DISSERTATION

UNDERSTANDING WEED BIOLOGY AND HERBICIDE RESISTANCE TO IMPROVE WEED MANAGEMENT

Submitted by

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ABSTRACT

UNDERSTANDING WEED BIOLOGY AND HERBICIDE RESISTANCE TO IMPROVE WEED MANAGEMENT

Weed management is essential in agriculture, natural areas, and rangelands. Weed control has mainly relied on herbicides. These chemical compounds are a low-cost option, easy to apply, and very efficient to eliminate weeds. However, as part of survival strategies weed species have evolved mechanisms to overcome herbicides and continue their life cycle. Thus, it is imperative that we increase our knowledge in weed biology and resistance mechanisms to develop better management strategies. Here I present three chapters that cover these areas of study. First, as an intent to promote more tools for management strategies in winter wheat, a field survey was conducted to identify the potential to implement harvest weed seed control for problematic winter annual grasses in this cropping system. The second chapter covers the results of a herbicide resistance survey to screen for imazamox and quizalofop resistance of troublesome winter annual grasses in winter wheat and rangeland areas. The third chapter aimed to determine the distribution of native and introduced *Phragmites australis* haplotypes which is a riparian species problematic in rangeland and natural areas.

Harvest weed seed control methods showed potential to manage downy brome, feral rye, and jointed goatgrass. Seed retention of these winter annual grasses was over 75% indicating that the majority of seeds could be collected during wheat harvest. After screening over 280 samples of winter annual grasses, only two feral rye populations showed resistance to imazamox. Further studies on resistance mechanisms showed that one population (A) can rapidly metabolize the

herbicide compared to a susceptible and the second population (B) contained a target site mutation in the imazamox target enzyme. Introduced *Phragmites australis* haplotypes were identified in Colorado using molecular markers. In addition, a low-cost and quick genotyping tool was developed to encourage land managers to conduct more frequent monitoring. Main results from this dissertation are expected to contribute with the big endeavor of promoting integrated weed management solutions and better weed biology understanding.

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BACKGROUND INFORMATION

Feeding the next generation in the face of climate change and a rapidly expanding world population is perhaps the biggest challenge of my generation. Plant pests are a wicked problem in agriculture and with increasing levels of herbicide resistance are one of the main threats to agricultural productivity. The dynamic and diverse nature of weed species in combination with their detrimental effects on crop productivity makes them a critical research challenge for the weed science community. Weeds are plants that establish in areas intended for agricultural crops and compete with crops for space, light, nutrients, and water (Van Heemst 1985). They use these resources much earlier in the growing season and with greater efficiency than crops, thus, will negatively affect the total yield that can be produced. Weeds also rapidly adapt and overcome any stressor such as drought, temperature, and any repetitive management activities. Weeds, unlike crops, have not gone through the same thousand years of human domestication. Because of this, weeds have not lost the ability to survive stressful xenobiotics such as herbicides. The scope of weed science also includes the management of invasive species, alike weeds they are plants that have thrived in undesirable ecological areas.

Rapid adaptation of weed populations to recurrent management practices is becoming a significant threat to agroecosystem productivity. Herbicide tools are the preferred method to manage weeds due to simplicity, convenience, consistent high efficacy, and relatively low cost. However, repeated use of similar herbicide programs has accelerated the natural selection process where individuals that are able to survive herbicide treatment can reproduce and thrive ensuring their continuing presence in the cropping system. Currently there are over 500 unique cases of weed species that have evolved resistance to at least one mode of action, and some

species have evolved multiple resistance to nearly all the available modes of action (Heap 2020). In addition, no new modes of action have been discovered in the past two decades (Duke 2012). Consequently, herbicide efficacy and crop productivity have been declining over time. Previous approaches to manage herbicide resistance have relied on alternative herbicide sites of action, although weed biotypes may rapidly evolve resistance to them as well. Therefore, a proactive approach is imperative to mitigate this evolutionary 'arms race' to ensure agroecosystem sustainability. Many species adapt and overcome abiotic and biotic stresses in a short period of time. The study of herbicide resistance mechanisms has greatly contributed to increase our understanding in weed biology, ecology, and evolutionary trends of these species (Busi et al. 2013).

The weed science community recommends that growers integrate a range of management strategies and practices that target different weed lifecycle stages to reduce population abundance (Beckie and Harker 2017). Integrated weed management (IWM) as a branch of pest integrated management seeks for a holistic approach to control weeds in agriculture. Multiple practices and weed biology knowledge integration are the IWM main components for a proactive weed management. This approach aimed to diversify control techniques and not rely only on 1-2 methods (Buhler 2002). IWM implementation reduces the speed of herbicide resistance, and provide alternative solutions without affecting the agroecosystem sustainability (Boydston 2010). This dissertation focuses on three different projects that aimed to contribute to the IWM field. The first chapter is focused on identifying the potential of harvest weed seed control as a non-herbicidal method to manage three problematic winter annual grasses in winter wheat. Results showed that there is a potential to implement these methods in Colorado winter wheat farms. Here we are proposing one more method to enrich the IWM portfolio in wheat. The second

chapter is the result of large winter annual grasses herbicide screening survey conducted through the years in Colorado. Our results showed the first two feral rye imazamox resistant populations and none to quizalofop. From the IWM perspective, this information is imperative for resistance management and improve the stewardship for the available tolerant wheat varieties. The third chapter is related to a survey for the invasive species *Phragmites australis*. In addition, this research provides a quick and low-cost genotyping protocol for introduced *Phragmites* decision-making tools for management. This is versatile species that can show morphological differences based on the soil and environmental conditions; thus, it is difficult to distinguish native and invasive haplotypes by visual estimation. There is an urgent need to manage invasive haplotypes as they can displace native species, reduce biodiversity, and change entire ecosystems.

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Chapter 1: Seed Retention of Winter Annual Grass Weeds at Winter Wheat Harvest Maturity Shows Potential for Harvest Weed Seed Control

SUMMARY

Downy brome, feral rye, and jointed goatgrass are problematic winter annual grasses in central Great Plains winter wheat production. Integrated control strategies are needed to manage winter annual grasses and reduce selection pressure exerted on these weed populations by the limited herbicide options currently available. Harvest weed-seed control (HWSC) methods aim to remove or destroy weed seeds, thereby reducing seed-bank enrichment at crop harvest. An added advantage is the potential to reduce herbicide-resistant weed seeds that are more likely to be present at harvest, thereby providing a nonchemical resistance-management strategy. Our objective was to assess the potential for HWSC of winter annual grass weeds in winter wheat by measuring seed retention at harvest and destruction percentage in an impact mill. During 2015 and 2016, 40 wheat fields in eastern Colorado were sampled. Seed retention was quantified and compared per weed species by counting seed retained above the harvested fraction of the wheat upper canopy (15 cm and above), seed retained below 15 cm, and shattered seed on the soil surface at wheat harvest. A stand-mounted impact mill device was used to determine the percent seed destruction of grass weed species in processed wheat chaff. Averaged across both years, seed retention (±SE) was 75% ± 2.9%, 90% ± 1.7%, and 76% ± 4.3% for downy brome, feral rye, and jointed goatgrass, respectively. Seed retention was most variable for downy brome, because 59% of the samples had at least 75% seed retention, whereas the proportions for feral rye and jointed goatgrass samples with at least 75% seed retention were 93% and 70%, respectively. Weed seed destruction percentages were at least 98% for all three species. These

results suggest HWSC could be implemented as an integrated strategy for winter annual grass management in central Great Plains winter wheat cropping systems.

INTRODUCTION

Weed control in wheat agroecosystems is imperative to prevent yield losses due to competition for light, nutrients, physical space, and water (Van Heemst 1985) Major winter annual grass weed species threatening wheat productivity in the western United States are downy brome, feral rye, and jointed goatgrass (Fleming et al. 1988; Lyon and Baltensperger 1995). For instance, feral rye densities at 40 plants m^{-2} and downy brome at 65 plants m^{-2} can cause 60% and 20% yield loss in winter wheat, respectively (Pester et al. 2000; Stahlman and Miller 1990). An additional threat posed by jointed goatgrass is the potential to hybridize with wheat. High densities of jointed goatgrass increase the risk of gene flow between these two species, leading to a potential for herbicide-resistance traits to transfer from wheat to jointed goatgrass (Donald and Ogg 1991; Gaines et al. 2008; Hanson et al. 2005; Mallory-Smith et al. 2018; Zemetra et al. 1998).

The most common weed-control practices in wheat cropping systems are tillage, crop rotation, and herbicides (Daugovish et al. 1999). Combining these strategies has substantially decreased winter annual grass densities and increased wheat yield (Lyon and Baltensperger 1995; Young et al. 1994). Selective POST herbicides available for feral rye and jointed goatgrass control in wheat are limited to imazamox (Tan et al. 2005) (Group 2, Clearfield® wheat) and quizalofop-p-ethyl (quizalofop) (Anonymous 2019; Ostlie et al. 2015) (Group 1, CoAXium® wheat). Multiple, selective Group 2 herbicides are registered for downy brome control in wheat, with resistance to several Group 2 herbicides documented (Mallory-Smith et al. 1999; Park et al.

2004). Integrated weed management (IWM) is a preventive approach to reduce the occurrence of individuals that evolved resistance to repeated practices (Buhler 2002). Variability in weed control practices diversifies the selection pressure in weed populations, which is expected to extend the utility of current methods. To maintain the efficacy of current weed management approaches, it is necessary to develop additional IWM alternatives.

Harvest weed seed control (HWSC) methods are conducted at crop harvest to reduce the input of weed seed into the soil seedbank (Walsh et al. 2013). Seedbank inputs were reduced from 80% to 95% for certain weed species by targeting the weed seed containing chaff fraction. The chaff fraction corresponds to the lemma, palea, and glumes and other light residual material after the grain has been threshed (Walsh et al. 2013; Walsh and Powles 2007). HWSC systems are widely used in Australia due to the high seed retention of dominant weed species, particularly annual ryegrass (*Lolium rigidum* Gaudin). Adoption in Australia is expected to double in the next 5 years to greater than 80% of growers using some form of HWSC (Walsh et al. 2017a).

There are six HWSC systems currently available, including chaff carts, narrow-windrow burning, bale direct system, chaff lining, chaff tramlining, and weed seed destruction using an impact mill system (Walsh et al. 2017b; Walsh and Powles 2007). In the central Great Plains, crop residues are used as erosion management and moisture retention; therefore, a suitable HWSC system in this area must return all residues to the field. Thus, systems compatible with retaining all residues (including chaff) are chaff lining, chaff tramlining, and impact mill systems for weed seed destruction (Walsh et al. 2013; Walsh et al. 2017b). Chaff lining and chaff tramlining involve modifications to the combine to redirect the chaff material in a single line (lining) or on the harvester wheel tracks (tramlining). These methods aim to reduce weed seed germination by concentrating the seeds in large amounts of chaff, thereby creating favorable

conditions to increase seed decay and reduce emergence (Ruttledge et al. 2018). Currently, there are two commercially available impact mill devices: the integrated Harrington Seed Destructor® (iHSD; De Bruin Engineering, PO Box 52, Mount Gambier, South Australia 5290, Australia) and the Seed Terminator® (Seed Terminator, 1284 South Road, Tonsley, South Australia 5042, Australia). These are attachments integrated into the combine that physically destroy up to 98% of weed seeds while returning the chaff to the field (Walsh et al. 2013; Walsh et al. 2012).

As a transformative IWM practice, there currently is much interest in the use of HWSC in cropping regions across the United States and Canada because a number of important weed species have high levels of seed retention at crop harvest (Walsh et al. 2017b). In addition, HWSC has become an integrated strategy to manage species with multiple herbicide-resistance such as Palmer amaranth (*Amaranthus palmeri* S. Watson) in soybean cropping systems (Schwartz et al. 2016). Although producers in the midwestern and southeastern United States have documented potential for HWSC, little is known about the effectiveness of HWSC in controlling weeds in central Great Plains winter wheat fields. Downy brome, feral rye, and jointed goatgrass have similar growth habits and maturity timing as wheat (Daugovish et al. 1999). Therefore, we hypothesized that the majority of downy brome, feral rye, and jointed goatgrass seeds are retained in the harvestable fraction of the wheat upper canopy. Our main objective was to assess the seed retention of downy brome, feral rye, and jointed goatgrass at wheat maturity as an indicator of potential HWSC efficacy. A secondary aim was to determine the effectiveness of an iHSD mill in destroying the seed of these species when processed in wheat chaff.

MATERIAL AND METHODS

Seed Retention and Plant Height

To determine whether weed-seed retention at wheat harvest would be sufficient to justify HWSC methods, a field survey was conducted at wheat maturity in eastern Colorado during the summers of 2015 and 2016 using a similar experimental approach as described by Walsh and Powles (2014) and Walsh et al. (2017b). Forty winter wheat sites were sampled at crop maturity. Sites were selected when one or more plant(s) from the studied weed species were present in the field. At each site, four replications of a 1 m^2 quadrat were collected. Sampling was conducted to simulate a crop harvest and was conducted when the wheat reached 18% to 20% moisture content. Wheat and weed species present in a 1 m^2 quadrat were hand cut at 15 cm above the soil surface and carefully placed in the same bag to prevent any seed shattering. No weed seed heads below 15 cm were identified across sites. Weed seeds on the soil surface were collected with a small broom and dustpan after the remaining wheat biomass was removed. Samples were air-dried and placed in dry storage conditions for processing. Weed plants from the upper canopy were separated and threshed by hand. Likewise, weed seed found on the soil surface was sorted by hand using multiple sieve sizes. Weed seed quantity was determined per sample by dividing the total weight by the 100 seed weight. Seed retention percentage is the proportion of weed seed retained in the upper canopy, calculated by the following equation:

$$seed\ retention\ \% = (total\ no.\ of\ seed\ upper\ canopy/\ (total\ no.\ of\ seed\ upper\ canopy + total\ no.\ of\ seed\ soil\ surface)) \times 100 \quad [1.1]$$

Wheat and the winter annual grass weeds downy brome, feral rye, and jointed goatgrass produce a single spikelet per tiller located near the top of the plant canopy. Plant height was used as a descriptive parameter to compare the harvest height of these winter annual grasses with wheat.

Height was measured of the tallest tiller of five plants from each weed species present in the sampling area and from five wheat plants in each site.

iHSD Efficacy

A stand-mounted iHSD unit was used to determine downy brome, feral rye, and jointed goatgrass seed destruction efficacy with the impact mill. In the summer of 2016, wheat chaff was obtained from weed-free wheat research plots grown at the Colorado State University Agricultural Research, Development, and Education Center, Fort Collins, CO. This wheat chaff was collected from a belt thresher. To replicate harvester-produced material, the chaff was passed through a combine. Similar to Walsh et al. (2018), before processing with the iHSD mill, 1,000 seeds of a weed species were mixed with 2 kg of wheat chaff. A single seed lot for each species was used, obtained from collections at the Colorado State University Weed Research Laboratory made in 2015. Seed germination was tested in Petri dishes, with average germination of 80%, 85%, 65% for downy brome, feral rye, and jointed goatgrass, respectively.

For each weed species, four samples following the previous description were prepared. A weed seed-containing chaff sample was then spread across the 2 m long conveyor belt that feeds samples into the mill. With the mill operating at 3,000 rpm, the conveyor belt was operated and delivered the chaff into the mill at a rate of 12 t hr^{-1}. A large (2 × 2 m) 0.5 mm mesh bag was attached to the outlet chute of the mill to collect the processed samples. After the samples were processed through the iHSD, seedling emergence was determined to assess seed destruction efficacy. A previous test was conducted with intact weed seed to determine the amount of chaff that could inhibit weed-seed germination. Results showed that 400 g of chaff did not reduce weed emergence across the three species. Processed samples were split in 400 g subsamples, mixed with 600 g of potting soil and placed in 60 × 30 cm trays. Trays were watered and

maintained at field capacity over 8 wk. During this time, seedlings were counted and removed. Control treatments consisting of the same proportion of iHSD processed chaff and potting soil were mixed with 100 intact seeds from each weed species from the same seed lot used for the iHSD tests to determine expected seedling emergence for each species. Destruction percentage was calculated by the following equation to account for seedling emergence in the seed lot when mixed with chaff and potting soil in the control:

% seed destruction = [1- (no. seedlings emerged in iHSD – processed sample/ no. seedlings emerged in control treatment)] x 100 [1.2]

Data Analysis

Seed retention and seedling emergence were analyzed with descriptive statistics using the R package 'plyr' (Wickham 2018). To determine the height difference between weed species and wheat, a linear mixed-effects model using the 'lme4' package in R, version 3.5.2, testing at an α of 0.05 was used (Bates et al. 2019). The fixed factor included in this model was weed species, whereas year and location where considered random effects. To obtain the comparisons from all least square means by species with a Tukey adjustment ($P < 0.05$), the R package 'emmeans' was used (Lenth 2019).

RESULTS

Seed Retention and Plant Height

HWSC systems have potential to reduce seed-bank inputs of winter annual grasses during the harvest of central Great Plains wheat crops, with the highest potential reduction for feral rye out

of the three species measured and lower potential for downy brome and jointed goatgrass. All three weed species had greater than 75% average seed retention at wheat crop maturity, indicating that a large proportion of total seed production could be targeted during harvest (Figure 1.1). Feral rye consistently produced the highest average seed retention (90%) and, therefore, has the greatest potential for HWSC. Seed retention of downy brome averaged 75% but was highly variable, ranging from 20% to 95% (Figure 1.1). Jointed goatgrass had an average of 76% seed retention. Approximately 60% of the downy brome samples had 75% or greater seed retention, whereas 70% and 93% of jointed goatgrass and feral rye samples, respectively, had 75% or greater seed retention. The percentages of samples that had 10% or less seed retention were 3%, 0%, and 8% for downy brome, feral rye, and jointed goatgrass, respectively. Additional work is necessary to understand if this variability could be related to an interaction between genotype and environment.

Plant height was considered as a measurement for potential weed-seed collection at harvest. Downy brome height was not different from wheat (Figure 1.2). Feral rye was 50% (approximately 40 cm) taller than wheat (Figure 1.2); consequently, it is highly likely that retained seed will be collected during harvest. Conversely, jointed goatgrass was 25% shorter than wheat (Figure 1.2). Weed species of similar or taller height compared to wheat would increase the likelihood of retained seed being collected with the combine at harvest. Therefore, downy brome and feral rye have a higher likelihood that the retained seed would be collected at the same time as wheat harvest, benefiting the HWSC system. Jointed goatgrass retained seed collection could be increased by lowering the combine harvest height.

iHSD Efficacy

Downy brome, feral rye, and jointed goatgrass seeds processed through the iHSD in wheat chaff had greater than 98% reduction in seedling emergence compared with untreated seeds germinated in wheat chaff (Figure 1.3). Average seedling emergence in the controls (i.e., untreated seed germination in iHSD-processed chaff and potting soil to mimic germination conditions in iHSD-treated samples) was 88% for downy brome, 16% for feral rye, and 75% for jointed goatgrass, with similar germination rates in potting soil alone. Visual examination of iHSD-processed seeds and chaff before planting in potting soil found only broken seed pieces and no intact seeds for all three species. These results indicated that iHSD efficacy is similar and very high across the studied weed species despite differences in seed density and weight for the three species (Figure 1.3).

DISCUSSION

Seed Retention and Plant Height

High seed-retention percentages indicate good potential impact for the use of HWSC systems during harvest (Walsh et al. 2013). Downy brome and jointed goatgrass had intermediate HWSC potential, whereas feral rye showed a higher potential (Figure 1.1), based on the total seed proportion retained above a 15 cm harvest height. Seed retention at plant maturity appears to be related primarily to weed species but also potentially to environmental conditions and location. Preliminary data collected in the Pacific Northwest region showed approximately 80% of downy brome seed had shattered by wheat harvest, whereas feral rye seed retention was greater than 60% (J. Barroso, unpublished data). Tidemann et al. (2017b) suggested that the differences in seed retention among wild oat (*Avena fatua* L.), false cleavers (*Galium spurium* L.), and

volunteer canola (*Brassica napus* L.) were due to shattering habits, growing degree days, and crop competition. For instance, Shirtliffe et al. (2000) reported a growing degree-day interval for wild oat with full seed shattering between 1,470 and 1,680. Different weed seed shattering patterns have been reported depending on the cropping system and harvest approach (swathing vs. direct harvest) (Beckie et al. 2017; Burton et al. 2016). In addition, wild oat and ryegrass species retained twice as much seed in Australia compared with the Great Plains region (Walsh et al. 2017b). Other species such as Palmer amaranth and tall waterhemp [*Amaranthus tuberculatus* (Moq.) J. D. Sauer] were reported to have a consistent seed retention between 94% to 100% across different regions (Schwartz et al. 2016).

Weed species of similar or taller height than the crop will increase the seed collection efficiency at harvest. Among the studied winter annual grasses, jointed goatgrass is the species that had more height disadvantage; downy brome and feral rye are optimal compared to wheat (Figure 1.2). However, Donald and Ogg (1991) found that even when growers tried to take advantage of the height difference between wheat and jointed goatgrass by raising the combine header, they were not able to avoid jointed goatgrass seed contamination in their grain. Jointed goatgrass and downy brome heights varied depending on the wheat variety and annual precipitation. These species can reach a similar or higher height than wheat when they are competing against semi-dwarf varieties and/or in dry conditions (Blackshaw 1994; Yenish and Young 2004). Feral rye height is also affected by wheat variety and growing conditions; however, the minimum height reported in previous research is 66 cm, which is taller than most commercial wheat varieties (Anderson 1998). Weed height can be modified by increasing planting density. Recent research showed that greater wheat planting densities can lead to increases in height and seed retention for

rigid ryegrass (Walsh et al. 2018), thereby potentially increasing the seed collection using a HWSC system.

Colorado winter wheat is mostly grown in no-till production systems. This farming practice favors downy brome, feral rye, and jointed goatgrass seed establishment. In a no-till system, these species have higher germination and lower dormancy when they are on the soil surface compared with a burial status (Donald and Ogg 1991; Stump and Westra 2000; Thill et al. 1984). HWSC as an IWM tool can disrupt the reproductive cycle for these species, thereby potentially increasing the seed collection using a HWSC system.

iHSD Efficacy

Previous research demonstrated that the impact mills are highly effective (>88% control) across several weed species and different chaff types (Walsh et al. 2017b). Impact mill efficacy can be affected by the mill speed, crop chaff type, chaff feeding rate, weed seed number, and density. Despite the significant effect of those factors on seed destruction, observed average destruction percentages are greater than 85% (Schwartz-Lazaro et al. 2017; Tidemann et al. 2017a; Walsh et al. 2018). Using a similar stationary prototype, Tidemann et al. (2017a) reported that weed seed destruction only decreased from 99% to 98.5% when the chaff volume was doubled. In addition, they showed a chaff-type effect where barley (*Hordeum vulgare* L.) and pea (*Pisum sativum* L.) had greater than 98.5% weed seed destruction, whereas canola chaff had a 5% reduction in efficacy; however, iHSD field trials in canola and barley crops showed no difference in seed destruction among several weed species (Walsh et al. 2018).

Proactive HWSC implementation in current weed-management practices in the central Great Plains is key to maintain POST herbicide efficacy on downy brome, feral rye, and jointed

goatgrass in winter wheat production systems. POST herbicides are at a high risk for resistance evolution due to their frequent use in these crop- ping systems. Currently, no cases of herbicide resistance in Colorado have been reported for downy brome, feral rye, or jointed goatgrass (Heap 2019); however, downy brome and jointed goatgrass imazamox resistance cases were reported in Montana and Washington, respectively (Kumar and Jha 2017; Mallory-Smith et al. 2018). A modelling study considering an integrated management approach (e.g., pre-emergence and post-emergence herbicides, and HWSC) indicated the frequency of resistance alleles could be eliminated or greatly reduced in weed populations and that weed density decreased to two plants m^{-2} (Somerville et al. 2018). Similar to herbicides, repetitive use of HWSC would increase natural selection pressure for escape traits such as early flowering, lodging, shattering, or shorter winter annual grass weed biotypes. Greenhouse experiments described that after five recurrent selection generations for early flowering, 77% of a wild radish (*Raphanus raphanistrum* L.) population flowered 30 d earlier than a non-selected population (Ashworth et al. 2016).

Our field studies investigating the potential for HWSC to be implemented in the central Great Plains wheat fields found that this practice could provide an important new tool for IWM practices. Downy brome, feral rye, and jointed goatgrass are troublesome winter annual grasses that affect winter wheat production. Harvest weed-seed control techniques are effective if the weed species has a high proportion of total seed production retained at crop maturity. Weed species with similar or taller plant height have higher weed-seed collection during harvest. On the basis of our results, HWSC can potentially reduce seedbank inputs for downy brome, feral rye, and jointed goatgrass, with higher potential for feral rye than for downy brome and jointed goatgrass. Our findings suggest HWSC methods could strengthen IWM practices in winter wheat fields to reduce winter annual grass interference.

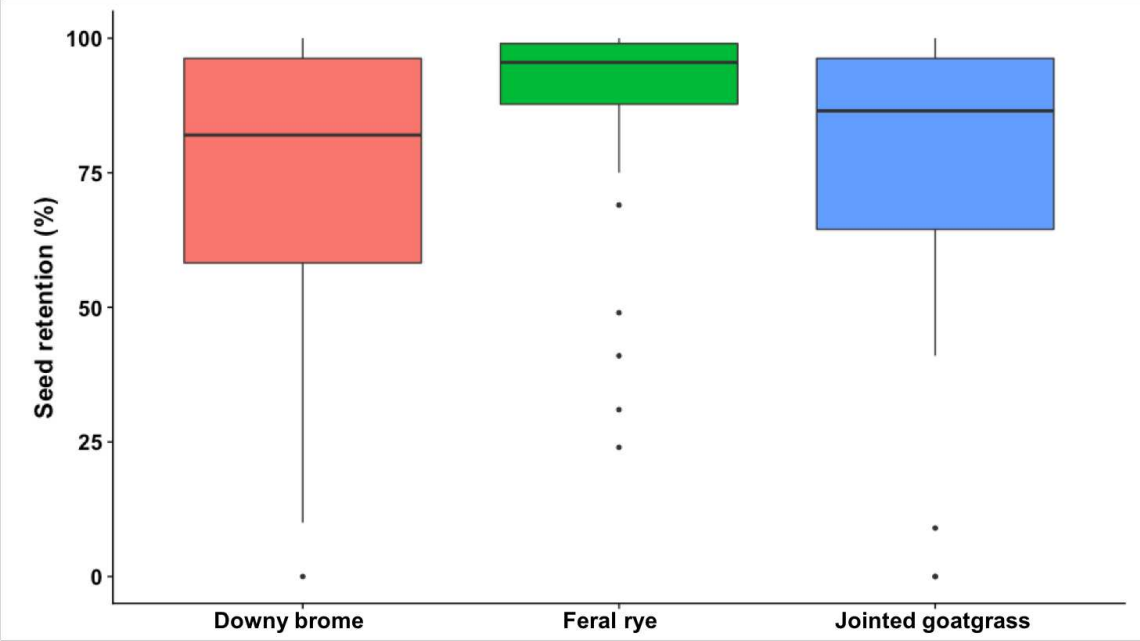

Figure 1.1: Box plot describing the seed retention percentage in the wheat canopy harvestable section (15 cm and higher) at crop maturity during the summers of 2015 and 2016 for downy brome (n = 17 sites), feral rye (n = 24 sites), and jointed goatgrass (n =10 sites).

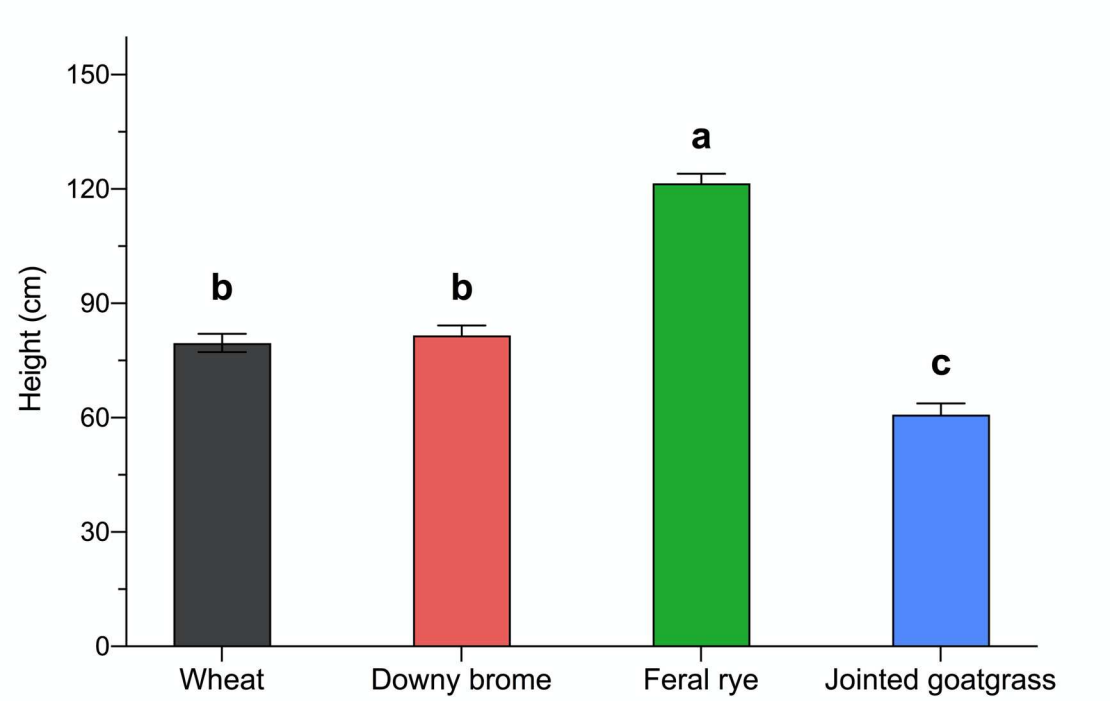

Figure 1.2: Plant height of wheat compared to downy brome (n=17 sites), feral rye (sites=24 sites), and jointed goatgrass (sites=10 sites) during the summers of 2015 and 2016. Letters indicate significant differences based on mixed-effects model ($\alpha \leq 0.05$).

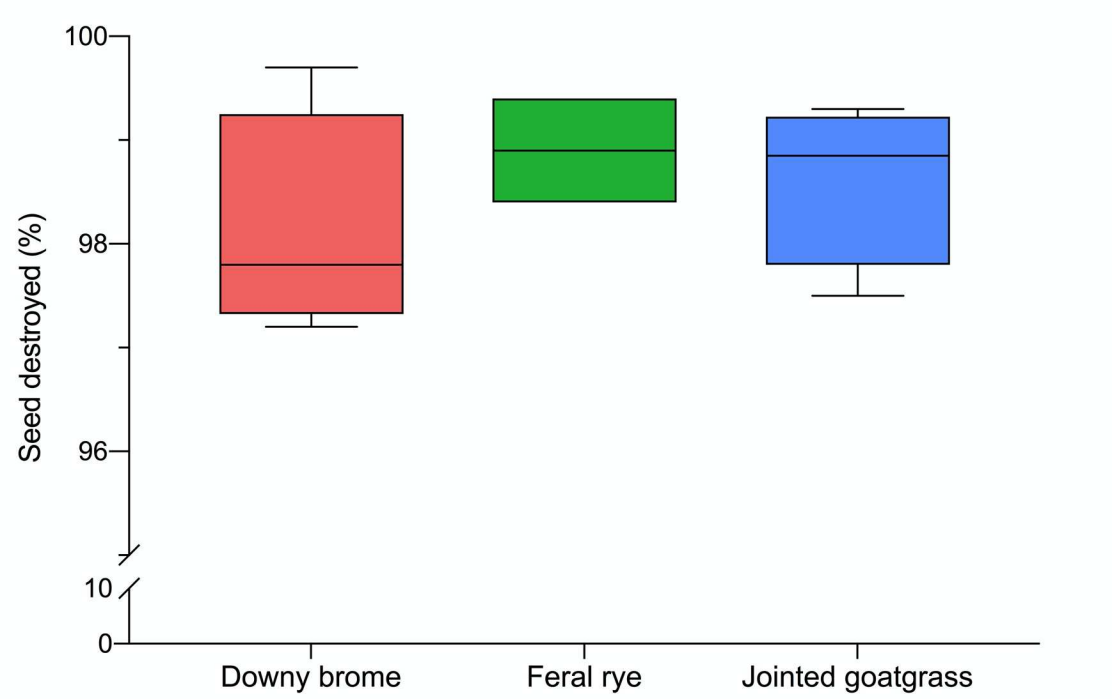

Figure 1.3: Box plot describing the percentage of seed destroyed by the integrated Harrington Seed Destructor (iHSD) for downy brome, feral rye, and jointed goatgrass, measured by reduction in seedling emergence compared with untreated controls (see Equation 1.2).

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Chapter 2: Survey of ACCase and ALS resistance in winter wheat identifies target-site and non-target-site imazamox resistance in *Secale cereale*

SUMMARY

Early detection of herbicide resistance in weeds is crucial for the successful implementation of integrated weed management. Feral rye (*Secale cereale*), downy brome (*Bromus tectorum*), and jointed goatgrass (*Aegilops cylindrica*) are problematic winter annual grasses in Colorado. Post-emergence selective control of feral rye and jointed goatgrass in wheat is limited to imazamox (Clearfield® wheat) and quizalofop (CoAXium® wheat). Currently, there is no information on the imazamox and quizalofop resistance status for feral rye, downy brome, and jointed goatgrass in Colorado. Our main objectives were to conduct an imazamox and quizalofop resistance survey for feral rye, downy brome, and jointed goatgrass and to identify the molecular mechanisms from the identified resistant biotypes. Greenhouse herbicide screening was conducted using labeled rates of imazamox and quizalofop to evaluate 287 collected samples across the three weed species. No resistance to imazamox or to quizalofop was identified in any downy brome or jointed goatgrass samples. No feral rye samples were resistant to quizalofop. Two feral rye populations (named A and B) were identified with resistance to imazamox. Acetolactate synthase (ALS) gene sequencing and *in-vitro* enzyme assays showed the known Ser653Asn mutation in population B conferring target-site resistance to imazamox, while population A had no ALS mutations and sensitive ALS enzyme, suggesting a non-target site mechanism. Enhanced metabolism was investigated by conducting an imazamox dose response experiment with and without malathion as a cytochrome P450 inhibitor. Additionally, intact imazamox and metabolites from susceptible and resistant feral rye individuals were quantified from susceptible

and resistant feral rye individuals with and without malathion. Dose response results for population A showed a biomass reduction of 2.7-fold when imazamox at 52.5 g ai ha^{-1} was mixed with malathion compared to imazamox alone. Metabolism data showed a T_{50} (time for 50% degradation of intact imazamox) of 1.1 d for population A, whereas the susceptible control had a T_{50} of 3 d. This is the first report of both target-site and metabolism-based imazamox resistance in feral rye.

INTRODUCTION

Herbicide resistance surveys are essential for early detection of resistant biotypes to inform proactive mitigation practices in agricultural fields (Beckie et al. 2000). Winter annual grasses such as (*Secale cereale* L.), downy brome (*Bromus tectorum* L.), and jointed goatgrass (*Aegilops cylindrica* Host) are troublesome weed species in winter wheat (Fleming et al. 1988; Lyon and Baltensperger 1995). In addition to competing with wheat for resources, seeds from feral rye and jointed goatgrass present in the harvested grain cause dockage penalty. Moreover, jointed goatgrass can hybridize with wheat as these two species are partially genetically compatible increasing the risk of gene flow and low grain quality (Gaines et al. 2008; Mallory-Smith et al. 2018).

Downy brome, feral rye, and jointed goatgrass management is challenging due to having similar growing cycles as winter wheat and the lack of post-emergence (POST) herbicide options. Downy brome can be controlled using with several Group 2 ALS herbicides, but selective control of feral rye and jointed goatgrass is limited to herbicide tolerant variety production systems. The first option available was Clearfield® wheat (Newhouse et al. 1992), which is tolerant to imazamox (Anonymous 2009) and has been utilized by growers since 2002.

Imazamox is part of the imidazolinone chemical family and acts as an acetolactate synthase (ALS) inhibitor (also referred as acetohydroxyacid synthase) (Group 2, HRAC) where the branched amino acids valine, leucine, and isoleucine are no longer synthesized (Shaner et al. 1984). Imazamox provides excellent control of downy brome and jointed goatgrass, whereas for feral rye it is labeled for suppression rather than control. Differences in efficacy are related to less translocation and a higher metabolism rate in feral rye than in jointed goatgrass (Pester et al. 2001). Most recently, CoAXium® (Ostlie et al. 2015) wheat tolerant to quizalofop (Anonymous 2019) has been commercialized. Quizalofop-p-ethyl (quizalofop) is part of the aryloxphenoxypropiate chemical family and its used to control many grass species. This herbicide mode of action is related to fatty acid biosynthesis where the acetyl-coenzyme A carboxylase (ACCase) is inhibited (Group 1, HRAC) (Burton et al. 1989). Quizalofop field results on CoAXium® wheat systems have shown an outstanding control of downy brome, jointed goatgrass, and feral rye with no crop injury (Westra et al. 2019a). Other methods such as harvest weed seed control have been proposed as an integrated method to decrease the seed bank of these winter annual grass species (Soni et al. 2019).

The resistance evolution likelihood based on application frequency and reported cases shows that ALS and ACCase inhibitors have the highest probability compared to other modes of action (Kniss 2018). These two modes of action are ranked as the first and third for the most resistance cases reported, with 165 and 49 weed species resistant to ALS and ACCase inhibitors, respectively (Heap 2020). Target site and non-target site resistance mechanisms to ACCase and ALS herbicides have been described in several weed species (Powles and Yu 2010). Target site mechanisms refer to a mutation that will lead to a change in the binding affinity between the herbicide and its enzyme target, whereas non-target site mechanisms are related to pathways for

the herbicide to not reach the target enzyme such as limited absorption and cellular transport, organelle sequestration, or degradation by enhanced metabolism (Gaines et al. 2020). Eleven mutations conferring resistance to one of more chemical families of the ACCase inhibitors have been identified in the homomeric plastidic ACCase carboxyltransferase domain, which corresponds to the herbicide site of action (Takano et al. 2019; Yu et al. 2010). Several mutations have been documented that confer resistance to ALS herbicides (Tranel et al. 2020). High mutation frequency and diversity associated with ALS and ACCase is because the binding site of these herbicides is located in the channel leading to the substrate active site; thus, the likelihood of evolving amino acid substitutions to prevent the herbicide interaction are high as the mutations outside of the catalytic domain and does not affect the normal protein function (McCourt et al. 2006). Non-target site mechanisms for ACCase and ALS herbicides are primary related to detoxification pathways (Jugulam and Shyam 2019). In the Western US winter wheat production region, there are no reports of quizalofop resistant weed species. Three *Bromus* sp. populations and one jointed goatgrass population have been reported resistant to imazamox in this cropping system (Heap 2020; Rodriguez et al. 2018). Currently, there is no information on the imazamox and quizalofop resistance status for feral rye, downy brome, and jointed goatgrass in Colorado winter wheat cropping areas. A herbicide resistance survey is necessary to establish a base line knowledge on resistance status after 18 yr of Clearfield® winter wheat systems and the recent release of quizalofop tolerant varieties winter wheat varieties. Our main objective was to conduct a herbicide resistance survey for early identification of winter annual grasses resistant to imazamox and quizalofop. In addition, we aimed to characterize the resistance mechanisms from the identified biotypes in the survey.

MATERIALS AND METHODS

Herbicide Resistance Survey

To determine the possible cases of resistance to quizalofop or imazamox a total of 44 downy brome, 22 jointed goatgrass, and 221 feral rye samples from winter wheat farms located in Colorado were screened. These samples were collected during the summers of 2012, 2015, 2016, and 2018 (Table 2.1). Although this resistance survey was focused in winter wheat cropping systems, some samples from 2012 and 2016 were collected from roadsides, rangelands, and natural areas in Colorado. One hundred and twenty seeds from each sample were planted in 60 × 30 cm trays filled with potting soil (Farfard #2-SV, American Clay Works, Denver, CO) for each herbicide. Plants were grown in a greenhouse under a photoperiod regimen of 14 h light/10 h dark and temperatures maintained between 22 and 26 C. Trays were watered daily at field capacity. Quizalofop (Aggressor™, Albaugh, LLC Ankey, IA) and imazamox (Beyond®, BASF, Research Triangle Park, NC) application rates were based on the commercial label recommendations to control downy brome, feral rye, and jointed goatgrass. Quizalofop and imazamox were applied at 32 and 52.5 g ai ha^{-1}, respectively. In addition, 0.25% (v/v) NIS (nonionic surfactant) and 2.5% (v/v) UAN (urea ammonium nitrate) were used as adjuvants for quizalofop and 1% (v/v) MSO (methylated seed oil) and 5% (v/v) UAN for imazamox. Plants were treated when they reached three true leaves. Herbicides were sprayed using a cabinet spray chamber (DeVries Generation III Research Sprayer, Hollandale, MN) with a XR TeeJet 11008 VS nozzle calibrated to deliver 187 L ha^{-1}. Survival percentage was determined at 3-4 wk after treatment (WAT). Plants that showed no or very low herbicide injury were subject to a second herbicide application using the same rates. A regrowth assessment was conducted by pruning

plants above 4 cm at 1 WAT and measuring the regrowth to rule out possible escapes and confirm the resistant phenotypes.

Feral Rye Imazamox Resistant Populations Characterization

Dose-response experiment

Based on the survey results two feral rye populations (named population A and population B) were identified as imazamox resistant. A dose response was conducted to characterize the resistance level from populations A and B compared to a susceptible biotype. Imazamox application rates were: 0, 13, 26, 52, 105, 210, 315, 420, and 525 g ai ha^{-1} combined with the same adjuvants as described above. Four seedlings were placed in a 3.8 cm × 3.8 cm × 5.8 cm pot filled with the same potting soil type used for the screening. Each pot was considered a biological replication. Each treatment included three replications for a total of 12 plants. In addition, the same imazamox rates were used in combination with malathion at 1000 g ha^{-1} as a P450 cytochrome inhibitor to identify possible metabolic resistance. The same procedure as described above was followed. Plants from imazamox and imazamox plus malathion dose-responses were pruned at 4 cm height 1 WAT to quantify fresh weight only of the regrowth biomass at 21 WAT. The imazamox dose-response was replicated twice and the imazamox plus malathion dose-response was conducted once.

ALS enzyme activity assay

An *in-vitro* assay was conducted to assess the imazamox ALS inhibition for populations A and B, compared to a susceptible control. This assay is a colorimetric estimation of acetoin, which is the reaction product after acetolactate decarboxylation. A modified procedure from Dayan et al. (2015) was followed. One gram of plant meristem tissue was collected. Tissue was flash frozen, ground until powder texture in liquid N_2, and stored at -80 C. An extraction buffer composed of deionized water, 25 mM potassium phosphate buffer (pH 7.5), 4 mM thiamine phosphate, 200

mM pyruvate, 5 mM magnesium chloride, 20 µM flavin adenine dinucleotide were continuously stirred and the pH was adjusted to 7. Tissue powder was mixed with 5 mL of the extraction buffer, vortexed vigorously for 1 min, and incubated on ice for 15 min. The homogenate was filtered through a cheese cloth and centrifuged at 16,000 × *g* at 4 C for 15 min. The supernatant containing the crude protein extract was pipetted out into a separate tube and used immediately for the enzyme assay. A 10 mM commercial imazamox (Anonymous 2009) stock solution was mixed with the buffer extraction to reach a 1 mM final herbicide concentration. The ALS inhibition assay was conducted in a 96-deep well plate, where 125 µL of the extraction buffer was added to all the wells. A herbicide concentration gradient of 0, 2, 4, 8, 16, 32, 63, 125, 250, and 500 µM was made by adding 125 µL of the 1 mM imazamox solution to the first row in the plate. The extraction buffer and herbicide solution were mixed thoroughly by pipetting and a 125 µL aliquot was used to repeat the same process in the following row to reach the desired concentrations. In each well 125 µL of the crude protein extract was added. The mixture was incubated for 1 h at 37 C. The ALS inhibition reaction was stopped by adding 62.5 µL of sulfuric acid at 5% and an incubation period of 15 min at 60 C. To continue the acetolactate derivatization to acetoin a fresh 2 N sodium hydroxide solution mixed with α-naphthol and creatine at 2.5% (v:w) and 0.25% (v:w), respectively, was prepared. Each well received 437.5 µL of this solution and an incubation period of 15 min at 60 C. After incubation the plate was centrifuged for 10 min at 4,000 × *g*. Two hundred and fifty µL were pipetted into UV-transparent microplate to measure absorbance at 530 nm with a spectrophotometer. Total crude protein was measured for each biological replication using the Bradford assay (Bradford 1976). The ALS activity as percentage of control was calculated by subtracting the background from the control

samples and normalized by total crude protein. Three biological replications per population were used.

Target-site and Non-Target Site Resistance Mechanism in Feral Rye

Target-site investigation: ALS gene sequencing

To identify possible target-site mutations in the ALS gene we sequenced a 160 bp region located in the conserved region of domain E where other ALS-resistance mutations have been reported in grasses. Genomic DNA was extracted using a modified CTAB protocol (Doyle 1991) from 15 and 39 imazamox resistant plants from populations A and B, respectively, and 3 individuals from a susceptible line. DNA quantity and quality were measured using a NanoDrop 2000™ (Thermo Fisher Scientific, Waltham, MA). Forward primer 5'AAGTCACTGCAGCAATCAAGAAG'3 and reverse primer 5'CAATACGCAGTCCTGCCAT'3 were designed to amplify the ALS region based on the published cereal rye genome (Bauer et al. 2017). A 50 µL master mix containing 25 µL of GoTaq® Green Master Mix (Promega, Madison WI), 2 µL of each forward and reverse primers at 10 µM, and 17 µL of sterile HPLC grade water was prepared. Four µL of DNA at 35 ng μL^{-1} were mixed with 46 µL of the master mix in a tube. Polymerase chain reaction (PCR) was conducted in a Bio-Rad T100 thermo cycler (Bio-Rad Laboratories, Inc., Hercules, CA) following the polymerase cycle recommendations that consisted of 95 C for 2 min, 35 cycles of 95 C for 30 s; 52 C for 30 s, 72 C for 30 s , and a final extension of 5 min at 72 C. Prior to sequencing, the amplicon size from each sample was assessed in a 1 % agarose gel using electrophoresis. PCR products were purified, and Sanger sequenced by Genewiz, Inc (South Plainfield, NJ, USA) using the same forward and reverse primers as described above. Resulting sequences were assembled and aligned to the cereal rye reference using Geneious R11 (Biomatters, Ltd., San Diego, CA).

Non-target site investigation: enhanced metabolism experiment

Non-target site resistance was investigated by measuring imazamox metabolites from populations A and a susceptible control. Measured metabolites were determined based on the compounds identified in imazamox wheat metabolism report (Friedrich et al. 2012). They correspond to parent imazamox (CAS # 114311-32-9), demethylated metabolite (CAS # 81335-78-6), glycosylated metabolite (CAS # 200111-50-8), and oxidized metabolite (CAS # 146953-32-4) (Figure 2.1). Feral rye plants at 3-4 true leaf stage were treated with imazamox at 62 g ai ha^{-1} alone and in combination with malathion at 1000 g ai ha^{-1} as a cytochrome P450 inhibitor using a spray cabinet calibrated to deliver 200 L ha^{-1}. Aboveground biomass was harvested at 0, 1, 3, and 7 d after treatment. Tissue was washed in a 1:1 water: acetonitrile solution to quantify the non-absorbed herbicide. Plant tissue homogenization was obtained after placing 2-3 g of diced tissue in a gentleMACS™ M tubes (Miltenyi Biotech, Auburn, CA) with 3 mL of 1:1 water, acetonitrile solution and processed twice in a gentleMACS™ Dissociator (Miltenyi Biotech, Auburn, CA). GentleMACS™ M tubes were centrifuged at 4,000 × *g* for 15 min at 4 C. Two mL of the supernatant were vacuum filtered using a 0.2 µm nylon 96-well plate. Remaining supernatant was stored at -80 C. Five hundred µL of plant extract were diluted to half of the concentration and placed in a microplate for mass spectrometry analysis. Metabolite quantification was conducted using a high-resolution mass spectrometry OrbiTrap Q Exactive HF (Thermo Fisher Scientific Inc, Waltham, MA). The settings used for electrospray ionization source were polarity (positive), spray voltage 2 kV, probe heater temperature 350 C, and capillary temperature 275 C. Chromatographic separation was conducted with a Nucleodur® C18 Pyramid column (150 mm × 3 mm, 3 µm) (Macherey-Nagel, Duren, Germany). The mobile phase was (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The gradient elution used was: 0 min (10% B) to 6 min (40% B) to 6.5 min (99% B) to 7.5 min (10%

B) until the run end at 10 min. A scan range of 70-800 *m/z* with a 30,000 resolution were used. The injection volume was 10 µL and the flow rate used was 600 µL min^{-1}. Calibration curves for each studied compound were prepared using analytical standards with acetonitrile and plant matrix as backgrounds. Data generated from the OrbiTrap were checked for quality and extracted using TraceFinder (Thermo Fisher Scientific Inc, Waltham, MA). The experiment was conducted twice. The first experiment had two biological replicates per time point and the second experiment contained three biological replicates. Intact imazamox and metabolites amounts were normalized to nmol g fresh $weight^{-1}$ (FW).

Statistical Analysis

Dose-response biomass data from the imazamox only dose-response was analyzed as percentage of untreated control fitting a 3 parameter log-logistic regression (Equation 2.1) in R using the "drc" package (R Core Team 2018; Ritz et al. 2015).

$$Y = d/1 + \exp\left[b(\log X - logE)\right] \qquad [2.1]$$

The equation parameters correspond to *Y* equal to the response variable, *d* is the upper limit, *b* the slope, *E* corresponds to the amount of herbicide required to inhibit 50% growth (GR_{50}), and *x* is the herbicide rate. The imazamox plus malathion dose-response biomass data were analyzed using a 2-way ANOVA and Tukey's multiple comparison ($\alpha = 0.05$) to identify significant differences among treatments with and without malathion. The ALS activity assay was analyzed as percentage of control. These data were subjected to nonlinear 3 parameter regression to calculate the IC_{50} (concentration required to inhibit 50% of the enzyme activity). Metabolism data were analyzed using a one-phase decay model for imazamox parent compound and a quadratic model for the three studied metabolites. The T_{50} (time for 50% compound degradation) of intact imazamox values were calculated based on the regression model. Imazamox dose-

response graph, and the rest of statistical analysis were conducted using GraphPad Prism (GraphPad Software 2020).

RESULTS

Quizalofop and Imazamox Resistance Survey

Herbicide screening of collected downy brome, feral rye, and jointed goatgrass showed that no sampled populations were resistant to quizalofop. Similarly, no downy brome or jointed goatgrass populations were resistant to imazamox. From the 221 feral rye screened samples only two populations were identified as imazamox resistant. Populations A and B were part of the 2018 collection (Table 2.1). These populations were collected from two farms with a conventional winter wheat – fallow rotation farms. They are separated by 52 km and located in Northern Colorado. Feral rye seed was collected from four and eight different locations from population A and B, respectively, in each farm. Survival mean was 40% and 80% for population A and B, respectively. Survival percentages within populations are variable (data not shown) as expected from a species that is considered predominantly cross-pollination as it has a rate of 70-90% (Vaquero et al. 1989).

Imazamox Resistance Characterization in Populations A and B

Dose-response using imazamox and the mixture of imazamox and malathion were conducted to characterize the resistance levels for both populations and with a cytochrome P450 inhibitor to identify possible enhanced metabolism. Imazamox only dose-response results showed that populations A and B survived higher herbicide doses compared to a susceptible biotype (Figure 2.2). The 3-parameter log-logistic regression resulted in a GR_{50} that was 40 and 247 times higher for population A and B compared to the susceptible control (Figure 2.2, Table 2.2). No significant differences were observed among the GR_{50} for susceptible control and populations A

and B. The susceptible population used for this experiment has not been previously exposed to imazamox applications; thus, sensitivity to this herbicide was higher than expected and the collected data did not provide the right fit for the model. However, these results are sufficient to confirm the resistant phenotype identified in the survey. In addition to a higher GR_{50}, biomass was reduced as the untreated control at the highest imazamox dose (equivalent to 10× label dose) for population B, indicating a higher resistance level compared to population A. Imazamox combined with malathion dose-response results were subject to high variability between treatments and within populations (Figure 2.3). However, imazamox doses of 13 and 53 g ha^{-1} had lower GR_{50} with malathion than without malathion for population A. Imazamox dose of 421 g ha^{-1} had lower GR_{50} with malathion than without malathion for population B. These results indicated that the malathion treatment likely inhibited cytochrome P450 enzymes and reduced imazamox metabolism, increasing sensitivity to the herbicide. Both dose response experiments were conducted with segregating populations; therefore, the population mean resistance response was variable.

An enzyme activity assay was conducted to assess the inhibition of ALS by imazamox *in-vitro*. Population B had IC_{50} of 247 μM, whereas population A was similar to the susceptible with an IC_{50} of 16 μM (Figure 2.4). These results suggested that population B might contain a target-site resistance mutation. On the other hand, population A had a similar trend as susceptible suggesting a non-target site resistance mechanism.

Target-Site Mechanism Investigation

Partial sequencing was conducted to identify possible non-synonymous mutations in the ALS gene. Sanger sequencing results depicted in Figure 2.5 show the alignment for populations A and B, susceptible control, and the cereal rye reference genome scaffold. The consensus sequence

from each collection point for population A and B were included. Population A did not contain the Ser653Asn mutation, whereas this mutation was present in population B in all the collection points except for number 7. These results support the findings from the ALS enzyme inhibition assay that suggested a target-site resistance mechanism in population B. In addition, they suggested that in population B both target and non-target site resistance mechanisms are co-existing.

Non-Target-Site Mechanism

Intact imazamox and three other metabolites (Figure 2.1) were measured in both the absence and presence of the P450 inhibitor malathion. Regression analyses are depicted in Figure 2.6. Susceptible plants treated with imazamox had a T_{50} intact imazamox of 3 d whereas population A was 1 d. When imazamox was applied with malathion, the T_{50} increased to 5 d for susceptible and to 2 d for population A. The demethylated, glycosylated, and oxidized metabolites followed a similar trend where population A had a greater concentration of these metabolites compared to the susceptible control, and the metabolite concentrations decreased in malathion treatments. These results suggested that population A may have enhanced metabolism mediated by cytochrome P450s as resistance mechanism.

DISCUSSION

Herbicide Resistance Survey

Herbicide resistance monitoring is indispensable for cropping systems that repeatedly use the same herbicide in their management plan. Resistance surveys are important as a stewardship tool. Based on a survey results it is possible to detect early resistant cases, prevent seed dissemination, and modify the herbicide program to reduce the dispersal of resistant biotypes (Beckie et al. 2000; Westra et al. 2019b). For instance, an *Avena* spp survey conducted in

Western Australia grain cropping systems showed the high frequency of ACCase resistant biotypes and provided suggestions on which modes of action should be included in their herbicide rotations based on number of cases identified (Owen and Powles 2009). Surveys are also important to identify the evolution of cross and multiple resistance cases in weed populations (Bagavathiannan and Norsworthy 2016; Owen et al. 2007). Based on these survey results management decisions can be made to develop solutions that are profitable for farmers. Survey results from *Lolium* sp. and *Amaranthus* spp have shown resistance to nearly all modes of action that can be used for their control, pointing in the direction that new strategies such as harvest weed seed control are needed (Norsworthy et al. 2016; Walsh and Powles 2007). Considering the importance of herbicide resistance surveys, none have been recently conducted in winter wheat cropping systems for downy brome, jointed goatgrass, and feral rye in Colorado. Here we summarized the quizalofop and imazamox screening results from four collection years in the Colorado area (Table 2.1). Quizalofop tolerant wheat varieties were recently available as part of the CoAXium® system for farmers. No quizalofop resistant biotypes were identified after screening 287 samples the three species (Table 2.1). This is important base line information for the CoAXium® stewardship program to lengthen the efficacy of this technology considering that ACCase resistance evolution occurs at a high frequency after selection pressure increases (Powles and Yu 2010). Imazamox selection pressure has been present for over 18 yr due to the use of Clearfield® wheat systems in winter wheat and only two feral rye populations were identified as resistant. Interestingly, very few winter annual grasses in winter wheat have been reported resistant to imazamox, despite the fact that ALS inhibitors have the most reported resistance cases (Heap 2020). Clearfield® wheat systems remain an important weed management tool for farmers; thus, creating preventative measures to slow the dissemination of these resistant

populations is imperative. In addition, these results are useful to spread awareness in extension meetings regarding the presence of imazamox resistance.

Target-Site Mechanism Investigation

Partial ALS gene sequencing indicated the presence of the Ser653Asn mutation (Figure 2.4) in population B, whereas population A had the same sequence as the susceptible reference. This result is supported by the ALS activity assay (Figure 2.3) where population A showed an IC_{50} similar to the susceptible control and population B required 230 µM more of imazamox to reach 50% inhibition. The Ser653Asn mutation confers resistance to imidazolinones, including imazamox, in winter wheat (Nakka et al. 2019) and has been reported in other three grass species (Tranel et al. 2020). Kumar and Jha (2017) demonstrated that this mutation provided a high imazamox resistance level of 110-fold compared to a susceptible biotype in downy brome. Other researchers have reported similar high resistance level in other grass species containing the same amino acid substitution (Beckie et al. 2012). Imazamox alone dose-response (Figure 2.2, Table 2.2) showed that population B has a high imazamox resistance with a 247-fold difference compared to the susceptible. Fitness cost associated to this specific single amino acid substitution has not been investigated in feral rye. However, research showed that reported mutations in the ALS gene have little to no negative fitness cost in plant development of resistant biotypes (Vila-Aiub et al. 2009; Yu and Powles 2014b). The Ser653Asn mutation confers cross resistance to other ALS chemical families such as sulfonylaminocarbonyltriazolinone and triazolopyrimidines but not to sulfonylureas in downy brome (Kumar and Jha 2017); however, *Setaria viridis* (L.) Beauv. with the same amino acid substitution was resistant to sulfonylureas (Laplante et al. 2009). Cross resistance due to this mutation in feral rye will be more problematic for herbicide control in rangelands than in winter wheat as the tolerant varieties are made only for

imidazolinones. No wheat-selective sulfonylureas provide control of feral rye. Target-site mechanisms like the amino acid substitution identified in population B represent a high risk for spreading in feral rye. It is estimated that such an outcrossing species should be physically separated by at least 200 m in order to prevent cross-pollination (Burger et al. 2006). ALS mutations are dominant and nuclear inherited, increasing the frequency of this resistance trait in the population (Tranel and Wright 2002).

Non-Target Site: Imazamox Enhanced Metabolism

The ALS enzyme of imazamox resistant population A was equally sensitive as a susceptible line (Figure 2.4). Moreover, the imazamox plus malathion dose-response showed a significant difference in biomass reduction among treatments for three imazamox doses with and without malathion (Figure 2.3). Sequencing data demonstrated the absence of Ser653Asn mutation in the ALS gene (Figure 2.5). These data strongly suggested that population A resistance mechanism is non-target site. Metabolite analysis with and without malathion treatment indicated that enhanced metabolism confers imazamox resistance in population A (Figure 2.6). Imazamox metabolism has been studied in several other species (Domínguez-Mendez et al. 2017; Vassios et al. 2011). Several researchers provide strong evidence that the imazamox metabolic pathway is mediated by cytochrome P450 monooxygenases (Iwakami et al. 2014; Wright et al. 2018). These are a large protein family that have a hemethiolate co-factor and are known to catalyze most of the reactions in the phase I of herbicide detoxification (Gaines et al. 2020). Malathion is an insecticide used to inhibit the activity of certain cytochrome P450 subfamilies (Powles and Yu 2010). Mixing malathion with imazamox increased the detection of the active ingredient 2.3 times for population A and remained similar for the susceptible control at 3 d after treatment (Figure 2.6). Imazamox compound underwent an *O*-demethylation reaction

where a methyl group was removed leading to a demethylated imazamox that will be either oxidized or conjugated to a glucose by a glycosyl transferase (Figure 2.1). The three metabolites quantified in population A concurred with the enhanced metabolism hypothesis, where the quantity of metabolites detected was higher when malathion was absent (Figure 2.6). Susceptible feral rye can metabolize imazamox and population A detoxifies imazamox faster than a susceptible biotype. The next steps to better understand this mechanism in population A will be looking into different transcriptomic and functional genetics approaches to identify the specific cytochrome P450s involved in this pathway for feral rye. Potential P450 candidates have been identified. *Arabidopsis thaliana* transformants with several members from the CYP81A sub-family from *Echinochloa phyllopogon* (Stapf) Koso-Pol showed that CYP81A24 conferred imazamox resistance (Dimaano et al. 2020). CYP81A9 endows tolerance in corn to a sulfonylurea ALS herbicide (Liu et al. 2019).

Metabolic resistance is a serious threat in winter wheat cropping systems. The risk of enhanced metabolism conferring resistance to multiple mode of actions is high as it has been documented for several cases (Yu and Powles 2014a). ACCase and ALS multiple resistance mediated by cytochrome P450s has been reported in other grass species (Zhao et al. 2019). Therefore, there is indication to cautiously manage and contain population A to prevent multiple resistance to quizalofop as this herbicide will be used more frequently in the future in winter wheat. Although inheritance and dominance of this enhanced metabolism in population A is not known, the risk of dissemination through pollen and seed mediated gene flow remains high as it is a predominantly outcrossing species.

This is the first report of imazamox resistance in feral rye. It is imperative to maintain integrated weed management practices to slow the evolution of resistance, reduce the spread of resistant

seed, and maintain the efficacy of herbicides used with tolerant varieties. Two feral rye populations (populations A and B) were identified as imazamox resistant from a herbicide resistance survey conducted in Colorado. Non-target site and target-site mechanisms were described for population A and B, respectively. Stewardship for Clearfield® and CoAXium® systems should incorporate this knowledge to promote practices among growers that will extend the viability of these technologies. Imazamox and quizalofop resistance scouting should be conducted every year in surrounding farms where populations A and B were collected as part of a monitoring program. This is the base line for future research related to develop quick field-testing tools based on antibodies linked to enhanced metabolism and identify the genes responsible for this trait to underline the design of techniques such as gene silencing.

Table 2.1:Quizalofop and imazamox resistance survey samples collection and resistance screening results for downy brome, jointed goatgrass, and feral rye by year.

	Winter annual grass species Number of samples			Resistant populations	
collection year	**downy brome**	**jointed goatgrass**	**feral rye**	**quizalofop**	**imazamox**
2012	0	0	106	0	0
2015	18	12	20	0	0
2016	26	10	38	0	0
2018	0	0	57	0	2
Total	44	22	221	0	2

Table 2.2: Imazamox only dose-response log-logistic 3-parameter for feral rye populations A, B, and susceptible control. Model lack of fit test p-value = 0.0637.
[a]*d* corresponds to the upper limit representing biomass at the lowest herbicide rate. *b* is the slope for each curve. *e* represents the required herbicide to inhibit 50% of biomass (GR_{50}).

Populations	Dose-response model parameter estimates Response variable: biomass (percent of untreated control)				
	***d*[a]**	***b*[a]**	***e*[a] (GR_{50})**	**R/S ratio**	**R/S p-value**
Susceptible control	100	5.0	0.6 ± 8.5		
Population A	102.3	0.3	24 ± 3.9	40	0.942
Population B	98.5	0.13	148.2 ± 51.5	247	0.944

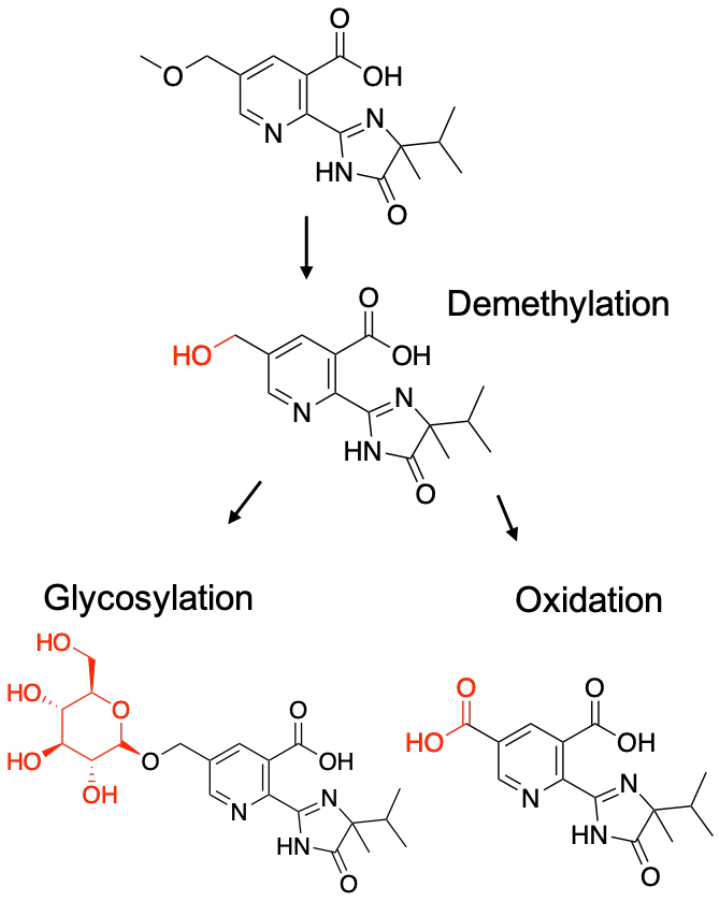

Figure 2.1: Imazamox metabolic pathway in spring wheat after a foliar application. Modified from Friedrich et al. (2012).

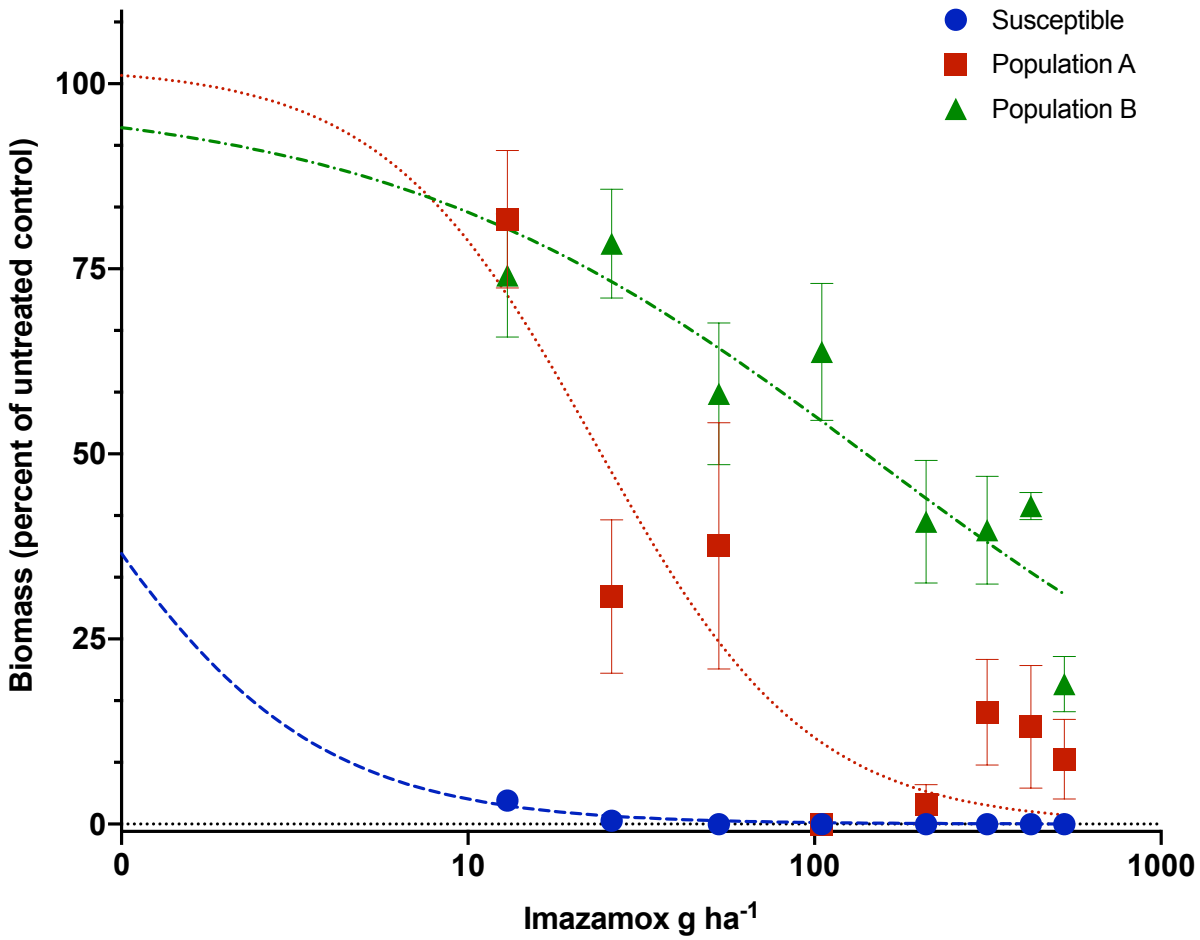

Figure 2.2: Imazamox dose-response curves comparing biomass reduction of feral rye from populations A (■) and B (▲) with a susceptible biotype (●) at 21 d after treatment. Each data point corresponds to the mean and standard error of six replications from two experimental replications.

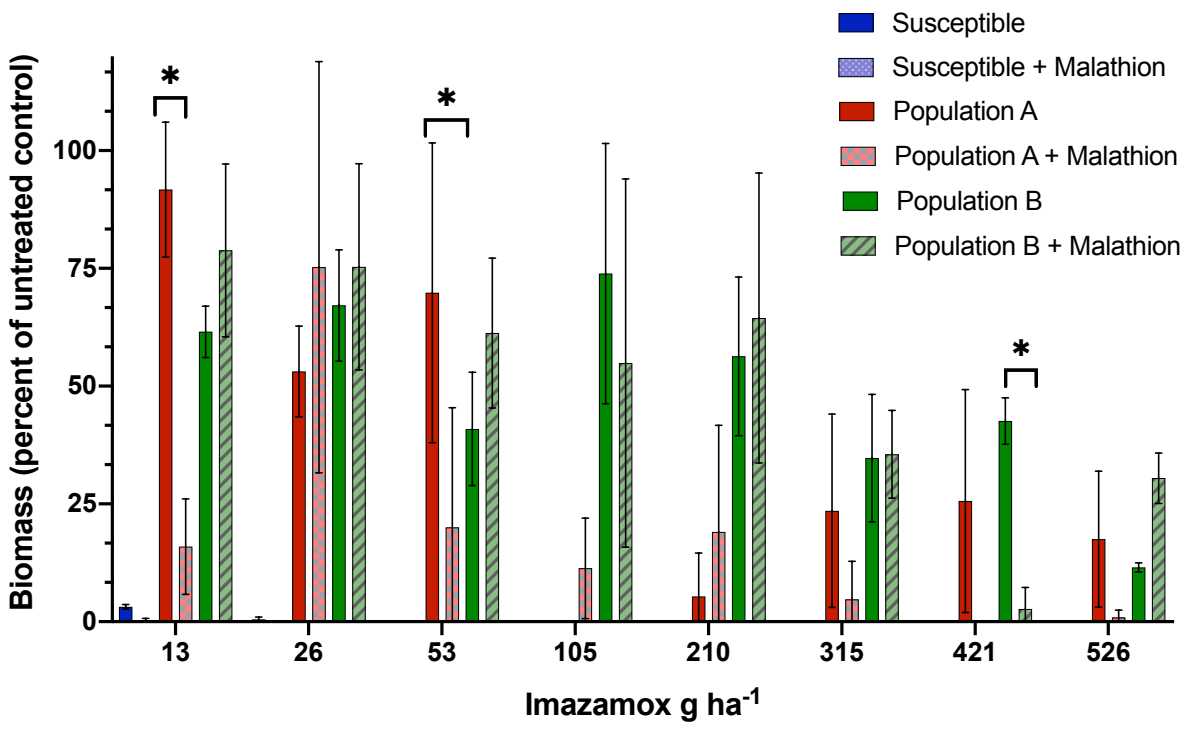

Figure 2.3: Imazamox alone and combined with malathion at 1000 g ai ha^{-1} dose-response depicting the differences in biomass as percentage of untreated control. Bars with * represent significant differences per population and among treatment based on Tukey's multiple comparison (α =0.05) (* = p-value < 0.005).

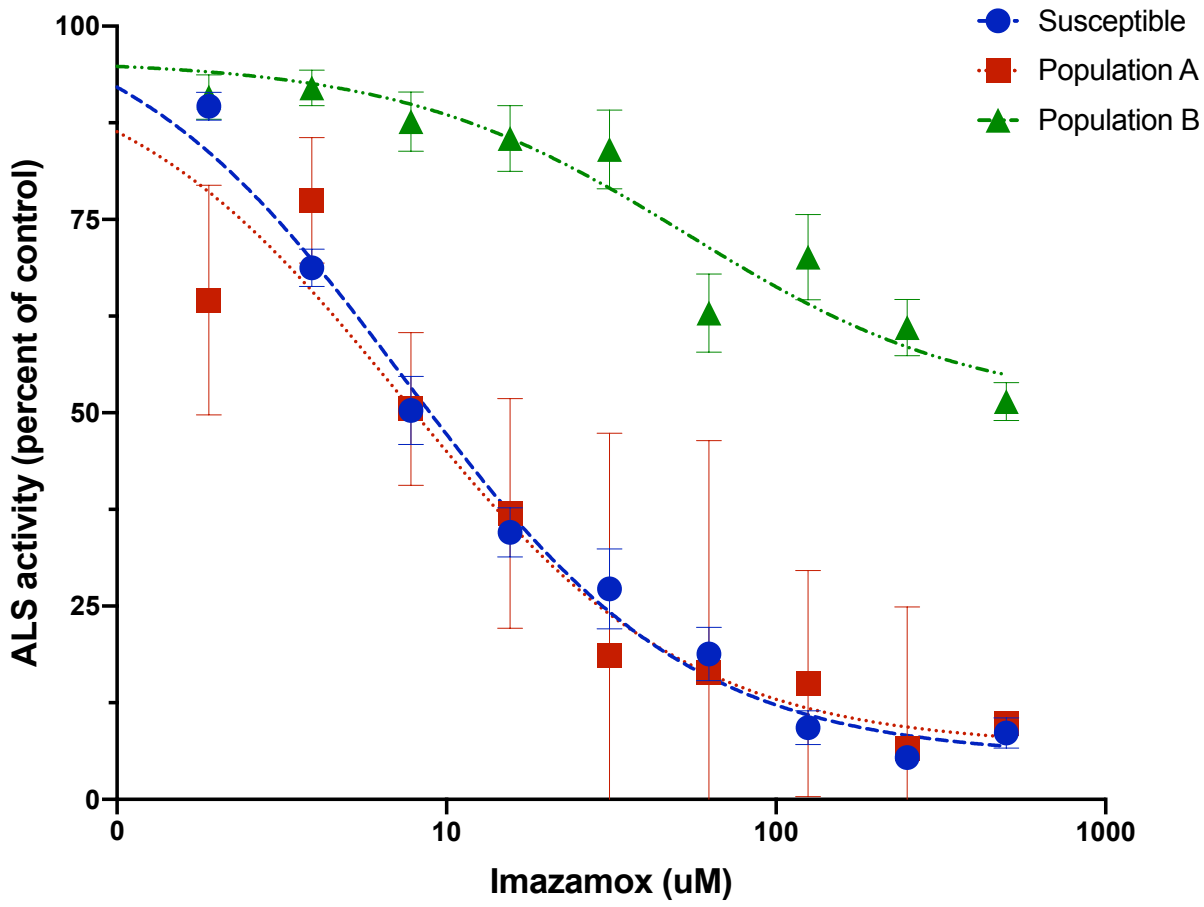

Figure 2.4: ALS enzyme activity in-vivo assay from feral rye from populations A (■) and B (▲), and a susceptible control (●). ALS activity was quantified as acetoin absorbance and calculated as a percentage based on the control. Each data point corresponds to the mean and standard error of three biological replications.

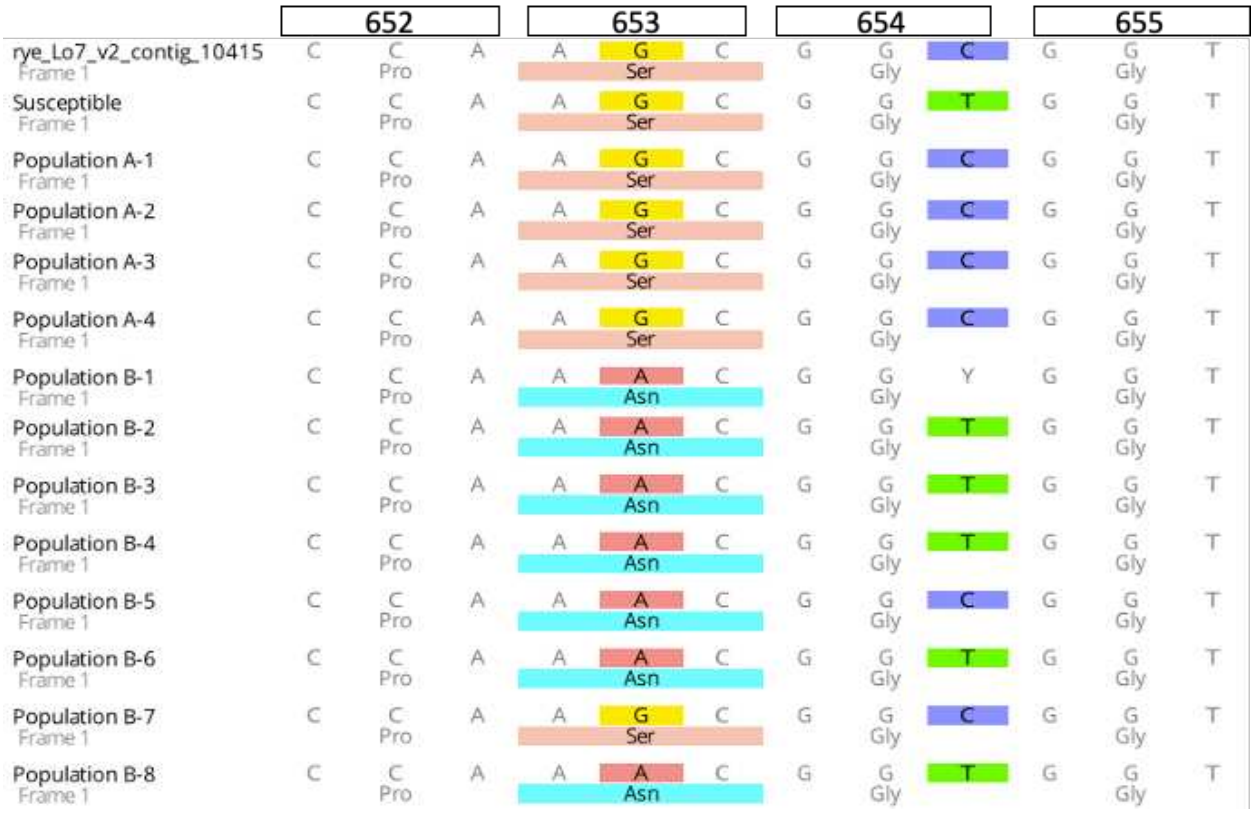

Figure 2.5: Consensus alignment from the partial ALS gene nucleotide and amino acid sequencing region showing the Ser653Asn mutation from each collection site per population A and B. Numbers next to population name indicated collection site. The numbers on top of the graph depict the residue number based on the *Arabidopsis thaliana* ALS amino acid sequence.

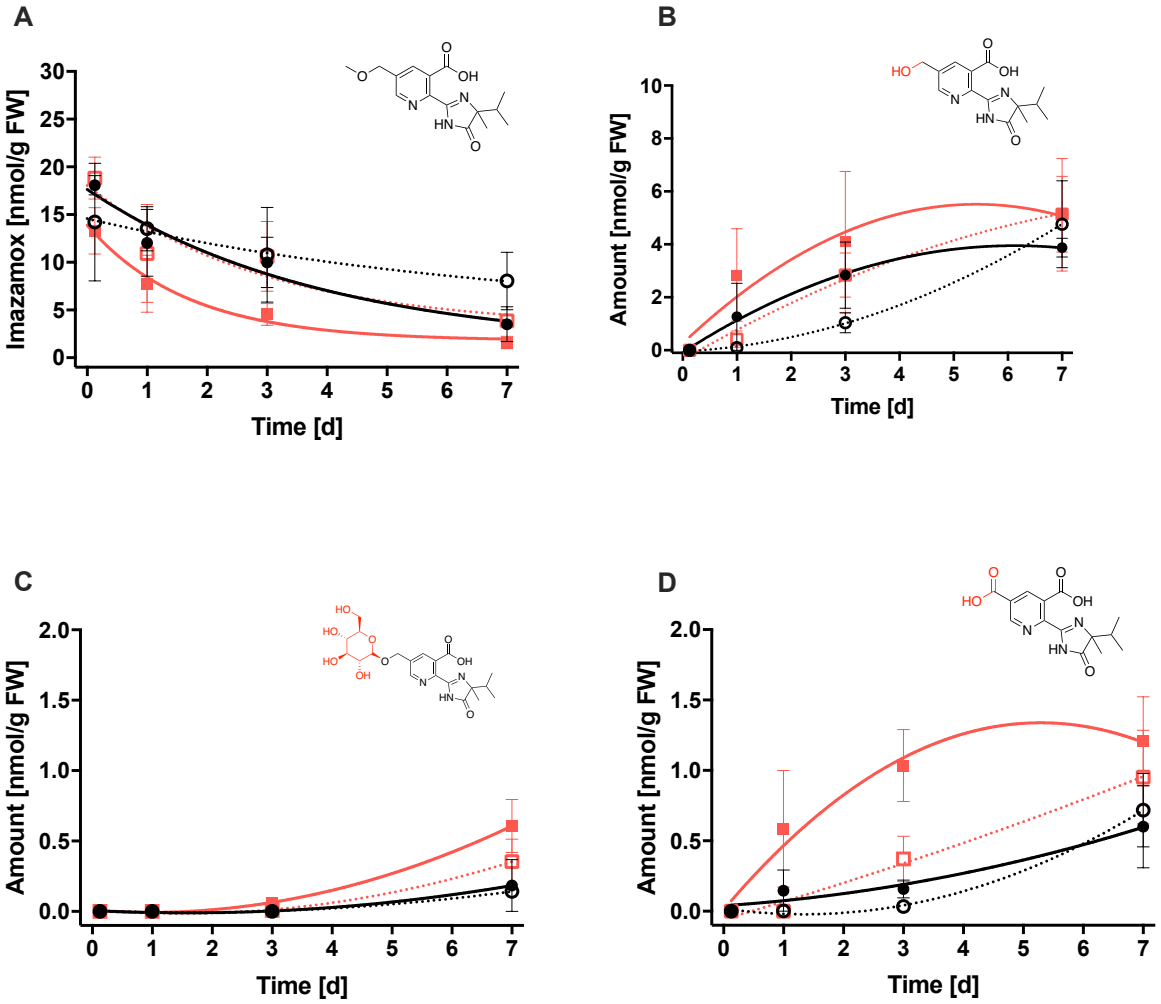

Figure 2.6: Quantification of intact imazamox (A), demethylated metabolite (B), glycosylated metabolite (C), and oxidized metabolite (D) as nmol per g fresh weight (FW) after treatment with imazamox at 62 g ai ha^{-1} or imazamox 62 g ai ha^{-1} plus malathion 1000 g ai ha^{-1} at 0, 1, 3, and 7 d after treatment. Curves correspond to feral rye susceptible control (●), susceptible control plus malathion (○), population A (■), and population A plus malathion (□). Bars represent the standard error of the mean from both experimental replications.

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Chapter 3: *Phragmites australis* in Colorado: haplotype distribution and molecular tools for management decisions

SUMMARY

Phragmites australis (common reed) is a cosmopolitan species distributed worldwide. Native *Phragmites* haplotypes represent an important component of the natural ecosystem, whereas introduced *Phragmites* haplotypes reduce biodiversity. While morphological characters are useful for visual classification of native and introduced haplotypes, they are not completely reliable when *Phragmites* is exposed to drastic environmental conditions due to its plasticity and adaptation abilities; thus, a genotyping method is preferred. The Colorado Department of Agriculture is considering whether to declare introduced *Phragmites* a noxious weed as a measure to reduce the ecological repercussions that this haplotype can cause. The occurrence of introduced *Phragmites* in the state remains unknown. Here we genotyped 186 samples collected across the state of Colorado, developed a cost-effective genotyping tool KASP (Kompetitive Allele Specific PCR) to discriminate among native and introduced haplotypes, and conducted a clustering analysis to identify possible new haplotypes. Based on the non-encoding chloroplast DNA regions (trnT-trnL and rcbL-psaI) we have identified 117 and 69 samples as native and introduced haplotypes, respectively. Samples were subject to Sanger sequencing or to Cleaved Amplified Polymorphic Sequence (CAPS) markers. The KASP assay accuracy compared to the results obtained by sequencing and CAPS classification was 90 and 98% for trnT-trnL and rcbL-psaI, respectively. Clustering analysis suggested that the existing haplotypes do not diverge from previously reported haplotypes. Results from this study provide land managers with accurate information and better genotyping tools for conservation and appropriate management of native *Phragmites*. We provide a rapid KASP assay for genotyping in plant diagnostic clinics and a new

approach to analyze the existence of new haplotypes by using a clustering approach for *P. australis*.

INTRODUCTION

Phragmites australis (Cav.) Trin. ex Steud. (common reed) is a polyploid cosmopolitan species distributed across continents worldwide. This perennial aquatic grass thrives in wetlands, littoral areas, irrigation canals, and standing waters (Brix 1999). In the United States, *P. australis* haplotypes are categorized based on their genetic diversity as: 1) native (subspecies *americanus*) originated from North America; 2) introduced (Eurasian origin); and 3) Gulf Coast (subspecies *berlandieri*), for which the origin remains cryptogenic (Saltonstall 2002). Native haplotypes are found across the country, whereas the Gulf Coast lineage has been identified to date only along the Florida coast and in California (Meyerson et al. 2010a). Introduced haplotypes have expanded aggressively across the US East Coast causing biodiversity loss and native *Phragmites* displacement. Introduced *Phragmites* grows rapidly in high density monoculture patches, leading to biodiversity reduction of insects, wildlife, and vegetation (Chambers et al. 1999; Saltonstall and Meyerson 2016). This expansion of introduced *Phragmites* haplotypes has been attributed to their ability to outcompete native haplotypes and greater range of adaptation (Silliman and Bertness 2004). For instance, introduced *Phragmites* are able to succeed in areas that had an anthropogenic disturbance while establishment of native lineages is limited under these conditions (Marks et al. 1994). Native haplotypes preservation is crucial to maintain an ecological balance in wetland ecosystems. Moreover, native *Phragmites* have an important socio-cultural role as ethnobotanic plant for Native Americans (Kiviat and Hamilton 2001).

Morphological differences among native and introduced haplotypes have been characterized in identification keys. However, due to *Phragmites* clonal and genetic diversity, and phenotypic plasticity, changes in environmental conditions can lead to different phenotypes adding more difficulty to accurately distinguish introduced versus native haplotypes based only on morphological characters (Saltonstall 2003a). Molecular tools are available to classify *Phragmites* haplotypes. The most common approach is based on non-coding regions (trnT-trnL and rcbL-psaI) in the chloroplast DNA as genetic markers (Saltonstall 2003c). This method requires amplification of these regions using PCR and Sanger sequencing of the amplicons. Later, these sequences are compared with the reference sequences to identify the haplotype for each region. Based on the Saltonstall (2016) naming system the trnT-trnL and rcbL-psaI regions are classified individually in a locus haplotype. The combination of these two loci determines the *Phragmites* haplotype name. Phylogenetic analyses reported elsewhere are used to place the haplotype name in the native, introduced, or Gulf Coast clades (Colin and Eguiarte 2016; Saltonstall 2002). Another molecular method is based on Cleaved Amplified Polymorphic Sequence (CAPS) markers (previously described as restriction fragment length polymorphism) based on differential restriction enzyme digestion at a single nucleotide polymorphism (SNP) in each of the two regions trnT-trnL and rcbL-psaI, to distinguish between native and introduced *Phragmites* (Saltonstall 2003c). Other methods are based on frequency of several microsatellites in genomic DNA (Saltonstall 2003b). These molecular tools have limitations due to the amount of time and cost required post-PCR if they were to be implemented as a high throughput approach for introduced *Phragmites* management decisions. Kompetitive Allele Specific PCR (KASP) is a cost-effective genotyping tool to distinguish SNPs, insertions, and deletions (He et al. 2014). Two forward primers are designed specific to the SNPs of interest and tagged with a

sequence that will hybridize with HEX or FAM fluorophores as quenchers. The reverse primer is designed using a common sequence between the two alleles. The PCR conditions are designed to increase binding specificity; thus, once the forward primer binds it releases the fluorophore and the real-time quantitative thermocycler can measure the fluorescence (He et al. 2014). KASP assays have been shown to produce reliable results for plant breeding, species classification, and pest identification (Patterson et al. 2017; Swisher Grimm and Porter 2020).

Introduced *Phragmites* expansion in the Western US remains at much lower frequency compared to the Eastern US. In the state of Colorado only native haplotypes have been reported (Lambert et al. 2016). However, these results do not cover most of the areas where *Phragmites* is found in the state; thus, the status of introduced haplotypes in Colorado remains unknown. Here we present a phylogenetic analysis describing the *Phragmites australis* haplotypes present in Colorado. This research is intended to provide an assessment of introduced haplotypes to direct management decisions in order to preserve native haplotypes in the state. In addition, we present a new KASP assay to distinguish between native and introduced *Phragmites* haplotypes that can be used as a cost-effective genotyping classification method.

MATERIAL AND METHODS

Plant Tissue Collection

Phragmites australis tissue collection was conducted by Colorado Department of Agriculture personnel from 20 counties in the state (Table 3.1). Each sample was documented following a field sample form that contained the following: collection date, GPS location, habitat type, number of samples per site, infestation size, population type (intermixed or a monoculture), and a visual classification. Approximately 1-5 g of plant tissue per individual was collected from the

top youngest leaf. Plant material was kept at 5 C and shipped overnight to Colorado State University for further analysis. A total of 186 samples were received from July through September 2017. Upon arrival *Phragmites* samples were assigned a code and stored at -80 C.

Native and Introduced *Phragmites* Haplotype Genotyping

Phragmites haplotype identification was conducted using sequencing of the two chloroplast DNA regions trnT-trnL and rbcL-psaI (Saltonstall 2003c) and the Cleaved Amplified Polymorphic Sequence (CAPS) markers (Saltonstall 2016) (Saltonstall 2003c; Saltonstall 2016). Plant tissue was ground to a fine powder with a mortar and pestle in liquid nitrogen for DNA extraction. Qiagen DNeasy plant mini kit® (Qiagen, Germantown, MD) was used following the manufacturer instructions to extract DNA from approximately 100 mg of ground plant tissue. DNA quantity were assessed using a NanoDrop 2000™ (Thermo Fisher Scientific, Waltham, MA). A master mix for each region was prepared using per reaction: 0.5 µL of Phusion™ High-Fidelity DNA polymerase, 10 µL 5X Phusion HF buffer (Thermo Fisher Scientific, Waltham, MA), 1 µL dNTPs at 10 mM, 2 µL of each forward and reverse primers at 10 µM, and 32.5 µL of sterile HPLC grade water. Two µL of DNA ranging from 20-40 ng μL^{-1} were added to 48 µL of master mix in a PCR tube. Polymerase chain reaction was conducted in a Bio-Rad T100 thermo cycler (Bio-Rad Laboratories, Inc., Hercules, CA) using the primers described by Taberlet et al. (1991) and (Saltonstall 2001) for the trnT-trnL and rbcL-psaI regions, respectively. Following the polymerase manufacturer recommendations, a 3-step PCR protocol was used for both loci that consisted of initial denaturation at 98 C for 30 s, 35 cycles of 98 C for 10 s; 58 C for 30 s, 72 C for 1:30 min, and a final extension of 10 min at 72 C. Amplicons were visualized in 1 % agarose gel using electrophoresis. PCR products were purified and Sanger sequenced by Genewiz, Inc (South Plainfield, NJ, USA) using the same forward and reverse primers as the

PCR. Forward and reverse sequences were assembled using Geneious R11 (Biomatters, Ltd., San Diego, CA). Resulting sequences were compared with published *Phragmites* haplotypes available in GenBank using BLAST (Basic Local Alignment Search Tool). Locus haplotype identification was made listing the highest four bit-scores obtained in the BLAST output for each region. Haplotype naming was assigned following the locus combinations listed in Saltonstall (2016).

Seventy-three samples had partial sequence data due to lack of complete sequence by Sanger sequencing for one or both loci. These samples were genotyped using CAPS (Cleaved Amplified Polymorphic Sequence). As described by Saltonstall (2003c), the restriction enzymes RsaI and HhaI (New England Biolabs Inc., Ipswich, MA) were used to digest the trnT-trnL and rbcL-psaI amplicons, respectively. Samples underwent the same PCR protocol and primers as described above. The digestion reaction was conducted using 5 µL of the PCR product, 1 U of the restriction enzyme, 5 µL of the 10X NEBuffer, and 39 µL of sterile HPLC grade water. After overnight incubation at 37 C, fragments were run in 1% agarose gel using electrophoresis and assigned as introduced or native according to the digestion results.

KASP Genotyping Tool for Native and Introduced Haplotypes

Kompetitive Allele Specific PCR (KASP) primers were designed based on a diagnostic single nucleotide polymorphism (SNP) for native and introduced haplotypes for each locus (Table 3.2). The SNPs correspond to *Phragmites australis* chloroplast sequence (GenBank accession: KJ825856) positions 48,707 and 57,974 for the trnT-trnL and rbcL-psaI regions, respectively. Following the KASP assay manufacturer's recommendations (LGC Genomics, Beverly, MA, USA), a primer master mix was prepared using 18 µL of each forward primer and 45 µL of reversed primer at 100 µM mixed with 69 µL of sterile water. A KASP master mix was prepared

using 12 µL of primer mix in 432 µL of 2X KASP Master mix (LGC Genomics, Beverly, MA, USA). Reactions were prepared in a 96-well plate where 4 µL of the KASP master mix and 4 µL of DNA template at 5-10 ng μL^{-1} per sample were mixed. A Bio-Rad CFX Connect (Bio-Rad Laboratories, Inc., Hercules, CA) real-time quantitative thermo cycler was used to conduct the PCR and read the HEX and FAM fluorescence. According to the KASP recommended protocol, the PCR conditions were initial activation at 94 C for 15 min; followed by 10 touchdown cycles of 94 C for 20 s, 61 to 55 C reducing 0.6 C per cycle for 60 s each cycle; followed by 35 cycles of 94 C for 20 s and 55 C for 60 s. The HEX and FAM readings were taken at 30 C for 10 s each cycle. Fluorescence data from cycles 23 and 30 were selected for the trnT-trnL and rbcL-psaI and regions, respectively. These cycles were selected based on optimal SNP discrimination. Data were standardized by calculating the percentage relative to the highest fluorescence per plate. These results were compared with haplotype classification obtained by sequencing and CAPS using the "mcnemar.test" function in R (R Core Team 2018). Haplotype classification obtained by CAPS and sequencing were regressed with the KASP fluorescence data to estimate the probability of native and introduced calling using the "glm" function in R (R Core Team 2018).

Phragmites australis Clustering Analysis

A clustering analysis was conducted with 105 and 80 sequences from the loci trnT-trnL and rbcL-psaI loci, respectively. These samples were selected based on the sequence quality and length. Microsatellites repeats in the loci trnT-trnL and rbcL-psaI are mainly where the haplotypes diverge from native, introduced, and Gulf Coast (Saltonstall 2002). Samples were clustered based on repeat sequences, for this, tandem repeats were identified using Tandem Repeats Finder (Benson 1999). Each sample was then coded as a sequence of unique repeats and Levenshtein (edit) distances were calculated for each pair of sequences using the "adist" function

in R (R Core Team 2018). Hierarchical clustering was then made based on these distances, and bootstrap probability values for the clustering were calculated using the pvclust R package (Suzuki and Shimodaira 2006). Hierarchical clustering trees and coded repeat sequences were visualized using ggtree package in R (Yu et al. 2017).

RESULTS

Both native and introduced haplotypes were identified in Colorado (Table 3.1 and Figure 3.1). From the total received samples 117 were classified as native and 69 as introduced based on the Sanger sequencing and CAPS results. No Gulf Coast haplotypes were identified. Native haplotypes were identified in eight counties, whereas introduced haplotypes were found alone in five counties. Both haplotypes co-existing in the same location were identified in five counties. Visual identification during plant tissue collection was reported for 158 samples. Twenty-seven samples did not match the genotyping results. Out of the total 27 individuals, 16 times the samples were classified as native haplotype when the CDA collector called invasive; and 11 times the samples were classified as invasive haplotype when the CDA collector called native. Native and introduced haplotypes were similarly distributed among monoculture and intermix systems. In addition, the majority of native and introduced haplotypes were present in riparian areas. Out of the 17 samples that were collected in roadsides, 15 were classified as native and two as introduced haplotypes. Following the combined haplotype naming described by Saltonstall (2016), 111 sequenced samples were assessed resulting in 54 individuals classified in the native clade as haplotypes: A (*n*= 17), B (*n*= 1), BI (*n*= 13), D (*n*= 3), E (*n*=10), and H (*n*= 10), and 58 samples in the introduced clade as haplotypes AD (*n*= 1), M (*n*= 44), and O (*n*= 12). Genotyping results from the KASP assay for each locus are depicted in Figure 3.2. The trnT-trnL locus assay had 90% accuracy compared to the sequencing and CAPS classification, whereas the

rbcL-psaI locus had 98% accuracy. Discrepancy between the methods was higher for the trnT-trnL region, where 5% of the native samples were called introduced and 18% of the introduced samples were classified as native. In contrast, the rbcL-psaI locus had no errors for native samples and only 4% of the introduced samples were called native. KASP assay genotyping results were not statistically different from what was obtained from sequencing and CAPS at both loci (trnT-trnL: McNemar $\chi 2$ = 1.38, p-value = 0.24; rbcL-psaI: McNemar $\chi 2$ = 1.33, p-value = 0.25); thus, KASP assay provided reliable results to distinguish native and introduced haplotypes. An estimated probability heatmap was built based on the logistic regression to show the decision boundaries among HEX and FAM for native and introduced calls, respectively (Figure. 3.2). In addition, based on this estimate probability the standard error of each observation was calculated and plotted to identify which samples in the data set have low confidence (Figure 3.2). To simplify these results and encourage the implementation of the KASP assay for *Phragmites* classification, we built a decision tree using conjectured values from the logistic regression output to rule out any low confidence observations that are less likely to be correct and for which an alternative approach should be used (Figure 3.3). This assay is based on chloroplast DNA; thus, it cannot detect hybridization among native and introduced haplotypes. Clustering analysis did not identify new locus haplotypes (Figures 3.4 and 3.5) as it indicated that the evaluated sequences are grouped with the reported haplotypes.

DISCUSSION

Native and Introduced *Phragmites* Haplotype Genotyping

Here we documented the first genetic assessment of *Phragmites* collected across the state. Previous research showed evidence for only native haplotypes in this area (Lambert et al. 2016;

Meyerson et al. 2010a). Introduced *Phragmites* haplotypes expansion in the Western US remains not as well described as it has been in the Eastern US. However, introduced haplotypes are predicted to out-compete and replace native haplotypes as has been documented in the Eastern US. Inland invasion reports in Utah are concerning, where despite finding five different native haplotypes the majority of the areas across the Great Salt Lake are being occupied by the introduced haplotype M. This information suggests that the introduced haplotype is rapidly displacing the native lineages (Kulmatiski et al. 2011). Introduced haplotypes are also found in other Western states such as California and Nevada, including Gulf Coast haplotypes in California and Arizona (Lambert et al. 2016). Introduced haplotype invasion in the West has been attributed to higher genetic diversity among individuals and successful viable seed dispersal as invasive characteristics (Kettenring and Mock 2012). Introduced *Phragmites* thrives in conditions exacerbated by climate change and in human disturbed areas; thus, proactive management is imperative to preserve native haplotypes.

Monitoring of introduced *Phragmites* is essential to identify the regions that need to be prioritized for management. This research identifies high risk invasion sites where introduced and native haplotypes are coexisting or locations where introduced lineages are predominant. Although native haplotypes are more frequent across the different sampled ecosystems, proactive management of introduced *Phragmites* patches is critical. For instance, out of the 17 samples that were collected on roadsides only two were characterized as introduced haplotypes. Roadside ecosystems are frequently disturbed, providing the required resources for introduced haplotype invasion. Characteristics such as linear shape, salinity, and nutrient accumulation favor introduced haplotypes over the native ones (Brisson et al. 2010). Introduced *Phragmites* management is a challenging task due to the extensive below ground rhizome network and seed

dispersal mechanisms. Control techniques are based on herbicide applications, prescribed fire, hand removal, and biological control (Lombard et al. 2012; Tewksbury et al. 2002). Long-term herbicide applications might not provide complete eradication of introduced haplotypes, yet this method may suppress this haplotype enough to increase plant biodiversity (Bonello and Judd 2020; Lombard et al. 2012). In Western states overall introduced haplotypes remain at lower frequency than native ones. Monitoring and early control might lead to possible full eradication as the introduced haplotypes expansion remains lower than what is documented in the Eastern US.

KASP Genotyping Tool for Native and Introduced Haplotypes

High-throughput and accessible techniques for *Phragmites* haplotypes classification are needed for management decisions. Here we propose the use of KASP as a genotyping tool to distinguish among native and introduced haplotypes. Results from this assay correctly classified most of the tested samples with a calling accuracy of 90 and 98% for trnT-trnL and rbcL-psaI, respectively. We developed a regression model to identify the decision boundaries between the two alleles and determined that the high likelihood regions for native and introduced calls are in the Cartesian plane (Figure 3.2). This information was used to derive a decision tree for implementation of this method in diagnostic laboratories (Figure 3.3). This fluorophore PCR type of assay has been widely used for plant breeding and species identification (Patterson et al. 2017; Semagn et al. 2014). Other commercial options are available that work under the same concept to identify SNPs such as TaqMan, and rhAmp (Broccanello et al. 2018). This assay is a cost-effective tool that can provide quick and reliable results for *Phragmites* genotyping.

The KASP assay that we described here does not aim to identify hybrids between native and introduced *Phragmites* haplotypes. Hybrids between these two haplotypes have been identified

in the Eastern and Western US (Lambert et al. 2016; Paul et al. 2010; Saltonstall et al. 2016). Although hybridization events can occur in nature, the frequency and establishment success of hybrids remains low. A greenhouse study where native and introduced haplotypes were artificially crossed showed that interspecific hybridization can occur; however, gene flow may be unidirectional with only the introduced haplotype as the pollen donor to produce viable offspring. Moreover, F1 plants might be subject to a fitness cost and less likely to compete and establish in a dense *Phragmites* stand (Meyerson et al. 2010b). Overall, the current state of knowledge regarding hybridization remains as a rare and low frequency event. Understanding hybridization events in *Phragmites* is difficult as ploidy levels varied among populations and dissemination of asexual reproductive structures can play a major role in hybrid dispersion. Regions where where native and introduced haplotypes are co-existing were identified (Figure 3.1), suggesting the importance of early management to prevent possible hybridization events.

Clustering Analysis

As the first Colorado *Phragmites australis* genotyping survey we analyzed the generated sequences for trnT-trnL and rbcL-psaI to identify haplotypes that have not been reported. We conducted a clustering analysis using the repeated minisatellites found in the non-encoding chloroplast DNA sequences for each locus (Figure 3.4, Figure 3.5).

Phragmites australis phylogeny studies have been based on two loci (trnT-trnL and rbcL-psaI) located in the large single copy (LSC) part of the chloroplast DNA (Saltonstall 2003c). These non-encoding regions are fairly conserved among and within species and provide enough information to build cladograms to better understand speciation in grasses (Davis and Soreng 1993). However, sequencing of trnT-trnL and rbcL-psaI in *Phragmites australis* does not consistently produce useful results. Our data showed that 75 samples were not able to obtain a

clear sequence in either one or both loci. Similar results have been reported elsewhere (Lambert et al. 2016). This lack of consistency might be attributed to heteroplasmy, where more than one haplotype variation is present in the chloroplast DNA at the individual level. Next generation sequencing in several angiosperms suggested that plant groups such as grasses have structural heteroplasmy, where the genes can be rearranged or in reverse complement (Wang and Lanfear 2019). Other instances of heteroplasmy are hypothesized to be related to parental leakage or biparental inheritance and a haplotype copy with polymorphisms within the same chloroplast will be present (Ellis et al. 2008). Lambertini (2016) documented several cases of heteroplasmy in different *Phragmites australis* populations collected from around the world. The author suggested that parental leakage originated the haplotype variations for each locus. Although we did not identify new haplotypes, a next-generation sequencing approach is warranted to further investigate possible heteroplasmy events and characterize any co-existing haplotypes in the same individual.

Phragmites australis introduced haplotypes invasion in Western states is occurring at a faster rate than expected. Monitoring and proactive management are essential to prevent introduced haplotypes large spread. Here we report the regions that need to be prioritized for management as introduced haplotypes are present and co-existing with native ones. In addition, we proposed a faster, low cost, and reliable genotyping tool to be implemented in plant diagnostic clinics as a service for land managers.

Table 3. 1: *Phragmites australis* tissue sample collection sites and haplotypes classification per county in the state of Colorado.

		Haplotypes classification	
County	**Total samples collected**	**Native**	**Introduced**
Adams	2	2	0
Arapahoe	14	8	6
Bent	3	0	3
Boulder	10	2	8
Chaffee	6	6	0
Delta	12	12	0
Denver	8	0	8
El Paso	5	4	1
Garfield	22	22	0
Jefferson	4	3	2
La Plata	3	3	0
Larimer	2	0	2
Mesa	53	36	17
Montrose	8	2	6
Morgan	1	1	0
Pueblo	17	4	13
Rio Blanco	2	0	2
San Miguel	2	0	2
Weld	10	10	0
Yuma	2	2	0

Table 3.2: Primers for trnT-trnL and rbcL-psaI regions used for Kompetitive Allele Specific PCR (KASP) assay. Forward primer (FP) and reverse primer (RP).

Locus	Primer name	Primer sequence (5'-3')	
trnT-trnL	SNP 48,707	Fluorophore probe	
	Native FP trnT-trnL	GAAGGTCGGAGTCAACGGATT	TTGAAACCAGGATTCCTTGTGTA**C**
	Introduced FP trnT-trnL	GAAGGTGACCAAGTTCATGCT	TTGAAACCAGGATTCCTTGTGTA**A**
	Universal RP trnT-trnL		GTATGGAAACCTGCTAAGTGGT
rbcL-psaI	SNP 57,974		
	Native FP rbcL-psaI	GAAGGTCGGAGTCAACGGATT	CTTAATCGATGGTATCTACCGGC**T**
	Introduced FP rbcL-psaI	GAAGGTGACCAAGTTCATGCT	CTTAATCGATGGTATCTACCGGC**G**
	Universal RP rbcL-psaI		TGTACAAGCTCGTAACGAAGG

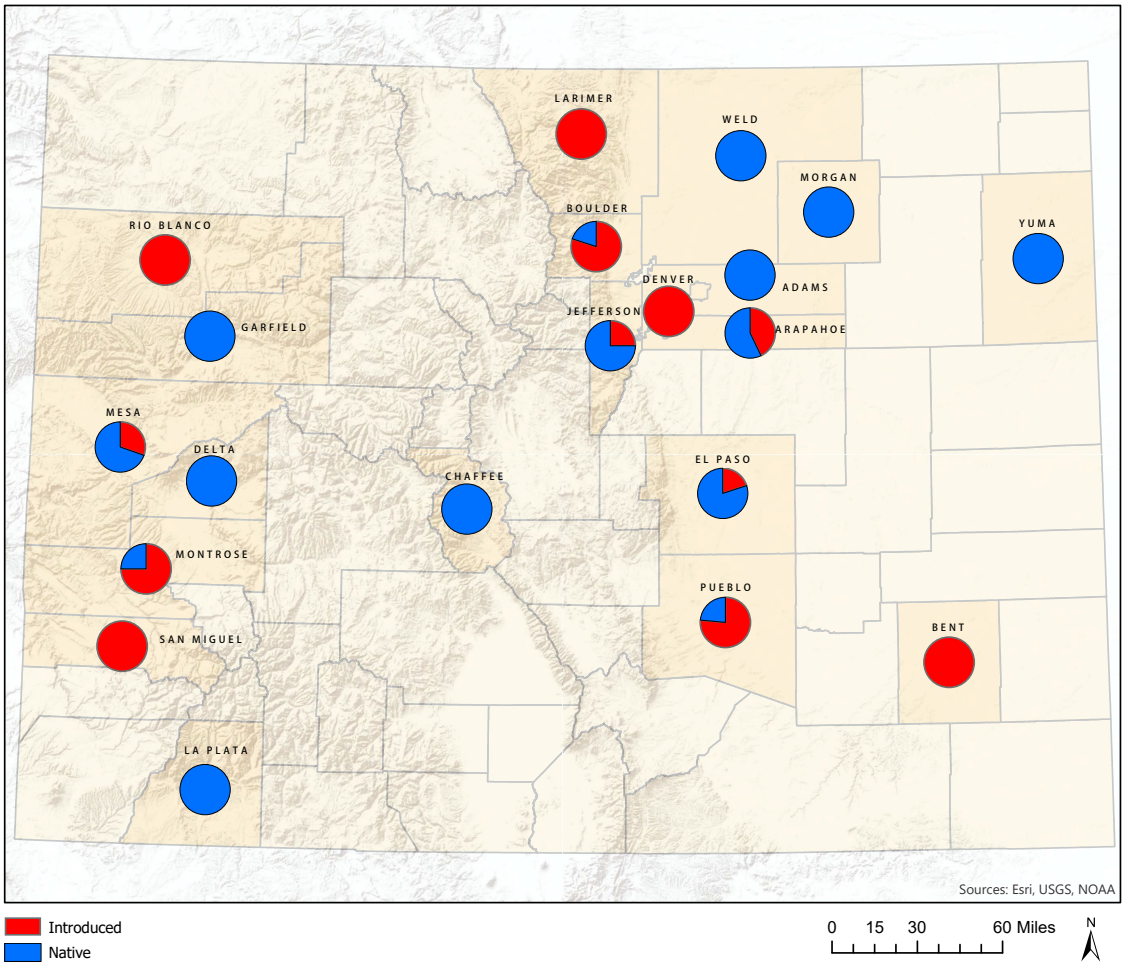

Figure 3.1: *Phragmites australis* haplotypes distribution in the state of Colorado based on different genotyping tools per county. Blue and red color represents native and introduced haplotypes, respectively.

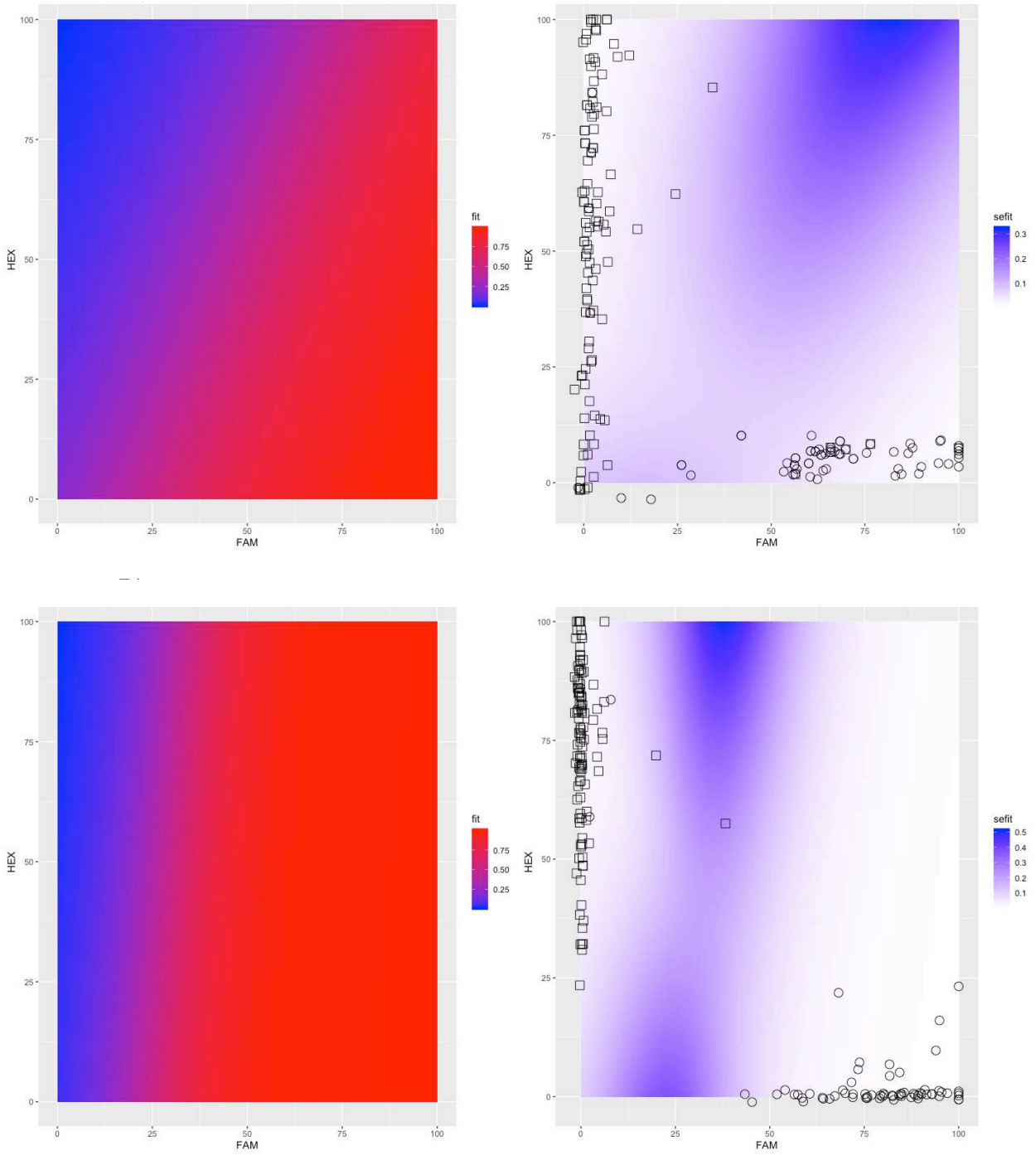

Figure 3.2: Kompetitive Allele Specific PCR (KASP) assay (A) trnT-trnL locus and (B) rbcL-psaI locus heatmap of estimated probability for native (HEX) and introduced (FAM) call based on the regression model. Standard error of the estimated probability heatmap for native (□) and introduced (○) haplotype distribution for (C) trnT-trnL locus and (D) rbcL-psaI locus.

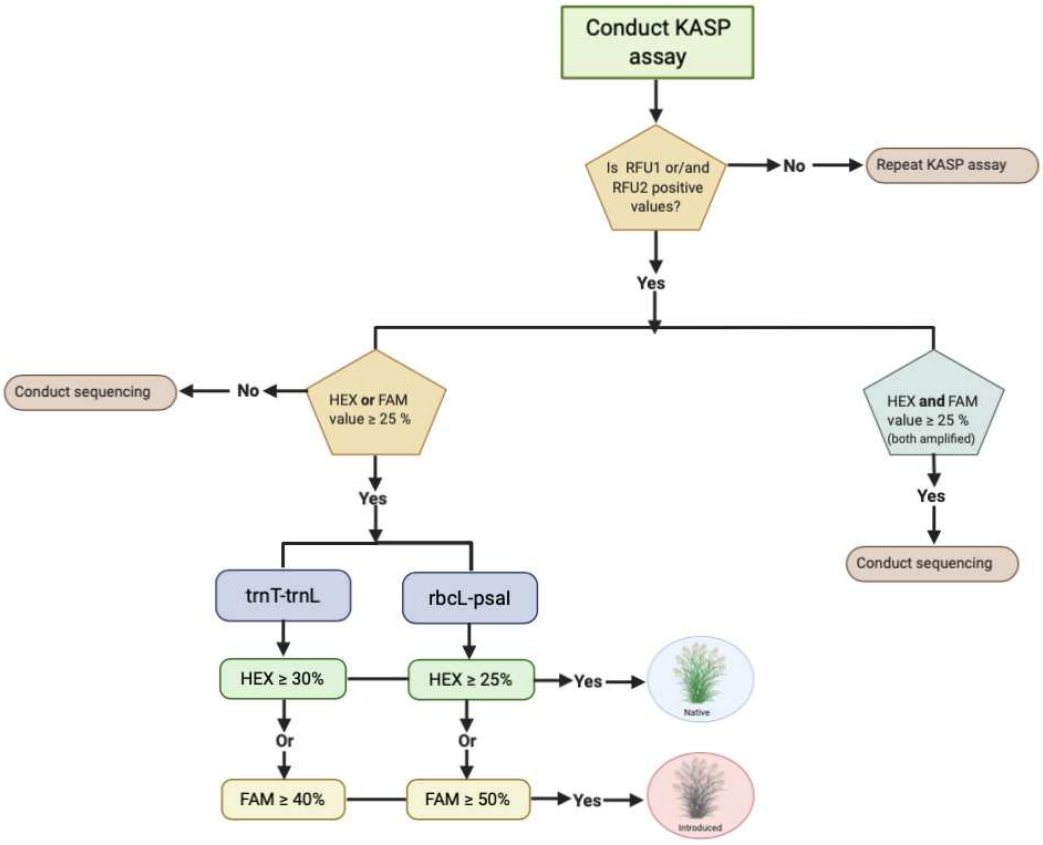

Figure 3.3: Decision tree for the Kompetitive Allele Specific PCR (KASP) assay outlining the procedure and values to determine if this genotyping tool is sufficient to discriminate among native and introduced haplotypes based on the non-encoding chloroplast regions trnT-trnL and rbcL-psaI.

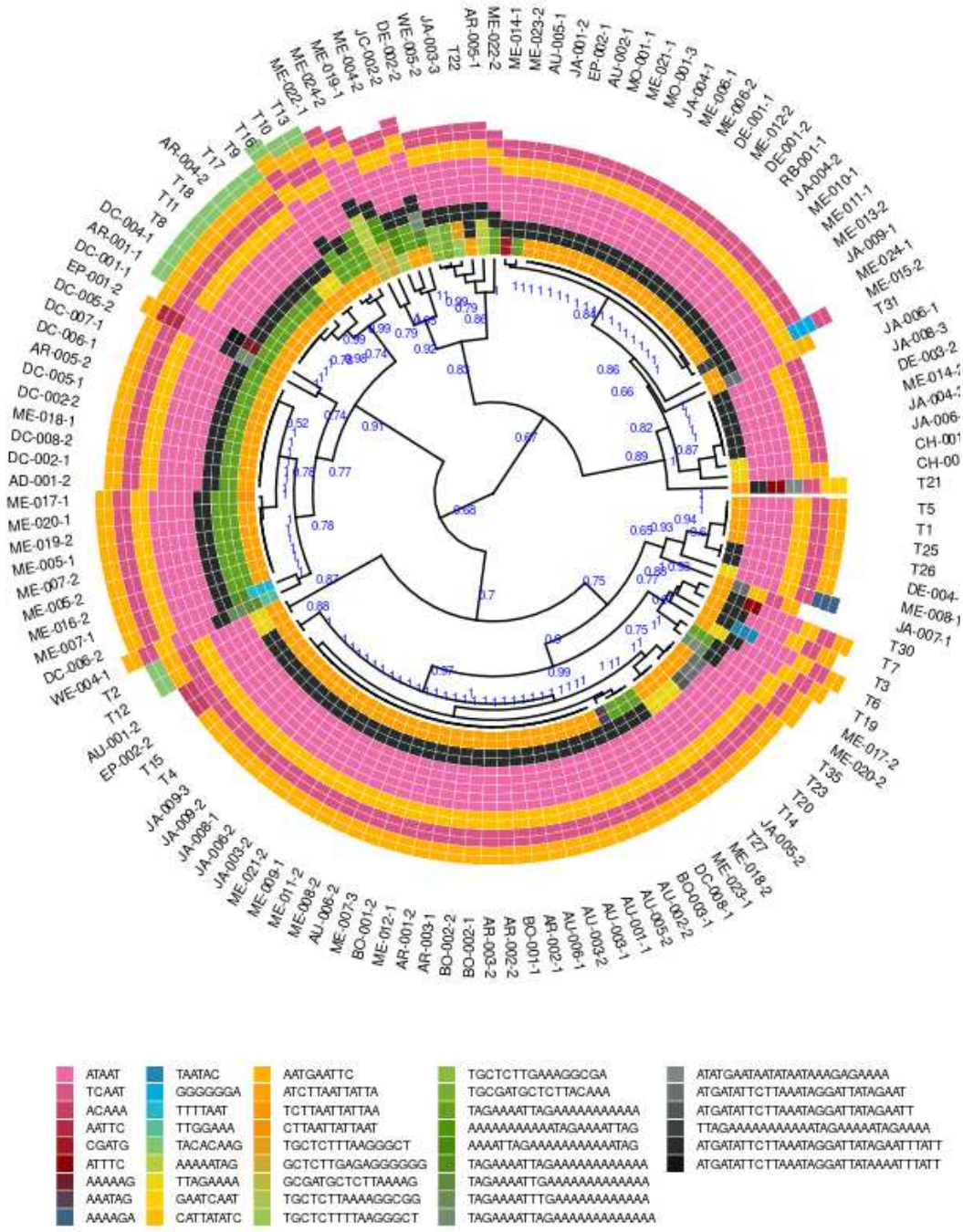

Figure 3.4: Clustering analysis of *Phragmites australis* samples for trnT-trnL. Central tree shows a hierarchical clustering based on edit distances between repeat sequences, numbers in blue indicated bootstrap probability values as calculated with pvclust (1000 boostraps). Colored squares represent unique tandem repeats found in sequential order in each sample

.

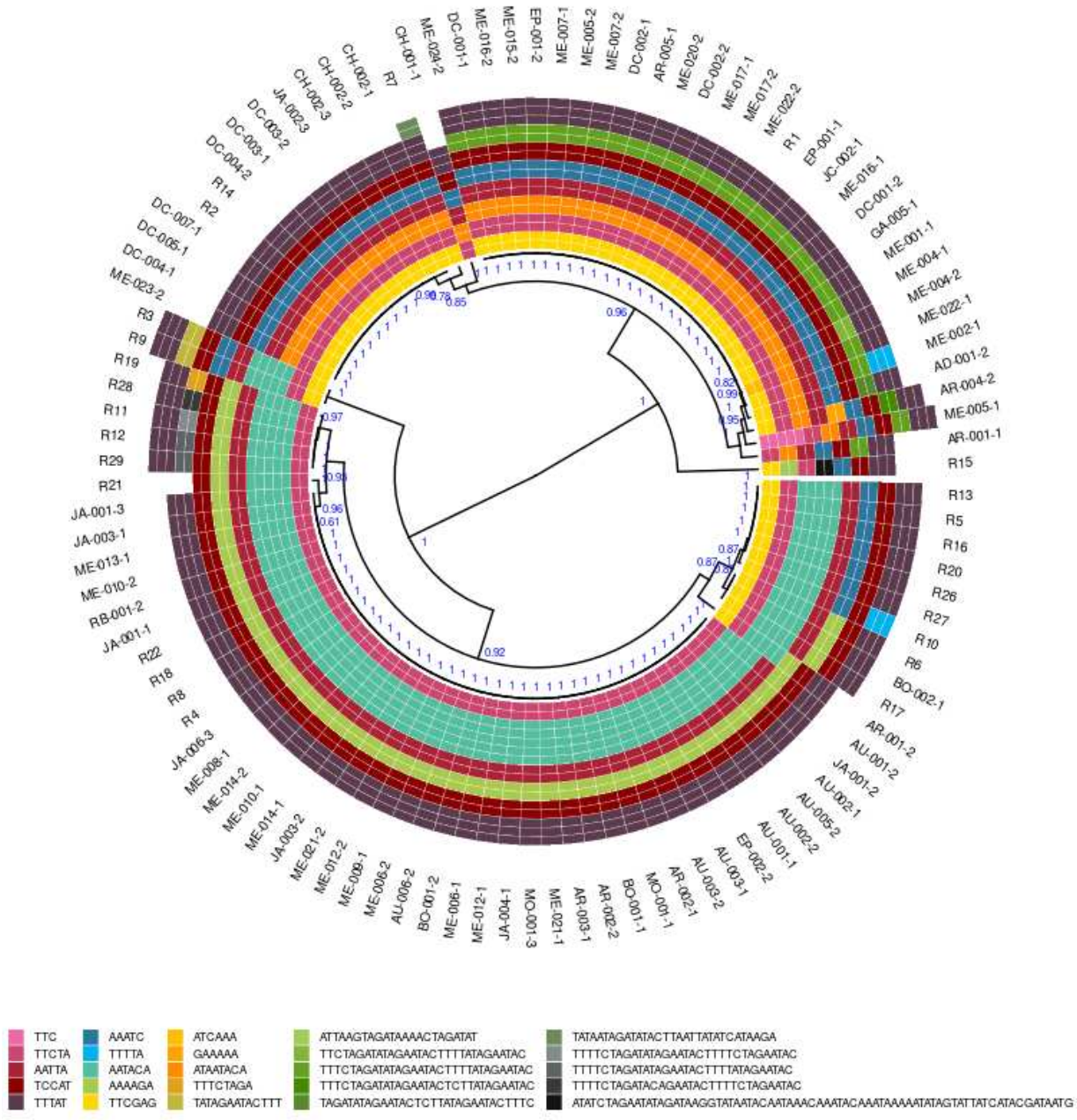

Figure 3.5: Clustering analysis of *Phragmites australis* samples for the and rbcL-psaI. Central tree shows a hierarchical clustering based on edit distances between repeat sequences, numbers in blue indicated bootstrap probability values as calculated with pvclust (1000 boostraps). Colored squares represent unique tandem repeats found in sequential order in each sample.

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