Indian J. Plant Physiol., Vol. 13, No. 3, (N.S.) pp. 217-223 (July-Sept., 2008)

ISOPROTURON INDUCED CHANGES IN PEROXIDASE AND CATALASE ENZYMES IN SUSCEPTIBLE AND RESISTANT BIOTYPES OF *PHALARIS MINOR* RETZ.

RUPA S. DHAWAN*, ROHTAS SINGH, A.K. DHAWAN AND J.P.S. DHINDSA

CCS Haryana Agricultural University, Regional Research Station, Uchani, Karnal-132 001, Haryana

Received on 12 Sept., 2006, Revised on 17 Sept., 2008

SUMMARY

Peroxidase and catalase activity was studied in isoproturon susceptible and resistant populations of *P.minor* with a view to observe differences in innate capacity of different populations under herbicide stress. Resistant populations differed from susceptible populations in exhibiting higher acitivity of these enzymes at the seedling stage. Isoproturon spray caused increase in peroxidase acivity in the susceptible biotypes 5 days after spray. Catalase activity remained low in the sprayed susceptible biotypes. Unsprayed susceptible and resistant biotypes did not differ in isoenzyme pattern of these enzymes. Sprayed resistant plants differ from sprayed susceptible plants in continued presence of isoperoxidases at Rfs 0.32 and 0.36 at 5 days of sampling and at Rfs 0.28, 0.30 and 0.58 at 10 days of sampling.

***Key words*: Catalase, isoproturon resistance, peroxidase, *Phalaris minor* Retz.**

INTRODUCTION

Phalaris minor Retz. (little seed canary grass) a weed of wheat fields has been exposed to urea herbicide isoproturon [N'(4-isopropyl phenyl) N,N dimethyl urea] for its management in the wheat growing regions of India since 1970's. In Haryana and Panjab as a result of exposure to this herbicide continuously for 10-15 years, some of the populations developed insensitivity to this category of herbicides (Dhawan and Malik 1995, Malik and Singh 1995, Walia *et al.* 1997, Yaduraju *et al.* 1995). In some areas of Uttaranchal, however, the development of resistance to the same herbicide could not be observed even after being exposed to an equivalent selection pressure by the same herbicide, thereby, providing indications regarding heterogeneity in the weed at different locations. A study of genetic variations amongst weed biotypes becomes important to devise sound weed management practices since genetically variable biotypes may show differential behaviour towards herbicides. Genetic variation has been assessed by studying morphological variations, variations in protein profiles and isoenzymes in different biotypes of a weed species (Gasquez and Compoint, 1976, Asins *et al.* 1999, Sterling *et al.* 2000). Since resistant biotypes are selected from the existing susceptible populations, it becomes important to assess the genetic variation amongst the resistant biotypes as well for their management. Electrophoretic data has been helpful in establishing homogeneity in triazine resistant *Chenopodium* populations (Mouemar and Gasquez 1983). A reduction in genetic variation in triazine resistant *Amaranthus retroflexus* and *Brassica rapa* biotypes has also been established by isoenzyme analysis (Warwick and Black 1988, Warwick and Black 1993).

Studies on the mode of evolution of resistance in this weed in two biotypes from Haryana, revealed that the target site is not affected (Singh *et al.* 1997). Rather detoxification of the herbicide by enhanced levels of

*Corresponding author, Present address: Department of Agronomy, CCS Haryana Agricultural University, Hisar-125 004 (Haryana), India, E-mail: rupadhawan@hotmail.com

oxidative enzyme cytochrome P450 monoxygenase is responsible for imparting resistance. Inhibitors of this enzyme like piperonyl butoxide and 1-aminobenzotriazole when used in combination with isoproturon could cause the reversal of resistance to isoproturon in these biotypes (Singh *et al.* 1998a, b). Since, herbicides especially photosynthetic inhibitors are known to cause oxidative stress (Arora *et al.* 2002, Grossman *et al.* 2001, Stajner *et al.* 2003), it was considered of interest to assess whether difference in activity and isoenzyme pattern of oxidative enzymes like peroxidase and catalase could provide leads to genetic diversity amongst the susceptible and resistant biotypes. This investigation was therefore, planned with a view to assess (i) if there existed any innate difference in activity of these enzymes in susceptible and resistant biotypes from different locations for early identification of the populations for early management (ii) to identify markers for the resistant trait in the form of specific isoenzymes of peroxidase and catalase and (iii) to assess if isoproturon spray caused any variable effects in susceptible and resistant biotypes.

MATERIALS AND METHODS

Two experiments were conducted. The first experiment involved a study of the levels of the enzymes catalase and peroxidase in susceptible and resistant biotypes at the seedling stage. In the second experiment effect of isoproturon spray on the activity and isoenzyme pattern of the enzymes peroxidase and catalase was studied. For first experiment, seeds of ten susceptible populations from different locations in India, viz. Madhya Pradesh [Jabalpur (1) and Gwalior (2)]; Uttaranchal (3); Himachal Pradesh [Palampur (4)]; Uttar Pradesh [Kanpur(5) and Faizabad (6)]; Bihar [Samastipur (7) and Ranchi (8)] and Haryana [Karnal (9) and Hisar (10)] and of 10 resistant populations, four from Panjab[Ludhiana (1), Amritsar (2), Sangrur (3) and Kapurthala (4)] and six from Haryana [Sagga (5), Hisar (6), Bhiwani (7), Ambala (8), Panipat (9) and Kaithal (10)] were allowed to germinate in Petriplates (22cms dia) lined with filter papers and soaked in distilled water. Ten day old seedlings were harvested for enzyme assay. For second experiment seeds of five susceptible biotypes viz. Uchani, Uttranchal, Madhya Pradesh, Ranchi and Panipat and 3 resistant biotypes viz. Uchana, Amritsar and Kapurthala were sown in pots in November, 2005. The plants were thinned to fifty per pot, two weeks after sowing. These were sprayed with 1 kg/ha isoproturon with the help of a knapsack sprayer 35 days after sowing when the plants had attained 2-3 leaf stage. Unsprayed plants were kept as controls. Upper two leaves were harvested for enzyme assay.

Enzyme extraction: Leaf tissue (100 mg) in 2 replicates was homogenized in 1ml phosphate buffer (0.1M, pH 7.0) in a pre-chilled glass pestle and mortar at 4°C. The homogenate was centrifuged at 5,600 g for 30 min. Pellet was discarded and the supernatant was used for enzyme assay and isoenzyme analysis.

Peroxidase assay: Peroxidase was assayed by the method of Chance and Maehley (1955). To 50 ml of enzyme extract in the cuvette, added 2.5 ml of benzidine reagent (1% bezidine in 25% glacial acetic acid and 1% H_2O_2 in 1:1 ratio, i.e. 1+1 ml diluted to 40 ml with distilled water) and rise in optical density (O.D.) was recorded at 620 nm against blank up to 3 min at 1 min interval. Enzyme activity was expressed in terms of rate of increase of absorbance per min per mg protein. One unit of enzyme was arbitrarily defined as 1.0 change in O. D. per min per mg protein.

Catalase assay: The method of Sinha *et al.* (1972) was adopted. To 100 µl of enzyme extract in the test tube, added 0.5 ml of H_2O_2 (0.2 M) and 0.7 ml phosphate buffer (0.1 M). The mixture was incubated at 37°C for 5 min in the incubator. To it 4.0 ml of potassium dichromate reagent (5% potassium dichromate and 100% glacial acetic acid in 1: 3 ratio) was added. The mixture was placed in a boiling water bath for 10 min and after cooling the O.D. was recorded at 570 nm against blank. The standard curve was prepared by using graded concentrations of H_2O_2. The enzyme activity was expressed as mg H_2O_2 hydrolyzed/min/mg protein.

Isoenzyme analysis: The isoenzymes were separated by polyacrylamide gel electrophoresis (PAGE) following the method of Laemmli (1970). Enzyme extract (60 µl) was mixed with 40 ml sample buffer (20% glycerol + 2% bromophenol blue) and loaded on to a discontinuous gel system with a 0.1 mm thick stacking gel of 4%

polyacrylamide in Tris - HCl (pH 6.8) and a resolving gel of 10% polyacrylamide in Tris - HCl buffer (pH 8.8). The gels were run at 25 mA in the stacking gel and 50 mA in the resolving gel. After electrophoresis the gels were stained with 100 ml benzidine reagent containing equal amount of 0.3% benzidine in 25% acetic acid and 0.5% H_2O_2 for 2-5 min (Guikema and Shermen 1980). Blue colored bands appeared which turned brown after 10-15 min. Rf was calculated by dividing the distance traveled by a band with the distance traveled by the tracking dye.

To stain for catalase 100 ml solution of H_2O_2 (3 mM) was poured on the gel in a tray, covered and incubated at 37°C in the incubator or at room temperature for 5 min. After that H_2O_2 solution was removed from the tray and a mixture of 100 ml solution containing equal amount of 2% potassium ferricyanide and 2% ferric chloride was added. Left the gel in it for 10 min in a dark and air-tight chamber. After that poured off the solution and washed the gel with distilled water. The gel was stained dark while clear/white bands of isoenzymes were spared out (Andersen *et al.* 1995).

RESULTS AND DISCUSSION

Peroxidase and catalase activity at the seedling stage: Peroxidase activity varied within a range of 5-10 enzyme units amongst the susceptible biotypes, lowest being in case of biotype from Samastipur and highest being in case of biotype from Jabalpur. In resistant biotypes it varied between a range of 13-32 enzyme units, the lowest being in case of biotype from Amritsar and highest being in case of biotype from Ambala. (Table 1).

Variation in catalase activity within the susceptible biotypes was between 10-12 mg H_2O_2 hydrolyzed / min /mg protein. In the resistant biotypes it was higher and varied between 15-27 mg H_2O_2 hydrolyzed /min /mg protein (Table 2).

Peroxidase and catalase activity as affected by isoproturon spray in susceptible and resistant biotypes: Peroxidase activity in the sprayed susceptible biotypes Uchani, Uttranchal and Ranchi remained the same as in the unsprayed ones till 5 days after spray and increased after this time. In the sprayed biotypes from Jabalpur and Panipat the activity was higher as compared to unsprayed biotypes at all the dates of sampling. In case of resistant biotypes no such difference could be seen (Fig.1).

A total of 15 bands with Rfs 0.009, 0.03, 0.06, 0.20, 0.24, 0.26, 0.28, 0.30, 0.32, 0.36, 0.38, 0.52, 0.56, 0.58 and 0.60 could be seen in all 8 biotypes at 1 day of sampling in the unsprayed lot indicating that the biotypes could not be differentiated on the basis of isoenzyme

Table 1. Peroxidase activity (enzyme unit, i.e. change in 1.0 O.D/min/mg protein) in isoproturon susceptible and resistant biotypes of *P. minor* in 10 days old seedlings.

Susceptible Biotypes		Resistant Biotypes	
Jabalpur	9.900 ± 0.104	Ludhiana	14.480 ± 0.277
Gwalior	9.009 ± 0.423	Amritsar	13.691 ± 0.092
Uttaranchal	8.042 ± 0.140	Sangrur	16.056 ± 0.596
Palampur	9.402 ± 0.249	Kapurthala	14.644 ± 0.544
Kanpur	6.747 ± 0.350	Sagga	20.765 ± 0.351
Faizabad	9.613 ± 0.803	Hisar (Dabra)	18.933 ± 0.186
Samastipur	5.349 ± 0.435	Bhiwani (Alakpura)	17.460 ± 0.303
Ranchi	7.708 ± 0.210	Ambala	32.744 ± 0.840
Karnal	8.021 ± 0.360	Panipat	14.661 ± 0.166
Hisar	6.845 ± 0.348	Kaithal	15.746 ± 0.084

Table 2. Catalase activity (mg H_2O_2 hydrolysed/min/mg protein) in isoproturon susceptible and resistant biotypes of *P. minor* in 10 days old seedlings.

Susceptible Biotypes		Resistant Biotypes	
Jabalpur	10.686 ± 0.067	Ludhiana	15.274 ± 0.239
Gwalior	10.804 ± 0.023	Amritsar	15.245 ± 0.238
Uttaranchal	10.636 ± 0.096	Sangrur	16.516 ± 0.695
Palampur	11.657 ± 0.042	Kapurthala	16.833 ± 0.245
Kanpur	11.100 ± 0.110	Sagga	19.535 ± 0.259
Faizabad	12.083 ± 0.230	Hisar (Dabra)	16.631 ± 0.280
Samastipur	12.797 ± 0.002	Bhiwani (Alakpura)	18.750 ± 0.261
Ranchi	10.834 ± 0.115	Ambala	27.879 ± 0.153
Karnal	10.096 ± 0.093	Panipat	16.100 ± 0.001
Hisar	10.476 ± 0.034	Kaithal	18.676 ± 0.162

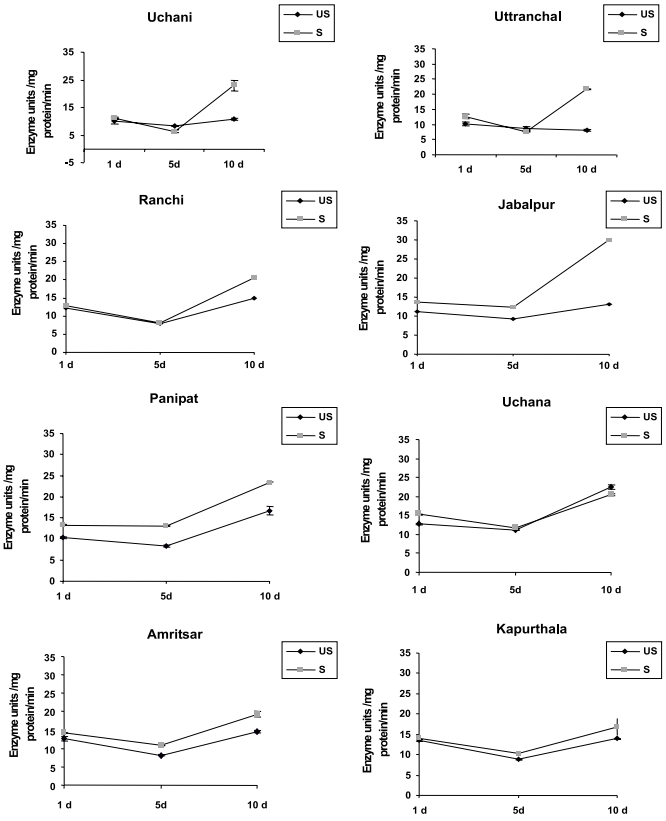

Fig. 1. Peroxidase activity in susceptible (Uchani, Uttranchal, Ranchi, Jabalpur and Panipat) and resistant (Uchana, Amritsar and Kapurthala) biotypes of *P. minor* at 1, 5 and 10 day of sampling after isoproturon spray. Data is the mean of 4 replicates. Bars indicate standard error.

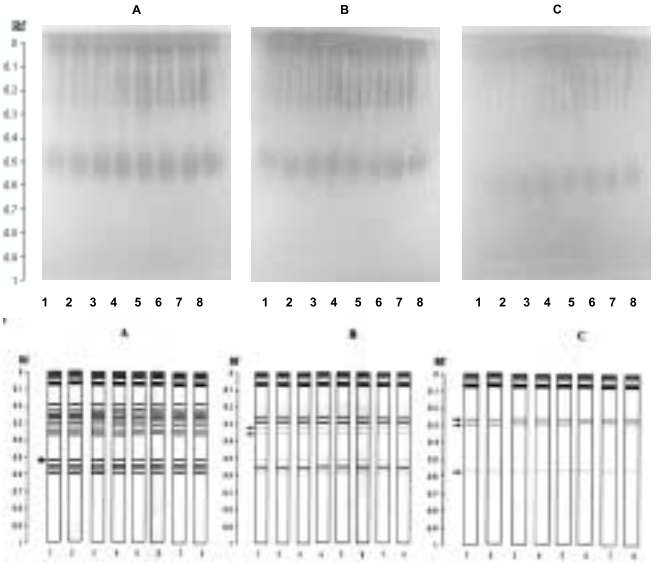

Fig. 2. Gel photograph and zymogram showing peroxidase isoenzymes in isoproturon susceptible and resistant biotypes of *P. minor* at 1(A), 5(B) and 10(C) day of sampling after isoproturon spray. 1=Uchani, 2=Uttranchal, 3=Jabalpur, 4=Ranchi, 5=Panipat, 6=Uchana, 7=Amritsar and 8=Kapurthala.

pattern of peroxidases. On 5th day after spray 14 bands could be seen in this lot. The band with Rf 0.38 was missing. At 10 days of sampling 6 bands with Rfs 0.009, 0.03 and 0.06, 0.28, 0.30 and 0.58 were observed in the same lot. In the sprayed lot, band with Rf 0.52 was missing in both the susceptible and resistant biotypes at

1 day of sampling. The sprayed resistant plants differed from sprayed susceptible plants in the presence of bands at Rfs 0.32 and 0.36 at 5 day of sampling and bands with Rfs 0.28, 0.30 and 0.58 at 10 days of sampling. No correlation of activity with the presence and absence of bands could be observed (Fig. 2).

Catalase activity in all the susceptible biotypes was low after isoproturon spray as compared to the unsprayed ones. In case of resistant biotypes the differences in activity of sprayed and unsprayed plants was negligible in case of biotype from Uchana. The activity was low in sprayed plants in case of biotype from Kapurthala and high in case of biotype from Amritsar (Fig. 3). Three bands with Rfs 0.007, 0.23 and 0.10-0.13 could be observed in the S and R biotypes at 1 day of sampling in unsprayed as well as sprayed lot.

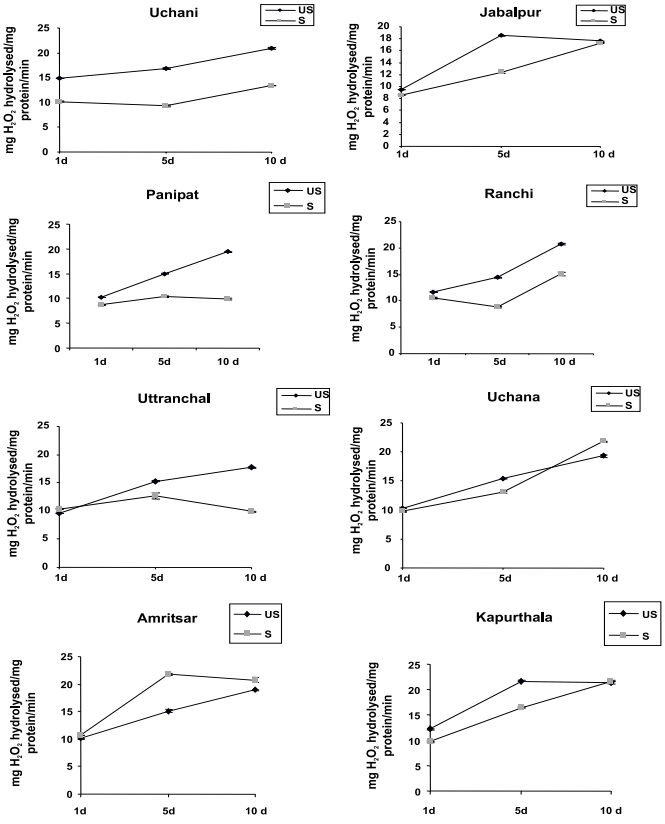

Fig. 3. Catalase activity in susceptible (Uchani, Uttranchal, Ranchi, Jabalpur and Panipat) and resistant (Uchana, Amritsar and Kapurthala) biotypes of *P.minor* at 1, 5 and 10 day of sampling after isoproturon spray. Data is the mean of 4 replicates. Bars indicate standard error.

At 5th day a band with Rf 0.044 appeared in the both the S and R biotypes in the unsprayed as well as sprayed lots. At 10 days again the three bands observed at day 1 could be seen. No difference could be observed within the S and R biotypes. Only the length of the band at Rf 0.10-0.13 was seen to decline in sprayed plants at all the stages (Fig. 4).

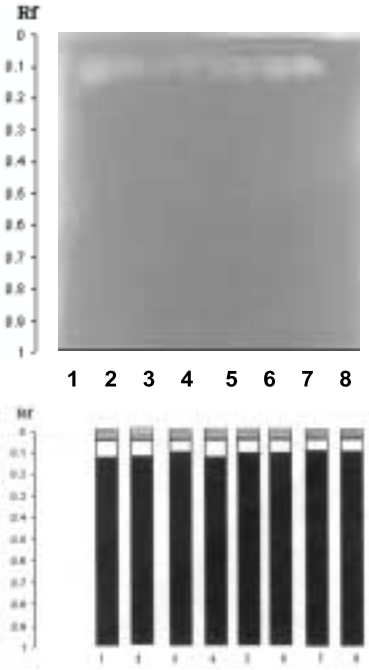

Fig. 4. Gel photograph and zymogram showing catalase isoenzymes in isoproturon susceptible and resistant biotypes of *P. minor* at 5 day of sampling after isoproturon spray. 1=Uchani, 2=Uttranchal, 3=Jabalpur, 4=Ranchi, 5=Panipat, 6=Uchana, 7=Amritsar and 8=Kapurthala.

Increased activity of peroxidase and catalase in isoproturon resistant biotypes of *Phalaris minor* is indicative of the fact that one of the adaptation mechanisms developed by resistant biotypes could be related to increased innate capacity to lower the levels of toxic compounds like H_2O_2. This is in line with the observations in paraquat resistant biotypes of *Erigeron canadensis* and *Conyza bonariensis* (Ye and Gressel 1994, Pyon *et al.* 2004) and is suggestive of the fact that antioxidant enzyme activity may be exploited as a parameter to evaluate herbicide resistance/tolerance in a biotype. Similar indications have been observed in case of triclopyr effects in aquatic plants and chlorosulfuron effects in transgenic tobacco plants (Sprecher *et al.*

1993, Sprecher and Stewart 1995, Kapchina-toteva *et al.* 2004). The levels of antioxidant enzymes in paraquat resistant and susceptible populations of *Lolium rigidum* from Australia, however, did not differ and the mechanism of resistance was found to be sequestration of paraquat limiting its translocation within the plants (Yu *et al.* 2004). In case of paraquat resistant red flower ragleaf (*Crassocephalum crepidioides* Benth S. Moore) high levels of superoxide dismutase could be correlated with resistance instead of peroxidase (Ismail *et al.* 2001).

An increase in peroxidase activity in susceptible biotypes 5 days after spray indicates that the plants are under stress and high peroxidase activity is induced to scavange the hydrogen peroxide produced as a result of the herbicide stress. No such change in the resistant biotypes indicates that the plants are free of stress and have developed a mechanism to evade herbicide stress. High peroxidase activity after exposure to atrazine in atrazine insensitive marsh plant *Spartina alternifolia* could be observed (Lytle and Lytle 1998). Lowered catalase activity in sprayed susceptible plants indicates lowered ability to scavange hydrogen peroxide in these biotypes. In the resistant biotype from Amritsar high activity might indicate an adaptation to the exposure to the herbicide. An increase in activity of catalase after exposure to isoproturon was also observed in aquatic worm *T. tubifex* demonstrating the occurrence of an oxidative stress response (Yahia- Mosieh *et al.* 2005).

ACKNOWLEDGEMENTS

This study was supported by a grant from Indian Council of Agricultural Research, New Delhi in the form of an ad-hoc research project. Seed material supplied by different AICRP-WC centres is gratefully acknowledged. Thanks are also due to Dr. N. T. Yaduraju, former Director, NRCWS, Jabalpur for his keen interest in the progress of the work.

REFERENCES

Andersen, M.D., Prasad, T.K., Stewart, C.R. (1995). Changes in isozyme profiles of catalase, peroxidase and glutathione reductase in acclimation to chilling in mesocotyls of maize seedlings. *Plant Physiol*. **109:** 1247-1257.

Asins, M.J., Carretero, J.L., Del Best, A., Carbenell, E.A. and De Barreda, D.G. (1999). Morphologic and isoenzyme variation in Barnyard grass (Echinocloa) weed species. *Weed Tech*. **13:** 200-215.

Arora, A., Sairam, R.K. and Srivastava, G.C. (2002). Oxidative stress and antioxidative system in plants. *Curr. Sci*. **82:** 1227-1238.

Chance, B. and Maehley, A.C. (1955). Assay of catalases and peroxidases. *Meth. Enzymol*. **2:** 764-775.

Dhawan, R.S. and Malik, R.K. (1995). Effect of isoproturon on reducing and non-reducing sugars in two biotypes of *Phalaris minor* Retz. *Indian J. Plant Physiol*. **38:** 171-172.

Gasquez, J. and Compoint, J.P. (1976). Apport de l'e electrophore'se en countrant pulse' a' la tazonomie d *Echinocloa crusgalli*(L).P.B. *Ann. Anelior. Plantes*. **26:** 345-3555.

Grossman, K., Kwiatkowski, J. and Tresch, S. (2001). Auxin herbicide induced H_2O_2 overproduction and tissue damage in cleavers (*Galium aparine* L.). *J. Exp. Bot.* **52:** 1811-1816.

Guikema, J.A. and Shermen, L.A. (1980). Electrophoretic profiles of cyanobacterial membrane polypeptides showing heme dependent peroxidase activity. *Biochem. Biophys. Acta*. **637:** 189-201.

Ismail, B.S., Chaoh, T.S., Salmijah, S. and Hussain, K.H. (2001). Role of superoxide dismutase and peroxidase activities in paraquat resistant redflower ragleaf (*Crassocephalum crepidioides* (Benth) Moore). *Aust. J. Agri. Res.* **52:** 5276-548.

Kapchina, Toteva,V., Slavov, S. Batchvarova, R., Krantev, A., Stefanov, D., Uzunova, A. (2004). Sress markers in chlorosulfuron tolerant trnagenic tobacco plants. *Bulg. J. Plant. Physiol*. **30:** 103-111.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of the bacteriophage Ty. *Natur*e. **277:** 680-685.

Lytle, J.S. and Lytle, T.F. (1998). Atrazine effects on estuarine macrophytes *Spartina alternifolia* and *Juncus roemarianus*. *Environ. Toxicol. Chem.* **17:** 1972-1978.

Malik, R.K. and Singh, S. (1995). Little seed canary grass (*Phalaris minor*) resistance to isoproturon in India. *Weed Tech.* **9:** 419-425.

Mouemar, A.A. and Gasquez, J. (1983). Environmental conditions and isoenzyme polymorphism in *Chenopodium album. Weed Res*. **23:** 141-149

Pyon, J.Y., Piao, R.Z. , Roh, S.W., Shin, S.Y. and Kwak, S.S. (2004). Differential levels of antioxidants in paraquat resistant and susceptible *Erigeron canadensis* biotypes in Korea. *Weed Biol. Management*. **4:** 114-121.

Sinha, A.K. (1972). Colorimeteric assay of catalase. *Analy. Biochem*. **47:** 2.

Singh, S., Kirkwood, R.C. and Marshall, G.C. (1997). Effects of isoproturon on photosynthesis in susceptible and resistant biotypes of *Phalaris minor* and wheat. *Weed Res.* **37:** 315-24.

Singh, S., Kirkwood, R.C. and Marshall, G. (1998 a). Effect of the monoxygenase inhibitor piperonyl butoxide on the herbicidal activity and metabolism of isoproturon in herbicide resistant and susceptible biotypes of *Phalaris minor* and wheat. *Pest Physiol. Biochem*. **59:** 143-53.

Singh, S., Kirkwood, R.C. and Marshall, G. (1998 b). Effect of ABT on the activity and rate of degradation of isoproturon susceptible and resistant biotypes of *Phalaris minor* Retz. and in wheat. *Pest. Sci*. **53:** 123-32.

Sprecher, S.L., Stewart, A. B. and Brazil, J.M. (1993). Peroxidase changes as indicators of herbicide induced stress in aquatic plants. *J. Aquatic Plant Manag*. **31:** 45-50.

Sprecher, S.L. and Stewart, A.B. (1995). Triclopyr effects on peroxidase activity in target and non-target aquatic plants. *J. Aquatic Plant Manag.* **33:** 43-48.

Stajner, D., Popovic, M. and Stajner, M. (2003). Herbicide induced oxidative stress in Lettuce, beans, pea seeds and leaves. *Biol. Plant*. **47:** 575-579.

Sterling, T.M. Murray, L.W. and Hou, Y. (2000). Morphological variation among *Gutierrezia sarothrae* populations. *Weed Sci*. **48:** 356-365.

Walia, U.S., Brar, L.S. and Dhaliwal, B.K. (1997). Resistance to isoproturon in *Phalaris minor* Retz in Punjab. *Plant Prot. Quart*. **12:** 138-140.

Warwick, S.I. and Black, L.D. (1986). Electrophoretic variation in triazine resistant and triazine susceptible populations of *Amaranthus retroflexus* L. *New Phytol*. **104:** 661-670.

Warwick, S.I. and Black, L.D. (1993). Electrophoretic variration in triazine resistant and susceptible populations of the allogamous weed *Brassica rapa*. *Weed Res*. **33:** 105-114.

Yahia Mosieh, Y., Paris-Palacios, S., Couderchet, M., Biagianti-risbong, S. and Vernet, G. (2005). Effect of herbicide isoproturon on metallothioneins, growth and antioxidative defenses in the aquatic worm *Tubifex tubifex*. *Ecotoxicology*. **14:** 559-571.

Ye, B. and Gressel (1994). Constitutive variation of ascorbate peroxidase activity during development parallels that of superoxide dismutase and glutathione reductase in paraquat resistant *Conyza*. *Plant Sci.* **102:** 147-151.

Yaduraju, N.T., Ahuja, K.N. and Bansal, K.C. (1995). *Phalaris minor* resistance to isoproturon. In: Abstracts "Biennial conference of Indian Society of Weed Science", pp 124. Annamalai University, Tamil Nadu.

Yu, Qin, Cairn, A. and Powels, S.B. (2004). Paraquat resistance in a population of *Lolium rigidum*. *Funct. Plant Biol.* **31:** 247-254.