Amaranthus tuberculatus (Mq. ex DC) J.D.Sauer: potential for selection of glyphosate resistance

Ian A. Zelaya and Micheal D.K. Owen
2104 Agronomy Hall, Ames, IA 50011, USA

Summary Reports alleging inconsistent common waterhemp (*Amaranthus tuberculatus* (Mq. ex DC) J.D.Sauer) control indicated that the species demonstrated an inherent variability to glyphosate. Correlation of tissue shikimic acid and phenotype in the field supported the tenet that the response was attributable, at least in part, to differences in glyphosate inhibition of 3-phosphoshikimate 1-carboxyvinyltransferase (EPSPS; EC 2.5.1.19).

Whole plant dose responses of the Everly, Iowa *A. tuberculatus* and a pristine population from Paint Creek, Ohio indicated that the Everly biotype demonstrated more variability to glyphosate than the unselected population. Isolation of resistant and susceptible plants through recurrent selection resulted in a 1.7 and 3.5 fold increase in population divergence in the first (F1) and second (F2) filial generations, respectively. While the selection method has increased the frequency of resistant individuals within the population, significant segregation for glyphosate efficacy was still apparent in the selected material. This limited segregation suggested that the response to glyphosate observed in *A. tuberculatus* may be governed by a polygenic event. At present we are attempting to reduce the genetic variability in *A. tuberculatus* and investigate possible resistance mechanisms in asexually propagated plantlets. Characterisation of the mechanism(s) of glyphosate resistance may be important in developing strategies to mitigate potential future problems.

Keywords Asexual reproduction, recurrent selection, log-logistic analysis, shikimic acid, resistance.

INTRODUCTION

Evolution of glyphosate resistance in plants is uncommon, despite of the prolonged and ubiquitous use of the herbicide worldwide (Caseley and Copping 2000). The rareness of this event was attributed to the limited metabolism of glyphosate in plants, the short-half life in the environment and the unique biochemical characteristics of the herbicide, and the fact that engineering glyphosate resistance in crops required of complex molecular modifications (Bradshaw *et al.* 1997). Nonetheless, inter- and intra-specific variability to glyphosate may enable the selection of pre-existent individuals within a population that demonstrate an improved fitness to glyphosate and result in weed population shifts (Baylis 2000).

In higher organisms, glyphosate resistance was engineered by amplification (Goldsbrough *et al.* 1990), enhanced transcription (Klee *et al.* 1987), or increased half-life of EPSPS (Holländer-Czytko *et al.* 1992) and by point mutations in EPSPS associated with an increased apparent dissociation constant (K_i) for glyphosate ($K_i^{glyphosate}$) (Padgette *et al.* 1991). An alternative resistance mechanism comprises glyphosate decarboxylation to α-aminomethylphosphonic acid (AMPA), a less-toxic compound to plants. The enzyme glyphosate oxido-reductase (*GOX*) mediates metabolism to AMPA by oxidation of the N-C_α bond in glyphosate (Barry *et al.* 1992). Concomitant expression of *CP4-EPSPS* and *GOX* instigated high resistance levels in glyphosate-resistant crops (Mannerlöf *et al.* 1997).

Recently, glyphosate resistance has been reported in Italian ryegrass (*Lolium multiflorum* Lam.) (Perez and Kogan 2002), rigid ryegrass (*Lolium rigidum* Gaudin) (Pratley *et al.* 1999), goosegrass (*Eleusine indica* (L.) Gaertner) (Lee and Ngim 2000), and horseweed (*Conyza canadensis* (L.) Cronq) (Van Gessel 2001). To date no cases of glyphosate resistant weed populations have been reported in the Mid West US. However, some Iowa producers have reported inconsistent control of *A. tuberculatus* with glyphosate in glyphosate-resistant crops. Two isolated incidents in Everly and Badger, Iowa suggested that *A. tuberculatus* plants were differentially affected by glyphosate. Plant samples were collected from Everly, Iowa to conduct an exhaustive assessment of the incident and evaluate the potential for glyphosate resistance.

MATERIALS AND METHODS

Evaluation of glyphosate efficacy Ninety-seven plants (53 ♀: 44 ♂) collected from the Everly field were grown to maturity and crossed without pollination restrictions. Glyphosate efficacy was evaluated in the progeny at the whole-plant and seedling levels. Seedling assessment comprised germinating 20 seeds per well in a 24 well cell culture cluster plate in 32mM, 10mM, 3.2mM, 1mM, 0.32mM, 0.1mM, and 0.032mM glyphosate. Seeds were grown at 30°C and 14 hrs light and 20°C 10 hrs dark conditions. Seedling germination, hypocotyl, and radicle length were recorded two weeks after establishment.

Whole plant assessment comprised three transplanted seedlings per 12 cm diameter pot in a peat: perlite:loam soil-mix (1:2:1) which were placed under natural light, and supplemented with 16 hrs of 600–1000 μmol^{-2} m s^{-1} PPFD. When 10–12 cm, plants were arranged in a complete randomised block design with three replications and sprayed with 0, 0.21, 0.52, 0.83, 1.25, and 1.37 kg a.e. ha^{-1} glyphosate. Plants were harvested two weeks after application and visual estimate of herbicide and biomass recorded. Plant biomass was dried at 35 C for 48 hrs; dry apices of treated plants were pooled for shikimic acid determination.

Recurrent selection A recurrent selection process was implemented to isolate resistant and susceptible individuals within the Everly population. This selection was conducted at the seedling level to expedite the recurrent selection process and deal with the prolific seed productivity of *A. tuberculatus*. Each generation was selected for susceptibility and resistance according to parameters determined by the seedling dose response (Table 1). Resistant individuals were considered those surviving the selection media, while susceptible seedlings comprised those demonstrating herbicide injury at low herbicide doses. Selected seedlings were rescued from the media by rinsing in distilled water, treating with Rootone (TechPac, Lexington, KY 40504), and transplanted in the potting soil-mix. Mature plants were intercrossed in plastic tents where pollen contamination was less than 0.01% (Brenner and Widrlechner 1998). Because all 12 plants surviving 10µM glyphosate differentiated to male plants (Table 1), F1-R individuals were backcrossed to female plants previously selected at 3.2mM glyphosate.

Asexual reproduction Perpetuation of specific genotypes was conduced asexually by cutting the main stem of plants, treating with Rootone, and providing 95% relative humidity and 16 hrs 1100 μmol^{-2} m s^{-1} PPFD. This process not only preserved and facilitated seed increase, but also instigated the re-differentiation of floral meristems back to vegetative development.

Shikimic acid assay Determination of shikimic acid was conducted spectrophotometrically with procedures modified from Cromartie and Polge (2000). Ten µl of the supernatant extract were mixed with 0.5% periodic acid, 0.5% sodium *meta*-periodate (w/w), incubated at 37°C for 30–45 min, and quenched with 1M NaOH: 0.056M Na_2SO_3 (3:2 v/v). Finally, absorbance was detected at 380 nm. Standard curves were constructed with shikimic acid (Fisher Scientific International) at a range of 1 to 10,000 µM.

Table 1. Dose parameters for the recurrent selection, frequency of resistant (R) and susceptible (S) individuals and sex ratios of selected *A. tuberculatus* populations.

Material	glyphosate	frequency[a]	sex ratio
P-R[b]	3.2mM	N/E[c]	7 ♀: 6 ♂
P-S	0.032mM	N/E	24 ♀: 29 ♂
F1-R	10mM	0.02–0.05	0 ♀: 12 ♂
F1-S	0.01mM	0.5–1.0	16 ♀: 21 ♂
backcross	8mM	0.02–0.05	7 ♀: 11 ♂

[a] = percentage relative to population. [c] = not estimated.
[b] = parental population.

Statistical analysis Plant biomass and shikimic acid data was subjected to analysis of variance, with mean separation and correlation coefficients determined by Fisher's least significant difference and Spearman correlation analysis, respectively (SAS 1996). In addition, biomass data was used to calculate GR_{50}, standard error, and 95% upper and lower confidence interval limits according to the log-logistic analysis (Seefeldt *et al.* 1995).

RESULTS AND DISCUSSION

***Amaranthus tuberculatus* inherent variably to glyphosate** Most reported cases of inconsistent *A. tuberculatus* control are attributed to application problems, sub-lethal dosage, or delayed glyphosate applications with respect to plants phenology. Nevertheless, our visual assessment of the Badger, Iowa *A. tuberculatus* population suggested that *A. tuberculatus* plants were differentially affected by glyphosate. Adjacent plants within the crop row that received a comparable glyphosate dose, displayed significantly different phenotypes. These differences were consistent with shikimic acid determinations made from the apices of putative susceptible and resistant phenotypes and from untreated *A. tuberculatus* plants. Putative susceptible plants consistently accumulated at least five folds more shikimic acid than the resistant phenotype (Figure 1). In addition, accumulation of shikimic acid in untreated *A. tuberculatus* plants was undetectable. While patterns of shikimic acid do not provide conclusive evidence with respect to the mechanism of resistance, the data supports the tenet that EPSPS in these plants was differentially inhibited by glyphosate.

Evaluations of *A. tuberculatus* seed from the Everly, Iowa population and other populations from agricultural environments suggested that the species was inherently variable to glyphosate, and thus may provide the genetic basis for the evolution of individuals with increased fitness to glyphosate. Genetic

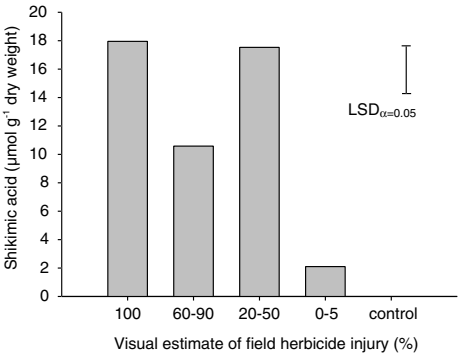

Figure 1. Shikimic acid accumulation in apex tissue of *A. tuberculatus* plants demonstrating different levels of field injury, two weeks after glyphosate application. Samples were segregated in susceptible (100%, 60–90%, and 20–50%) and resistant (0–5%) and a control (not sprayed). Data represent the mean of six plants.

diversity is a common trait in Amaranthaceae (Chan and Sun 1997). Because *A. tuberculatus* is an obligate outcrosser, genetic recombination during mitosis could explain the diversity observed in the species.

The recurrent selection increased the frequency of resistant individuals within the Everly, Iowa population; this corresponded to a 3.5 fold resistance increase after two generations of selection (Table 2). With the exception of backcross of F1-R and F2-R (F2B-R), the selection procedure also reduced the variability to glyphosate as estimated by the standard error of the log-logistic analysis. Furthermore, the variability of selected material was comparable to that of the pristine Ohio population (Table 2). Regardless of attempts to isolate stable-homogeneous resistant and susceptible populations, significant segregation for glyphosate efficacy was still apparent after two generations of recurrent selection. Variable responses of Amaranthaceae to glyphosate have been documented (Baylis 2000). Reports indicate that individual *A. tuberculatus* plants can tolerate field rates of at least 6.72 kg of glyphosate per ha (Smeda 2000).

Potential for selection of glyphosate resistance in *A. tuberculatus* The lack of segregations during recurrent selection suggests that the variability to glyphosate in *A. tuberculatus* may be attributed to a polygenic character of the trait. Thus, recombination of resistant and susceptible alleles during mitosis would result in progeny with different allele combinations and may explain the phenotype observed at the whole plant level. Alternatively, variable response to glyphosate could be explained by the differential expression of *EPSPS* polymorphs. At least two EPSPS isoforms

Table 2. Dose-response parameters for the pristine, parental, and selected *A. tuberculatus* populations.

Gnotype	GR_{50}[a]	STE[b]	95% CI[c]	R/S[d]
Parental	0.50	0.15	0.20/0.80	N/A[e]
Pristine	0.39	0.09	0.22/0.57	
F1-S	0.52	0.06	0.39/0.65	1.7
F1-R	0.89	0.09	0.70/1.09	
F2-S	0.27	0.10	0.08/0.46	3.5
F2B-R	0.96	0.37	0.23/1.69	

[a] kg a.e. ha^{-1} that reduced 50% biomass accumulation.
[b] standard error of the log-logistic analysis.
[c] 95% upper and lower confidence intervals (kg a.e. ha^{-1}).
[d] resistance to susceptibility ratio.
[e] not applicable as material was unselected.

with similar structural and kinetic properties exist in maize (Forlani *et al.* 1994). In addition, the expression of *EPSPS* in plants was tissue-specific and developmentally regulated (Benfey *et al.* 1990). To date, no clear resistance mechanism has been elucidated from the reported cases of resistant weeds. However, studies in field bindweed (*Convolvulus arvensis* L.) suggest that cellular and metabolic processes may concomitantly act to determine glyphosate tolerance in plants (Westwood *et al.* 1997).

Our current data suggests that the potential for the evolution of glyphosate resistance in *A. tuberculatus* is considerable. Nevertheless, several cycles of selection may be required to isolate resistant individuals. Reported cases of glyphosate resistance in *L. multiflorum*, *L. rigidum*, *E. indica* and *C. canadensis* required of several years of persistent glyphosate selection (Pratley *et al.* 1999, Lee and Ngim 2000, Van Gessel 2001). This requirement for prolonged selections may be attributed to a low frequency of resistant individuals within the population or to a physiological penalty associated with the resistance trait. Point mutations in the binding domain of EPSPS may result in a kinetically less efficient enzyme (Padgette *et al.* 1991).

While no cases of glyphosate resistant weeds have been reported in the Mid West US, increases in the area planted with glyphosate-resistant crops may enhance the selection pressure for glyphosate and instigate weed population shifts. Farmers typically rely on multiple glyphosate applications to meet weed control expectations. Thus frequent and ubiquitous use of glyphosate in field crops will likely provide enough selection pressure for the evolution of glyphosate resistant weed populations.

CURRENT WORK

Evaluations of mechanisms of resistance The recurrent selection of *A. tuberculatus* was based on the

seedling assay. This approach elucidated resistant and susceptible individuals, however the method has potential for misidentification of phenotypes. Thus, a three level selection approach will be conducted to isolate plant material suitable for translocation, metabolism, and *EPSPS* DNA sequence analysis. Plants will be characterised by their response to glyphosate at the seedling and whole-plant level and their shikimic acid accumulation patterns. The mechanisms of resistance will be investigated in asexually-propagated resistant, susceptible, and pristine *A. tuberculatus* plantlets.

ACKNOWLEDGMENTS

This investigation was possible by the economic support of Monsanto and Syngenta companies. The authors wish to thank Dr. Jonathan Gressel for his comments and recommendations to this investigation.

REFERENCES

Barry, G., Kishore, G., Padgette, S., Taylor, M., Kolacz, K., Weldon, M., Re, D., Eichholtz, D., Fincher, K. and Hallas, L. (1992). Inhibitors of amino acid biosynthesis: strategies for impairing glyphosate tolerance to crop plants. *In* 'Biosynthesis and molecular regulation of amino acids in plants,' eds B.K. Singh, H.E. Flores and J.C. Shannon, pp. 139-45. (American Society of Plant Physiologists, Vol. 7, Rockville, MD).

Baylis, A.D. (2000). Why glyphosate is a global herbicide: strengths, weaknesses and prospects. *Pest Management Science* 56, 299-308.

Benfey, P.N., Takatsuji, H., Ren, L., Shah, D.M. and Chua, N.H. (1990). Sequence requirements of the 5-enolpyruvylskikimate-3-phosphate synthase 5'-upstream region for tissue-specific expression in flowers and seedlings. *Plant Cell* 2, 849-56.

Bradshaw, L.D., Padgette, S.R., Kimball, S.L. and Wells, B.H. (1997). Perspectives on glyphosate resistance. *Weed Technology* 11, 189-98.

Brenner, D.M. and Widrlechner, M.P. (1998). *Amaranthus* seed regeneration in plastic tents in greenhouses. *Plant Genetic Resources Newsletter* 116, 1-4.

Caseley, J. and Copping, L. (2000). Twenty-five years of increasing glyphosate use: the opportunities ahead. *Pest Management Science* 56, 297.

Chan, K.F. and Sun, M. (1997). Genetic diversity and relationships detected by isozyme and RAPD analysis of crop and wild species of *Amaranthus*. *Theoretical and Applied Genetics* 95, 865-73.

Cromartie, T.H. and Polge, N.D. (2000). An improved assay for shikimic acid and its use as monitor for the activity of sulfosate. *WSSA Abstract* 40, 121.

Forlani, G., Parisi, B. and Nielsen, E. (1994). 5-*enol*-pyruvyl-shikimate-3-phosphate synthase from *Zea mays* cultured cells: purification and properties. *Plant Physiology* 105, 1107-14.

Goldsbrough, P.B., Hatch, E.M., Huang, B., Kosinsky, W.G., Dyer, W.E., Herrmann, K.M. and Weller, S.C. (1990). Gene amplification in glyphosate tolerant tobacco cells. *Plant Science* 72, 53-62.

Holländer-Czytko, H., Sommer, I. and Amrheim, N. (1992). Glyphosate tolerance of cultured *Corydalis sempervirens* cell is acquired by an increased rate of transcription of 5-enolpyruvylshikimate 3-phosphate synthase as well as by a reduced turnover of the enzyme. *Plant Molecular Biology* 20, 1029-36.

Klee, H.J., Muskopf, Y.M. and Gasser, C.S. (1987). Cloning of an *Arabidopsis thaliana* gene encoding 5-enolpyruvylshikimate-3-phosphate synthase: sequence analysis and manipulation to obtain glyphosate-tolerant plants. *Molecular and General Genetics* 210, 437-42.

Lee, L.J. and Ngim, J. (2000). A first report of glyphosate-resistant goosegrass (*Eleusine indica* (L.) Gaertn.) in Malaysia. *Pest Management Science* 56, 336-9.

Mannerlöf, M., Tuvesson, S., Steen, P. and Tenning, P. (1997). Transgenic sugar beet tolerant to glyphosate. *Euphytica* 94, 83-91.

Padgette, S.R., Re, D.B., Gasser, C.S., Eichholtz, D.A., Frazier, R.B., Hironaka, C.M., Levine, E.B., Shah, D.M., Fraley, R.T. and Kishore, G.M. (1991). Site-directed mutagenesis of a conserved region of the 5-enolpyruvylshikimate-3-phosphate synthase active site. *The Journal of Biological Chemistry* 266, 22364-9.

Perez, A. and Kogan, M. (2002). Glyphosate-resistant Italian ryegrass (*Lolium multiflorum*) in Chile. *WSSA Abstract* 42, 21.

Pratley, J., Urwin, N., Stanton, R., Baines, P., Broster, J., Cullis, K., Schafer, D., Bohn, J. and Krueger, R. (1999). Resistance to glyphosate in *Lolium rigidum*. I. Bioevaluation. *Weed Science* 47, 405-11.

SAS, Institute (1996). 'The SAS system for windows'. (SAS Institute, Cary, NC).

Seefeldt, S.S., Jensen, J.E. and Fuerst, E.P. (1995). Log-logistic analysis of herbicide dose-response relationships. *Weed Technology* 9, 218-27.

Smeda, R.J. (2000). Insensitivity of a common waterhemp population to glyphosate. *NCWSS Proceedings* 55, 90.

VanGessel, M.J. (2001). Glyphosate-resistant horseweed from Delaware. *Weed Science* 49, 703-5.

Westwood, J.H., Yerkes, C.N., DeGennaro, F.P. and Weller, S.C. (1997). Absorption and translocation of glyphosate in tolerant and susceptible biotypes of field bindweed (*Convolvulus arvensis*). *Weed Science* 45, 658-63.