THE EVOLUTION OF TOLERANCE IN THE COMMON MORNING GLORY IPOMOEA PURPUREA: FROM GENETICS TO GEOGRAPHY

by

REGINA S. BAUCOM

(Under the Direction of Rodney Mauricio)

ABSTRACT

The evolution of a trait in nature is dependent on the amount of genetic variation underlying the trait, the presence of selection on the trait, and the inheritance pattern of the trait. I investigated each of these components using a trait that has important implications for agriculture: the ability of Ipomoea purpurea, a noxious crop weed, to tolerate glyphosate, or the active ingredient in the herbicide RoundUp®. In every investigation, I found the presence of genetic variation underlying tolerance, suggesting that adequate fuel for the evolutionary process exists in nature. In addition, I found the presence of positive, directional selection on tolerance, providing evidence that glyphosate is a potent force of selection on tolerance in populations of I. purpurea. I also found that, in the absence of glyphosate, a significant fitness cost exists such that the continued evolution of tolerance might be mitigated by removing use of glyphosate in cropping systems. Finally, I uncovered an inheritance pattern for tolerance, in that it is primarily under additive nuclear genetic control, and is not under the direct influence of a maternal or paternal effect.

The work presented here also shows the presence of genetic variation for tolerance in pre-glyphosate lines, suggesting that tolerance is a pre-adaptation, and that the evolutionary dynamics controlling its increase or decrease within populations is not as simple as previously thought. However, a geographical survey of tolerance in the Southeastern US indicates that variation for tolerance exists at multiple scales of study, from within-populations to among-regions. This broad-scale investigation of glyphosate tolerance in *I. purpurea*, from its genetics to its geographical partitioning, represents the first in-depth evolutionary consideration of tolerance to an herbicide.

INDEX WORDS: tolerance, genetic variation, fitness costs, geography, selection, evolution, *Ipomoea purpurea*

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INTRODUCTION

LITERATURE REVIEW

Human beings represent a significant evolutionary force (Palumbi 2001). As such, one of the greatest challenges for biologists is to understand human-mediated evolutionary change. The introduction of pesticides in the past century has significantly intensified agricultural production (Cousens and Mortimer 1995). Use of chemical methods for pest control is now the major method of eradicating weed infestations in crops (Powles et al. 1997). Currently, use of the herbicide RoundUp® is on the rise world-wide and is projected to increase given the wide-spread adoption of RoundUp-Ready® crops (Shaner 2000). Unfortunately, almost since pesticides were put into wide use, insect and plant species have evolved mechanisms allowing them to avoid eradication (Gould 1995). In particular, the evolution of herbicide resistance in weedy plants has occurred many times (Heap 1997). Despite the importance of understanding this trait, we still know relatively little about the evolution of herbicide resistance (Darmency 1994; Jasieniuk et al. 1996; Jordan and Jannink 1997).

The scale of resistance

First, in order to understand the evolution of herbicide resistance, we must understand the genetic basis of the trait. Several studies have investigated the genetics of resistance, but these studies account for only approximately twenty percent of all known cases of herbicide resistance. In specific cases where the genetics of herbicide resistance has been described, inheritance is predominantly *via* nuclear transmission (Jasieniuk et al. 1996). Resistance is most commonly due to a single, dominant or partially dominant gene (Cousens and Mortimer 1995; Jasieniuk et al. 1996). However, there are exceptions to these findings. Resistance to the triazine class of herbicides is controlled by the chloroplast genome and thus is inherited primarily maternally (Warwick 1991). Because genes inherited through the chloroplast do not undergo recombination, the resistance mutation is likely to sweep through a population at a much faster rate than resistance inherited through the nuclear genome (Darmency 1996).

Second, in order to understand the evolutionary dynamics of a resistance trait, we must understand the action of selection in natural populations. It is generally accepted that the increase in herbicide resistance in a weed population is due to the herbicide directly selecting for individuals that are more resistant (Maxwell and Mortimer 1994). In order for selection to increase the frequency of such a trait over time genetic variation for herbicide resistance must exist and there must be a relationship between resistance and fitness (Endler 1986). Although variation for herbicide resistance is well documented as a phenotype (Whitworth and Muzik 1967; Ellis and Kay 1975; Holliday and Putwain 1980; Warwick and Marriage 1982; Lior et al. 2000), it is often not clear how much of this variation is due to genetic rather than environmental causes. In addition, many studies attempting to link resistance and fitness are confounded by a lack of control for the genetic background and may often ignore the possibility of resistance induced by non-genetic and thus non-heritable factors such as maternal effects (Bergelson and Purrington 1996).

Various authors have argued that the level of resistance present within a population reflects a compromise between the benefits of reduced herbivory and the costs of resistance (Janzen 1973a; Mooney and Gulmon 1982; Simms and Rausher 1987; Fagerstrom 1989; Simms 1992; Zangerl and Bazzaz 1992; Mauricio 1998). Similar to herbivory resistance studies, it is likely that the distribution of herbicide resistant phenotypes in a plant population is impacted not only by the genetic control and inheritance of resistance but also the potential for fitness costs to herbicide resistance.

Empirically, fitness costs are often reflected in fewer offspring produced by the resistant individual in the absence of the selective agent. Theoretically, fitness costs are proposed to arise by antagonistic pleiotropy or trade-offs between two traits. These trade-offs exist if genetic change leading to an increase in the value of one trait cannot occur without a decrease in the value of another trait (Mauricio 1998). If the plant benefits by both characters, a negative genetic correlation will constrain evolutionary change (Lande 1980; Rausher 1992). The benefit gained from selection to increase the value of one character will be countered by the cost of the correlated decrease in the value of the other character.

In addition to understanding the genetics of resistance and selective forces governing resistance, it is of interest to know how variation for resistance is partitioned over geography. This is largely attributable to the recognition that populations are rarely genetically isolated from one another, calling into question the scale at which resistance should be considered. Variation in resistance can exist among individuals within a single population or among populations physically structured across geography. Variation can also exist among aggregations or groups of populations found over geography, giving the appearance of spatial ‘clumping,’ or a mosaic of trait values over a wide geographical area (Thompson 1997; Brodie and Ridenhour 2002). Larger-scale regional trends can also exist such that populations from ecologically-distinct, widely-separated areas exhibit differences in resistance. An initial assessment of where variation in resistance exists, whether within or among populations, among aggregations of populations leading to a ‘mosaic’ of trait values, or among ecologically distinct regions will first provide an idea of the potential for the evolution of the resistance trait in nature.

Tolerance to herbicide

Although simple genetic mechanisms of herbicide resistance have been studied, tolerance to herbicide, or the ability of a plant to experience damage and produce progeny after herbicide application, has not been widely considered. Populations of plants are rarely completely resistant or completely susceptible to herbicide application (Cousens and Mortimer 1995). This apparent polymorphism among individuals has also been detected by those investigating plant resistance to herbivory (Berenbaum et al. 1986; Rausher and Simms 1989; Simms and Rausher 1989; Mauricio et al. 1997), and as such has been the focus of much theoretical work (Simms and Rausher 1987; Fagerstrom 1989). There are few studies concerned with the phenomenon of herbicide tolerance, however, and only one study, by Duncan and Weller (1987) that analyzes the genetics controlling tolerance to herbicide. And, although these authors use the term tolerance, their trait is a measure of plant damage after herbicide application. This is at odds with the rich literature of empirical work on tolerance, and is more similar to those who study resistance to herbicide as a function of plant damage. Tolerance, as defined in this dissertation, is a function of plant fitness.

The distinction between resistance and tolerance was initially enumerated by agricultural scientists. Painter (1951) first described herbivore tolerant plants as those that could survive “under levels of infestation that would generally kill or severely injure susceptible plants”, but then expanded his definition to include a plant that “shows an ability to grow and reproduce itself or repair injury” (1958). Inherent in this definition is that a comparison must be made between plants in the level of tolerance versus susceptibility. In addition, the individuals under examination must be classified according to their genetic relationship, whether full or half-sibs belonging to the same genetic line, or individuals belonging to the same population. Then, within each genetic class, the fitness of individuals must be measured at different levels of damage. The fitness response of the individuals of the genetic class, across the range of damaging environments, is then an estimate of the tolerance of that group (Stowe et al. 2000).

Thus, tolerance is defined as the reaction norm of fitness across a damage gradient, and it can be treated as a phenotypically plastic trait (Simms 2000). The fitness function is probably best modeled as a polynomial equation (Pilson 2000), but for simplicity, a linear function is frequently used to describe tolerance (Mauricio et al. 1997; Tiffin and Rausher 1999; Simms 2000; Stinchcombe and Rausher 2002; Baucom and Mauricio 2004) and is often a good approximation. Although this definition and subsequent estimation of tolerance to herbivory and pathogen is well treated within the literature, tolerance to herbicide is not.

Study system and objectives

The common morning glory, or *Ipomoea purpurea*, is often found infesting agricultural crops. In interviews we have conducted in the Southeast, farmers report that the morning glories in their fields are no longer being effectively killed by applications of the herbicide RoundUp®. RoundUp® was introduced in 1974 and its use has increased dramatically in North America since the mid 1990's (Shaner 2000). In addition, as more acres of genetically transformed RoundUp® resistant crops are planted each year the role of RoundUp® as a major selective force will undoubtedly increase. The seeming increase in frequency of tolerance to RoundUp® represents a unique opportunity to examine the evolutionary genetics of a novel trait.

The work presented here examines the potential for the evolution of herbicide tolerance in nature by addressing the following subjects: (1) *The force of selection on tolerance* – In this chapter I ask if there is genetic variation underlying tolerance in the field-collected, once-selfed progeny of maternal lines. I assess the potential for fitness costs and fitness benefits associated with tolerance, and provide information about the net selection acting on tolerance, (2) *The genetics of tolerance* – In this section I attempt to uncover how much of the observable tolerance is due to additive gene action rather than non-genetic factors such as maternal effects, gene dominance and epistatic interactions, (3) *The origin of tolerance* – This section assesses if tolerance is a relatively new trait or could be considered a pre-adaptation, and (4) *The geographical variation of tolerance* – In this chapter I assess the level of tolerance among two species of morning glory, *Ipomoea purpurea* and *I. hederacea* in an attempt to understand how the species house variation in tolerance over geography.

Significance of this work

The widespread and increasing use of chemical herbicides to control weeds is a large, uncontrolled, experiment in evolutionary biology. As such, this dissertation represents the first large-scale examination of tolerance to an herbicide as estimated as a function of plant fitness. Furthermore, it is the first detailed examination of the evolutionary ecology underlying RoundUp® tolerance in *Ipomoea purpurea*. This work specifically provides much-needed consideration of fitness costs or lack thereof in plants that are tolerant to herbicide rather than resistant. In addition, this work provides insight into the genetics underlying tolerance, information about the origin of tolerance, and a preliminary view into how variation for this trait is partitioned over geography. This broad view of the genetics, selective forces, and geographical structure underlying tolerance to RoundUp® in *I. purpurea* will both further our knowledge of the evolutionary ecology of a human-mediated trait, and will provide information to those who are working to control its continued evolution in crop systems.

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CHAPTER 2

FITNESS COSTS AND BENEFITS OF NOVEL HERBICIDE TOLERANCE IN A NOXIOUS WEED

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ABSTRACT

Glyphosate, the active ingredient in the herbicide RoundUp®, has dramatically increased in use over the past decade and constitutes a potent anthropogenic source of selection. In the Southeastern U.S., weedy morning glories have begun to develop tolerance to glyphosate, representing a unique opportunity to examine the evolutionary genetics of a novel trait. We found genetic variation for tolerance, indicating the potential for the population to respond to selection by glyphosate. However, a significant evolutionary constraint exists: in the absence of glyphosate, tolerant genotypes produced fewer seeds than susceptible genotypes. The combination of strong positive directional selection in the presence of glyphosate and strong negative directional selection in its absence may indicate that the selective landscape of land-use could drive the evolutionary trajectory of glyphosate tolerance. Understanding these evolutionary forces is imperative for devising comprehensive management strategies to help slow the rate of the evolution of tolerance.

INTRODUCTION

Strong selection exerted by human technological innovations has wide-ranging evolutionary consequences and, as such, has caused accelerated cases of evolution in the natural world (Palumbi 2001). For example, the introduction of herbicides and pesticides in the past century has significantly intensified agricultural production (Cousens and Mortimer 1995). However, the repeated use of herbicides exerting strong selection pressure on crop weeds has led to over 250 documented cases of herbicide resistance (http://www.weedscience.org), and this process is likely to accelerate with increased reliance on herbicides.

Since its introduction in 1974, glyphosate, the active ingredient in the herbicide RoundUp®, has dramatically increased in use (Shaner 2000), particularly with the advent in the 1990's of crops genetically engineered to be tolerant of RoundUp® (e.g., Roundup Ready® canola, corn, cotton, soybeans and sugar beets). Our analysis of United States Department of Agriculture (USDA) statistics shows that not only is the use of glyphosate increasing in U.S. soybean crops, but there is a concomitant decrease in the use of other herbicides (Fig 2.1) (NASS-USDA 2003). In addition, glyphosate is being increasingly used in a conservation context as a component of the management of invasive weeds (Matarczyk et al. 2002). This widespread pattern of increased usage suggests that glyphosate is fast becoming the predominant herbicide in managed systems.

To date, only six cases of glyphosate resistance have been reported in plants out of the 250 cases of herbicide resistance (http://www.weedscience.org). If one considers the trajectory of evolution to every other major pesticide (Gould 1995; Palumbi 2001), more cases of glyphosate resistance will likely follow. However, tolerance to glyphosate, or the ability to sustain damage without a corresponding reduction in fitness (Crawley 1983) is also likely to be an important evolutionary strategy of weedy plants to circumvent the damaging effects of herbicide.

The distinction between tolerance and resistance was first identified in the plant-herbivore literature (Painter 1958). Resistance traits are defined as traits that reduce the amount of damage a plant experiences whereas tolerance, or compensation, is the ability of a plant to sustain a fixed amount of herbivore damage without a corresponding reduction in fitness (Mauricio et al. 1997). Unlike resistance, tolerance does not prevent herbivory, but allows the plant to compensate for damage that herbivores have already inflicted. We have borrowed these definitions and applied them to the plant-herbicide system. Thus, resistance to an herbicide would involve a trait that prevented the plant from experiencing the damaging effects of the herbicide. For example, a plant enzyme that detoxified the herbicide would be considered a resistance trait. Tolerance to an herbicide is simply the ability of a plant to compensate for the damaging effects of the herbicide. Like that of tolerance to herbivores, there are likely to be a myriad of mechanisms that confer tolerance to herbicides (Cousens and Mortimer 1995). In the Methods, we describe our operational definition of tolerance.

In the southeastern United States, *Ipomoea purpurea* (L.) Roth (the tall or common morning glory) is a noxious crop weed whose negative effects on agriculture have been largely mitigated by the use of glyphosate (Baylis 2000; Hoss et al. 2003). Our interviews with farmers in the Southeast suggest that morning glories can tolerate applications of glyphosate. In some cases, increasing concentrations of the herbicide have been required to control *I. purpurea* infestations. Such an increase in tolerance to glyphosate represents a unique opportunity to examine the evolutionary genetics of a novel trait, especially with regards to the constraints on the evolution of tolerance in natural plant populations. Understanding these constraints is also imperative for devising comprehensive management strategies to help slow the rate of the evolution of tolerance (Rausher 2001). In this report we describe experimental evidence indicating the presence of genetic variation for tolerance to RoundUp® in wild-collected *I. purpurea* maternal lines, as well as the presence of fitness costs associated with this tolerance. We also show both significant positive selection for tolerance in the presence of RoundUp® and significant negative directional selection against tolerance in the absence of RoundUp®.

MATERIALS AND METHODS

We collected seeds from 32 randomly-selected, individual plants growing in an agricultural field in Oconee County, Georgia, that has been consistently sprayed with RoundUp® for approximately 8 years. Since *I. purpurea* possesses a mixed-mating system of outcrossing and selfing (Ennos 1981) all seeds collected from each plant share the maternal genetic contribution. We used the maternal line as the unit of our genetic analysis, which involved some design tradeoffs. Although using this maternal line design does not allow us to determine the additive genetic variance of tolerance, it does provide a broad-sense genetic measure. Of course, differences among these maternal lines represent both genetic differences and the effects of the common parental environment. Since the main mode of action of glyphosate is to inhibit an enzyme (5-enolpyruvalshikimate-3-phosphate synthase) which occurs in the chloroplasts, including the maternal effect seemed appropriate. In a parallel study we are investigating the maternal and paternal contributions to tolerance. In order to minimize these environmentally-derived differences, we planted five seeds from each grandmaternal line and selfed them for one generation. Maternal individuals were randomized in the greenhouse to account for potential environmentally-induced differences within the greenhouse. Seeds were then collected from each plant and seeds from each of the five maternal lines were bulked according to grandmaternal line.

We planted this experiment into an agricultural field at the University of Georgia's Plant Sciences Farm in Oconee County, Georgia. This site supports a natural population of *I. purpurea* and was in the same area from which the maternal lines were collected. We randomized ten replicates of each of the 32 maternal lines among five spatial blocks to account for habitat heterogeneity. To ensure germination we scarified each seed prior to planting and marked planted seeds with plastic straws. Within each block we planted seeds 1-m^2 from the next experimental individual. We removed vegetation surrounding experimental individuals once to deter herbivory from cotton rats, *Sigmodon hispidus*, but otherwise let competitive weeds grow undeterred. Each plant was allowed to grow up a 1-m tall bamboo stake which mimics *I. purpurea* growth in agricultural fields and allows for easy identification of experimental plants. We applied glyphosate (RoundUp®, Monsanto, St. Louis, MO) at a rate of 1.121 kg Ha^{-1} with a hand-held CO_2 pressurized plot sprayer calibrated to a spray volume of 20 gallons per acre to half of the experimental individuals on 17 July 2002. This concentration of glyphosate has been found to reduce *I. purpurea*'s biomass by 90% (Culpepper et al. 2001). In *I. purpurea*, the physical symptoms of damage after glyphosate application are generally chlorosis and necrosis of the leaves and death of the apical meristem.

We collected mortality and damage data by assessing death of sprayed individuals and by counting the total number of leaves per plant and the number of leaves exhibiting symptoms of glyphosate damage. We collected fruits during ten rounds of collection and counted all viable seeds. Relative fitness was calculated by dividing all fitness values by overall mean fitness.

Individuals that died before glyphosate application were not included in the analysis, and individuals that died as a result of glyphosate application were given a fitness of 0.

Our operational definition of tolerance is the ability of plants to reproduce after experiencing damage by herbicide. Tolerance was estimated for each maternal line because a single plant cannot be both damaged and undamaged. The mean relative fitness of each maternal line was regressed on environment (no herbicide; herbicide), and the level of tolerance was determined as the slope of relative fitness on environment after the effects of block were removed. A slope of zero would mean the line was completely tolerant, whereas a significant negative slope would indicate low tolerance. A line exhibiting a positive slope would be overcompensating for damage (Stowe et al. 2000). This method defines tolerance as a norm of reaction to glyphosate, analogous to studies that assess tolerance to herbivory (Abrahamson and Weis 1997; Mauricio et al. 1997; Tiffin and Rausher 1999; Stinchcombe and Rausher 2002). If the maternal lines respond differently in the two environments, *i.e.*, the slopes of the lines between fitness and environment differ across maternal lines, we conclude that the lines exhibit genetic variation for tolerance. The statistical significance of such a response is tested with an analysis of variance (ANOVA) by examining the magnitude of the genotype by environment interaction with fitness as the dependent variable. For this analysis, we used the GLM procedure of the SAS statistical software package (version 8.0, SAS Institute, Cary, NC) to conduct the ANOVA. In this analysis, the response variable was $\log_e$-transformed relative fitness, which we calculated by dividing each individual's fitness by overall mean fitness (Tiffin and Rausher 1999; Stinchcombe and Rausher 2002). We used the residuals of relative fitness after the effects of block had been removed as the dependent variable in the model to reduce the effect of spatial heterogeneity.

To detect costs of tolerance, we tested for the presence of a significant genetic covariance between relative fitness and mean level of tolerance for each maternal line. Using the same set of data to estimate both the slope (tolerance) and the fitness of undamaged plants produces an artifactual covariance (Mauricio et al. 1997; Tiffin and Rausher 1999) that was subtracted from the calculated covariance for an unbiased estimate of the covariance. Standard errors of the covariances were made by jackknifing maternal line estimates (Gray and Schucany 1972), with a one-tailed *t* statistic then used to calculate a confidence interval.

To assess the pattern and magnitude of selection on tolerance to glyphosate, the partial regression analysis described by Rausher (Rausher 1992; Mauricio and Mojonnier 1997) was used to determine coefficients of selection in both treatment environments. Again, we used maternal line means as our unit of analysis. Before conducting the analyses, tolerance was standardized to a mean of zero and a variance of one. The response variable was the residual of relative fitness after the effects of block were removed to minimize the effects of spatial variation. Selection gradients in both treatments were estimated from the regression of fitness on tolerance using maternal-line means, following standard methods (Rausher 1992; Mauricio and Mojonnier 1997; Tiffin and Rausher 1999). Only linear terms were included in the regressions for each maternal line because the initial analysis revealed no evidence of any nonlinear effects of treatment environment on fitness. As the same artifactual covariance between fitness and tolerance in the fitness cost analysis also applies in the selection analysis, standard errors of the covariances were made by jackknifing maternal line estimates (Gray and Schucany 1972) with a one-tailed *t* statistic then used to calculate a confidence interval to assess statistical significance of the selection gradients.

RESULTS

Maternal lines differed in tolerance to glyphosate damage, as revealed by the significant genotype by environment interaction in an ANOVA (Table 2.1), meaning that there is significant genetic variation for tolerance in this study population. In this analysis, $\log_e$-transformed fitness was the response variable, maternal line was the independent variable and the treatment environment was the covariate. The maternal line by environment term was significant, indicating that the slopes of the relationship between fitness and environment (no herbicide, herbicide) differ among maternal lines. The fitness norm of reaction, which is the fitness of each maternal line regressed on treatment environment, illustrates the fitness trade-off across environments (Fig 2.2).

As indicated by a negative correlation between fitness and tolerance in the control treatment, substantial fitness costs are associated with tolerance to glyphosate (Fig 2.3A). The corrected covariance between tolerance to glyphosate (slope) and the relative fitness of undamaged plants was equal to -0.0200. Using the jackknife, we calculated the 95% confidence interval of this covariance to be ±0.0147. This corrected covariance was significantly different from zero at $P = 0.002$, indicating that there is a negative covariance between tolerance and fitness in the absence of glyphosate, and thus evidence of a fitness cost of tolerance to glyphosate.

The magnitude of selection against tolerance associated with this cost, estimated as the coefficient of a standardized regression of fitness on tolerance using maternal line means (Mauricio and Mojonnier 1997; Rausher 1992) is $\beta_2 = -0.0806$ ($P < 0.0001$). The corrected covariance between fitness and tolerance using maternal line means is $\beta_2 = -0.0967$. The jackknife procedure showed that this covariance was significantly different from zero at $P = 0.03$ with a confidence interval of ±0.0776.

In contrast, in the herbicide treatment, there is net selection for increased tolerance, as indicated by a positive correlation between fitness and tolerance. Using the same coefficient of a standardized regression of fitness on tolerance, we estimate the strength of positive selection on tolerance to be $\beta_1 = 0.0945$ ($P < 0.0001$; Fig 2.3B). After correcting for the artifactual covariance, $\beta_1 = 0.0882$ with a $P = 0.015$. There was no evidence of stabilizing or disruptive selection on tolerance in either environment.

DISCUSSION

One prerequisite for the evolution of widespread tolerance to glyphosate is the presence of genetic variation for tolerance. Moreover, the rate of evolution of tolerance to selection imposed by glyphosate is expected to be proportional to the amount of genetic variation for tolerance that exists in natural populations (Fisher 1930). Consequently, a lack of appreciable genetic variation for tolerance could serve as a constraint on the evolution of tolerance. Unfortunately, this does not appear to be the case: substantial genetic variation for tolerance already appears to exist in at least one natural population. Given the continued presence of glyphosate, the number of tolerant individuals should increase within the population over time, as might the overall level of tolerance of the population. This result is also significant in that glyphosate is an extremely effective, novel, selective agent put into widespread use recently, yet this plant species already exhibits a genetically-based ability to tolerate the herbicide.

Of course, the presence of genetic variation in and of itself does not guarantee that tolerance will evolve. A second prerequisite for the evolution of widespread tolerance to glyphosate is the presence of net selection favoring increased tolerance. The net selection acting on tolerance is determined by two components: fitness costs and benefits. Costs of resistance or tolerance are fitness reductions that are thought to arise from the diversion of limiting resources away from present and future growth and reproduction (Simms and Triplett 1994). Such costs are common, but not universal, for resistance and tolerance to herbivores (Simms and Rausher 1987; Simms and Triplett 1994; Mauricio et al. 1997; Tiffin and Rausher 1999). Benefits of tolerance are increases in fitness that result from the ability to reduce the detrimental effects of damage on survival and reproductive success. Tolerance can evolve only if there is a net benefit, *i.e.,* if the magnitude of fitness benefits exceeds the magnitude of the costs (Simms and Rausher 1987; Simms and Triplett 1994; Mauricio et al. 1997; Lennartsson et al. 1998; Tiffin and Rausher 1999; Juenger and Lennartsson 2000; Roy and Kirchner 2000).

We found that the most tolerant line produced 35% fewer seeds in the absence of RoundUp® than the most susceptible line. Although this type of fitness cost has been well-documented for genetically engineered resistance to the herbicide chlorsulfuron in *A. thaliana* (Bergelson and Purrington 1996; Purrington 2000) and resistance to the triazine herbicides (Warwick 1991), reports of fitness costs associated with tolerance to herbicide, more specifically natural tolerance to glyphosate, are lacking. However, the magnitude of the fitness cost in our system was similar to the 34% reduction in fitness in transgenic *A. thaliana* (Bergelson and Purrington 1996; Purrington 2000). These costs indicate that, in the absence of herbicide, natural selection would tend to minimize levels of tolerance.

Our data on the benefits of tolerance in the presence of glyphosate suggest that there is likely to be strong positive selection for tolerance in areas where glyphosate is sprayed. However, in areas where glyphosate is not sprayed, the costs of tolerance we measured suggest that the trait will be strongly selected against. Clearly, glyphosate use is increasing dramatically in the U.S. We obtained a preliminary estimate of the *net* selection for tolerance in *I. purpurea* by weighting the estimates of the magnitude of selection in each of our treatment environments by the proportions of U.S. crop acreage that were sprayed and not sprayed with glyphosate. We used the mean direction and magnitude of selection on glyphosate tolerance for *I. purpurea*, β, a weighted average of the magnitude of selection with and without herbicide: $\beta = p_1\beta_1 + p_2\beta_2$, where p_1 and p_2 are the proportions of U.S. crop acreage sprayed and not sprayed and β_1 and β_2 are the coefficients of selection corresponding to the environment with and without glyphosate, respectively. To estimate p_1 and p_2, we used USDA data on acreage planted in soybeans, cotton and corn, and acreage sprayed with glyphosate (NASS-USDA 2003).

Our analysis revealed that, from 1991 through 2001, estimates of β are negative, indicating that net selection acted against an increase in tolerance (Fig. 2.4). During this period, the high cost of tolerance provided a successful evolutionary constraint. However, in 2002, β becomes positive, indicating net selection for increased tolerance (Fig. 2.4). Apparently, in this year, the continued increase in glyphosate use (Fig. 2.1) caused the proportion of acreage sprayed to cross a threshold such that herbicide use is common enough to tip the balance towards selection for the evolution of tolerance.

Admittedly, these calculations are crude. The analysis makes a number of simplistic assumptions: that we can extrapolate the results from a single study population in a single year, that there is little gene flow between sprayed and non-sprayed fields and that the species is localized to agricultural fields. However, this analysis does serve as a useful starting point for mathematical models of the spread of this important agricultural trait. Taking these calculations at face value, the analysis suggests that the amount of land experiencing a certain selective regime could influence the continued evolution of traits which then impact the efficacy of modern-day agriculture.

These calculations do suggest that serious and immediate consideration should be given to developing regional strategies for managing the evolution of tolerance in *I. purpurea*. Heretofore, little attention has been given to such efforts, particularly in contrast to the multitude of models that attempt to manage the evolution of *Bt* resistance (Adkisson, et al. 2000). For glyphosate, such strategies could involve something as simple as periodically spraying with alternate herbicides, as long as there is little cross-tolerance with glyphosate. If however, there is cross-tolerance with other causes of plant damage, such as hail, herbivores, or pathogens, alternating spraying regimes may not be a viable mechanism for controlling the evolution of glyphosate tolerance.

An additional complication in modeling the evolution of tolerance to herbicide is the presence of a persistent seed bank. *Ipomoea purpurea* is known to sustain a viable seed bank of at least 7 years (Baskin and Baskin 1998). Seed banks can preserve genetic variation (Morris et al. 2002) and can act as a buffer that could retard the evolution of traits in response to recent selection (Templeton and Levin 1979).

In conclusion, whether an evolutionary threshold has been crossed or not, the main findings of this work stand: there is evidence for genetic variation for tolerance in this species; in the presence of glyphosate, there is strong selection for tolerance, but in the absence of glyphosate, there is a significant cost to being tolerant. The estimates of these parameters will be critical for any serious attempt to model the evolutionary trajectory of this trait. Furthermore, our study illustrates the continuing relevance of basic evolutionary studies as a foundation for developing effective management strategies (Gould 1995; Rausher 2001).

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Table 2.1. Analysis of variance for relative fitness ($\log_e$-transformed). The maternal line by treatment interaction demonstrates the existence of genetic variation for tolerance.

Source of Variation	df	Type III SS	F	P
Maternal Line	31	13.0788	2.08	0.0005
Treatment	1	1.1354	5.59	0.0182
Maternal Line × Treatment	31	9.9309	1.58	0.0235
Error	1201	255.3189		

FIGURE LEGENDS

Fig. 2.1. The proportion of soybean acreage sprayed with glyphosate from 1991 to 2002 relative to other herbicides. Data from (NASS-USDA 2003).

Fig. 2.2. Relationship between relative fitness and treatment environment for the 32 maternal lines. Residuals of fitness were used after the effect of block was removed. On the x-axis, 0 = glyphosate absent and 1 = glyphosate present. Slopes of the lines represent tolerance.

Fig. 2.3. (A) Costs of tolerance indicated by a significant negative genetic correlation between fitness and tolerance in the absence of glyphosate. (B) The benefits of tolerance in the presence of glyphosate as measured by a standardized selection gradient. In both figures, the y-axis depicts the residuals of relative fitness after the effects of block had been removed and the x-axis depicts the level of tolerance for each maternal line standardized to a mean of zero and a variance of one.

Fig. 2.4. Relative proportion of US agricultural land planted in soybean, cotton and corn subject to net selection (calculated by weighting acreage with a measure of selection) for tolerance to glyphosate over the past 12 years. A negative value indicates that the costs of glyphosate tolerance outweighed the benefits and we would predict that tolerance would decrease in the weed populations growing in US soybean, cotton and corn agricultural lands. A positive value indicates that the acreage subject to benefits of glyphosate tolerance outweighed the costs and we would predict that tolerance would increase in weed populations. Data from (NASS-USDA 2003).

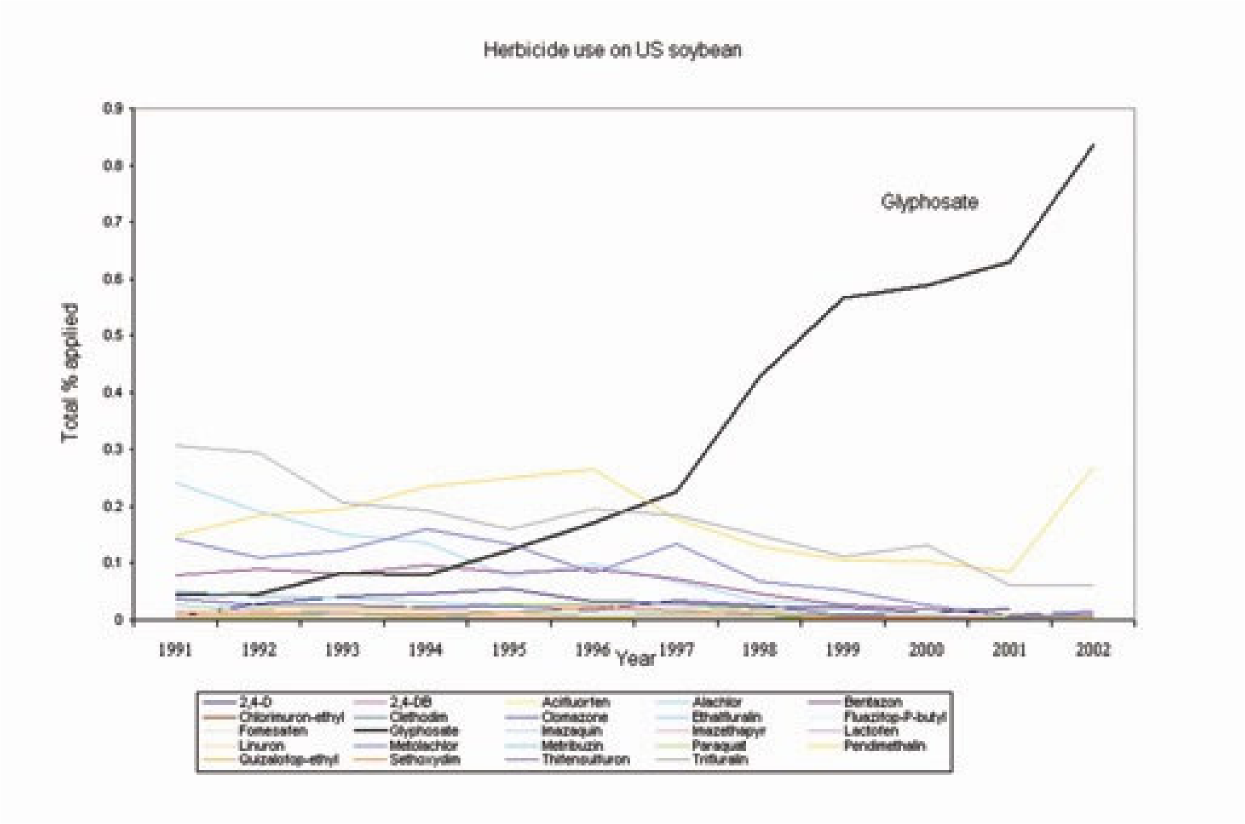
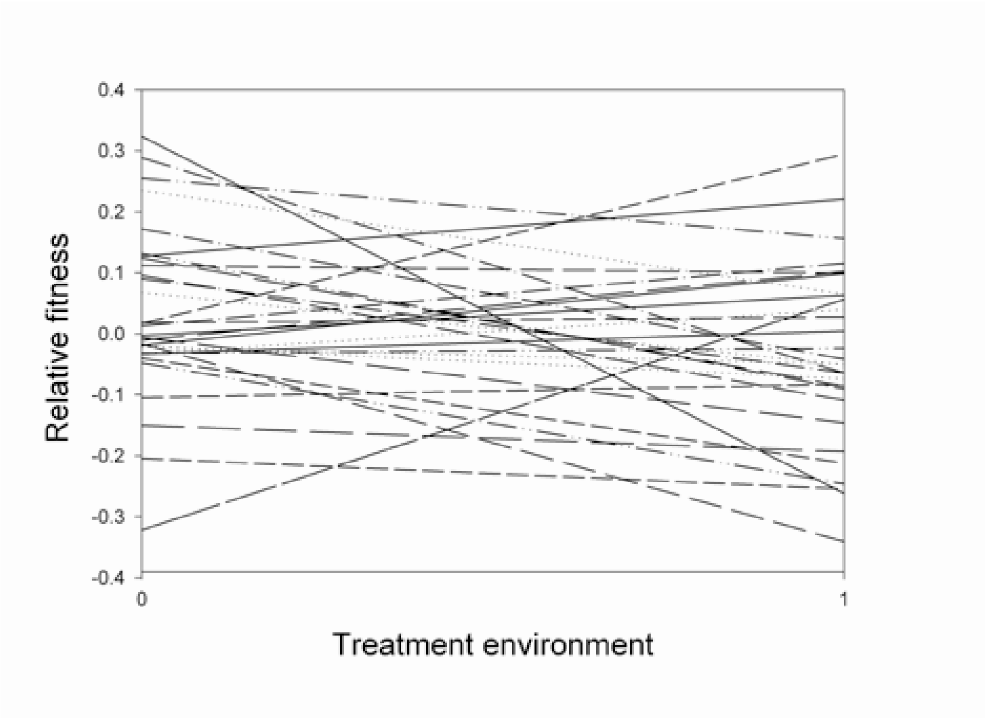

Herbicide use on US soybean
Total % applied
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Glyphosate
1991
1992
1993
1994
1995
1996
1997
1998
1999
2000
2001
2002
Year
2,4-D
2,4-DB
Acifluorfen
Alachlor
Bentazon
Chlorimuron-ethyl
Clethodim
Clomazone
Ethalfluralin
Fluazifop-P-butyl
Fomesafen
Glyphosate
Imazaquin
Imazethapyr
Lactofen
Linuron
Metolachlor
Metribuzin
Paraquat
Pendimethalin
Quizalofop-ethyl
Sethoxydim
Thifensulfuron
Trifluralin

Figure 2.1



Relative fitness
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
0
1
Treatment environment

Figure 2.2

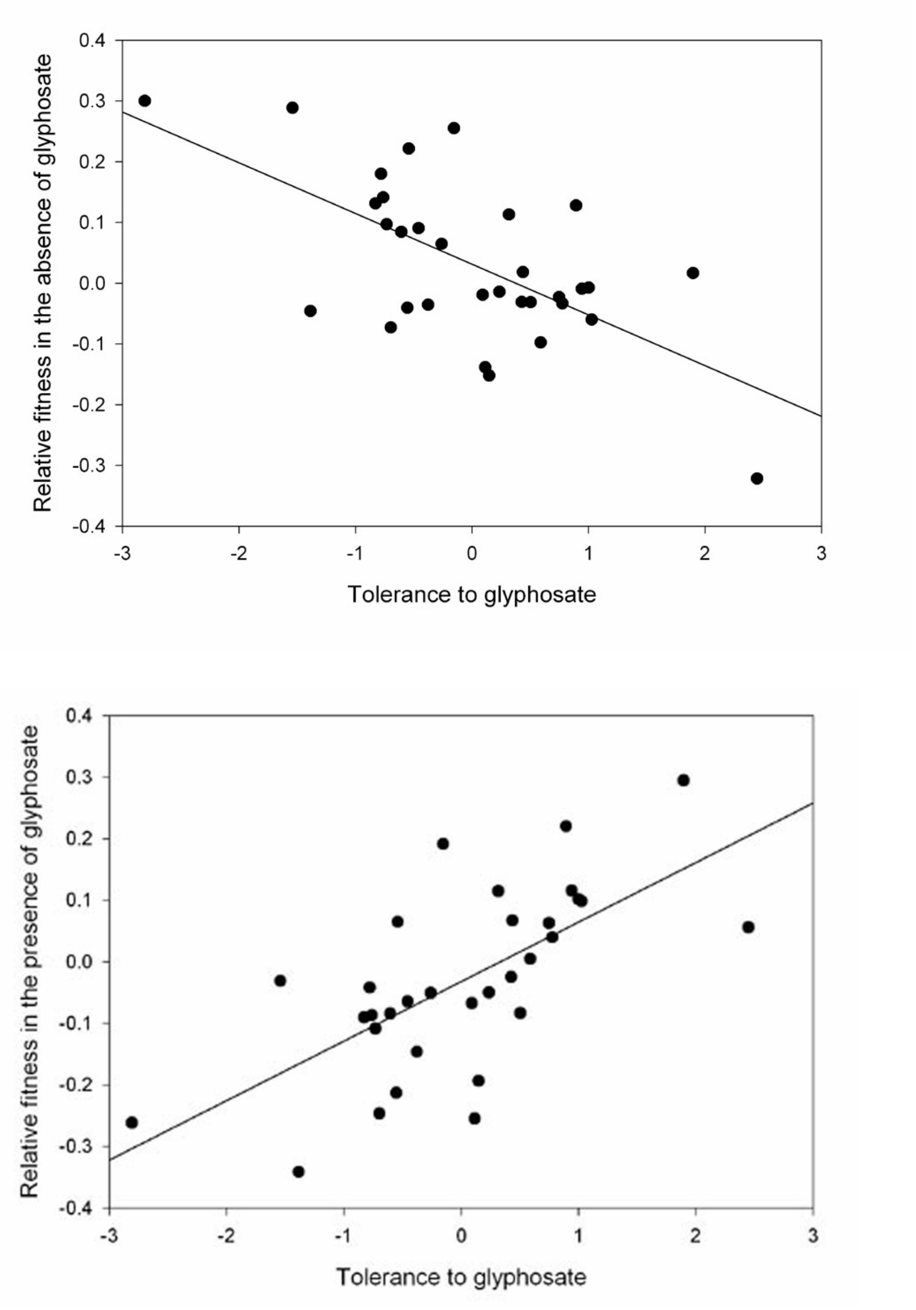


Figure 2.3(A) and 2.3(B)

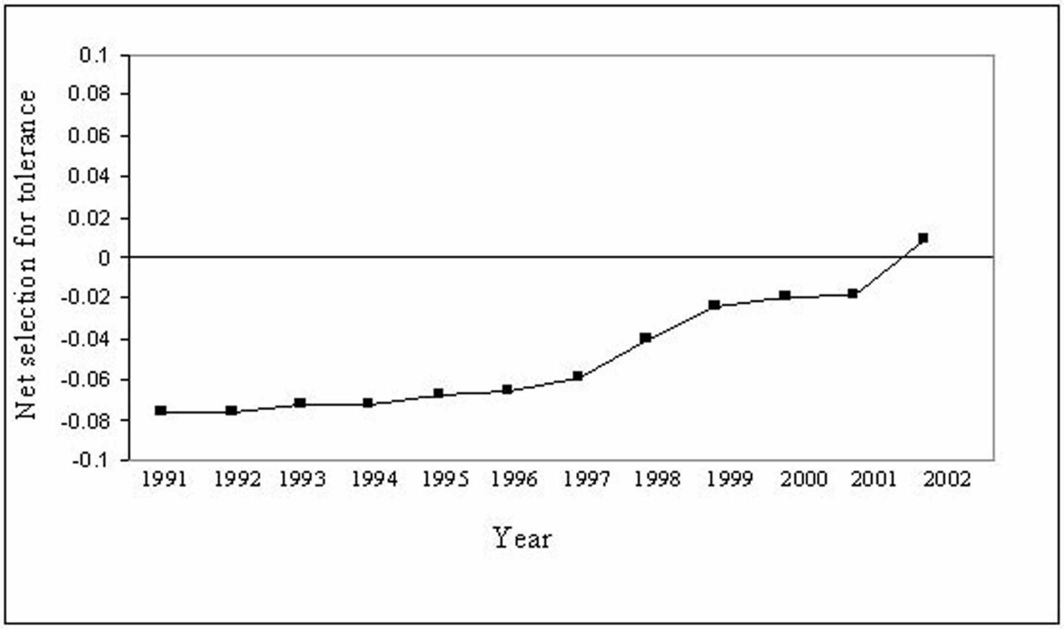

Net selection for tolerance
0.1
0.08
0.06
0.04
0.02
0
-0.02
-0.04
-0.06
-0.08
-0.1
1991
1992
1993
1994
1995
1996
1997
1998
1999
2000
2001
2002
Year

Figure 2.4

CHAPTER 3

THE GENETICS OF TOLERANCE

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ABSTRACT

Genetic parameters underlying herbicide tolerance are of immense interest to those studying its evolution and spread in a weed species. This is because the response to selection by herbicide is dependent on the proportion of phenotypic variance that is due to the additive genetic variance for tolerance, or the heritability of tolerance. To get an accurate estimation of the additive genetic variance, it must be separated from other factors such as parental effects and dominance. We report here the use of a diallel crossing scheme designed to decompose the phenotypic variance of tolerance into its causal components in the common morning glory, *Ipomoea purpurea*. We found that tolerance to the herbicide glyphosate was determined primarily as an additive genetic effect with significant nuclear-extranuclear interactions.

INTRODUCTION

The genetics underlying herbicide tolerance are of immense interest to those studying its evolution and spread in a weed species. Factors such as the intensity of selection by herbicide and the potential for fitness differentials between tolerant and susceptible individuals are pertinent to the study of tolerance evolution, yet it is the estimation of genetic parameters such as the heritability of a trait that allows prediction of the evolutionary response to selection (Falconer and Mackay 1996). Heritability is a ratio that describes the amount of phenotypic variation of a trait that can be ascribed to the genotype of the parent, or that portion of the observed variance that is inherited from one generation to the next (Falconer and Mackay 1996). Thus, the heritability or genetic variance underlying a trait is a fundamental parameter often investigated by evolutionary biologists.

There are a variety of experimental designs employed for the estimation of heritability. It is often that these methods of partitioning the phenotypic variance into genetic and environmental variance allow only the estimation of broad-sense heritability, such that factors are included in the genetic variance. Certain factors, such as parental effects, and specifically maternal effects, can affect the phenotype of the offspring yet do not conform to quantitative genetic models of inheritance (Kirkpatrick and Lande 1989). This is because parental effects, while often due to a genetic basis in the parent, are an environmental source of variation from the standpoint of the progeny (Lynch and Walsh 1998). This means that traits influenced by parental effects are dependent not only on selection in the current generation, but also on the evolutionary response in the previous generation (Falconer 1965; Kirkpatrick and Lande 1989). As a consequence, maternal and paternal modes of inheritance are thought to produce time lags in the response of a population to selection, causing unusual evolutionary dynamics (Kirkpatrick and Lande 1989).

There are two ways offspring can be influenced by parental effects (Hohenboken 1985). First, the phenotype can be influenced by nonnuclear DNA inherited through the cytoplasm via the organellar genomes of the chloroplast or mitochondria. Second, parental care or aspects of the parental phenotype that are influenced by its environment can also impact the phenotype of the offspring. Because progeny develop in the maternal environment, the maternal parent is thought to be the greatest source of these non-nuclear influences on the phenotype. Although maternal effects are thought to be ubiquitous in seed plants (Galloway 2001), paternal effects have also been documented (Avni and Edelman 1991; Simms and Triplett 1996).

In addition to parental effects, other factors such as dominance, epistasis, and nuclear-extranuclear or organeller interactions are also included in the non-additive portion of the genetic variance. Quantitative geneticists employ crossing designs to effectively separate these effects from the additive genetic variance and give a true estimate of the heritable genetic variation underlying a trait. By specifically using a reciprocal crossing design in a hermaphrodite, the genetic variation among groups of interrelated individuals can be separated into the factors of parental effects and dominance as well as any additive causal components (Cockerham and Weir 1977).

Previously, we have documented the presence of genetic variation for tolerance to glyphosate, the active ingredient in the herbicide RoundUp$^{®}$, in the common morning glory, *Ipomoea purpurea* (Baucom and Mauricio 2004). However, it was unknown if the genetic variation was attributable to additive genetic variance or if there were significant influences such as maternal effects underlying tolerance. Although a common garden experiment was employed such that a portion of the phenotypic variance of tolerance could be attributed to a genetic basis, we used the selfed progeny of field-collected maternal lines as our unit of replication. This means that it was unknown if the trait could be ascribed to additive genetic variance or if the maternal parent significantly influenced the observed tolerance in the progeny population. The purpose of this experiment was to determine the inheritance pattern of tolerance in *I. purpurea*, and to separate potential nuclear additive effects on tolerance from factors. A reciprocal crossing design without self-fertilizations was employed so that the variance of tolerance could be partitioned into additive genetic nuclear effects and non-additive nuclear effects that included maternal effects.

MATERIALS AND METHODS

Experimental Organism

The common morning glory, *Ipomoea purpurea* (L.) Roth. (Convolvulaceae), is a weedy annual vine that grows in disturbed habitats throughout the southeastern United States. Germination occurs from mid-May to late August. Flowering typically occurs about six weeks after germination and continues until the first hard frost. Individual flowers open for a single morning and are pollinated almost exclusively by bumblebees (Ennos 1981), although this species is also capable of self fertilization. Fruits mature four to six weeks after pollination and produce from one to six seeds each. The average outcrossing rate for this species has been estimated as approximately 70% in natural populations (Brown and Clegg 1984; Ennos 1981).

Previously, we examined a single population of *I. purpurea* and found genetic variation for tolerance to glyphosate (Baucom and Mauricio 2004). After application of the herbicide, *I. purpurea* individuals appeared stunted and damaged, but continue to re-grow and produce flowers.

Glyphosate is a non-specific post-emergence herbicide (Grossbard and Atkinson 1985). It enters the plant through the stems and leaves by diffusion and is mobile throughout the plant in the phloem (Caseley and Coupland 1985). Glyphosate accumulates in the apical meristems and other sites of sugar utilization (Franz et al. 1997), and causes plant death by inhibiting the biosynthesis of aromatic amino acids (Amrhein et al. 1980; Steinrucken and Amrhein 1980)by inhibiting 5-enol-pyruvylshikimate-3-phosphate synthase, a key enzyme in the shikimate pathway. Approximately 1.5 weeks after application, leaves of sprayed plants begin to exhibit yellowing and necrosis. On many plants, the leaves and the apical meristem completely die, rendering the plant stunted or dead. After being sprayed, plants that survive and produce flowers appear to do so from new stem growth (pers. observation).

Experimental Design

Assessment of natural variation – In fall 2000, seeds were haphazardly collected from 122 individuals growing on the University of Georgia's Plant Sciences Farm and a field located within Oconee County, Georgia. Since *I. purpurea* employs a mixed-mating system, seeds collected from each plant are considered progeny from a maternal line. In the greenhouse, we randomly planted five replicate seeds from each maternal line within four glyphosate treatments: 1.121 kg a.i. ha^{-1}, 0.56 kg a.i. ha^{-1}, 0.28 kg a.i. ha^{-1}, and 0 kg a.i. ha^{-1} as part of an initial investigation designed to assess the level of variation in response to being sprayed by glyphosate. The height of each plant was measured to the nearest cm immediately before the application of glyphosate, and mortality and height of each plant was recorded after glyphosate application. Maternal lines that exhibited the most change in height and the least change in height in the highest level of glyphosate were retained for use as parents in two complete six-by-six diallel crosses for generation of experimental families. A single seed (not used in the initial experiment) from each of the 12 field-collected maternal lines was grown in 12-inch pots and fertilized every other week in the greenhouse. Once individuals began flowering, reciprocal pollinations were performed among all individuals within a diallel by removing an anther from a pollen parent and touching it to the stigma of the seed parent. Anthers were removed from each pollen donor and pollen recipient the night before crosses were made to prevent self-pollination. These crosses produced 60 full-sib families within 12 maternal and 12 paternal half-sib families.

Field protocol – On July 9 2004 we planted fourteen replicates of each family, including reciprocals, among two spatial blocks in an agricultural field on the University of Georgia's Plant Sciences Farm. To ensure germination we nicked each seed prior to planting and marked planted seeds with straws. Within each block we planted seeds 1-m^2 from the next experimental individual. We removed vegetation surrounding experimental individuals once to deter herbivory from the cotton rat, *Sigmodon hispidus*, but otherwise let competitive weeds grow undeterred. Each plant was allowed to grow up a 1-m tall bamboo stake which mimics *I. purpurea* growth in agricultural fields and allows for easy identification of experimental plants. We applied glyphosate at a rate of 1.121 kg a.i. ha^{-1} with a pressurized CO_2 plot sprayer which keeps droplet size and spray intensity constant (R & D Sprayers, Opelousas, LA) on August 15, 2004 to half of the experimental plants.

Data collection – We collected mortality and damage data by assessing death of sprayed individuals and by counting the total number of leaves per plant and the number of leaves exhibiting symptoms of glyphosate damage. We collected fruits during three rounds of collection and counted all viable seeds. The relative number of seeds produced by each individual was our estimate of fitness, and only individuals that survived to produce at least one seed were included in the analysis.

Statistical Analysis

Genetic variation for tolerance – To test for the presence of genetic variation for tolerance to glyphosate, we used the GLM procedure of the SAS statistical software package (SAS Institute) to conduct a nested ANOVA. In this analysis, the response variable was $\log_e$-transformed relative fitness which we calculated by dividing each individual's fitness by overall mean fitness (Stinchcombe and Rausher 2002; Tiffin and Rausher 1999). We used the residuals of relative fitness after the effects of block had been removed as the dependent variable in the model to reduce the effect of spatial heterogeneity. The predictor variables in the model were diallel, treatment, sire and dam nested within diallel, and the interactions between treatment and the main effects. The term of interest with regard to tolerance was the interaction between dam and treatment and sire and treatment; a significant interaction effect indicates that glyphosate did not affect the fitness of all lines equally and is evidence of genetic variation for tolerance.

Diallel analysis – We used the diallel analysis developed by Cockerham and Weir (1977) to detect the nuclear and non-nuclear effects of tolerance. To perform this analysis, we first had to estimate 'pseudo-tolerance' for each replicate within families. This is because an individual cannot be both damaged and undamaged; it is for this reason that tolerance is most often empirically estimated at the family level as the difference in fitness between a group of related individuals planted in both the damaging environment and a control. To do this, we first jackknifed the residual fitness for each replicate within family for both treatments which gave us 14 estimates of residual fitness within each family/treatment combination. We then randomly subtracted each jackknifed value of fitness in the spray environment from a jackknife value of fitness in the control environment, which produced 14 pseudo-tolerance estimates per family. We then performed the Cockerham and Weir diallel analysis (1977) to estimate what proportion of the variance in tolerance was attributable to four possible types of effects (see Simms and Triplett 1996 for a detailed explanation of the analysis). For example, using the relationships between individuals *i* and *j*, the variance can be partitioned into the following: the "nuclear general effect," which is the average effect of individual *i* as parent (also known as the general combining ability, or GCA), and results from additive effects of nuclear genes on the level of tolerance. The "nuclear specific effect" is the average effect of crossing individuals *i* and *j*, $i \neq j$ (also known as the specific combining ability, or SCA), and results from non-additive effects of nuclear genes on tolerance. The "reciprocal general effect" is the difference between individual *i* as sire and individual *i* as dam, (RGCA) and reflects differences among parental plants in offspring tolerance that is independent of their nuclear contribution to the zygote, and thus is the portion of variance corresponding to maternal and paternal effects inherited through cytoplasmic or mitochondrial DNA. Finally, the "reciprocal specific effect" is the difference between full-sib families (from dam *i* and sire *j* vs. sire *i* and dam *j*; RSCA), and includes all possible interactions of parental and progeny genomes (i.e., progeny nuclear genome plus maternal chloroplast genome).

Sums of squares for each effect were calculated according to the Cockerham-Weir model [Lynch and Walsh 1998, p. 615]. Using the method of symmetrical differences (Cockerham and Weir 1977), the observational variance component associated with the reciprocal general effect was broken down into separate maternal and paternal variances. The relative magnitudes of these maternal and paternal variances should reflect the influence of each parent on tolerance; however, the statistical significance of these separate components could not be determined because the two are confounded in the error mean square. These analyses were performed separately for each diallel.

RESULTS

We found significant genetic variation for tolerance among paternal half-sib families, as indicated by the significant Sire × Treatment interaction in the nested ANOVA (Table 1, Fig. 1). This suggests that there is heritable variation for tolerance in the source population from which parent plants were obtained. However, the Dam × Treatment interaction in the ANOVA was not significant ($P = 0.0981$), suggesting that the variation among paternal half-sib families could be due to a non-nuclear paternal effect. In addition, progeny from different crosses did not appear to respond similarly to the application of glyphosate, in that progeny from different Sire × Dam combinations responded differently to treatment with the herbicide.

A Cockerham and Weir (1977) diallel analysis corroborates the findings of the initial ANOVA in Table 1. In both diallels, significant additive genetic variance was found for tolerance, suggesting that the Sire × Treatment interaction of the ANOVA in Table 1 reflects heritable variation for tolerance, and is not due to a non-additive paternal effect (Tables 2, 3). In addition, if tolerance were controlled by a non-additive paternal effect, a significant reciprocal general term would be expected, since that is the term that encompasses both maternal and paternal effects on the offspring. However, in neither diallel was this term significant, further supporting the hypothesis that tolerance is a trait under additive genetic control and is inherited through the nuclear genome. Diallel 2 did, however, exhibit a significant reciprocal specific term, indicating that the progeny from these crosses exhibited different levels of tolerance given their parental background. While this effect is not caused by a maternal or paternal effect per se, it is caused by all possible interactions of the parents and offspring in their effects on tolerance, in that it represents any nuclear-extranuclear interactions (Cockerham and Weir 1977).

DISCUSSION

Tolerance to glyphosate in *I. purpurea* is inherited as a nuclear additive effect. This result confirms previous findings that tolerance is under genetic control, and further clarifies that the trait is heritable in our source population. This is because the nuclear general effect is estimated from the covariance of half-sibs, and thus it is an approximation of heritability.

There were no significant parental effects in either diallel. This supports our finding that tolerance is a trait under nuclear genetic control, and is not inherited primarily via the maternal contribution of the chloroplast nor is it impacted by the maternal environment. We had reason to suspect that maternal effects on tolerance to glyphosate in *I. purpurea* could be an important source of variation due to glyphosate's mechanism of action in plants. Glyphosate binds to 5-enol-pyruvl-shikimate-3-phosphate synthase (EPSP synthase), effectively preventing phosphoenol pyruvate (PEP) from catalyzing EPSP synthase to form the precursor amino acids in the shikimate acid pathway (Amrhein et al 1980). While the gene encoding EPSP synthase resides on the nuclear genome, the catalysis of EPSP synthase occurs in the cytosol of the chloroplast. Although the mechanism of tolerance in *I. purpurea* is unknown, it is possible that it could be due to the proximity of this reaction to the chloroplast. In addition, there are examples from the herbicide resistance literature that have found strictly maternal inheritance of resistance, but this is a finding most often specific to the triazine class of herbicides (Warwick 1991).

Another reason to suspect that maternal effects might impact the inheritance of tolerance comes from a quantitative genetic analysis of field bindweed, or *Convolvulus arvensis*. This study uncovered significant maternal effects in tolerance to glyphosate (Duncan and Weller 1987), and is one of few studies that have investigated the genetics underlying tolerance to glyphosate. Similar to our results, the authors also reported the presence of nuclear general effects, or additive gene action underlying their measure of tolerance, and no evidence of nuclear specific effects, which is the component attributable to dominance. Although there were similarities between the two studies, their measure of tolerance was a visual rating of damage to the plant rather than the difference in fitness between related individuals in a spray and a control environment as is our estimate. It is unclear how interrelated these two measures of tolerance might be, and is a topic currently under further consideration.

While no evidence of parental effects or dominance effects were uncovered by the current study per se, there was a significant reciprocal specific effect in one diallel, or the effect due to all interactions of the nuclear and extranuclear genomes (Cockerham and Weir 1977). Any effect that the extranuclear genomes might have on the nuclear genome would thus be estimated under this effect, such as traditional maternal or paternal effects, extranuclear organelles such as plastids (Cockerham and Weir 1977), and any epigenetic effects such as genomic imprinting (Li et al. 1993; Razin and Cedar 1994). Mojonnier attributed a significant reciprocal specific effect as being due to variation among maternal parents in the degree to which they provisioned offspring sired by different paternal parents (Mojonnier 1998). Because only one diallel in our experiment exhibited significant reciprocal specific effects, it seems likely that the effect is due to nuclear-extranuclear interactions, and is a result of the particular background from which the progeny derived. If nuclear-extranuclear interactions are the cause of the reciprocal specific effect, then that would suggest tolerance is impacted by equilibrium between genomes, and that certain combinations between individuals could eventually outcompete other combinations or crosses given continued selection by glyphosate.

CONCLUSION

This study is the first to attempt an estimation of the inheritance patterns of tolerance as measured by differential fitness. There is a rich literature of investigations of tolerance to herbivory (Agrawal et al. 1999; Fineblum and Rausher 1995; Juenger and Bergelson 2000; Juenger and Lennartsson 2000; Juenger et al. 2000; Mauricio 2000; Mauricio et al. 1997; Pilson 2000; Roy and Kirchner 2000; Simms 2000; Simms and Triplett 1994; Stinchcombe 2002a; Stinchcombe 2002b; Stinchcombe and Rausher 2002; Stowe 1998; Stowe et al. 2000; Tiffin and Inouye 2000; Tiffin and Rausher 1999; Weinig et al. 2003)yet none of these works have explicitly attempted to analyze the pattern of the inheritance of tolerance. This is most likely due to the method by which tolerance is estimated in that it is a family level estimate rather than an individual replicate estimate. While our method of estimating tolerance per each individual is not as satisfying as it would be with a non-permutated value, this is one of the most parsimonious ways of overcoming the problem. Another, more circuitous possibility would be to find a tolerance trait that was highly and positively correlated to tolerance and then performing the diallel analysis on that measure. A tolerance trait is defined as a trait of the plant phenotype that helps to increase fitness when damage occurs, but has no function in the absence of damage (Juenger and Bergelson 2000). However, it would be difficult to be certain that this trait was truly a tolerance trait, and was not a function of resistance, especially since the possibility exists that resistance and tolerance are mutually exclusive traits (Mauricio et al. 1997).

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FIGURE LEGEND

Figure 3.1. Variation among paternal half-sib families in their level of tolerance. Standard error bars were created using individual 'pseudo-tolerance' estimates.

Table 3.1. Nested analysis of variance for relative fitness ($\log_e$-transformed). The Treatment by Sire interaction term indicates significant heterogeneity of paternal lines in response to application of glyphosate.

Source	Df	Type III SS	Mean Square	F Value	P
Diallel	1	64.4309	64.4309	95.45	<.0001
Treatment	1	720.0804	720.0804	1066.76	<.0001
Sire(dial)	10	11.2441	1.1244	1.67	0.0834
Dam(dial)	10	19.0150	1.9015	2.82	0.0018
Sire*Dam(dial)	37	20.5497	0.5554	0.82	0.7666
Treatment*Sire(dial)	10	14.7181	1.4718	2.18	0.0167
Treatment*Dam(dial)	10	10.8658	1.0866	1.61	0.0981
Treatment*Sire*Dam(dial)	37	41.4539	1.1204	1.66	0.0080
Error	1510	1019.2744	0.6750		

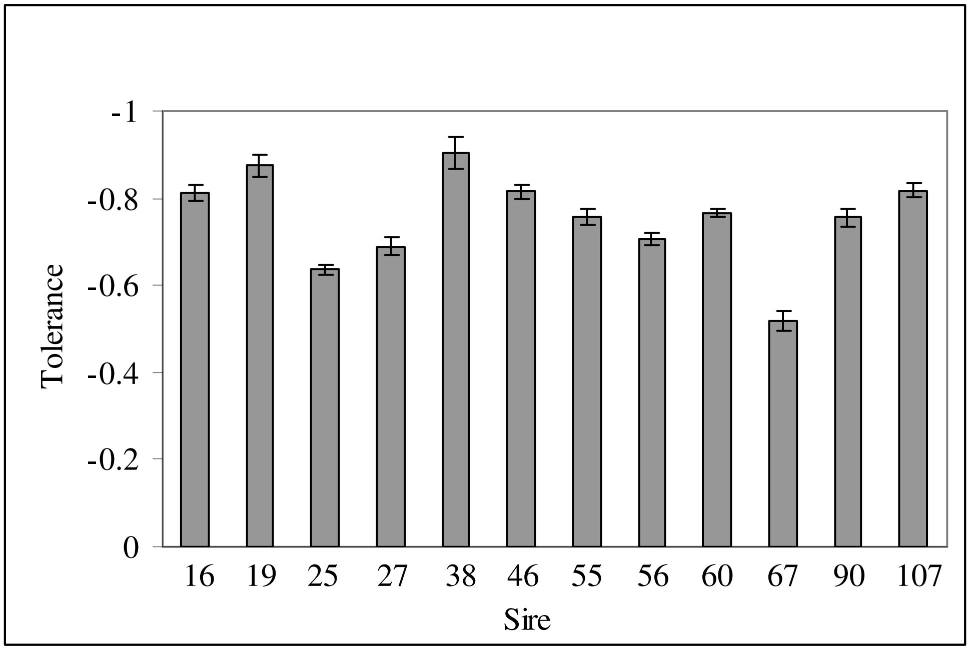

Figure 3.1

Table 3.2. Cockerham and Weir (1977) diallel analysis of tolerance for the individuals in Diallel 1

Source	df	F statistic	Variance	Interpretation
Nuclear general	5	12.1112****	0.0049	Significant additive effects underlying tolerance
Nuclear specific	9	0.0000	0.0000	No nonadditive effects controlling tolerance
Reciprocal general	5	ne[†]	0.0018	No maternal or paternal effects controlling tolerance
Maternal	…	…	-0.0022	
Paternal	…	…	0.0040	
Reciprocal specific	10	0.0000	-0.0004	No nuclear-extranuclear interactions
Error	390		0.0058	Within-cross variation

† Non-estimable due to a '0' reciprocal specific MS term, which would be the denominator in the F-statistic

Table 3.3. Cockerham and Weir (1977) diallel analysis of tolerance for the individuals in Diallel 2

Source	df	F statistic	Variance	Interpretation
Nuclear general	5	37.6285****	0.0061	Significant additive effects underlying tolerance
Nuclear specific	9	0.0000	-0.0131	No nonadditive effects controlling tolerance
Reciprocal general	5	0.0765	-0.0081	No maternal or paternal effects controlling tolerance
Maternal	…	…	-0.0100	
Paternal	…	…	0.0019	
Reciprocal specific	10	52.7010****	0.0258	Significant nuclear-extranuclear interactions
Error	390		0.0070	Within-cross variation

**** $P < 0.0001$

CHAPTER 4

PRE-EXISTING GENETIC VARIATION FOR GLYPHOSATE TOLERANCE

Baucom, R.S. and R. Mauricio. To be submitted to *Science*.

ABSTRACT

A central tenet of evolutionary biology is that the process of evolution is fuelled by the naturally occurring genetic variation underlying a trait. We found the presence of genetic variation for tolerance to the herbicide RoundUp® in *Ipomoea purpurea* seed accessions collected before common use of the herbicide in agricultural fields. This suggests tolerance to RoundUp® in *I. purpurea* is a pre-adaptation to another agent of selection. We also found significant genetic variation for tolerance in lines exposed to RoundUp® for at least 10 years, indicating the potential for tolerance to respond to selection by use of the herbicide.

INTRODUCTION

A central tenet of evolutionary biology is that the process of evolution by selection takes advantage of naturally existing variation within populations. Without variation underlying a trait, selection has no fuel and the evolutionary process cannot proceed. Documented cases of antibiotic resistance show that genetic variation for resistance existed well before the discovery of antibiotics (Palumbi 2001), suggesting that the trait evolved in response to some other, and unknown selective force. If an adaptation conferring defense evolved in response to selection via another agent, then the evolutionary dynamics governing the increase and extinction of a resistance mutation are less certain.

Ipomoea purpurea, the tall morning glory, can tolerate applications of glyphosate, which is the active ingredient in RoundUp®, an herbicide of immense importance to the agricultural industry. It is unknown if *I. purpurea's* ability to tolerate applications of this herbicide is due to a relatively new mutation conferring tolerance, or if the tolerant phenotype is an adaptation to another damaging agent. Here we provide evidence that genetic variation for glyphosate tolerance in *I. purpurea* pre-dated the widespread adoption and use of glyphosate in crops by assessing the fitness of *I. purpurea* accessions collected in the late 1980's, well before the widespread adoption and use of glyphosate in the early 1990's (Fig. 4.1).

MATERIALS AND METHODS

We acquired pre-glyphosate *I. purpurea* source populations from the Plant Genetic Resources Conservation Unit of the USDA in Griffin, GA. These populations represent a world-wide sample of *I. purpurea*, and were collected in the early 1980's, prior to the widespread adoption of glyphosate in the early 1990's. Seeds of each population were maintained at a constant temperature until germination trials in August, 2003. Of the seventeen populations assayed for germination (> ~20,000 seeds across populations), 5 produced at least 2 viable source individuals. We planted these ten pre-glyphosate lines in the greenhouse and allowed the plants to self in order to produce enough experimental individuals for the field. To generate post-glyphosate lines, or lines that experienced selection by glyphosate, twelve individuals collected in August, 2000 from Oconee County, Georgia were germinated and used in 2 sets of complete diallel crosses without self-fertilization in the greenhouse. The initial twelve individuals were collected from populations that experienced glyphosate application for at least 10 years. Thirty lines were established from these crosses for use in the field experiment.

The field experiment was planted July 9, 2004 in an agricultural field at UGA's Plant Sciences Farm in Oconee County. We randomized 7 replicates of each line among 2 spatial blocks to take into account habitat heterogeneity. We followed established field protocols for performing the field study (Baucom and Mauricio 2004) and applied glyphosate at a rate of 1.121 kg Ha^{-1} with a CO_2 plot sprayer calibrated to a spray volume of 20 GPA (R&D Manufacturers, Inc. Opelousas, LA.) to half of the experimental individuals on 15 August 2004.

We collected fruits and counted all viable seeds from each individual for our estimate of fitness. Individuals that expired before the application of glyphosate were not included in the analysis; those that died as a result of glyphosate application were given a fitness score of '0'.

To test for the presence of genetic variation for tolerance to glyphosate, we used the GLM procedure of the SAS statistical software package (SAS Institute, Inc. 2002) to conduct ANOVA. In this analysis, the response variable was $\log_e$-transformed relative fitness (Tiffin and Rausher 1999; Stinchcombe and Rausher 2002; Baucom and Mauricio 2004). We used the residuals of relative fitness after the effects of block had been removed as the dependent variable in the model to reduce the effect of spatial heterogeneity. The term of interest with regard to tolerance was the interaction between genetic line and treatment; a significant interaction indicates that glyphosate did not affect the fitness of all genetic lines equally and is evidence of genetic variation for tolerance. This ANOVA was performed separately for the pre- and post-glyphosate lines.

RESULTS AND DISCUSSION

Our results indicate that genetic variation for tolerance exists in both the pre-glyphosate (Line × Treatment F-value = 2.72; p-value = 0.0048) and in the post-glyphosate (Line × Treatment F-value = 2.08; p-value = 0.0008) lines (Table 4.1). The presence of genetic variation for this trait in the pre-glyphosate lines provides evidence that tolerance existed in the species before wide-spread use of the herbicide. Thus it is likely an adaptation to another type of selective agent in nature, or a pre-adaptation, that has been co-opted for defense against glyphosate application. That genetic variation is present in the post-glyphosate lines suggests continued selection by glyphosate in agriculturist's fields could lead to an increase in the frequency of tolerance.

In fact, previous work has shown positive directional selection on glyphosate tolerance in an extant population of *I. purpurea*, as well as an approximate 85% increase in use of the herbicide in agriculture since the early 1990's (Baucom and Mauricio 2004). The combination of strong, positive directional selection on tolerance, the increased use of glyphosate in recent years and the presence of genetic variation for tolerance suggests that it should increase in frequency in crop systems, as per the evolutionary process.

Similar to this study, assays of populations previously unexposed to current-day selective agents have found the presence of defensive capability (Houndt and Ochman 2000). Other work has uncovered a higher than expected frequency of the defense mutation underlying the trait (Gould et al. 1997). This, as well as the findings in our study, would suggest that ample polymorphism for defense exists in pest populations in nature. Our study, while unable to identify previous selective agents that may have maintained glyphosate tolerance, presents a rare opportunity to see that a trait of immense importance to current-day agriculture has been present within agricultural crops well before the common use of glyphosate.

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Tiffin, P., and M. D. Rausher. 1999. Genetic constraints and selection acting on tolerance to herbivory in the common morning glory Ipomoea purpurea. American Naturalist 154:700-716.

FIGURE LEGEND

Figure 4.1. The proportion of soybean acreage sprayed with glyphosate from 1991 to 2002. Data are taken from (NASS-USDA 2003).

Table 4.1. ANOVA tables showing presence of genetic variation for tolerance in A) Lines collected and stored prior to the use of glyphosate, and B) Lines collected from an agricultural field that had experienced selection by glyphosate for ~ 10 years.

(A) Source	df	Type III SS	MS	F Value	P
Pre-glyphosate lines	9	1.5399	0.1711	1.74	0.0804
Treatment	1	14.9280	14.9280	151.74	<0.0001
Line×Treatment	9	2.4050	0.2672	2.72	0.0048
Error	262	25.7751	0.0984		

(B) Source	df	Type III SS	MS	F Value	P
Post-glyphosate lines	29	19.2687	0.6644	5.04	<0.0001
Treatment	1	111.3391	111.3391	845.29	<0.0001
Line×Treatment	29	7.9455	0.2740	2.08	0.0008
Error	785	103.3974	0.1317		

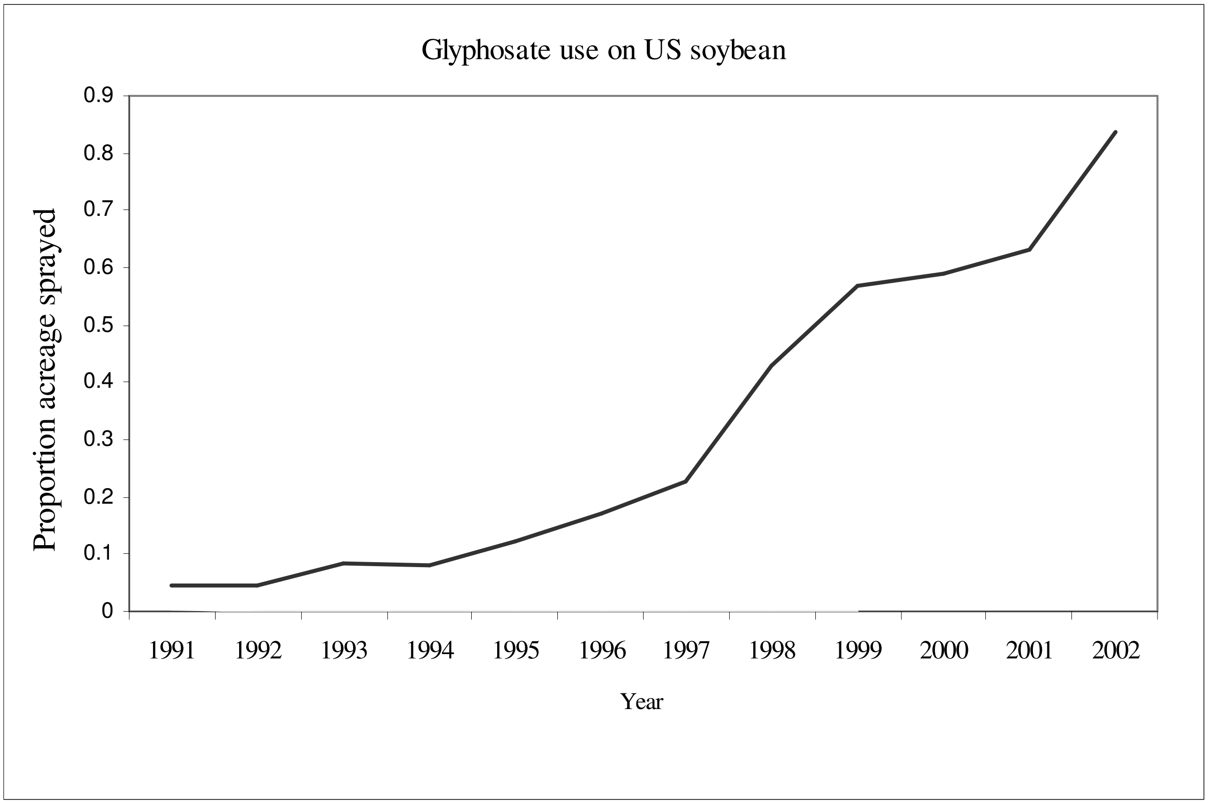
Glyphosate use on US soybean
Proportion acreage sprayed
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1991
1992
1993
1994
1995
1996
1997
1998
1999
2000
2001
2002
Year

Figure 4.1

CHAPTER 5

THE EVOLUTION OF NOVEL HERBICIDE TOLERANCE IN A NOXIOUS WEED: THE GEOGRAPHIC MOSAIC OF SELECTION

Baucom, R.S. and R. Mauricio. *Submitted to Evolution*. 9/15/2005

ABSTRACT

Understanding how genetic variation is organized over geography has long been of interest to evolutionary biologists given that traits can vary within and among populations, across regions, and at continental or global scales. The pattern of regional variation can have an important impact on trait evolution at the local or population level. Using a common garden, we ascertained whether a geographically variable mosaic of tolerance to the widely applied herbicide RoundUp® existed in two closely related co-occurring species of morning glory, *Ipomoea purpurea* and *I. hederacea*. We assayed RoundUp® tolerance in over 3700 plants representing 310 families from 29 populations in the southeastern United States. Our findings suggest that the two species of morning glory partition their respective levels of genetic variation for tolerance to glyphosate differently. Variation for tolerance in *I. purpurea* appears to exist among maternal lines, populations, and regions, whereas in *I. hederacea*, variation in tolerance existed only among populations. Despite this difference, the species collected within the same site exhibit similar levels of tolerance in one region of the Southeast. In addition, we find that there is a trend for positive spatial aggregation of this trait on a local scale, but evidence of negative spatial autocorrelation at greater distances. Given our results, we conclude that the evolutionary trajectory of this trait cannot be considered without prior knowledge of both the ecology and selective history of individual populations, groups of populations, ecological regions, and species.

INTRODUCTION

Ecologists and evolutionary biologists have long recognized the need to incorporate the concepts of spatial scale and geographic structuring as important factors influencing the demographic and genetic patterns of adaptive traits in natural populations (Hutchinson 1959; Wright 1943). This is largely attributable to the recognition that populations are rarely genetically isolated from one another, calling into question the scale at which adaptive traits should be considered. Variation in adaptive traits can exist among individuals within a single population or among populations physically structured across geography. Variation can also exist among aggregations or groups of populations found over geography, giving the appearance of spatial 'clumping,' or a mosaic of trait values over a wide geographical area. The resulting pattern of evolutionary 'hotspots' or 'coldspots,' where neighboring populations exhibit similar high or low trait values, respectively, could be produced by similar regimes of selection within aggregations of populations (Brodie and Ridenhour 2002; Thompson 1997). Alternatively, this pattern of spatial variation might be seen if populations are connected by gene flow where the level of variation at one site might potentially be a determinant of evolutionary potential at another site. Larger-scale regional trends can also exist such that populations from ecologically-distinct, widely-separated areas exhibit differences in trait values.

Despite the recognition that different levels of population geographic structure exist and can influence the scale at which adaptive trait variation should be considered, it is most often within a single population that the evolutionary dynamics of an adaptive trait are studied. Considering adaptive trait variation in a broad geographical context will allow greater insight into the evolutionary process; incorporating trait variation data from within and among populations, population aggregations, and ecologically distinct regions will aid further predictions about the evolutionary trajectory of important plant traits.

In a previous study, genetic variation in tolerance to glyphosate, the main ingredient in the herbicide RoundUp$^{®}$, was found within a population of the common morning glory, *Ipomoea purpurea*, a noxious agricultural weed in the southeastern United States (Baucom and Mauricio 2004). Glyphosate use is common in current farming management (Dill 2005) and intense selection by this herbicide could impact the scale at which variation in tolerance resides. An initial assessment of where variation in tolerance to glyphosate exists, whether within or among populations, among aggregations of populations leading to a ‘mosaic’ of tolerance, or among ecologically distinct regions will first provide an idea of the potential for the evolution of tolerance in nature. This information will also inform the weed manager of how to best control the continued evolution of the trait. Second, comparing the level of tolerance among groups that have experienced a similar selection regime by glyphosate will provide evidence for the repeatability of the evolutionary process, in that it will provide an idea of how often the same evolutionary outcome might result in nature given a similar selection regime; a microevolutionary version of Stephen J. Gould’s “replaying the tape of evolution” metaphor (Gould 1989).

In this paper we investigate variation in tolerance within and among populations of two morning glory species that have experienced strong selection by glyphosate over the past 25 years. We address the following questions: Over what spatial scale does variation in tolerance to glyphosate exist among populations of two morning glory species: within populations, among populations, or among topographically distinct regions? Is there a true geographical mosaic of tolerance in that local populations of each species are found to spatially aggregate for their level of tolerance, such that there are ‘hotspots’ and ‘coldspots’ of tolerance across a wide geographical area? Do closely related species exhibit similar levels of tolerance when found to co-occur, such that parallel evolution can be inferred, or are the two species exhibiting dissimilar levels of tolerance?

MATERIALS AND METHODS

Natural History

The common morning glory, *Ipomoea purpurea* (L.) Roth. (Convolvulaceae), is a weedy annual vine that grows in disturbed habitats throughout the southeastern United States. Germination occurs from mid-May to late August. Flowering typically occurs about six weeks after germination and continues until the first hard frost. Individual flowers open for a single morning and are pollinated almost exclusively by bumblebees (Ennos 1981), although this species is also capable of self fertilization. Fruits mature four to six weeks after pollination and produce from one to six seeds each. The average outcrossing rate for this species has been estimated as approximately 70% in natural populations (Brown and Clegg 1984; Chang and Rausher 1998; Ennos 1981).

The ivy-leaf morning glory, *Ipomoea hederacea* (L.) Jacquin, is also a weedy annual vine which both morphological and molecular data suggests is closely related to *I. purpurea* (Miller et al. 2002; Miller et al. 2004). In the southeastern US, populations of *I. hederacea* are found in disturbed areas and agricultural fields, often interdigitated within populations of *I. purpurea*. Patterns of seed germination and flower and fruit production are similar to those of *I. purpurea*, although *I. hederacea* appears to flower and senesce earlier than *I. purpurea* in natural populations (*pers. observation*). Flowers of *I. hederacea* range from light to dark blue in color and are often visited by bumblebees (Ennos 1981) although the selfing rate for one population has been estimated at 93% (Ennos 1981). The two species do not form viable hybrid offspring from crosses in the greenhouse (Guries 1978) and there is no evidence that the two species successfully mate in the wild.

A review of weed species in the Southeast places annual morning glory species as most troublesome weed in soybean and the number two most troublesome weed in cotton and corn (Webster 2004). Both *I. purpurea* and *I. hederacea* are considered noxious crop weeds that decrease crop yield and lead to harvesting difficulties. Both species are able to tolerate glyphosate in that they are able to survive application of the herbicide, re-grow and produce progeny for the next generation. Previously, we examined a single population of *I. purpurea* and found genetic variation for tolerance to glyphosate (Baucom and Mauricio 2004). After the application of the herbicide, *I. purpurea* individuals appeared stunted and damaged, but continue to re-grow and produce flowers.

Glyphosate is a non-specific post-emergence herbicide (Grossbard and Atkinson 1985). It enters the plant through the stems and leaves by diffusion and is mobile throughout the plant in the phloem (Caseley and Coupland 1985). Glyphosate accumulates in the apical meristems and other sites of sugar utilization (Franz et al. 1997), and causes plant death by inhibiting the biosynthesis of aromatic amino acids (Amrhein et al. 1980; Steinrucken and Amrhein 1980) by inhibiting 5-enol-pyruvl shikimate-3 phosphate synthase, a key enzyme in the shikimate pathway. Approximately 1.5 weeks after application, leaves of sprayed plants begin to exhibit yellowing and necrosis. On many plants, the leaves and the apical meristem completely die, rendering the plant stunted or dead. After being sprayed, plants that survive and produce flowers appear to do so from new stem growth (pers. observation).

Experimental Design

Twenty-nine populations of *I. purpurea* and 17 populations of *I. hederacea* were collected from cotton, soybean or corn fields located in either the Coastal Plain in North and South Carolina or from the Cumberland Plateau in middle Tennessee (Figs. 5.1, 5.2). The two species were found at a common set of seventeen collection sites. Ripened seeds were sampled from up to 20 maternal individuals per species and site.

All populations had a recent history of glyphosate exposure, whether glyphosate was sprayed the same year of seed collection or in the previous year. Available farmers were censused about the rate and frequency of glyphosate application within their fields. In counties where farmers were not available for questioning, the county USDA extension agent was queried. In general, there were no reported differences in the rate of glyphosate application, which was 1 quart per acre and approximates 1.121 kg a.i. ha^{-1}, the highest rate of glyphosate used in this study. Variation did exist among the types of crops from which seeds were collected: soy fields were sprayed twice per year, whereas cotton fields were sprayed once during the growing season, and corn was sprayed before the beginning of the growing season. Although variation among crop types could produce variation among populations in level of tolerance, all farmers and extension agents reported the use of crop rotation programs, such that every crop was rotated at least every two years. Given the diversity of particular crop rotation regimes, and given that no farmer reported spraying more than 1 qt per acre, we chose to analyze all collection sites together regardless of crop type under the assumption that all sites experienced consistently variable selection regimes.

On January 23, 2004, 3720 seeds (310 maternal plants, 207 from *I. purpurea* and 103 from *I. hederacea*, 3 replicates per line, 4 spray treatments) were scarified with a razor blade and planted in a pine-bark soil mixture in a completely randomized design in the University of Georgia Plant Biology greenhouses. Plants were watered daily and kept on a 12:12 day/night light regime with sodium vapor supplementary lights (Energy Technics, York PA). Plants were fertilized once with a 10-30-20 fertilizer (Peter's Blossom Booster) but otherwise allowed to grow until treatments were applied. On March 1, 2004 glyphosate was applied to individuals at the following rates: 0, 0.4, 0.8 and 1.6 kg active ingredient per hectare with a pressurized CO_2 plot sprayer which keeps droplet size and spray intensity constant (R & D Sprayers, Opelousas, Louisiana).

On March 17 2004, we recorded mortality of the sprayed individuals. We also recorded the date of first flower and the number of flowers produced by each individual daily after glyphosate application in the four treatments. No individuals flowered prior to glyphosate application. In all analyses, total number of flowers was used as our estimate of individual fitness. Although in a previous study we used the total number of seeds as the estimate of fitness (Baucom and Mauricio 2004), number of flowers is a more appropriate predictor of reproductive effort in the greenhouse, given that *I. hederacea* is almost a complete selfer whereas *I. purpurea* is primarily outcrossing. In addition, there is variability among *I. purpurea* genotypes for anther-stigma distance (Chang and Rausher 1998), meaning that the use of seed number in the greenhouse would be a measure of anther-stigma distance rather than of reproductive effort. For analysis, total number of flowers was relativized by dividing by the maximum flower number of each species.

Variation for tolerance

To determine if there is genetic variation among maternal lines for tolerance, and variation among populations and regions for tolerance, we assessed if fitness responded differently across the glyphosate treatments separately for each species. Specifically, we determined if the slopes of the lines between fitness and treatment environment differ across maternal lines, populations, and regions. The significance of such genetic variation is tested with a nested analysis of variance (ANOVA) by examining the significance of the maternal line by treatment, the population by treatment, and the region by treatment interactions. For this analysis, we used the GLM procedure of the SAS statistical software package (version 8.0) to conduct the ANOVA with relative fitness as the dependent variable. Region, populations nested within regions, maternal line nested within populations and regions, and the interactions between treatment and the main effects were our independent variables. The interaction term between region and treatment, and population and treatment will determine if there is variation in tolerance among the Coastal Plain (CP) and Cumberland Plateau (CU) regions, and the populations collected within these regions, respectively. The interaction term between maternal line and treatment determines if the variation in tolerance is genetic.

Measurement of tolerance

Individuals collected within 29 populations of *I. purpurea* and 17 populations of *I. hederacea* form the basis of our measure of tolerance. It is not possible to obtain values of tolerance on an individual plant because an individual cannot be both damaged and undamaged (Mauricio et al. 1997; Rausher 1992; Stinchcombe and Rausher 2002; Tiffin and Rausher 1999). Thus we estimated a population-level tolerance value by using replicates of individuals collected within populations and assessed the fitness of those replicates in the different treatments.

Tolerance is most often modelled as a norm of reaction by regressing fitness on damage see (Baucom and Mauricio 2004; Mauricio et al. 1997; Simms and Triplett 1994; Tiffin and Inouye 2000; Weinig et al. 2003) and is referred to as the plastic response of fitness on damage

(Abrahamson and Weis 1997). The slope of this regression is the estimate of tolerance; a small slope indicates low sensitivity to damage, or high tolerance, whereas a large negative slope indicates a high sensitivity to damage, or low tolerance. A positive slope indicates overcompensation. For our analyses, we used the REG procedure in SAS to conduct a multiple regression of relative fitness on the treatment and maternal line variables and retained the slope of the relationship between fitness and treatment as our estimate of tolerance. Initial analyses testing for the presence of genetic variation for tolerance indicated there was within-population variation (a significant Maternal Line × Treatment interaction) for tolerance in *I. purpurea* but not *I. hederacea*, and significant among-population variation for tolerance (a significant Population × Treatment interaction) in both species. Thus we performed our regression by species and population, including maternal line as a variable to obtain a tolerance value for each species/population combination that included any potential effect of maternal line on the estimate of tolerance.

Variation among species and regions

To determine if tolerance values varied among species over the Southeast we performed an ANOVA with tolerance level as the dependent variable, and species, region and their interaction as the independent variables (N = 29 *I. purpurea*, N = 17 *I. hederacea*). The analysis was then performed by species, and the Tukey method was used to test for differences among the two regions for each species (SAS Institute 1990).

We employed the nonparametric Wilcoxon signed-rank sums test for matched pairs to determine if values of tolerance were similar among the two species found co-occurring within the same collection sites (N = 17). Each species' trait value was matched with the other species trait value from the same collection site, and the test was performed over all populations collected from within the Southeast and then separately for each of the regions. We chose to do the analysis per region given the differences between edaphic factors within the Cumberland Plateau and Coastal Plain.

'Hotspots' analysis

To test for positive or negative spatial autocorrelation of tolerance over the Southeast, we calculated Moran's *I* (Moran 1950) over mutually exclusive spatially lagged distance classes. A high positive value of Moran's *I* would indicate the spatial clustering of similar values, whereas a high negative value indicates dissimilar values. Unlike other studies that assess if populations spatially aggregate for high or low levels of a trait value, this analysis determines if populations aggregate for similar or dissimilar values of tolerance, regardless of the level of tolerance. The higher the value of Moran's *I*, the more the observation is similar (positive) or dissimilar (negative) to its neighbors. The analyses were performed for each species separately within either the Coastal Plain or the Cumberland Plateau. We performed the analyses per each region because populations within each of the regions were widely separated from one another (Fig. 5.1, 5.2), and Moran's *I* values are affected by discontinuities in sampling patterns (Epperson and Li 1996). The first quartile of nearest neighbor statistics was estimated in the program ROOKCASE (Sawada 1999) for each species within each region and was used to determine the size of the lag distances. For example, if the first quartile of nearest neighbor statistics was 7000 m, each lag distance was set at 7000 m and a global Moran's *I* statistic was calculated for each distance. Significance in the lag distance classes was tested in ROOKCASE by using 10,000 Monte Carlo permutations of Moran's *I*, and a 99% confidence envelope was generated from the permutations. Values of Moran's *I* within 99% confidence envelope represent cases where the null hypothesis of no spatial autocorrelation cannot be rejected. Values outside of this range indicate significant spatial autocorrelation, or evidence of either ‘hotpots’ or ‘coldspots’ of tolerance.

RESULTS

Genetic and geographical variation for tolerance

We first determined whether genetic variation existed for tolerance among maternal lines within populations of both species. For this analysis, we performed a nested ANOVA in which fitness in the spray (treatment) environment was the dependent variable, and region, population nested within region, maternal line nested within population and region, treatment, and the interactions between the main effects and treatment were the independent variables. The maternal line by treatment interaction term was significant for *I. purpurea* (Maternal Line × Treatment effect; F = 1.14, P = 0.0401; Table 5.1), but not *I. hederacea* (Maternal Line × Treatment effect F = 0.82, P = 0.9568; Table 5.2), indicating that the maternal individuals of *I. hederacea* did not differ in the regression of fitness on the treatment environments, and that genetic variation for tolerance was present only within *I. purpurea* populations.

We next determined if variation existed among populations and regions for both species. In the same nested ANOVAs, the population by treatment and region by treatment interactions assessed if there was variation among populations and regions for tolerance, respectively. The population by treatment interaction term was significant for both *I. purpurea* (Population × Treatment F = 1.43, P = 0.0087; Table 5.1) and *I. hederacea* (Population × Treatment F = 2.09, P <0.0001; Table 5.2), indicating that the populations of both species differed in the regression of fitness on the treatment environments and thus exhibited variation over geography for tolerance. At the regional level *I. purpurea* exhibited significant variation among regions for tolerance

(Region × Treatment F-value = 5.11, P = 0.0006; Table 5.1) but *I. hederacea* did not (Region × Treatment F-value = 0.80, P = 0.4925; Table 5.2).

Variation among species and regions

The two species are not exhibiting different levels of tolerance in the Southeast overall (average tolerance, *I. purpurea*: -2.04, *I. hederacea*: -2.42, F-value = 1.27, P = 0.2683, Table 5.3), but there was a significant species by region interaction (F-value = 4.81, P = 0.0339, Table 5.3). When tolerance values are compared among regions within species, *I. purpurea* populations from the Cumberland Plateau are significantly more tolerant to glyphosate than populations collected from the Coastal Plain (Fig. 5.3), but the level of tolerance among regions was similar for populations of *I. hederacea* (Fig. 5.3).

Variation among species pairs

When each species' trait value was matched with the other species trait value from the same collection site and tested for rank similarity, it was found that *I. purpurea* and *I. hederacea* individuals were not exhibiting similar tolerance levels in the 17 collection sites in the Southeast where the two species co-occurred (Table 5.4). This result was also found when using only collection sites within the Coastal Plain. In collection sites within the Cumberland Plateau, however, the rank order between the species was preserved, meaning that the two species were exhibiting similar levels of tolerance. This suggests that the species collected from within the same site in this region of the Southeast have potentially responded to the selection regime in a similar manner (Table 5.4). This pattern could be found if outlier sites with either high tolerance or low tolerance were negating the effect. This appeared to not be the case; when tolerance values of each species were plotted and a line was drawn connecting the same site, the rank order between species appeared conserved across sites of the Cumberland Plateau (Fig 5.4).

'Hotspots 'and 'coldspots' of tolerance in the Southeast

There was no evidence of significant positive spatial autocorrelation among *I. purpurea* populations from the Coastal Plain (Fig. 5.5A) or the Cumberland Plateau (Fig. 5.5B), suggesting that tolerance values were not similar among closely situated populations. However, in the Coastal Plain, the first lag distance produced a trend toward positive spatial autocorrelation (Moran's I = 1.70, P = 0.07). In addition, the third lag distance, or 21 km, showed a significant negative association among populations, meaning that, at greater distances from the central reference point, populations were significantly dissimilar for their level of tolerance (Moran's I = −1.81). Similarly, in the Cumberland Plateau, a significant negative spatial autocorrelation was found at 96 km in *I. purpurea* (Moran's I = -1.94). *Ipomoea hederacea* populations did not show any significant spatial autocorrelations from either region (Fig. 5.5C, 5.5D).

DISCUSSION

Geographic patterns of phenotypic variation

There is growing data to suggest that populations vary according to defensive trait such as resistance to predators and pathogens (Brodie and Ridenhour 2002; Burdon and Thrall 1999), and level of resistance to herbicides (Warwick 1991). Our results support this conclusion on multiple levels of geography in that we find evidence for within- and among-population variation, as well as among-region variation in the level of tolerance. Specifically, we found evidence of significant maternal line, or within-population variation in *I. purpurea* for tolerance to glyphosate. This was expected, given that within-population variation for tolerance has previously been documented in one population from the Southeast (Baucom and Mauricio 2004). This suggests that populations of the common morning glory remain unfixed for this highly adaptive character, and that further bouts of selection by use of the herbicide could result in the continued evolution of this trait.

Unlike *I. purpurea*, there was no evidence of within-population variation for tolerance in *I. hederacea*. However, there was evidence of among-population variation within this species. The lack of within-population variation, but the presence of among-population variation could be attributed to this species' highly selfing mating system. In fact, this pattern is typical of predominantly selfing species, which tend to house more neutral genetic variation among populations than within populations (Hamrick and Godt 1996), and could occur given population establishment by a single or few individuals tolerant to glyphosate. Those individuals would predominate genetically within populations, especially given selection by glyphosate, and lead to more among- than within-population variation.

Although we found evidence of among- population variation for *I. hederacea*, there appeared to be no among-region variation in tolerance for this species, unlike that of *I. purpurea*. Again, the difference between the two species in how they house their respective level of genetic variation for tolerance might be due to the difference in mating system. The lack of gene flow between populations of *I. hederacea* could lead to more variation among populations, whereas gene flow among populations within regions in *I. purpurea* could effectively homogenize trait values, and lead to one region being more tolerant than the other. Seeds of both species are gravity dispersed, although it is equally likely that farm machinery could transport seeds of both species among fields. Gene flow by pollen movement is potential mechanism by which *I. purpurea* populations could be interacting; however, there was still evidence for significant population variation within *I. purpurea*, meaning that a complete admixture of genes is not occurring.

The variation among regions in *I. purpurea* could also be due to random processes not related to the use of glyphosate, such as drift in the process of population establishment or selection on a trait correlated to tolerance. Adaptation to other abiotic or biotic factors not examined by this common garden study could also potentially explain variation among regions in tolerance. The average elevation of Coastal Plain populations was about 50 m above sea level, whereas populations from the Cumberland Plateau were found at an elevation greater than 300 m above sea level. The regions also vary in the length of their growing season (CP 185-200 d; CU 175 d), and their average annual temperature (CP 55-57 F; CU 55 F), but not their average precipitation (1,170 mm).

Alternatively, variation in farmer's practices among the two regions could cause the apparent differentiation among the Cumberland Plateau and the Coastal Plain. From our census of farming practices, there were no indications given of major differences in glyphosate use between the regions; however, variation in crop rotations and the number of times per season glyphosate was sprayed existed among all farmers. If selection on tolerance by glyphosate produced the apparent differentiation among regions in *I. purpurea*, then the amount of adaptive genetic variation for this trait should be greater than that of neutral genetic variation. A measure of the regional quantitative trait variation in tolerance, or a 'Q_{st}', was estimated as 0.6913, following the method of Spitze (Spitze 1993). Although we do not have an estimate of F_{st}, or measure of neutral genetic variation for this particular species, the F_{st} of species with a similar mating system and seed dispersal mechanism to *I. purpurea* has been shown to be 0.248 (Hamrick and Godt 1996), which is much lower than our estimate of quantitative trait variation, and suggestive of directional selection as promoting variation among regions in *I. purpurea*. While this is circumstantial evidence for selection producing the apparent pattern of trait variation in this study, it highlights the need for further study of this system. Reciprocal transplants of the species among the Coastal Plain and Cumberland Plateau, as well as monitoring herbicide use, may help to clarify the exact nature and cause of the differences among regions.

Variation among species

Initially we found no evidence of one species being more tolerant than the other. However, when using populations of the species collected within the same 17 sites, we found that the rank order between the species was not preserved in sites within the Coastal Plain, meaning that the species exhibited different levels of tolerance within populations, and that *I. hederacea* appeared the more tolerant species. This result was not found within the Cumberland Plateau, suggesting that the two species within sites in this region of the Southeast are exhibiting similar levels of tolerance and thus could have responded in parallel to selection by glyphosate.

The geographic mosaic

We found no clear evidence of tolerance 'hotspots' or positive spatial autocorrelation of tolerance in the Southeast for either species, although we did see a trend toward a hotspot in *I. purpurea* populations from the Coastal Plain. We also found evidence of significant negative spatial autocorrelation in *I. purpurea* in both regions. In practice, a negative autocorrelation is produced by tolerance levels being significantly dissimilar among populations. This would be expected at greater population distances, and is often caused by significant positive autocorrelation on a very local scale. Based on simple migration models, genetic distance should increase with geographic distance (Nei 1972). If the trend towards positive spatial autocorrelation is a real effect, then our finding of positive autocorrelation on a local scale and negative spatial autocorrelation at greater distances seems to support the isolation by distance model. Again, an analysis of gene flow among populations of this species would further help to clarify the size of the genetic neighborhood of these populations.

There was no evidence of significant aggregation of tolerance in either region for *I. hederacea*. The lack of populations positively aggregating for their level of tolerance could be due to the variation apparent among populations. Taken at face value, it is apparent that the populations comprising these designations are independent units undergoing distinct evolutionary forces and responding on their own evolutionary trajectory. This lack of trait value 'clumping' suggests that the populations sampled closest to one another were not part of a metapopulation, or a larger population connected by gene flow.

Conclusion

The two species of morning glory investigated in this study are both partitioning their respective levels of genetic variation for tolerance to glyphosate among populations, yet *I. purpurea* shows significant variation within populations as well as differentiation among ecological regions for tolerance. In addition, the species appear to be responding in parallel in one region of the Southeast for their respective level of tolerance, in that the two species found within the Cumberland Plateau exhibit similar levels of tolerance. It is apparent in at least one morning glory species that tolerance can spatially aggregate on a local scale and thus produce a pattern of tolerance, albeit a negative pattern of association.

Spatial and geographic patterns of genetic variation have long been of interest to genetics since spatial structuring can influence mating system dynamics, gene flow and patterns of selection (Endler 1977). Although we did not find clear evidence of positive spatial autocorrelation, we did find evidence that the further the populations were from one another, the less similar their respective trait values. In sum, our data suggests that the continued evolution of tolerance to glyphosate is, in large part, dependent on the individual species, agricultural field, region in question, as well as units in the geographical hierarchy that often are ignored: the potential for population aggregation of trait values. Prior knowledge of the ecology, selective history, and geographic patterning of populations and regions of species experiencing strong, human-mediated selection need to be assessed before management decisions are made.

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FIGURE LEGENDS

Figure 5.1. Geographic patterns across the Southeast of glyphosate tolerance in *I. purpurea*. The symbols are graded in color for the average level of tolerance among populations.

Figure 5.2. Geographic patterns across the Southeast of glyphosate tolerance in *I. hederacea*. The symbols are graded in color for the average level of tolerance among populations.

Figure 5.3. Differences in the level of tolerance between regions within species. A lower bar indicates a higher level of tolerance.

Figure 5.4. Values of tolerance for each species within A) the Coastal Plain and B) the Cumberland Plateau. Lines connect the species collected within the same site.

Figure 5.5 Correlograms of Moran's *I* for A) *I. purpurea* populations within the Coastal Plain, B) *I. purpurea* populations within the Cumberland Plateau, C) *I. hederacea* populations within the Coastal Plain, and D) *I. hederacea* populations from the Cumberland Plateau. Values outside of the 99% confidence intervals indicate significant levels of either positive or negative spatial autocorrelation. 10,000 Monte Carlo permutation tests were performed to construct the confidence intervals.

Table 5.1. ANOVA for relative fitness indicating maternal line, population and regional variation in tolerance to glyphosate in *I. purpurea.*

Source of variation	df	Type III SS	Mean Square	F	p-value
Region	1	32.7500	32.7500	15.58	<0.0001
Population (Region)	27	119.5746	4.4287	2.11	0.0008
Maternal Line (Population Region)	167	369.6913	2.2137	1.05	0.3158
Treatment	3	2458.2281	819.4094	389.90	<0.0001
Maternal Line×Treatment	467	1119.2027	2.3966	1.14	0.0401
Population×Treatment	80	240.9637	3.0120	1.43	0.0087
Region×Treatment	3	32.2149	10.7383	5.11	0.0016
Error	1293	2717.3663	2.1016		

Table 5.2. ANOVA for relative fitness indicating population variation in tolerance to glyphosate in *I. hederacea.*

Source of variation	df	Type III SS	Mean Square	F	p-value
Region	1	1.5094	1.5094	0.86	0.3537
Population (Region)	15	61.0920	4.0728	2.32	0.0031
Maternal Line (Population Region)	77	184.3346	2.3940	1.37	0.0255
Treatment	3	1942.4472	647.4824	369.55	<0.0001
Maternal Line×Treatment	222	320.5140	1.4438	0.82	0.9568
Population×Treatment	45	164.4624	3.6547	2.09	<0.0001
Region×Treatment	3	4.2207	1.4069	0.80	0.4925
Error	655	1147.6214	1.7521		

Table 5.3. ANOVA with each population's tolerance value as the dependent variable.

Source of variation	df	Type III SS	Mean Square	F	p-value
Region	1	0.5522	0.5522	1.46	0.2336
Species	1	0.4818	0.4818	1.27	0.2653
Region × Species	1	1.8172	1.8172	4.81	0.0339
Error	42	15.8752	0.3780		

Table 5.4. Wilcoxon signed rank sums test for matched pairs assessing whether the species found within the same collection site were exhibiting similar levels of tolerance.

Trait	Region	N	Z	p-value
Tolerance	ALL	17	2.272	**0.0115**
	Cumberland	6	0.5246	0.3001
	Coastal Plain	11	2.578	**0.0050**

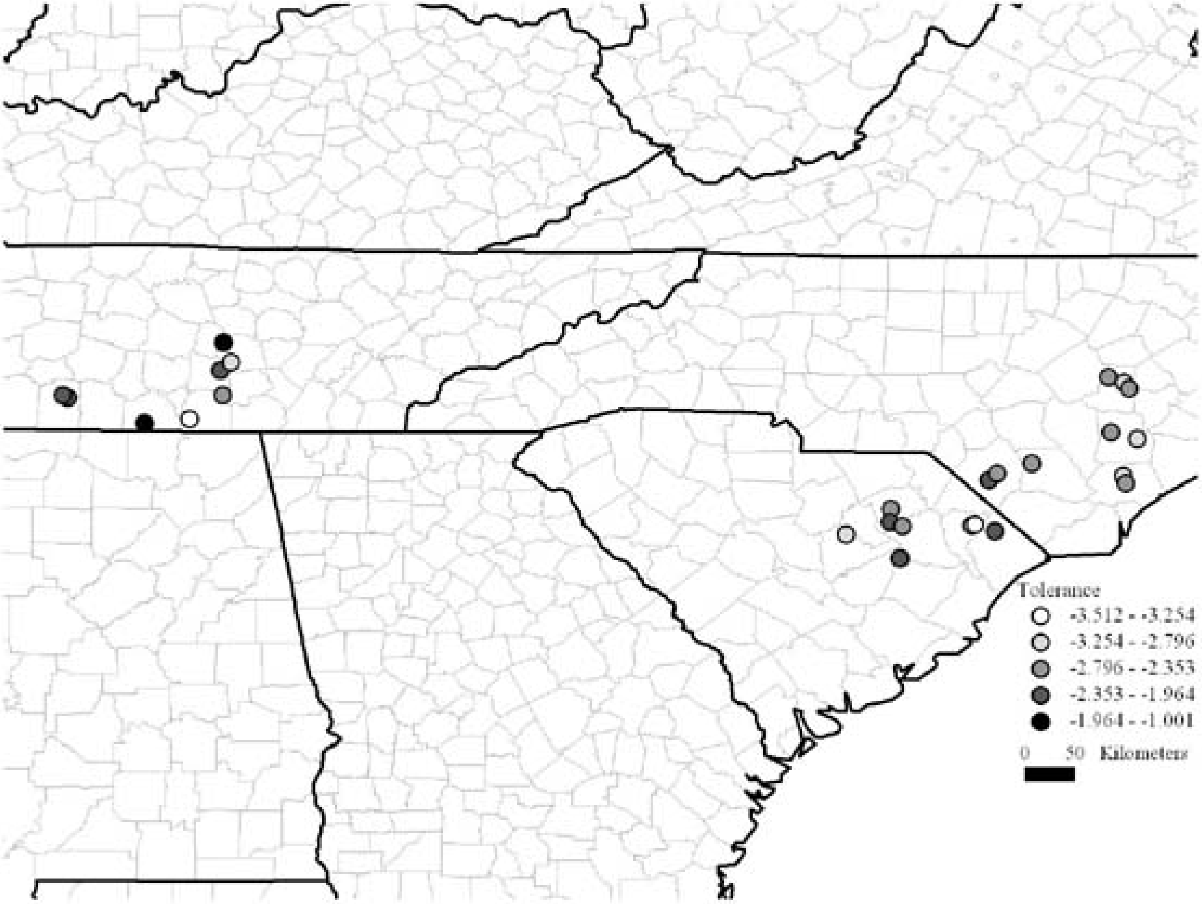

Tolerance
○ -3.512 - -3.254
○ -3.254 - -2.796
● -2.796 - -2.353
● -2.353 - -1.964
● -1.964 - -1.001
0 50 Kilometers

Figure 5.1

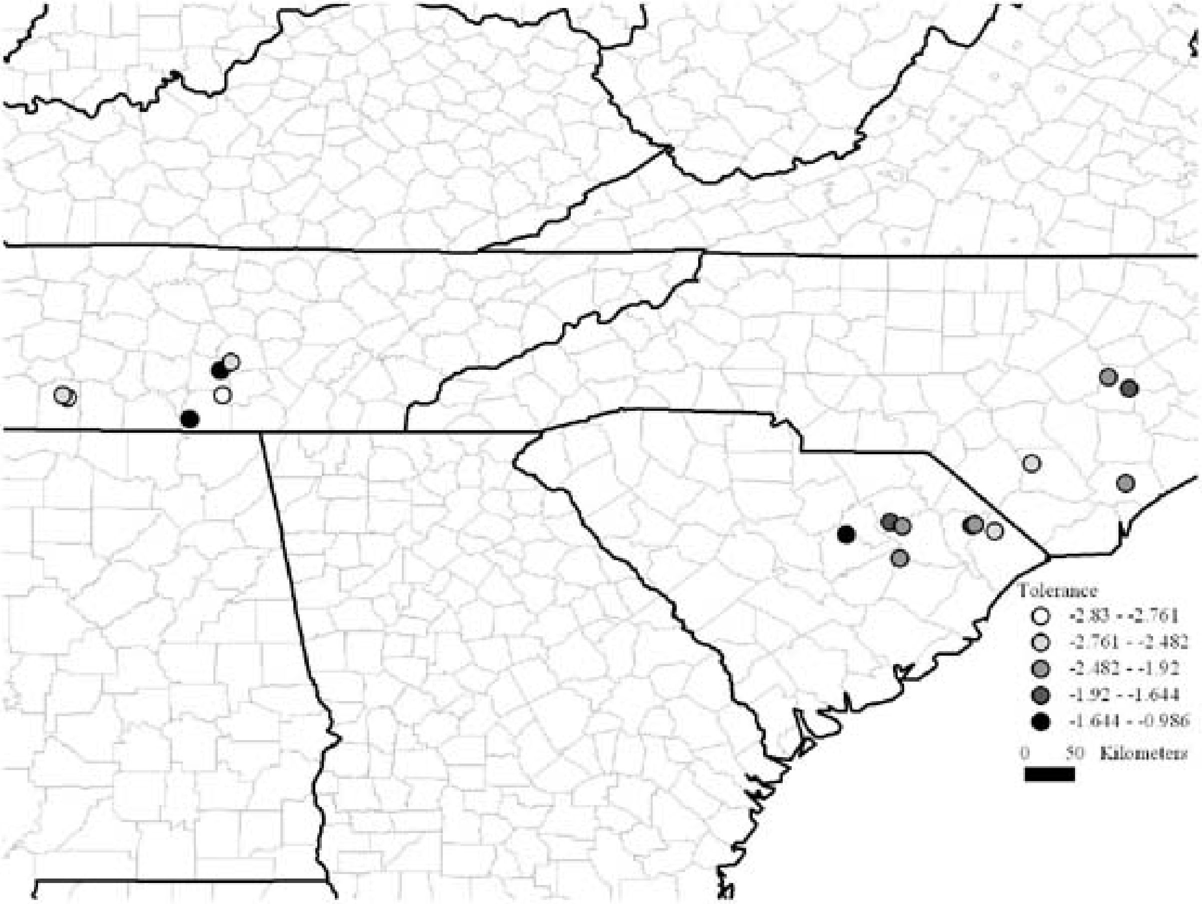

Tolerance
○ -2.83 - -2.761
○ -2.761 - -2.482
● -2.482 - -1.92
● -1.92 - -1.644
● -1.644 - -0.986
0 50 Kilometers

Figure 5.2

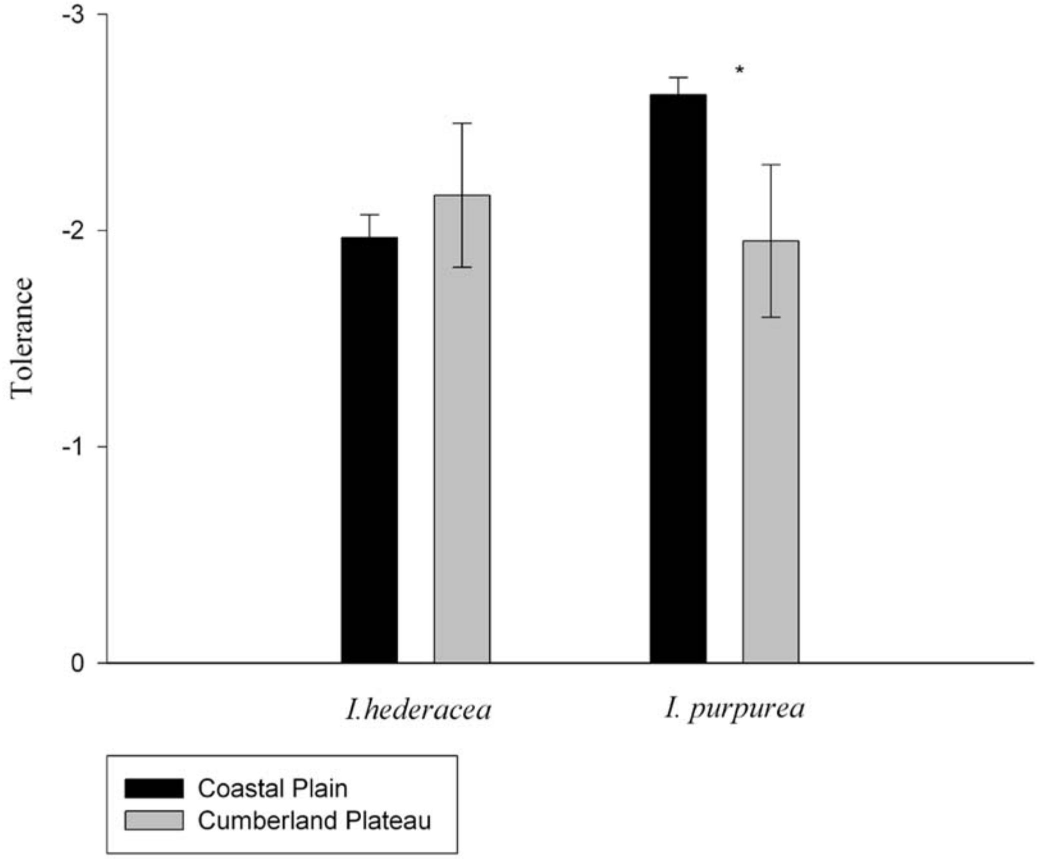
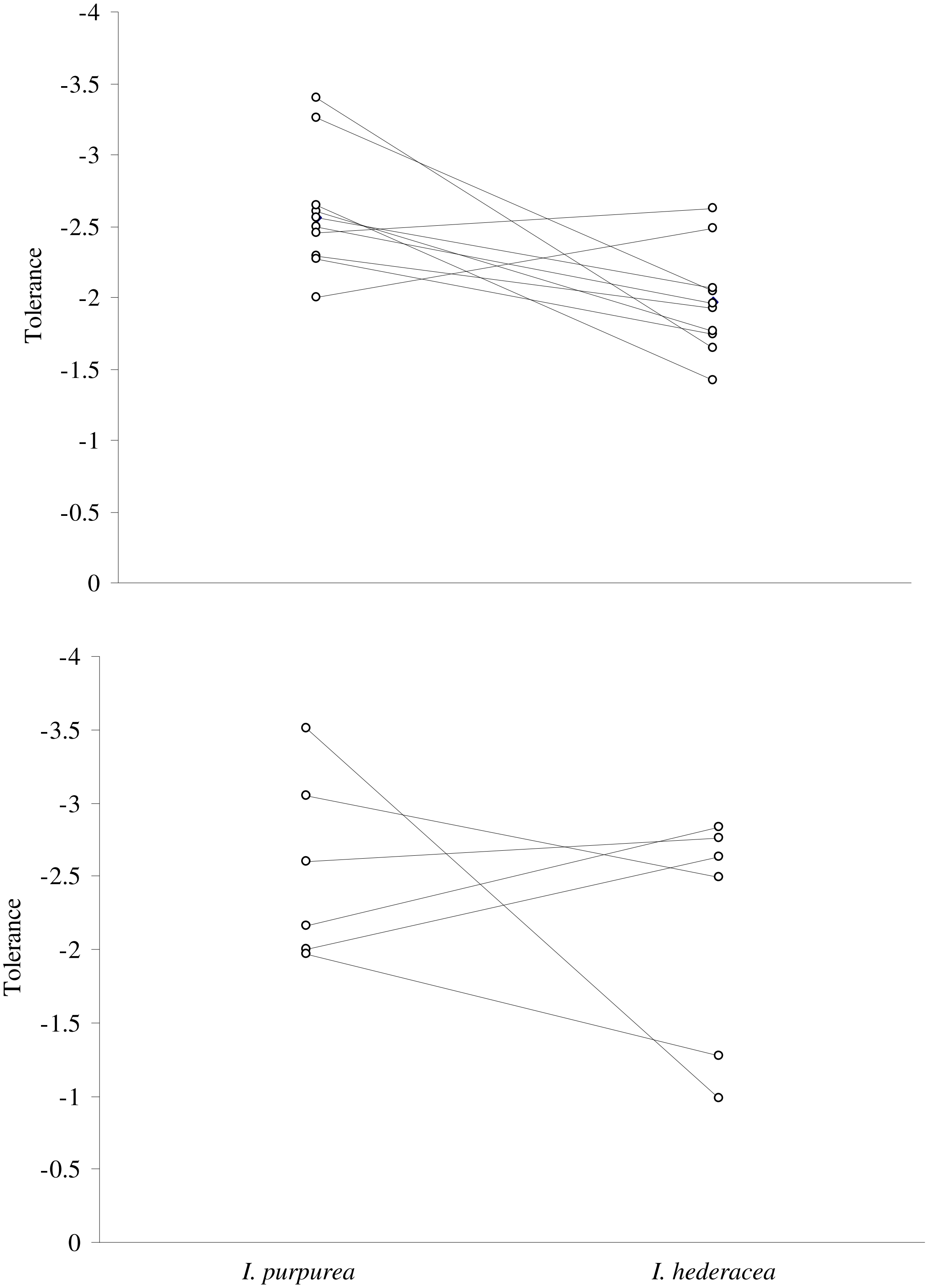

-3
-2
-1
0
Tolerance
*
I.hederacea
I. purpurea
Coastal Plain
Cumberland Plateau

Figure 5.3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-3
-2.5
-2
-1.5
-1
-0.5
0
Tolerance
-4
-3.5
-3
-2.5
-2
-1.5
-1
-0.5
0
Tolerance
I. purpurea
I. hederacea

Figure 5.4.

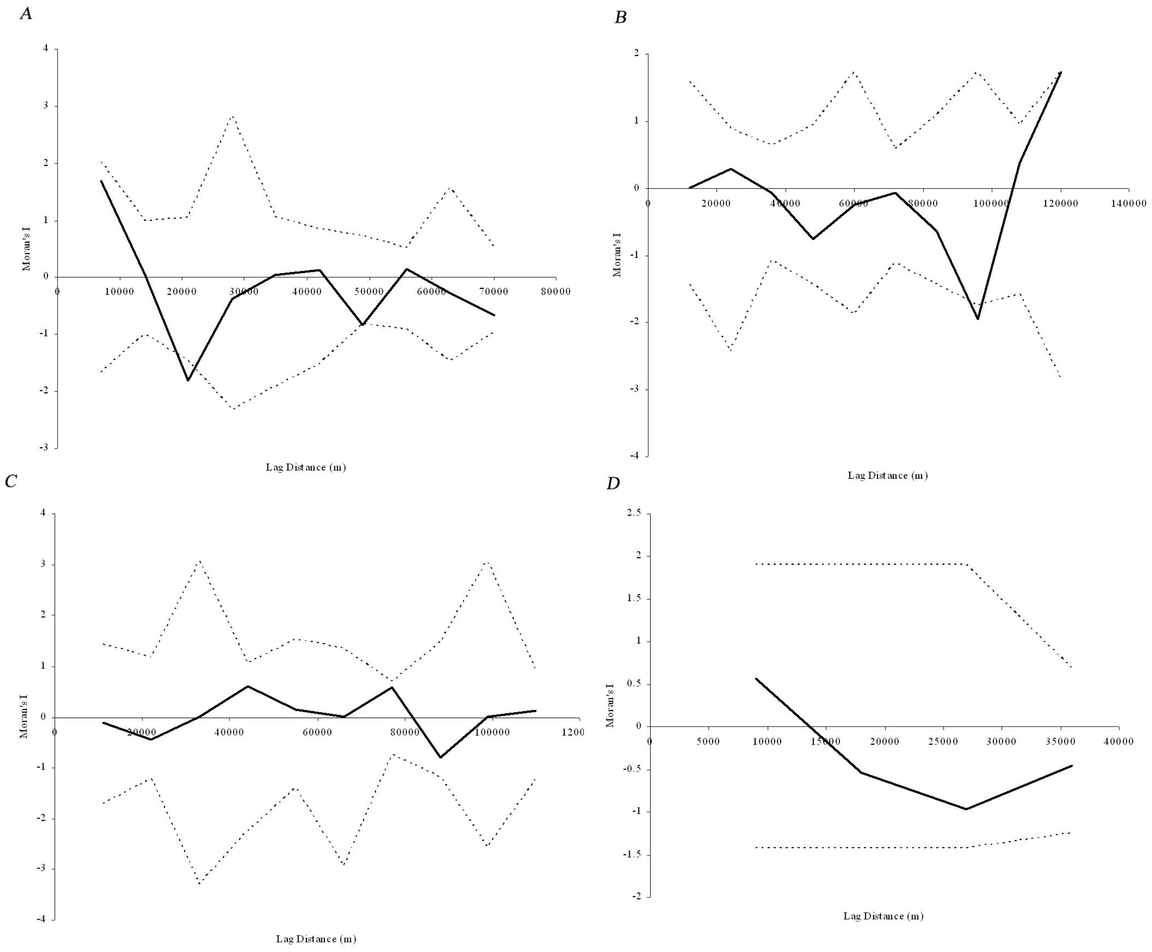
A
4
3
2
1
0
-1
-2
-3
Moran's I
0
10000
20000
30000
40000
50000
60000
70000
80000
Lag Distance (m)
B
2
1
0
-1
-2
-3
-4
Moran's I
0
20000
40000
60000
80000
100000
120000
140000
Lag Distance (m)
C
4
3
2
1
0
-1
-2
-3
-4
Moran's I
0
20000
40000
60000
80000
100000
1200
Lag Distance (m)
D
2.5
2
1.5
1
0.5
0
-0.5
-1
-1.5
-2
Moran's I
0
5000
10000
15000
20000
25000
30000
35000
40000
Lag Distance (m)

Figure 5.5.

CHAPTER 6

CONCLUDING REMARKS

The evolution of a trait in nature is dependent on the amount of genetic variation underlying the trait, the presence of selection on the trait, and the inheritance pattern of the trait. I investigated each of these components on a trait that has important implications for agriculture: the ability of *Ipomoea purpurea*, a noxious crop weed, to tolerate glyphosate, or the active ingredient in the herbicide RoundUp®. In every investigation, I found the presence of genetic variation underlying tolerance, suggesting that adequate fuel for the evolutionary process exists in nature. In addition, I found the presence of positive, directional selection on tolerance, providing evidence that glyphosate is a potent force of selection on tolerance in populations of *I. purpurea*. Finally, I uncovered an inheritance pattern for tolerance, in that it is primarily under additive nuclear genetic control, and is not under the direct influence of a maternal or paternal effect. All of these findings suggest that tolerance can and should increase in nature over time; especially given the finding that glyphosate is becoming one of the most heavily-relied upon herbicides.

As use of glyphosate increases in cropping systems, so too should selection for increased tolerance in agricultural crops, as long as the benefit of tolerance outweighs a cost of tolerance. Costs are fitness reductions that are thought to arise from the diversion of limiting resources away from present and future growth and reproduction (Simms and Triplett 1994). Fitness costs are common, but not universal for resistance and tolerance to herbivores (Mauricio et al. 1997; Simms and Rausher 1987; Simms and Triplett 1994; Tiffin and Rausher 1999). Benefits of tolerance are increases in fitness that result from the ability to reduce the detrimental effects of damage on survival and reproductive success. Tolerance can evolve only if there is a net benefit, *i.e.,* if the magnitude of fitness benefits exceeds the magnitude of the costs (Juenger and Lennartsson 2000; Lennartsson et al. 1998; Mauricio et al. 1997; Roy and Kirchner 2000; Simms and Rausher 1987; Simms and Triplett 1994; Tiffin and Rausher 1999). In our study population, I found that the benefit of tolerance in the presence of glyphosate outweighed the cost. I also found that the cost in the absence of glyphosate was significant enough to suggest that crop rotations might be a potential mechanism by which the rise of tolerance can be mitigated. However, this is strictly true only if there is no cross-tolerance to herbivores, pathogens or frost damage, such that the mechanism influencing tolerance to glyphosate is not the same mechanism controlling tolerance to these other sources of damage.

Unfortunately, this appears to be a plausible scenario. I found that tolerance to glyphosate exists in accessions of *I. purpurea* collected and stored before the wide-spread use of the herbicide. This suggests that tolerance to glyphosate is a pre-adaptation to some type of damaging agent in nature, whether it is damage inflicted by herbivory, frost, or some other unknown selective agent. These results suggest that the evolutionary dynamics underlying the increase in tolerance to glyphosate are not as simple as previously described.

Regardless of the original agent of selection on tolerance, or perhaps because of it, tolerance to glyphosate is widespread across the Southeastern US. There is growing data to suggest that populations vary according to defensive trait such as resistance to predators and pathogens (Brodie and Ridenhour 2002; Burdon and Thrall 1999), and level of resistance to herbicides (Warwick 1991). Our results support this conclusion on multiple levels of geography in that we find evidence for within- and among-population variation, as well as among-region variation in the level of tolerance. Our data suggests that the continued evolution of tolerance to glyphosate is, in large part, dependent on the individual species, agricultural field, and region in question. Prior knowledge of the ecology, selective history, and geographic patterning of populations and regions of species experiencing strong, human-mediated selection need to be assessed before management decisions are made.

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Warwick, S. I. 1991. Herbicide resistance in weedy plants: Physiology and population biology. Ann. Rev. Ecol. Syst. 22:95-114.