of weed species and thus is more reliable than a single herbicide The mixture is cheaper in each herbicide used separately and what is even more important this mixture reduces the input of each herbicide into the environment

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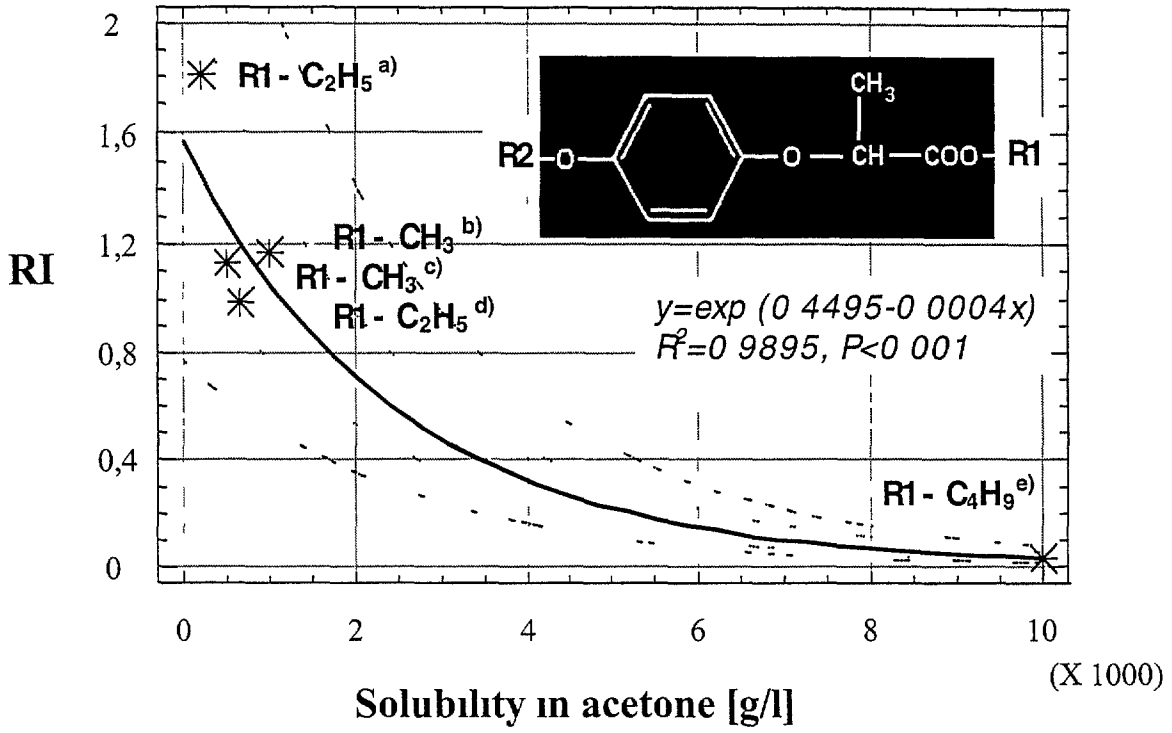

Fig 1 The most acetone soluble arylophenoxy-propionates exert the greatest negative cross resistance The resistance index exerted by arylophenoxy-propionates (RI) is plotted against the solubility of these herbicides in acetone Letters indicating herbicides (a) fenoxaprop-ethyl, (b) haloxyfop-methyl, (c) diclofop-methyl, (d) quizalofop-ethyl, (e) fluazifop-butyl Additional lines indicate the 95% confidence intervals of the regression (dotted) and the 95% confidence intervals of the prediction (dot-dashed)

Table 1 Negative cross resistance exerted by selected herbicides on *Echinochloa crus-galli* L

Target of action	Chemical family[a]	Herbicide	GR_{50}[b]		RI_{50}[c,d]
			suseptible	resistant	
Photosystem II	triazines	atrazine	0 60	32 00	53 33
ACCase	aryloxyphenoxy- propionates	fluazifop-butyl	0 21	0 01	0 03a
		quizalofop-ethyl	0 03	0 03	0 99d
		diclofop-methyl	0 28	0 31	1 13de
		haloxyfop-methyl	0 03	0 03	1 17de
		fenoxaprop-ethyl	0 17	0 31	1 81f
	cyclohexanediones	sethoxydim	0 09	0 04	0 47b
		cycloxydim	0 07	0 12	1 65e
EPSP synthase	glycines	glyphosate	0 20	0 19	0 95c
Cell division	chloracetamides	metolachlor	1 30	0 64	0 49b
ALS	sulfonylureas	chlorsulfuron	0 00	0 00	0 67bc
Glutamine synthase	phosphinic acids	glufosinate	0 32	0 20	0 63bc

[a] Accase-acetylCoA carboxylase, EPSPsynthase-enolphosphate, shikimate phosphate synthase, ALS-acetolactate synthase

[b] GR_{50} – rate of herbicide [$kg*ha^{-1}$] reducing plant biomas by 50%

[c] RI_{50} = GR_{50} of resistant biotype / GR_{50} of susceptible biotype

[d] The RI for herbicides followed by the same letter are not significantly different at the P<0 05 level using Duncan,s Multiple Range Test data for atrazine have not been included

Table 2 Negative cross resistence exerted for selected herbicides on *Conyza canadensis* Cronq

Target of action	Chemical family [a]	Herbicide	GR_{50} [b] susceptible	GR_{50} [b] resistant	RI_{50} [c,d]
Photosynthesis at PSII	triazines	atrazine	0 95	32 00	33 68
	benzothiadazole	bentazone	0 19	0 10	0 53c
	phenyl-pyridazine	pyridate	1 69	0 23	0 14ab
Gluthamine synthetase	phosphinic acids	glufosinate	0 11	0 16	1 45e
Auxinic	phenoxycarboxylic acids	2,4-D	0 11	0 11	0 95d
		MCPA	0 08	0 01	0 12a
	pyridinecarboxylic acids	fluoroxypyr	1 38	0 72	0 52c
EPSP synthase	glycines	glyphosate	1 20	0 46	0 38bc
Photosystem I	bipyridyliums	diquat	0 04	0 02	0 54c
Herbicide mixture (1 1 w/w)					
PSII	benzothiadazole	bentazone			
Synthetic auxins	pyridinecarboxylic acids	fluoroxypyr	0 60	0 29	0 48c

[a b c d] Symbols as in table 1

Appendix B

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Synthesis, Properties, and Use of Copper-Chelating Amphiphilic Dithiocarbamates as Synergists of Oxidant-Generating Herbicides

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The effect of herbicides that act by generating active oxygen species is often mitigated by the enzymes of the Halliwell Asada oxygen detoxification pathway Use of compounds interfering with this pathway synergizes herbicides allowing the killing of weeds at lower herbicide doses We have used different dithiocarbamate chelators (Dtcs) capable of removing copper from superoxide dismutase the first enzyme of the pathway suppressing its action Dtcs with different hydrophilic lipophilic properties (sodium salts of ethyl (2 (2-ethoxyethoxy)ethyl) butyl (2 (2-ethoxyethoxy)ethyl) hexyl (2 (2 ethoxyethoxy)ethyl) and dibutyldithiocarbamic acids) were synthesized so that they might penetrate plant cuticles better than sodium diethyldithiocarbamate Octanol water distribution ratios and the stabilities of their copper complexes were determined All Dtcs tested had a high affinity for copper The Dtcs did not prevent iron-catalyzed Fenton reaction leading to hydroxyl rather production *in vitro* Therefore they would not protect plants from the oxidative damage caused by herbicide as do compounds that complex iron and thereby prevent the phytotoxic iron catalyzed Fenton reaction These amphiphilic compounds show some synergistic activity *in vivo* with paraquat as well as lactofen herbicides that generate active oxygen species by different mechanisms The results demonstrate that dithiocarbamates have potential as synergists for oxidant generating herbicides ©1998 Academic Press

radical

INTRODUCTION

One way to use less herbicide is to ensure that weeds cannot detoxify the herbicide or the phytotoxic compounds generated by herbicide use (1), by using otherwise inactive compounds to block the detoxification pathways (1, 2) The effects of the inhibitors of metabolism together with the herbicides are well beyond the sums of their activities alone Thus the metabolic inhibitors act as "synergists ' Major herbicides (bipyridilliums that interact with photosystem I the many herbicides that block photosystem II, and others that block protoporphyrinogen oxidase (3)) have one property in common, they cause the production of active phytotoxic oxygen species (4–7) There are wide differences among plant species in their susceptibility to these herbicides plant resistance can be a result of two major factors *in vitro* degradation of the herbicide, and/or detoxification of the active oxygen species by one or more plant enzyme pathways such as the Halliwell-Asada pathway (8, 9)

Compounds inhibiting the enzymes of the Halliwell-Asada pathway, in both *in vitro* and *in vivo* systems, allow the killing of weed species at minimal doses of oxidant-generating herbicides, i e , they have "synergized" the herbicides (2, 10, 11) The chelators are capable of removing copper and/or zinc from superoxide dismutase and copper from ascorbate peroxidase, both key enzymes in the oxygen detoxification pathway are cogent examples The ability to bind copper from oxidant-detoxifying enzymes opens a possibility of improving crop protection with smaller amounts of herbicides

Oxine (8-hydroxyquinoline) and oxine class chelators failed as "synergists" (2), because they have higher affinity for iron than for copper,

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[2] Abbreviations Dtc sodium salts of dithiocarbamic acid OTS *n* octadecyltrichlorosilane NTS hydrolyzed 15 nonadecenyltrichlorosilane

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preferentially binding iron in low copper plant systems Many iron chelators such as hydroxyquinoline are effective at preventing herbicide action instead of synergizing herbicide activity (10), presumably because they prevent the iron-catalyzed formation of hydroxyl radicals by the Fenton reaction As the iron content of the plants is at least two orders higher than the content of copper, this preferential affinity of oxines for iron leads to the protection of the plants from oxidative damage

Dithiocarbamates (Dtc)[2] are powerful copper chelators, having far greater affinity for copper than iron (12) Sodium diethyldithiocarbamate is used in analytical chemistry for the estimation of copper in iron-containing biological materials such as plants and blood (13, 14), as well as in steel and ferrous alloys (15) Sodium diethyldithiocarbamate has been used as an inhibitor of superoxide dismutase (16, 17), being tested as a synergist for oxidant-generating herbicides (2, 10, 11) High levels of this hydrophilic compound were required (10) as it could not pass easily through leaf cuticles, and most remained on the surface of the leaves Compounds that are too lipophilic will stay inside the cuticle, so a balance between lipophilic and hydrophilic properties must be obtained

We chose disubstituted dithiocarbamates as potential synergists for two reasons (1) Disubstituted dithiocarbamates are more stable than the monosubstituted ones (18), and (2) the presence of two substituents bound to nitrogen provides good opportunities for varying the hydrophilic-lipophilic balance of the compounds

Disubstituted dithiocarbamates with polyether and aliphatic side chains were synthesized (Fig 1) The ether chain is responsible for the hydrophilic properties, and the aliphatic chain allows for varying the lipophilicity The hydrophobic dibutyldithiocarbamate was used for comparison The hydrophilic-lipophilic balance, the ability to inhibit the Fenton reaction, and the potential of these compounds to act as synergists were tested, as reported below

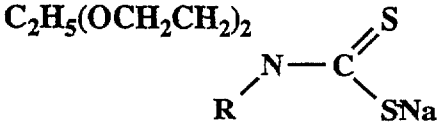

FIG 1 *Amphiphilic dithiocarbamates synthesized during this study R is an aliphatic chain of different lengths (R = ethyl butyl or hexyl)*

MATERIALS AND METHODS

Synthesis of Glycol(alkyl)dithiocarbamates

Strategy *N*-Alkyl-*N*-(2-(2-ethoxyethoxy)-ethyl)dithiocarbamates were synthesized according to the scheme shown in Fig 2 The method used for the preparation of 2,3-dioxypropyl(alkyl)amines (reaction of 2,3-dioxypropylchloride with 8–10 times excess of alkylamine) (19) was adapted for the synthesis of alkyl(ethoxyethoxyethyl) amines Relatively pure secondary amines in good yields were prepared from ethoxyethoxyethylchloride using a 10 times excess of primary amines The preparation of dithiocarbamates (20) results from the reaction of a secondary amine with CS_2 in the presence of NaOH Ethyl alcohol was used for the preparation of sodium salts of butyl- and hexyl(2-(2-ethoxyethoxy)ethyl)dithiocarbamic acids However, the synthesis of the sodium salt of ethyl(2-(2-ethoxyethoxy)ethyl)dithiocarbamic acid in ethanol also resulted in the formation of ethylxanthate by *O*-alkylation of the solvent The synthesis of I-Et-Dtc in tetrahydrofuran with sodium hydride was preferred for this reason Dibutyl dithiocarbamic acid was synthesized from dibutylamine in water according to Ref (21) The water-soluble dithiocarbamate salts were converted to the corresponding methyl esters by alkylation with methyl iodide prior to analytical characterization by TLC, NMR and elemental analysis

Chemicals and instrumentation for synthesis of Dtcs The following chemicals were used for syntheses thionylchloride (E Merck, for synthesis) diethylene glycol monoethyl ether (Fluka AG) butylamine (Fluka, AG), sodium carbonate (E Merck, for analysis) hexylamine (Fluka AG), ethylamine (Fluka, AG) potassium

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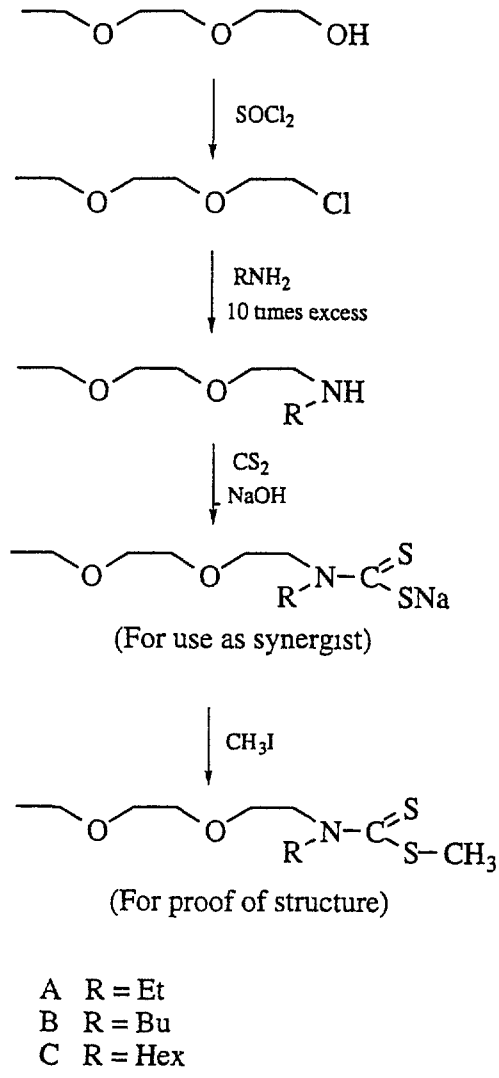

FIG 2 *General scheme for the synthesis of amphiphilic dithiocarbamates and their methyl esters*

hydroxide (E Merck, GR, for analysis), sodium hydroxide (E Merck, GR, for analysis), ethyl alcohol absolute (BIO-LAB, AR), carbon disulfide (Fluka, puriss), tetrahydrofuran (BIO-LAB, A R) sodium hydride suspension (80% in paraffin oil E Merck, for synthesis) calcium chloride anhydrous (Fluka AG), dibutylamine (Fluka AG), methyliodide (E Merck, for synthesis)

Proton NMR spectra were measured on a Varian FT-80 Bruker WH-270, or a Bruker AMX 400 NMR spectrometer All chemical shifts are reported in delta units downfield from tetramethylsilane as internal standard, or the H_2O signal was used as a reference The following abbreviations have been used s, singlet, d, doublet, t, triplet, q, quartet, m, multiplet Deuterated $CDCl_3$ (Aldrich) and D_2O (E Merck) used for NMR were of 99 8% isotope purity UV/VIS spectra were measured on a Hewlett-Packard 8450A diode array spectrophotometer Flash column chromatography separations were performed on silica gel Merck 60 (230–400 mesh ASTM) TLC was performed on E Merck Kieselgel 60 F_{254} plates Staining of TLC plates was done by (i) basic aqueous 1% $KMnO_4$, and (ii) 0 3% ninhydrin in $EtOH_{abs}$ Tetrahydrofuran was distilled under $LiAlH_4$ and passed through an Al_2O_3 column

Synthesis of 2 (2 ethoxyethoxy)ethyl chloride A solution of $SOCl_2$ (66 8 g 561 mmol, 10% excess) in 50 ml of chloroform was added dropwise at the rate of 1 drop / s for 2 h to a cooled, stirred solution of diethylene glycol monoethyl ether (68 ml, 508 mmol) in 68 ml of chloroform The mixture was stirred for 4 h at room temperature followed by 1 h at 50°C After the removal of chloroform by distillation, the residue was distilled at low pressure (105°C 30 mm Hg) to yield 58 1 g (75% yield) of colorless liquid TLC R_f = 0 47 ($CHCl_3$, staining with $KMnO_4$)

^{1}H NMR δ ($CDCl_3$, 80 MHz) = 3 85–3 5 (8H, m), 3 54 (2H, q), 1 21 (3H, t)

Synthesis of ethyl (2-(2 ethoxyethoxy)ethyl)amine Ethylamine (75% aqueous solution, 86 ml, 1 06 mol, 10 times excess) and 9 4 g of K_2CO_3 were placed in a 850-ml flask fitted with a magnetic stirrer, a dropping funnel, an ice bath, and a reflux condenser 2-(2- Ethoxyethoxy)ethyl chloride (15 g, 98 4 mmol) was added dropwise The mixture was stirred at room temperature for 50 h, after which 300 ml of diethyl ether and 30 g of KOH were added and stirred for 20 min The ether solution was decanted and the residue was extracted twice with ether The ether solutions were combined

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and dried over KOH Ether and the excess ethylamine were removed by distillation at 26°C, and the product was distilled twice under vacuum (66–68°C, 1 mm Hg) Of the product 11 7 g (74% yield) was obtained TLC R_f = 0 1 ($CHCl_3$ MeOH = 9 1 staining by ninhydrin) ^1H NMR δ ($CDCl_3$, 400 MHz) = 3 71–3 57 (6H, m), 3 54 (2H, q), 2 80 (2H, t), 2 66 (2H, q), 1 816 (1H, s), 1 22 (3H, t) 1 12 (3H, t)

Synthesis of butyl (2 (2 ethoxyethoxy)ethyl)amine *n*-Butylamine (150 ml 1 52 mol, 9 times excess) and 12 2 g of sodium carbonate were placed in a 500-ml two-necked flask, fitted with a magnetic stirrer and a dropping funnel 2-(2-Ethoxyethoxy)ethyl chloride (25 6 g, 167 8 mmol) was added dropwise at room temperature The reaction mixture was heated in an oil bath at 110°C for 30 h and cooled at room temperature overnight A gel-like substance formed An excess of *n*-butylamine was removed by distillation under vacuum, and 200 ml of diethyl ether was added to the residue After homogenization of the mixture, the ether solution was filtered under argon and dried over K_2CO_3 The ether was removed by distillation, and the product was distilled under vacuum (89–94°C/1 mm Hg) to yield 21 6 g (75 3%) of colorless liquid TLC R_f = 0 36 ($CHCl_3$ MeOH NH_3(25% aq) 9 1 0 1 staining with ninhydrin ^1H NMR δ ($CDCl_3$ 270 MHz) = 3 69–3 56 (6H m), 3 55 (2H, q) 2 79 (2H, t), 2 60 (2H, t,), 1 860 (1H, s), 1 57–1 4 (2H, m) 1 42–1 27 (2H m), 1 22 (3H, t), 0 91(3H, t)

Synthesis of hexyl (2-(2 ethoxyethoxy)ethyl)amine The synthesis was the same as for butyl (2-(2-ethoxyethoxy)ethyl)amine except that *n*-hexylamine was used instead of *n*- butylamine TLC R_f = 0 37 ($CHCl_3$ MeOH NH_3(25% aq) 9 1 0 1 staining with ninhydrin) ^1H NMR δ ($CDCl_3$, 270 MHz) = 3 8–3 5 (6H m), 3 53 (2H, q), 2 80 (2H t), 2 60 (2H t), 1 81 (1H, s), 1 55–1 40 (2H m) 1 42–1 25 (6H, m) 1 22(3H, t) 0 88 (3H, t)

Synthesis of ethyl (2 (2-ethoxyethoxy)ethyl) dithiocarbamic acid sodium salt (compound I Et Dtc) Ethyl (2-(2-ethoxyethoxy)ethyl)amine (0 966 g 6 mmol) 6 ml of THF and 80% NaH (0 171 g, 5 7 mmol of pure compound) were placed in a 50-ml round-bottom flask, equipped with a magnetic stirrer and a $CaCl_2$ outlet CS_2 (0 343 ml, 5 7 mmol) was added to the rapidly stirred, cooled mixture Ten minutes after addition of carbon disulfide the ice bath was removed and the mixture was stirred for 2 5 h at room temperature THF (30 ml) was added and the solution was filtered THF was evaporated under vacuum (25°C / 1 mm Hg), and the residue was washed with three 50-ml portions of dry diethyl ether The product was dried under vacuum (25°C / 1 mm Hg) The yield was 0 971 g (65 8%) of white powder UV log $\varepsilon_{(258\ nm)}$ = 4 14, log $\varepsilon_{(286\ nm)}$ = 4 12 ^1H NMR δ (D_2O, 270 MHz) = 4 20 (2H, t), 4 08 (2H, q), 3 86 (2H, t), 3 74–3 62 (4H, m), 3 58 (2H q), 1 21 (3H t), 1 18 (3H, t)

Synthesis of butyl (2-(2 ethoxyethoxy)ethyl) dithiocarbamic acid sodium salt (compound I-Bu-Dtc) Butyl (2-(2-ethoxyethoxy)ethyl)amine (1 647 g, 8 7 mmol), NaOH (0 349 g, 8 7 mmol) and ethyl alcohol (96%, 10 ml) were placed in a 50-ml flask fitted with a magnetic stirrer and an ice bath CS_2 (0 577 ml, 9 6 mmol, 10% of excess) was added to the rapidly stirred, cooled mixture Ten minutes after addition of carbon disulfide, the ice bath was removed and the mixture was stirred for 2 h at room temperature After the removal of the liquid phase by distillation under vacuum (1 mm Hg), dry ethyl ether (40 ml) was added The reaction mixture was stirred for several minutes, and the ether was removed The residue was dried under vacuum to constant weight The yield was 2 32 g (93%) of white powder UV log $\varepsilon_{(258\ nm)}$ = 4 15, log $\varepsilon_{(286\ nm)}$ = 4 13 ^1H NMR δ (D_2O, 270 MHz) = 4 20 (2H, t), 4 03 (2H, t), 3 85 (2H, t), 3 73–3 60 (4H m), 3,57 (2H, q), 1 75–1 60 (2H, m), 1 38–1 22 (2H, m), 1 17 (3H, t), 0 90 (3H t)

Hexyl (2-(2-ethoxyethoxy)ethyl)dithiocarbamic acid, sodium salt (compound I Hex Dtc) The synthesis was the same as for compound I-Bu-Dtc except that hexyl (2-(2- ethoxyethoxy)ethyl)amine was used instead of butyl (2-(2 ethoxyethoxy)ethyl)amine UV log $\varepsilon_{(258\ nm)}$ = 4 16, log $\varepsilon_{(286\ nm)}$ = 4 14 ^1H NMR δ (D_2O,

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270 MHz) = 4 22 (2H, t) 4 05 (2H, t), 3 87 (2H, t), 3 75–3 62 (4H, m), 3 60 (2H, q), 1 72(2H, m), 1 31 (6H, m), 1 20 (3H, t) 0 88 (3H, t)

Synthesis of dibutyl dithiocarbamic acid sodium salt (compound Dibutyl-Dtc) NaOH (1 N water solution, 15 853 ml, 15 8 mmol) and freshly distilled dibutylamine (2 25 g, 17 4 mmol, 10% of excess) were placed in a 100-ml flask, fitted with a magnetic stirrer CS_2 (0 953 ml, 15 8 mmol) was added dropwise to the rapidly stirred, cooled mixture Ten minutes after addition of the reagents, the ice bath was removed The mixture was stirred for 2 h at room temperature and lyophilized for 1 5 days The yield was 3 324 g (93%) of white powder UV log $\varepsilon_{(260\ nm)}$ = 4 21 log $\varepsilon_{(282\ nm)}$ = 4 18 ^{1}H NMR δ (D_2O, 270 MHz) = 3 98 (4H, t), 1 77–1 62 (4H, m) 1 41–1 23 (4H, m), 0 92 (6H t)

Synthesis of butyl (2-(2-ethoxyethoxy)ethyl)-dithiocarbamic acid, methyl ester Butyl (2- (2-ethoxyethoxy)ethyl)amine sodium salt (1 482 g 5 16 mmol) and 10 ml of absolute ethanol were placed in a 50-ml round-bottom flask, equipped with a magnetic stirrer and an ice bath CH_3I(0 347 ml, 5 57 mmol, 8% of excess) was added to the rapidly stirred, cooled mixture Five minutes after addition of the reagents, the ice bath was removed and the mixture was stirred for 7 h at room temperature The ethanol was removed under vacuum The residue was rinsed with chloroform (40 ml) after which the chloroform solution was filtered The filtrate was evaporated under vacuum (1 mm Hg) to yield 1 331 g (92 4%) of yellow oil TLC R_f = 0 39 ($CHCl_3$ staining by $KMnO_4$) ^{1}H NMR δ ($CDCl_3$, 400 MHz) = 4 2–3 65 (6H m) 3 48–3 58 (4H, m) 3 46 (2H q) 2 57 (3H s) 1 68–1 59 (2H m), 1 35–1 23 (2H m) 1 15 (3H t), 0 90 (3H t) Element analysis calculated for $C_{12}H_{25}NO_2S_2$ N 5 23%, S 23 97%, found N 5 6% S 22 41%

Hexyl (2-(2-ethoxyethoxy)ethyl)dithiocarbamic acid methyl ester The synthesis was the same as for methyl ester of butyl (2-(2-ethoxyethoxy)ethyl)dithiocarbamic acid except that sodium salt of hexyl (2-(2-ethoxyethoxy)ethyl)amine was used instead of sodium salt of butyl (2-(2-ethoxyethoxy)ethyl)amine TLC R_f = 0 40 ($CHCl_3$ staining by $KMnO_4$) ^{1}H NMR δ ($CDCl_3$ 270 MHz) = 4 22–3 70 (6H, m), 3 67–3 51 (4H m) 3 53 (2H, q), 2 63 (3H, s) 1 8–1 64 (2H, m), 1 31 (6H, m), 1 22 (3H, t,), 0 90 (3H, m) Elemental analysis calculated for $C_{14}H_{29}NO_2S_2$ C 54 68%, H 9 51%, N 4 56% S 20 85%, found C 54 38%, H 9 27%, N 4 80%, S 21 27%

Ethyl (2-(2 ethoxyethoxy)ethyl)dithiocarbamic acid, methyl ester The synthesis was the same as for methyl ester of butyl (2-(2-ethoxyethoxy)ethyl)dithiocarbamic acid except that sodium salt of ethyl (2-(2-ethoxyethoxy)ethyl)amine was used instead of sodium salt of butyl (2-(2-ethoxyethoxy)ethyl)amine TLC R_f = 0 38 ($CHCl_3$ staining by $KMnO_4$) ^{1}H NMR δ ($CDCl_3$, 270 MHz) = 4 24–4 1 3 96–3 72 (6H, m) 3 66–3 52 (4H m), 3 52 (2H, q), 2 64 (3H s) 1 28 (3H, t), 1 22 (3H t) Elemental analysis calculated for $C_{10}H_{21}NO_2S_2$ N 5 85%, S 26 78% found N 5 51%, S 25 49%

Influence of Dithiocarbamates on Fenton Reaction (*in Vitro*)

The ability of Dtcs to inhibit or enhance the rate of Fenton reactions was measured using two different substrates and solvent systems as follows

1 Deoxyribose/Malondialdehyde Assay for the Determination of Hydroxyl Radicals Using Thiobarbituric Acid

Chemicals The following chemicals were used Copper (II) sulfate pentahydrate (E Merck, GR, for analysis), potassium dihydrogen phosphate (Aldrich, ACS), sodium phosphate dibasic heptahydrate (Aldrich, ACS), ferric chloride solution, 60%, w/v (BDH, Analar), sodium dodecyl sulfate (Sigma, GC), L-ascorbic acid (BDH, Analar), deoxyribose (Sigma), 2-thiobarbituric acid (Aldrich), thiourea (E Merck, for analysis), acetic acid glacial (BIOLAB), hydrogen peroxide (30%, E Merck, for synthesis)

Fenton reaction kinetics A total of 0 05 M Na/K phosphate buffer, pH 7, was used for the

preparation of the aqueous solutions Components of the Fenton reaction (2 ml of a 0 4 mM solution of $FeCl_3$ (or $Fe(Dtc)_3$, or $CuSO_4$, or $Cu(Dtc)_2$) in 4% sodium dodecyl sulfate, 2 ml of a 8 mM solution of ascorbic acid in the buffer, and 2 ml of a 40 mM solution of deoxyribose in the buffer were added to 10-ml polypropylene tube The free radical Fenton reaction was started by the addition of 2 ml of 8 mM hydrogen peroxide solution in the buffer with shaking The formation of malondialdehyde was measured by its reaction with thiobarbituric acid and thiourea, forming a pink chromophore 400 μl of the reaction mixture was added between 10 min and 5 h to the test tube containing 1 5 ml of 20%, v/v, acetic acid, 1 5 ml of 0 8%,w/v thiobarbituric acid, and 0 6 ml of 0 5 M thiourea The test tubes were closed, shaken, and placed in an oil bath at 95°C The reaction mixtures were incubated for 1 h and cooled to room temperature One milliliter of the solution was taken after cooling and diluted with water to 5 ml and the absorbance of the resulting chromophore was measured at 532 nm against water

2 *Methanol/Formaldehyde Assay for the Determination of Hydroxyl Radicals Using the Nash Reagent*

Chemicals The following chemicals were used Copper (II) sulfate pentahydrate (E Merck GR, for analysis), potassium dihydrogen phosphate (Aldrich, ACS), sodium phosphate, dibasic heptahydrate (Aldrich ACS), ferric chloride solution, 60%, w/v (BDH Analar), L-ascorbic acid (BHD, Analar), thiourea (E Merck, for analysis) acetic acid glacial (BIO-LAB), hydrogen peroxide (30%, E Merck, for synthesis), methanol absolute (BIO-LAB, AR), ammonium acetate (E Merck, for analysis), acetylacetone (E Merck, for synthesis) formaldehyde solution (min 37%, E Merck, GR, for analysis)

Fenton reaction kinetics A total of 0 05 M Na/K phosphate buffer, pH 7, was used The Nash reagent for the color test (20, 22) contains 15 g of ammonium acetate 300 μl of acetic acid and 200 μl of acetylacetone mixed together and the volume was adjusted to 100 ml with water Ascorbic acid (7 5 ml of 4 mM solution in 50% methanol/buffer) was added to 3 75 ml of 0 4 mM solution of $FeCl_3$ (or $Fe(Dtc)_3$, $CuSO_4$ or $Cu(Dtc)_2$) in 50% methanol/water The Fenton free radical reaction was started by the addition of 3 75 ml of 8 mM hydrogen peroxide solution in 50% methanol/buffer with shaking The formaldehyde formed by reaction of hydroxyl radicals with methanol was measured by adding 750 μl of the reaction mixture at various times between 10 and 300 min to tubes containing 1 25 ml of 0 16 M water solution of thiourea After the incubation was finished, 3 ml of Nash reagent was added to each of the test tubes, which were incubated at 37°C for 1 hr The absorption of diacetyldihydrolutidine formed in the reaction was measured at 412 nm

Hydrophilic and Lipophilic Distribution Ratios

1-Octanol (Riedel-deHaen, synthesis grade (λ250–270 nm < 0 06) and double-distilled water were used as the partitioning solutions A Hewlett-Packard 8450A diode array spectrophotometer was used for the quantitative determination of dithiocarbamates in the standard solutions and in the partitioned solutions The aqueous phase stock solutions were shaken with an excess of octanol to presaturate them and were then allowed to stand overnight before use The octanol stock solutions were also presaturated with water and allowed to settle overnight The experiments were performed in 10-ml stoppered centrifuge tubes The tubes were inverted gently for 5 min and then, to assure complete phase separation, they were centrifuged for 20 min at 1000–2000*g* The aqueous and organic phases were removed separately and analyzed by UV spectrophotometry

For the experiments on the kinetics of absorbance of dithiocarbamates on the glass surface, the octanol and water solutions were centrifuged separately for several hours and the UV spectra of the solutions were measured through particular periods of time

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Oxidative Properties (*in Vivo*)

Solutions of the herbicides paraquat or lactofen containing putative synergists (different dithiocarbamates) were sprayed to run off on *Stellaria media*, at the 4 true-leaf stage, and *Sinapis alba*, at the 2 true-leaf stage of development

Plants were allowed to dry and kept for 24–48 h at room temperatures ranged from 22 to 35°C with light intensity around 500 μEin s^{-1} m^{-2} Visual assessment of damage was made on a 0–4 scale (0 no damage to the plant, 4, death) The main effect was a rapid desiccation of the leaves due to water loss There was no major pigment bleaching or dry weight loss within this time, so visual assessment was the only reliable parameter that could be measured While statistics are often presented on such data, they are inaccurate, as the visual measurements are inherently nonlinear All treatments were performed on four pots with plants and the reported values are the average of four trials Various concentrations of herbicide with synergist were used together with two controls herbicide alone and synergist alone

RESULTS AND DISCUSSION

Complexation Studies

The stability of the $Cu(Dtc)_2$ complexes was determined by the competition method described by Janssen (23) This method is based on the competition of dithiocarbamate with 8-hydroxyquinoline ligands for copper, and are described in detail in another paper (Rogachev *et al*, in preparation) The competitional (K_c) value, presented in this article shows the relative competitive stability of $Cu(Dtc)_2$ over Cu(hydroxyquinoline)$_2$ (Table 1) The higher the K_c value, the stronger the copper-dithiocarbamate complex

The relationships between the K_c values and the structure of dithiocarbamates are shown in Table 1 Decreasing the size of the R_2 substituent from Hex to Bu and to Et, while retaining the same Et-O-CH_2CH_2-O-CH_2CH_2-group, decreases the overall electron density on the nitrogen and hence reduces the activity of the sulfur atoms toward coordination of metal ions Dibutyldithiocarbamate was used as a more hydrophobic compound for comparison The dialkyl dithiocarbamates are stronger ligands for copper than their diglycol analogs

Distribution Ratio

The partition coefficient (P) or the distribution ratio (D) between water and octanol are most commonly used measures of the hydrophobicity of compounds (24) The hydrophobic parameter P is of great practical utility in predicting the distribution of the compounds *in vivo* through cuticles D can depend on the concentration of solute, but at reasonably low concentrations, D becomes constant and is called the partition coefficient P

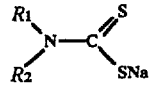
Initially we tried to use relatively low concentrations of Dtcs (0 1 mM) for the direct determination of P Partitioning was performed by the shake-flask method according to Leo (24) with 10 mM NaOH in the aqueous phase, added to prevent the decomposition of dithiocarbamates Sodium dithiocarbamates are amphiphilic molecules with polar $-CS_2^-$ groups and hydrophobic aliphatic tails They behave as detergents and form emulsions with even gentle shaking of water/octanol mixtures which hampers the phase separation in the shake-flask method Complete phase separation may be achieved only by centrifugation of these emulsions

At first, the measurements of the partition coefficients P using low Dtc concentrations were carried out in standard polypropylene or glass tubes The solutes adsorbed onto the surface of the tubes during centrifugation, which greatly decreased the accuracy, rendering the determination of P impossible The adsorption of polar solutes onto glass surfaces is known (25) treatment of the test tubes with polymethacrylate ester greatly diminished the surface adsorption of quaternary ammonium salts (25)

Special glass tubes coated with a hydrophobic monolayer of either *n*-octadecyl-trichlorosilane (OTS) or hydrolyzed 15-nonadecenyl-trichlorosilane (NTS) were tested A low concentration (100 μM) I-hexyl-Dtc was dissolved in octanol and centrifuged and the absorbance at 290 nm

TABLE 1
Correlation among Structure Lipophilicity of the Synthesized Dithiocarbamates and Relative Stabilities of Their Copper Complexes

Compound



	R_1	R_2	log K_c[a]	HLB (D)[b]
Dibutyl Dtc	Bu	Bu	3 64	0 21
I Hex Dtc	Et O CH_2CH_2 O CH_2CH_2	Hex	2 03	0 14
I Bu Dtc	Et O CH_2CH_2-O CH_2CH_2	Bu	1 95	0 05
I Et Dtc	Et O CH_2CH_2 O CH_2CH_2	Et	1 53	0 025

[a] K_c is the competitional stability constant which shows the relative stability of $Cu(Dtc)_2$ over $Cu(Ox)_2$ where Ox is 8 hydroxyquinoline

[b] HLB is the hydrophilic lipophilic balance as measured in the octanol/water system The distribution ratio $D = C_{org}/C_{aq}$ The linear slope of a plot of log K_c against HLB is $y + 8\ 1621^{-2} + 8\ 2129^{-2}$ and $r^2 = 0\ 804$

was measured to evaluate the success of this method to reduce surface adsorption (Fig 3) The adsorption of the solute from aqueous phase was not observed Adsorption onto the coated surface was less than onto the unmodified glass (Fig 3) NTS was the best surface-modifying agent for this particular compound The OTS-coated glass seems to be too hydrophobic to prevent the adsorption of I-Hex-Dtc this compound probably has some adherence to the hydrophobic surface of the modified glass via its hydrophobic site

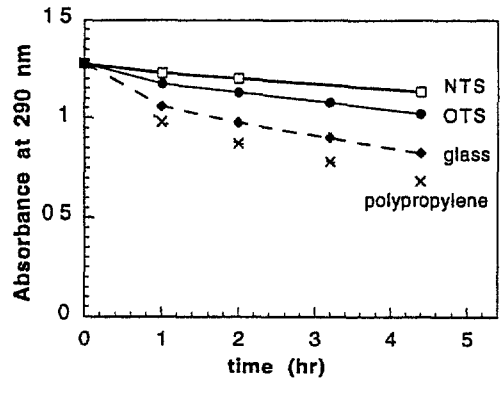


FIG 3 *Prevention of adsorption of Dtc to surface during partitioning by coating glass tubes The absorbance of 0 1 mM I Hex Dtc in octanol after several hours of centrifugation in polypropylene tube an uncoated glass tube and glass tubes coated with OTS or NTS*

As all Dtcs have different liphophilicities, each Dtc may need a specific glass coating agent For simplicity, we decided to measure octanol/water distribution ratio D instead of partition coefficient P All distribution measurements for different Dtcs were performed at 100-fold higher concentrations of dithiocarbamates (10 mM solutions) in polypropylene tubes to avoid the need for a surface modification procedure Absorbance of Dtcs on the surface is then negligibly small compared to their concentrations, as the surface absorptive capacity quickly becomes saturated The experiments were performed in opposite directions for each compound extraction of the Dtc, dissolved in octanol by 10 mM NaOH aqueous solution, and extraction of the Dtc, dissolved in 10 mM NaOH aqueous solution, by octanol The agreement of these two values and a material balance of more than 98% recovery validated the use of high Dtc concentrations

Results of partition measurements by the shake-flask method are presented in Table 1 Changing the aliphatic substituent from ethyl to hexyl leads to a sixfold increase in hydrophobicity of the dithiocarbamates There was a very strong positive linear correlation between the log K_c and the HLB values the correlation coefficient (r^2) was 0 804

Increasing or Suppressing *in Vitro* Fenton Activity of Metals by Complexing with Dithiocarbamates

Chelators such as oxines can decrease the yield of hydroxyl radicals by effectively rendering the metal catalyst unavailable for Fenton reactions, they function indirectly as antioxidants Some chelators such as EDTA act indirectly as prooxidants, they increase the yield of hydroxyl radicals by binding metals in such a way that there availability to Fenton reactions is enhanced Hence, the influence of metal chelators on Fenton-dependent hydroxyl radical formation provides information on the indirect prooxidative (or antioxidative) properties of these compounds

The capacity of the synthesized dithiocarbamates to act as indirect prooxidants when complexed with copper or iron was evaluated in two test systems that measure oxidation of organic molecules due to Fenton reaction-generated hydroxyl radicals The first used deoxyribose as the radical scavenger (26, 27), and the second used methanol (22)

1 Deoxyribose as Scavenger for Fenton Generated Radicals

Fenton oxidation of aqueous deoxyribose is a commonly used method for measuring hydroxyl radical production (28 29) The copper and iron complexes of Dtcs are barely soluble in water and several organic solvents such as 1-methyl-2-pyrrolidinone, dimethylformamide acetone, and ethanol, which are supposed to be relatively weak scavengers of free radicals, were examined as potential solvents for the deoxyribose assay The drastic decrease in absorption band of the pink pigment due to scavenging of hydroxyl

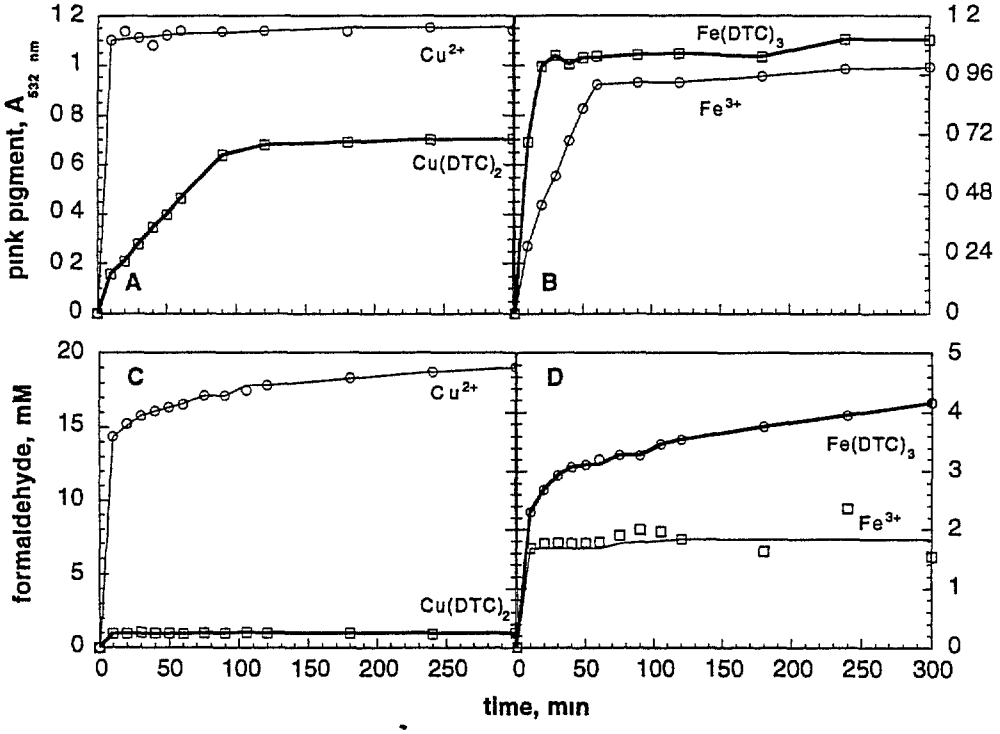


FIG 4 *Effect of Dtc in suppressing copper catalyzed Fenton reaction and stimulating iron catalyzed Fenton reaction Kinetics of Fenton type reaction with (A) Cu^{2+} and $Cu(I\ Hex\ Dtc)_2$, (B) Fe^{3+} and $Fe(I\ Hex\ Dtc)_3$ measured by the detergent–modified deoxyribose assay and kinetics of the Fenton type reaction with (C) Cu^{2+} and $Cu(I\ Hex\ Dtc)_2$, (D) Fe^{3+} and $Fe(I\ Hex\ Dtc)_3$ measured with the Nash reagent*

radicals by these solvents (data not shown) prevented a quantitative determination of the indirect antioxidative properties of Dtc complexes by the standard deoxyribose method

The problem of the determination of the influence of Dtcs on the Fenton reaction was solved by using a modified deoxyribose assay, using solubilization of $Cu(Dtc)_2$ and $Fe(Dtc)_3$ in water by a 1% solution of sodium dodecyl sulfate to increase the water solubilities of $Cu(Dtc)_2$ and $Fe(Dtc)_3$ The sodium dodecyl sulfate did not significantly decrease the absorption signal at 532 nm and, hence, the deoxyribose assay can be performed under these detergent conditions

Kinetic data were obtained by stopping the reaction using thiourea as a hydroxyl radical scavenger, as it quenches the reaction of hydroxyl radicals with deoxyribose and allows measurement of the amount of malondialdehyde at the time of the stoppage Results of the kinetic measurements of the Fenton (formed with iron) and Fenton-type (with copper) reactions using this modified deoxyribose assay are presented in Figs 4A and 4B

2 *Methanol as a Solvent and as a Scavenger for Fenton-Generated Radicals*

A 50% aqueous methanol solution was used to dissolve $Cu(Dtc)_2$, $Fe(Dtc)_3$, and the Fenton reagents and act as the scavenger The oxidation of methanol to formaldehyde was used to detect hydroxyl radical production The kinetics of the formaldehyde production were monitored by the specific reagent described by Nash (22), producing a yellow chromophore (λ_{max} = 412 nm) Results of the kinetic measurements using the Nash reagent are presented in Figs 4C and 4D

Both methods show that Dtc is an indirect prooxidant with iron, stimulating iron-dependent Fenton reactions (Figs 4A and 4C) The same chelator is an antioxidant with copper, suppressing copper-dependent Fenton reactions (Figs 4B and 4D) The magnitude of suppression or stimulation differs between methods, which may be due to different conformations of the complex in detergent vs methanol

The presence of dithiocarbamates should not protect plants from the oxidative damage due to iron-catalyzed Fenton reaction Dtc chelates copper better than iron, and only small amount of iron ions will be bound to a Dtc, but even this complexation will increase the rate of Fenton reaction and slightly increase the production of hydroxyl radicals The complexation with copper leads to reduction of hydroxyl radical generation This is not relevant as copper has little role in Fenton-type reactions in plants, as plants contain two orders of magnitude less copper than iron The minuscule reduction of hydroxyl

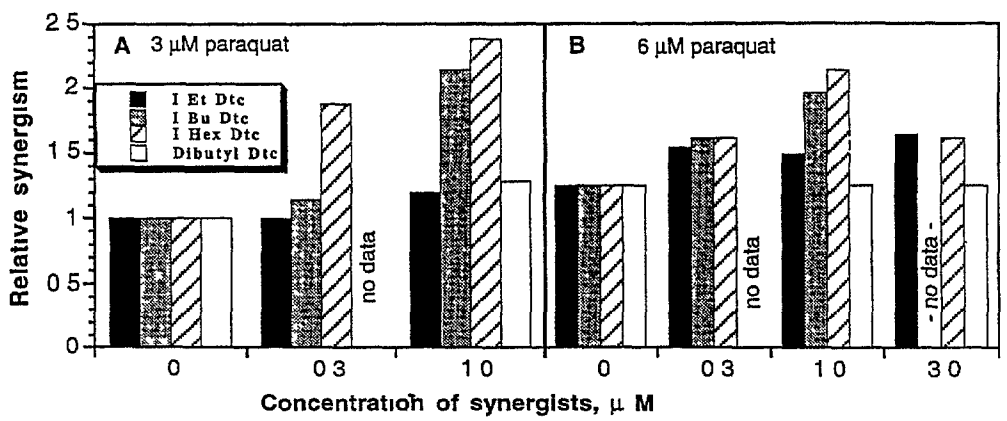


FIG 5 *Enhancement of damage caused by paraquat by varying amounts of synergist The relative synergism is the ratio between destruction due to the herbicide together with synergist and destruction due to the herbicide alone for the concentration of herbicide equal to (A) 3 μM (B) 6 μM (for Stellaria media)*

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radical generation due to copper binding should not influence phytotoxicity

Oxidative Properties (*in Vivo*)

The ability of the Dtcs to synergize the activity of the oxidant-generating herbicide paraquat was assayed using *S alba* and *S media* The results of many of these *in vivo* tests for different concentrations of paraquat and synergists (Dtcs) are summarized in Table 2 The large number of pots and plants required for each data point precluded simultaneously measuring the effects of more than one chelator at a time A 0–4 visual scale was used for evaluation of plant damage (0, no damage to the plant 4, death) as no other parameter could reliably or adequately measure the wilting damage, which was so rapid that there was no loss of chlorophyll or dry weight in the two weed species used Statistical analyses could not be used due to the intrinsic nonlinearity of such observations Still, the results consistently reoccurred in the various repeat experiments, despite their not being astoundingly great synergists The K_c values of dithiocarbamates are also presented for comparison The ratios between damage due to the herbicide together with synergists and damage due to the herbicide alone are presented for easy comparison of the data (Fig 5) Data for 3 μM and 6 paraquat are shown

There is some correlation between the synergistic properties of dithiocarbamates and their

TABLE 2
Damage to Plants Caused by the Use of Paraquat together with Synergists (Dtcs)

			Concentration of paraquat μM			
Species Synergist	log K_c	μM	0	3	6	10
			damage to plant			
Stellaria media						
I-Et Dtc	1 53	0	0	1 3	2 6	3 6
		0 3	0	1 2	3 2	
		1	0	1 6	3 1	
		3	0	1 6	3 4	
I Bu Dtc	1 95	0	0	0 7	1 4	3 5
		0 3	0	0 8	1 8	
		1	0	1 5	2 2	
I Hex Dtc	2 03	0	0	0 8	1 4	3 5
		0 3	0	1 5	1 8	
		1	0 1	1 9	2 4	
		3	0	1 6	1 8	
Dibutyl Dtc	3 64	0	0	1 4	1 9	2 5
		0 3				
		1	0	1 8	1 1	2 3
		3	0		1 4	3 2
Sinapis alba						
		0	0	0 5	1 6	3 9
I Bu Dtc	1 95	0 3	0	1 1	2 2	
		1	0	1 4		
		0	0	0 7		
I Hex Dtc	2 03	0 3	0	0 7		
		1	0 1	2 0		
		3	0	2 2		
		0	0	1 3	1 5	
Dibutyl Dtc	3 64	0 3				
		1	0	1 13	2	
		3	0	1 5	1 4	

complexation properties for I-Et-Dtc, I-Bu-Dtc and I-Hex-Dtc compounds (Table 2, Fig 5) The stronger the Cu-Dtc complex (higher value of K_c), the greater the damage to the plants due to the presence of the dithiocarbamates with paraquat The chelators alone caused no visible damage at the concentrations tested Copper-dibutyl-Dtc complex, which has the highest K_c value, demonstrates weaker synergistic properties, possibly due to its higher hydrophobicity and lower penetration through the cuticle of the leaf

The ability of Dtcs to synergize herbicides is not limited to paraquat, which generates superoxide after accepting electrons from (or in place of) ferredoxin in photosystem I of photosynthesis Diphenyl ether (and other) herbicides cause an accumulation of photodynamic protoporphyrin IX, generating active oxygen species (3), which can be quenched by the same plant enzymes that quench the hydroxyl radicals generated in the presence of paraquat We thus tested whether there was a synergism between the diphenyl ether herbicide lactofen and one of the amphiphilic Dtc chelators (Fig 6) The Dtc had no effect alone, but enhanced the effect of lactofen, as expected

CONCLUSIONS

1 Amphiphilic dithiocarbamates with different substituents can be synthesized by the method described in this paper

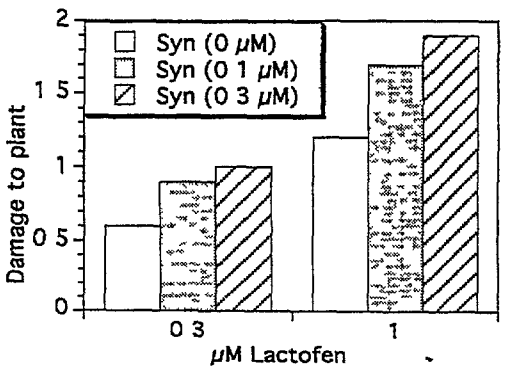

FIG 6 *Enhancement of damage caused by lactofen on Sinapis alba by the synergist I Bu Dtc The synergist by itself had no discernible effect on the weed Open bars no synergist dotted bars 0 1 μM synergist simultaneously with the herbicide hatched bars 0 3 μM synergist*

2 Dithiocarbamates can be considered for use as potential synergists of oxidant-generating herbicides for the following reasons

a. They have the ability to strongly chelate copper

b They stimulate iron-dependent Fenton reaction (*in vitro*) generating phytotoxic hydroxyl radicals

c The compounds synthesized show a modicum of synergistic activity (*in vivo*)

The Dtcs that we have synthesized so far do not have a strong enough synergizing activity to be considered for further development

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