ABSTRACT

BROOKS, ASHLEY MEREDITH. Allelopathy in Rye (*Secale cereale*). (Under the direction of David A. Danehower.)

Allelopathy is an ecological phenomenon in which chemicals produced by and released from a plant affect the germination or growth of another plant. A possible exploitation of allelopathy is the use of allelopathic cover crops for weed management. Organic farming systems can utilize allelopathy as an alternative to synthetic herbicides and conventional farming can reduce reliance upon pre-emergence herbicides. Rye (*Secale cereale*) is a cover crop species known to be allelopathic to many weeds. In addition to allelopathic activity, rye is a successful cover crop because of prolific biomass, high germinability and winter hardiness. The objective of this research was to investigate the potential to develop a rye cultivar with increased allelopathy through a conventional breeding approach. A population of 150 half-sib families of rye was grown at two North Carolina locations. Above ground tissue was utilized to assess rye allelopathic activity. To assess allelopathy in the population, we aimed to develop a greenhouse bioassay which utilized a rye incorporated soil media and redroot pigweed (*Amaranthus retroflexus*) as the indicator species. It is necessary to identify a screening protocol to quantify variation in allelopathic activity and to identify high performing lines. The greenhouse bioassay was fast, inexpensive and able to screen the large number of genotypes in the rye population. Results of redroot pigweed fresh weight biomass were reproducible and were utilized to estimate genetic parameters for allelopathy in the rye population. Estimates of genetic variation, genotype x environment interaction and narrow sense heritability help plant breeders develop an appropriate breeding program for the trait of interest. The estimates also give an idea of the rapidity at which progress can be made through selection. Genetic variation for rye allelopathy was not significant across locations but was significant within each location. Redroot pigweed fresh weight biomass was normally distributed indicating that allelopathy in rye is a quantitative trait. Heritability estimates were low on a per-plot basis and moderately low on an entry mean basis. A petri dish bioassay was also utilized to estimate genetic parameters for allelopathy in rye. Redroot pigweed germination and root length measures were utilized to quantify allelopathic activity. Genetic variation was not significant across locations for germination or root length.

Analysis of variance within each location detected variation among the genotypes grown at the Kinston location but not at the Clayton location. Measures of redroot pigweed germination and root length were normally distributed. Heritability estimates were low on a per-plot basis and on an entry mean basis. This study demonstrates that allelopathy in rye is under genetic control and that it is a quantitative trait. Results suggest that a conventional breeding approach may be used for the development of a highly allelopathic rye cultivar.

Allelopathy in Rye (*Secale cereale)*

by
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BIOGRAPHY

Ashley Brooks was born in Pensacola Beach, FL and has lived within the southern United States her entire life. Whether imagining ways to fix the hole in the ozone layer or trying to influence other neighborhood kids to meet under the park picnic table every Saturday to study leaves, her focus has always leaned towards interest and concern for the environment. Towards the end of her undergraduate study at the University of North Carolina at Greensboro, an anthropology course entitled 'The Globalization of the Agro-Food Sector' exposed her to new ways of thinking about agriculture and opened a world of interests. A few years after graduating, a rye allelopathy project was designed in the Crop Science Department at North Carolina State University which managed to encompass both of her passions: the environment and agriculture.

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Chapter I

Allelopathy in Rye (*Secale cereale*) Cover Crops

Introduction

Weed Control with Cover Crops

Weeds are an important threat to agriculture because they compete with crops thereby reducing yield and causing significant economic losses. The most dependable source of weed control for over 50 years has been synthetic herbicides. Some of the reasons for this are that herbicides are effective, easy to apply, relatively cheap and they reduce the need for mechanical means of weed control which enhance soil erosion (Gianessi and Reigner 2007).

Despite growth in the use of herbicides over the past 50 years, there are nevertheless, drawbacks to heavy dependence upon them. A key problem is the shift in weed population structure resulting in the increased presence of herbicide-resistant weed biotypes (LeMerle 2001; Owen and Zelaya 2005). The International Survey of Herbicide Resistant Weeds (2008) reports 318 resistant species worldwide. Proliferation of these biotypes has been amplified by the development of herbicide resistant crops such as glyphosate-resistant cotton, maize, soybean and canola (Owen and Zelaya 2005). Currently, there are nine resistant weed biotypes in North Carolina, six in South Carolina, 11 in Tennessee and nine in Virginia (International Survey of Herbicide Resistant Weeds 2008). Heap (1997) pointed out that herbicide-resistant weeds pose the greatest threat to farmers when there are few or no other alternatives to control them.

Negative impacts on human, wildlife and environmental health are additional concerns related to the heavy use of synthetic herbicides (Bertholdsson 2005). The release of Rachel Carson's book, "Silent Spring" in the 1960s alerted the public to the potential risks associated with herbicide use. By the 1980s public concern over their use pressured regulatory agencies to develop more strict protocols (Carson 1962; Foley 1999; Dayan et al. 1999). The FDA reported in 1989 that 97% of the public was concerned about the use of pesticides. The Food Quality Act of 1996 requires that alternatives to synthetic herbicides be

developed and that agriculture adopt integrated management strategies for weed control (USEPA 1996).

One such alternative is the use of cover crops. Traditionally, cover crops have been used in rotation with food crops or in polycultural systems as an integrated component of small scale agriculture (Singh et al. 2003). Many plant species are used as cover crops around the world, depending upon geographical location, choice of management strategies and the needs of the cropping system. Historically, as farming shifted towards reliance on external inputs and monoculture, cover crop use declined. Agricultural scientists are beginning to re-evaluate the importance of diversity in agriculture and the potential for use of cover crops. Cover crops offer many advantages besides the ability to suppress weeds. They are known to increase the overall environmental health of agricultural systems (Lal et al. 1991). Soil moisture levels are improved due to reduced evaporation and improved soil structure, which allows more infiltration of water. Nutrient recycling is improved and, in the case of legume crops, cover crop can contribute nitrogen to the soil (Worsham 1991). Cover crop residues can reduce leaching of chemicals through soil and water (Barnes and Putnam 1983). They contribute organic matter, thereby improving soil fertility. They can reduce chemical inputs into surface and groundwater by reducing or eliminating the application of herbicides and fertilizers. This is important in areas of intensive farming where soil nutrients become depleted and in areas of the world where organic matter decomposes quickly, such as in the tropical lowlands. In addition, cover crops protect soil from wind and water erosion, important factors in soil loss (Anderson et al. 2001).

The use of cover crops for weed control is a weed management strategy that is particularly useful in organic farming systems where synthetic herbicides cannot be used (Olofsdotter 1998; Gawronska et al. 2006). Based on USDA estimates, organic cropland increased from 161,600 ha to 565,600 ha from 1992 to 2003. Walz (1999) stated that organic farmers rely most heavily on mechanical and hand-weeding methods for weed management. She reported that of 30 research areas, weed control is of the highest concern among organic farmers. The

demand for alternatives to herbicides, such as the use and improvement of cover crops, will continue to increase as organic farming increases (Olofsdotter 1998).

Whether the focus is on organic or conventional agricultural production systems, cover crops can be used as a part of an integrated weed management system. Integrated systems of weed control do not attempt to eradicate weeds but instead employ one or more biological, chemical, preventative or physical methods for long term weed management (Grundy and Froud-Williams 1997; Olofsdotter 1998; Mortensen et al. 2000). While cover crops cannot eliminate all competition from weeds, they might be useful in a reduced herbicide management system (Ateh and Doll 1996).

One mechanism of weed suppression by cover crops is through competitive effects imparted by the physical presence of the cover crop. Live cover crops compete with weeds for space, sunlight, moisture, nutrients and water (Shilling et al. 1985; Lehman and Blum 1997). Cover crop residues can control weeds through production of mulch (Bhowmik and Inderjit 2003). Once the crop is mowed, ploughed or dessicated, the residues provide mulch which can physically impede weed seedling emergence and can interfere with light interception (Teasdale and Mohler 2000).

In addition to physical competition with weeds, some cover crops are allelopathic. The term allelopathy was first defined in 1937 by Dr. Hans Molisch as "the biochemical interactions between all types of plants including microorganisms". This phenomenon includes the stimulation or suppression of germination and/or growth of a plant species, the receiver, by chemicals produced in and released from another plant, the donor. It is possible that allelopathic cover crops can also be used as an alternative to herbicides or in conjunction with reduced inputs of herbicides (Putnam and Duke 1974; Singh et al. 2003).

What is Allelopathy?

As previously stated, allelopathy is an ecological process in which a chemical released by a "donor" plant has some effect upon the germination or growth of a "receiver" plant. For experimental purposes, a "receiver" species is often referred to as an "indicator" because there is some measureable effect upon the species that is indicative of an allelopathic interaction. To begin a discussion of allelopathy, is helpful to explain some of the terms used describing plant-plant interactions. Competitive, allelopathic and indirect effects can be categorized as "interference" (Muller 1969). A competitive effect (i.e. "competition") is physical interference in which one plant has reduced or removed factors such as nutrients, light, water and space, rendering them less available to a neighboring plant (Fuerst and Putnam 1983). An allelopathic effect (i.e. "allelopathy") is chemical interference of one plant by another plant (Muller 1969; Fuerst and Putnam 1983). The effect can be positive or negative. An indirect effect is the result of interference with the surrounding environment of a plant. (Muller 1969; Fuerst and Putnam 1983). An indirect effect is interference which cannot be attributed to physical or chemical interactions. They are usually related to soil biotic factors such as mycorrhizal associations and nematode activity or abiotic factors such as soil organic matter or hydrological conversions (Kobayashi 2004; Anaya 1999). For example, a fungus may have a symbiotic relationship with a host plant but may be parasitic to another plant. If the host and the susceptible plant grow near each other the fungus can attack the susceptible plant. In this case, the host plant has interfered, albeit passively or indirectly, with the health of the susceptible plant.

Though there is extensive literature on allelopathy, the phenomenon is still somewhat controversial among some scientists. Scientists have struggled to prove its existence and have often incorrectly attributed plant-plant interference to allelopathy when interference could actually be explained by competition or indirect effects (Willis 2007; Kobayashi 2004). Much of the controversy surrounding allelopathy is based upon lack of well thought out methodologies for detecting and measuring allelopathic effects and the fact that most

allelopathic bioassays are in vitro systems while relatively little work has been done using in vivo bioassay systems and even less than that in field studies. Some scientists argue that allelopathy cannot be proven but as Willis (2007) states "the same can be said of most ecological phenomena".

Although the concept of allelopathy is not new, it has yet to be exploited to any extent. The potential to utilize allelopathy rests upon our ability to understand the complex relationships that exist between plants and to then manipulate that relationship through genetics and agronomic practices. In developing this understanding, it is important to bear in mind that allelopathic relationships are often species specific, concentration dependent and environmentally influenced. Ignoring any of these considerations can hinder research and lead to faulty interpretation of results.

Exploitation of Allelopathy

Scientist discussed several possibilities for the exploitation of allelopathy for weed control. A possible exploitation is the use of allelochemicals as herbicides (Duke et al. 2000). This approach is, however, somewhat limited for reasons including toxicity to non-target species (plant and animals), high costs associated with synthesis and manufacturing and short half-lives in the environment.

Allelochemicals have served as template molecules in herbicide development. For example, the herbicide Cinmethylin was developed based upon structure of the allelochemical, 1,4-cineole, a compound often found in aromatic species (Bhowmik 1988). Mesotrione, a hydroxyphenylpyruvate (HPPD) inhibiting herbicide, is an analog of leptospermone, an allelochemical produced by lemon bottlebrush (*Callistemon citrinus*) (Bhowmik and Inderjit 2003).

Another area of interest is the genetic manipulation of allelochemical biosynthetic pathways via biotechnology. The use of biotechnology to improve allelopathic potential is limited

because gene sequencing is often incomplete for the enzymes involved in the allelochemical biosynthetic pathways (Duke et al. 2002). An exception to this is sorgoleone, the primary allelochemical produced in sorghum which is known to inhibit photosystem II and HPPD. Yang et al. (2004) identified and associated the *SOR1* gene with sorgoleone production in sorghum root hairs. Understanding gene regulation of the biosynthetic pathway may enable scientists to manipulate gene expression and allelochemical production.

Allelopathic cover crops are another way that allelopathy can potentially be exploited to suppress weeds. Rye (*Secale cereale*), sorghum (*Sorghum* spp.), buckwheat (*Fagopyrum esculentum*), clovers (*Trifolium* spp.), alfalfa (*Medicago sativa*), hairy vetch (*Vicia villosa*) and velvet bean (*Mucuna pruriens*) are all cover crops which are known to be allelopathic (Singh et al. 2003).

The use of conventional breeding to improve crop allelopathy was suggested by Putnam and Duke (1974) after they discovered significant variation among 538 accessions of the *Cucumis* genus for allelopathic activity against white mustard (*Brassica hirta* Moench.) and wild proso millet (*Panicum miliaceum* L.). The presence of variation led to the conclusion that a gene pool exists for allelopathy and therefore, development of allelopathic cultivars should be possible. Selection for an allelopathy trait could give crops a competitive advantage over weed interference (Putnam and Duke 1974). Since that time, the germplasm of some crop species has been assessed for variation in allelopathic activity (Wu et al. 1999). For example, Macias (1999) reported that significant variation in allelopathy existed among 26 cultivars of sunflower (*Helianthus annuus*). Xuan and Tsuzuki (2002) showed that significant variation for allelopathic activity existed in eight varieties of Japanese alfalfa (*Medicago sativa* L.).

Genetic Improvement in Cereal Crops

It has been suggested that cereal crops are more allelopathic and have better application potential in agriculture (Sánchez-Moreiras et al. 2004). Studies within the last few decades have found that cereal species including rice, wheat, barley, oats and sorghum contain

significant genetic variation for allelopathy (Olofsdotter et al. 2002; Wu et al. 2000a; Bertholdsson 2004; Nimbal et al. 1996).

Rice (*Oryza sativa*) allelopathy is the most extensively studied of all crop plants. Research into rice allelopathy began over two decades ago and significant progress has been made toward the development of a cultivar with high weed suppressive ability. Dilday et al. (1994) evaluated nearly 10000 USDA-ARS rice accessions for allelopathic activity against duck salad (*Heteranther limosa*), a problematic aquatic weed in U.S. rice fields. Through a series of experiments they identified 12 accessions of rice that were capable of 80-90% weed control within a 0.18-0.20m radius of the rice plants and another 12 accessions with 50-85% weed control within a 0.13-0.18m radius (Dilday et al. 1994). In developing molecular markers for allelopathy in rice, Jensen et al. (2001) mapped four main-effect and epistatic quantitative trait loci (QTL) on three chromosomes that are correlated with a reduction in barnyardgrass (*Echinochloa crus-galli*) root growth. The QTL accounted for 35% of the phenotypic variation in the population. Epistatic effects explained much less of the phenotypic variation which supports quantitative genetic control of the trait. The identification of the QTL indicates that marker assisted selection could be utilized for genetic enhancement. The broad sense heritability for barnyardgrass root inhibition was estimated to be 0.85, which is moderately high (Olofsdotter 2001). This estimate indicated that 85% of the variation in root length could be explained by genetic effects and supports that the allelopathy trait is under genetic control (Bernardo 2002). Subsequently, Ma et al. (2006) developed a rice line, K21, which was highly allelopathic against barnyardgrass and displayed desirable agronomic traits. K21 was derived from a cross between Kouketsumochi, an allelopathic cultivar with poor agronomic traits, and Dongjinbyeo, a non-allelopathic cultivar with desirable agronomic traits. This was the first reported attempt at developing an allelopathic rice cultivar (Ma et al. 2006).

Wheat has also been evaluated for variation in allelopathic activity. Spruell (1984) found differential activity among 286 wheat accessions against (*Bromus japonicas*) and common

lambsquarters (*Chenopodium album*). Wu et al. (2000a) screened 453 wheat (*Triticum aestivum*) genotypes and reported variation in control of ryegrass shoot length varied up to 11 fold. Analysis of 15 wheat accessions commonly grown in the Loess Plateau in China resulted in a mean heritability estimate of 0.83 and significant genetic variation (Zuo et al. 2007). Wu et al. (2003) examined a wheat population derived from a cross between a cultivar with low allelopathic activity, Suncov, and the highly allelopathic cultivar, Tasman. Using restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSRs) markers, they uncovered two QTL associated with the allelopathic effects of wheat on ryegrass (*Lolium rigidum*). Allelopathic activity, as measured by root length of ryegrass relative to a control, was normally distributed, indicating that allelopathy is likely a quantitative trait (Wu et al. 2003).

Progress has been made in estimating genetic parameters associated with barley allelopathy. Based on a study with 127 barley cultivars, Bertholdsson (2004) reported that estimates of heritability and genetic variation for allelopathic potential in barley are sufficient to permit a breeding program. Lin et al. (2005) screened 65 barley accessions for allelopathic activity against lettuce. Results indicated significant variation among the accessions for lettuce root growth inhibition. Inter-simple sequence repeat (ISSR) analysis detected genetic polymorphisms in the barley accessions that indicated that accessions of the same geographical origin could be grouped together based on allelopathic activity (Lin et al. 2005).

Fay and Duke (1977) studied the allelopathic activity of scopoletin, an allelochemical produced by oats (*Avena sativa*), against the annual weed species Italian ryegrass (*lolium multiflorum* Lam.), barnyardgrass, redroot pigweed, and wild mustard. They found that at concentrations of 1.0mM and 0.5mM, scopoletin resulted in significant reduction in radicle growth of all species. The grass species tended to be more suspectible to the toxic effects than the broadleaf species. They also screened 3000 USDA accessions and found that 25 accessions exuded higher levels of scopoletin from the roots and four accessions exuded as

much as three times the rate exuded by the common variety, 'Garry Oats' (Fay and Duke 1977).

Rye Allelopathy

Rye (*Secale cereale*) is a cereal cover crop known to be allelopathic to many weed species. There is evidence that the weed suppressive ability of rye is comparable to standard herbicide treatments (Shilling et al. 1986; Fujii 2001). Both the living cover crop and mulch of rye have been shown to suppress many broadleaf and grass weeds in crops such as maize, tobacco, snap beans cabbage and other vegetable species (Shilling et al.. 1986; Dhima et al. 2006; Masiunas et al. 1997; Creamer et al. 1997).

This species is a particularly good candidate for genetic improvement of allelopathy for several reasons which will be discussed in more detail throughout this paper. First, rye is already a successful cover crop utilized in the southern United States. Prolific biomass, which impart a competitive advantage over weeds, and allelochemical activity are two reasons for rye success. Additionally, rye is easy to grow, has high germinability and is winter hardy. The second reason that rye should be considered for allelopathy improvement is that rye allelochemicals have been isolated, identified and their phytotoxicity proven in bioassays. The genetics underlying control of the allelochemical biochemical have been elucidated in other species which may improve selection ability. The final reason for the interest in rye is the finding that rye was allelopathic to three triazine resistant biotypes of barnyaradgrass, willowherb (*Epilobium ciliatum*) and horseweed (*Conyza canadensis*) (Przepiorkowski and Gorski 1994). This supports the idea that rye allelopathy could be applicable as an integrated approach to weed control where herbicides have failed.

Competitive Ability of a Living Cover of Rye

Barnes and Putnam (1983) found 98%, 42% and 90% reductions, respectively, in early season biomass production of common lambsquarter (*Chenopodium album*), large crabgrass (*Digitaria sanguinalis* (L.) Scop.) and common ragweed (*Ambrosia artemisifolia*) when

grown with a living rye cover. A study by Perez and Ormeno-Nunez (1993) found that dry-weight of several broad-leaved weed species were 94% less in fields planted with rye than with wheat and 77% less than in fields planted with forage oats. The dry weight of wild oats (*Avena fatua* L.), a grass weed in cereal crops, was 81% less in fields planted with rye than with wheat and 86% less than in field planted with forage oats. Weed biomass represented 19.4% of the total biomass in a field of wheat, 14.3% in oats and only 3.7% in rye. Ateh and Doll (1996) investigated the control of weeds by a living cover crop of rye in soybean (*Glycine max*). In 1992, 1993 and 1994, total weed shoot biomass was reduced by 90, 82 and 60%, relative to a no-rye weed control, respectively (Ateh and Doll 1996).

Competitive Ability of Rye Debris

Shilling et al. (1986) evaluated rye, wheat, barley and oats debris for control of broadleaf and grass species. They found that rye was the most suppressive of the cover crops, controlling broadleaf weeds by 85% and grass species by 70%. Masiunas et al. (1997) compared a conventional system of fall-tillage using the herbicide trifularin to hairy vetch mulch, a rye mulch and a living perennial ryegrass cover, in cabbage and snap beans. The fall- planted rye residues were found to be the most promising for weed suppression. Results were similar to those obtained with the conventional system which utilizes herbicides (Masiunas et al. 1997). They also reported that the control of late season weeds is less effective with cover crop residues and may require the use of conventional herbicides. Nagabhushana et al. (2001) found that rye mulch with no tillage resulted in 76% control of several early season annual broadleaf weeds compared to only 43% weed control in no tillage with rye mulch removed. They also report 80-95% control of broadleaf weeds including sicklepod (*Cassia obtusifolia* L.), cocklebur (*Xanthium strumarium*), prickly sida (*Sida spinosa*), common purslane (*Portulaca oleracea* L.) and pigweed (*Amaranthus* spp.). A study was performed to compare growth of several weed species in nine vegetable crops between traditional cultivation (rotary tillage) and no-tillage cultivation using plant residues from a rye cover crop (Jelonkiewics and Borowy 2005). Averaged over a three year period, a total of 41 weeds were present in the no-tillage rye plots compared to 664 weeds in the traditional tillage

plots, three weeks after vegetable planting. Ninety seven weeds were present in rye covered plot compared to 235 weeds in conventional plots seven weeks after planting (Jelonkiewics and Borowy 2005).

Allelopathic Activity of Rye

In field, greenhouse and laboratory studies, the use of proper controls can allow weed suppression to be attributed to allelopathic activity as opposed to physical interference caused by rye cover crop residues. One way to account for the physical effects is through the use of chemically inert mulches. Using an inert mulch of poplar excelsior as a control, Barnes and Putnam (1983) found up to a 74% reduction in barnyardgrass and a 55% reduction in pigweed (*Amaranthus retroflexus*) relative to the control. Lehman and Blum (1997) studied the effects of rye residues at various levels on the emergence of ivy-leaf morning glory (*Ipomoea hederacea* L.) and redroot pigweed seedlings. Weed suppression of both species was significant and suppression of weed seedling emergence followed a typical dose-response curve (Lehman and Blum 1997). The use of chemically inert mulches to support allelopathic activity also applies to greenhouse and laboratory studies.

Aqueous extractions can be used in bioassays to separate the physical effects of plant debris from allelopathic chemical effects on weed growth. Aqueous extracts of rye inhibited goosegrass (*Eleusine indica*) and pigweed root length and have inhibited root length, seedling fresh weight and germination of barnyardgrass and bristly foxtail (*Setaria verticillata*) in petri dish bioassays (Reberg-Horton et al. 2005; Dhima et al. 2006). Bioassays using rye aqueous extracts, in conjunction with chemical analyses, also support the phytoxicity of the aqueous extracts (Burgos and Talbert 2000; Reberg-Horton et al. 2003).

Rye Allelochemicals

Several compounds implicated in rye allelopathy have been identified. The benzoxazinoids are the primary group of compounds believed to impart allelopathic activity to rye and other members of the Poaceae including wheat and maize. The derivatives of the benzoxazinoids

can be subdivided into the hydroxamic acids and their methyl derivatives, lactams and benzoxazolinones (Figure 1) (Villagrasa 2006). The specific benzoxazinoids in rye are: benzoxazilin-2-one, 'BOA' (hydroxamic acid); 2-hydroxy-1,4-benzoxazin-3-oneHBOA (lactam); 2,4-Dihydroxy-1,4-benzoxazin-3(4H)-one, 'DIBOA (benzoxazilinone); and 2-(2,4-dihydroxy-1,4(2H)-benzoxazin-3(4H)-one)-β-D-glucopyranoside, 'DIBOA-glycoside' (benzoxazilinone). The specific benzoxazinoids in wheat and maize are: 6-methoxy-benzoxazolin-2(3H)-one 'MBOA' (hydroxamic acid); 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one 'HMBOA', (lactam); 2,4-Dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4)-one, 'DIMBOA (benzoxazilinone); and 2-(2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one)-β-D-glucopyranoside, 'DIMBOA-glycoside' (benzoxazilinone) (Villagrasa 2006; Macías et al. 2006; Morant et al. 2008).

Hydroxamic acids accumulate in the vacuole of cereal plants as glycosides: DIBOA-glycoside in rye and DIMBOA-glycoside in maize and wheat. Upon injury, the vacuole and other compartments of the cell are disrupted and a β-glucosidase is released, cleaving the glucoside and resulting in the production of the more toxic aglycones, DIBOA (rye) and DIMBOA (wheat and maize) (Fomsgaard 2004; Hofman and Hofmanová 1971). Cuevas et al. (1992) reported that the β-glucosidase enzyme present in maize has a high affinity for hydroxamic acid-glycosides. The same group later found that in wheat, rye and maize seedlings, this enzyme is present in epidermal cells of the roots. Activity of the root enzyme was significantly higher in maize than in wheat or rye. In wheat and rye, the enzyme was found in the epidermal cells of the shoots. The glucosidase was observed in vascular bundles of all three species. The coleoptiles of all species exhibited the highest β-glucosidase activity although it was not detected in the vascular bundles of the rye coleoptile (Nikus and Jonsson 1999). Once DIBOA or DIMBOA are liberated by the glucosidase they immediately degrade to their respective benzoxazilinones, BOA and MBOA (Nikus and Jonsson 1999). The transformation of the aglycone to the benzoxazilinone is the result of non-enzymatic hydrolysis (Virtanen and Hietala 1960). In aqueous solutions, DIBOA and DIMBOA have a half-life of less than 24 hours (Bredenberg et al. 1962).

Experiments utilizing High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) have shown that hydroxamic acids are exuded from rye roots and above ground tissue (Pérez and Ormeño-nuñez 1991). Nikus and Jonsson (1999) used an immunohistological experiment to investigate the location of β-glucosidase in seedling tissues of rye. Results of their study indicated that the enzyme was located in the epidermal cells of the roots (Nikus and Jonsson 1999). Further research indicated that the enzyme was located within the cortical cells of rye roots (Nikus et al. 2001).

In the shoots, the enzyme was localized in the vascular bundles and in the epidermal cells of rye (Nikus and Jonsson 1999). Investigation at the sub-cellular level revealed that the enzyme was primarily localized in cells walls and the cytoplasm but it was also present in the plastids and proplastids (Nikus et al. 2001). Wheat displayed the same localization trends as rye but the enzyme was more likely to be found in the plastids and protoplastids in maize (Nikus et al. 2001).

Once released from the plant, BOA, MBOA and HBOA are subjected to microbial conversions. There are three major transformation groups: aminophenoxazinones, malonamic acids and acetamides (Understrup et al. 2005). Gagliardo and Chilton (1992) showed that BOA is transformed into 2-amino-3H-phenoxazin-3-one (APO) which contradicted earlier reports that 2,2'-oxo-1,1'-azobenzene (AZOB) was a transformation product of BOA (Chase et al 1991). They also reported that APO was more toxic to barnyardgrass than BOA. The most likely route for the conversion is that BOA is hydrolyzed to o-aminophenol and then oxidation of o-aminophenol leads to APO (Gagliargo and Chilton 1992). This conversion took place in the presence of the fungal pathogen *G. graminis* var. tritici but BOA was not transformed in sterile soil (Fomsgaard et al 2004). Further research indicated that APO was further degraded to 2-acetylamino-(3H)-phenoxazin-3-one (AAPO) (Understrup et al. 2005). BOA transformation to HPMA, a malonamic acid, took place in the presence of *G. graminis* var. tritici and *G. graminis* var.

graminis (Understrup et al. 2005). Acetamides were formed in the presence of fungal pathogens *P. tabacinum* and *G. cibotti* when incubated with the lactam HBOA but not with BOA. However, in the pathway leading to the acetamides from HBOA, 2-aminophenol was an intermediate which suggests that BOA could be transformed to an acetamide (Fomsgaard et al. 2004).

Evidence for Allelochemical Activity

Isolation of benzoxazinoid allelochemicals and subsequent test in bioassays has provided direct evidence for their toxicity. Bioassays using rye aqueous extracts, in conjunction with chemical analysis, support the phytoxicity of BOA and DIBOA (Burgos and Talbert 1999; Reberg-Horton et al. 2005). After isolating BOA and DIBOA from rye shoot tissue, Barnes et al. (1987) provided evidence for the phytotoxicity of these compounds against cress (*Lepidium sativum* L. 'Curly') root growth. Singh et al. (2005) used multiple bioassays to investigate the activity of BOA on mung bean (*Phaseolus aureus*). BOA interfered with primary metabolism resulting in reduced germination and inhibition of early root growth and development (Singh et al. 2005). Pérez and Ormeño-Núñez (1993) used a petri dish bioassay to test toxicity of BOA, DIBOA and DIBOA-glycoside to wild oats (*Avena fatua* L). All compounds resulted in a reduction of root length at 0.25mM concentrations. Suppression increased with increasing concentrations of the compounds. Reberg-Horton et al. (2005) reported that levels of DIBOA were correlated with phytoxicity levels of aqueous extracts of 10 rye cultivars. Chase et al. (1991) investigated the activity of DIBOA and BOA against two weed species, garden cress (*Lepidum sativum* L.) and barnyard grass and two vegetable crop species, cucumber (*Cucumis sativas* L.) and snap beans (*Phaseolus vulgaris* L.). They found that DIBOA and BOA reduced barnyardgrass, garden cress and cucumber root and shoot length. Root and shoot length reduction increased as DIBOA and BOA concentrations increased (Chase et al. 1991).

Yenish et al. (1995) studied the rate of rye residue decomposition and the rate of decline of the benzoxazinoids. They found that the rate of allelochemical disappearance coincided

more closely with a decline in weed suppressive ability than did the decomposition of rye residue. Rye residues decayed by 50% in 15 weeks and benzoxazinoid concentrations declined by 50% in only 10-12 days. Rye is known to suppress weeds for about 4 weeks following cutting. These results indicate that chemical interference is more important than physical interference, and are supported by Barnes and Putnam (1986, 1987). Using aqueous extracts from eight winter rye cultivars, Burgos et al. (1999) found that high levels of DIBOA and BOA correlated with high levels of goosegrass inhibition and low levels of the allelochemicals correlated with low inhibition.

Wolf et al. (1985) extracted BOA and DIBOA from *Acanthus mollis* and DIBOA-glycoside from rye seedlings. These compounds were then utilized in petri dish bioassays to measure inhibition of velvetleaf (*Abutilon theophrasti*) seed germination. DIBOA completely inhibited velvetleaf germination at higher concentrations (2mM). There was a decline in germination inhibition with lower concentrations of DIBOA. BOA (5 mM) was less effective and stimulated germination of velvetleaf at lower concentrations. DIBOA-glycoside was the least active with velvetleaf germination reaching 89% two days after treatment. In an experiment to study the effect of kill date, cultivar and nitrogen rate on the weed suppressive ability of rye, Reberg-Horton et al. (2005) also found a correlation between DIBOA levels and weed suppressive ability.

The activity of DIMBOA and MBOA, allelochemicals primarily produced by wheat and maize, has also been demonstrated in experiments. Root growth of wild oats (*Avena fatua*) was inhibited by 50% when treated with DIMBOA and MBOA at 0.7mM and 0.5mM concentrations, respectively (Perez 1990). Seed germination was also inhibited by MBOA (Perez 1990). A study by Blum et al. (1992) indicated that MBOA was more toxic than DIMBOA. They also demonstrated suppression of germination, and radicle and hypocotyl growth of ivy-leaf morning glory (*Ipomoea hederacea*), a troublesome weed in the U.S.

Genetic control of Allelochemicals

The genetic control of hydroxamic acid production in rye has not been well studied. However, the genetics underlying the biochemical pathways producing hydroxamic acids in other cereal species have become clearer in recent years. Niemeyer and Jerez (1997) suggested that the biosynthesis of hydroxamic acids in hexaploid wheat (*Triticum aestivum*, 2n=6x=42) is under multigenic control. The biosynthesis of benzoxazinones in hexaploid wheat is controlled by five homoeologous genes, *TaBx1-TxBx5,* from each of the three genomes (Nomura et al. 2005). Studies using monosomic lines indicated that chromosomes 4A, 4B, 5B and 4D were involved in differential accumulation rates of DIBOA and DIMBOA (Niemeyer and Jerez 1997). Nomura et al. (2005) reported that the B-genome in wheat contributes more than the A or D genomes to benzoxazinoid biosynthesis. These genes are orthologous to barley genes, *HlBx1-HlBx5,* and maize genes, *Bx1-Bx5* (Nomura et al. 2005). In maize, BX1 is the branch enzyme which converts indole-3-glycerol phosphate to indole. The genes *Bx2-Bx5* take part in the synthesis of DIBOA from indole (Frey 1997). A strong homology is shared between the *HlBx1-HlBx5* genes of barley and the *Bx1-Bx5* genes of maize - 72%,80%, 76%, 81%, and 78%, respectively (Grün et al.. 2005).

The accumulation of DIMBOA in maize has been studied by several researchers. Quantitative inheritance of DIMBOA production was suggested by Klun et al. (1970) while Dunn et al. (1981) proposed a monogenetic model. In a diallel breeding experiment to study the relationship between European corn borer and DIMBOA levels, Klun found that 90.8% of maize hybrid variance in DIMBOA levels could be attributed to general combining ability (GCA) and that specific combining ability was also significant. The high GCA indicates that production of DIMBOA might be controlled by additive or additive x additive effects (Klun et al. 1970).

Genetic Variation in Benzoxazinoids

Evidence exists for the differential production of the benzoxazinoids within cereal species (Villagrasa et al. 2006). Burgos et al. (1999) found that total hydroxamic (DIBOA) content

ranged from 137 to 1469µg/g dry tissue among eight cultivars of rye. Niemeyer et al. (1988) screened 55 accessions of wheat for production of hydroxamic acids (DIMBOA) and found that concentrations ranged from 0.2 -16.0 mmol/kg fresh weight. A study of 52 Chilean accessions of wheat found that DIMBOA levels ranged from 1.4 to 10.8mmol/kg fresh weight (Copaja et al. 1991). Nicol et al. (1992) screened 47 *Triticum* accessions from international sources and found that DIMBOA concentrations ranged from 0.99 to 8.07 mmol/kg fresh weight. The similarity in results between the Chilean and the international germplasm screening indicates that the range of DIMBOA levels may not exceed these concentrations (Nicol et al. 1992). Wu et al. (2001b) found significant variation in root and shoot production of DIMBOA among 58 wheat accessions. DIMBOA levels ranged from 39.3-730.4 mg/kg dry weight in shoot tissue and 48.3-734.1mg/kg dry weight in root tissue. DIMBOA was not detected in shoots of two accessions and roots of one accession (Wu et al. 2001b). Burgos et al. (1999) reported significant variation in DIBOA levels among eight rye cultivars**.** The differential production of hydroxamic acids in the cereals supports arguments that selection for genotypes producing higher levels could lead to more highly allelopathic cultivars as has been achieved for pest and disease resistance.

Variability in Rye Allelopathy Reports

There is conflicting evidence in the scientific literature on the levels of rye allelopathic activity (Reberg-Horton et al. 2005). This is due, in part, to the genetic make-up of varieties as well as environmental influences (Burgos et al. 1999; Foley 1999). Further compounding the confusion is the differential accumulation of allelochemicals in various plant parts at different developmental stages (Burgos et al. 1999; Copaja et al. 1999; Wu et al. 2001b; Mogensen et al. 2006*;* Villagrasa et al. 2006). To study maturity effects on allelochemical production, Burgos et al. (1999) quantified shoot levels of hydroxamic acids in a rye cultivar (e.g. Bates) at 30, 45, 60 and 75 days after planting (DAP). Hydroxamic levels increased from 30 days until peaking at 60 days, and then declined. These results contrasted with other studies which showing hydroxamic levels peaking at early seedling growth stages before declining (Argandona et al. 1980). Reberg-Horton et al. (2005) found that DIBOA

concentrations declined at varying rates among 10 rye cultivars. DIBOA levels differed within individual cultivars depending upon plant age and developmental stage.

Effect of Developmental stage on Benzoxazinoid Production

Copaja et al. (1999) studied production of DIBOA and DIMBOA over time in three wheat cultivars. They reported that the highest levels of the compounds in all three cultivars occurred within the first week of germination and began to decline after seven days. DIBOA levels were lower than DIMBOA levels in the leaves of the three cultivars and were lower in the roots of two of the three cultivars (Copaja et al. 1999). Mogensen et al. (2006) reported that concentrations of Bx derivatives in the roots remained relatively constant while concentrations steadily declined in the shoot tissue at later growth stages.

Wu et al. (2001b) studied the accumulation of DIMBOA in roots and shoots of 17 day old seedlings of 58 wheat accessions. Fifty of the 58 accessions had higher levels of DIMBOA in their roots than in the shoots with an average of 643.0mg/kg in roots and 439.4mg/kg in shoots. In contrast to these results, Mogensen et al. (2006) found that DIMBOA levels among three winter varieties of wheat were higher in leaf tissue than in root during early growth stages. Only one of several studies has reported the detection of benzoxazinoid derivatives in ungerminated wheat seed (Argandona et al. 1980; Copaja et al. 1999; Villagrasa et al. 2006).

Allelochemical Mode of Action

Different modes of action in plants have been suggested for the hydroxamic acids. In *Avena sativa* and *Vicia faba,* DIBOA and BOA inhibited H^+-ATPase activity in the plasma membrane at concentrations ranging from 0.25 to 2.0 mM. This inhibition was correlated with reduced radicle elongation in *A. sativa*. Friebe et al. (1997) hypothesized that radicle length was reduced due to altered nutrient uptake through the plasma membrane. Sanchez-Moreiras et al. (2004) found that growth inhibition of *L. sativa* seedlings by hydroxamic acids could likely be attributed to mitotic interference. Singh et al. (2005) reported that BOA

interfered with respiratory and photosynthetic processes within mung bean (*Phaseolus aureus*). The same group reported that BOA generated reactive oxygen species, which induced oxidative stress, in the roots and shoots of mung bean (Batish et al. 2006). If this class of chemicals does indeed have such diverse modes of action, their utilization as an in vivo weed control agent could make it more difficult for weed species to develop resistance to these allelochemicals.

Non-allelopathic Activity of Benzoxaxinoids

In addition to herbicidal bioactivity, the benzoxazinoids are known to have insecticidal, anti-bacterial and anti-fungal properties (Rice 1984; Barnes et al. 1987; Niemeyer 1988). Virtanen and Hietala (1955) identified BOA as the compound extracted from rye seedlings which had anti-fungal properties against a strain of the pathogen *Fusarium nivale.* BOA was isolated and experiments confirmed that it was most likely the compound responsible for the inhibition of *Fusarium nivale* growth and *Sclerotinia trifoliorum*, or clover-rot fungus. The hypothesis that DIMBOA was involved in maize resistance to the European corn borer was confirmed through experiments which correlated resistance and hydroxamic acid levels (Klun et al. 1970). Principal component analysis, a multivariate statistical approach, found a correlation between fusarium head blight resistance and levels of benzoxazinoids in winter wheat varieties (Søltoft et al. 2008). Based on this knowledge, Rice (1984) pointed out that breeding for increased weed suppressive ability using these chemicals should be possible just as breeders select for pest resistance.

Allelopathy Screening

Analytical Chemistry Screening

As discussed above, chemical screening has been used to assess the quantity of allelopathic components in plants (Wu et al. 1999). A benefit of quantitative chemical analysis is the ability to measure the purely chemical characteristic associated with allelopathy without the confounding aspects of competition or indirect interference and the variability associated with field and bioassay systems. Direct quantification of allelochemicals is a measure that

can also be used to estimate genetic variability of allelopathy among genotypes (Bertholdsson 2004). When allelochemicals content is correlated to field performance, it may be possible to reduce expensive and lengthy field experiments (Wu et al. 1999). Of course, the use of chemical analysis requires the identification and isolation of the purported allelochemicals (Fay and Duke 1977).

As Fuerst and Putnam (1983) point out, allelochemicals must first be characterized, isolated and synthesized to provide evidence of their phytotoxicity before large scale analysis. Evidence for differential accumulation of benzoxazinoids among the Poaceae indicate that selection for this allelochemical trait could be successful. Allelopathy, by definition, is based upon the production of allelochemicals and their activity upon receiver species. This implies that allelochemical concentrations within a plant could be indicative of the potential of allelopathic activity, i.e. plants with higher levels of allelochemicals should have better weed suppressive ability than plants with lower levels (Wu et al. 1999).

Most allelochemical analyses utilize high performance liquid chromatography (HPLC) or gas chromatography (GC) to quantify chemical content. Recently, Finney et al. (2005) developed a GC method which allowed simultaneous analysis of benzoxazinoids and two other compounds implicated in rye allelopathy, β-hydroxybutyric acid and β-phenyllactic acid. While quantification of allelochemicals can be a useful tool, the interactive or cumulative effect of the multitude of allelochemicals present in a plant may not be realized through analytical techniques (Inderjit and Nilsen 2003). It is important to keep in mind that how allelochemicals affect neighboring plants depends not only upon their concentration in the donor plant but also upon the complex interactions after they are produced (Einhellig 1989; Blum 1993). Plants exude numerous compounds. Understanding the behavior of the chemicals in nature will allow a better analysis of chemicals. The best approach for chemical analysis is to demonstrate that strong relationships exist between the quantity of allelochemical measured analytically and the effectiveness of plant extracts containing the chemical in bioassays and ultimately in field experiments (Wu et al. 2001c).

Bioassay Screening

Before genetic parameters can be estimated for allelopathy, it is important to identify a phenotypic screen which captures the bioactivity ascribed to the allelochemicals. To date, no such bioassay exists for rye. In the development of a bioassay, it is important to address challenges such as choice of indicator species, transport of allelochemicals to the species, mechanism of inhibition, importance of environmental factors and allelochemical conversions by the environment or by microbes.

In its early years, allelopathy research lacked solid methodologies. Much improvement has been made in the development of sound, reproducible methods for study of the phenomenon. Bioassays are the most commonly employed techniques to screen for allelopathic potential within a species. A bioassay can be defined as a "determination of the relative strength of a substance (such as a drug) by comparing its effect on a test organism with that of a standard preparation" (http://www.merriam-webster.com). Several different bioassays have been employed to assess allelopathy including such systems as petri dish-based (in vitro) systems, the plant-box method, the relay-seeding technique and the equal-compartment-agar method (Fuji 1992; Navarez and Olofsdotter 1996; Wu et al. 2000b). There is no "best" bioassay to assess allelopathy so ideally researchers should employ a combination of laboratory, growth chamber, greenhouse and field designs for study (Gawronska 2006).

Bioassays to evaluate allelopathy are complicated by many factors. Despite awareness of flaws in early research, the most difficult task is still the proper demonstration that effects on receiver species can be attributed to allelopathy instead of some other plant-plant interaction (Romeo and Weidenhamer 1998). To effectively study allelopathic effects, competitive and indirect effects of interference should be controlled or minimized so that weed suppression can be attributed primarily or exclusively to chemical activity (Fuerst and Putnam 1983; Rice 1984; Weidenhamer 1996, Foley 1999; Kobayashi 2004).

Proper controls can be useful in distinguishing between physical and allelopathic effects. For example, mulch from a cover crop has a competitive, physical effect on weed seed germination and seedling growth through light interception and/or lowering of soil temperatures (Williamson and Richardson 1988). The use of an inert mulch in a control plot can account for physical effects that might be present in the treatment plots (Barnes and Putnam 1983). Barnes and Putnam (1983) found that poplar (*Populus*) excelsior, which has mulch properties similar to that of rye, could be used as a control in rye allelopathy studies. Indirect effects can be related to soil biotic factors such as mycorrhizae associations and nematode activity (Kobayashi 2004; Anaya 1999). For the purpose of a bioassay, it might be useful to reduce these effects through the use of sterilized soil or soil-less planting media. Importantly, these biotic components may play and important role in suppression or enhancement of plant growth and therefore may be essential for an ecologically meaningful bioassay (Romeo and Weidenhamer 1998).

In addition to separation of interference effects from allelopathic effects, there are other key tenets to consider in the development of an allelopathy bioassay. The study should simulate the release of the allelochemicals from the donor plant and the subsequent mode of transport to the receiver plant. Some of the ways in which allelochemicals are excreted into the environment include tissue leaching, root exudation, volatilization and release of allelochemicals from plant debris (Rice 1984). A bioassay which mimics the natural mechanism of release of allelochemicals as it occurs in a nature is more meaningful than one that uses extracts of plant material or isolated allelochemicals to test activity. For example, plant debris of sorghum may not be indicative of the allelopathic potential because sorgoleone, the primary allelochemical in sorghum, is released from the roots. Transport is an important consideration to bioassay development because of the wide array of allelochemical conversions that can occur due to microbial, chemical and phytolytic indirect effects. If the suspected mode of transport is through the soil then an appropriate bioassay would utilize soil as the media because of the known biochemical transformations that can occur and may result in more or less toxic derivatives of the chemicals (Romeo and

Weidenhamer 1998; Fomsgaard et al. 2006). There is another school of thought, which is that all indirect effects should be eliminated. In this case the scientist(s) may choose to use sterilized soil or media. The bioassay should also consider the flux of allelochemicals into the environment. Allelochemicals released from roots (e.g. sorghum) are continually released into the soil. On the other hand, allelochemical levels in soil may decline rapidly after they are released from plant debris (e.g rye straw) (Wickcliffe 1999; Nagabhushana 1993).

Choice of an indicator species varies widely among bioassays (Inderjit and Dakshini 1995). Some studies utilize indicator species, such as tomato and lettuce, that tend to have dependable, high germination rates and are readily available, which is useful for preliminary screening for allelopathic activity (Leather and Einhellig 1986). On the other hand, scientists have argued that these species have minimal ecological relevance (Injderjit and Nilsen 2003). More ecologically relevant indicator species include those which co-occur with the donor species. This approach can be difficult because it difficult to obtain sufficient amounts of clean weed seed with high germinability. Ultimately, it is necessary to confirm the laboratory results with the weed species of interest (Inderjit and Dakshini 1995). The scientific literature is full of field and laboratory evidence for allelopathic activity on weed species. In utilizing weed species as indicators, consideration should be give to the agro-ecological significance of the species in the system under study. In some cases it may be desirable to develop bioassays for specific problematic species such as invasive weeds or weeds showing resistance to herbicides.

The mechanism of absorption of allelochemicals into the receiver species could prove to be a useful adjunct to bioassay systems. In addition to absorption, it is beneficial to understand the mode of action of the allelochemicals in weed species (Romeo and Weidenhamer 1998). Knowledge of the mode of action could make it easier to distinguish between allelopathic and competitive effects. For example, investigation of sorghum allelochemicals has uncovered sorgoleone whose structure is related to important quinones in plants. It has a

higher affinity for (ubi/plasti) quinone binding sites and therefore disrupts electron transport during photosynthesis, leading to chlorosis in susceptible plants (Nimbal et al. 1996). Since sorgoleone can induce chlorosis and allelochemical effects are dose dependent the degree of chlorosis in a susceptible species might be a useful phenotypic response parameter to quantify in a sorgoleone bioassay system (Belz et al. 2005). Response parameters to be used as measures of allelopathic activity in a bioassay are another important consideration (Inderjit and Nilsen 2003). Seed germination rate, relative to a control, is commonly employed to assess allelopathic potential (Leather and Einhellig 1986). Measures of plant growth are also useful response parameters and include can include seedling root, coleoptiles and radicle length, and seedling fresh weight (Gawronska 2006).

A meaningful bioassay should consider the influence of environmental factors upon allelopathic effects (Romeo and Weidenhamer 1998). The response of indicator species can vary depending upon abiotic environmental conditions such as moisture, nutrient levels, and temperature. Environmental conditions can also impact production of allelochemicals in the donor species (Rice 1984; Einhellig 1996; Romeo and Weidenhamer 1998).

This brings us to a frequently disregarded aspect of allelopathy. Most reported research is based upon the inhibition of growth of one plant by another. However, allelopathy can also refer to the stimulation of plant growth. The phenomenon of allelopathy is concentration dependent and stimulation of growth can occur when the chemicals are at relatively low concentrations. For example, the effects of aqueous extracts of alfalfa at different concentrations upon lettuce growth were investigated using a serial dilution bioassay (Xuan and Tsuzuki 2002). Fresh and dry tissue of eight varieties of alfalfa were extracted, diluted to various concentrations and transferred to petri dishes. Lettuce germination and hypocotyl and radictle elongation were recorded as measures of allelopathic activity. The most dilute concentration resulted in a promotion of hypocotyl elongation for all varieties, reaching a maximum of 210% promotion of hypocotyl elongation did not occur with the higher concentration treatments. Lettuce radicle length was promoted by four varieties at the lowest

extract concentration *(1/20)* and by three varieties at next to lowest concentration *(1/10).* Lettuce seed germination increased under the lowest concentration treatment for five varieties (Xuan and Tsuzuki 2002). Leather (1983) found that aqueous extracts of sunflower (*Helainthus annus* L.) leaf tissue inhibited wild mustard (*Brassica kaber*) seed germination by 75%. At 10 and 100 fold dilutions, germination was stimulated by as much as 15% (Leather 1983). The same pattern was observed in a physiological study of the activity of DIBOA and DIMBOA against *Avena sativa* and *Vicia faba* growth. Plasma membrane H+-ATPase activity in the roots was suppressed at high concentrations of DIBOA and DIMBOA but stimulated at lower concentrations (Friebe et al. 1997).

The Genetic Exploitation of Rye: Strategies and Considerations

Breeding for increased allelopathic crops holds potential and is deserving of further investigation. An important component is the identification of highly allelopathic parental germplasm (Belz and Hurle 2004). Once parental lines have been identified, mating designs can be utilized to develop appropriate study populations. If significant genetic variation for allelopathy does exist then enhancement of the trait through selection could be successful (Wu et al. 1999a). Bertholdsson (2005) discusses the use of molecular markers for selection of allelopathy. Marker assisted selection could save time and money, often associated with exhaustive field screening methods. Phenotypic screening for the allelopathic effect can assist researchers in the identification of QTL (quantitative trait loci). Linkage maps for rye have been created and should be useful in identifying QTL.

The importance of accurate and quantifiable measures of allelopathy at the beginning of a selection experiment cannot be overstated. The ability to discern performance of genotypes will allow selection to progress. It is best to include both bioassay and chemical assay data so that direct correlations can between made between weed suppressive ability and allelochemical levels. Data from such studies are used to estimate genetic parameters such as genotypic variance, genotype x environment interaction and heritability. These parameters help the breeder identify suitable selection methods for the allelopathy trait and are also an

indication of expected gain through breeding (Bernardo 2002). An estimate of genetic variance explains how much of the variation in weed suppressive ability is actually due to genetic variation for the trait in the population. The effect of environment on weed suppressive variability observed in the population can be assessed by the significance of the genotype x environment interaction. Finally, the heritability of the allelopathy trait in rye will be useful to predict the ease with which progress will be made using selection.

Allelopathic cultivar development should include long terms goals to sustain genetic enhancement. Once identified, it will be necessary to monitor cultivars for decline or changes in performance of allelopathic activity. Weed population shifts and development of allelopathy resistant weeds are important topics to address for future progress. Another consideration is the critical weed free period in crops which might utilize rye allelopathy. Yenish et al. (1995) carried out a field study to determine the rate of rye residue and allelochemical decline after cutting. They found that 50% of rye residue declined after 105 days. However, benzoxazinoid levels declined by 50% of day 0 levels after only 10-12 days. This suggests that, in addition to allelopathy, other methods of weed control may be necessary for control of late season weeds.

Screening of rye germplasm should not be limited to cultivated accessions. Scientists have suggested that older or uncultivated germplasm might contain more variation for allelopathic activity than cultivated accessions. (Putnam and Duke 1974; Bertholdsson 2004). There may be some benefit to use of uncultivated parental germplasm in the development of rye populations to be screened for potential allelopathic variation. For example, Bertholdsson (1994) reported more allelopathic variation in older races of barley and wheat than those which have been cultivated. Escobar and Niemeyer (1993) found that modern high yielding cultivars of Swedish wheat cultivars exuded less of the allelochemical, DIMBOA, than older cultivars. Hashem and Adkins (1996) screened 19 wild wheat (*Triticum speltoides*) accessions and found allelopathic variation against wild oats (*Avena fatua*) and Indian hedgemustard (*Sisymbrium orientale*). Czarnota et al. (2003) screened seven accessions of

sorghum for sorgoleone concentrations. They found that the lowest and highest levels were found in two weedy accessions, Shattercane (0.50 mg/g freshweight) and Johnsongrass (14.75 mg/g fresh weight). There was less variation in sorgoleone levels among the five cultivated accessions (1.33-1.85 mg/g fresh weight) (Czarnota et al. 2003). There is concern that allelopathy has a negative correlation with plant biomass yield which is another component of a successful cover crop. It is possible that selective breeding for agronomic traits over time may have reduced the genetic variability for allelopathy in crops (Putnam and Duke 1974; Bertholdsson 2004; Olofsdotter 2002).

Rye is an obligate out-crossing, diploid species with 14 chromosomes (2n=14). It is believed to have originated in southwestern Asia as did wheat, oats and barley (Bushuk 2001). The rye genome is homologous to all three wheat genomes (A, B, D) and there is a high level of synteny between rye and wheat chromosomes. Among two rye, four barley and seven wheat maps, there were only 12 inconsistencies between loci in the group one chromosome linkage group (Bushuk 2001). The significance of this is that the extensive knowledge of wheat genetics could be useful for identification of loci for the allelopathy trait in rye. The recent development of rye genetic maps will also be useful for identifying loci (Devos et al. 1993). Rye inbred lines became available during the 1980s and since then, genetic analysis of the rye genome has improved (Bushuk 2001). Before this development, the self-incompatible nature of rye has made it difficult to work with without the use of special breeding techniques.

Perhaps to fully exploit the use of in vivo natural products for weed control through allelopathy we could take a lesson from the methods used to develop and evaluate new herbicides. Before herbicides are released, intensive screening trials must occur. Absorption, translocation and mode of action are determined for variety of weeds and susceptible or resistant species are identified. Performance in various environments is evaluated. Often the performance of herbicides is based upon very specific criteria including proper choice of environment and target species. It is difficult to distinguish between

allelopathy and resource competition. One can argue that all plants produce toxic chemicals and are therefore "allelopathic" (Harper 1994). Instead of thinking in terms of an 'allelopathic plants' we should think more holistically in terms of 'allelopathic relationships'. Understanding this relationship between crop plants and the weeds with which they compete under various environmental conditions will allow the exploitation of allelopathy.

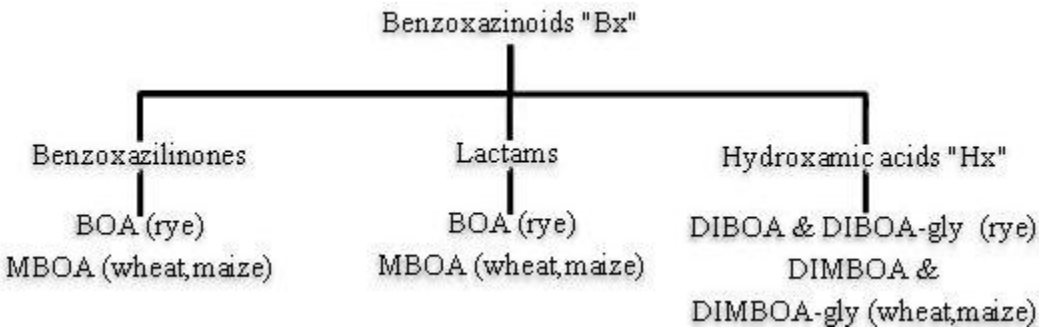

Figure 1. Allelochemicals identified in the Poaceae.

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Chapter II

A GREENHOUSE BIOASSAY TO ASSESS VARIATION IN RYE (*SECALE CEREALE*) ALLELOPATHIC ACTIVITY

For submission to: Journal of Chemical Ecology

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Abstract

Key problems in conducting bioassays of potential allelopathic cover crops are separation of interference components, identifying sensitive, quantifiable measures of allelopathic activity and reproducibility of experiment results. Bioassays should be simple, inexpensive and equipped to handle large numbers of genotypes. A greenhouse bioassay system was developed to assess variation in the allelopathic activity of soil-incorporated rye (*Secale cereale*) debris against redroot pigweed (*Amaranthus retroflexus*). Redroot pigweed emergence and fresh weight biomass were quantified as measures of allelopathic activity. Two bioassays were conducted and reproducible results were obtained for fresh weight biomass but not for emergence. A population of 150 half-sib families of rye grown in two locations in North Carolina was utilized to obtain estimates of genetic variance and heritability for rye allelopathic activity against redroot pigweed fresh weight biomass. Genetic variation among the half-sib families was not significant across locations but was significant within each location. Heritability estimates for the allelopathy trait were low based on this bioassay. Redroot pigweed fresh weight biomass data displayed a normal distribution indicating allelopathy in rye is a quantitative trait. The greenhouse bioassay eliminated physical interference so that weed suppression could be attributed to allelopathy alone and it was successful in handling the large number of genotypes for screening. This bioassay system could be used to screen other species for which allelopathic action is imparted through plant debris.

Keywords: Allelopathy, weed suppression, Rye, *Secale cereale*, Allelopathic activity, Redroot pigweed, *Amaranthus retroflexus*

Introduction

One of the most important challenges that farmers face is crop competition with weeds. Weeds reduce crop yield, leading to economic losses if not properly managed. Herbicides have been a successful means of weed control in developed countries for several decades. However, the occurrence of herbicide resistant weeds, as well as concerns for human, wildlife and environmental health, have put pressure upon scientists to develop more sustainable methods of weed control (Pimentel and Greiner 1997). A sustainable approach to weed management would incorporate a variety of control methods and could reduce or eliminate heavy dependence upon herbicides.

One option for weed control in agricultural settings is the use of cover crops. Cover crops are used to impede the germination and growth of weeds and they offer a wide range of other benefits including the addition of organic matter to the soil, enhancement of soil moisture, nutrient recycling and protection from erosion. Live cover crops compete with weeds for light, nutrients, water and space. Cover crop residues provide mulch which can physically impede weed seedling emergence and can interfere with light interception (Teasdale and Mohler, 2000). Some cover crops release chemicals which are toxic to certain weeds, a phenomenon referred to as allelopathy (Bhowmik and Inderjit, 2003). Numerous studies have shown that allelopathic activity can be a significant component of the weed suppressive ability of crops (Creamer et al., 1996; Lehman and Blum, 1997; Dhima et al., 2006). It has been suggested that the use of allelopathic crops, including cover crops, may be a viable option for weed control. Further, there may be opportunity to enhance the weed suppressive ability of cover crops through conventional breeding for the allelopathy trait.

The potential for enhancement of allelopathic activity through plant selection has been investigated in several crops including rice (*Oryza sativa*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), sorghum (*Sorghum bicolor*), sunflower (*Helianthus annuus*) and cucumber (*Cucumis sativas*) (Dilday et al., 1998; Wu et al., 2000a; Leather, 1983; Putnam and Duke, 1974). Extensive germplasm screening has revealed significant variation in

allelopathic activity within these species. This suggests that there is a genetic pool for selection and thus, improvement of the trait is feasible. Most progress towards allelopathy improvement has occurred in wheat and rice. Quantitative trait loci (QTL) have already been identified which are associated with allelopathy in both of these cereal crops (Wu et al. 2003; Jensen et al., 2001). Identification of QTL will allow the use of marker assisted selection to develop cultivars improved for allelopathy.

The existence of highly allelopathic parental germplasm is the basis of selection for allelopathy (Belz and Hurle, 2004). To assess variation in germplasm, it is essential to identify a sound phenotypic screen for the trait (Courtois and Olofsdotter, 1998). Elaborate bioassays were developed to assess genetic variation for allelopathy including the relay-seeding technique and the equal compartment-agar method (Fuji, 1992; Wu et al 2000b). One of the most difficult tasks in allelopathy screening is the separation of various components of interference (Fuerst and Putnam, 1983; Romeo and Weidenhamer, 1998). Fuerst and Putnam (1983) define interference as "the adverse effect that neighboring higher plants can exert on each other's growth". Competitive, allelopathic and indirect effects can be categorized as "interference". Competition is defined as physical interference in which one plant competes with another plant for nutrients, light, water and/or space. Allelopathy is a form of chemical interference in which one plant (donor species) produces a chemical that affects the growth of another plant. Indirect interference effects are caused by the surrounding environment of a plant and can usually be attributed to microbial, physiochemical or phytolytic activity in the soil (Rice, 1984; Inderjit and Nilsen, 2003).

To study allelopathy, the competitive and indirect effects of interference should be accounted for or minimized so that weed suppression is attributed primarily to allelopathic interactions (Fuerst and Putnam, 1983). Some field and greenhouse studies have utilized inert mulches to account for physical effects of plant debris on indicator species growth (Barnes and Putnam 1983; Creamer et al., 1996). Elaborate laboratory protocols have also been developed which attempt to separate physical and chemical effects (Olofsdotter, 1996; Fujii, 1992). For

example, the equal compartment-agar method physically separates wheat (i.e. donor species) seedlings from annual ryegrass (i.e. indicator or receiver species) and utilizes an agar medium for diffusion of allelochemicals released from wheat roots (Wu et al., 2000b). The relay-seeding technique is a laboratory technique developed to assess rice allelopathy (Navarez and Olofsdotter, 1996). Results from a study utilizing barnyardgrass (*Echinochloa crus-galli*) as the test species were correlated with field experiments. Allelopathy was responsible for 34% of the reduction in barnyardgrass growth (Olofsdotter, 2001).

Allelopathy bioassays should mimic field conditions as closely as possible (Barnes and Putnam, 1983). This includes simulation of the natural release of allelochemicals from the donor species and transport to the receiver species (Romeo and Weidenhamer, 1998). Allelochemicals can be excreted into the environment through live tissue leaching, root exudation, volatilization and leaching of plant residues or debris (Rice, 1984). Once released from the plant, allelochemicals are subjected to transformation through microbial, chemical and phytolytic processes as they are transported through various mediums (Romeo and Weidenhamer, 1998; Fomsgaard, 2006). Scientist disagree as to whether allelochemical transformations should be permitted in a bioassay system. On one hand, these interactions can alter the magnitude of suppression in the field and so these processes should not be excluded in bioassays. On the other hand, exclusion of these processes removes indirect effects and gives a more direct measure of the level of allelochemical activity of the initial allelochemical metabolite(s). The use of sterile soil can help reduce indirect effects while a non-sterile or perhaps an inoculated soil might give a better approximation of field conditions.

Choice of indicator species varies among bioassays (Inderjit and Dakshini, 1995). The goals of the bioassay should determine species choice. Studies have utilized cultivated indicator species, such as tomato *(Solanum lycopersicum*) and lettuce (*Lactuca sativa*), because of dependable germination and ready availability, both of which are useful for preliminary screening of allelopathic activity (Leather and Einhellig, 1986). Some scientists argue that

these species do not co-occur with the donor species and thus may not have ecological relevance (Injderjit and Nilsen, 2003). Despite problems with consistent germination, seed uniformity and availability, the scientific literature is full of field and laboratory evidence for allelopathic activity on weed species. The choice of weed species for bioassay development is somewhat limited, however, by the availability of high quality weed seed. Regardless of the plant species chosen for use in the bioassay, it is important that appropriate parameters are chosen to indicate allelopathic activity on the receiver species (Inderjit and Nilsen, 2003). Reduction in weed seedling emergence and growth performance, such as seedling root length or seedling fresh weight, may be selected as phenotypic measures of allelopathy when compared to a control (Gawronska, 2006). The measures should be sensitive enough to detect variation in allelopathic activity among different donor plants. It is also important to control environmental conditions such as moisture, nutrient levels, temperature, and presence of pathogens in order to minimize sources of experimental variability (Injderjit and Nilsen, 2003). The influences should be closely evaluated and controlled for in bioassay development.

Despite efforts to develop appropriate allelopathy bioassays, there are still conflicting reports of allelopathic activity within a given species. This is, in part, due to the fact that variability in allelopathic activity can be attributed to the genetic make-up of the donor species. A characteristic of quantitative genetic traits is that phenotypic expression is often influenced by environment. Changes in environmental conditions under which the bioassay is conducted can alter production of allelochemicals by interacting with allelochemical biosynthetic pathways (Rice, 1984; Einhellig, 1996; Romeo and Weidenhamer, 1998). The differential accumulation of allelochemicals in various plant parts at various developmental stages creates an even more complex interaction (Rice, 1984; Burgos et al., 1999; Copaja et al., 1991; Wu et al., 2001; Mogensen et al., 2006). Because of these complexities, it is essential to control environmental variation in a bioassay. Similarly, just as environment can alter the allelochemical output of the donor species, variation in the environment can also affect the growth and susceptibility of the receiver species. Thus, environmental variability

becomes an especially daunting obstacle for field and even greenhouse studies. This and the costs of such experiments, is undoubtedly one of the reasons that a large majority of bioassay systems for allelopathy are conducted in vitro in a laboratory or within environmental chambers.

Rye (*Secale cereale*) is an allelopathic cover crop grown in the southern United States. Various assay systems have been used to study rye allelopathy. Field and greenhouse experiment were conducted to test allelopathic activity against weeds such as common lambsquarters (*Chenopodium album* L.), large crabgrass (*Digitaria sanguinalis*), common ragweed (*Ambrosia artemisifolia* L.), wild oats (*Avena fatua*), redroot pigweed (*Amaranthus retroflexus*) and ivyleaf morningglory (*Ipomoea hederacea* L.) **(**Barnes and Putnam 1983; Pérez and Ormeño-Núñez 1993; Lehman and Blum 1997). Seed germination bioassays have been employed to test allelopathic activity of rye against species such as goosegrass (*Echinochloa crus-galli*), redroot pigweed, common lambsquarters and barnyardgrass (Shilling et al., 1986; Barnes and Putnam, 1987; Reberg-Horton et al., 2005).

Significant evidence exists for allelopathy in rye, yet, very little research has been conducted in terms of potential for genetic improvement. Studies have shown differential production of allelochemicals among different genotypes and some have reported correlation of allelochemical levels to levels of bioactivity against a susceptible species (Burgos et al., 1999; Reberg-Horton et al., 2005). However, very few studies have been published which report large scale germplasm screening utilizing bioassays (Foley, 1999).

The goal of this experiment was to develop a greenhouse bioassay to assess genetic variation for allelopathic activity with two half-sib populations of rye. The use of a sound bioassay screening protocol is necessary to understand genetic variability and genetic control of allelopathy (Courtois and Olofsdotter, 1998). In addition to reproducibility, the bioassay should be able to screen a large number of genotypes and should be carried out in a time and cost efficient manner.

Methods and Materials

Test material Seed of 15 rye accessions, which were previously identified as being highly allelopathic, were obtained from the USDA's National Small Grains Collection (unpublished data). These accessions were each crossed with Wrens Abruzzi, a rye variety commonly grown as a cover crop in the southern United States. An equal number of hybrid seeds from each cross were bulked. This bulked population was advanced for two generations by open pollination in isolation. During the second generation of open pollination 150 plants were randomly chosen and harvested individually to obtain seed of 150 half-sib families.

In October 2006, the 150 half-sib families and checks were planted in duplicate randomized complete block design experiments at research stations in Kinston and Clayton, NC. Soil in Kinston, NC is characterized as fine loamy sand. Soil in Clayton, NC is characterized as Norfolk loamy sand. In spring 2007, shoot tissue was harvested at flag leaf emergence. Plant material was forced-air dried at 54°C for 3-4 days, ground to pass through a 2-mm mesh screen and stored in airtight bags in the dark prior to analyses.

Indicator species Previous studies have indicated sensitivity of redroot pigweed (*Amaranthus retroflexus*) to the allelopathic activity of rye (Putnam and DeFrank, 1983; Barnes and Putnam, 1986). Additionally, it is a problematic weed in agricultural fields in the southern United States and was a successful indicator species in allelopathy bioassays (SWSS 2006, 2008; Steinsiek et al., 1982). A redroot pigweed growth study determined that two weeks was the optimal time of growth because all viable seed should have germinated Also, based on visual observations of allelopathic activity using a limited number of rye varieties, variation in weed vigor was evident (unpublished data).

Dose response A preliminary dose-response study was carried out to determine the optimal amount of rye tissue to incorporate into the soil (Inderjit and Nilsen, 2003). Rates tested included 2, 5, 10 and 20 grams of Wrens Abruzzi rye tissue per 600 grams of soil. Emergence and fresh weight biomass of redroot pigweed were the measured response

parameters to rye allelopathic activity. The study indicated that 20g tissue/600g soil resulted in nearly 50% inhibition of emergence and fresh weight biomass.

Experimental design The greenhouse bioassay was a completely randomized block design. Twenty grams of ground rye tissue from each plot was weighed into one gallon plastic bags. Six hundred grams of dried, steam sterilized soil was added to the bags containing rye tissue. The plastic bags were sealed and mixed thoroughly to ensure even distribution of the rye in the soil media. The soil/rye mixture was placed into 5x7" planting trays on top of a one inch layer of proprietary soil blend (Fafard Inc., Agawam, MA). One hundred seed of redroot pigweed were planted in each tray at a one centimeter depth. Seed were evenly dispersed and care was taken to avoid soil clumping. The trays were randomized five days after planting (DAP) and 10 DAP. Seedling fresh weight biomass (shoot) and total emergence were chosen as the response parameters to allelopathic activity of the rye tissue. Redroot pigweed seedlings, including roots, were carefully removed from the soil and cut directly above the white/red pigmentation line and shoot portions were immediately weighed. Fresh weight biomass was calculated on a per seedling basis and the calculation for each half-sib family was:

total shoot fresh weight of the indicator / # of indicator seedlings emerged

A capillary mat and drip hose system (Hummert International) was used for irrigation. This system was chosen to prevent leaching of allelochemicals from the soil which could occur with overhead watering. Evaporation in conjunction with capillary action from below allowed the soil to remain saturated while preventing leaching. Samples were randomized once during the two week growth period.

Bioassay reproducibility A set of 25 randomly chosen genotypes were selected from each location to test reproducibility of the greenhouse bioassay. The bioassay was conducted twice. The second bioassay was carried out under similar experimental conditions as the first

bioassay. The two bioassays differed in the following ways: 1) slight adjustments were made to the irrigation system in the second bioassay to improve drainage and thus minimize microbial growth and 2) the first bioassay was conducted in June 2007 and the second one was conducted in October 2007. Daily temperatures in the greenhouse ranged from 17-27°C in June and 16-42°C in October.

Full scale experiment The greenhouse bioassay was employed to assess variation among the half-sib families of rye for allelopathic activity against redroot pigweed. This bioassay included all genotypes grown in both locations and was conducted in October 2007.

Data analysis All data were subjected to analysis of variance using PROC GLM and PROC MIXED in SAS version 9.1 (SAS Institute 2002).

The following model was used to test reproducibility of the bioassay:

$$y = \mu + R_i + E_j + (RE)_{ij} + G_k + (GR)_{ik} + (GE)_{jk} + e_{ijk}$$

where y is the phenotypic value for the allelopathy trait of a genotype, μ is the overall experimental mean, R_i is the block effect, E_j is the experimental effect, (RE_{ij}) is the block x experiment interaction effect, G_k is genotype effect, $(GR)_{ik}$ is the genotype x block interaction effect, $(GE)_{jk}$ is the genotype x experiment effect and e_{ijk} is the residual error. Reproducibility of the greenhouse bioassay was quantified by the Pearson correlation coefficient using PROC CORR in SAS.

The model used for the analysis of variance in the full scale bioassay was:

$$y = \mu + E_i + R_{ji} + G_k + GE_{ik} + e_{ijk}$$

where y is the phenotypic value for the allelopathy trait of a genotype, μ is the overall experimental mean, E_j is the location effect R_{ji} is the block effect, G_k is genotype effect, $(GE)_{ik}$ is the genotype x environment interaction effect and e_{ijk} is the residual error. Variance components were estimated. Narrow sense heritability and standard errors were estimated on a per-plot basis and on an entry mean basis according to Holland et al. (2003).

Results

Preliminary study Results indicated that 20g of rye tissue resulted in 63% reduction in redroot pigweed emergence while the lower concentrations resulted in less than 50% control (data not shown). Fresh weight biomass of redroot pigweed biomass was reduced by 35% at the 20g rye tissue level. Emergence and fresh weight biomass followed a typical dose-response trend (i.e. responses decreased as rye tissue concentration increased).

Bioassay reproducibility Pearson correlation coefficients indicating the relationships between the bioassays 1 and 2 were weak (Figures 1 and 2). The correlation coefficient between the two bioassays for redroot pigweed fresh weight biomass was 0.34 and was significant at α=0.10 for the 25 Clayton genotypes (Table 1). The correlation coefficient between the two bioassays was 0.37 for the Kinston genotypes and was significant at α=0.10. Genotype x experiment interaction effects were not significant indicating that genotype performance across the bioassays was not statistically different (data not shown).

Correlations between the two bioassays were not significant for redroot pigweed germination in the Clayton or the Kinston data sets (Table 1). This indicates that germination was not reproducible across the two greenhouse bioassays and therefore, may not be a good measure of the allelopathic activity of the rye genotypes. Genotype x experiment interaction effect was significant for the Clayton and Kinston data sets indicating unstable genotype performance across the two bioassays (data not shown).

Full scale experiment Across location analysis of variance did not detect a significant genotype main effect for redroot pigweed fresh weight biomass (Table 2). Location effect was significant.

Clayton and Kinston data each displayed an approximately normal distribution (Figures 3 and 4). Within location analysis detected significant variation among genotypes grown at both locations at α=0.05 (Table 3). Narrow sense heritability estimates on a per-plot basis were low for fresh weight biomass at the Clayton location (h^2=0.17, s.e.=0.09) and the Kinston location (h^2=0.21, s.e.=0.11). Narrow sense heritability estimates on an entry mean basis were moderately low for the Clayton location (h^2=0.29, s.e.=0.13) and the Kinston location (h^2=0.35, s.e.=0.15).

Redroot pigweed fresh weight biomass, expressed as percent of control, ranged from 1.59-34.40% (mean=16.75% ± 5.80) and 2.32-22.72% (mean=8.22% ± 3.07) for the genotypes grown at the Clayton and Kinston locations, respectively (Table 4). Mean fresh weight biomass was lower at the Kinston location than the Clayton location indicating a higher suppressive ability among genotypes grown in that location.

Discussion

The primary focus of this research was to develop a simple, rapid procedure to screen rye populations for allelopathic activity. Results for redroot pigweed fresh weight biomass were reproducible which suggests that the bioassay was able to detection differences among the half-sib families and that genotype performance was somewhat stable across the two bioassays. Redroot pigweed emergence results were not reproducible. One reason for this may be that genotype performance, as measured by emergence, was masked by variation in the viability of redroot pigweed seed. The calculation for fresh weight biomass accounts for such variability thereby allowing a more sensitive measure of allelopathic activity. The presence of significant variation among the half-sib families in the two populations under study indicates that selection for allelopathy in rye could be successful. Although heritability

estimates were low, they were similar to that of yield in maize. Heritability estimates are expected to increase as the screening protocol is improved.

In the development of this greenhouse bioassay we attempted to address some of the challenges outlined such as transport of allelochemicals to the target species, mechanism of inhibition, importance of environmental factors and allelochemicals conversions by the environment or by microbes (Romeo and Weidenhamer, 1998). In the field, rye is usually plowed, mowed or dessicated, resulting in a mulch which suppresses germination and growth of weeds through physical and chemical effects. Evidence suggests that the allelochemicals present in rye can be transported to weed species through leaching of rye debris. Through the use of ground tissue and the capillary irrigation system, this bioassay successfully mimicked field conditions and at the same time, minimized the physical effects of the mulch upon weed germination and growth. Effects of light competition were removed by incorporating the rye mulch into the soil. In an attempt to remove any obvious indirect effects, such as microbial activity, steam sterilized soil was utilized. Despite the use of sterilized soil, there was abundant microbial growth. Since live donor plants were not utilized in this bioassay, there was no concern for nutrient competition between the donor and the indicator species. Choice of redroot pigweed as an indicator species was based upon the fact that it is problematic in the southeastern US and studies have demonstrated that these species are sensitive to rye aqueous extracts. The parameters to assess allelopathic activity among the rye genotypes are easy to measure and are suitable for rapid, high throughput screening. Many studies utilize pre-germinated seeds in order to reduce error associated with germination variability. For the sake of simplicity and rapidity, pre-germinated seed were not utilized. However, lack of reproducible results for emergence suggests that pre-germinated seed may allow better detection of variation among the genotypes.

Although this bioassay was able to detect differences in genotype performance for suppression of redroot pigweed fresh weight biomass there are improvements to be made to the design. It is important that the protocol is reproducible. The following changes will

improve the greenhouse bioassay: 1) An inert mulch should be utilized to separate any minute physical effects from chemical effects. 2) A more stringent randomization procedure should be used help reduce experimental error by balancing environmental effects in the greenhouse. 3) Soil media should be sieved to reduce clumping, thereby improving rye tissue dispersal in the media to prevent micro-environment conditions in the planting trays. 4) Attempts should be made to reduce microbial growth. Microbial growth on the soil surface of some flats may have resulted in allelochemical conversions which can effect growth of indicator species. 5) Slight changes in irrigation components are required to ensure even distribution of water through the soil column. 6) The use of a different media may be warranted if the response parameter of interest involves roots. Root growth is not suitable as a response to allelopathic activity in this study because of damage that may be incurred when removing seedlings from the soil.

In addition to allelopathy, high cover crop biomass is an important component of the weed suppressive ability of rye. Its environmental versatility and its potential use against herbicide resistant weeds suggest that investigation of genetic improvement for rye allelopathic activity is important (Przepiorkowski and Gorski, 1994). Future efforts should include incorporating rye varieties with high biomass into a breeding program (Burgos et al. 1999). Studies involving release, transport and uptake of known allelochemicals should be undertaken. Concurrent work for improvement of rye for allelopathy includes a petri dish bioassay and allelochemicals analysis.

Table 1. Pearson correlation coefficient, ρ, and associated P-values, indicating the relationship between results of two greenhouse bioassays.

Allelopathy measure	Location	ρ	P-value	r^2
emergence	Clayton	-0.02	0.92	-0.04
	Kinston	-0.05	0.81	0.00
fresh weight biomass	Clayton	0.34	0.10	0.12
	Kinston	0.37	0.07	0.10

Table 2. Analysis of variance table for the across location analysis of Redroot pigweed (*Amaranthus retroflexus*) fresh weight biomass.

Source	df	Mean square	p-value
loc	1	3.43×10^{-3}	<0.0001
block(loc)	2	1.17×10^{-5}	0.86
genotype	149	1.10×10^{-4}	0.28
loc*genotype	132	9.92×10^{-5}	0.06

Table 3. Etimates of variance components[1] and heritabilities (h^2) and standard errors for rye (*Secale cereale*) allelopathy based upon redroot pigweed (*Amaranthus retroflexus)* fresh weight biomass.

Location	σ_G^2	p-value	h^2 (per-plot basis) ± s.e.	h^2 (entry mean basis) ± s.e.
Clayton	1.70×10^{-5}	0.03	0.17 ±0.09	0.29 ±0.13
Kinston	1.90×10^{-5}	0.04	0.21 ±0.11	0.35 ±0.15

[1] $\sigma^2_{G,}$ genetic variance with p-value associated with the appropriate mean square

Table 4. Mean and range of redroot pigweed (*Amaranthus retroflexus*) fresh weight biomass. Data are presented as percent of control.

Location	Mean ± SD	Range
Clayton	16.75% ± 5.8	1.59-34.40%
Kinston	8.22% ± 3.07	2.32-22.72%

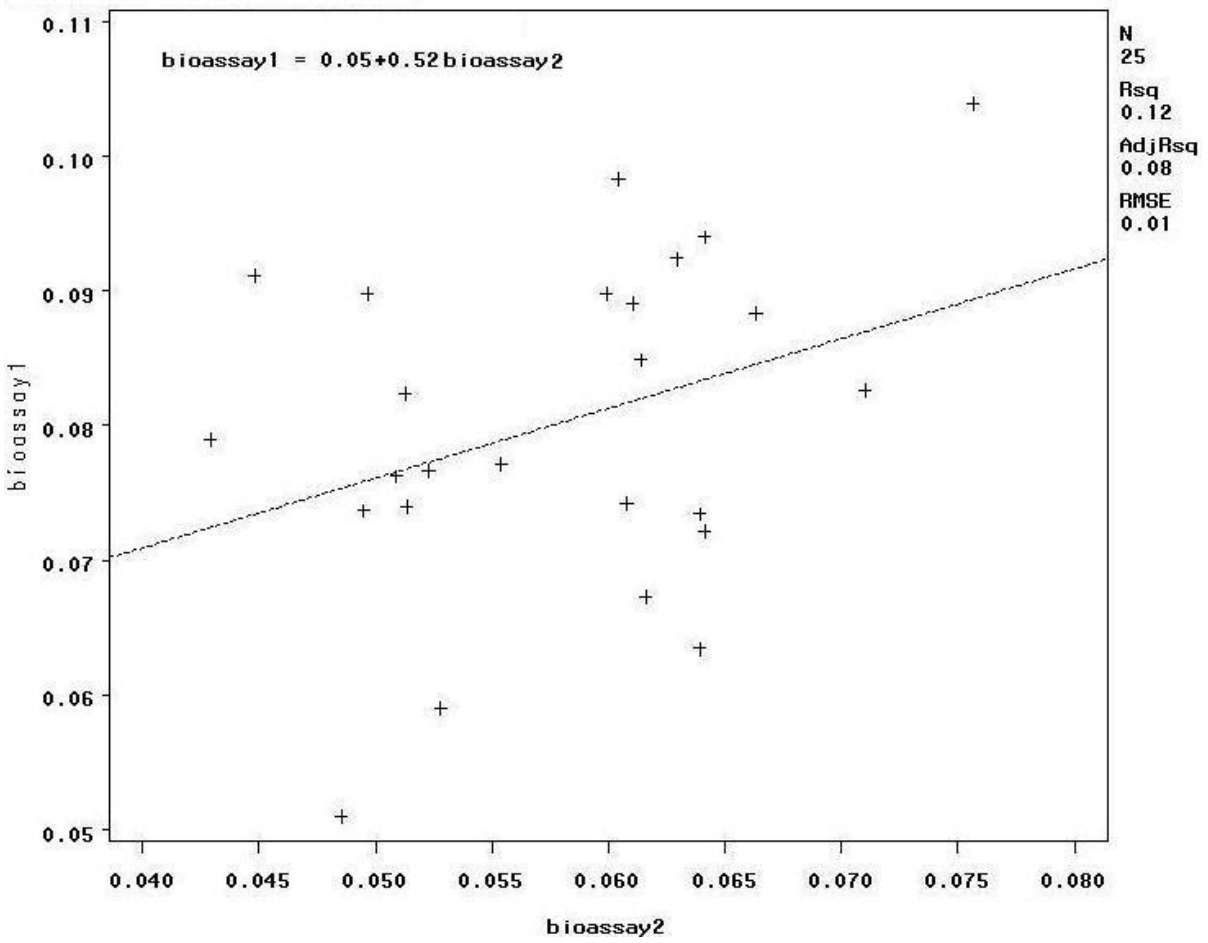

Figure 1. Correlation between the two greenhouse bioassays based on 25 genotypes grown at the Clayton location.

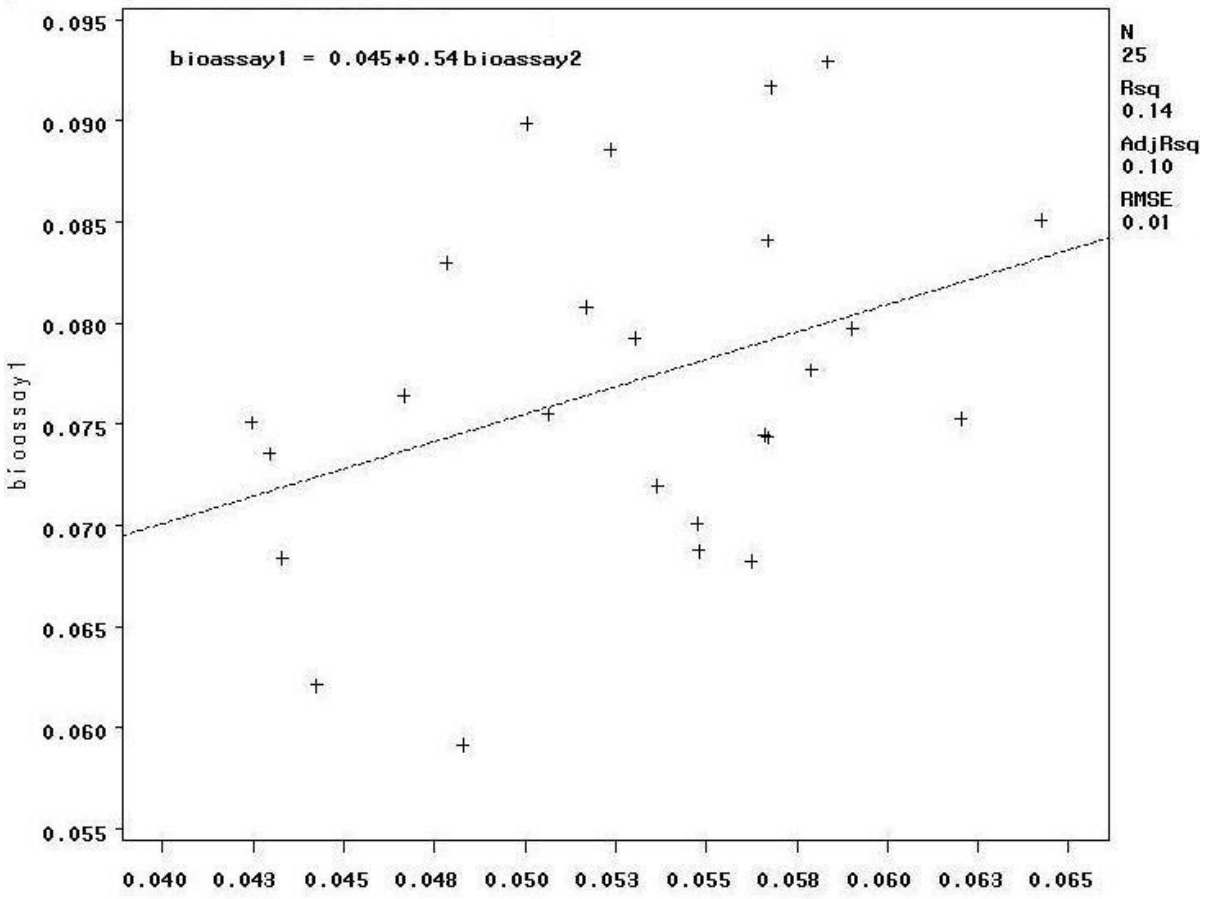

Figure 2. Correlation between the two greenhouse bioassays based on 25 genotypes grown at the Kinston location.

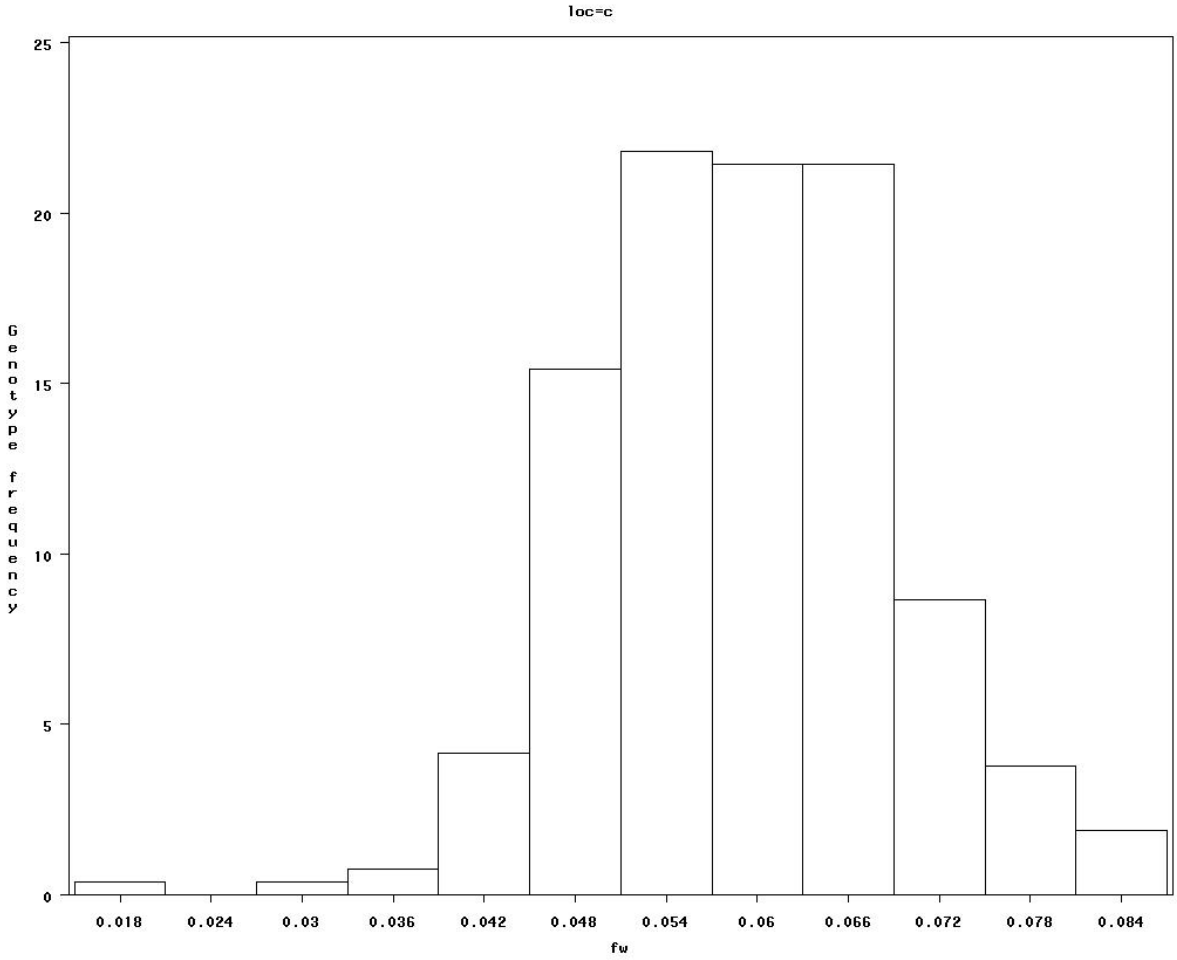

Figure 3. Frequency distribution displaying allelopathic effects of rye half-sib families on redroot pigweed (*Amaranthus retroflexus*) fresh weight biomass. The distribution represents genotypes grown at the Clayton location.

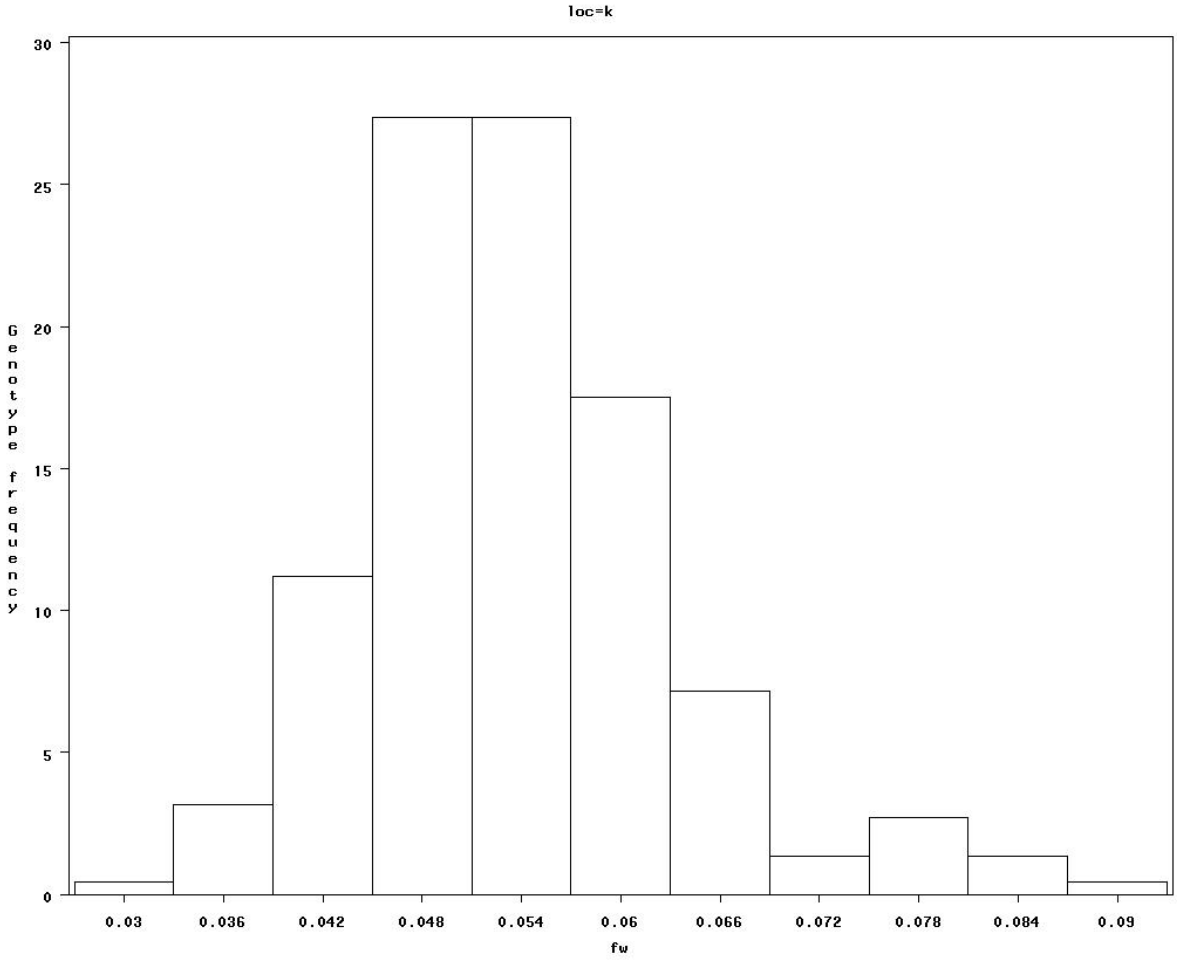

Figure 4. Frequency distribution displaying allelopathic effects of rye half-sib families on redroot pigweed (*Amaranthus retroflexus*) fresh weight biomass. The distribution represents genotypes grown at the Kinston location.

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Chapter III

Estimation of Genetic Parameters for Rye Allelopathy (*Secale cereale*)

Abstract

Variation in allelopathy has been observed in rye (*Secale cereale*), suggesting that improvement through conventional breeding methods could be successful. Redroot pigweed (*Amaranthus retroflexus*) germination, root length and fresh weight biomass were quantified to assess variation in allelopathic activity of rye. Half-sib families were grown at two North Carolina locations and were utilized to obtain estimates of genetic variance and heritability. All measures displayed normal distributions indicating that allelopathy in rye is a quantitative trait. Genetic variation among half-sib families was significant for most measures of redroot pigweed control. Estimates of narrow sense heritability ranged from 0.17-0.21 on per plot basis and 029-0.35 on an entry mean basis. Standard errors for heritability were 0.09-0.11 on a per plot basis and ranged from 0.13-0.15 on an entry mean basis. Based on the presence of significant variation and moderately low heritabilities, it was concluded that selection for allelopathy in rye may be effective. It was also concluded that methods for assessment of allelopathic activity in rye should be improved.

Keywords: Allelopathy, Rye, *Secale cereale*, crop breeding

Introduction

Allelopathy is an ecological phenomenon in which a "donor" plant species releases chemicals that effect germination and/or growth of a "receiver" species. Research has begun to exploit the allelopathic weed suppressive ability of some crop species (Olofsdotter 2001; Wu et al. 2000**)**. The possibility of exploiting allelopathy as an alternative to herbicides for weed management is important because of the occurrence of herbicide resistant weeds worldwide and growing concerns over the environmental effects of herbicide usage. Upon discovering significant variation among 538 accessions of cucumber (*Cucumis sativas*) for allelopathic activity on white mustard (*Brassica hirta*), Putnam and Duke (1974) concluded that a genetic pool exists for allelopathy and thus, selective breeding could be utilized to develop highly allelopathic cultivars. This finding led to germplasm screening efforts for allelopathic activity in several crops.

Cereal crops such as rice (*Oryza sativa*), wheat (*Triticum aestivum*), rye (*Secale cereale*), sorghum (*Sorghum bicolor*) and barley (*Hordeum vulgare*) are known to be allelopathic to several agronomic weed species and the potential for improvement of their allelopathic activity through selective breeding is currently being researched (Olofdotter 1996; Wu 2000; Chase et al. 1991; Nimbal et al. 1996; Bertholdsson 2005; Kim and Shin 2008). Although allelopathic activity was observed in other non-cereal crop species including alfalfa (*Medicago sativa*), sunflower (*Helianthus annuus*) and cucumber, it has been suggested that cereals are more allelopathic and have better potential for application in agricultural systems (Xuan and Tsuzuki 2002; Leather 1983; Putnam and Duke 1974; Sánchez-Moreiras et al 2004).

To date, the greatest success towards development of an allelopathic crop cultivar has been achieved in rice (Belz 2007). Evidence exists for variation in allelopathic activity of rice against several weed species including barnyardgrass (*Echinochloa crus-galli*), ducksalad (*Heteranthera limosa,* redstem (*Ammannia coccinea*) and variable flatsedge (*Cyperus difformis*) (Dilday et al. 1998; Ebana et al. 2001b; Hassan et al 1997). Four main-effect and

epistatic QTL have been located on three chromosomes that are correlated with a reduction in barnyardgrass root growth (Jensen et al. 2001). The QTL accounted for 35% of the phenotypic variation in the population. Epistatic effects explained much less of the phenotypic variation (Jensen et al 2001). The broad sense heritability for allelopathic activity against barnyardgrass was estimated to be 0.85 which indicates that selection could be effective for developing more allelopathic varieties (Olofsdotter 2001). Ma et al. (2006) developed a rice line, K21, which was highly allelopathic against barnyardgrass and also displayed desirable agronomic traits. This was the first reported attempt to develop an allelopathic rice cultivar (Ma et al 2006).

There is evidence for differential production of allelochemicals among wheat genotypes (Copaja et al. 1991; Nicol et al. 1992; Wu et al. 2001). Variation in allelopathic activity of wheat has been reported against ryegrass (*Lolium perenne*), Japanese brome (*Bromus japonicas*), common lambsquarters (*Chenopodium album* L.) and redroot pigweed (*Amaranthus retroflexus*) (Wu et al. 2000; Spruell 1984; Zhang et al. 2005). Wu et al. (2003) examined a wheat population derived from a cross between a cultivar with low allelopathic activity, Suncov, and the highly allelopathic cultivar, Tasman. With the use of restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and microsatellite (SSRs) markers, they found two QTL associated with the allelopathic effects of wheat root exudates on ryegrass. Allelopathic activity, measured as ryegrass root length as percent of control, was normally distributed, indicating that allelopathy is a quantitative trait (Wu et al. 2003). Analysis of 15 wheat accessions commonly grown in the Loess Plateau in China resulted in a mean heritability estimate of 0.83 and significant genetic variation was detected for allelopathic activity on ryegrass (Zuo et al. 2007).

Sorghum germplasm screening indicated significant variation exists for production of sorgoleone, a toxic allelochemical exuded from roots, among 25 sorghum genotypes (Nimbal et al. 1996). Sorgoleone concentrations among the genotypes ranged from 0.7-17.8 mg/g root fresh weight (Nimbal et al. 1996). Another study found differences in the amount of

root exudate from seven accessions of sorghum ranging from 0.5-14.8 mg/g root fresh weight (Czarnota et al. 2003). It is interesting to note that the lowest and highest levels were found in the two weedy accessions, Shattercane and Johnsongrass, respectively, while the five cultivated accessions ranged only from 1.33-1.8 mg/g freshweight (Czarnota et al. 2003). These results support the suggestion that allelopathic variation has been reduced in cultivated germplasm because of selection of other agronomic traits such as yield (Putnam and Duke 1974; Bertholdsson 2004).

Lin et al. (2005) screened 65 barley accessions for variation in allelopathic activity against lettuce (*Lactuca sativa*). Results indicated significant variation among the accessions for inhibition of lettuce growth. Inter-simple sequence repeat (ISSR) analysis detected genetic polymorphisms within the barley accessions that indicated that accessions of the same geographical origin could be grouped together based on allelopathic activity (Lin et al. 2005). Significant variation has also been detected for allelopathic activity of barley on annual ryegrass (Bertholdsson 2004).

Rye is a known to be allelopathic to several weed species including wild oats (*Avena fatua* L.), proso millet (*Panicum miliaceum* L.), barnyardgrass, palmer amaranth (*Amaranthus palmeri*), large crabgrass (*Digitaria sanguinalis)*, goosegrass (*Eleusine indica*) and redroot pigweed (*Amaranthus retroflexus*) (Pérez and Ormeno-Núnez 1993; Barnes and Putnam 1986; Burgos and Talbert 1999; Reberg-Horton et al. 2005). Despite substantial evidence for rye allelopathy, little research has been carried out to determine the potential for genetic enhancement of allelopathic activity. Rye is already a successful cover crop because of abundant biomass which can physically impede weed seedling emergence and can interfere with light interception (Teasdale and Mohler 2000). High allelopathic activity could improve the overall effectiveness of rye as a cover crop. There is also evidence for rye allelopathic against some herbicide resistant weeds. Przepiorkowski and Gorski (1994) found three triazine resistant biotypes of barnyardgrass, willowherb (*Epilobium ciliatum*) and horseweed (*Conyza canadensis*) were susceptible to the allelopathic activity of rye. Last, the study of the

genetics of rye allelopathy could lead to important findings for future research in rye or other Poaceae species.

The suspected allelochemicals in rye and wheat belong to the same family of compounds and share the same biosynthetic pathways. This suggests that the observed variation in allelochemical production and activity in wheat might also be present in rye. Cyclic hydroxamic acids have been implicated as the primary allelochemicals produced by rye, wheat and maize (*Zea mays*) (Niemeyer 1988). The most abundant hydroxamic acids found in rye are the benzoxazinoids, (2-O-β-D-glucopyranosyl-4-hydroxy-(2H)-1,4-benzoxazin-3(4H)-one (DIBOA-glycoside), 2,4-dihydroxy-(2H)-1,4-benzoxazin-3(4H)-one (DIBOA) and benzoxazilon-2-one (BOA). The primary benzoxazinoids produced in maize and wheat are DIMBOA-glycoside, DIMBOA and MBOA. The glycoside form is most stable and is stored in plant tissue. Upon injury to the plant, the glycoside is cleaved by a β-glucosidase, releasing the less stable and more toxic DIBOA. Hydrolytic conversion of DIBOA produces BOA (Fomsgaard 2004; Hofman and Hofmanová 1971).

Niemeyer and Jerez (1997) suggested that biosynthesis of hydroxamic acids in hexaploid wheat (*Triticum aestivum*, 2n=6x=42) is under multigenic control. Nomura et al. (2005) later reported that the biosynthesis of benzoxazinoids in hexaploid wheat is controlled by five homoeologous genes, *TaBx1-TxBx5,* from each of the three genomes. These genes are orthologous to barley genes, *HlBx1-HlBx5,* and maize genes, *Bx1-Bx5* (Nomura et al. 2005). In maize, BX1 is the branch enzyme which converts indole-3-glycerol phosphate to indole. The genes *Bx2-Bx5* take part in the synthesis of DIBOA from indole (Frey et al. 1997). A strong homology is shared between the *HlBx1-HlBx5* genes of barley and the *Bx1-Bx5* genes of maize - 72%, 80%, 76%, 81%, and 78%, respectively (Grun et al. 2005).

To study the genetics underlying the biosynthesis of DIMBOA, Niemeyer and Jerez (1997) substituted chromosomes of cv. Cheyenne into monosomic lines of cv. Chinese Spring, a high DIMBOA producing cultivar. They found that genes on chromosomes 4A and 4B may

be involved in the conversion of DIBOA into DIMBOA. They also suggest that gene(s) on chromosome 5B may be involved in the accumulation of DIMBOA through a pathway without DIBOA as a precursor. The substitution of chromosome 5D led to reduced accumulation of DIBOA and DIMBOA which suggests that gene(s) on this chromosome might inhibit biosynthesis of benzoxazinoids (Niemeyer and Jerez 1997).

Understanding the genetic control of allelochemical production is important because the biosynthesis and bioactivity of allelochemicals is the basis for allelopathy. Quantified levels of allelochemicals could be an indication of the suppressive ability of a donor species. However, there are other factors involved in allelopathic activity which must be included for a thorough investigation of genetic control. Interactive or cumulative effects in nature may not be captured through analytical chemistry techniques. The best approach for study is not only chemical analysis but also the simultaneous screening of germplasm through bioassays and/or field experiments (Wu et al 2001).

The objective of this research was to determine genetic parameters associated with allelopathy in rye (*Secale cereale)* against redroot pigweed through the evaluation of a synthetic population of half-sib families. For this study, a petri dish bioassay and a novel greenhouse bioassay were used to screen rye in order to obtain estimates of variance components and narrow sense heritability for the allelopathy trait. In conjunction with a rye linkage map, this study could assist in understanding the genetic control of allelopathy in rye and thereby lead to manipulation of the trait and improved selection methods for cultivar development.

Materials and Methods

Test material

Seed of 15 rye accessions, which were previously identified as being highly allelopathic, were obtained from the USDA's National Small Grains Collection (Table 1) (Reberg-Horton,

unpublished data). These accessions were each crossed with Wrens Abruzzi, a rye variety commonly grown as a cover crop in the southern United States. An equal number of hybrid seeds from each cross were bulked. This bulked population was advanced for two generations by open pollination in isolation. During the second generation of open pollination 150 plants were randomly chosen and harvested individually to obtain seed of 150 half-sib families.

In October 2006, the 150 half-sib families and checks were planted in duplicate randomized complete block design experiments at research stations in Kinston, and Clayton, NC. Soil in Kinston, NC is characterized as fine loamy sand. Soil in Clayton, NC is characterized as Norfolk loamy sand. In spring 2007, shoot tissue was harvested at flag leaf emergence. Plant material was forced-air dried at 54°C for 3-4 days, ground to pass through a 2-mm mesh screen and stored in airtight bags in the dark prior to analyses.

Redroot pigweed (*Amaranthus retroflexus*) was chosen as an indicator (i.e. receiver) species for rye allelopathy in this study because it is problematic weed in the southeastern United States and has been shown to be susceptible to rye allelochemicals (SWSS 2006; Reberg-Horton et al. 2005). The measures utilized to quantify allelopathic activity of rye included redroot pigweed germination, root length and above ground fresh weight biomass.

Petri dish bioassay

A petri dish bioassay was utilized to compare differences among the half-sib families in their ability to reduce root length and germination of redroot pigweed. Aqueous extracts were prepared by weighing one gram of tissue into a 50 mL plastic centrifuge tube, adding 40 mL of deionized water and mixed on a shaker for one hour. Samples were centrifuged at 3800rpm for eight minutes and vacuum filtered through medium porosity filter paper (Fisherbrand, Pittsburg, PA). The rye extract was stored at 5°C and remaining plant material was discarded.

Petri dish bioassays were carried out in growth chambers in the Phytotron at North Carolina State University. Eight mL of extract were placed into a glass petri dish containing seed germination paper. Twenty five redroot pigweed seeds were arranged at equidistance on the soaked paper. Deionized water was used as a control. Each petri dish was sealed with parafilm to prevent moisture loss and placed into a growth chamber. Environmental conditions inside the chamber were 30°C at 75% relative humidity and constant light. After 96 hours, pigweed germination and root length to the nearest millimeter were recorded. Root length was calculated on a per seedling basis and the calculation for each half-sib family was:

total root length / # of seedlings germinated

Greenhouse bioassay

A greenhouse bioassay system was utilized to assess variation in redroot pigweed fresh weight biomass among the half-sib rye families. The greenhouse bioassay was a complete randomized block design. Twenty grams of ground dry rye tissue was mixed with six hundred grams of steam-sterilized soil. The soil-rye mixture was placed into 5 x 7" planting trays on top of a one inch layer of proprietary soil blend (Fafard Inc., Agawam, MA).

One hundred seed of redroot pigweed were planted in separate furrows in each tray at a depth of one centimeter. Seed were evenly dispersed and care was taken to avoid clumping. A capillary irrigation system was employed to prevent leaching of allelochemicals while maintaining uniformly moist conditions throughout the soil.

Data were collected two weeks after planting. Fresh weight biomass (grams) was calculated on a per seedling basis and the calculation for each half-sib family was:

total shoot fresh weight / # of seedlings emerged

Data analysis

All data were square root transformed to remove heterogeneity of variance. Data were subjected to analysis of variance using PROC GLM and PROC MIXED in SAS version 9.1 (SAS Institute 2002**)**. The model used for the analysis of variance was:

$$y = \mu + E_i + R_{ji} + G_k + GE_{ik} + e_{ijk}$$

where y is the phenotypic value for the allelopathy trait of a genotype, μ is the overall experimental mean, E_j is the location effect R_{ji} is the block effect, G_k is genotype effect, $(GE)_{ik}$ is the genotype x environment interaction effect and e_{ijk} is the residual error. The model used for within location analysis of variance was:

$$y = \mu + R_i + G_j + e_{ijk}$$

where y is the phenotypic value for the allelopathy trait of a genotype, μ is the overall experimental mean, R_i is the block effect, G_j is the genotype effect and e_{ij} is the residual error. Heritability estimates were made on a per-plot basis and on an entry mean basis according to Holland et al. (2003). Correlations between redroot pigweed germination, root length and fresh weight were calculated using PROC CORR and PROC REG in SAS.

Results

Petri dish bioassay: Redroot pigweed germination

Across location analysis of variance detected a significant genotype x environment interaction (Table 2). The genotype x environment interaction was caused by unstable genotype performance across locations (data not shown). The genotype main effect was not significant for redroot pigweed germination. The block within location effect was significant.

Significant variation for germination was detected among the genotypes at the Kinston location (Table 3). The frequency distribution of redroot pigweed root length was approximately normal which suggests that weed suppressive ability may be a quantitative trait (Figure 1). Narrow sense heritability estimates were low on a per plot basis (h^2=0.19, s.e.=0.11) and moderately low on an entry mean basis (h^2=0.31, s.e.=0.15). The block effect was highly significant for the Clayton location and may have prevented detection of variation among the genotypes (data not shown). The coefficient of variation was higher and R^2 lower at the Kinston location than at the Clayton location (Table 4). This indicates a higher magnitude of variability among the Clayton genotype responses relative to the mean response than that which was measured in the Kinston location.

The Kinston location data for redroot pigweed germination, expressed as percent of control, ranged from 0.00=154.55% (mean=80.69% ±51.35) (Table 5).

Petri dish bioassay: Redroot pigweed root length

Across location analysis of variance detected a significant genotype x environment interaction (Table 2). The genotype x environment interaction was caused by unstable genotype performance across locations (data not shown). The genotype main effect was not significant for redroot pigweed root length. The block within location effect was significant.

The results of the within location analysis of redroot pigweed root length were very similar to results obtained in the germination analysis. Within location analysis of variance detected significant variation among the genotypes at the Kinston location (Table 3). The frequency distribution of redroot pigweed root length for the genotypes grown at the Kinston location was approximately normal (Figure 2).

Narrow sense heritability estimates for the Kinston location were low on a per plot basis (h^2=0.20, s.e.=0.10) and moderately low on an entry mean basis (h^2=0.32, s.e.=0.14). The block effect was highly significant for the Clayton location (data not shown). The coefficient

of variation was notably higher and the R^2 lower at the Clayton location than at the Kinston location (Table 4).

Redroot pigweed root length data, expressed as percent of control, ranged from 0.00=155.63% (mean=46.95% ± 31.99) (Table 5). Although the range is similar to germination, the overall mean root length was lower (71.06% ±33.55). This suggests that root length may be more susceptible to allelopathic activity of rye extracts than germination and thus, may be a better measure for quantifying allelopathy in a petri dish bioassay.

Greenhouse bioassay: Redroot pigweed fresh weight biomass

Across location analysis of variance did not detect a significant genotype main effect for redroot pigweed fresh weight biomass (Table 2). Location effect was significant.

Clayton and Kinston data each displayed an approximately normal distribution (Figures 3 and 4). Significant variation was detected among genotypes grown at both locations (Table 3). Narrow sense heritability estimates on a per-plot basis were low for fresh weight biomass at the Clayton location (h^2=0.17, s.e.=0.09) and the Kinston location (h^2=0.21, s.e.=0.11). Narrow sense heritability estimates on an entry mean basis were moderately low for the Clayton location (h^2=0.29, s.e.=0.13) and the Kinston location (h^2=0.35, s.e.=0.15).

Redroot pigweed fresh weight biomass, expressed as percent of control, ranged from 1.59-34.40% (mean=16.75% ± 5.80) and 2.32-22.72% (mean=8.22% ± 3.07) for the genotypes grown at the Clayton and Kinston locations, respectively (Table 5). Mean fresh weight biomass was lower at the Kinston location than the Clayton location indicating a higher suppressive ability among genotypes grown in that location.

Correlations

Correlations were positive and significant between redroot pigweed germination and root length (Figure 5). There were no significant correlations between fresh weight biomass and germination or root length (Table 6). This suggests that genotype performance based on fresh weight biomass is not a good indicator of results for germination or root length. The lower coefficient of variation and higher R^2 value for fresh weight biomass indicates that this method of quantifying allelopathic activity may be more appropriate than germination or root length.

Discussion

The purpose of this research was to determine genetic parameters associated with allelopathy in a population of rye half-sib families. This type of preliminary research is useful to assist plant breeders in developing breeding strategies. Estimations of genetic variance and heritability can be used to predict expected genetic gain from selection (Bernardo 2002). Our results indicated that selection for allelopathy in rye could be successful. Although genotype effect was not significant across locations for any measure of allelopathy, genetic variation was significant in one or both locations for each measure.

The significant genotype x environment interaction detected in the redroot pigweed germination analysis may be due to biological differences between the two locations in which the rye families were grown. The various abiotic and biotic factors present in dissimilar environments may differentially influence regulation of genes involved in biosynthetic pathways (Bernardo 2002). This interaction can result in differential accumulation of allelochemicals in the rye tissue grown at the two environments (Rice 1984). The phenotypic expression of these environmental influences on allelochemical biosynthesis is evident in the significant genotype x environment interaction effect for redroot pigweed germination.

Significant genetic variation was detected at the Kinston location for all measures of allelopathy but only fresh weight biomass variation was significant at the Clayton location. One possible explanation for this result is that the fresh weight biomass is a more sensitive measure of allelopathy than seedling germination and root length and thus, is more likely to detect slight variations in activity against redroot pigweed growth. The notion that one screening method may be more sensitive than another is important to a breeding program. It is the ability to assess variation in activity and to rank genotype performance which will allow progress to be made in selection. Fresh weight biomass data was obtained from the greenhouse bioassay. This system subjected redroot pigweed seeds and seedlings to rye tissue, and allelochemicals which leached into the soil, for two weeks. Seed germination and root length were obtained from the petri dish bioassay which exposed redroot pigweed seeds and seedlings to rye allelochemicals for only 96 hours. The longer allelochemical exposure time may have been necessary to discern true allelopathic potential of each genotype.

Narrow sense heritability estimates for allelopathy in rye were low to moderately low but were similar to those found for yield in maize (Hallauer and Miranda 1988). Standard errors were all near 50% of their associated heritability estimates. Heritability estimates and standard errors are expected to improve as phenotypic screening techniques are refined. In addition, improvement in screening methods will allow the detection of associations between allelopathic phenotypes and molecular markers. This is important to locate QTL involved with allelopathy and for the use of marker assisted selection (MAS). MAS will reduce time and labor involved with phenotypic screening of crops for allelopathic activity against agronomic weed species.

Future work for this research includes quantification of hydroxamic levels through gas chromatograph analysis. In addition, a complete analysis of a second year of field grown tissue will be used to estimate genotype x year and genotype x location x year interaction.

Table 1. List of highly allelopathic USDA accessions utilitized to develop the population of rye (*Secale cereale*) half-sib families.

Accession	Origin
PI 241285	Brazil
PI 254820	Austria
PI 260055	Ukraine
PI 280834	Russian Federation
PI 280836	Russian Federation
PI 290444	Hungary
PI 294794	Bulgaria
PI 323370	Spain
PI 326286	Kazakhstan
PI 349913	Macedonia
PI 374458	Macedonia
PI 390353	Yugoslavia
PI 205222	Turkey
PI 362400	Yugoslavia
PI 535821	Germany

Table 2. Analysis of variance for allelopathy in rye (*Secale cereale*) half-sib families grown at Kinston and Clayton , NC. Redroot pigweed (*Amaranthus retroflexus)* growth parameters were utilized to assess rye allelopathy.

	Germination			Root length			Fresh weight biomass		
Source	df	Mean square	p-value	df	Mean square	p-value	df	Mean square	p-value
loc	1	6.35	0.08	1	0.03	0.79	1	3.43×10^{-3}	<0.0001
block(loc)	2	107.80	<0.0001	2	18.33	<0.0001	2	1.17×10^{-5}	0.86
genotype	149	2.33	0.83	149	0.37	0.68	149	1.10×10^{-4}	0.28
loc*genotype	141	2.73	0.04	141	0.47	0.11	132	9.92×10^{-5}	0.06

Table 3. Estimates of variance components[1] and heritabilities (h^2) and standard errors for rye (*Secale cereale*) allelopathy based upon redroot pigweed (*Amaranthus retroflexus)* growth parameters.

Allelopathy measure	Location	σ_G^2	p-value	h^2 (per-plot basis) ± s.e.	h^2 (entry mean basis) ± s.e.
Germination	Kinston	0.11	0.03	0.19 ±0.11	0.31 ±0.15
Root length	Kinston	0.07	0.05	0.19 ±0.10	0.32 ±0.14
Fresh weight biomass	Clayton	1.70×10^{-5}	0.03	0.17 ±0.09	0.29 ±0.13
	Kinston	1.90×10^{-5}	0.04	0.21 ±0.11	0.35 ±0.15

[1] $\sigma^2_{G,}$ genetic variance with p-value associated with the appropriate mean square

Table 4. Coeffecients of variation and R^2 values associated with the within location analyses of rye (*Secale cereale*) allelopathy. Redroot pigweed (*Amaranthus retroflexus)* growth parameters were utilized to assess rye allelopathy.

Allelopathy measure	Location	C.V.	R^2
Germination	Clayton	34.04	0.64
	Kinston	27.49	0.70
Root length	Clayton	39.43	0.60
	Kinston	31.57	0.69
Fresh weight biomass	Clayton	15.30	0.61
	Kinston	15.75	0.72

Table 5. Mean and range of rye (*Secale cereale*) allelopathic activity against redroot pigweed (*Amaranthus retroflexus)*. Data are expressed as percent of control.

Allelopathy measure	Location	Mean ± SD	Range
Germination	Kinston	80.69 ± 51.35	0.00-154.55
Root length	Kinston	46.95 ± 31.99	0.00-155.63
Fresh weight biomass	Clayton	16.75 ± 5.8	1.59-34.40
	Kinston	8.22 ± 3.07	2.32-22.72

Table 6. Correlation and (in parentheses) p-values of redroot pigweed (*Amaranthus retroflexus)* growth measures indicating rye (*Secale cereale*) allelopathy.

	length	fresh weight biomass
germination	0.92 (<0.0001)	0.01 (0.94)
length		0.01 (0.94)

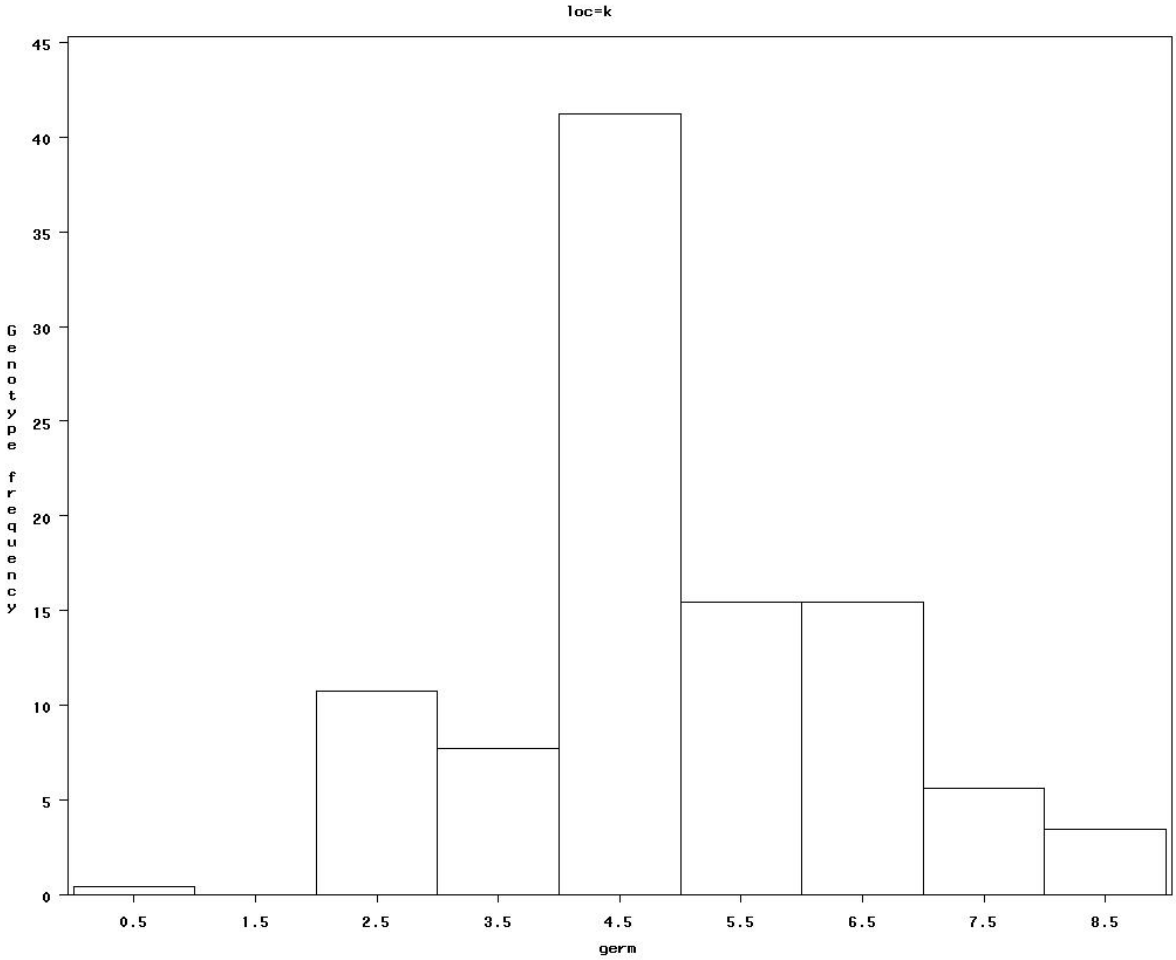

Figure 1. Frequency distribution displaying allelopathic effects of rye (*Secale cereale*) half-sib families on redroot pigweed (*Amaranthus retroflexus*) germination. The distribution represents genotypes grown at the Kinston location.

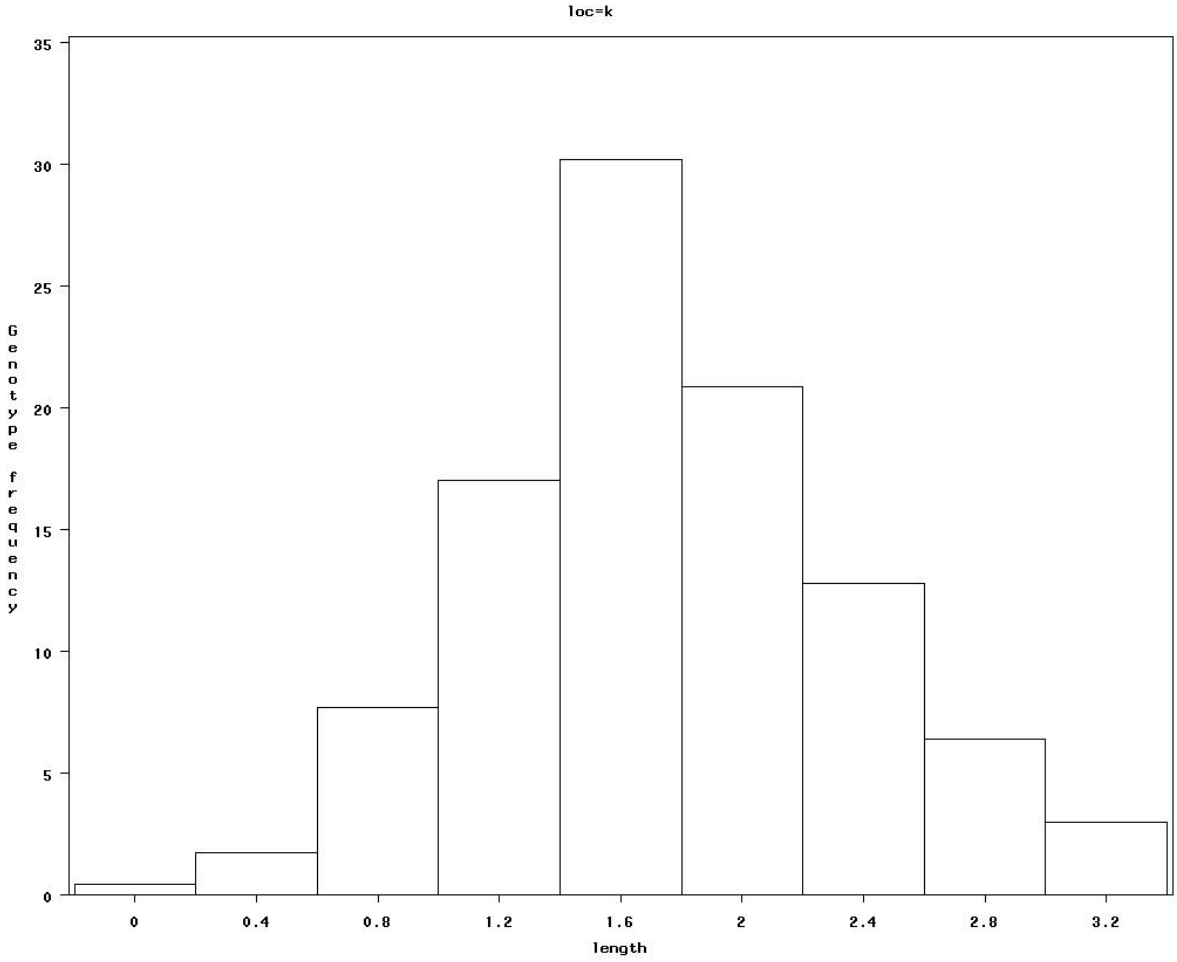

Figure 2. Frequency distribution displaying allelopathic effects of rye (*Secale cereale*) half-sib families on redrood pigweed (*Amaranthus retroflexu)s* root length. The distribution represents genotypes grown at the Kinston location.

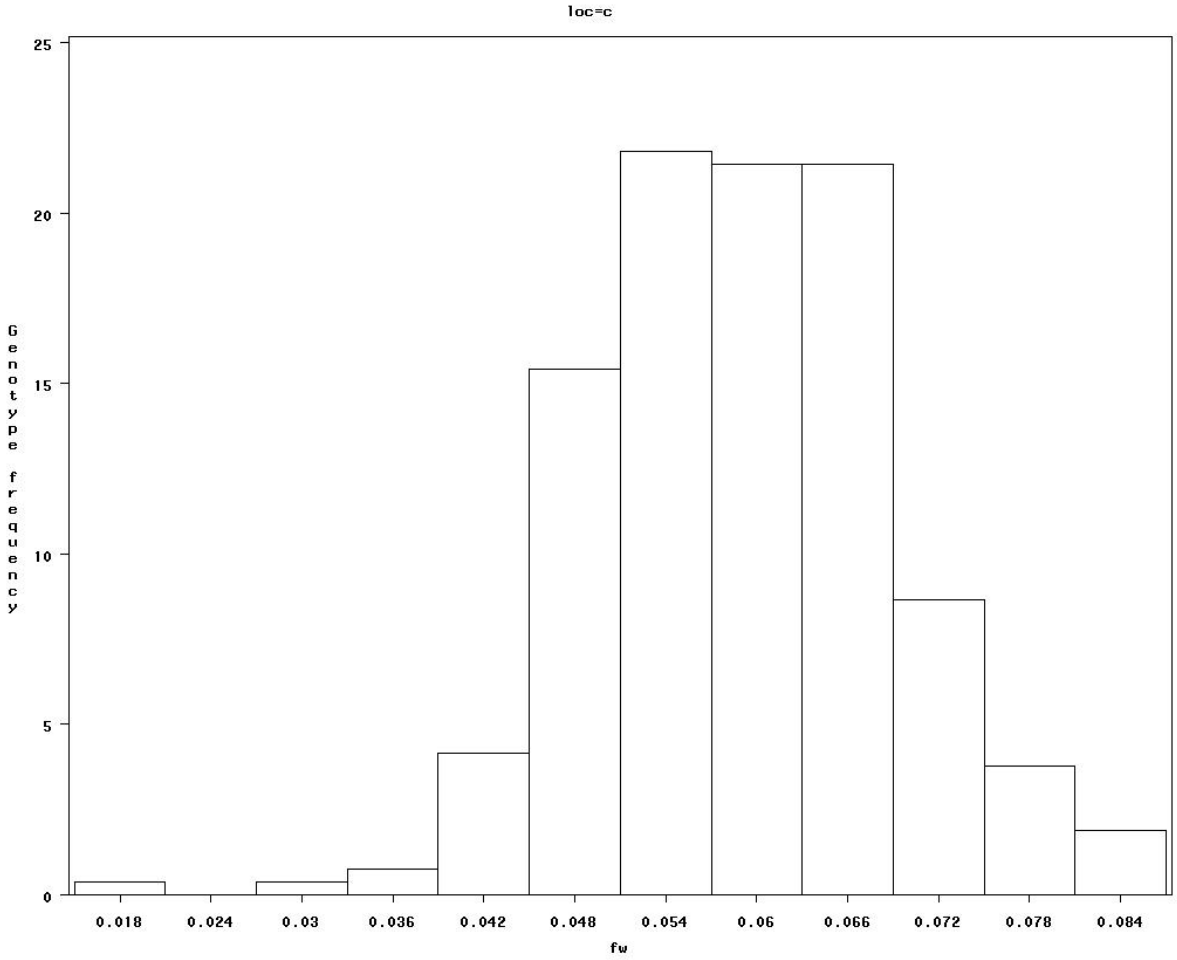

Figure 3. Frequency distribution displaying allelopathic effects of rye (*Secale cereale*) half-sib families on redroot pigweed (*Amaranthus retroflexus)* fresh weight biomass. The distribution represents genotypes grown at the Clayton location.

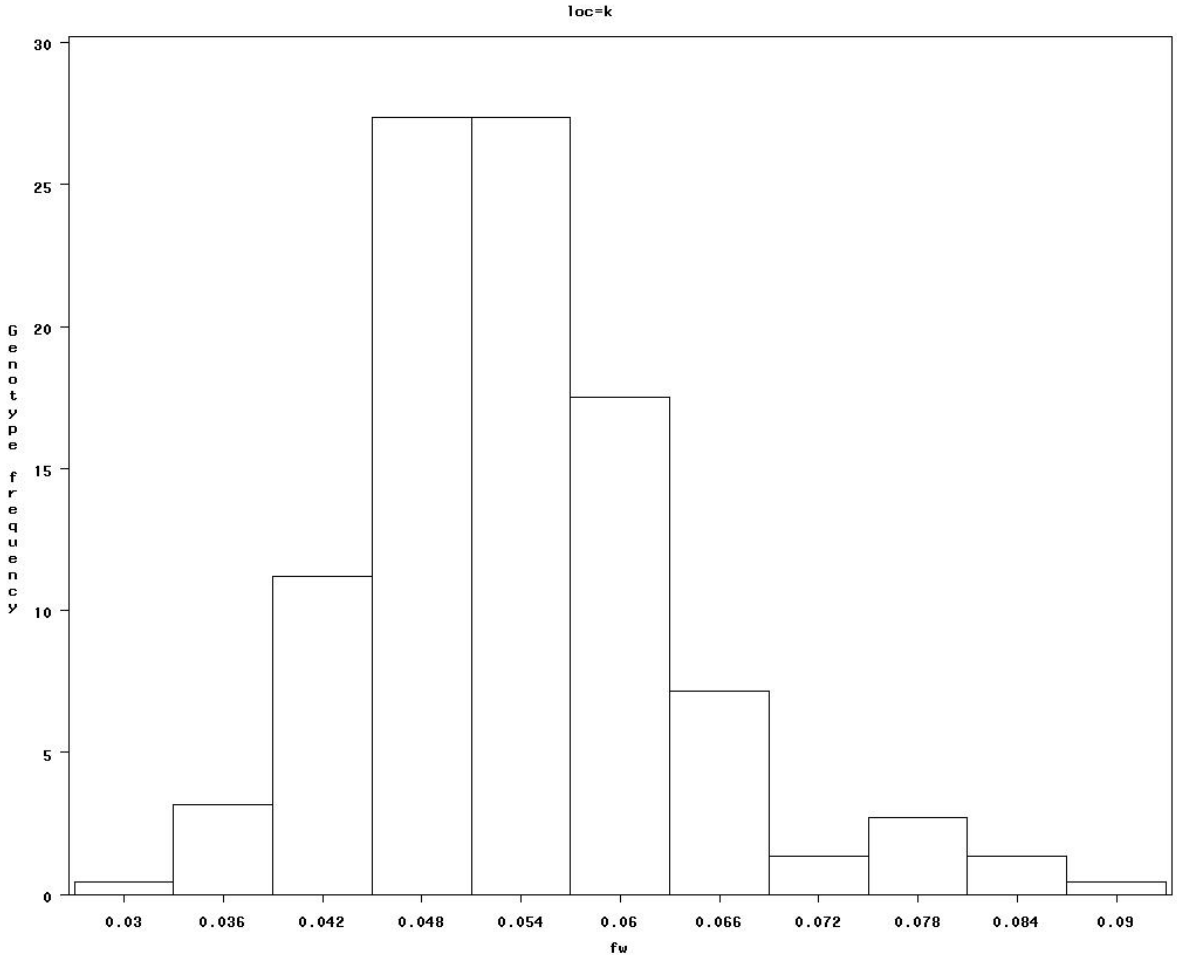

Figure 4. Frequency distribution displaying allelopathic effects of rye (*Secale cereale*) half-sib families on redroot pigweed (*Amaranthus retroflexus)* fresh weight biomass. The distribution represents genotypes grown at the Kinston location.

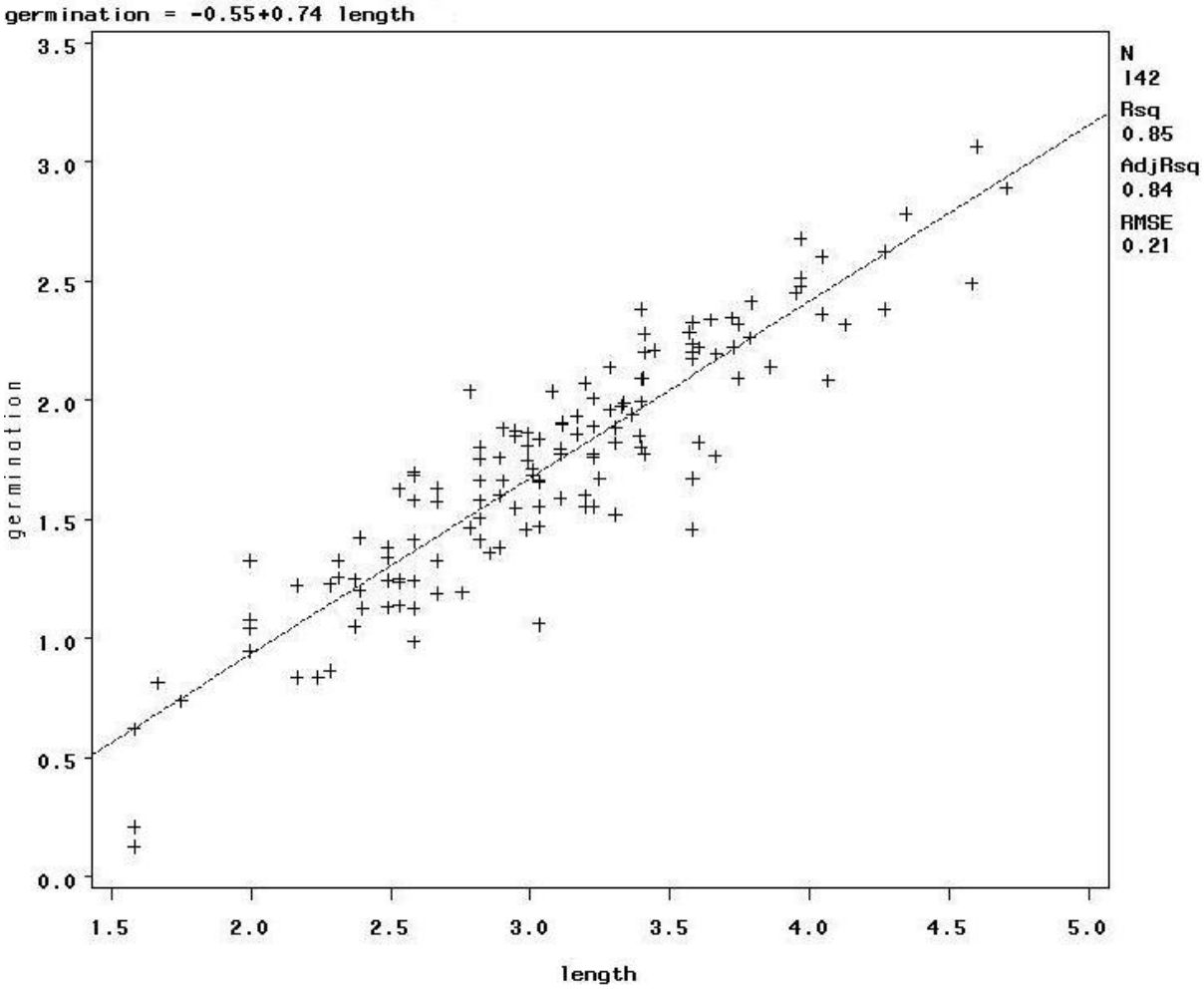

Figure 5. Correlation between redroot pigweed (*Amaranthus retroflexus)* germination and root length measured in the half-sib population of rye using the petri dish bioassay.

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APPENDIX

Appendix A. Correlation between the greenhouse bioassays based upon redroot pigweed (*Amaranthus retroflexus*) emergence (Clayton data set).

The CORR Procedure

2 Variables: bioassay1 bioassay2

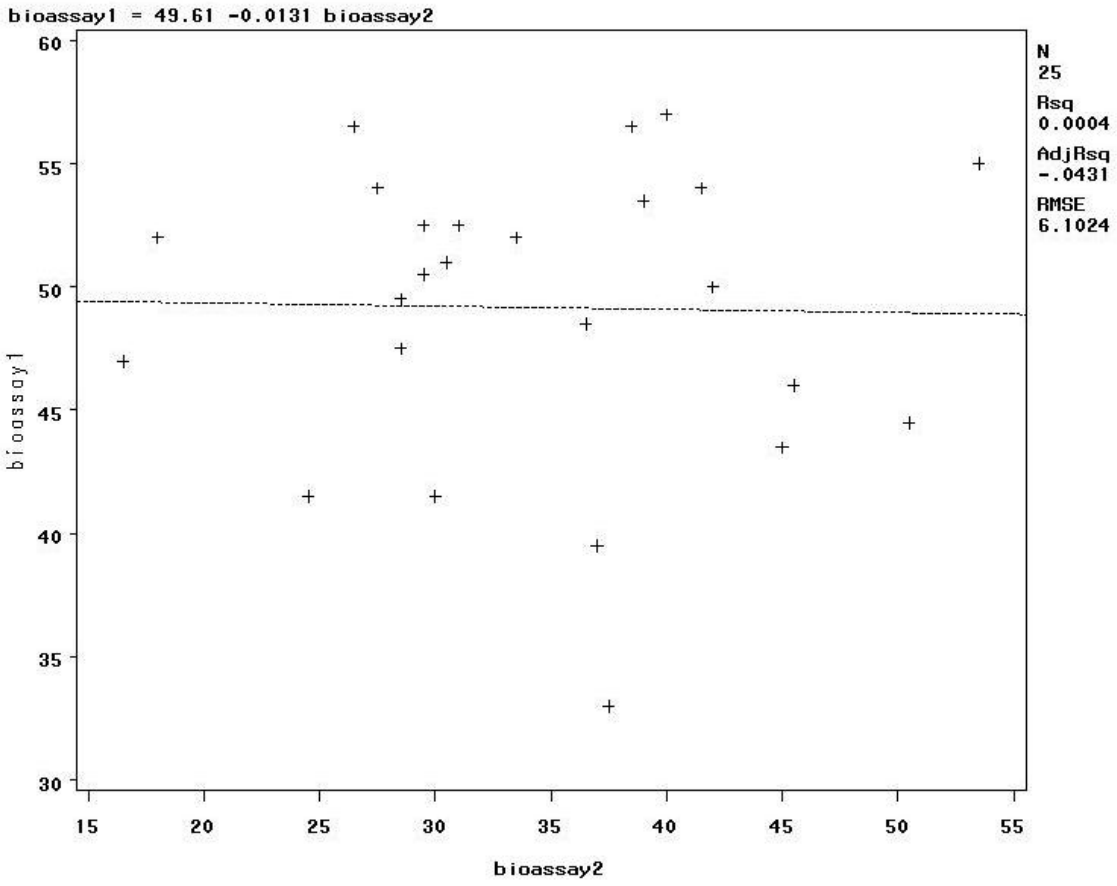
Simple Statistics

Variable	N	Mean	Std Dev	Sum	Minimum	Maximum
bioassay1	25	49.1600	5.9752	1229.0000	33.0000	57.0000
bioassay2	25	34.4200	9.1739	860.5000	16.5000	53.5000

Pearson Correlation Coefficients, N = 25
Prob > |r| under H0: Rho=0

	bioassay1	bioassay2
bioassay1	1.0000	-0.0201 0.9241
bioassay2	-0.0201 0.9241	1.0000

Appendix A cont. Regression analysis of the greenhouse bioassays based upon redroot pigweed (*Amaranthus retroflexus*) emergence (Clayton data set).



Appendix A cont. Correlation between the greenhouse bioassays based upon redroot pigweed (*Amaranthus retroflexus*) emergence (Kinston data set).

The CORR Procedure

2 Variables: bioassay1 bioassay2

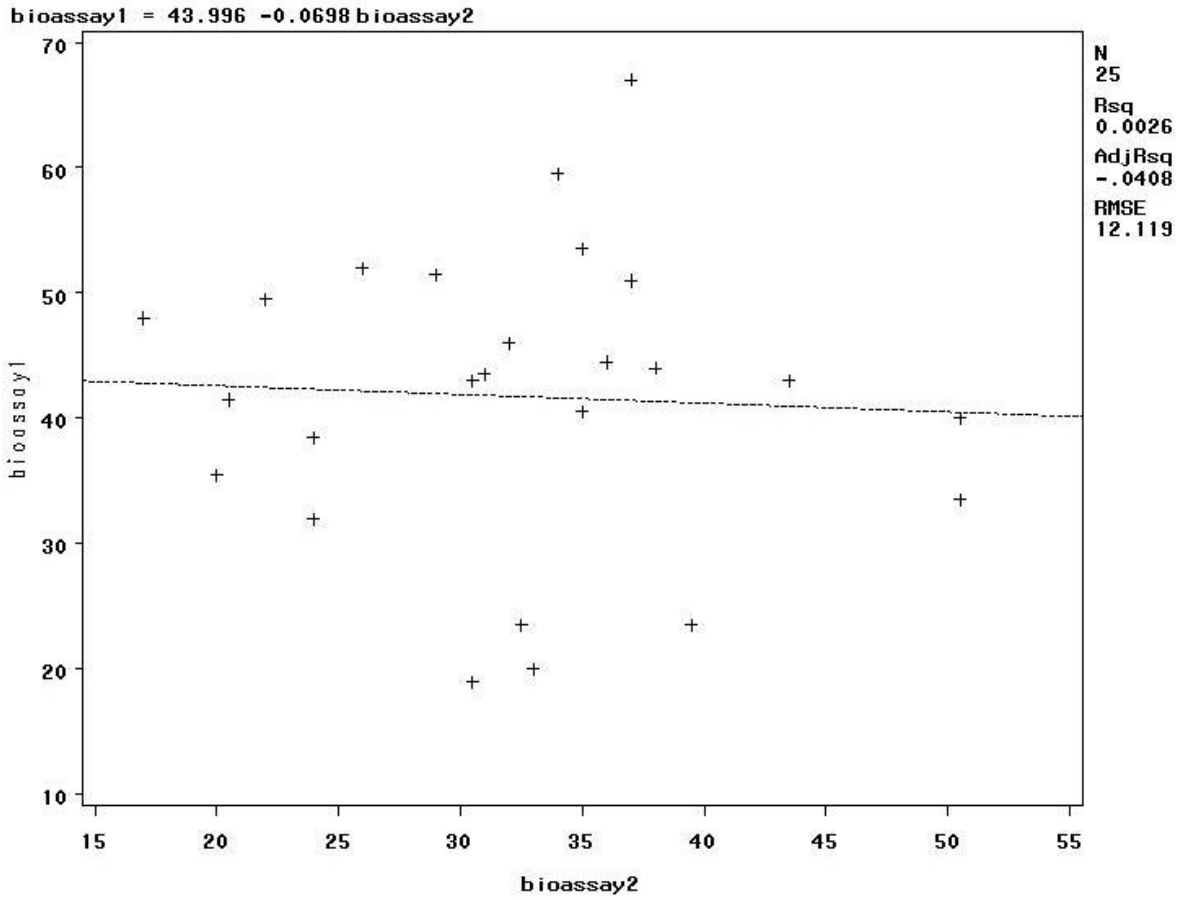
Simple Statistics

Variable	N	Mean	Std Dev	Sum	Minimum	Maximum
bioassay1	25	41.7400	11.8788	1044.0000	19.0000	67.0000
bioassay2	25	32.3200	8.6010	808.00000	17.0000	50.5000

Pearson Correlation Coefficients, N = 25
Prob > |r| under H0: Rho=0

	bioassay1	bioassay2
bioassay1	1.0000	-0.0505 0.8104
bioassay2	-0.05054 0.8104	1.0000

Appendix A cont. Regression of the greenhouse bioassays based upon redroot pigweed (*Amaranthus retroflexus*) emergence (Kinston data set).



Appendix A cont. Correlation between the greenhouse bioassays based upon redroot pigweed (*Amaranthus retroflexus*) fresh weight biomass (Clayton data set).

The CORR Procedure

2 Variables: bioassay1 bioassay2

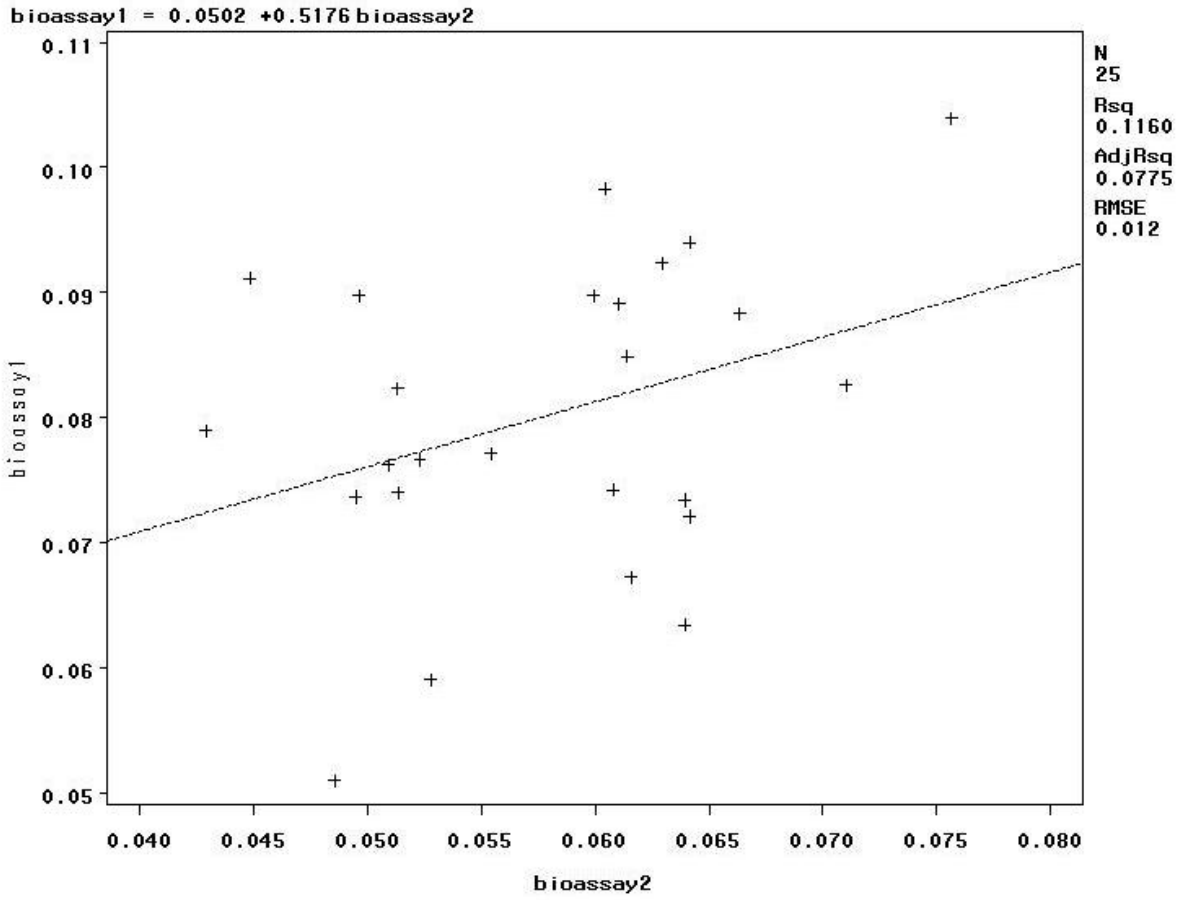
Simple Statistics

Variable	N	Mean	Std Dev	Sum	Minimum	Maximum
bioassay1	25	0.0802	0.0125	2.0042	0.0501	0.1040
bioassay2	25	0.0579	0.0082	1.4469	0.0429	0.0757

Pearson Correlation Coefficients, N = 25
Prob > |r| under H0: Rho=0

	bioassay1	bioassay2
bioassay1	1.0000	0.3405 0.0958
bioassay2	0.3405 0.0958	1.0000

Appendix A cont. Regression of the greenhouse bioassays based upon redroot pigweed (*Amaranthus retroflexus*) fresh weight biomass (Clayton data set).



Appendix A cont. Correlation between the greenhouse bioassays based upon redroot pigweed (*Amaranthus retroflexus*) fresh weight biomass (Kinston data set).

The CORR Procedure

2 Variables: bioassay1 bioassay2

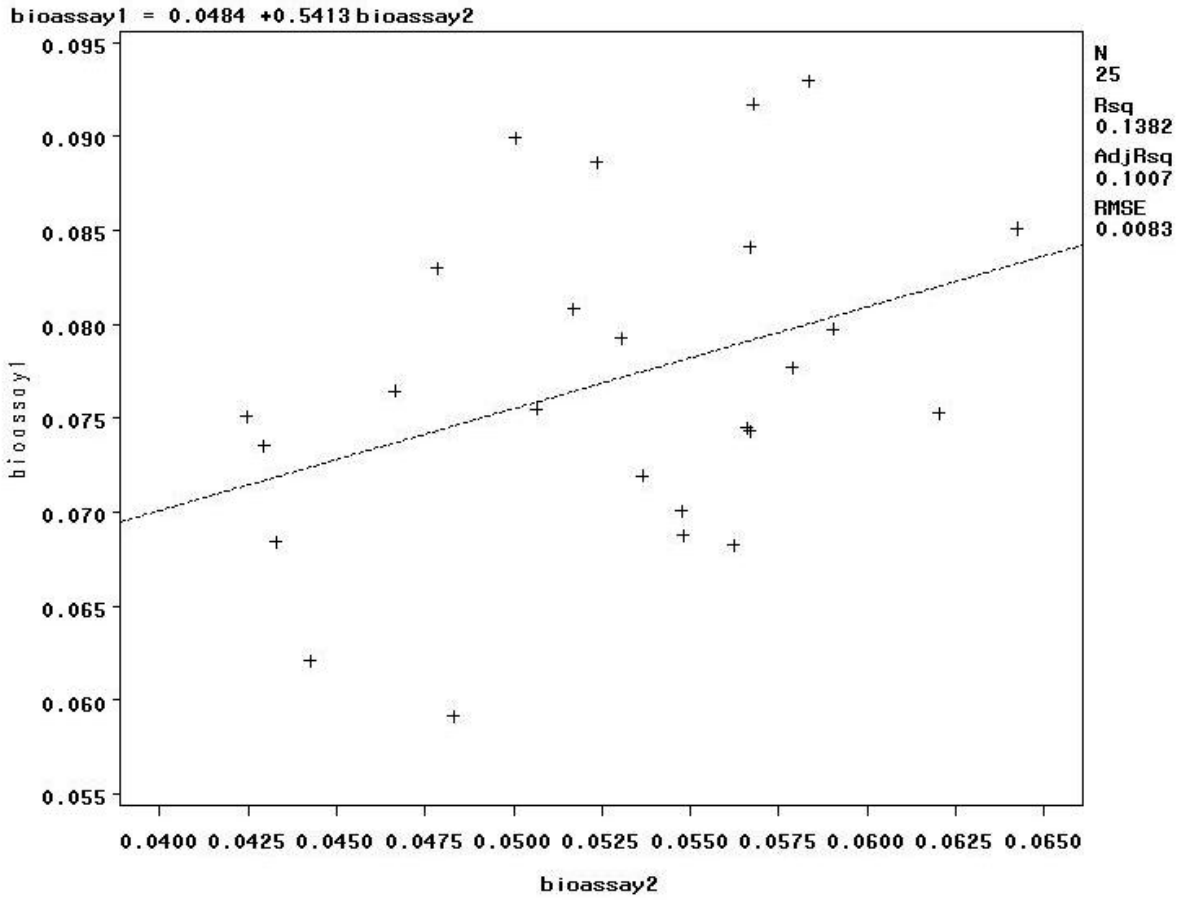
Simple Statistics

Variable	N	Mean	Std Dev	Sum	Minimum	Maximum
bioassay1	25	0.0771	0.0087	1.9263	0.0592	0.0930
bioassay2	25	0.0529	0.0060	1.3214	0.0425	0.0643

Pearson Correlation Coefficients, N = 25
Prob > |r| under H0: Rho=0

	bioassay1	bioassay2
bioassay1	1.0000	0.3718 0.0673
bioassay2	0.3718 0.0673	1.0000

Appendix A cont. Regression of the greenhouse bioassays based upon redroot pigweed (*Amaranthus retroflexus*) fresh weight biomass (Kinston data set).



Appendix B. Across location ANOVA for redroot pigweed (*Amaranthus retroflexus*) fresh weight biomass in the greenhouse bioassay.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	284	0.0343	0.0001	1.55	0.0005
Error	204	0.0159	0.0001		
Corrected Total	488	0.0502			

R-Square	Coeff Var	Root MSE	fw Mean
0.6830	15.5724	0.0088	0.0567

Source	DF	Type I SS	Mean Square	F Value	Pr > F
loc	1	0.0037	0.0037	47.73	<.0001
block(loc)	2	0.0002	0.0001	1.07	0.3436
genotype	149	0.0173	0.0001	1.49	0.0042
loc*genotype	132	0.0131	0.0001	1.27	0.0617

Source	DF	Type III SS	Mean Square	F Value	Pr > F
loc	1	0.0034	0.0034	43.98	<.0001
block(loc)	2	0.0000	0.0000	0.15	0.8608
genotype	149	0.0164	0.0001	1.41	0.0118
loc*genotype	132	0.0131	0.0001	1.27	0.0617

Tests of Hypotheses Using the Type III MS for loc*genotype as an Error Term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
genotype	149	0.0164	0.0001	1.11	0.2753

Appendix C. Clayton location ANOVA for redroot pigweed (*Amaranthus retroflexus*) fresh weight biomass in the greenhouse bioassay.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	141	0.0161	0.0001	1.39	0.0307
Error	124	0.0102	0.0001		
Corrected Total	265	0.0263			

R-Square	Coeff Var	Root MSE	fw Mean
0.6124	15.3040	0.0091	0.0593

Source	DF	Type I SS	Mean Square	F Value	Pr > F
block	1	0.0000	0.0000	0.45	0.5057
genotype	140	0.0161	0.0001	1.40	0.0290

Source	DF	Type III SS	Mean Square	F Value	Pr > F
block	1	0.0000	0.0000	0.07	0.7882
genotype	140	0.0161	0.0001	1.40	0.0290

The Mixed Procedure

Covariance Parameter Estimates

Cov Parm	Estimate
block	0.0000
genotype	0.0000
Residual	0.0001

Appendix C cont. Clayton location heritability estimates for redroot pigweed (*Amaranthus retroflexus*) fresh weight biomass in the greenhouse bioassay.

Clayton Heritability on a Per-Plot Basis

H
0.1723

SE
0.0876

Clayton Heritability on an Entry Mean Basis

H
0.2939

SE
0.1276

Appendix C cont. Kinston location ANOVA for redroot pigweed (*Amaranthus retroflexus*) fresh weight biomass in the greenhouse bioassay.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	142	0.0145	0.0001	1.42	0.0415
Error	80	0.0057	0.0001		
Corrected Total	222	0.0202			

R-Square	Coeff Var	Root MSE	fw Mean
0.7166	15.7457	0.0085	0.0537

Source	DF	Type I SS	Mean Square	F Value	Pr > F
block	1	0.0001	0.0001	1.83	0.1798
genotype	141	0.0143	0.0001	1.42	0.0425

Source	DF	Type III SS	Mean Square	F Value	Pr > F
block	1	0.0000	0.0000	0.24	0.6226
genotype	141	0.0143	0.0001	1.42	0.0425

The Mixed Procedure

Covariance Parameter Estimates

Cov Parm	Estimate
block	2.88E-7
genotype	1.90E-5
Residual	0.0001

Appendix C. Kinston location heritability estimates for redroot pigweed (*Amaranthus retroflexus*) fresh weight biomass in the greenhouse bioassay.

Kinston Heritability on a Per-Plot Basis

H
0.2089

SE
0.1077

Kinston Heritability on an Entry Mean Basis

H
0.3456

SE
0.1474

Appendix D. Across location ANOVA for redroot pigweed (*Amaranthus retroflexus*) germination in the petri dish bioassay.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	293	238.0537	0.8125	1.47	0.0010
Error	231	127.2522	0.5509		
Corrected Total	524	365.3060			

R-Square	Coeff Var	Root MSE	germ Mean
0.6517	32.0660	0.7422	2.3146

Source	DF	Type I SS	Mean Square	F Value	Pr > F
loc	1	4.9907	4.9907	9.06	0.0029
block(loc)	2	50.4108	25.2054	45.76	<.0001
genotype	149	89.5659	0.6011	1.09	0.2746
loc*genotype	141	93.0863	0.6602	1.20	0.1119

Source	DF	Type III SS	Mean Square	F Value	Pr > F
loc	1	1.9368	1.9368	3.52	0.0620
block(loc)	2	51.2938	25.6469	46.56	<.0001
genotype	149	91.9773	0.6173	1.12	0.2181
loc*genotype	141	93.0863	0.6602	1.20	0.1119

Tests of Hypotheses Using the Type III MS for loc*genotype as an Error Term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Genotype	149	91.9773	0.6173	0.94	0.6573

Appendix D cont. Across location ANOVA for redroot pigweed (*Amaranthus retroflexus*) root length in the petri dish bioassay.

Source	DF	Type I SS	Mean Square	F Value	Pr > F
loc	1	0.2044	0.2044	0.52	0.4724
block(loc)	2	37.0251	18.5126	46.94	<.0001
genotype	149	54.9695	0.3689	0.94	0.6691
loc*genotype	141	66.5004	0.4716	1.20	0.1148

Source	DF	Type III SS	Mean Square	F Value	Pr > F
loc	1	0.0279	0.0279	0.07	0.7903
block(loc)	2	36.6633	18.3317	46.48	<.0001
genotype	149	54.7999	0.3678	0.93	0.6766
loc*genotype	141	66.5004	0.4716	1.20	0.1148

Tests of Hypotheses Using the Type III MS for loc*genotype as an Error Term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
genotype	149	54.8000	0.3678	0.78	0.9326

Appendix E. Clayton location ANOVA of redroot pigweed (*Amaranthus retroflexus*) germination in the petri dish bioassay.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	149	141.3848	0.94890	1.54	0.0051
Error	142	87.7357	0.6179		
Corrected Total	291	229.1204			

R-Square	Coeff Var	Root MSE	germ Mean
0.6171	35.2874	0.7860	2.2275

Source	DF	Type I SS	Mean Square	F Value	Pr > F
block	1	49.9590	49.9590	80.86	<.0001
genotype	148	91.4267	0.6177	1.00	0.5009

Source	DF	Type III SS	Mean Square	F Value	Pr > F
block	1	50.0291	50.0291	80.97	<.0001
genotype	148	91.4267	0.6177	1.00	0.5009

Appendix E cont. Kinston location ANOVA of redroot pigweed (*Amaranthus retroflexus*) germination in the petri dish bioassay.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	143	91.6784	0.6411	1.44	0.0305
Error	89	39.5165	0.4440		
Corrected Total	232	131.1948			

R-Square	Coeff Var	Root MSE	germ Mean
0.6988	27.4917	0.6663	2.4238

Source	DF	Type I SS	Mean Square	F Value	Pr > F
block	1	0.4528	0.4528	1.02	0.3153
genotype	142	91.2255	0.6424	1.45	0.0299

Source	DF	Type III SS	Mean Square	F Value	Pr > F
block	1	1.2647	1.2647	2.85	0.0950
genotype	142	91.2255	0.6424	1.45	0.0299

Covariance Parameter Estimates

Cov Parm	Estimate
block	0.0011
genotype	0.1056
Residual	0.4602

Appendix E cont. Kinston location heritability estimates for redroot pigweed (*Amaranthus retroflexus*) germination in the petri dish bioassay.

Kinston Heritability on a Per-Plot Basis

H
0.1867

SE
0.1059

Kinston Heritability on an Entry Mean Basis

H
0.3146

SE
0.1504

Appendix E cont. Kinston location ANOVA of redroot pigweed (*Amaranthus retroflexus*) root length in the petri dish bioassay.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	144	60.8048	0.4223	1.39	0.0454
Error	90	27.3316	0.3037		
Corrected Total	234	88.1364			

R-Square	Coeff Var	Root MSE	length Mean
0.6899	31.5705	0.5511	1.7455

Source	DF	Type I SS	Mean Square	F Value	Pr > F
block	1	0.4994	0.4994	1.64	0.2030
genotype	143	60.3054	0.4217	1.39	0.0462

Source	DF	Type III SS	Mean Square	F Value	Pr > F
block	1	0.9513	0.9513	3.13	0.0801
genotype	143	60.3054	0.4217	1.39	0.0462

Covariance Parameter Estimates

Cov Parm	Estimate
block	0.0026
z*entry	0.0714
Residual	0.3045

Appendix E cont. Kinston location heritability estimates for redroot pigweed (*Amaranthus retroflexus*) root length in the petri dish bioassay.

Kinston Heritability on a Per-Plot Basis

H
0.1899

SE
0.1014

Kinston Heritability on an Entry Mean Basis

H
0.3191

SE
0.1432