ABSTRACT

WARRIS, ALIYAH. Quantification of Allelopathy Activity and Heritability in Cereal Rye (*Secale cereale* L.) Germplasm. (Under the direction of Dr. Ramon G. Leon).

The adoption of cereal rye (*Secale cereale* L.) as a cover crop is increasing in conventional and organic farming systems due to its weed suppressive ability, benefits to soil health, and overall contribution to sustainable agriculture. Cereal rye allelopathy, which is the production and release of allelochemicals that can impede surrounding weed emergence and growth, is an important component of its weed suppressive ability. However, the focus of most cereal rye breeding programs for cover cropping has been primarily on breeding for increased biomass production, with few programs concentrated on breeding for increased allelopathy. A known challenge to farmers is that weeds have evolved and adapted to certain weed management practices, necessitating an integrated weed management approach involving the combined use of multiple weed management practices including chemical control, mechanical control and cultural practices like crop rotation and cover cropping. Cereal rye allelopathy could be valuable to integrated weed management strategies that involve the use of cereal rye as a cover crop and the justification and proposal for breeding increased allelopathy in rye is explored in the proceeding chapters.

Allelopathic activity has been screened in cereal rye germplasm by commonly using lettuce (*Lactuca sativa* L.) as the bioindicator species of injury *in vitro* and in soil, but the germination and growth responses to allelochemicals by weed species has not been widely studied. Additionally, screenings that have been performed using lettuce and other bioindicator species have utilized fresh, vigorous seed. Using fresh seed can be an inaccurate representation of seedbank communities that would be affected by cereal rye allelopathy, as weed seeds vary in

age and vigor in the field. In the present research, I studied the responses of weed seeds with varying vigor to allelochemicals in the following chapters.

In the first study, I conducted *in vitro* and greenhouse screenings of allelopathy to characterize the variation that existed in the cereal rye germplasm available and lines characterized as having high allelopathy, causing > 50% lettuce growth, were identified. Broad-sense heritability (H^2) estimates of allelopathy were quantified and genetic correlations between allelopathy activity and vegetative growth traits were estimated. The results of the H^2 estimates and positive genetic correlation between tiller number, leaf area (cm^2), and increased allelopathy suggest that cereal rye has a high enough genetic component to breed for increased allelopathy and that selecting for increased allelopathy will not negatively affect certain rye vegetative growth traits like tiller number and leaf area (cm^2), which are important traits influencing cereal rye biomass. The second study examined the allelopathic effects of cereal rye on the germination of *Amaranthus palmeri* L., *Digitaria sanguinalis* L., *Setaria faberi* Herrm., and *Lactuca sativa* L. seeds. Additionally, the response of these four species to accelerated aging in conjunction with the allelopathic influences on germination was also investigated. There were differences in germination among species, which was dependent on seed vigor and allelochemical exposure. The findings indicate that the integration of cereal rye into crop rotations has the potential to diminish weed populations, but its efficacy may be contingent upon the species and age distribution of the seedbank.

Having the ability to concurrently breed for increased allelopathy and increased vegetative growth in cereal rye would make it easier for breeders to generate weed suppressive varieties. Further research on the response of weed species to allelochemicals and the effect of seed age and vigor is required to augment understanding of the relationship between seed age and allelopathic activity, and its implications on weed control by cereal rye allelopathy.

Quantification of Allelopathy Activity and Heritability in Cereal Rye (*Secale cereale* L.) Germplasm

by
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A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Science

Crop Science

Raleigh, North Carolina
2024

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DEDICATION

To my amazing family,

I would not be here without you.

BIOGRAPHY

Aliyah Warris was born on July 15th, 1999, in Winston-Salem, North Carolina where she graduated from Parkland High School. Aliyah received her Bachelor of Arts in Biology at the University of North Carolina at Greensboro in 2021. While at the University of North Carolina at Greensboro, she joined the Beta Iota Omicron Biology Club, where she served as the head officer and president her sophomore and junior year. She led a few community outreach and garden projects, which developed her interest in agronomy and the impact on food security here in North Carolina. Aliyah served as an undergraduate researcher within the Osena Research Group, directed by Dr. Ayalew L. Osena. In this capacity, she contributed to research initiatives that utilized molecular-genetic tools to enhance yield, nutritional quality, and stress resilience in orphan crops, ultimately aiming to improve food security through the advancement of these underutilized species. After graduating, Aliyah was hired to work at the LabCorp Research Triangle Park Center for Molecular Biology and Pathology, where she worked as a molecular genetics laboratory technologist, performing PCR based genetic testing for the diagnosis and treatment of various genetic conditions.

In the Spring of 2023, Aliyah embarked on her M.S. journey in the Crop Science program at North Carolina State University under the direction of Dr. Ramon G. Leon. During her M.S. Aliyah further developed her interests in genetics by engaging in research focused on the heritability and genetic correlations of traits beneficial to cover crop use for weed suppression. Aliyah anticipates continuing her research in plant genetics and integrated weed management in her Ph.D. under the direction of Dr. Ramon G. Leon at North Carolina State University.

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Ramon G. Leon, for providing me with an exceptional opportunity and for his encouragement in pursuing my research interests, as well as for equipping me with the necessary tools to achieve my goals. I would also like to thank my committee, Dr. James B. Holland, Dr. Chris Reberg-Horton, and Dr. Virginia Moore, for their invaluable guidance, instruction, and support throughout my program.

I would like to express my heartfelt gratitude to my husband, Justin, and my mother and grandparents, Kelley, Thomas, and Maxine, for their unwavering support, along with my close family.

Finally, I would like to express my appreciation to all past and present members of the Weed Ecology and Biology Lab for their technical expertise, assistance in the greenhouse, and their guidance. Thank you to April Dobbs, Avi Goldsmith, Caroline Barrett, Dr. Democ Rebong, Dr. Carlos Sousa, Dr. Chathura Perrera, and Dr. Igor Almeida for your teamwork, and most importantly, your friendship.

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Chapter 1 General Introduction

Cereal Rye (*Secale cereale* L.): An Overview

Cereal rye (*Secale cereale* L.) is an annual cereal grass (Cyperales: Poaceae) that originated from wild rye species like *Secale montanum* Guss. in the Near East and Central Asia (Hillman, 2013), and its domestication coincided with small grains production and their spread throughout Russia and Scandinavia, later reaching Poland and Germany, gradually expanding across Europe by 2000 to 3000 BCE and introduced to North America and western South America during the 16th and 17th centuries (1501 to 1700 CE). Rye remains were found with wheat and barley in archaeological sites suggesting that its domestication took place at about 6000 BCE (Sencer & Hawkes, 1980). In fact, in 1917 Russian plant geneticist Nikolai I. Vavilov proposed that cereal rye developed as a secondary crop in wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) fields, where wild rye populations gradually infiltrated, producing weedy ryes that were brittle. Ongoing selection for non-brittle ryes with larger grains eventually led to the cultivation of cereal rye (Feldman & Levy, 2023). Miedaner et. al. (2016) reported that cereal rye is mainly a European cereal in the present day, with Russia, Belarus, Poland, Germany, and Ukraine responsible for 75% of the global production. In that region, cereal rye is mostly cultivated for food, animal feed, bioethanol, and biogas (Miedaner & Laidig, 2019), whereas in the United States, most cereal rye is utilized for forage and cover crops (Newell & Butler, 2013).

Cereal rye is a diploid species with a chromosome number of 2n = 14, consisting of seven pairs of chromosomes, and has a large genome of 1C = 8.1 Gb, which is about 50% larger than the barley genome (Martis et al., 2013). In the U.S., cereal rye is often grown primarily as a forage crop. It is valued for its rapid growth, high biomass production, and ability to provide grazing or silage for livestock, particularly in the cooler months (Ates et al., 2017). Many of the rye cultivars used are tall, which can lead to lodging—where the plants fall over, typically due to wind or rain. Lodging can make harvesting difficult and reduce yield and quality (Shah et al., 2017). A challenge for breeding cereal rye is its strong self-incompatibility (SI) system preventing self-fertilization necessitating the presence of different plants for outcrossing and successful pollination (Lundqvist, 1957). This SI system is a genetic mechanism that promotes genetic diversity but also makes it challenging for breeders to develop stable, high-yielding cultivars since controlled breeding is more complex. Because of the traits—tall growth, lodging, and obligate outcrossing—breeding programs face challenges in consistently producing desirable cultivars. These difficulties necessitate innovative breeding strategies, such as using molecular markers or developing hybrids that can overcome some of these issues. Cereal rye has significant hybridization potential, both within its own species and with related species (e.g., wheat), which contributes to the plant's adaptability to various climates and soils (Miedaner & Laidig, 2019). This intraspecific and intergeneric hybridization potential, which contributes to greater genetic diversity is crucial for breeding programs focused on enhancing traits like disease resistance and overall productivity (Hackauf et al., 2022). Although hybridization offers advantages, it also brings challenges. Balancing genetic diversity with the need for consistency in traits is a key concern for breeders.

Weed Suppressive Ability of Cereal Rye

Cereal rye is widely recognized for its effective weed-suppressive abilities when used as a cover crop. A cover crop is a plant grown primarily to manage soil erosion, enhance soil health, and suppress weeds between cash cropping seasons (Sharma et al., 2018). Cover crops have been increasingly adopted by farmers primarily for sustainability reasons, as cover crops provide many benefits to soil health and contribute to the longevity of farmland (Glaze-Corcoran et al., 2023; Golden et al., 2024; Han & Niles, 2023; Kaye & Quemada, 2017). There is also a contribution to soil carbon sequestration by cover crops, which has a direct impact on climate change (Kaye & Quemada, 2017; Lal, 2015; Novara et al., 2019). Additionally, there have been incentives offered to farmers to use cover crops, which has aimed to promote sustainable agriculture and benefits to the environment (Bergtold et al., 2019; Repetto, 1987). Cereal rye's rapid growth and dense foliage create a competitive environment that inhibits weed germination and establishment (Mirsky et al., 2013). Its extensive root system not only helps outcompete weeds for essential resources like water and nutrients but also effectively scavenges nitrogen from the soil (Brandi-Dohrn et al., 1997; Mirsky et al., 2013; Snapp & Surapur, 2018; Teasdale & Mohler, 1993). This ability allows cereal rye to absorb excess nitrogen that might otherwise contribute to nutrient leaching, particularly during the off-season when crops are not actively growing. Incorporating cereal rye as a cover crop also improves soil structure and health, contributing to its weed management capabilities. When the rye is terminated, it forms a mulch layer that reduces soil disturbance and further suppresses weed emergence (Williams et al., 2018). This is because the biomass generated by cereal rye adds creates a physical barrier that prevents weed seeds from emerging (Camargo Silva & Bagavathiannan, 2023). Aside from its advantages to soil health, cereal rye can grow longer into the fall and can be planted earlier in the spring than other cover crops due to its winter-hardiness and its adaptation to a wide range of environments (Kantar & Porter, 2014; Larsen et al., 2018).

Cereal rye is recognized for its effective weed suppression through several mechanisms, including allelopathy, in addition to its vigorous growth and nitrogen scavenging abilities.

Allelopathy refers to the biochemical interactions among plants, whereby one plant releases allelochemicals that inhibit the growth of neighboring plants (Hickman et al., 2021; Nikus & Jonsson, 1999). Cereal rye produces a range of allelochemicals, such as benzoxazinoids (BX) like 2,4-dihydroxy-1,4(2H)-benzoxazine-3-1 (DIBOA) and 2,3-benzoxa- zolinone (BOA), which have been demonstrated to suppress the germination and seedling growth of several weed species (Barnes & Putnam, 1987; Rice, 1984) . These compounds are exuded from the plant's roots and released by shoot residues when decomposing. The structural characteristics of cereal rye further enhance its allelopathic potential. Its dense root system and substantial biomass not only allow for competitive resource uptake but also facilitate a continuous release of allelochemicals into the soil. As the rye decomposes, these allelochemicals persist, continuing to affect weed seed germination and seedling growth (Otte et al., 2020). This dual approach—combining physical competition with chemical inhibition—positions cereal rye as a valuable component of integrated weed management (IWM) strategies. By leveraging these natural processes, agricultural systems can reduce dependency on synthetic herbicides, promote biodiversity, and foster a more sustainable approach to enhancing soil health and crop productivity.

Screening for Allelopathy

Recent studies aimed at understanding the factors that determine the allelopathic activity of cereal rye by quantifying the concentration of allelochemicals in cereal rye tissues using high-performance liquid chromatography and gas chromatography (Brooks et al., 2012; Reberg-Horton et al., 2005) as well as *in vitro* and field screenings that use a bioindicator species to measure injury (Rebong et al., 2024). However, a drawback of this methodology is its potential to have low predictive power of actual weed suppression observed in field conditions (Brooks et al., 2012; Mwaja et al., 1995). For allelochemicals to be effective, they must be released into the soil, a process influenced by factors like root exudation and the breakdown of leaves after rye termination (Schulz et al., 2013). This means that a rye plant may possess a high concentration of allelochemicals in its tissues but may not release them effectively, leading to minimal weed suppression. The dynamics of allelochemical release and their availability in the soil are critical factors that influence weed suppression, making it essential to consider both the concentration of these compounds and their biological activity in the field.

Laboratory or *in vitro* screenings of allelopathy have provided valuable insights into the potential of cereal rye, to suppress weeds through allelochemical interactions. By conducting controlled experiments, like the equal-compartment-agar-method (ECAM), researchers can assess the efficacy of allelochemicals released by roots, allowing for a systematic evaluation of their allelopathic properties (Rebong et al., 2024). Additionally, *in vitro* studies can help elucidate the mechanisms of allelochemical action, such as their impact on seed germination and seedling development of target weed species (Cheng & Cheng, 2015). This information can advise breeding programs aimed at enhancing allelopathic traits in cereal rye. By combining laboratory findings with field trials, researchers can better understand the environmental factors that influence allelochemical release and effectiveness, ultimately leading to the development of more sustainable weed management strategies that rely on natural plant interactions.

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Chapter 2 Screening strategies and heritability estimation for allelopathy in cereal rye (*Secale cereale* L.)

Abstract

The demand for cereal rye as a cover crop is rising in organic and conventional farming systems due to its benefits to soil conservation and health as well as for weed suppression. Cereal rye produces allelochemicals that reduce weed emergence and growth. However, breeding programs for cover cropping have focused on biomass production, and little attention has been paid to breeding for increased allelopathy. In this study, 38 crosses derived from lines previously identified as highly allelopathic accessions and commercial cereal rye varieties 'ND Gardner' and 'Aroostook' were screened for allelopathic activity using the *in vitro*, equal-compartment-agar method with lettuce as the bioindicator. After *in vitro* phenotyping, allelopathic activity was also assessed in a loam soil under greenhouse conditions. Lettuce growth suppression was used as a surrogate of weed suppression, and vegetative growth of lettuce was characterized. Fourteen lines demonstrated high allelopathic activity causing > 50 % lettuce growth reduction. *In vitro*, broad-sense heritability (H^2) estimates were 0.69 and 0.48, for visually estimated plant injury and lettuce root reduction, respectively. In soil, H^2 for lettuce injury and shoot biomass reduction was 0.18 and 0.23, respectively. Cereal rye allelopathic activity was genetically correlated with plant height (-0.59), leaf area (0.10), and tiller number (0.47). The H^2 estimates indicate that allelopathy has a genetic component large enough to justify breeding for increased allelopathy in cereal rye and that tiller number can be part of the selection criteria for this trait.

Introduction

In Poaceae species including cereal rye (*Secale cereale* L.) and wheat (*Triticum aestivum* L.), benzoxazinoid compounds such as 2(3H)-benzoxazolinone (BOA), 2,4-Dihydroxy-(2H)-1,4-benzoxazin-3(4H)-one (DIBOA), and 6-Methoxy-2-benzoxazolinone (MBOA), are produced as secondary metabolites, which are allelochemicals that can act as defenses against insect pests (Barnes & Putnam, 1987; Rice, 1984; Wu et al., 2001), and inhibit seedling emergence and growth of surrounding plants when released to the soil (Hickman et al., 2021; Nikus & Jonsson, 1999). Due to the ability of cereal rye to produce relatively large amounts of benzoxazinoids, breeding cereal rye for increased allelopathy might be a valuable addition to integrated weed management programs that incorporate cover crops to outcompete weeds (Barnes & Putnam, 1987; Brooks et al., 2012; Putnam & DeFrank, 1983; Putnam et al., 1983; Rice, 1984). Historically, screening strategies to assess allelopathy activity include *in vitro* and greenhouse tests in which a bioindicator species is grown with cereal rye plants or residues and bioindicator injury is rated after a few days (Putnam & DeFrank, 1983; Putnam et al., 1983; Wu et al., 2001). For example, the Equal-Agar-Compartment-Method (ECAM) is an *in vitro* screening that was implemented to assess allelopathic interactions between wheat cultivars and annual cereal ryegrass (*Lolium rigidum* Gaudin) seeds, with the latter being the bioindicator of injury by allelopathy (Wu et al., 2000). In this method, allelochemicals are released to the agar media by cereal rye roots and interact with the roots of the bioindicator causing injury symptoms such as reductions in root and shoot growth, chlorosis, necrosis, and death. Injury level depends on the amount and bioactivity of the allelochemicals. This method also separates allelopathic interference from competitive interference by implementing a volume of agar that allows roots to grow freely and by creating compartments that prevent the shoots of the plants from shading each other (Fuerst & Putnam, 1983; Wu et al., 2000). Despite the practicality of *in vitro* bioassays, there is a need to validate these methods in soil and to develop practical bioassays that better predict allelochemical dynamics in the field.

In addition to the allelopathic characteristics of cereal rye, the demand for cereal rye as a cover crop has increased in conventional and no-till farming systems due to farmers' efforts to augment soil organic matter, improve water infiltration, reduced soil erosion, and minimize nitrate leaching (Brandi-Dohrn et al., 1997; Mirsky et al., 2013; Snapp & Surapur, 2018; Teasdale & Mohler, 1993). There are few cover crop breeding programs, and those working with cereal rye have focused on biomass production for weed suppression and other ecosystem services. However, there seems to be an opportunity to improve cereal rye weed suppression by breeding for increased allelopathy as well. Also, the relationship between the level of allelopathy and biomass produced by cereal rye at the individual level has not been well studied. Therefore, there is an additional opportunity to investigate differences in vegetative growth of cereal rye lines with different levels of allelopathy.

This study was conducted to determine the heritability of allelopathy in cereal rye breeding material *in vitro* and in soil to establish whether the allelopathic trait has a sufficiently high genetic control to breed for increased allelopathy. Furthermore, because of the demand for cereal rye as a cover crop due to its large biomass production and winter hardiness, this study aimed to determine any genetic correlations between level of allelopathy and vegetative growth of rye lines previously selected for high allelopathy. The objectives of the present research were 1) to characterize variation in allelopathy among cereal rye lines using *in vitro* and in soil tests,

2) to quantify the heritability of this trait, and 3) to estimate genetic correlations with vegetative morphological traits.

Materials and Methods

Plant Material

Cereal rye lines were selected from the North Carolina State University Cover Crop Breeding Program. The lines used were generated by crossing germplasm that had been previously identified having different levels of allelopathic activity under *in vitro* conditions (Table 1.1). For a detailed description of the pedigree of the lines used here see Rebong et al. (2024). Briefly, 268 accessions from the USDA-National Small Grains Collection were screened for allelopathy activity based on weed germination in petri dishes containing water extracts from leaves. The 15 most allelopathic accessions and varieties ‘Wrens Abruzzi’ and ‘Wheeler’ were outcrossed in open pollinated field nurseries for two generations. After seed pooling, six thousand lines were subjected to a preliminary screening for allelopathy in a field nursery observing weed seedling emergence variation around cereal rye plants. The most allelopathic half-sib families (i.e., seed from single plants) were selected for the present study.

Controlled crosses were made between the 10 most allelopathic lines and ‘ND Gardner' and 'Aroostook'. Aroostook cereal rye was developed and released by the New York State College of Agriculture and Life Sciences, Cornell University, and the Maine Department of Agriculture (USDA NRCS Big Flats Plant Materials Center, 2015). ND Gardner cereal rye was developed and released by the North Dakota State University Carrington Research Extension Center in (Berti et al., 2020). The crosses with ND Gardner and Aroostook were made using the allelopathic line as male and the commercial line as female under controlled greenhouse conditions due to the reproductive biology of cereal rye (i.e., self-incompatible and obligated outcrossing; Langridge & Baumann, 2008; Lundqvist, 1957; Sencer & Hawkes, 1980; Yang et al., 2008). After harvest, seeds were stored at 3˚C until experiments were initiated. The following laboratory and greenhouse experiments were split into three separate experiments due to time and space constraints. The first experiment included 14 ND Gardner rye lines and four commercial rye varieties, the second experiment included 15 ND Gardner rye lines and four commercial rye varieties, and the third experiment included nine Aroostook rye lines and three commercial rye varieties.

Laboratory bioassay

The ECAM (Wu et al., 2000) was used to assess allelopathic interactions between cereal rye and lettuce (*Lactuca sativa* L.), with this latter species serving as bioindicator. The experiment had a completely randomized design with three to five replications per cross. Twenty-nine crosses from ND Gardner maternal lines and nine crosses from Aroostook maternal lines were selected after the first screening. Five replications of a negative control with lettuce-only, or the rye-free controls, were included for comparison. Furthermore, 'Wrens Abruzzi', 'Wheeler', ND Gardner, and Aroostook, commercial varieties used as cover crops were included as positive controls. Each experimental unit was a single cereal rye individual per cross.

A single pre-germinated cereal rye seed per experimental unit was aseptically sown in a 0.3% water-agar medium in a 500 mL beaker, which was then covered with plastic wrap to avoid contamination and reduce evaporation. Beakers were placed in growth chambers (Achieva Console Germinator, Model A3920/B, Seedburo Equipment Co.) set to provide a 25˚C/13˚C and light/dark cycle of 13/11 h (Wu et al., 2000).

After seven days of cereal rye growth, at the one leaf stage, five lettuce seedlings (cotyledon stage) were placed into the agar on one-half of the beaker opposite to the cereal rye seedling. An autoclaved filter paper was used as a divider within the beaker to create two equal compartments. The divider served to prevent interference of the plant shoots of each species while allowing cereal rye roots to release allelochemicals to the agar. The beakers were then returned to the growth chamber and maintained there for ten days (Wu et al., 2000).

Cereal rye allelopathic activity was quantified by visually estimating lettuce injury 10 days after lettuce planting (DAP) with a rating of 0% meaning no visible injury compared with cereal rye-free control and 100% plant death. Injury symptoms included stunting, shoot deformity, chlorosis, necrosis, and wilting. Following injury ratings, the longest root of lettuce plants was measured using ImageJ (Abràmoff et al., 2004). Data for the five individuals were averaged for each beaker. Relative root length reduction in the ECAM bioassay (RLR-E) was quantified based on a cereal rye-free control (Worthington et al., 2015).

Greenhouse experiment

After *in vitro* assessment of lettuce plants, cereal rye seedlings at the two-leaf stage were removed from beakers and transplanted to 15-cm clay pots filled with loam soil (Soil Science Division Staff, 2017). The experiment was arranged in a randomized complete block design with the same number of replications used in the laboratory bioassay. Cereal rye plants were grown to the boot stage, which occurred between 1228 – 1279 growing degree days (base temperature = 3.3 ˚C). At this point, cereal rye shoots were harvested and dry biomass (g), tiller number, leaf area (cm^2), and plant height (cm) were quantified. Next, 50 lettuce seeds were sown in the pots. Also, rye-free pots were included as negative controls. Cereal rye allelopathic activity for the greenhouse experiment was quantified by visually estimating lettuce injury 14 and 21 DAP using the same criteria as in the laboratory bioassay and using the average for the analysis. At 21 DAP, total lettuce shoot growth was quantified measuring total fresh weight (g) per pot. Relative lettuce shoot biomass reduction was also quantified in the greenhouse (SBR-G) based on the cereal rye-free control (Worthington et al., 2015).

Statistical analysis

All statistical analyses were carried out using the R software v. 4.3.1 (R Core Team, 2023). The 'ggplot2' package was used for visualization of results (Wickham, 2016). Heritability estimates for traits evaluated in the laboratory bioassay and greenhouse experiment were calculated using the 'ASReml' package (Butler et al., 2023). This package was also used to estimate the genetic correlations (r_G) among dry biomass (g), tiller number, leaf area (cm^2), and plant height (cm) (Butler et al., 2023). The analyses were performed on the ND Gardner and Aroostook cereal rye lines individually for each data set and combined. A multivariate mixed linear model of variance was used to separate the means and correlation parameters among the cereal rye lines and to partition the total genetic variance and the total phenotypic variance for the RLR-E and SBR-G data. The broad-sense heritability (H^2) was estimated by dividing the total genetic variance by the total phenotypic variance. Best linear unbiased predictions (BLUPs) were estimated for the cereal rye lines, and H^2 on the family mean basis was estimated using the Cullis estimator of family mean of heritability (Cullis et al., 2006; Goldberger, 1962) which is defined as:

$$H^2{}_{fam} = 1 - \frac{(BLUP_{avsed})^2}{2 \cdot V_g} \qquad \text{Equation 1}$$

where H^2_{fam} is the proportion of total phenotypic variance due to genetic variance among the families, $BLUP_{avsed}$ is the average standard error of the BLUPs for the lines which captures the variance in the estimated means for each family, and V_g is the variance attributable to the genetic differences among families.

Results

ECAM and greenhouse allelopathy screening

The ECAM bioassay allowed discarding 55-65% of lines from the screening with low allelopathic activity based on a threshold of either $\geq$ 65% injury and/or $\geq$ 50% RLR-E. Furthermore, 10 highly allelopathic lines were selected among the ND Gardner crosses including NCR22-013, NCR22-016, NCR22-028, NCR22-029, NCR22-033, NCR22-049, NCR22-073, NCR22-089, NCR22-096, and NCR22-107 (Tables 1.2 and 1.3). Three of these lines (i.e., NCR22-016, NCR22-029 and NCR22-049) met the high allelopathy criteria for injury also in the greenhouse. For Aroostook crosses in the ECAM, four lines were identified as highly allelopathic, which included NCR22-156, NCR22-170, NCR22-191, and NCR22-194 (Table 1.4). These lines also exhibited SBR-G values that met the criteria for high allelopathy in the greenhouse experiment. From the ECAM bioassay, lines NCR22-033, NCR22-073, and NCR22-107 from ND Gardner crosses and NCR22-156, NCR22-170, NCR22-191, and NCR22-194 from Aroostook crosses were identified as being more allelopathic than Wheeler, ND Gardner, Aroostook, and Wrens Abruzzi. Significant variation in weed suppressive ability by SBR-G was found among the individuals of the 29 ND Gardner and 9 Aroostook crosses and their respective controls (Tables 1.2, 1.3, and 1.4). Significant variation in weed suppressive ability by RLR-E was found among the individuals of 15 ND Gardner and 9 Aroostook crosses and their respective controls, excluding the 14 ND Gardner crosses and controls used in the second experiment in which the weed suppressive ability by RLR-E was not significant ($P > 0.05$).

Heritability estimates

Data from ND Gardner and Aroostook crosses were pooled because sample size for the latter was too small for heritability analyses. Results from the ECAM bioassay demonstrated that variation in allelopathy among cereal rye lines has an important genetic component. *In vitro* and in soil, H^2 for RLR-E, SBR-G and plant injury ranged from 0.47 to 0.90 on a family mean basis (Table 1.5). These H^2 values also support that the trait of allelopathy has a genetic component high enough to breed for increased allelopathy.

Allelopathy and cereal rye vegetative growth

Genetic correlation (r_G) results (Table 1.6) indicate that cereal rye plants with more tillers and larger leaf area tended to be more allelopathic in the greenhouse screening than cereal rye plants with fewer tillers and smaller leaf area. For example, there was a positive genetic correlation between SBR-G and cereal rye leaf area and tiller number of 0.10 and 0.47, respectively (Table 1.6). Based on the r_G data in this study, it is possible to breed for increased allelopathy, tiller number, and leaf area in cereal rye lines concurrently. However, there was a negative genetic correlation between lettuce biomass shoot reduction (SBR-G) and cereal rye plant height of -0.59 in the greenhouse experiment. This result suggests that cereal rye might sacrifice plant height to favor increased allelopathy.

Discussion

Allelopathy screening strategies and rye breeding program

The purpose of using the ECAM coupled with the greenhouse experiment for an allelopathy screening, was to compare results and identify consistencies with the rating of allelopathy. The overall goal of incorporating an *in vitro* screening is to make the cereal rye breeding program for increased weed suppression more efficient. Thus, by eliminating *a priori* lines that do not exhibit increased allelopathy, the number of lines that must be screened in field nurseries is dramatically reduced saving time, space, and money. The efficiency of the ECAM procedure is demonstrated in this experiment, as the time required for this method of screening for levels of allelopathy in cereal rye lines is four weeks in the laboratory, and more than half of the lines can be potentially removed from the screening as a result.

There is a concern with the efficacy of using *in vitro* screenings and how representative the screenings are of natural systems. The young age of the cereal rye during the test and the possible saturation of allelochemicals in the agar media causing an overestimation of general phytotoxicity caused by allelochemicals are both factors that cause critics of this method to question whether *in vitro* allelopathy screenings simulate field screening conditions (Reberg-Horton et al., 2005). However, the goal of this study was to mitigate this limitation by the greenhouse screening of allelopathy, in which cereal rye plants are larger and at the boot stage, and therefore are more similar to plants that would be screened in the field than plants screened *in vitro*. Although the greenhouse screening conditions are largely controlled, there is more variation in temperature and light than the *in vitro* screening, a larger area in pots for cereal rye roots to release allelochemicals, and the interaction between allelopathic compounds and soil particles. In future experiments, it would be valuable to test how consistent is the ranking of allelopathic activity when transplanting cereal rye plants to the field after the greenhouse screening.

Breeding for increased allelopathy

It is important to establish the genetic component of a trait of interest before developing processes to breed for the trait (Acquaah, 2007), and this can be done by estimating the heritability of the trait in a population on an individual or a family mean basis. In the present study, heritability estimates of allelopathy were derived to justify breeding for increased allelopathy for the overall goal of increased weed suppression by cereal rye. The H^2 estimates for the different parameters (Table 1.5) clearly indicate that allelopathy is heritable and that it is feasible to breed for the trait based on data from allelopathy screenings *in vitro* and in soil. There is no universally accepted categorization of heritability, but there have been numerous studies conducted on wheat that categorize heritability values as low, moderate, and high, with low H^2 <0.4, moderate H^2 ranging from 0.4 to 0.7, and high H^2 > 0.7 (Heffner et al., 2011; Plavšin et al., 2021). In applying this H^2 categorization, on a family mean basis, the H^2 estimates *in vitro* and in soil provided that allelopathy is moderate to highly heritable, with the *in vitro* screenings having H^2 estimates of 0.79 and 0.9 for RLR-E and the visual rating of allelopathy *in vitro*, respectively and the soil screenings having H^2 estimates of 0.47 and 0.55 for SBR-G and the visual rating of allelopathy in soil, respectively (Table 1.5). Family mean-based heritability is often used more than single plant-based heritability in plant breeding due to reduced environmental variability, replication allowing a more accurate assessment of genetic potential, and because breeders often make selections based on the performance of families and not individual plants (Beavis et al., 2023; Schmidt et al., 2019; Wray & Visscher, 2008). Heritability estimates are also used to predict genetic gain achievable by selecting for a specific trait in a population and higher broad-sense heritability estimates indicate greater potential for genetic gain through selection (Holland et al., 2002; Rutkoski, 2019). The moderate to high H^2 estimates for *in vitro* RLR-E and visual rating, and SBR-G and visual rating in the greenhouse experiment on a family mean-basis indicate that selection will be more effective in improving the trait of allelopathy, thus also resulting in greater genetic gain.

Importance of r_G between allelopathy and vegetative growth traits

Since cereal rye breeding for cover crop use has primarily focused on breeding for increased biomass production, an aim for the present study was to determine whether there were genetic correlations between allelopathy and vegetative growth (Reberg-Horton et al., 2005; Ruis et al., 2019). More specifically, the objective was to determine whether breeding for increased allelopathy in cereal rye had any direct impact on the vegetative morphological traits on a single plant basis. One of the most attractive traits of cereal rye as a cover crop is its prolific biomass production, therefore it is important to rule out the possible caveat of whether breeding for increased allelopathy negatively impacts rye vegetative growth. In the present study, there was a positive genetic correlation between SBR-G and cereal rye leaf area and tiller number (Table 1.6) which suggests that cereal rye plants with high tillering and larger leaf area would have more groundcover, which is desired in conventional and organic cover-crop systems (Boyd et al., 2009; Liebert et al., 2017; Mirsky et al., 2013). Enhanced groundcover coupled with increased weed suppression by allelopathy has the potential to improve integrated weed management programs that include cereal rye as a cover crop (Reberg-Horton et al., 2005). Though tiller number and leaf area were positively correlated with allelopathy, there was a negative genetic correlation between allelopathy and plant height. Researchers have suggested that the synthesis of allelochemical compounds constitutes a trade-off with plant growth (Hickman et al., 2021). Nevertheless, the results from this study challenges prior research in that specific plant growth traits are affected differently, as tiller number and leaf area have a positive genetic correlation and plant height has a negative correlation to SBR-G.

The high genetic correlation between allelopathy and cereal rye dry biomass (g) obtained in the present study must be considered cautiously. Because the standard error was out of bounds, it is possible that the result is an overestimation. This limitation is likely due to the sample size and data set size used in this study. Larger data sets would be required in the future for the most accurate r_G between allelopathy and biomass. Nevertheless, biomass is likely to be positively related to allelopathy because tiller number is directly related to biomass and root production (Boe & Beck, 2008; Liu et al., 2017; Severini et al., 2020).

Conclusions

Significant variation in weed suppressive ability by RLR-E and SBR-G was found among all of the cereal rye lines screened, along with their respective controls (Table 1.2), and the efficiency of allelopathy screenings *in vitro* and in soil were demonstrated in this study. Results of the H^2 estimates indicate that allelopathy is moderately to highly heritable *in vitro* and in soil on a family mean basis. This result suggests that it is not only feasible to breed for increased allelopathy in cereal rye, but breeders could also have better success generating weed suppressive varieties by combining allelopathy and vegetative traits because both traits are positively related.

Funding

The North Carolina Small Grains Grower's Association; USDA-NIFA; USDA-NRCS

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Tables

Table 1.1: Crosses made by the North Carolina State University Cover Crop Breeding Program from previously identified allelopathic lines with different levels of allelopathic activity under *in vitro* conditions as the male parent and the commercial lines 'ND Gardner' and 'Aroostook' as the female parent, under controlled greenhouse conditions. 'P1 – P10' denotes individual crosses of the same parents.

Cross Designation	**Male Parent (USDA-NSGL)**	**Female Parent**
NCR22-008	NC20-A117-1 P6	ND Gardner
NCR22-010	NC20-A117-1 P7	ND Gardner
NCR22-011	NC20-A117-1 P8	ND Gardner
NCR22-013	NC20-A117-1 P9	ND Gardner
NCR22-015	NC20-A122-2 P1	ND Gardner
NCR22-016	NC20-A122-2 P2	ND Gardner
NCR22-025	NC20-A122-3 P1	ND Gardner
NCR22-027	NC20-A122-3 P3	ND Gardner
NCR22-028	NC20-A122-3 P4	ND Gardner
NCR22-029	NC20-A122-3 P4	ND Gardner
NCR22-030	NC20-A122-3 P5	ND Gardner
NCR22-033	NC20-A122-3 P7	ND Gardner
NCR22-035	NC20-A122-3 P9	ND Gardner
NCR22-037	NC20-A129-2 P1	ND Gardner
NCR22-039	NC20-A129-2 P3	ND Gardner
NCR22-040	NC20-A129-2 P4	ND Gardner
NCR22-049	NC20-A130-2 P1	ND Gardner
NCR22-059	NC20-A133 P1	ND Gardner
NCR22-063	NC20-A133 P7	ND Gardner
NCR22-073	NC20-R02-1 P5	ND Gardner
NCR22-085	NC20-R101-3 P6	ND Gardner
NCR22-086	NC20-R101-3 P7	ND Gardner
NCR22-089	NC20-R101-3 P8	ND Gardner
NCR22-093	NC20-R103-2 P2	ND Gardner
NCR22-096	NC20-R103-2 P4	ND Gardner
NCR22-100	NC20-R103-2 P9	ND Gardner
NCR22-107	NC20-R114 P4	ND Gardner
NCR22-108	NC20-R114 P5	ND Gardner
NCR22-109	NC20-R114 P6	ND Gardner
NCR22-146	NC20-A130-2 P8	Aroostook

Table 1.1 (continued.)

NCR22-149	NC20-A130-2 P10	Aroostook
NCR22-156	NC20-A133 P6	Aroostook
NCR22-166	NC20-R02-1 P9	Aroostook
NCR22-170	NC20-R101-3 P2	Aroostook
NCR22-172	NC20-R101-3 P3	Aroostook
NCR22-191	NC20-R114 P7	Aroostook
NCR22-194	NC20-R114 P8	Aroostook
NCR22-196	NC20-R114 P10	Aroostook

Table 1.2*:* Visual estimate of lettuce injury and relative root length reduction (RLR-E) of lettuce seedlings grown with cereal rye seedlings *in vitro* and visual estimate of lettuce injury and relative shoot biomass reduction (SBR-G) of lettuce seedlings grown with cereal rye plants in soil compared with the rye-free control for the first experiment. The first experiment included 14 ND Gardner rye lines and 4 commercial rye varieties.

Line	**Visual Injury *in vitro***	**RLR-E**	**Visual Injury in soil**	**SBR-G**
NCR22-008	24	26	56	51
NCR22-010	44	36	46	57
NCR22-011	54	38	55	56
NCR22-015	38	24	52	61
NCR22-016	72	53	52	58
NCR22-025	38	38	51	54
NCR22-027	54	37	42	43
NCR22-028	68	48	50	44
NCR22-029	45	53	62	69
NCR22-030	36	35	65	63
NCR22-035	48	33	31	45
NCR22-037	60	42	54	49
NCR22-039	40	30	46	52
NCR22-049	60	58	74	84
Wheeler	42	52	50	62
ND Gardner	83	75	54	39
Aroostook	66	50	45	40
Wrens Abruzzi	50	43	72	71
Rye-free Control	0	0	0	0
p-value	< 0.001	< 0.001	0.109	0.020

Table 1.3: Visual estimate of lettuce injury and relative root length reduction (RLR-E) of lettuce seedlings grown with cereal rye seedlings *in vitro* and visual estimate of lettuce injury and relative shoot biomass reduction (SBR-G) of lettuce seedlings grown with cereal rye plants in soil compared with the nil rye-free control for the second experiment. The second experiment included 15 ND Gardner rye lines and 4 commercial rye varieties.

Line	**Visual Injury *in vitro***	**RLR-E**	**Visual Injury in soil**	**SBR-G**
NCR22-013	74	50	36	39
NCR22-033	88	60	56	43
NCR22-040	35	35	38	48
NCR22-059	39	38	56	49
NCR22-063	55	45	63	61
NCR22-073	78	55	66	56
NCR22-085	46	33	44	29
NCR22-086	44	36	51	47
NCR22-089	68	49	61	51
NCR22-093	54	42	39	37
NCR22-096	68	43	46	49
NCR22-100	51	39	35	29
NCR22-107	79	52	43	41
NCR22-108	53	36	36	40
NCR22-109	58	38	49	37
Wheeler	23	34	16	18
ND Gardner	78	55	48	37
Aroostook	65	56	56	46
Wrens Abruzzi	68	54	73	63
Rye-free Control	0	0	0	0
p-value	< 0.001	< 0.001	0.013	0.096

Table 1.4: Visual estimate of lettuce injury and relative root length reduction (RLR-E) of lettuce seedlings grown with cereal rye seedlings *in vitro* and visual estimate of lettuce injury and relative shoot biomass reduction (SBR-G) of lettuce seedlings grown with cereal rye plants in soil compared with the nil rye-free control for the third experiment. The third experiment included 9 Aroostook rye lines and 3 commercial rye varieties.

Line	**Visual Injury *in vitro***	**RLR-E**	**Visual Injury in soil**	**SBR-G**
NCR22-146	56	22	63	75
NCR22-149	46	16	34	61
NCR22-156	70	38	62	77
NCR22-166	64	23	68	82
NCR22-170	74	37	55	70
NCR22-172	57	39	58	80
NCR22-191	65	31	61	72
NCR22-194	77	46	54	63
NCR22-196	56	34	54	72
Wheeler	48	30	49	73
ND Gardner	64	44	81	84
Aroostook	60	28	42	52
Rye-free Control	0	0	0	0
p-value	< 0.001	0.007	0.001	< 0.001

Table 1.5: Broad-sense heritability (H^2) estimated on a family mean basis for visual estimate of lettuce injury and relative root length reduction (RLR-E) of lettuce seedlings grown with cereal rye seedlings *in vitro* and visual estimate of lettuce injury and relative shoot biomass reduction (SBR-G) of lettuce seedlings grown with cereal rye plants in soil.

Variable	**H^2 family**
RLR-E	0.79
Visual rating *in vitro*	0.90
SBR-G	0.47
Visual rating in soil	0.55

Table 1.6: Genetic correlations (r_G) of the combined ND Gardner and Aroostook data between the level of allelopathy in a cereal rye individual, indicated by lettuce shoot biomass reduction (SBR-G) and vegetative morphological traits within the same individual, on a plant-by-plant basis. 'NA' represents an out-of-bounds standard error.

Trait	**r_G**	**SE**
Plant height (cm)	-0.59	0.24
Leaf area (cm^2)	0.10	0.29
Tiller number	0.47	0.31
Dry biomass (g)	0.99	NA

Chapter 3 Seed aging reduces weed germination more than allelochemicals

Abstract

Cereal rye (*Secale cereale* L.) is grown as cover crop due to its ability to enhance soil health and suppress weeds through allelopathy, but the germination responses of different weed species to allelochemicals have not been studied for seeds varying in vigor. This study investigated the allelopathic effects of cereal rye on the germination of *Amaranthus palmeri* L., *Digitaria sanguinalis* L., *Setaria faberi* Herrm., and *Lactuca sativa* L. Seeds were germinated *in vitro* in media with allelochemicals secreted by roots of cereal rye lines varying in allelopathic activity. Seeds were subjected to an accelerated aging treatment to modify their vigor. Results showed that aged seeds exhibited 31% lower total germination than non-aged seeds. The magnitude of the germination reduction due to the presence of allelochemicals was species dependent. *Setaria faberi* exhibited hormesis when germinated in the low allelopathy treatment increasing total germination more than 20% compared with the non-treated control. *Digitaria sanguinalis* did not respond to seed aging, and the high allelopathy treatment reduced total germination less than 10%. *Amaranthus palmeri* germination exhibited the highest total germination and was the only species that was not affected by the seed aging and allelopathy treatments. Germination rate was reduced by seed aging and to a less extent by allelochemicals. Results suggest that incorporating allelopathic *S. cereale* varieties in cover crop rotations can reduce weed populations, but the age structure of the seed bank might determine the importance of allelochemicals for weed emergence.

Introduction

As weeds evolve, they can develop several adaptations that make them more resilient and challenging to control, like herbicide resistance, resistance to environmental stress, and increased competitive ability (Clements et al., 2005; Harker, 2013; Owen et al., 2015). The implementation of integrated weed management (IWM) is increasing in conventional and no-till farming because of its value to control weeds in a more robust and sustainable manner (Bajwa, 2014; Farooq & Pisante, 2019; Monteiro & Santos, 2022). IWM methods include the use of chemical herbicides, biological controls, mechanical tillage, and cultural practices such as crop rotation, row spacing optimization, and cover cropping (Gupta et al., 2022; Mendes & Alberto Da Silva, 2022; Scavo & Mauromicale, 2020). The demand for cereal rye (*Secale cereale* L.) as a cover crop has increased due to farmers' efforts to supplement soil organic matter, improve water infiltration, control erosion, and reduce nitrate leaching (Brandi-Dohrn et al., 1997; Mirsky et al., 2013; Snapp & Surapur, 2018; Teasdale & Mohler, 1993). Cereal rye is also known for its allelopathic effects in which benzoxazinoid compounds are produced as secondary metabolites, which are allelochemicals that when released, can defend against insect pests (Barnes & Putnam, 1987; Rice, 1984; Wu et al., 2001), and can suppress seedling emergence and growth of nearby weeds (Carlsen et al., 2009; Hickman et al., 2021; Nikus & Jonsson, 1999).

Laboratory and field screenings to determine the level of allelopathy in various cereal rye lines have been performed using species like lettuce (*Lactuca sativa* L.), annual ryegrass (*Lolium rigidum* Gaudin) and white mustard (*Sinapis alba* L.) as the bioindicator species of injury (Belz & Hurle, 2004; Rebong et al., 2024; Wu et al., 2000). However, little has been done to make direct comparisons of how different species respond to allelochemicals, especially in the case of weed species such as *Amaranthus palmeri* L., *Digitaria sanguinalis* L., *Setaria faberi* Herrm., which are of economic importance in many crops (Basinger et al., 2019; Ethridge et al., 2023; Oreja et al., 2022). As part of the integrated management of *A. palmeri*, *D. sanguinalis*, and *S. faberi* incorporating allelopathic cereal rye varieties in the cover crop rotation could be beneficial for increasing weed suppression (Scavo & Mauromicale, 2021).

Most studies have evaluated the effect of allelopathy testing seeds recently produced and that exhibit high vigor. However, the composition of weed seed banks includes seeds of different age and vigor, and these factors have not been considered in allelopathy studies. It is certainly challenging to study seed age responses to allelochemicals *in situ*. A practical alternative is to conduct laboratory studies that modify seed vigor using methods for accelerated aging (Baker, 1974; Reed et al., 2022; Turner et al., 2014). In an accelerated aging test, seeds are subjected to controlled high temperature and humidity conditions for a set period of time, which simulates an aging process in which energy reserves and cell membrane integrity are reduced, and afterward seeds are germinated to assess viability and vigor. The purpose of seed vigor tests are to assess viability, predict performance, and to provide insight into seed germination potential under less than optimum conditions (Fenollosa et al., 2020; Marcos-Filho, 1998; Mcdonald, 1993; TeKrony, 1983, 2005).

This study was conducted to characterize the effects of seed vigor and exposure to allelochemicals secreted by cereal rye roots on germination of *A. palmeri*, *D. sanguinalis*, *S. faberi* and *L. sativa*. Thus, the objectives of the present research were 1) to characterize differences in germination after accelerated aging among the mentioned species, and 2) to determine if weed seed vigor influences susceptibility to allelochemicals.

Materials and Methods

The study compared the germination of *A. palmeri*, *D. sanguinalis*, *S. faberi*, and *L. sativa* under six treatments based on factorial combinations of three levels of allelopathy (none, low, and high) and two aging conditions (aged and not aged).

Allelopathic matrix

Samples of agar were obtained from a collection of agar media in which multiple cereal rye (*Secale cereale* L.) lines were grown in water-agar to freely release allelochemicals for 17 days, and a bioindicator of injury, which was lettuce, was used to visually assess percent injury. Agar extracts from the previous characterization of allelopathy in cereal rye lines (Chapter 2) were selected for high allelopathy based on a threshold of either ≥ 70% injury and/or ≥ 50% lettuce root growth reduction and were selected for low allelopathy based on a threshold of either ≥ 45% injury and/or ≥ 35% lettuce root growth reduction (Warris et al. unpublished; Table 1).

Seed accelerated aging and germination

Amaranthus palmeri, *D. sanguinalis*, *S. faberi*, and *L. sativa* seeds, collected fresh from the field, greenhouse studies, and obtained commercially, respectively, were previously stored at 3˚C until experiments were initiated. The seeds of the four species were artificially aged to change their vigor by placing them on a filter paper in a sealed, glass container with a paper towel saturated with water to maintain a high relative humidity environment. The containers were then placed into a germinator (Achieva Console Germinator, Model A3920/B, Seedburo Equipment Co.) set to provide 45˚C for 48 hours with no light.

For the germination test, all seeds were surface sterilized and placed onto the surface of the allelopathic agar extracts in a petri dish, with each species being in a separate petri dish. Twenty five seeds that underwent the accelerated aging were placed in on top of the agar in one-half of the petri dish and 25 non-aged seeds were sown on the other half. Petri dishes were sealed with parafilm and placed in the germinator set to provide a 22 $\pm$ 2˚C/20 $\pm$ 2˚C and light/dark cycle of 12/12 h. Germination counts were collected daily for 21 days. Seeds were considered germinated when protruded radicles were at least 2 mm long.

Experimental design and statistical analysis

The experiment was a completely randomized design with five replications, and it was conducted twice. Due to limited agar extracts, a single line extract was used for each treatment. Therefore, the five cereal rye lines for high and low allelopathy were considered replications per species for the statistical analysis. All statistical analysis were performed using the R software 4.4.1 (R Core Team, 2024, n.d.). The 'ggplot2' package was used for visualization of results (Wickham, 2016). The 'emmeans' and the 'agricolae' packages were used for ANOVA and post-hoc testing (De Mendiburu, 2006; Lenth, 2016).

Results and Discussion

Every species responded differently to the six treatments when considering total seed germination and germination rate (Figure 2.1). *Amaranthus palmeri* was less affected by allelochemicals and aging when considering the total germination, which reached 80% regardless of the treatments. Conversely, the germination rate of this weed was slower for aged seeds than non-aged seeds. This result is likely due to the small seed size of *A. palmeri* notwithstanding the high temperatures and humidity of the accelerated aging test (Walters et al., 2010). With *A. palmeri* being one of the more problematic weed species due to its rapid evolution and resistance to herbicides and current methods of weed management, this result indicates that cereal rye allelopathy alone might not be as effective inhibiting seedling emergence and growth as other factors such as cover crop biomass (Roberts & Florentine, 2022).

In Figure 2.1, Control_NA treatment has the highest germination rate for *D. Sanguinalis*, which is also consistent for three other species, and total germination in all treatments ranged from 51 to 70%. However, there are no clear differences in the response of *D. sanguinalis* to the other five treatments, as there are no statistical differences between the two aging treatments and the three allelopathy treatments (Figure 2.2). Results suggest that it is unclear whether aging and allelochemicals have a true impact of the germination rate of *D. sanguinalis*. This finding suggests that *D. sanguinalis* may not be directly suppressed by allelochemicals, irrespective of seed age. However, the characteristics of cereal rye as a cover crop, such as substantial biomass and dense foliage, may still provide the ability to suppress weeds by enhancing competitive advantages against this species.

Accelerated aging decreased germination of both *L. sativa* and *S. faberi* more than the other species with reductions of approximately of 50 to 70% when compared to the Control_NA (Figure 2.1). *L. sativa* was the only species in which there was a clear interaction between aging and allelopathy. Thus, aged seeds germinated in allelopathic media (high or low) had up to 70% reduction in total germination and took five more days to reach maximum germination compared with the other treatments. There was significant variation across all species and treatments and their interaction at 21 days (Table 2.2). Furthermore, the estimated marginal means (EMMs) comparison of groups showed that there is high variation across the means for each treatment by species group (Figure 2.2), but the impact of aging on the mean germination of *S. faberi and L. sativa* presented the most striking results. *Setaria faberi* Low_AA and High_AA treatments exhibited 67% and 51% lower total germination than Low_NA and High_NA treatments, respectively and *L. sativa* Low_AA and High_AA treatments exhibited 56% and 67% lower total germination than the Low_NA and High_NA treatments, respectively.

The present study demonstrated that there are clear differences in germination among the four species that are dependent on seed vigor (i.e., accelerated aging) and allelochemical exposure. Furthermore, for species in which there is an interactive response between these two factors, there can be synergistic reductions in germination. However, with the caveat that we just studied a small number of species, this seems to be the exception. The response to allelochemicals by each weed species is important to *S. cereale* allelopathy research for its incorporation into integrated weed management practices and the results suggest allelochemicals released in the field could reduce or slow germination of some weed seeds (Singh et al., 2003). Furthermore, the response of *L. sativa* to allelochemicals in the present study is consistent with the injury seen in ECAM bioassay and field screenings of S. cereale allelopathy in which *L. sativa* is the bioindicator of injury.

The germination study demonstrated significant variability in the responses of different weed species to allelochemicals, with seed age exerting an influence on these responses. While most germination studies typically utilize fresh, vigorous seeds, the results of this germination study indicate that the responses of weed seeds are contingent upon the specific aging treatments applied. This finding suggests that relying exclusively on fresh seeds in germination studies may not accurately represent the effect of allelochemicals in within seed banks, which have considerable variation in age composition. Additionally, these results underscore the potential of cereal rye allelopathy to influence weed community dynamics, implying that the implementation of allelopathic weed control could result in shifts in species composition and density. Further research on weed seed vigor and allelochemical interactions will enhance understanding of sustainable weed management practices that involve *S. cereale* as a cover crop, and its implications in agronomy.

Funding

The North Carolina Small Grains Grower's Association; USDA-NIFA; USDA-NRCS

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Tables

Table 2.1: Five cereal rye (*Secale cereale* L.) lines selected for high allelopathy and five selected for low allelopathy. Lines were selected for high allelopathy based on a threshold of either $\geq$ 70% injury and/or $\geq$ 50% lettuce root reduction and were selected for low allelopathy based on a threshold of either $\geq$ 45% injury and/or $\geq$ 35% lettuce root reduction.

Cross	Level of Allelopathy
NCR22-013	High
NCR22-016	High
NCR22-033	High
NCR22-073	High
NCR22-107	High
NCR22-008	Low
NCR22-015	Low
NCR22-040	Low
NCR22-085	Low
NCR22-086	Low

Table 2.2: Results from a factorial analysis of variance (ANOVA) used to determine significant differences between the group means of the three allelopathy treatments, the two aging treatments, the four species, and their interactions, with run as a random effect at the 21-day time-point.

Source	DF	SS	MS	F value	Pr(>F)
Allelopathy (Al)	2	3618	1809	34	<0.001
Aging (Ag)	1	12090	12090	225	<0.001
Species (Sp)	3	13070	4357	81	<0.001
Al x Ag	2	1196	598	11	<0.001
Al x Sp	6	2392	399	7	<0.001
Ag x Sp	3	9587	3196	59	<0.001
Al x Ag x Sp	6	2607	434	8	<0.001
Residuals	150	8179	55		

Figures

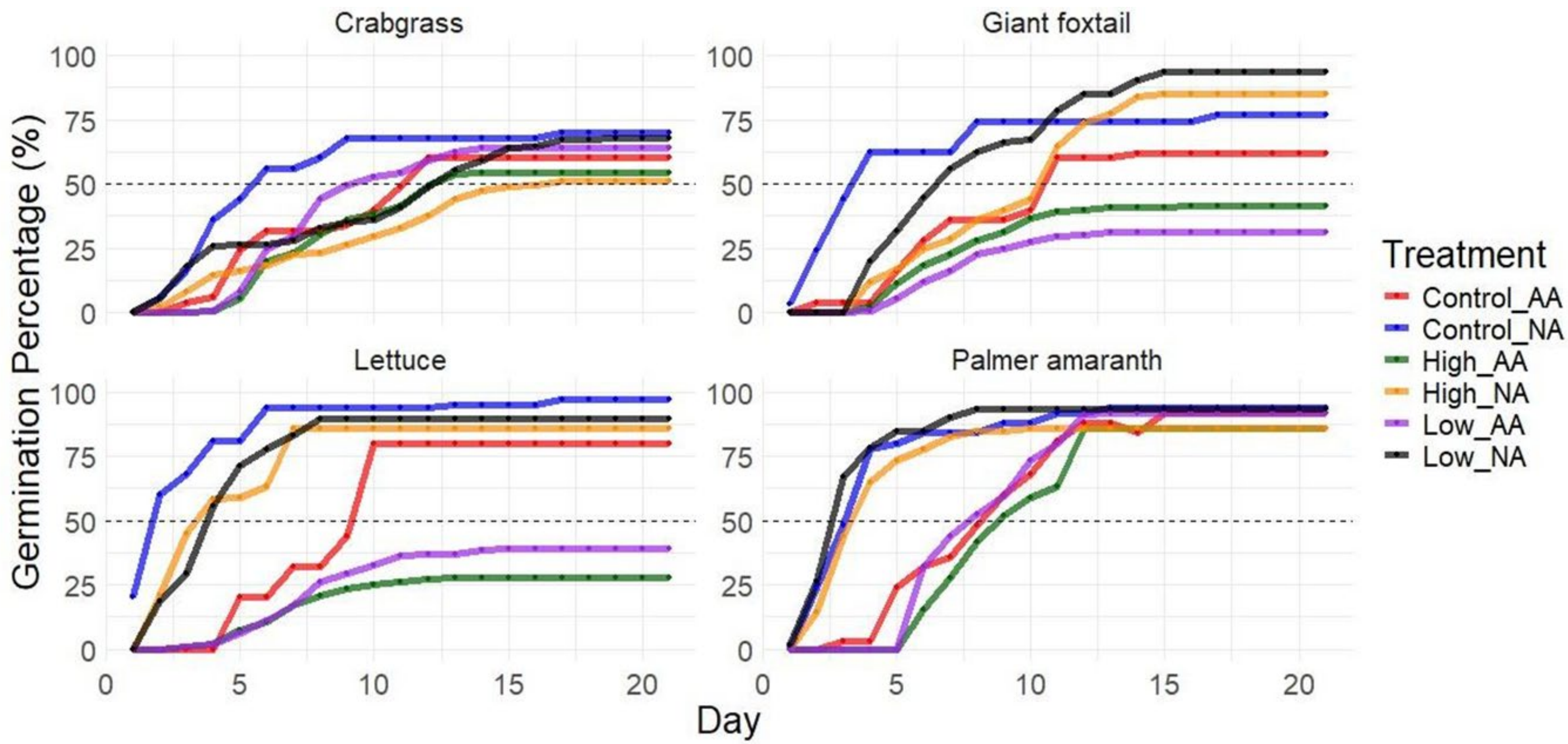

Figure 2.1. 21-day germination of crabgrass (*Digitaria sanguinalis*), giant foxtail (*Setaria faberi*), lettuce (*Lactuca sativa*), and Palmer amaranth (*Amaranthus palmeri*), treated with (AA) and without (NA) an accelerated aging seed vigor test, germinated in agar containing high and low levels allelochemicals and allelochemical-free agar (control).

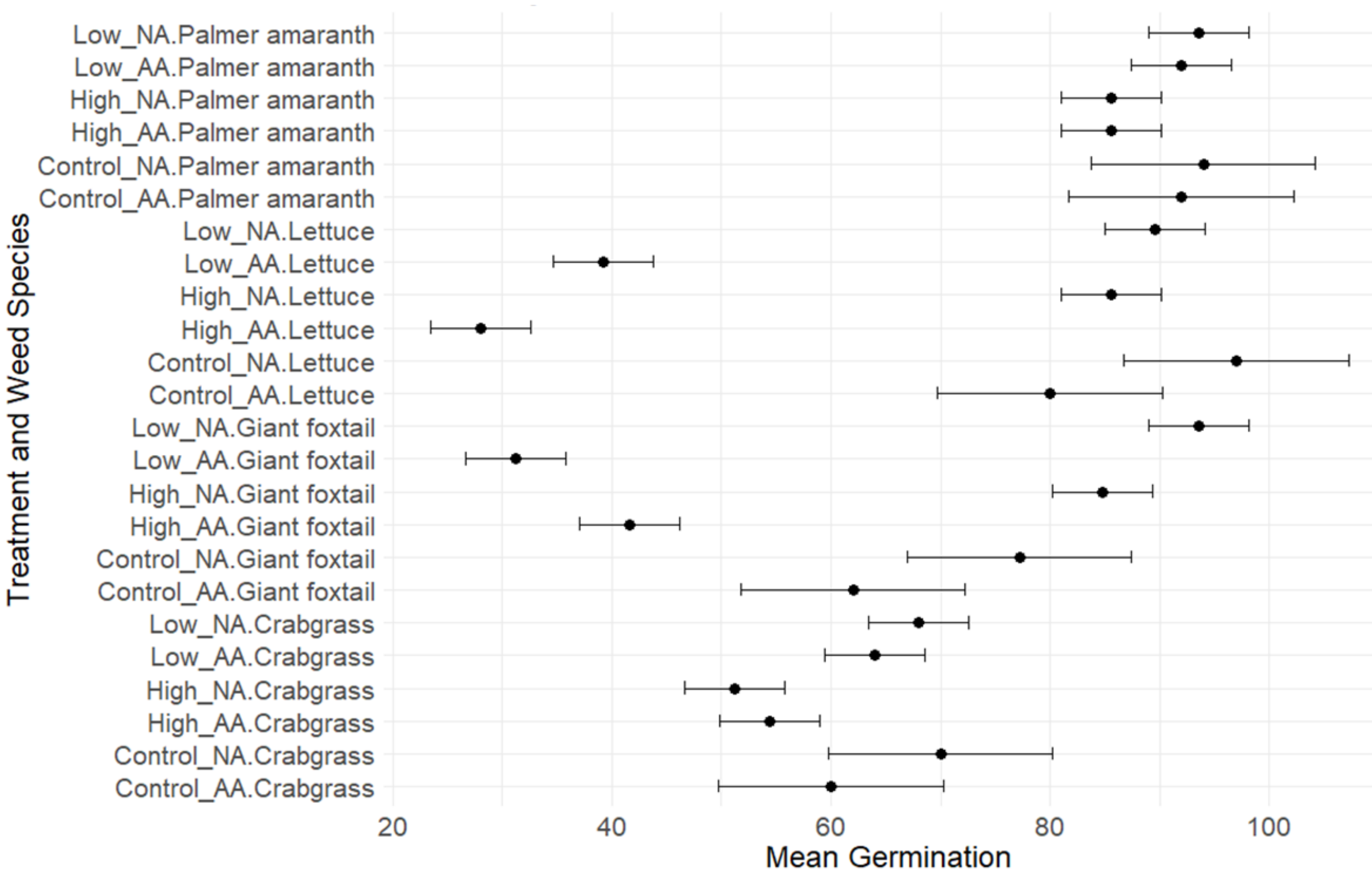

Figure 2.2: Estimated marginal means (EMMs) that show the average value germination at 21 days at each factor level of treatment and bioindicator species which includes, crabgrass (*Digitaria sanguinalis*), giant foxtail (*Setaria faberi*), lettuce (*Lactuca sativa*), and Palmer amaranth (*Amaranthus palmeri*). Error bars indicate the 95% confidence interval of the LS mean and means that overlap are not significantly different from each other.

Chapter 4 General Conclusion

Incorporating allelopathy screenings in cereal rye can significantly enhance integrated weed management strategies. While breeding efforts have traditionally focused on increasing biomass in cereal rye, the potential for breeding specifically for enhanced allelopathic properties remains underexplored. However, recent advancements in screening methods for allelopathy, both *in vitro* and in soil within greenhouse settings, suggest that it is possible to accelerate the breeding timeline for this beneficial trait. The heritability of allelopathy in cereal rye has been shown to be sufficiently high on a family-mean basis, making it a promising target for breeders. Given the obligate outcrossing nature of cereal rye and the associated challenges it presents for breeding, the heritability estimates obtained in this study are encouraging.

Significant variation in weed suppressive abilitiy was observed among the cereal rye lines screened and this variation underscores the efficiency of the allelopathy screenings conducted, as they demonstrated clear differences in weed suppression capabilities. Also, by screening lines *in vitro* and in soil, the number of lines required for field screenings is significantly reduced. Importantly, estimates of heritability indicate that allelopathy is moderately to highly heritable, which further supports the feasibility of breeding for this trait. Breeders can maximize their efforts by targeting both allelopathy and vegetative biomass simultaneously, as genetic correlation data suggest these traits are positively related.

However, the results from SBR-G and visual injury assessments conducted in the greenhouse indicate that certain lines, which were not identified for high allelopathy *in vitro*, demonstrated high allelopathy in soil. This can be problematic because those lines would have been screened out and eliminated from the breeding material based on the *in vitro* assessment when selections were made for high allelopathy. One consideration for cereal rye breeders to circumvent this, would be to have the resources to immediately screen breeding material in the greenhouse to make selections for high allelopathy based on the genetic correlation data collected in this study, which is selecting for plants that have more tillers and larger leaf area.

The germination studies reinforced the idea that incorporating allelopathic varieties of cereal rye into cover crop rotations can effectively reduce weed populations. However, the effectiveness of allelochemicals may be influenced by the age structure of the weed seed bank, indicating that the timing and conditions of weed emergence play crucial roles in the overall efficacy of allelopathy. Additionally, the interaction between allelochemicals and seed aging highlights the complexity of weed management strategies. Ultimately, these findings suggest that breeding for increased allelopathy in cereal rye not only has the potential to improve weed suppression but could also lead to the development of more effective cover crop systems. By integrating allelopathic traits with traditional biomass breeding, researchers and farmers alike can work toward more sustainable agricultural practices.

The germination study revealed considerable variation in the responses of weed species to allelochemicals, with some responses influenced by the age of the seeds. Most germination studies typically use fresh, vigorous seeds. However, the findings of this study clearly indicate that weed seeds respond differently depending on the aging treatment. This suggests that relying solely on fresh seeds for germination studies may not accurately reflect the age structure present in seed banks. These results also highlight the potential for cereal rye allelopathy to alter the dynamics of weed communities, indicating that the application of weed control by allelopathy could lead to shifts in species composition and abundance. For example, *Amaranthus palmeri* was less affected by allelochemicals than *Setaria faberi* for example, but was mostly affected by aging. *Setaria faberi* germination was affected by an interaction between allelochemicals and aging. These results indicate that cereal rye allelopathy could cause different responses in weed communities, such that germination and growth of weeds like *Setaria faberi* would be slowed over time, while weeds like *Amaranthus palmeri* would not be as affected by allelochemicals and would remain in the seedbank. This scenario is unfavorable, as *Amaranthus palmeri* is one of the more challenging weeds to control due to its herbicide resistance, rapid growth, high seed production, and capacity to germinate across a wide range of temperatures (Borgato et al., 2024; Reinhardt Piskackova et al., 2021; Ward et al., 2013).

For future consideration, when evaluating the use of cereal rye as a cover crop for its allelopathic properties to potentially reduce weed emergence and growth, it is crucial to consider the age of the existing seed bank. The age of weed seeds can significantly influence their viability and response to allelochemicals. Older seeds may exhibit reduced germination rates or altered physiological responses compared to fresher, more vigorous seeds (Dekker, 1999; Rajjou et al., 2012; Reed et al., 2022). Understanding the composition of the seed bank—specifically whether the weed species present are aged or vigorous—can provide valuable insights into their likely responsiveness to the allelopathic effects of cereal rye. Aged seeds may be more susceptible to allelopathy, potentially leading to decreased germination and growth, while vigorous seeds could demonstrate resilience, thereby necessitating targeted management strategies. By assessing the age structure of the seed bank, practitioners can better predict the effectiveness of cereal rye in suppressing specific weed populations. This knowledge allows for a more informed approach to cover crop selection and management, ultimately enhancing the efficacy of integrated weed management practices.

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