Breeding allelopathy in cereal rye for weed suppression

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Abstract

Rapid increase in the hectarage and agricultural systems that use cover cropping for soil conservation and improvement, soil moisture retention, and weed management has highlighted the need to develop formal breeding programs for cover crop species. Cereal rye (*Secale cereale* L.) is preferred by many growers due to high biomass production and weed-suppression potential, which is believed to be partially due to allelopathy. Rye germplasm exhibits large variability in allelopathic activity, which could be used to breed rye with enhanced weed suppression. Here, we provide an overview of rye history and breeding and describe a strategy to develop rye lines with increased allelopathic activity. The discussion focuses on ways to deal with important challenges to achieving this goal, including obligate cross-pollination and its consequent high segregation levels and the need to quantify allelopathic activity under field conditions. This review seeks to encourage weed scientists to collaborate with plant breeders and promote the development of cover crop cultivars better suited to reduce weed populations.

Introduction

The adoption of cover cropping has increased during the last few years to replace winter fallow and thus reduce soil erosion, decrease nitrogen leaching, add soil organic matter, improve soil moisture and health, and suppress weeds (NRCS 2015). In the United States, federal and state conservation programs expanded support for cover crops, which led to unprecedented adoption by farmers (Wallander et al. 2021). According to the 2017 Census of Agriculture, in 2017, there were 6.2 million ha of cover crops, which represented a 50% increase compared with the 4.2 million ha reported in 2012 (USDA 2019).

Historically, cover crops were grown predominantly in organic and low-input farming operations. However, the evolution of resistant weeds and the concomitant reduction in herbicide efficacy throughout the United States resulted in rapid adoption of cover crops in conventional farms as an avenue for mitigating this problem (SARE 2020). Winter cereal rye (*Secale cereale* L.) is the most widely planted cover crop in the United States, favored by farmers due to its winter hardiness, abundant biomass, ability to scavenge extra nitrogen from the soil, erosion control, and weed suppression (Jabran et al. 2015; Miedaner and Laidig 2019; Sustainable Agriculture Network 1998). In the United States, rye is typically grown during the winter before summer crops such as maize (*Zea mays* L.) for grain or silage, soybean [*Glycine max* (L.) Merr.], and cotton (*Gossypium hirsutum* L.) (Wallander et al. 2021). The most common cultivars of rye used in the United States were released decades ago (e.g., 'Elbon' in 1956, 'Wrens Abruzzi' in 1970, 'Wheeler' in 1972, 'Maton' in 1975) and more recent releases derive from those (e.g., 'Oklon' from Maton, 'Wrens 96' from Wrens Abruzzi) (Casey 2012; Morey 1970; Moore Seed Farm, www.msfseeds.com; Newell and Butler 2013). Since then, investment in rye breeding has been minimal, especially for cover crop uses. Furthermore, growers frequently purchase seed that does not specify the variety. Increased cover crop adoption highlights the lack of cover crop breeding programs and limited cultivars available to farmers, especially cultivars bred for cover cropping–related goals such as weed suppression.

Even though one of the attractive features of rye is its potential to reduce weed problems in summer crops, this trait has not been a core component of breeding programs. Developing cultivars with superior weed suppression would be beneficial, not only for organic production but also for conventional farms to reduce their reliance on herbicides and manage resistant weed populations (Schulz et al. 2013; Worthington and Reberg-Horton 2013). Nevertheless, rye cultivars have been bred mainly for grain and forage production. There have been no clear and

consistent efforts to breed specifically for weed suppression. Although high biomass production is recognized as perhaps the most important factor for the ability of cover crops to reduce weed emergence and establishment, allelopathy seems to be also an important trait influencing rye's weed-suppression ability, especially under conditions that limit biomass production (e.g., limiting rainfall, low temperatures, and short growing seasons). Despite allelopathy's metabolic complexity and potential difficulty for breeding, researchers have successfully introduced this trait into rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), and barley (*Hordeum vulgare* L.) cultivars (Worthington and Reberg-Horton 2013), albeit at lower levels than in rye.

Here, we provide an overview of the biology, genetics, and breeding history of rye. Also, we discuss potential breeding strategies to increase weed suppressiveness in rye focusing on allelopathy. The main goal of this article is to prompt discussion about the need for collaborations between weed scientists and plant breeders and the importance of breeding cover crops to generate varieties that can address priority issues that growers are facing.

Rye Description

Center of Origin and Global Distribution

Rye is an annual, cross-pollinating, self-incompatible cereal grass (Cyperales: Poaceae) that was domesticated after wheat, barley, and oats (*Avena sativa* L.). According to Sencer and Hawkes (1980), paleoethnobotanical evidence suggests that rye was transported from northeastern Anatolia to the west and northwest of the Black Sea in mixtures with wheat and barley as early as the 8th and 5th centuries BCE. The locations in Europe where rye was discovered, as well as the age of archaeological remains, correspond to the dates of Celtic occupation of those areas. However, Schlegel (2013) reported that archeological remnants of rye were found among wheat and barley from at least 6,000 yr ago in the Anatolian regions of Turkey, indicating the existence and cultivation of rye in the region during prehistoric times. In 1917, the Russian plant geneticist Nikolai I. Vavilov reported that rye evolved only as a secondary crop in wheat and barley as wild populations of rye invaded fields growing these crops in southwestern Asia (Middle East) resulting in weedy ryes with varying degrees of rachis brittleness. The continuous selection by man of weedy ryes for nonbrittle rachis and bigger grain gave rise to cultivated rye, which became a secondary crop under conditions not suited for wheat and barley. Based on phytogeographic, genetic, paleoethnobotanical, linguistic, and other evidence, Sencer and Hawkes (1980) concluded that the center of origin of rye is located between the slopes of Mt Ararat to Lake Van area in eastern Turkey. From there, it migrated to Russia and Scandinavia, and then to Poland and Germany, where it gradually spread throughout most of Europe from 2000 to 3000 BCE (Schlegel 2013). It was later brought from Europe to North America and western South America to the settlements established in the 16th and 17th centuries (1501 to 1700 CE). However, the region around Tabriz (Iran) toward the Black Sea in the west and toward Afghanistan in the east was also considered as a center of origin, as those areas coincide with the recent distribution of weedy rye (Schlegel 2013). A recent study using simple sequence repeat marker analysis by Maraci et al. (2018) revealed that domesticated populations from southwestern Asia (Middle East) have the highest genetic diversity, supporting the idea of this area being the true center of origin for cultivated rye. To this date, it is not known where and when allelopathy evolved and whether it occurred naturally or was selected for during domestication; characterizing the presence of this trait in rye germplasm from areas with high genetic diversity is still a necessary but pending task.

The Food and Agriculture Organization reported in 2017 that 74% of the world's harvest for rye grains was produced by six countries in northeastern Europe, namely Germany (3.173 billion kg), the Russian Federation (2.54 billion kg), Poland (2.19 billion kg), Belarus (650 million kg), Denmark (577 million kg), and Ukraine (391 million kg). In these countries, rye is produced for human food, animal feed, bioethanol, and biogas (Miedaner and Laidig 2019). The United States uses less than 15% of its rye hectarage for grain, which represented 341 million kg in 2017. The majority of rye hectarage is used as pasture, hay, and cover crop (Newell and Butler 2013; Oelke et al. 1990).

Taxonomy and Evolution

The revised taxonomy by Frederiksen and Petersen (1998) divides the genus *Secale* into three species: the annual self-compatible wild species *Secale sylvestre* Host; the outcrossing and perennial wild species *Secale strictum* (C. Presl) C. Presl (also known as *Secale montanum* Guss. and *Triticum strictum* C. Presl), and *Secale cereale*, which includes cultivated, weedy, and wild rye subspecies. It is also suggested that although the domestication history of rye remains unclear, *Secale cereale* subsp. *vavilovii* (Grossh.) Kobyl. is the putative wild progenitor of domesticated rye (Zohary et al. 2012). Results of a recent study on the genetic diversity and relationship between domesticated rye and its wild relatives using genotyping by sequencing confirmed Frederiksen and Petersen's (1998) taxonomic classification. The three species formed well-defined clusters in the principal component analysis, resided on distinct branches in a neighbor-joining tree, and were assigned predominantly to different ancestry components (Schreiber et al. 2019). In all analyses, *S. cereale* subsp. *vavilovii* was grouped together with *S. cereale* subsp. *cereale*, *afghanicum*, *segetale*, *ancestrale*, and *dighoricum*, indicating high relatedness and close genetic affinity. Results also showed a closer genetic relationship between *S. cereale* subspecies and *S. strictum* than with *S. sylvestre*, supporting early reports that *S. cereale* diverged from *S. strictum* (Ren et al. 2011; Zohary 1971). While information about allelopathy is available for *S. cereale*, none exists for *S. sylvestre* and *S. strictum*. There are studies reporting high weed-suppression levels in *S. strictum*, but no information about the suppression mechanisms is available (Andersen et al. 1992; Buman et al. 1988; Tang et al. 2011).

Genetics and Breeding

Rye is diploid with 14 chromosomes ($2n = 2x = 14$) and a relatively large genome size of 7.86 Gb (with 90% repetitive sequences) compared with other cereals such as sorghum [*Sorghum bicolor* (L.) Moench] (0.7 Gb), barley (5.0 Gb), maize (2.4 Gb), and rice (0.43 Gb), but smaller than oat (12.5 Gb), and wheat (17 Gb) (Eckardt 2000; Flavell et al. 1974; Haberer et al. 2005; Li 2021; Paterson 2008; Schreiber et al. 2020; Shi and Ling, 2018).

In the United States, rye is grown primarily as forage, and the cultivars commonly used are tall, with lodging tendencies, obligate outcrossing, and poor seed production making it difficult to breed (Barnett et al. 2002). Outcrossing is the result of a strong self-incompatibility (SI) system (Lundqvist 1957). In Poaceae species such as rye, SI is controlled by two unlinked loci, *S*- and *Z*-, which

are both gametophytically expressed (Langridge and Baumann 2008). When both pollen S and Z alleles are matched in the pistil, incompatibility occurs, inhibiting pollen growth; otherwise, pollen is compatible and fertilization proceeds (Yang et al. 2008). Because of this reproductive characteristic, most cultivars released in the United States have been bred as open-pollinated varieties (OPVs), resulting in materials with high heterogeneity and heterozygosity (Miedaner and Laidig 2019; Newell and Butler 2013). Furthermore, selection of rye lines has not been conducted extensively using molecular markers due to this poorly characterized high allelic diversity.

Hybrid rye breeding started in the early 1970s at the University of Hohenheim, Germany (Geiger and Miedaner 1999). With access to diverse germplasm, researchers were able to identify and develop the three parental lines needed to produce a hybrid rye: (1) self-fertilizing inbred lines, (2) cytoplasmic male sterile lines, and (3) fertility-restoring lines. Today, hybrid rye represents 70% to 80% of German production. It is also being planted in Austria, Poland, the Russian Federation, Denmark, Estonia, the United Kingdom, Ireland, Canada, and the United States; although in all these cases, hybrid rye varieties were developed by Germany. With a 15% to 20% grain yield advantage of hybrids over traditional OPVs, hybrid rye breeders have started using single-nucleotide polymorphism arrays for large-scale genotyping of elite lines and their crosses, making it possible to breed for yield, grain quality, pest and disease resistance, and other important agronomic traits more efficiently.

Development of a Rye Breeding Program for Weed Suppression

Weed-Suppression Mechanisms

Harper (1977) referred to a cultivar's weed suppressive ability as its "interference potential." It is important not to confuse weed-suppression ability with weed competition tolerance (Cahill et al. 2005; Lemerle et al. 2001; Watson et al. 2006). In the former, the crop can reduce the growth and even survival of weeds with which it is interacting. The latter refers to the capacity of the crop to mitigate reductions in growth and yield when experiencing weed interference (Leon et al. 2016). Although both traits are desirable, weed suppressive ability is preferred, because it reduces the risk of yield losses while decreasing the potential reproduction of weeds, thus contributing to weed management (Cosser et al. 1997). Interference of resource acquisition and allelopathy are two mechanisms by which weed suppression may operate. The former mechanism is dependent on a crop cultivar's capacity to acquire water, nutrients, and light in a constrained environment, inhibiting the growth and reproduction of surrounding weed species, while allelopathy is the process by which plants release phytotoxins into the environment, limiting the growth of nearby plants (Muller 1969).

Cover crop residues suppress weeds by (1) physically obstructing weed emergence and growth (Teasdale and Mohler 2000); (2) acting as a physical barrier limiting light penetration to the soil surface, where many weed species rely on light exposure and soil warming to aid germination; or (3) releasing allelopathic compounds near the soil surface that damage embryo and seedling tissue during seed germination and seedling emergence (Barnes and Putnam 1983; Weston 1996). Although the physical effects of cover crops are considered the main mechanisms for weed suppression, allelopathy can also contribute meaningfully to weed suppression (Shilling et al. 1985). In one case of pea (*Pisum sativum* L.) growing with rye residue, the biomass of barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.] and redroot pigweed (*Amaranthus retroflexus* L.) was reduced by 74% and 55%, respectively, and the majority of that reduction was attributed to allelochemicals when compared with a non-allelopathic mulch (Barnes and Putnam 1983).

Allelopathy

The mechanism of allelopathy in rye has been widely studied, and although rye synthesizes and secretes many molecules that exhibit allelopathic activity, a few compounds explain the majority of the allelopathic effects. Shilling et al. (1986) found 17 phytotoxic compounds in 'Abruzzi' rye, a common cultivar in the southeastern United States, but the most phytotoxic were 2,4-dihydroxy-1,4(2H)-benzoxazine-3-1 (DIBOA) and 2,3-benzoxa-zolinone (BOA) (Barnes and Putnam 1987; Shilling et al. 1985). DIBOA and BOA are classified as benzoxazinoids (BX), with DIBOA being the most biologically active of this chemical family (Barnes et al. 1986; Burgos and Talbert 2000; Niemeyer 2009; Shilling et al. 1985). BOA is the breakdown product of DIBOA (Barnes and Putnam 1987; Burgos and Talbert 2000). Dicotyledonous and monocotyledonous species are both sensitive to BX molecules, suggesting that rye allelopathic activity has potential for broad-spectrum weed suppression. However, there are differences in weed sensitivity depending on the type of BX, with dicots exhibiting higher sensitivity to BOA and monocots to DIBOA (Barnes and Putnam 1987; Nair et al. 1990).

Rye allelochemical production dramatically exceeds that of other cereals. For example, DIBOA concentration in rye roots can be up to 3 times higher than in triticale ($\times$*Triticosecale* Wittm. ex A. Camus [*Secale* $\times$ *Triticum*]) and 48 times higher than in wheat. In the case of shoots, the differences are even more dramatic, with rye exhibiting DIBOA concentrations higher than triticale and wheat shoots by factors of 90 and 937, respectively (Reiss et al. 2018a). Allelopathic activity also varies widely in rye germplasm. Several studies have documented up to 10-fold differences in allelochemical production among commercially available rye cultivars (Burgos et al. 1999; Reberg-Horton et al. 2005; Rice et al. 2005). This variability among rye cultivars suggests that there is potential to enhance weed suppression via breeding. It has been suggested (but not explicitly tested) that growth traits such as rye early vigor and canopy height can be positively related with BX concentration in rye tissue (Reiss et al. 2018a). It will be important to determine whether there are fitness benefits or costs associated with allelopathic activity (Reiss et al. 2018b) to identify any trade-offs that will affect a breeding program focused on weed suppression. In field settings, these two factors are difficult to differentiate without an adequate phenotyping strategy.

Extensive work has been conducted to quantify the production of allelochemicals in rye (Brooks et al. 2012; Burgos et al. 1999; Reberg-Horton et al. 2005), and this information can inform the selection of phenotyping parameters. However, the large number of potential allelochemicals could make selection difficult and more expensive. Fortunately, correlations between the concentration of DIBOA, BOA, and total hydroxamic acids, all members of the BX chemical family are high ($R^2 > 0.8$). Thus, differentiating between allelopathic compounds might be unnecessary from a breeding perspective (Rice et al. 2005). Another positive aspect of the BX biosynthetic pathway for breeding is that its phenotypic

variation has a clear genetic component. Previous research documented narrow-sense heritability of DIBOA production between 0.2 and 0.6, which are levels comparable to maize yield. Furthermore, this indicates that the production of allelopathic compounds is a quantitative trait likely controlled by multiple genes, but with enough heritability to make genetic improvement (Brooks et al. 2012; Hallauer et al. 2010). In addition to allelochemical concentration, maturity is also an important physiological trait that impacts allelopathy in rye. The maximum concentration of allelopathic chemicals occurs at the boot stage or the transition from vegetative to reproductive growth (La Hovary 2011). Therefore, this period of growth should occur close to cash crop planting to maximize weed suppression. In this regard, flowering time and biomass yield at the boot stage are both critical traits associated with allelopathic activity in the field. Green planting is a strategy that may help extend the allelopathic effect during the cash crop establishment phase (Ficks et al. 2022; Reed et al. 2019).

Challenges for Allelopathy Screening and Breeding

There are multiple challenges for breeding allelopathy in rye, but among the most important are (1) allelopathic activity quantification; (2) obligate cross-pollination; and (3) low genetic gain, which is related to the first two.

Most recent efforts to quantify allelopathic activity have focused on measuring allelochemical concentration in rye tissues using analytical chemistry with complex methods such as high-performance liquid chromatography and gas chromatography (Brooks et al. 2012; Burgos et al. 1999; Reberg-Horton et al. 2005). The main limitation of this approach is its low correlation with actual weed suppression in the field (Brooks et al. 2012; Khanh et al. 2009; Mwaja et al. 1995). Because allelochemicals must be released to the soil, processes such as root secretion and release during leaf decomposition after rye termination (i.e., killing by herbicides, mowing, or roller-crimper) can modulate how much of the allelochemicals are biologically available for weed suppression. Thus, it is possible to have a rye plant with a high concentration of allelochemicals in its tissue but with limited release, which ultimately will result in low weed suppression. Conversely, a plant with moderate concentration or production but high root secretion or fast release of allelochemicals during leaf degradation might be more effective in reducing weed seedling establishment. For these reasons, although convenient for processing large numbers of plants in a short time, relying solely on analytical quantification of allelochemical concentration in plant tissue does not seem to be an adequate approach for selection of allelopathic lines.

Obligate cross-pollination is one of the most important limitations for rapid development of allelopathic rye cultivars. This issue is particularly challenging during the initial phases of selection for identification of the germplasm of interest and transfer of the trait to lines with desirable agronomic performance. Open-pollinated germplasm has high levels of segregation after crossing, even within closely related families. This is particularly problematic when selecting for quantitative traits such as allelopathy, because uniformity of the selected material is much lower than for self-pollinating germplasm. This challenge, together with the difficulty of quantifying allelopathic activity, can reduce the efficiency of a breeding program by requiring large field experiments, recurrent selection for multiple generations to increase the frequency of allelopathic alleles in the population, and isolation and rogueing of selected germplasm in each round of selection.

Breeding Strategy

With all these considerations and previous experiences in mind, we propose increasing the efficiency of a rye breeding program for allelopathy based on two operational principles. First, allelopathic activity must be quantified under field conditions to improve selection accuracy. Second, the number of lines screened in the field must be reduced as much as possible to limit resource needs and improve selection efficiency. However, implementation of these principles must be complemented with actions to maintain the necessary number of lines in a breeding program to identify and increase frequency and expression of the desired traits. Next, we provide an example of a strategy to operationalize these principles (Figure 1). Although we recognize that allelochemical content and release from shoots is important, this proposal is focused on describing root allelopathic activity in vivo. We prioritized roots, because in rye, they are responsible for the majority of allelochemical production and release to the soil (Otte et al. 2020; Rice et al. 2022). Also, there are important confounding effects between leaf tissue decomposition and the release of allelochemicals, making it more difficult to phenotype the lines and correctly isolate and identify the factor responsible for weed suppression.

Initial Population Development

An initial detailed screening of diverse germplasm to identify materials with allelopathic potential might be a time-consuming step, but one that is critical to set the basis of a robust breeding program (Figure 1). For example, Reberg-Horton (2002) evaluated 268 rye accessions from the USDA National Small Grain Library and 10 commercially available cultivars for allelopathy in laboratory conditions. This was done by aqueous extraction from rye tissue grown in the field. Bioassays were done by adding the extracts to petri dishes lined with filter paper and using the seeds of *A. retroflexus* or goosegrass [*Eleusine indica* (L.) Gaertn.] as bioindicators. Reduction of radicle elongation was the main variable for selection, considering previous studies reporting this as one of the most common effects of allelopathy (Burgos and Talbert 2000; Wu et al. 2000).

All accessions that were both late maturing (field observations) and highly allelopathic (laboratory bioassay) were selected to generate an initial rye population. The former criterion was part of the selection process or protocol because flowering time influences biomass accumulation and late-maturing lines tend to maximize this trait (Reberg-Horton 2002). Based on these criteria, 15 accessions were selected to advance in the breeding population along with two publicly available cultivars. Wrens Abruzzi, a southeastern adapted cultivar, was chosen because of its allelopathic activity, which was among the highest of all rye entries tested. Wheeler was also chosen because of its late maturity and allelopathic activity, although it exhibits less allelopathic activity than Wrens Abruzzi. These 17 genotypes were allowed to cross-pollinate in isolation over two generations under field conditions, and their progeny were bulked to form the base population for future selection for enhanced allelopathic activity. This outcrossing strategy between entries of the germplasm bank and lines with proven adaptation to local conditions is critical to generate a starting population with a rich combination of allelopathy and agronomic traits.

The bulked population provides the material for the first round of phenotyping and selection under field conditions. For this purpose, thousands of plants (from individual seeds) will have to be screened. Each rye plant is a unique genotype, so at this stage
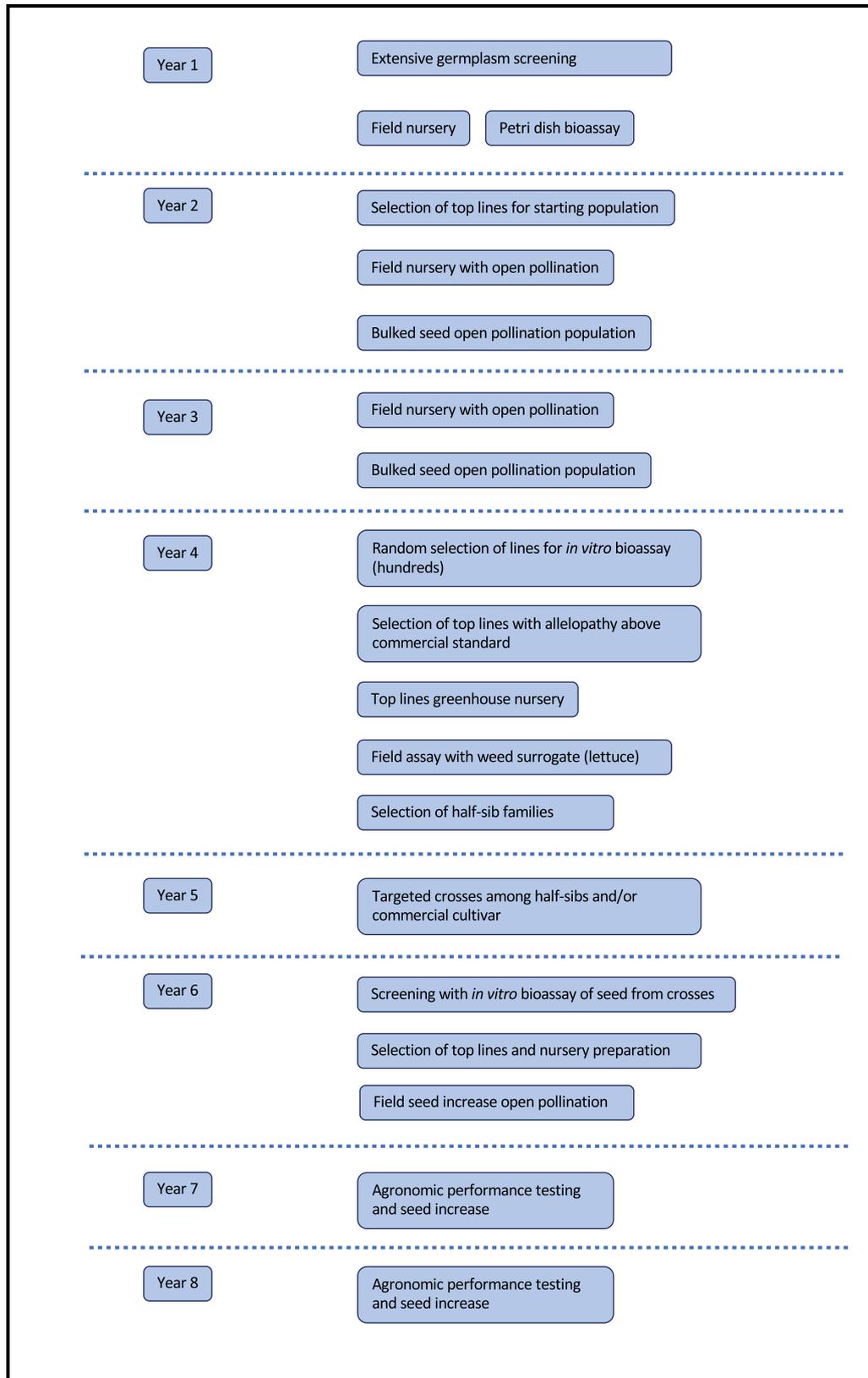
Year 1 — Extensive germplasm screening

Field nursery — Petri dish bioassay

Year 2 — Selection of top lines for starting population

Field nursery with open pollination

Bulked seed open pollination population

Year 3 — Field nursery with open pollination

Bulked seed open pollination population

Year 4 — Random selection of lines for *in vitro* bioassay (hundreds)

Selection of top lines with allelopathy above commercial standard

Top lines greenhouse nursery

Field assay with weed surrogate (lettuce)

Selection of half-sib families

Year 5 — Targeted crosses among half-sibs and/or commercial cultivar

Year 6 — Screening with *in vitro* bioassay of seed from crosses

Selection of top lines and nursery preparation

Field seed increase open pollination

Year 7 — Agronomic performance testing and seed increase

Year 8 — Agronomic performance testing and seed increase

Figure 1. Diagram of breeding steps to increase allelopathic activity in rye.

there are no replications, unless a nursery is generated and individual lines are vegetatively cloned (i.e., tiller splitting). Because of the scale of this screening, multiple individuals of

control lines (e.g., original commercial parents) should be randomized throughout the field and used as reference checks. Lines that exhibit weed suppression inferior to these commercial
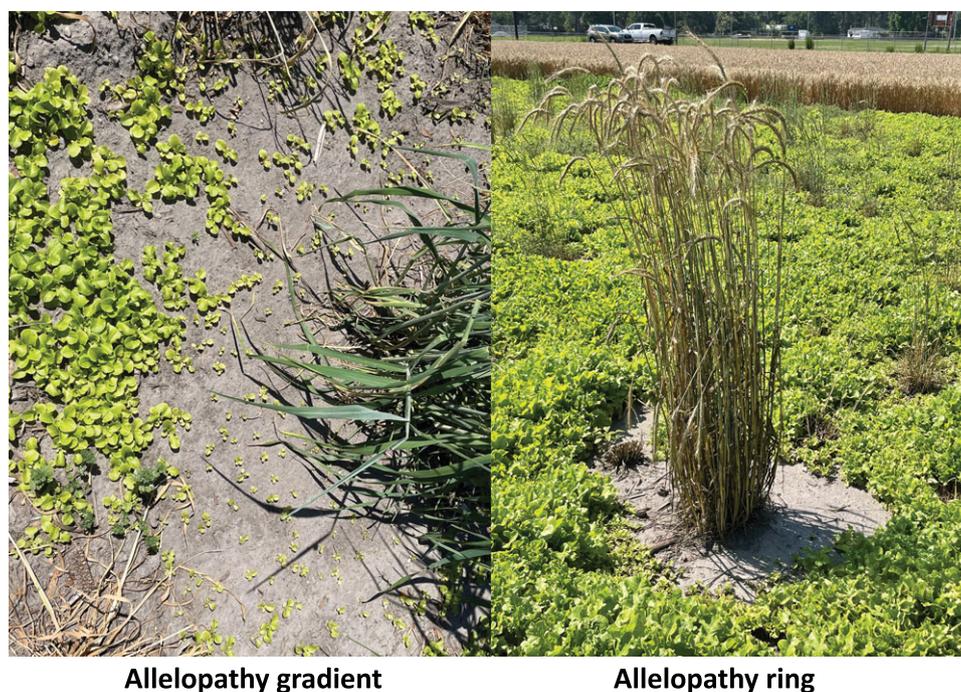
Allelopathy gradient   **Allelopathy ring**

Figure 2. Gradient of allelopathic activity of rye on lettuce causing chlorosis and stunting (left). Inhibition of lettuce emergence by a highly allelopathic rye line forming a bare ring at the base of the plant.

cultivars must be eliminated, to reduce the frequency of low- or non-allelopathy alleles. Furthermore, rye-free controls (negative controls) should also be randomly placed throughout the field to determine weed pressure and growth in the absence of weed suppression.

Some Considerations for Field Testing

Raised beds should be avoided, because shading, depending on orientation and sun angle, as well as variation in soil moisture along the bed, could affect weed growth, making it difficult to rate allelopathic activity. Therefore, preparation of a flat and uniform seedbed is recommended. Plant spacing should be 1 to 1.5 m to avoid confounding effects and allow proper growth of weeds or indicator plants. Uniformity throughout the field of the indicator weed species is of the utmost importance for accurate and consistent assessment of allelopathic activity. Unless the seedbank is very dense, weeds might exhibit patchiness and irregular germination over time, affecting the assessment. For this reason, using a crop as a surrogate for weeds will increase accuracy of the phenotyping. After testing multiple species, our preference is lettuce (*Lactuca sativa* L.) seeded at a high rate (44 kg ha^{-1}) (Figure 2). Although we considered several crops for this purpose, lettuce has two critical advantages. First, it is sown at shallow depths from which weeds tend to germinate, allowing radicle growth through the rye root system and increasing the chances for exposure to allelochemicals (Smith et al. 1973; Zahara 1969). Second, lettuce is a late-winter, early-spring crop adapted to cool conditions (Dufault et al. 2009; Wagenvoort and Bierhuizen 1977). Therefore, it can be seeded to coincide with the peak of allelopathic activity in rye (i.e., boot stage).

Using small rows as plots might seem intuitively desirable, especially for the initial work with half-sib families (i.e., head-rows, see Some considerations for field testing section), but row plots will make phenotyping more difficult, because even half-sibs can show significant levels of segregation. Therefore, overall ratings per plot might result in inaccurate estimations of the traits. Another unintended consequence of row plots is the reduction of seed production per plant due to intraspecific competition (Juskiw et al. 2000). Obtaining as much seed as possible from each maternal plant is critical for advancing lines to following selections in more locations and environments. For all these reasons, until there is certainty that a line is consistently and highly allelopathic, single-plant evaluations are a more effective approach for screening and seed production.

In Vitro Bioassay for Selection Efficiency

The challenges associated with creating a uniform weed population in the field increase as the number of lines and area needed for the screening increase. Therefore, ensuring that the material that is screened in the field, especially in later stages of the breeding program, has real potential for allelopathic activity is critical to maintain the efficiency of a breeding program. Developing ways to identify those lines before they are grown in the field can reduce the cost of the screening and enhance throughput considerably. It is in this type of situation that low-cost in vitro bioassays are valuable. In this regard, the equal compartment agar method (ECAM) is particularly helpful for rye breeding, because it is economical, technically simple, and requires a short time frame. Adapting this method, a single rye individual is grown in a beaker with a water-agar medium. After the plant has produced several leaves and roots (approximately 2 wk), seeds of a susceptible indicator species are added to determine allelopathic activity (Wu et al. 2000). The rye and bioindicator plants are separated by a sterile filter paper, ensuring only allelochemicals released by rye to the agar medium should be responsible for any reductions in growth of the bioindicator (Figure 3). This should be done comparing against an allelopathic standard and a rye-free negative control. Lines causing less injury to the bioindicator than the reference standard are

Figure 3. In vitro bioassay to identify rye lines meeting the minimum target allelopathic activity before field screening. The photos illustrate the reduction in growth and injury caused to lettuce seedlings by two experimental entries of rye and two commercial cultivars ('ND Gardner' and 'Aroostook') compared with control treatments without rye.

eliminated. Those that pass this test are transplanted for nursery preparation and then field screening (Year 4 in Figure 1). Based on our research, implementing the ECAM allows reduction of the number of lines that must be taken for field evaluation by 30% to 60%. This approach is particularly important for conducting targeted crosses, because it allows confirmation of allelopathic potential before conducting the cross. Another advantage is that after the bioassay, the plant can be vegetatively propagated (i.e., tiller splitting). Clones can be used for field screening while a nursery is maintained with the other clones for either crossing or seed increase in isolation.

Because the duration of the bioassay is very short, rye lines exhibit little to no differences in root length and density. Therefore, variation in allelopathic activity among lines should be predominantly due to differences in BX biosynthesis and/or secretion. Thus, incorporating an in vitro bioassay might help maintain selection for BX production and reduce the risk of selecting only for morphological changes of BX-producing tissue.

Phenotyping and Selection

Some of the traits that should be considered include growth habit, leaf shape, biomass production, flowering time, and susceptibility to foliar diseases [e.g., rust (*Puccinia recondita*), bacterial leaf blight (*Xanthomonas campestris*)] (Crespo-Herrera et al. 2017; Wen et al. 2018). Growth habit can range from prostrate to upright. Once prostrate individuals reach reproductive growth, the growth habit becomes upright, resulting in plants with a short, wide crown. Leaf width also differs among genotypes, ranging from 0.75 to 2.0 cm. Measurements or visual assessments of canopy volume before anthesis are important to capture potential biomass differences (Dobbs et al. 2023). Waiting until harvest to determine biomass is not possible in this setting, because lines that do not meet the

desired criteria for allelopathic activity and other agronomic traits should be eliminated before pollination starts.

Allelopathic activity should be evaluated to coincide with the booting stage or soon after, when allelochemical release peaks (Brooks et al. 2012). This can be done in fields with weed species that are pervasive across the field forming evenly distributed and dense populations, which should be reduced around rye plants that are allelopathic (Figure 4). Parameters that indicate allelopathic intensity include weed emergence inhibition, chlorosis, stunting, and necrosis (Figures 2 and 4). All these parameters inform the preliminary selections, with a final visual decision made at harvest when seeds from each selected line are collected and maintained separately.

An important decision for selection is whether to maintain lines exhibiting high levels of one of the traits of interest but lacking others. For example, there will be lines with low or no detectable allelopathic activity but with high biomass production and disease resistance. Similarly, breeders will find highly allelopathic lines with low biomass accumulation. At early stages of selection, not knowing the nature of the genetic correlation between allelopathy and other agronomic traits with certainty, it might be advantageous to maintain lines with high performance of one or a few traits in the gene pool (Condon et al. 2009), at least until more targeted crosses are implemented or lines with a robust level of all traits of interest are identified.

Half-Sib Families

Once the phenotyping data from this first large-scale field study are available, rather than bulking the seed of all the selected lines, as done previously, harvesting seed on an individual plant basis and keeping the seeds separate will increase heritability and accelerate improvement for the trait. Because of the
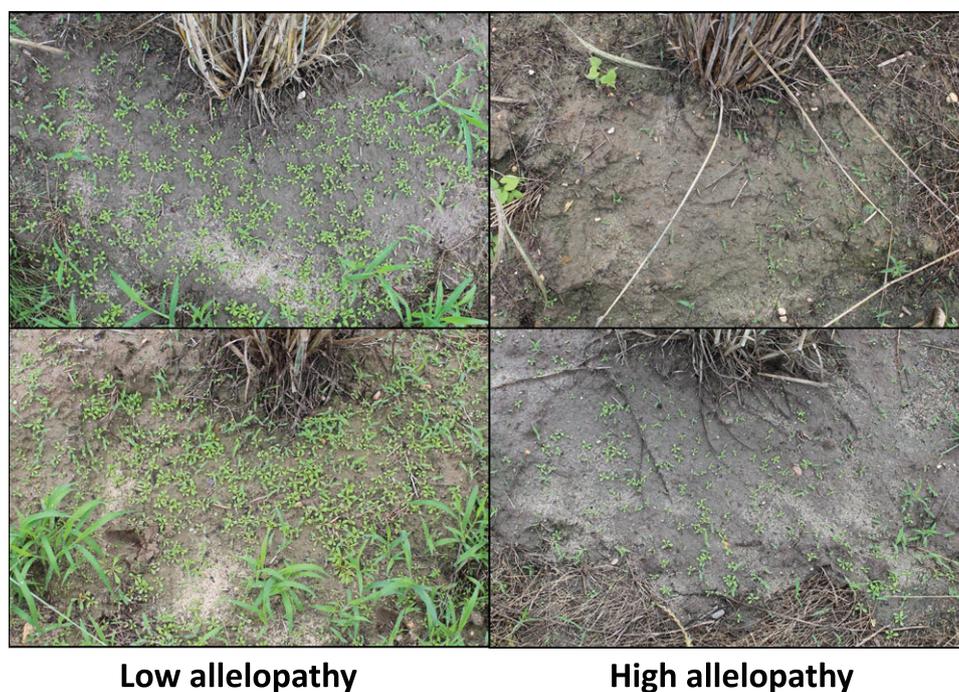
Low allelopathy **High allelopathy**

Figure 4. Examples of suppression of carpetweed (*Mollugo verticillata* L.) populations under field conditions by rye lines with low and high allelopathic activity.
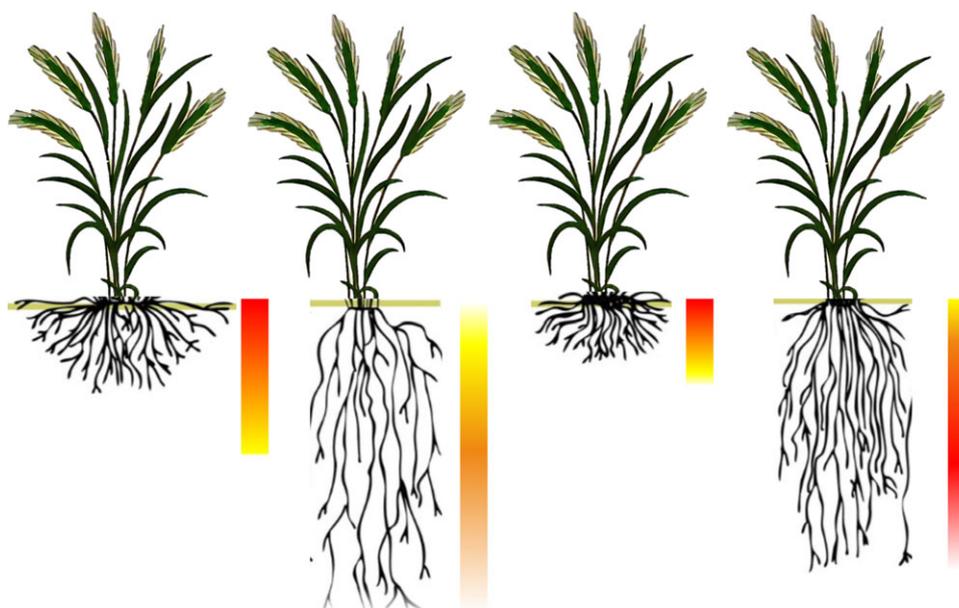

Figure 5. Rye root system architecture and its effect on allelochemical distribution in the soil profile. Red, orange, yellow, and white indicate high, medium, low, and no allelochemical concentration, respectively.

outcrossing nature of this crop, the seeds from each plant are considered to be predominantly half-sibs, for which there are detailed phenotypic information of the maternal line (Year 4 in Figure 1). An advantage of using half-sib families is that they can be replicated in the following screenings while maintaining control over the maternal genetic effect, which contributes to reducing segregation as the rounds of selection increase (Bernardo 2020).

Half-sib families meeting the desirable phenotype can be used for targeted crosses among themselves under controlled conditions

to pursue a further increase in allelopathy or growth and to reduce segregation. Furthermore, half-sib families with evident enhanced allelopathy can be crossed with locally adapted cultivars to integrate the trait (Year 5 in Figure 1).

Allelopathy Selection and Root Architecture

Under field conditions, some lines do not reduce weed emergence, but they can cause high levels of injury to established weeds (SHI and DR, personal observation). These different behaviors suggest

Figure 6. Rye allelopathy-mediated lettuce (weed surrogate) suppression at 4 wk after seeding. Shoots were eliminated before lettuce seeding and when rye reached the boot stage.

that emergence inhibition and injury of emerged weeds might be traits controlled by different genes, which poses the question of whether those differences are due to variation in their allelochemical profiles. Although this is possible, the extensive research done on rye pointing at DIBOA and BOA as the main allelochemicals make this option unlikely. It seems more plausible that the depth within the soil where allelochemicals are released might be the factor driving the differences in emergence inhibition and injury of emerged weeds. If the allelochemicals are released close to the soil surface, it is more likely that germinating weed seeds will be killed before they can emerge. Conversely, if most of the allelopathic activity occurs below their maximum germination depth, weeds will be able to germinate and emerge and they will not be affected until the radicle reaches the soil layer where allelochemicals are in high concentration. If this is indeed correct, it is possible that allelopathic activity might be most efficiently selected for by modifying root system architecture (e.g., increasing root density, favoring shallower roots) (Figure 5). These types of morphological changes might also affect nutrient and water acquisition in ways that are not desirable (e.g., lower fertilizer scavenging, less drought tolerance).

Caveats and Future Needs

There is a need to develop rye lines that are uniform and stable, but the outcrossing nature of this crop is a major obstacle for meeting this goal in a timely fashion. Crossing a proven enhanced allelopathic line with a self-compatible breeding line to develop a system to easily move the trait to locally adapted cultivars might be an important step to take advantage of allelopathy in rye.

Another important future step is to determine how allelopathy increased. As discussed earlier, it is not known to what extent the gain in allelopathy was due to the selection of more efficient BX biosynthesis and release or to changes in plant architecture (Figure 5). Answering this question will allow development of better and more effective phenotyping and selection methods. Yet another important step is estimating genetic correlations between allelopathy and desirable morphological and growth traits, which will be critical for identifying trade-offs that might have to be considered to ensure both chemical and physical weed suppression. Similarly, the predictive potential of in vitro bioassays for

allelopathic activity in soil and field conditions must be determined. If these two types of phenotyping strategies are not related, bioassays might have to use substrates other than agar, such as sterile soil. Also, a greenhouse assay with soil might be necessary between in vitro and field testing.

The proposed breeding strategy leaves a major question unanswered, which is whether selecting for root secretion of allelochemicals will also have a positive effect on shoot allelochemical concentration. When rye is used as mulch, it is possible that allelochemicals will be released to the soil as residues degrade. Determining whether such release is biologically significant and strong enough to suppress weeds is not easy, but if indeed it occurs, it could be important to extend the period during which weed emergence and survival are reduced. We prioritized selection based on root-mediated allelopathy, because root and shoot allelochemical concentrations are similar (Otte et al. 2020). More importantly, recent studies quantifying allelochemicals in soil from natural tissue (not extracts) demonstrated that rye roots, not shoots, are the main source of these compounds (Otte et al. 2020; Rice et al. 2022). As shown in Figure 6, rye can exhibit allelopathy even after shoot removal.

Here, we proposed a breeding strategy for rye to increase allelopathic activity for weed suppression. This is just one possibility, and there are many others that can be implemented according to germplasm availability; laboratory, greenhouse, and field space; and budget. Weed scientists can use this information to start a conversation with plant breeders to address the great need for new cover crop cultivars.

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