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

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Executive Summary

We are using 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 the *psbA* gene in resistant and susceptible biotypes of *Conyza canadensis* and of *Echinochloa crus-galli* was performed using SSCP and nucleotide sequencing techniques, and the results showed that the mutation was in the 264-th codon for the resistant biotypes in both species. This mutation did not always lead to high level of resistance to atrazine. Further changes in the ratio of resistant and susceptible populations and in the composition of weed species were noted as a results of the influence of both herbicide treatment and weed competition, with an increasing role played by perennial weed species. The expression of negative cross resistance in this year's studies was also confirmed for both species.

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 one additional enzyme - enhanced glutathione peroxidase activity correlates with resistance, as do higher levels of the polyamine putrescine. Evidence is presented for elevated transcript levels of the mRNAs for the enzymes 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 year's experiments on both sides are the basis for collaboration, which was further discussed during scientific meetings attended by both p.i.'s and during a visit by the Polish p.i to the laboratories and greenhouse of the Israeli collaborators.

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)

In last year's studies, the presence of triazine resistance in *C. canadensis* and *E. crus-galli* was shown with restriction enzyme BstXI (that cuts *psbA* gene at the mutation point). This year molecular analysis of *psbA* gene mutation was performed with more precise techniques: Single Strand Conformational Polymorphism (SSCP) and base sequencing using Genetic Analyser ABI Prism 310 model (Perkin Elmer). For each analysis five plants were used. Plants were cultivated in the greenhouse in automatically controlled conditions. The above ground parts of the plants in the 7-9 leaf stage were harvested for DNA extraction. The DNA from a each plant was extracted individually by the CTAB protocol with some modifications. Extracts from single plants were used in parallel both for SSCP and sequencing analysis. Sequencing and SSCP was performed on the ABI Prism 310 capillary electrophoresis genetic analyser.

Strand Conformational Polymorphism Analyses

Since a one base difference leading to a simple mutation is able to change conformation and electrophoretic mobility in the SSCP technique, it was selected for analysis. This technique is based on the principle that under non-denaturing conditions single strand DNA fragments assume unique conformations that depend on their nucleotide sequences. Two primers designed with Primer 3 software: primer A 5'-CAGGGAAACCACTGAAAACG -3' and primer B 5'-AGTACTAATACCTAAAGCGG-3' were added to the PCR mixture. The primers: A and B were labelled on the 5' end with FAM and TET dyes respectively. The GeneAmp PCR System (Perkin Elmer) was programmed for touchdown (TD) PCR. The amplified DNA was concentrated and purified with Centricon-100 (Amicon) columns. The PCR products together with internal size standards labelled with a third dye: TAMRA, were denatured at 90°C in formamide/NaOH and loaded to ABI Prism 310 Analyser for native electrophoresis. The migration of DNA fragments was analysed with a Power PC Macintosh computer using Gene Scan Software.

PCR amplification of extracted DNA resulted in 165 bp fragments of the *psbA* gene (without used primers). Using the Single Strand Conformational Polymorphism technique were able to show evident differences in migration of the analysed DNA fragments. The A and B strands of 165 bp fragments of *psbA* gene both of *E. crus-galli* and of *C. canadensis* were easily distinguished. The pair of separated strands from *E. crus-galli* samples moved through the native gel faster than those of *C. canadensis* ones. FAM labelled strands were detected slightly earlier than TET labelled ones in both species. However, it was more clear

for *E. crus-galli*. Although the single base substitution in 264'th codon of the *psbA* gene bearing the triazine resistance did not alter mobility of the strand on the gel when compared with susceptible forms, minimal delay was observed for resistant biotypes. Nevertheless, one base substitution might significantly change the molecular shape of ssDNA, but this depends on the proper conditions of electrophoresis. If so, we should be able to develop fast and simple protocol for triazine resistance mutation screening within plant population. Therefore, the precise protocol (the temperature of gel running, sample concentration and denaturation conditions) is under investigation.

Sequencing

The next step, the nucleotide sequencing reaction that includes DNA fragment amplification, cycle sequencing and electrophoresis, is the most precise technique for identification of mutation. The desired 413 base-pair fragments of the *psbA* gene was first amplified by PCR with the following oligonucleotides: "primer 1- forward" 5'-CTTATGCACCCATTTCACAT-3' and "primer 2- reverse" 5'-ACTTCCATACCAAGGTTAGC-3'. The GeneAmp PCR System (Perkin Elmer) was programmed for touchdown (TD) PCR. Next the amplified DNA was concentrated and purified with Centricon-100 (Amicon) columns. The cycle sequencing technique was performed as described in Perkin Elmer protocol. One, either forward or reverse primer, was added for a single reaction. Two reactions for each individual plant were performed to get both DNA strands sequenced. Four different fluorescent dyes : FAM, JOE, ROX and TAMRA (Perkin Elmer) were used for identification of A, C, G, and T bases respectively. These dyes were used for dideoxynucleotide triphosphate terminator ddNTPs labelling. With this procedure the growing chain was simultaneously terminated and labelled with dye. The sequencing products were purified with Centri - Sep spin columns, mixed with Template Suppression Reagent (TSR) and placed on to the Genetic Analyser autosampler. Data were processed on Power PC Macintosh computer using Sequencing Analysis 2.1.2. software and Clustal W software to find alignments (to compare the sequences). Additionally the results were compared with information of the Gene Bank Database.

According to manufacturer the level of inaccuracy for Gene Analyser ABI Prism 310 during dye ddNTP reaction is 1.5%. In our case we were able to lower this level by comparison of both strands of the analysed fragments, which shows that the working conditions of our system are very good. Nucleotide sequences of *Nicotiana tabacum* (dicotyledons) and *Oryza sativa* (monocotyledons) were used as a standards for *C. canadensis* and *E. crus-galli* respectively. The standard data were taken from the Gene Bank Database.

Forty changes in nucleotides were found among all six genotypes compared 15 in resistant form (R) and 14 in susceptible (S) in case of comparison of *C. canadensis* with *N. tabacum* and 13 (R) and 12 (S) in case of comparison of *E. crus-galli* with *O. sativa* (Figure 1). When compare *C. canadensis* vs. *E. crus-galli* 31 differences in nucleotide sequence were noted both in susceptible and resistant biotypes of these two species. Most of the differences between *E. crus-galli* and *C. canadensis* did not relate to the amino acid sequence. In total comparison there were five transversions and twenty five transitions. Only one transition in position 713 resulted in change of amino acid in case of *E. crus-galli* . The codon AGA (arginine) present in all other genotypes has been replaced by AAA (lysine). This difference exists even between *E. crus-galli* and *O. sativa*, evolutionary close-

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                 L  G  V  A  G  V  F  G  G  S  L  F  S  A  M  H  G  S  L  V
               598 *         *         *         *         *         *
N. tabacum       TTAGGCGTAGCTGGTGTATTCGGCGGCTCCCTATTCAGTGCTATGCATGGTTCCTTGGTA
C. canadensis S C    C                   C        T                 T
C. canadensis R C    C                   C        T                 T
E. crus-galli S T    T                   T        C                 C
E. crus-galli R T    T                   T        C                 C
O. sativa        TTAGGTGTAGCTGGTGTATTCGGCGGTTCCCTATTCAGTGCTATGCATGGTTCCTTGGTA
                 T  S  S  L  I  R  E  T  T  E  N  E  S  A  N  E  G  Y R/K F
               658 *         *         *         *         *         *
                 ACTTCTAGTTTGATCAGGGAAACCACAGAAAATGAATCTGCTAATGAAGGTTACAGATTC
                   C                     A        A        C  A      AGA  C
                   C                     A        A        C  A      AGA  C
                   C                     T        G        T  G      AAA  T
                   C                     T        G        T  G      AAA  T
                 ACCTCTAGTTTGATCAGGGAAACCACTGAAAACGAATCTGCTAATGAGGGTTACAGATTT
                  G  Q  E  E  E  T  Y  N  I  V  A  A  H  G  Y  F  G  R  L  I
               718 *         *         *         *         *         *
                 GGTCAAGAGGAAGAAACTTATAACATCGTAGCCGCTCATGGTTATTTTGGCCGATTGATC
                         A     A        T  C  A  C     C           C     G
                         A     A        T  C  A  C     C           C     G
                         A     G        T  T  G  T     C           T     A
                         A     G        T  T  G  T     C           T     A
                 GGTCAAGAGGAAGAAACTTATAATATTGTGGCCGCTCATGGTTATTTTGGCCGATTAATC
                  F  Q  Y  A S/G F  N  N  S  R  S  L  H  F  F  L  A  A  W  P
               778 *         *         *         *         *         *
                 TTCCAATATGCTAGTTTCAACAACTCTCGTTCGTTACACTTCTTCCTAGCTGCTTGGCCT
                            AGT                 T     T      C A
                            GGT                 T     T      C A
                            AGT                 T     C      T G
                            GGT                 T     C      T G
                 TTCCAATATGCTAGTTTTAACAACTCTCGTTCTTTACACTTCTTCTTGGCTGCTTGGCCT
                  V  V  G  I  W  F  T  A  L  G  I  S  T  M  A  F  N  L  N  G
               838 *         *         *         *         *         *
                 GTAGTAGGTATCTGGTTTACCGCTTTAGGTATCAGCACTATGGCTTTCAACCTAAATGGT
                         T        C  T           C  C        T
                         T        C  T           C  C        T
                         G        C  T           T  T        A
                         G        C  T           T  T        A
                 GTAGTAGGAATTTGGTTTACCGCTTTAGGTATTAGTACTATGGCTTTCAATCTAAACGGA
   F  N  F  N  Q  S  V  V  D  S  Q  G  R  V  I  N  T  W  A  D  I  I  N  R
898 *         *         *         *         *         *                   969
  TTCAATTTCAACCAATCTGTAGTTGACAGTCAAGGCCGTGTAATTAATACTTGGGCTGATATCATTAACCGT
                   A        T  T     C  T  A     C                 T
                   A        T  T     C  T  A     C                 T
                   C        T  C     T  C  T     T                 C
                   C        T  C     T  C  T     T                 C
  TTCAATTTCAACCAATCTGTAGTTGATAGCCAAGGTCGCGTTATTAATACTTGGGCTGATATCATCAACCGT
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Figure 1. Nucleotide sequences of a 372bp fragment of chloroplast *psbA* gene for atrazine susceptible (S) and resistant (R) biotypes of *C. canadensis* and *E. crus-galli*. The noncoding strands for *N. tabacum* and *O. sativa* are also shown. Numbers are bp relative to 5' end of the coding region. The deduced amino acid sequence is indicated above the nucleotide sequence.

species. When comparisons are made between susceptible and resistant biotypes within *E. crus-galli* and within *C. canadensis,* only one change is noted. In both species the transition occurring in position 790 resulted in the substitution of glycine 264 (GGT) for serine 264 (AGT), which is commonly correlated with atrazine resistance. These results further validate data presented in the last report where we showed that endonuclease BstXI digested the *psbA* gene only in the resistant plants. It also confirmed results of other authors related to triazine resistance as a consequence of this mutation.

These studies also illuminated a very interesting phenomenon; i.e. there are transitions and transversions in the *psbA* gene, which as a part of chloroplast genome yet is recognized as being very conservative.

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

Last year's study indicated some changes in the expression of resistance to atrazine herbicides in the following generation of originally resistant plants. In both species seedlings germinated from seeds collected from resistant plants (grown on field plots with high level of atrazine as a result of four years treatment with 2 $kg \cdot ha^{-1}$) that should be resistant as well, were killed in greenhouse test in the range of 25 to 50%. Therefore, a more detailed study including molecular analysis of plants expressing changes in the level of resistance to atrazine in the next generation was undertaken this year.

Greenhouse test

The level of the resistance was estimated using greenhouse test for all herbicide treatments. Seeds of *E. crus-galli* and of *C. canadensis* collected (in 1995) from 5 (randomly chosen) plants per plot were germinated on blotting paper in Petri dishes at 25°C. One thousand uniform seedlings were transplanted to the soil mixture that contained 10 $kg \cdot ha^{-1}$ of atrazine. Thus the numbers of surviving seedlings reflected the level of resistance. Results of these studies are presented in Tables 1 and 2. In both experiments, in any treatments where atrazine was used, the number of resistant plants increased significantly, showing the role of atrazine in selecting for and maintaining the resistant biotype in the population. These results also showed that the negative cross resistance was exerted in *C. canadensis* by glyphosate and pyridate and in *E. crus -galli* by metolachlor, glyphosate, and fluazifop-butyl. In the experiment with *E. crus-galli* (Table 1) 195 seedlings out of 1000 from control plots were resistant (surviving 10 kg of atrazine$\cdot ha^{-1}$). Since these plots were not treated with herbicides during the past 4 years (the whole experimental period), the relatively high number of resistant seedlings must be the result of wind and/or bird spreading seeds from neighboring plots. The most effective treatment in controlling of both biotypes of *E. crus-galli* was with metolachlor (complete control). Good control was obtained with glyphosate (42 seedlings survived) and but to a lesser degree with fluazifop-butyl (119 seedlings survived). Treatments with atrazine alone or in combination with another herbicide led to significant increase in the proportion of resistant seedlings, with the exception of combination of atrazine with metolachlor, where the level of resistance was lowest of all atrazine treatments.

A similar trend was observed in the study with *C. canadensis* (Table 2). In control plots 221 seedlings survived this very high rate of atrazine. The most effective treatment against the resistant population of *C. canadensis* was pyridate where only 19 of the resistant seedlings survived. Second was glyphosate with 125 of resistant plants, and next 2,4-D, at the recommended rate (with 228 surviving seedlings). Whenever atrazine was used (both alone or with another herbicide) the number of resistant seedlings increased significantly (above 600). Only when atrazine treatment was followed by pyridate was the level of resistance slightly lower (479 of surviving seedlings). Despite the fact that these results confirmed the

Table 1. Survival of *Echinochloa crus-galli* seedlings treated with 10 kg·ha^{-1} of atrazine after pretreatment with other herbicides [a].

Herbicides applied [kg·ha^{-1}]				Seedlings survived		
first	Application	second		number [b]	% of control	% of atrazine 2.0
control				195a	100.0	22.5
atrazine	2.0			866c	444.1	100.0
glyphosate	1.6			42a	21.5	4.8
metolachlor	1.6			—	—	—
fluazifop-butyl	0.25			119a	61.0	13.7
atrazine	1.0	glyphosate	0.8	655bc	335.9	75.6
atrazine	1.0	metolachlor	0.8	592bc	303.5	68.4
atrazine	1.0	fluazifop-butyl	0.12	912c	467.7	105.3
atrazine	0.5	glyphosate	0.4	759c	389.2	87.6
atrazine	0.5	metolachlor	0.4	278ab	142.6	32.1
atrazine	0.5	fluazifop-butyl	0.06	663bc	340.0	76.6

[a] 1000 uniform seedlings from 20 plants pretreated with herbicides were used in the testing.
[b] Means followed by the same letter are not significantly different at the 5% level according to Duncan's multiple Range Test.

Table 2. Survival of *Conyza canadensis* seedlings treated with 10 kg·ha^{-1} of atrazine after pretreatment with other herbicides [a].

Rate of herbicide [kg·ha^{-1}]				Seedlings survived		
first	Application	second		number [b]	% of control	% of atrazine 2.0
control				221ab	100.0	36.0
atrazine	2.0			614c	277.8	100.0
glyphosate	1.6			125a	56.6	20.4
pyridate	1.0			19a	8.6	3.1
2,4-D	1.8			228ab	103.2	37.1
atrazine	1.0	glyphosate	0.8	611c	276.5	99.5
atrazine	1.0	pyridate	0.5	479bc	214.9	78.0
atrazine	1.0	2,4-D	0.9	641c	290.0	104.4
atrazine	0.5	glyphosate	0.4	634c	286.9	103.3
atrazine	0.5	pyridate	0.25	605c	273.8	98.5
atrazine	0.5	2,4-D	0.45	609c	275.6	99.2

[a] 1000 uniform seedlings from 20 plants pretreated with herbicides were used in the testing.
[b] Means followed by the same letter are not significantly different at the 5% level according to Duncan's multiple Range Test.

role of atrazine in the selecting for and maintaining the resistance, changes in the level of resistance was also noted. A similar phenomenon was observed by Dr. H. Darmency (INRA, France, personal communication) in *Solanum nigrum* which together with our results convinced us to study this phenomenon at the molecular level.

Molecular analysis

Seedlings that germinated from seeds collected from the resistant plants in the field, but having about 50% lowered resistance in the following generation as shown by greenhouse test, were used for this study. Seeds used in this study were collected from the same plants as for greenhouse test. Uniform seedlings were transplanted to the pots that contained soil without herbicides. Molecular analysis was performed for 60 resistant and 30 susceptible seedlings both of *E. crus-galli* and of *C. canadensis*. DNA was extracted separately from each seedling at the few leaf stage by the CTAB protocol, with some modifications. The presence of a mutation in the 264th codon of the *psbA* gene was checked by the BstXI restriction enzyme technique. In total, 120 resistant and 60 susceptible plants were tested.

The analysis showed that each individual in the progeny of resistant plants was cut by the restriction enzyme. This means that the *psbA* gene was mutated in the 264th codon in each of the plants of all the progeny of the resistant plants, which should have resulted in resistance. However, as shown in greenhouse tests (Tables 1 and 2) of the progeny of the same resistant plants, contained some seedlings that survived and some that were killed by 10 $kg \cdot ha^{-1}$ atrazine. Susceptible plants of both species were not cut by the BstXI restriction enzyme with the exception of one plant of *E. crus-galli* . The results indicate that in the resistant population there are plants with different levels of resistance. This suggests that the mutation of the *psbA* gene does not always give very high resistance to atrazine, and that another factor is involved in the expression of resistance. The phenomenon described above requires further study and will be one of our main topics next year.

c. The expression of negative cross resistance in field conditions

The expression of negative cross-resistance by *E. crus-galli* and *C. canadensis* was studied in field conditions in a separate experiment for each species. This year is the fourth one of the five year experimental program. Greenhouse tests carried out during winter allowed us to predict the probable infestation of the plots by resistant and susceptible populations during 1996 from specific treatments in 1995. Data of the greenhouse tests are presented in Table 1 and 2. In the field experiments soil was not cultivated (as in orchard) and the herbicides were applied using typical farmers' recommendations: atrazine on May 17th, metolachlor on June 3 , and the leaf penetrating compounds: fluazifop-butyl, 2,4-D, glyphosate, and pyridate on June 4th. Similarly to last year, seed drop from a few randomly chosen plants in each plot was prevented and fully mature seed were harvested for greenhouse tests that will be run during winter 1997. The effect of four years of treatments with herbicides on the weed infestation, community structure and changes in the number of plants per unit area were evaluated on 1 m^{-2} plots on August and the results are presented in Tables 3 and 4.

In this year's studies, the number of weed species noted on the plots decreased as compared with last year from 16 to 13 in *E. crus-galli* and from 19 to 17 in case of *C. canadensis* . Further changes in community structure were observed leading to the increased role of the perennial species. Based on the number of resistant plants of *E. crus-galli* and of *C. canadensis* recorded on plots we may assume that negative cross resistance was involved in the reduction of the resistant form of these weeds.

The *E. crus-galli* plots were very heavily infested with *Poa annua* (Table 3) which practically eliminated other weed species. Only a few plants appeared of

Table 3. Species structure of weed community (number of plants) after the fourth year of herbicide treatment for *Echinochloa crus-galli* control.

Species		Herbicides [kg·ha^{-1}]									
						atrazine 1.0 plus			atrazine 0.5 plus		
	control	atrazine 2.0	glyphosate 1.6	metolachlor 1.6	fluazifop-butyl 0.25	glyphosate 0.8	metolachlor 0.8	fluazifop-butyl 0.25	glyphosate 0.4	metolachlor 0.4	fluazifop-butyl 0.06
	plants·m^{-2}										
Achillea millefolium				0.8							
Capsella bursa-pastoris				2.0							
Chenopodium album	0.3		1.3	1.0	0.8						
Cirsium arvense	0.5						0.5				1.0
Conyza canadensis		0.5					1.3		0.5		3.3
Echinochloa crus-galli	1.0	4475.0	73.0	4.5	30.8	997.5	6.8	427.8	1953.3	1250.0	1709.8
Lamium sp.			13.3	2.0	2.8				0.5		
Poa annua	825.0		68.0	1.5	4.0		128.3	195.5		1675.0	0.5
Polygonum aviculare	0.5				0.3						
Rorippa sylvestris					0.3						
Senecio vulgaris			2.8		1.3						
Stellaria media			3.5								
Taraxacum officinale	1.0		30.5	44.8	968.8				5.8		
Total [a]	**828.3a**	**4475.5c**	**192.3a**	**56.5a**	**1008.8a**	**997.5a**	**136.8a**	**623.3a**	**1960.0ab**	**2925.0bc**	**1714.5ab**
Percent of control	**100.0**	**542.3**	**23.3**	**6.9**	**122.3**	**1208.6**	**16.6**	**75.7**	**237.5**	**354.4**	**207.6**

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

Table 4. Species structure of weed community (number of plants) after the fourth year of herbicide treatment for *Conyza canadensis* control.

Species	Herbicides [kg·ha^{-1}]										
	control	atrazine 2.0	glyphosate 1.6	2,4-D 1.8	pyridate 1.0	atrazine 1.0 plus glyphosate 0.8	atrazine 1.0 plus 2,4-D 0.9	atrazine 1.0 plus pyridate 0.5	atrazine 0.5 plus glyphosate 0.4	atrazine 0.5 plus 2,4-D 0.4	atrazine 0.5 plus pyridate 0.25
	plants*m^{-2}										
Achillea millefolium	10.5									0.3	
Amaranthus retroflexus									13.0		0.5
Chenopodium album	0.8		3.3	1.0	4.0						1.0
Cirsium arvense		0.3		0.8	0.8			0.5		0.5	0.3
Conyza canadensis	60.0	99.5	20.8	17.0	5.8	128.3	58.8	57.8	90.3	59.3	47.5
Echinochloa crus-galli			27.0	0.3		25.5	5.0	12.5	19.5	30.8	
Epilobium adenocaulon	1.0	0.3	13.6		0.5	9.5		1.3	3.8	0.3	4.3
Lamium sp.	1.0		15.4	1.8	2.3						
Plantago lsp.	1.0				0.5						
Poa annua	8.6	1601.		2650.0		2025.0	2925.0	1403.8	760.5	1043.5	459.3
Polygonum sp.				0.3	2.6						
Rorippa sylvestris					1.0						
Rumex acetosella		0.3			1.5				0.5	6.3	4.8
Senecio vulgaris			4.0	0.3	0.3						
Sonchus arvensis	0.8		0.5	0.3					0.3		0.8
Stellaria media	3.0		0.8							0.5	0.3
Taraxacum officinale	1125.	1.3	2976.0	86.5	2426.0	0.5	0.3	2.0	373.0	62.8	988.0
Total [a]	**1211.a**	**1703.a**	**3061.4 a**	**2758.3a**	**2445.3a**	**2188.8a**	**2989.1a**	**1477.9a**	**1260.9a**	**1204.3a**	**1506.8a**
Percent of control	**100.0**	**140.5**	**252.7**	**227.6**	**201.8**	**180.6**	**246.7**	**122.0**	**104.1**	**99.4**	**124.4**
Total without *Poa annua*	**1203.a**	**101.7a**	**3061.b**	**108.3a**	**2445.3b**	**163.8a**	**64.1a**	**74.1a**	**500.4a**	**160.8a**	**1047.5a**
Percent of control	**100.0**	**8.5**	**254.5**	**9.0**	**203.2**	**13.6**	**5.3**	**6.2**	**41.6**	**13.4**	**87.1**

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

annual *Chenopodium album* and of perennials *Cirsium arvense, Polygonum aviculare* and *Taraxacum officinale.* Negative cross resistance was exerted on *E. crus-galli* in the field in the plots that were treated with metolachlor, glyphosate and in combination with atrazine (1.0 $kg \cdot ha^{-1}$) plus metolachlor (0.8 $kg \cdot ha^{-1}$). In the case of *C. canadensis* a strong increase of perennial *Taraxacum officinale* was recorded (Table 4). As a consequence, the number of other weeds on the control plots were similar or even lower than on plots treated with herbicides. There were no significant differences in effectiveness of the herbicides used. Due to the extreme infestation of some of the plots by *Poa annua* and *Taraxacum officinale,* the presence of other species was limited. In order to combat the resistant plants of *C. canadensis* application of a reduced rate of atrazine with a second herbicide was more effective than single treatment with higher rate. This, in addition to better control of resistant plants allowed us to reduce the herbicide input to the environment. Negative cross resistance was also observed, but was much less evident than in the case of *E. crus-galli.* It is worthwhile to note that the species composition of the herbicide treated weed communities was also influenced by more rainy and cooler weather, which as we assume favors the resistant biotype over the susceptible biotype.

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 last reports we discussed how chelators that remove copper from the first enzymes of the pathway can prevent oxidant detoxification. The synthesis program continued, based on dithiocarbamate derivatives. In the past year much was done with four such compounds. We previously reported the physical, chemical and biochemical characterization of four of these compounds, to better understand the system. As none of these proved to have a sufficient level of synergy to consider for use in the field, the applicable side of such studies has been terminated, and the graduate student working on this is now 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.

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)

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 (9-10 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>

Last year we reported at length how 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.

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 (Ye and Gressel, unpublished) and we indeed found that there were constitutively elevated levels of this enzyme in the paraquat-resistant biotype of *Conyza bonariensis* (Fig. 2). Furthermore, a 24 h prestressing of the plants with 1μM paraquat induced even higher levels of this enzyme.

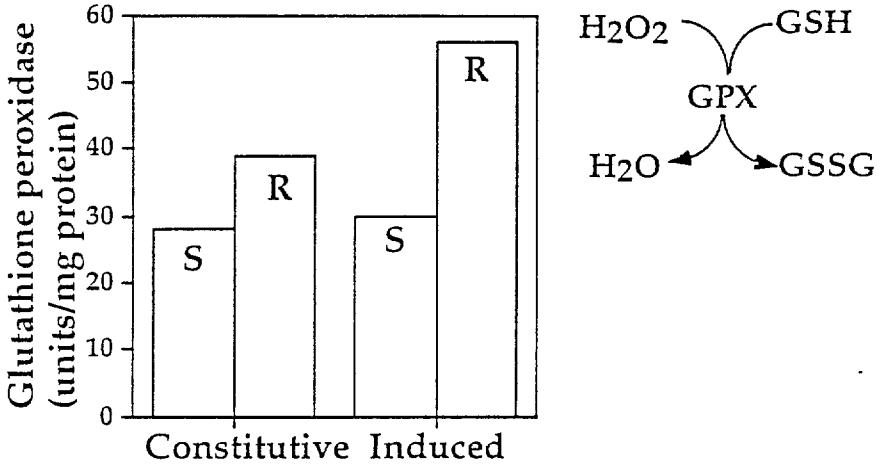

Figure 2. Elevated constitutive levels of glutathione peroxidase and its inducibility in paraquat-resistant *Conyza*. GSH-peroxidase activity was assayed by fluorometry with NAM based on the principle of monitoring the increase of GSSG caused by the enzymatic peroxidation of GSH (Kamata,T. et al. Biosci. Biotech. Biochem. 58, 878-880, 1994). Unreacted GSH was eliminated with N-ethylmaleimide, and the GSSG formed reduced to GSH with $NaBH_4$, which was assayed by fluorometry with NAM. The reaction mixture contained 0.6 ml 0.1 M sodium phosphate buffer,(pH 7.5), 0.1 ml 35 mM NaN_3, 0.1 ml 50 mM GSH in 40 mM EDTA), 0.1 ml of crude enzyme extract. The enzyme reaction was started with 0.1 ml 4 mM tBHPO. The 1.0 ml mixture was incubated at 25 °C for 10 min. Then a 0.1-ml aliquot was added to 0.1 ml 100 mM of NEM (*n*-ethylmaleimide solution dissolved in 0.5 M HEPES buffer, pH, 7.5. The mixture was shaken at room temperature for 30 min. NEM and GSSG were reduced by 0.1 ml of 8.0 M of $NaBH_4$ containing 0.15 N NaOH under the same conditions. 10 μl of methanol/*n*-octanol (7:3) were adding for defoaming. Excess $NaBH_4$ was decomposed by 0.2 ml of 20% metaphosphoric acid under cooling. A 0.1-ml aliquot was added to 3.0 ml of 0.5 M boron carbonate buffer, pH 8.8, and 0.1 ml of 0.25 mM NAM in acetone was added. The solution was left for 60 min in the dark and fluorescence intensity was measured at 435 nm with excitation at 360 nm. The fluorescence increase by nonenzymatic oxidation was assayed as the blank. One unit of GPX specific activity was defined as the amount of activity need to oxidize 1 μmol of GSH per min at pH 7.0 at 25 °C

We can now summarize our cumulative enzyme data, for all the enzymes measures so far in Table 5.

The newly added enzyme, glutathione peroxidase can directly accept electrons from peroxide, augmenting the previously reported effects of ascorbate peroxidase and mono and hydroascorbate reductases (Fig. 3).

Table 5. Constitutive activities of antioxidant enzymes in *Conyza* and induced activities 24 hours after 1.0 μM paraquat pretreatment

	Sensitive biotype			Resistant biotype		
			Enzyme Activities			
Plant age	cont.	treat.	% of cont.	cont.	treat.	% of cont.
Chloroplast SOD (U/mg protein)[a]						
2 weeks	68.4±4.7	63±6.9	**93**	71±6.6	81±10.2	**115**
10 weeks	110±10.6	113±9.2	**103**	122±7.7	164±9.6	**134**
Cytosolic SOD (U/mg protein)						
2 weeks	80±5.8	64±9.4	**80**	74.4±9.8	89.2±12.6	**120**
10 weeks	93±5.9	118±12.6	**126**	139±11.8	170±11.8	**122**
APX						
2 weeks	3.5±0.2	2.3±0.24	**66**	2.0±0.06	2.6±0.06	**130**
10 weeks	2.3±0.16	1.9±0.16	**83**	3.2±0.24	4.5±0.27	**141**
DHAR (U/μg protein)[c]						
10 weeks	8.6±0.12	7.7±2.4	**90**	10.7±1.1	18.2±7.1	**170**
MDAR (U/μg protein)[d]						
10 weeks	7.4±0..6	7.7±1.1	**104**	6.7±0.4	9.1±0.4	**136**
GPX (U/mg protein)[e]						
2 weeks	18.8±1.2	24±1.6	**127**	21.5±1.6	24±0.5	**114**
10 weeks	23.3±2.3	25±2.4	**107**	42.7±2.6	57±3.2	**133**
GR (U/mg protein)[f]						
2 weeks	12.3±0.4	30.3±3.3	**246**	11.4±1.4	24.4±3.6	**216**
10 weeks	17.7±1.7	30.6±3.5	**170**	22.4±2.4	26±2.4	**116**

Abbreviations: SOD=superoxide dismutase; APX=ascorbate peroxidase; DHAR=dehydroascorbate reductase; MDAR=monodehydroascorbate reductase; GPX= glutathione peroxidase; GR=glutathione reductase; con.=control; treat.=pretreatment.

a. One unit of superoxide dismutase activity is defined as the amount of enzyme that inhibits the rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C.

b. One unit of ascorbate peroxidase activity is defined as the amount of enzyme that oxidizes 1 mmol of ascorbate per minute at 25° C.

c. One unit of DHAR activity is defined as the amount of enzyme that reduces 1 mmol of ascorbate from DHA per minute at 25° C.

d. One unit of MDAR activity is defined as the amount of enzyme that oxidizes 1 mmol of NADH per minute at 25° C.

e. One unit of GPX activity is defined as the amount of enzyme that oxidize GSH per min per min at pH 7.0 at 25°C. The data of the ten week are from only one experiment.

f. One unit of glutathione reductase activity is defined as the amount of enzyme that oxidizes 1 nmol of 5,5'-dithiobis(2-nitrobenzoic acid)(DTNB) per minute at 25°C.

Methods: As per last year's report and Figure 3.

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 (Figure 4). 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

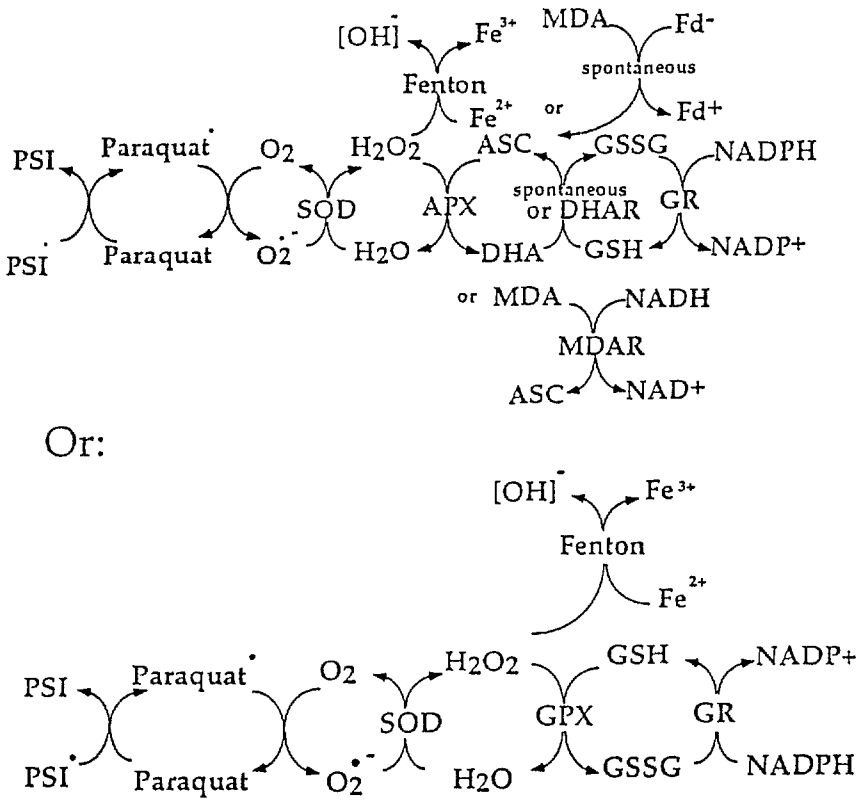

Figure 3. A schematic diagram showing the function of the various enzymes thought to be up-regulated in oxidant resistant *Conyza.*

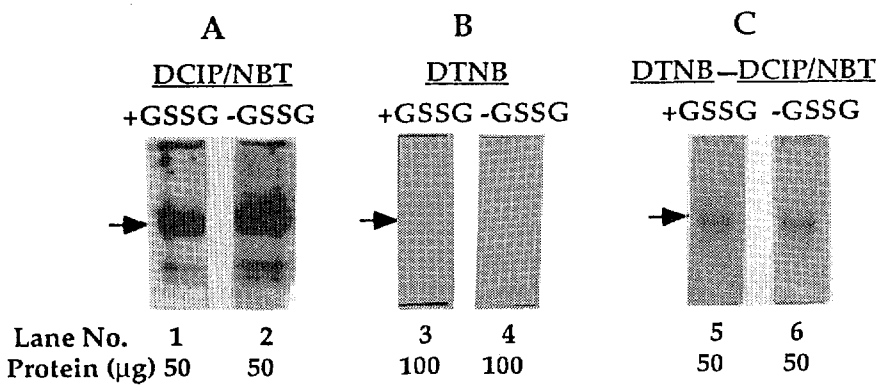

Figure 4. Comparison of old and new methods for staining glutathione reductase on gels. (A) NADPH-DCIP/NBT staining with GSSG (lane 1) and without GSSG (lane 2); (B) NADPH-DTNB staining with GSSG (lane 3) and without GSSG (lane 4); (C) Sequential method first staining with NADPH-DTNB and later staining with DCIP/NBT, with GSSG (lane 5) and without GSSG (lane 6). A crude enzyme extract was prepared from *Conyza bonariensis*, and aliquots were loaded on a standard gel. Fifty µg protein per slot were applied, except in B, where 100 µg were loaded. The gel was cut into strips for each method of analysis. The arrows show the location of the GSSG-specific glutathione reductase band. For further details see Ye et al., 1997.

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 (Figure 5).

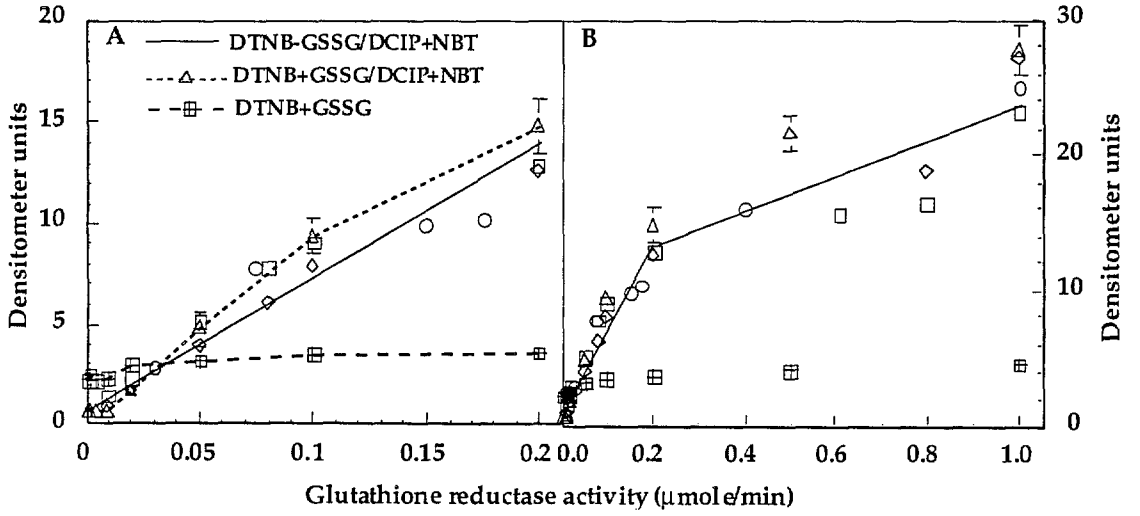

Figure 5. *Densitometric quantification of glutathione reductase staining before and after counter-staining with DCIP/NBT.*
The major band was scanned from positive prints of gels in experiments performed at different times. (A) The averages of three different experiments were normalized based on the densitometer values for 0.2 µmole/min of GSSG reductase activity; and (B) for 1.0 mmole/min of enzyme activity to correct for differences in photographic developing. The solid regression line is from the average data of three different experiments (o, ◊, and □) performed without GSSG. The regression equations and r^2 of the lines (- - ■ - -), (--Δ--), and (—) from 0.02 to 0.2 µmole/min of glutathione reductase activity (A) are Y=7.13X+2.5, r^2=0.77; Y=74.97X+0.52, r^2=0.98; and Y=62.2X+0.73, r^2=0.98, respectively. For further details see Ye et al., 1997.

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 Table 6. 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 (Table 6) 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 (Table 7). 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

Table 6. Transcript abundance of antioxidant enzymes 24 hours after 1μM paraquat pretreatment in *Conyza*

	Relative mRNA					
	Sensitive biotype			Resistant biotype		
	Cont.	Pretreat.	% of Cont.	Cont.	Pretreat.	% of Cont.
Chloroplast SOD						
2 weeks	100	150	**150**	112	132	**118**
10 weeks	138	124	**90**	153	173	**113**
Cytosolic SOD						
2 weeks	100	95	**95**	114	126	**111**
10 weeks	91	75	**82**	113	187	**166**
Cytosolic APX						
2 weeks	100	126	**126**	122	134	**110**
10 weeks	187	116	**62**	194	276	**142**
GSH-peroxidase						
10 weeks	100			134		
Chloroplast GR						
2 weeks	100	157	**157**	151	181	**120**
10 weeks	231	247	**107**	263	328	**125**

The relative mRNA levels were compared with those of the sensitive controls of 2 week old plants. The data of 2 weeks and 10 weeks were from the same northern blotting experiments. The cDNA probe for chloroplast and cytosolic superoxide dismutases from tomato were kindly provided by E. Galun The probe for cytosolic ascorbate peroxidase from pea was kindly provided by R. D. Allen. The cDNA probe for glutathione peroxidase from citris was kindly provided by Dr. D. Holland. The cDNA probe for chloroplast glutathione reductase from pea was kindly provided by G. Creissen. About 15 μg of total RNA extracted from leaf tissue were used for each blot. A plant tubulin cDNA probe from pea (kindly provided by G. Segal) was used as a non variable, native mRNA control. Densitometic analysis were performed by using Molecular Analyst™ /Macintosh Image Analysis Software.

Table 7. Free polyamine contents in *Conyza* leaves at 2 and 10 weeks of age

Polyamines	2 weeks			10 weeks		
(μM/g fw)	Sensitive	Resistant	R/S	Sensitive	Resistant	R/S
Putrescine	7	15	**2.5**	9.5±2	54±6	**5.7**
Spermidine	25	20	**0.8**	33±3	61±9.6	**1.85**
Spermine	240	270	**1.1**	360±14	365±110	**1.0**
Ratio of putrescine/spermidine + spermine						
	0.0264	0.0517	**2.0**	0.0242	0.127	**5.3**

Perchloric acid soluble dansylated polyamines were separated by TLC and spots dissolved in ethylacetate. The concentrations were determined by measuring fluorescence at 495 nm with an activating wavelength of 350 nm. Standards putrescine, spermidine and spermine were used for calibration.

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.

f. Strategies for resistance management.

A key part of this project is to design strategies for resistance management. Our colleagues in Poland 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., submitted - see section B5). 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.5 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 t riazine 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.

Gardner, S. N., J. Gressel, M. Mangel ( ) A strategy to delay the evolution of pesticide and drug resistances when they can be both polygenic and monogenic. (submitted).

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

Gressel, J. (1996) Fewer constraints than proclaimed to the evolution of glyphosate-resistant weeds. Resistant Pest Management (in press)

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.

Ye, B., C. Gitler and J. Gressel (1997) A high sensitivity, single gel, PAGE method for quantification of glutathione reductases. Anal. Biochem. 245:(in press).

C. Scientific Impact of Collaboration.

The senior investigators met at meetings 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 under the auspices of the project. Below is his report:

Report on Scientific Visit to the Israel (April 22 - May 10, 1996) As a part of collaboration, Co-Principal Investigator S. Gawronski in 1996 visited Weizmann Institute of Science at Rehovot, Israel under the auspices of this project. This visit provided an opportunity to meet Prof. J. Gressel and other members of the Department of Plant Genetics as well as for discussion of all aspects of studied problems i.e. of parasitic weeds, weed resistance to herbicides including negative cross- resistance and synergism as a main topics. Additionally, there was a chance for detailed discussion with Prof. Gressel's colleagues on other aspects of research areas covered in the Department such as plant reaction to oxidative, UV and pathogen stresses which are on the top world level. Taking a part in the Life Science Open Day (08.05.1996) was a good background for S. Gawronski for general orientation in the research directions of Weizmann Institute. The 24h/daily access to well organised scientific library allowed S. Gawronski to up-date with the literature in case of less-available in Poland journals. S. Gawronski visited also the Weed Science Laboratory, Faculty of Agriculture Food & Environment, The Hebrew University of Jerusalem headed by Prof. Baruch Rubin where weed control, weeds resistance to herbicides (monitoring, biology and mechanisms of resistance) were discussed including teaching of weed science on the academic level. Also meeting with Dr.Tuvia Yaacoby, Chief Herbologist from Plant Protection & Inspection Services, Ministry of Agriculture was arranged in order to get orientation in the procedure of registration of new herbicides as well as in the policy and strategy in that area of agriculture in Israel. An interesting part was the exposure to practical agriculture by visiting individual farms in the north of the country and kibbutz in the Negev Desert with contrasting climatic conditions.

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.

E. Strengthening Developing Country Institutions

The ability to perform the project and its interim success strengthen the academic aspects of the Warsaw lab. The modest size of the grant precludes anything but modest addition to their physical facilities.

F. Future Work

Because the project was arbitrarily extended to five years, instead of the three requested, we are clearly on schedule.

Section II

A. Managerial Issues.

No issues beyond those discussed in earlier reports.

B. Budget

No major changes were made

C. Special Concerns

None at present

D. Collaboration, Travel, Training and Publications.

See travel report (Section IC). There was also considerable collaboration by phone, letter and fax and email. No joint publications as yet, but we have discussed each others work at length.

E. Request for A.I.D. / BOSTID Actions

None.