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Allelopathy in weedy rice as a genetic resource for developing competitive rice varieties

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Allelopathy in weedy rice as a genetic resource for developing competitive rice varieties

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Weedy rice is of the same species as cultivated rice, *Oryza,* and is a common rice weed exhibiting allopathic characteristics. Incorporation of allelopathic traits from weedy rice into cultivated rice may have a positive impact on rice yield. The overall objective of this study was to characterize weedy rice accessions based on the allelopathic potential to suppress barnyardgrass. These accessions, once discovered, may be used in identifying phenotypic traits and genes associated with their weed suppressive trait. Ten weedy rice accessions, and five rice cultivars (Rex, CL163,Rondo, PI312777, PI338046) were screened using a stair-step technique. The allelopathic potential was calculated based on the percent inhibition of barnyardgrass. The best performing accessions were phenotyped for root characteristics in a lab setting. Lastly, 30 SSR markers were used to evaluate the weedy rice to identify markers associated with the trait. Accessions identified in this study should be considered in the development of weed-suppressive rice cultivars.

DEDICATION

This thesis is dedicated to everyone who supported me during this process. Specifically, my committee members Dr. Tseng, Dr. Fox, Dr. Peterson and Dr. Popescu who invested time and energy into my education. To Dr. Bohach and Dr. Willard without whom I would not have been at Mississippi State. Lastly, this work is dedicated to all my fellow graduate students who encouraged me and struggled with me throughout my master's journey and kept me going.

ACKNOWLEDGEMENTS

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CHAPTER I

LITERATURE REVIEW

Background

Weedy rice is of the same species as cultivated rice, Oryza, and is a common rice weed exhibiting allopathic characteristics. The demand for rice is increasing as the global population increases to a projected 8.27 billion by 2030 (Muthaya et al., 2014; Olofsdotter, 2000). Weeds cause majority of yield losses in rice production and increased production cost (Chopra et al., 2017). Herbicide resistance in weed populations introduce a need for supplemental weed management strategies (Gealy, 2013). Without sustainable weed management, troublesome weeds like barnyardgrass may continue to reduce yields.

Purpose of the Study and Hypothesis

The goal of this study was to identify the markers associated with allelopathic root traits in weedy rice. Information on the root architectural differences between allelopathic and non-allelopathic phenotypes will help identify root traits associated with allelopathy. Once known, it may be possible to employ weedy rice accessions with the allelopathic ability for suppressing barnyardgrass in rice breeding programs.

In this study, the hypothesis was that barnyardgrass would exhibit significant reduction when exposed to allelopathic effects from weedy rice. Moreover, measurements taken from images would describe a set of root characteristics associated with allelopathic weedy rice accessions. Finally, markers associated with the allelopathic root phenotypes would be identified.

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Objectives

Chemicals produced from allelopathy are secondary chemicals. Previous studies lack confidence concerning which chemicals are allelopathic in wild-type species of rice. Studying phenotypic traits may reduce the knowledge gap about chemical components related to allelopathy in weedy rice. It is difficult to observe root systems accurately without disrupting their natural architecture, and since they are not visible in the field, techniques for studying them accurately still need development. Phenotyping allelopathic root traits with germination pouches is a promising method to identify these characteristics of roots and compare genetic factors that may be incorporated into breeding programs. Therefore, the objectives of this study are to:

1. Confirm allelopathic weedy rice accessions through height, chlorophyll, and biomass suppression measurements on barnyardgrass,

2. Identify root characteristics associated with allelopathic phenotypes through noninvasive images, and computer-based calculations, and

3. Identify markers associated with allelopathic weedy rice and root systems architecture in allelopathic accessions.

This study took place between June 2018 and Feburary 2020. The screening was conducted at the RR Foil Plant Science Research Center, Mississippi State University (88.7847°, 33.4552°). The second and third objectives were conducted in Dorman Hall Laboratory 306, 32 Creelman St. Mississippi State MS 39762 (-88.7944° 33.4539°).

Weed Impact in Rice

Rice Production takes place in more than one hundred countries and supplies over 715 million tons of crop annually (Muthayya et al., 2014). Fifteen countries account for the majority of the world's rice harvest and 90% of those countries within the Asian continent. In Asia, rice is a vital nutritional food crop providing up to 50% of the dietary caloric supply, and it is a

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significant part of the protein consumption for an estimated 520 million people (Muthayya et al., 2014). Rice production demand is projected to increase up to 70% by the year 2050 according to the global population growth (Muthayya et al., 2014; Olofsdotter et al., 2000).

A variety of weed species infest rice fields and paddies affecting the health, food supply, and economy of those consuming and producing the crop (Smith, 1983). Historically, weed competition is the most critical constraint to upland rice production. Weeds are a significant factor in yield losses, causing increased production cost and decreased rice quality (Chopra et al., 2017; Jensen et al., 2001). Yield loss by weeds is due to several factors including the species present, degree of infestation, rice ecosystem, growing season, cultivar raised, and finally, cultural and management practices (Raj and Syriac, 2017).

Over 350 weed species infest rice crops systems including bearded sprangletop, Amazon sprangletop, broadleaf signalgrass, duck salad, eclipta, and more. Some of the most troublesome weeds in rice production are barnyardgrass (*Echinocloa crus-galli*) and weedy rice or "red rice" (*Oryza sativa*) (Smith, 1983). Arkansas growers spend more than $100 million in weed control annually. Approximately 15-20% investment in rice weed control is herbicide application costs (Hardke et al., 2016).

Production Constraints

Weed control efforts give rise to other complications in cost and labor practices explicitly mechanical or hand weeding, seedbed preparation, crop rotation, land leveling, levee construction, seed selection, water management, and herbicide cost (Matsunaka, 1980). In many systems, nearly half of the production effort is dedicated to weed control and yet yield losses, due to weeds, in a given field can be estimated at 40-100% (Galinato et al., 1999; Raj and Syriac, 2017). In addition to the yield loss potential, weeds in rice increase the cost of production and

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lower the quality of the crop itself (Jensen et al., 2001). Rice is an aquatic crop yet 45% of rice is grown in unflooded cropland (IRRI, 2002). The genetic diversity of the crop allows for it to survive in many environmental situations, but this factor also opens opportunities for a wide variety of weeds to intrude rice production areas (Smith, 1983). Each method of rice farming, whether it be upland, lowland, direct-seeded, transplant, or others, has unique weed problems.

Transplanting rice into puddled systems is the traditional method rice producers employ worldwide. Transplanting into flooded paddies is the most effective way to control weeds because of the weed suppression in the initial stages of rice development (Matsunaka, 1980; Chauhan et al., 2013; Dass et al., 2017). Water conservation efforts such as intermittent flooding and shallower water depths in fields cause ineffective control of weeds (Olofsdotter et al., 2000). Due to water consumption and economic struggles in the predominant rice-producing countries, located primarily in Asia, there has been a shift from transplanting to direct seeding (Raj and Syriac, 2017). A direct seeding rice system drawback is noted when practices are favorable, not only for the rice crop, but also for its competitor weeds; thus, allowing weeds to thrive (Raj and Syriac, 2017).

Water and labor conserving planting methods have increased the use of herbicide control (Durga et al., 2019). Chemical controls are efficient but have ecosystem health concerns associated with their use, such as its effect on animals and soil quality (Durga et al., 2019). Furthermore, herbicide resistance may develop in weeds like weedy rice, that have a close genetic relationship with the rice crop (Olofsdotter 2002; Sudianto et al., 2014). Repeated use of herbicides may cause weeds found in rice to develop resistance to the herbicides, making them less effective over time (Gealy 2013; Olofsdotter et al., 2000).

Herbicide resistance in weeds was reported in Japan and Korea, beginning in the early 1980s (Datta and Herdt, 1983). In Arkansas, the occurrence of ALS-resistant sedges has increased since 2012. The development of rice lines that can tolerate certain herbicides is a recent milestone in mitigating weed incumbrances (Sudianto et al., 2014). Researchers developed Clearfield rice lines as herbicide-tolerant breeds of cultivated rice. These rice lines allow producers to apply imidazoline, an ALS inhibitor, to the crop while killing its co-habitations. However, five years after the commercialization of Clearfield rice, producers reported ALS-resistant weeds in Clearfield rice systems (Zhang et al., 2006; Sudianto et al., 2014). Herbicide-resistant breeding may provide a temporary solution to a lingering problem that compounds with every chemical application as resistance develops at an increasing rate in the weedy relatives due to natural crossing (Sudianto et al., 2014).

Barnyardgrass, Most Problematic Weed in Rice

Barnyardgrass (*Echinochloa crus-galli*) is one of the troublesome, widespread weeds in rice production (Zhang et al., 2017). The biological similarities of barnyardgrass and commercial rice enable them to tolerate flooded culture and co-exist in similar growing environments. Barnyardgrass possesses characteristics that contribute to its success and vigor such as swift development to maturity, phenotypic variability, a high amount of seed production with low seed dormancy, and canopy height (Galinato et al., 1999). If not controlled, season-long interference of barnyardgrass may result in as much as a 70% loss in grain yield (Smith, 1988).

The evolution of herbicide-resistant weed populations has produced an interest in breeding weed suppressive crops. Additionally, many small rural farms and organic producers require alternative weed management strategies and strategies beyond conventional herbicides (Worthington and Horton, 2013). Herbicide resistance is defined as:

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"The inherited ability of a plant to survive and reproduce following exposure to a dose of

herbicide normally lethal to the wild type. Resistance may be naturally occurring or

induced by genetic engineering" (Nandula, 2010).

Herbicides have been the primarily management strategy against barnyardgrass in U.S. rice production for over 6 decades. Barnyardgrass herbicide formulations include butachlor, propanil, quinclorac, pendimethalin, and others (Kwesi and Datta., 1991). In 2011, barnyardgrass was first reported in Mississippi to have resistance to four sites of action being ACCase inhibitors, ALS inhibitors, Cellulose inhibitors, and PSII inhibitors (Ureas and Amides) (Heap, 2019). Recently, barnyardgrass populations with multiple resistance to propanil and quinclorac have been reported (Heap, 2019). Today, barnyardgrass has resistance to six modes of action in 34 countries. The widespread distribution and ability of barnyardgrass to evolve resistance to the herbicides is a major concern to both producers and researchers.

Weedy Rice as Genetic Resource for Superior Traits

Weedy rice is also difficult to manage weed in rice production systems (Norsworthy et al., 2013). Weedy rice, known commonly as "red rice," is of the same genus and species as white rice and is characterized by red seed coat. The hull morphology of weedy rice varies in color and the species is considered a noxious weed due to its undesirable color traits decreasing quality in cultivated rice (Hardke, 2016). This genus encompasses twenty-one wild species and two cultivated *O. sativa* and *O. glaberrima* commonly known as Asian rice and African rice, respectively (Khush, 1997). Like cultivated rice, weedy rice is a self-pollinating, annual grass, with a complete flower and awned husks. Its distinguished red pericarps shatter easily similar to cultivated rice, but early on in development. Early shattering allows weeds to have a better chances of survival and embedment in the soil bank where they have a long period of dormancy

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(Thurber et al., 2011; Chin, 2001). Weedy rice is a product of hybridization between cultivated rice and wild relatives (Delouche and Labrada, 2007). Due to the natural crossing along with multiple introductions of the weed population, which are not native to the U.S., weedy rice has developed a vast germplasm with many varieties (Burgos et al., 2008). Weedy rice is a subspecies of rice that exhibits highly successful weedy characteristics (Delouche and Labrada, 2007). Some of the weed's characteristics include: 1) exceptional adaptation, 2) a similar lifecycle to that of rice, 3) high seed population, 4) rapid emergence, 5) taller canopy than cultivated rice, 6) tendency to lodge because of weak culm, 7) high tillering capacity, 8) spreading growth habit with long, 9) drooping leaves, 10) vigorous absorption of fertilizer, 11) tolerance to shade, 12) simultaneous maturation of grains, and 13) seed dehiscence (Shivrain et al., 2010; Delouche and Labrada, 2007).

Weedy rice's morphological and physiological similarity to cultivated rice, and its extensive germplasm allow it to survive in a variety of ecological niches, thus making it one of the most difficult-to-control weeds in rice fields globally (Shrestha et al., 2020; Chuhan, 2013). The first weedy rice species was recorded in the 19th century was a seed contaminant from an imported Asian seed source (Allston, 1846). Weedy rice has been hybridizing with cultivated rice and is evolving into the troublesome and diverse weed that it has become today (Londo and Schaal, 2007). In some cases, weedy rice may reduce rice crop yields up to 87% in the U.S. (Hardke, 2016). Arkansas is the highest rice producing state in the U.S. and has measured losses at $274/ hectare due to weed infestation (Burgos et al., 2008).

Trends in farming such as direct seeding support weedy rice establishment because the size differential and maturity gap are absent between the weed and the rice population (Delouche and Labrada, 2007). Herbicides used to treat weedy rice also kill the cultivated rice it is infesting

(Pantone and Baker, 1991). Due to the crossing of high yielding varieties and some of the Oryza species, new herbicide-resistant weedy rice's have emerged (Sudianto et al., 2014) After Clearfield® technologies were integrated into rice production, outcrossing between the weed and the new rice varieties created new generations of ALS-resistant weeds. The spread of these resistant weedy rice could nullify chemical treatments and Clearfield technologies; the only known effective means of control for this particular pest in rice to rice cropping systems (Hardke, 2016; Delouche and Labrada, 2007). Provisia™ rice was introduced to compliment the Clearfield system by adding another mode of action to control weeds (Camacho et al., 2019). With rotation, the development of ALS resistance in weeds may be slowed or significantly retarded. Genetically improving varieties is the common strategy for combatting resistance in weeds.

Allelopathy

Research has noted synergistic and suppressive interactions between plants for centuries. In chickpea and the black walnut tree, some adverse effects on the growth of the surrounding vegetation have been observed (Weston, 2005). Allelopathy is a natural and complex phenomenon that has become the study of many crop scientists in the past few decades. Allelopathic mechanisms for use in crops has been the subject of much research since its definition in the 1930s as:

"Any direct or indirect harmful or beneficial effect by one plant on another through the production of chemical compounds that escape into the environment"

(Khanh et al., 2007; Rice, 1984). Released allopathic chemical compounds help provide the donor plants with a competitive advantage by adding phytotoxins to the environment around them (Pratley, 1996). Allelopathic activity is selective in its effectiveness and varies between

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varieties, environmental conditions, growth stage, and species contact (Weston, 2005). The chemicals produced are secondary products and, therefore, it is yet unknown what compounds are the most active in suppression and under what conditions (Weston, 2005).

Allelopathy as Alternative Weed Control Strategy

Allelopathy may potentially serve as a biological means to control weeds that are particularly challenging to manage. The selection of an allelopathic characteristic followed by incorporation into the desired crop variety is one option to "allelopathically enhance" weed management strategies Chung et al., 2003). Advantageous allelopathic residues improve weed control when applied as straw mulch or found growing an allelopathic variety in a rotational sequence (Chung et al., 2003). Both living and dead tissues of allelopathic rice varieties can release chemicals (Kong et al., 2006). Mulching with green manure crops possessing bioherbicidal characteristics or weed-smothering capability is one effective technique (Singh et al., 2007). Rye, sorghum, rice, sunflower, rapeseed, and wheat are critical allelopathic crops (Jabran et al., 2015). Allelopathy is, therefore, a promising method to suppress weeds and has the potential to provide significant value due to the impracticality of rotating crops in a paddy field (Khanh et al., 2007). This weed control method has shown potential as a possible supplemental strategy for the weed crisis in rice (Chopra et al., 2017; Jabran et al., 2015). The economic importance of rice allelopathy is potentially significant due to research on allelopathic cultivars that gave 30% more weed control and 44% less yield loss than non-allelopathic cultivars (Gealy et al., 2013). Efforts to apply allelopathy as a weed management strategy have been slow. Challenges arise due to the physiological nature of allelopathy, the influence the environment holds in its effectiveness, as well as its genetic characteristic as a quantitative trait (Zhang et al., 2019).

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Allelopathy in Rice

In commercial rice, hundreds of accessions demonstrate herbicidal activity against different weed species in a variety of ways. Since the 1980s, during studies of allelopathic rice varieties in duck salad, field experiments provided valuable data on the allelopathic potential of a diverse germplasm of rice showing different levels of weed inhibition (Jensen et al., 2001; Dilday et al., 1989). The allelopathic potential of rice differs depending on its origin, plant size, developmental stage, plant part, and hull color (Shrestha et al., 2020; Khanh et al., 2007).

Some allelochemicals identified in rice varieties are momilactone B, 3-isopropyl-5-acetoxycyclohexene-2-one-1, and 5, 7, 4¢-trihydroxy- 3¢, 5¢-dimethoxyflavone; all of which are allelopathic products released into the soil at phytotoxic levels (Kong et al., 2006). Understanding what allelochemicals weedy rice produce would provide insight into the mechanisms taking place, and if these chemicals contribute to the competitiveness these weedy types. Significant differences exist between the allelopathic and non-allelopathic cultivars in their production of three of these six compounds identified in solutions containing root products (Seal et al. 2004). Trans-ferulic acid, p-hydroxybenzoic acid, and caffeic acid from the exudates of allelopathic rice cultivars were concentrated in higher amounts than non-allelopathic varieties (Seal et al. 2004). Genetics studies have recently revealed differences in the expression of biosynthetic pathways between allelopathic and non-allelopathic rice lines, particularly PI312777, an allelopathic variety grown outside the U.S. Some secondary metabolites from the shikimate and acetic pathways expressed in allelopathic rice were fatty acids, phenolic acids, and flavonoids. Furthermore, phenylalanine ammonia lyase is hypothesized to be an important regulator in chemical production (Zhang et al., 2019).

Weedy Rice Allelopathy

Weedy rice is a primary weed in rice paddies (Kraehmer et al., 2016). The weed exhibits superior traits allowing it to thrive among rice cultivars such as high cold tolerance at the seedling stage, an ability for shoot emergence even in deep water, the long span of seed dormancy, and usually is taller than rice cultivars (Chin, 2001; Suh et al., 1997). Current studies tend to focus on crop allelopathy, but little is known about the potential of the weedy species to produce more or different chemicals that contribute to a weed's success (Bertin et al., 2003). According to R. J. Smith Jr, assuming that; a) allelopathic traits existed in wild-type rice, b) that these traits can be incorporated into currently used or just as aggressive cultivars, and c) weed control methods can be adopted from the activity of allelopathy, allelopathy may be a valuable new form of weed control in rice (Smith, 1983). Although current rice varieties may possess allelopathic abilities, wild type rice may have a higher activity that is not lost through selective breeding and hybridization (Zhang et al., 2019; Olofsdotter, 1998).

Studies are underway to identify the genetic basis of proven allelopathic rice cultivars (Zhang et al., 2019; Ebana et al., 2003; Jensen et al., 2001). Weedy rice has the potential through root mechanisms to keep weeds species like barnyardgrass and Amazon sprangletop under control (Shrestha et al., 2020; Gealy et al., 2013). Bioassays conducted on wild rice and domestic rice found that wild rice had a greater ability to inhibit seedling germination and root growth on test species such as lettuce and barnyardgrass (Wedger et al., 2019). Tests found barnyardgrass height suppression by various weedy rice accessions to be higher than cultivated rice varieties, thus indicating the weed's potential as a gene pool for allelopathic traits (Shrestha et al., 2020).

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Root Phenotyping for Allelopathic Traits

Roots release various compounds into the soil at the same time they uptake nutrients. Root exudates secreted into the soil may influence plant growth patterns as well as soil composition and ecology (Jabran et al., 2015; Bertin et al., 2003). There are several modes in which a donor plant can release these allelochemicals. Allelochemicals may spill off leaves and stems of plants, or leach through the degrading parts of the plant or the roots (Bertin et al., 2003). Microorganisms transport the root exudes released by diffusion, ion channels, and vesicle transport to a target plant (Jabran et al., 2015; Bertin et al., 2003).

Sustainable farming aims at utilizing fewer resources and inputs, including lower levels of applied chemicals for weed control (Zhu et al., 2011). Until the past few decades, most phenotyping research focused on aboveground plant parts contributing to yield increase. Research is needed to improve the potential of plant parts not visible to the naked eye. Roots are complex systems with many parts that differentiate during development. Many factors contribute to the development of the roots; the type of plant, the soil structure, and soil environment (Zhu et al., 2011; Bertin et al., 2003). Root architecture is a term that refers to distinct aspects of the shape of root systems (Lynch, 1995). The nutrient concentration of the rhizosphere is directly affected by the status of the root architecture. Roots exploit soil structure and secret their exudates or secondarily metabolic deposits from photosynthesis (Bertin et al., 2003). A significant, practical example of root phenotyping for structural traits is the advantage of shallow rooting in some soil types vs. deep in others due to nutrient availability (Walter et al., 2015; Zhu et al., 2011).

Research suggests that roots secrete more allelochemicals into the environment than the aboveground plant parts (Bertin et al., 2003). The root structure is essential in understanding

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what characteristics of the plant underground contribute to the allelopathic abilities that weedy rice exhibits to advance weed control efforts. Species such as sorghum, wheat, and Oak have all exhibited allelopathic soil activity similar to several rice varieties (Kong et al., 2006; Bertin et al., 2003). Root systems architecture and its plasticity are directly associated with the plant's ability to thrive in response to the diverse environments and stressors, including weeds, as they are other species competing for recourses (Sanduh et al., 2016).

Weed suppressive genes in rice

Rice root systems genetics have been studied thoroughly to increase stress tolerances such as drought. Allelopathy has not been a focus of efforts in terms of root morphology in rice (Courtois et al., 2009). Dilday et al. (1998) deduced that allelopathy in rice is a qualitative trait meaning it is dependent on the actions of many genes as well as the environment (Jensen et al., 2001). It has also been suggested that epistasis, the suppression of a gene by another, may be involved in allelopathy (Jensen et al., 2001). Selecting for quantitative traits which are traits that are a product of the cumulative actions of multiple genes as well as the environment based on phenotyping alone is inefficient because of environmental influence. DNA markers assist in gene identification by providing a tool to sort through the variation in the expression of quantitative traits by using quantitative trait loci (QTL). The foundation behind QTL mapping is to identify links between phenotypes and molecular markers. With the assistance of DNA markers, genes can be located, which are linked to traits, and those genes can then be utilized through marker-assisted selection techniques (Jensen et al., 2001).

Allelopathic mechanisms are unknown, therefore research focus has shifted to root morphology and not allelopathic activity. Early root system traits in plant seedlings are important for stability, nutrient uptake, and competition with surrounding weeds (Courtois et al., 2009).

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Rice root development is governed by fundamental inherent pathways that determine the plant's architecture as an expression of its genetic potential. Root development is additionally an effect of response pathways that determine development reactions to stress (Courtois et al., 2009). Regarding root characteristics in rice, prolific amounts of genetic variation have been observed. Changes in characteristics exist between the six isozyme groups in terms of root morphology. These groups include Indica, tropical Japonica, temperate japonica, aus from southern Asia, double haploid populations, and recombinant inbred lines (Jensen et al., 2001; Lafitte and Courtois, 2002). Genotypic associations between different measurements of root architecture have been linked together. Root length and root thickness are closely related, while root length and tiller number are not strongly associated (Courtois et al., 2003). Variation in allelopathic activity seems to be independent of the variation in root mass, although it may contribute to overall competitive advantage (Jensen et al., 2001). Quantitative trait loci associated with allelopathy have been identified in all regions of the rice genome except for Chromosome 2 (Worthington, 2013). Quantitative trait loci mapping is useful in marker-assisted selection (MAS) but is not efficient in gene discovery because hundreds of genes could be located on a region 5-10 centimorgan (cM) long (Worthington, 2013). Association mapping posed a promising means of fine-tuning the extensive regions associated with allelopathy. Marker trait associations can be made using simple sequence repeat (SSR) markers to clarify what has been found through QTL research. The development of effective markers is still needed to aid marker-assisted selection for traits associated with allelopathy (Worthington, 2013).

CHAPTER II

SCREENING WEEDY RICE FOR ALLELOPATHIC POTENTIAL USING A STAIR-STEP SYSTEM

Abstract

Rice supplies a significant portion of the daily diet of millions worldwide. The demand for rice production is projected to increase by up to 70% in the coming years due to population growth. Unfortunately, weed competition is the most limiting factor to rice yields. Supplemental and sustainable weed management strategies are needed to compensate for herbicide resistance. Weedy rice is a common rice weed of the same species as cultivated rice, Oryza, and exhibits allopathic characteristics, giving it an advantage over its neighbors. Identification and incorporation of allelopathic traits from weedy rice into cultivated rice lines may have a positive impact on rice yield. The objective of this study is to rank weedy rice accessions based on allelopathic potential to suppress a prevalent weed in rice production systems, barnyardgrass (Echinochloa crus-galli). These highly allelopathic accessions, once identified, may be used in phenotypic studies to identify characteristics associated with their weed suppressive trait and serve as candidates for genetic studies. In this study, ten weedy rice accessions, two non-allelopathic rice cultivars (Rex, CL163), and three allelopathic rice cultivars (Rondo, PI312777, PI388046) were screened using a stair-step method. Height, chlorophyll, and biomass measurements on 14-day-old seedlings were used to calculate the reduction percentage of barnyardgrass seedlings. The accessions were categorized based on allelopathic potential. Some

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weedy rice was found to be more suppressive than the cultivated rice lines (B2) screened in this study. Allelopathic traits found in weedy rice, being the same species as rice, can easily be used in rice breeding programs. These accessions should be considered in the development of weed-suppressive rice cultivars using marker-assisted selection.

Introduction

Over 350 weed species have been reported in rice crops, the most numerous being grasses that include barnyardgrass, junglerice, and weedy rice or red rice (Smith, 1981). In many systems, nearly half of the farmer's time is taken up by weed control, and yet yield loss due to weeds in rice has been estimated at 40%-100% (Galinato et al., 1999). On top of the loss potential, weeds in rice make the cost of production higher and lower the crop quality (Jensen et al., 2001). Manual weeding, although used in rural production, is not as efficient as modern chemical controls. These chemical controls or herbicides cause heavy selection pressure on weeds and cause herbicide resistance to develop. Herbicide resistance is a major challenge, especially in weeds like red rice that have a close genetic relationship with the crop itself (Olofsdotter et al., 2002). It has become apparent that new methods of weed control need to be developed and incorporated into rice production (Chung et al., 2003).

Genetically based weed control methods are being explored to enhance chemicals application efficacy or to serve as alternatives to herbicide application. Utilizing allelopathy in variety development has shown much potential as a supplemental weed management strategy to chemical and cultural practices (Jabran e al., 2015; Chopra et al., 2017; Kim and Shin, 1998). Allelopathy is the ability of a plant to release chemicals into the environment giving it an advantage over its neighbors by inhibiting the growth and development of surrounding plants (Rice, 1984). Allelopathy has been exhibited in some rice cultivars and in weedy rice (Gealy et al

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2013). Weedy rice is listed as one of the major weeds in rice paddies and is of the species Oryza sativa (Kraehmer et al., 2015). Weedy rice exhibits superior traits allowing it to thrive among rice cultivars such as high cold tolerance at the seedling stage, an ability for shoot emergence even in deep water, a long period of seed dormancy, and is normally taller than rice cultivars (Chin et al., 2001). The competitive nature of weedy rice, therefore, serves as a potential gene pool for weed suppressive traits that can be used in breeding elite rice cultivars. Identifying characteristics of weed rice, both above and below ground, associated with allelopathic abilities, is the first step to tapping into the plant's potential. Once identified, genes associated with these characteristics can be located and be used in breeding programs as a biological weed control method (Ebana et al., 2001). Improving the weed suppressive ability of crops will benefit farmers in rice-cultivating countries by reducing labor costs while increasing yield without any detrimental effect on the environment and playing an essential role in sustainable weed management (Khanh et al., 2007).

There are many methods for screening plants for allelopathy, some in the field, and some in a greenhouse, demonstrating allelopathic abilities being present in both sets of conditions (Chung et al., 2003; Jensen at al., 2001; Li et al., 2015). Allelopathy is a complicated phenomenon and is a quantitative trait meaning multiple factors, both known, and unknown contribute to its production and intensity. These factors include genes and the joint action of secondary metabolites that may act together with the environment (Chung et al., 2003; Ebana et al., 2001; Olofsdotter et al., 2002). Additionally, it is difficult to separate allelopathic activity from competition during screenings.

Traditionally, field experiments are conducted to screen for allelopathy in rice. A study conducted by Dilday et al (1994, 2000) compared rice accessions allelopathic potential to

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different weed species finding variability in susceptibility among species. Field experiments use the percentage reduction in the number of weed plants in a test plot relative to a no allelopathic control (Jensen et al., 2001). These studies found valuable information about the allelopathic potential in the diverse germplasm, which is exhibited at different levels in different varieties (Jensen et al., 2001). The restrictions with field experiments include labor cost, the amount of space needed, the large amount of time they consume, the difficulty when large amounts of plants need to be screened, and the influence of the environment (Li et al., 2015).

Greenhouse methods are preferred as they have a more controlled area and, at the same time, closely mimic field conditions, in comparison to lab screenings (Wu et al. 2001). The inhibitory circle method has been used to assess allelopathy in weedy rice against barnyardgrass and Palmer amaranth again finning variability in the germplasm or weedy rice (Shrestha et al., 2018). The difficulty with field and a few greenhouse experiments is that resource competition and allelopathy cannot be confidently separated; thus, there is a need for laboratory screening methods (Olofsdotter et al., 2002; Jensen et al., 2001). Although simple and quick, questions arise concerning how lab results alone compare in field settings as environmental factors must be considered (Wu et al., 2001). To overcome these drawbacks, of eliminating competition and mimicking field conditions, allelopathy screenings using plant seedings in a stair-step system is applicable (Quasem & Hill, 1989; Liu & Lovett, 1993).

The method of screening in this study creates a stair-step system modified by Bel and Kope where the nutrient solution from the top reservoir flows by gravity to an incubation tray through a few lines of pots with replications in each step to a collector tank from which it is automatically recycled by an electric pump (Liu & Lovett, 1993). This screening method is efficient in time, space, and recourses while providing confidant results. It creates similar field

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conditions for the plant and eliminates any fear of competition. The objective of this study, therefore, is to confirm that the allelopathic potential previously observed in weedy rice accessions against barnyardgrass (*Echinochloa crus-galli*) are in fact allelopathic using a stair-step apparatus.

Materials and methods

Planting and settings

The weedy rice allelopathy screening was conducted in 2018 -2019 in a greenhouse located at the RR Foil Plant Science Research Center, Mississippi State University (-88.7944°, 33.4552°). Weedy rice (*Oryza sativa)* represents the donor weed species, while barnyardgrass (*Echinochloa crus-galli)* is the recipient weed species. The weedy *O. sativa* donor accessions consisted of B2, S33, B84, S97, S94, B81, B8, B34, B14, B84, and five cultivated accessions (Rex PI312777, PI338046, CL163, and Rondo) were included for comparison (Table 2.1).

All weedy rice was selected form a collection of accessions gathered from across Arkansas (Tseng et al., 2013). Five suppressive (>40% barnyardgrass reduction) accessions, B2, B84, B81, B83, B14, and five non-suppressive (<30% barnyardgrass reduction) accessions, S33, B8, B34, S94, S97 were selected based on their performance in previous screenings against barnyardgrass using a different method and a larger sample size of 54 accessions (Shrestha, et al 2020). Allelopathic rice (PI338046, PI312777, Rondo) and non-allelopathic rice lines (Rex, CL163) served as comparisons to weedy rice.

The temperature was maintained at 28°C during the day and 24°C at night, respectively, with a 16/8 hour day/night cycle. The humidity in the greenhouse was maintained at 53%. This experiment was replicated three times consecutively and run twice, making a total of 6 replications. The growth medium used in this screening was sand (silicon dioxide). All pots,

15.24 cm (6 in) diameter, 10.795 cm (4.25 in) deep (Pöppelmann TEKU ® GmbH &Co. KG), were lined with two coffee filters (Great Value Basket ,Walmart ®) to prevent the medium from falling out and filled with sand. The donor pots contained six plants each, while the receiver pots held three plants. All seeds were surface sterilized in 70% ethanol for 30 seconds, followed by 5% bleach soak for 10 minutes and rinsed thoroughly. Seeds were pregerminated in petri dishes lined with filter paper placed in a growth chamber until seedling emergence, after which they were transplanted to pots. Donor accessions were three weeks old at the start of the experiment which is when plants were placed in the system. The day the receiver plant emerged and was transplanted corresponds to day 1.

Stand construction

An 2.4 m x 0.9 m x 0.9 m wooden bleacher stand with six steps was constructed to hold plant samples. The structure was designed to fit the number of samples needed for the study at hand. The middle four steps contain plastic dishes (15.74 cm circumference, 4.31 cm deep, 29.5 mL liquid hold) with PVC tubes (2.54 cm long, 0.35 cm inner diameter, 0.64 cm outer diameter) coming out of the bottom edge of each dish. The top-most step contained plastic bottles (1000 mL, spray-painted black) with PVC tubes coming out of the bottom edge of each bottle. All tube connections were lined with a waterproof sealant to prevent water leakage. The bottom-most step consists of plastic canisters (2270 mL, spray-painted black) containing a submersible pump (21 W 1000 L hr^{-1}) connected to a PVC tube (1.07 m long, 1.27 cm inner diameter, 1.59 cm outer diameter) that outlets into the 1000 mL bottle in the top-most step. Each canister in the bottom-most step has a small hole on the upper back that connects to the dish above via PVC tubes. All pumps operated on a digital timer and set to circulate solution for 1 minute every three hours per day.

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Screening Samples

For this screening, each replication consisted of one column (four pots) of the receiver barnyardgrass as a control, and 15 treatments or donor accessions with their corresponding control of a single column without a receiver species. The columns consist of 4 pots placed down the steps of the system. Ten weedy rice accessions as donors with corresponding controls adding up to 20 columns, five cultivated rice samples as donors with their corresponding controls adding up to 10 columns, and one barnyardgrass control totaling 31 columns were included in this screening as one replication (Figure 2.1).

A solution of half strength Hoagland's No. 2 basal salts (Caisson Laboratories, INC. Smithfield, UT) was set to flow for one minute every three hours throughout the 21 days of the screening. A baseline of 1500 mL half strength Hoagland's solution was added to the bottom tank on day 1, and additional solution was added to the tanks every two days, respectively, thus keeping the tank full of solution. All plant heights were measured in cm on 1, 7, 14, 21 dy after the initiation of the system, or here onwards referred to as days after treatment (DAT). Chlorophyll measurements of all plants were recorded at 7 and 14 DAT. On 21 DAT, all plants were cut at the base and dried for one week in an oven at 60 °C, after which the dry weight was recorded in grams. Comparisons among weedy rice/rice accessions were based on percent inhibition data.

Height reduction percentage of recipient plant and donor plant samples was calculated as:

$$\text{Height reduction (\%)} = [\text{height of Control cm} - \text{height of treated (cm)} / \text{height of Control (cm)}] \times 100. \qquad (2.1)$$

Whereas, the height of the control is the mean height of all the plants in the four control pots combined, the height of the treated is the mean heights of all the receiver plants in the two

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pots in the treatment column, and the height reduction percentage is the mean height reduction of the receiver plant species per accession or treatment. The highest and lowest ranking accession were determined by calculating the mean inhibition percentage of each accession on barnyardgrass based on height, chlorophyll, and biomass reduction, all calculated using the equation above. The height reduction of weedy rice and rice was calculated to identify any adverse effect the barnyardgrass may have on the receiver plants.

Statistical Analysis

Accessions were considered as the fixed effect, while replications and runs were considered as random effects. The experimental design was a randomized complete block with three replications. Data were analyzed using a general linear model with mean values separated using Fisher's Protected Least Significant Difference at or below a 0.05 probability level in JMP 14 (JMP®, Version 13. SAS Institute Inc., Cary, NC, 1989-2007). Principal Component Analysis and hierarchal clustering were used to visualize correlation among the original variables and between the variables and components in JMP. The hierarchal clustering method was used to group accessions into different clusters based on the overall inhibition of *E. crus-galli*.

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Table 2.1   Plant accessions screened listed by name with description of biotype

Accession name	Description
B2	Black Hull
B84	Black Hull
B81	Black Hull
B8	Black Hull
B34	Black Hull
B83	Black Hull
B14	Black Hull
S97	Straw Hull
S94	Straw Hull
S33	Straw Hull
PI312777	Cultivated Line
PI338046	Cultivated Line
Rondo	Cultivated Line
Rex	Cultivated Line
CL163	Cultivated Line
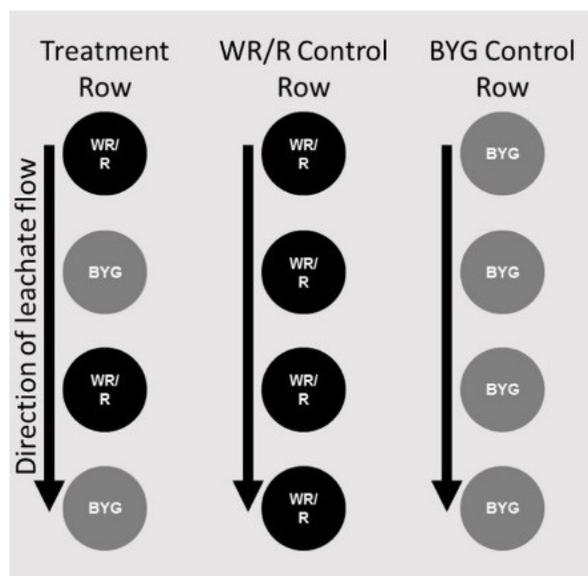
Figure 2.1   Example layout of one treatment in the stair-step system

The label WR/R represents a donor rice or weedy rice accession. The first column (far left) contains the donor alternating with the receiver plant labeled (BYG). Each accession is coupled with its corresponding control in the second column of only WR/R. Finally, the third column consists of only barnyardgrass, the receiver species, and serves as a control for the entire replication.

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Results and Discussion

The allelopathic potential of *O. sativa* accessions was calculated based on mean height reduction, dry biomass reduction, and chlorophyll reduction of the receiver weed, barnyardgrass. The stair-step system was conducted for 21 days. Biomass data from 21 DAT was kept in order to calculate biomass reduction percentage. The height reduction percentage, although not statistically different, was highest 14 DAT. Additionally plants began to outgrow the system by 21 days; therefore, height and chlorophyll data at 14 DAT was used to calculated reduction percentage (Figure 2.2).

Allelopathic potential of weedy rice based on height reduction

Differences were found among accessions in their allelopathic ability ($p < 0.05$) based on the barnyardgrass height reduction percentage. The height reduction percentages ranged from 10-57% among weedy rice accessions. The heights of treated barnyardgrass ranged from 10-19 cm where the mean control height was 20 cm. Weedy rice accession B81 reduced barnyardgrass height the most out of all samples in the screening with 57% mean reduction similarly to accessions S94 (50%), B2 (48%) and allelopathic rice PI312777 (42%). Accession B83 had the least reduction percentage with only 10% following cultivar PI338046 with 12% (Table 2.2).

Allelopathic potential of weedy rice based on biomass and chlorophyll reduction

The biomass reduction caused by weedy rice accessions ranged from 8 – 51%. Weedy rice B83 reduced barnyardgrass biomass by 51%, which was the most of all weedy rice in this study (Table 2.2). The chlorophyll reduction due to weedy rice ranged from 3- 34%, while among cultivated rice, it ranged from 1-18%. Weedy rice, B84, reduced barnyardgrass chlorophyll by 34%, which was the highest reduction among all accessions in this screening.

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Rice cultivar, Rondo, had the least mean chlorophyll reduction among all accessions (1%), while weedy rice, B14, caused the lowest chlorophyll reduction among the weedy rice accessions (3%) (Table 2.2).

Adverse effects caused by barnyardgrass

The reduction percentage of *O. sativa* accessions was calculated based on the mean height and chlorophyll to check for any adverse effect the receiver weed may have had on the donor. The mean height reduction percentage of weedy rice accessions from the receiver weed ranged from 0-12 %, and for cultivated rice, it ranged from 0-10%. The weedy rice accession B2 was most affected by barnyardgrass based on height reduction (12%). Weedy rice B84 and B34, and cultivated rice PI338046, PI312777, and Rondo did not show any height reduction in the presence of barnyardgrass. The mean chlorophyll reduction of weedy rice accessions ranged from 2-20%, while for cultivated rice, it ranged from 1-13%. Weedy rice B43 was most affected by barnyardgrass in terms of chlorophyll reduction (20%), while cultivated rice PI338046 was least effected by barnyardgrass based on chlorophyll reduction (1%) (Table 2.3).

Principal Component Analysis and dendrogram

Principal Component Analysis (PCA) based on height reduction, biomass reduction, and chlorophyll reduction of barnyardgrass revealed that 66% of the variation in allelopathic potential was related to component 1 and 22% was related to component 2. All the parameters used (height reduction, biomass reduction, and chlorophyll reduction) were found to be positively correlated with component 1. Weedy rice accessions B81, B2, B8, S94, clustered together in the PCA indicating high allopathic potential in these weedy rice biotypes in this cluster (Figure 2.3).

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A dendrogram was created based on three parameters, height reduction, chlorophyll reduction, and biomass reduction of barnyardgrass. Two primary clusters were formed: the first consisted of accessions showing moderate to low reduction including weedy rice B14, B34, S97, and B83 as well as rice cultivars Rondo, PI388046 and CL163; the second group of highly suppressive and moderately suppressive weedy rice (B2, B8, B81, S94, S33, and B84) as well as cultivated rice Rex and PI312777 (Figure 2.4).

A dendrogram with weedy rice alone created three groups. Those with high allelopathic potential based on the three parameters were B2, B81, and S94.The accessions with low allelopathic potential grouped together and included B34, B83, B14, S33, B8 and S97. Accession B84 clustered alone with high reduction percentage based on biomass and chlorophyll reduction but low height reduction of barnyardgrass. (Figure 2.5).

Utilizing the allelopathic ability from weedy rice may increase the competitive ability of rice varieties that are being outcompeted by troublesome weeds such as barnyardgrass. This tool can supplement current weed control efforts, both chemical and manual, and may help overcome yield losses due to weeds. Screening weedy rice accessions to identify the best candidates for further gene studies is an essential step in furthering breeding efforts. This screening identifies weedy rice accession that can be the subjects of further investigation to understand allelopathic production and genetic controls. Allelopathy is inherent in rice because of adaption and is spread through the germplasm (Jensen et al., 2001). Weedy rice B2 was the most allelopathic weedy rice accession of the samples screened. This weedy rice accession has shown to have highly competitive ability and suppression of barnyardgrass in previous screenings (Shrestha et al., 2020). Results from this study suggest this accession's success in the field and ability to

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overcome competitive weeds like barnyardgrass is due to allelopathic activity which until now, these interactions could have been contributed to the plants vigor.

Variation in the allelopathic potential of the weedy rice accessions to suppress barnyardgrass seedlings was found based on three parameters. Height was the primary parameter measured as weedy rice often is taller than the crop, produces more reproductive tillers, and grows much faster than the crop smothering it (Dai et al., 2016). PI312777 reduced barnyardgrass height the greatest in this screening, being an allelopathic rice cultivar. This cultivar has exhibited high suppression of a wide variety of weed species in field screenings (Okuno et al., 2003). All weedy rice accessions exhibited some degree of barnyardgrass suppression. Weedy rice is a product of outcrossing with cultivated rice. Its weed suppressive activity is associated with origin and cultivar (Chung et al. 2003). The high genetic diversity in weedy rice could account for the range in the allelopathic potential against barnyardgrass. Additionally, accessions that are not considered highly suppressive have some allelopathic activity dependent on accession and weed species (Jensen et al., 2001). All accessions in this screening caused less than 50% mean reduction of barnyardgrass growth. This study eliminated recourse competition leaving any residue from barnyardgrass roots to be the primary means of simulating allelopathic production from the rice accessions. The lack of competition for space and nutrients may have decreased the overall inhibition potential that would have been observed in natural field settings where plant to plant interaction is present. Both of these components, competition, and allelopathic ability contribute to the suppression of weeds (Worthington et al., 2013). The characterization of allelopathy by different parameters may provide an insight into the genetic capabilities of the distinct accessions. The weedy rice accessions found to be the most

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suppressive in this study were primarily blackhull type, which had been found to be more allelopathic than strawhull types (Shrestha et al., 2020).

Variability in allelopathic potential within rice cultivars was also observed. The allelopathic rice PI312777 was highly suppressive in this screening as expected (Worthington 2013; Gealy et al., 20012). However, Rex, a non-allelopathic southern US variety, performed well in this study against barnyardgrass. For this screening, Rondo was set as the allelopathic rice standard as it is a rice cultivar with US grain quality standards suggested for its weed suppressive ability (Worthington et al., 2013). Rondo and PI338046, two other known moderately allelopathic cultivars, did not perform well in this study and clustered away from PI312777 and Rex on the basis of their allelopathic potential. Rex and CL163 are two southern rice cultivars that had similar height reduction percentages on barnyardgrass, while PI312777 is a progeny of TN1 and Taichung 65 cultivars, and PI338046 is a Philippine cultivar (Ebana et al., 2001). This genetic background of the cultivars may be a reason for the observed variability in allelopathic potential. Literature has also found differences in the expression of biosynthetic pathways between allelopathic and non-allelopathic rice cultivars, particularly PI312777 an allelopathic variety which performed the best in this screening (Zhang et al., 2019). Domesticated rice possesses traits that contribute to their adaptability to the environment. Domestication causes the rice genome to prioritize reproduction, not survival, and therefore a decrease in fitness of the population (Sun et al., 2012). It is possible that the performance of the cultivated rice under greenhouse conditions and unnatural soil media for rice in this experiment contributed to the allelopathic performance of the domestic rice screened in this study.

Barnyardgrass has been studied extensively for its allelopathic activity. Due to the known allelopathic activity of barnyardgrass the inhibition of the weedy rice and rice in this study was

calculated to check for susceptibility of the donor plants. Mature barnyardgrass plants also have germination effects on rice via root released secondary metabolites (Khanh et al., 2018). Barnyardgrass in this screening was tested at the seedling stage to minimize potential adverse impact on the donor plants. The plant density in the receiver pots was also low to maximize effects from the donor and to decrease any concentration of allelochemicals the barnyardgrass may have produced. Weedy rice like cultivated rice is affected by barnyardgrass root exudates. The donor plants exhibited no more than 12% susceptibility or reduction due to barnyardgrass. The best performing allelopathic weedy rice's (B2, S94, B81) in this screening were reduced 8% or less when exposed to compounds from barnyardgrass. Both donor and receiver species exhibited some degree of effects from allelochemicals in this system. Some studies suggested that the presence of barnyardgrass induces higher amounts of chemical release from rice similarly to a stress response (Zhang et al., 2019; Khanh et al., 2018). For this reason, the weedy rice and rice in this study had greater effect on barnyardgrass than vice versa but this could differ between growing stages. Overall, barnyardgrass at the seedling stage had little effect on the weedy rice and rice cultivars and yet was still susceptible to rice residue further suggesting allelopathy as a weed control method.

Differences in the characteristics and concentrations of allelochemicals produced by the two plant species may present results similar to nutrient stress (Quasem et at., 1998). The use of nutrient additives is essential to ensure adequate conditions. Species differ in their responses to different minerals and weed species may respond better than a crop to the nutrients provided (Schuamker et al., 2020; Quasem et at., 1998). When inhibitory effects are observed even in the presence of a nutrient solution, it confirms the production or presence of allelopathic chemical(s), as was observed in this screening (Quasem et at., 1998).

Dendrogram results showed two outlying weedy rice accessions: B84, which grouped with suppressive rice in the full dendrogram. When weedy rice was analyzed alone, this accession fell in a single cluster. Accession B84 caused moderate height reduction, high biomass reduction, and high chlorophyll reduction of barnyardgrass. On the other hand, accession B84 caused little height and biomass reduction but high chlorophyll reduction of barnyardgrass. These two accessions were variable in how they suppressed barnyardgrass, suggesting different allelopathic mechanisms. All accessions varied between each parameter measured. Secondary metabolites have different effects on plant processes depending on the compound present. Some compounds effect plant height and some can target photosynthesis. The variation between reduction within an accession between parameters suggests multiple compounds produced as well as differences in production of compounds between accessions (Zhang et al., 2019; Yang et al., 2002) Weedy rice B2, B81 and S94 were highly suppressive when factoring in all three parameters and were grouped together in both dendrograms.

Conclusion

The weedy rice accessions found in this study serve as subjects for studying allelopathic traits as well as the mechanisms related to the phenomena. Allelopathic cultivars PI312777, Rondo, and PI338046 should also be considered for further studies as they are known inhibitory rice in field conditions. Accessions B2, B84, S94 and B81 exhibited the highest suppression of barnyardgrass based on the three parameters measured confirming their strong allelopathic activity against barnyardgrass. Investigations into the physiology of the responses observed and the mechanisms associated with allelopathy will provide sustainable strategies to manage weeds in rice production.

Table 2.2   Suppression of barnyardgrass, based on height, chlorophyll, and biomass by weedy rice and rice accessions

Accession	Heights		Chlorophyll		Biomass		Mean Allelopathic Potential (%)	
	value (cm)	reduction (%)	value (cci)	reduction (%)	value (g)	reduction (%)	BYG reduction (%) stair-step screening	BYG reduction (%) Pot screening
Control	20 a	0	387 a	0	0.29 a	0	0	0
B83	19 a	10 g	362 ab	4 b	0.22 ab	51 a	12.6	43.7
PI338046	18 ab	12 g	342 a-c	6 b	0.24 ab	8 b	8.6	56.6
B34	18 ab	20 e-f	358 ab	4 b	0.18 a-c	36 ab	20.0	27.3
Rondo	17 a-c	25 d-g	382 ab	1 b	0.23 ab	28 ab	18.0	51.2
S33	16 a-d	26 d-g	308 b-e	12 ab	0.11 bc	40 a	26.0	18.3
S97	16 a-d	26 d-g	343 a-c	5 b	0.12 bc	43 a	24.7	27.3
B84	16 a-e	27 c-g	208 e	34 a	0.13 bc	49 a	36.7	42.5
B14	14 b-e	34 b-f	369 ab	3 b	0.1 bc	37 ab	24.7	40.4
B8	13 c-e	35 b-f	306 b-e	13 ab	0.1 bc	42 a	30.0	28.1
CL163	13 de	36 b-e	315 b-d	9 b	0.18 a-c	30 ab	25.0	18.6
Rex	12 de	38 b-e	237 de	18 ab	0.06 c	44 a	33.3	21.6
PI312777	11 ef	42 a-d	251 de	17 ab	0.04 c	39 ab	32.7	54.0
B2	10 e-g	48 a-c	263 c-e	15 ab	0.08 bc	34 ab	32.3	61.3
S94	10 e-g	50 a-b	224 de	19 ab	0.2 a-c	20 ab	29.7	22.6
B81	8 fg	57 a	224 de	19 ab	0.1 bc	31 ab	35.7	54.2

Mean separated with Fisher's protected LSD ($\alpha = 0.05$), means with different letters denote a significant difference

Table 2.3   Mean reduction percentage of donor rice and weedy rice based on height, and chlorophyll by the receiver weed barnyardgrass at 14 DAT

Accession	Mean Height Reduction (%)	Mean Chlorophyll Reduction (%)	Mean Susceptibility (%)
B2	12 a	6 b-d	8.0
B8	1d	11 a-c	6.0
B14	0.4 d	9 b-d	5.0
B34	0.0 d	5 b-d	3.0
B81	4 a-d	10 a-d	7.0
B83	2 cd	11 a-c	7.0
B84	0.0 d	2 b-d	1.0
CL163	4 b-d	7 b-d	5.0
PI338046	0.0 d	1 d	0.3
PI312777	0.0 d	2 cd	1.0
Rex	10 ab	13 a-c	11.0
Rondo	0.0 d	11 a-d	5.0
S33	10 a-c	6 b-d	8.0
S94	3 b-d	20 a	12.0
S97	1d	14 ab	7.0

Mean separated with Fisher's protected LSD ($\alpha = 0.05$), means with different letters denote a significant difference.
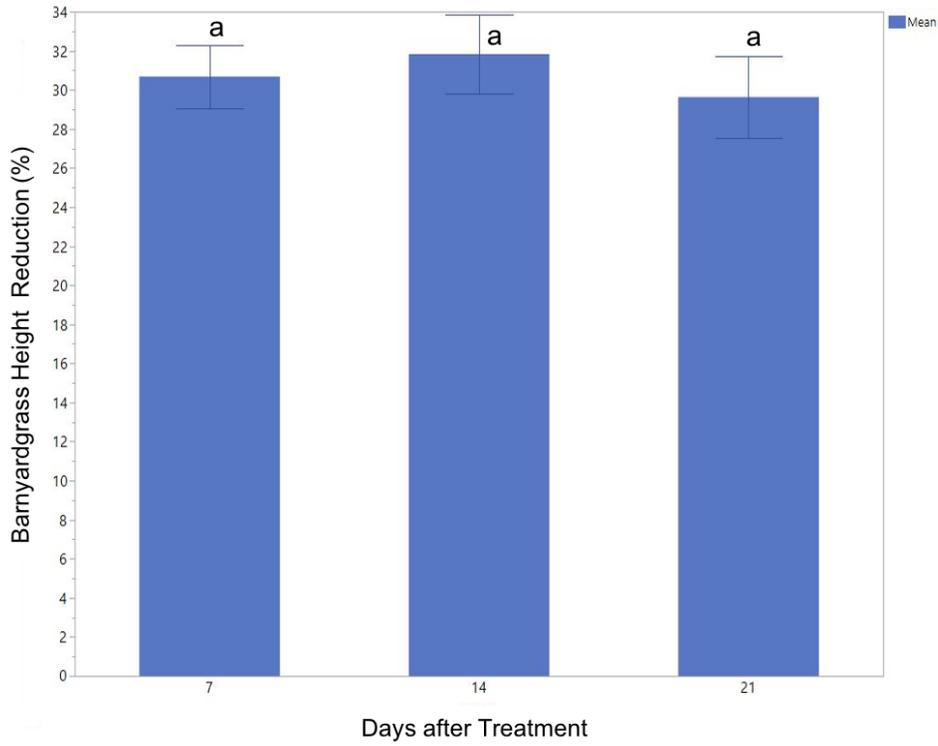
Figure 2.2   Height reduction of barnyardgrass 7, 14 and 21 days after treatment in the stair-step system

Means separated with Fisher's protected LSD ($\alpha = 0.05$), means with different letters denote a significant difference.
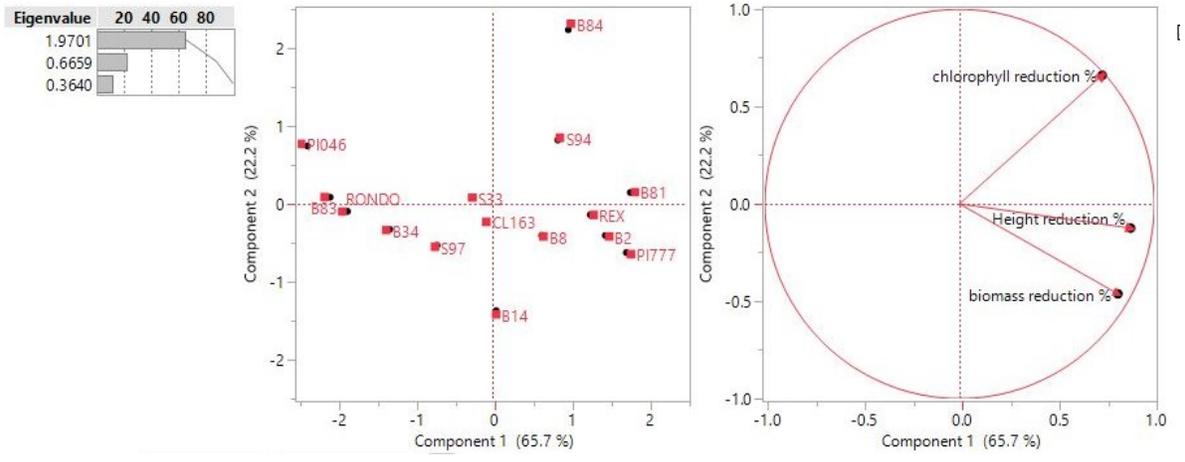
Figure 2.3   Principal Component Analysis with three components, height, chlorophyll, and biomass inhibition of barnyardgrass

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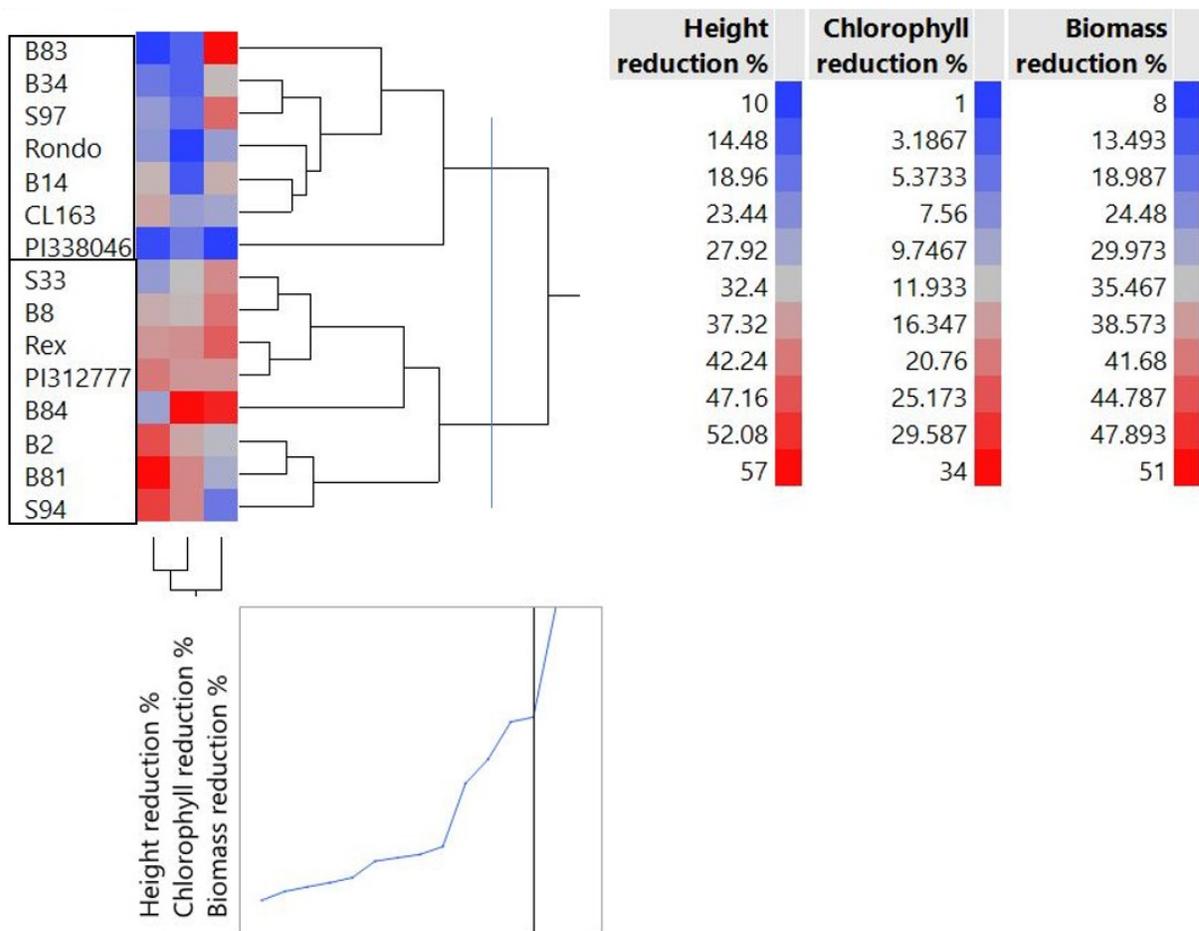
Figure 2.4   Dendrogram of Allelopathic potential of weedy rice and rice accessions to suppress barnyardgrass screened in the stair-step system.

Clustering of all accessions based on three parameters: height, chlorophyll, and biomass reduction of barnyardgrass at 14 DAT. Blue indicates lower reduction percentage while red indicate higher reduction percentage. Accessions grouped based on overall allelopathic potential. Group one represented by the top box with the least mean allelopathic potential, followed by group two with the most allelopathic potential.
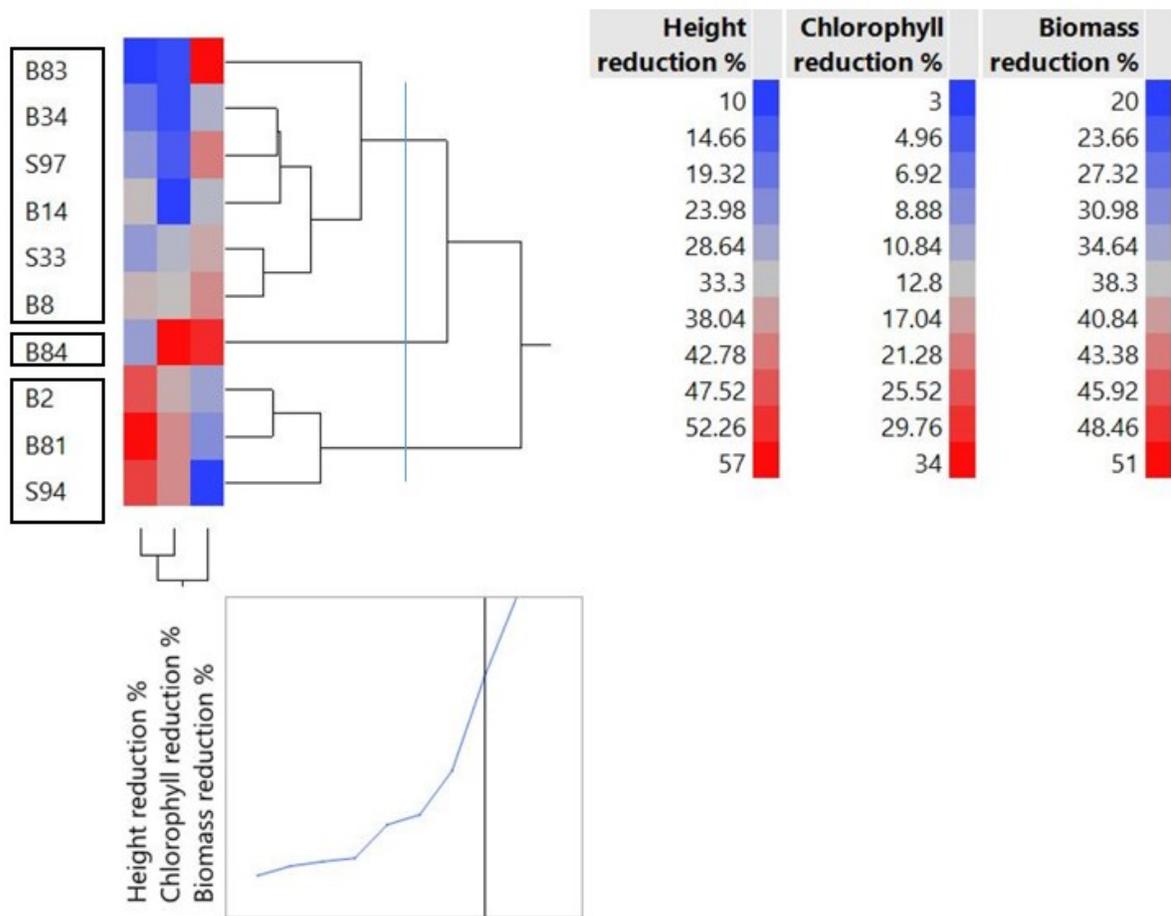
Figure 2.5   Dendrogram of allelopathic potential to suppress barnyardgrass of weedy rice
accessions alone screened in the stair-step system.

Clustering of all accessions based on three parameters: height, chlorophyll, and biomass
reduction of barnyardgrass at 14 DAT. Blue indicates lower reduction percentage while red
indicate higher reduction percentage. Accessions grouped based on overall allelopathic potential.
Group one represented by the top box with the least mean allelopathic potential, followed by
group two with moderate allelopathic potential, and group three with the highest mean
allelopathic potential to suppress barnyardgrass.

CHAPTER III

ROOT SYSTEM ARCHITECTURAL TRAITS ASSOCIATED WITH ALLELOPATHY IN WEEDY RICE

Abstract

Weedy rice is of the species *Oryza* and is a wild relative of cultivated rice. These weedy species possess unique hardiness that allows it to thrive in dynamic and stressful environments. Traits such as the potential to grow taller, produce more tillers, and consume more nutrients than its crop competitor exist in weedy rice. These characteristics suggest that weedy rice is a stored source of novel genes for competitive traits. One such trait is allelopathy, which is a physiological process that occurs when a species releases secondary metabolites that suppress the growth and development of neighboring species. Weed competition is a limiting factor in rice production systems; therefore, it is critical to identify specific allelopathic weedy rice accessions to determine the genetic pathways and mechanisms associated with allelopathy to be used in breeding programs. Due to the complex nature of the production of allelopathic chemicals and the lack of knowledge of the mechanisms surrounding allelopathy in weedy rice, phenotypic traits, particularly root traits, can be used to overcome this limitation and serve as target characteristics for breeding weed suppressive rice varieties. Five weedy rice accessions chosen from preliminary screenings of larger sample sizes with the ability to significantly suppress barnyardgrass weed seedling growth, and another five weedy rice with low barnyardgrass suppression were chosen for the current phenotypic root study. Five cultivated rice lines were

used as a comparison. These accessions were propagated in a transparent growth pouch for four weeks. Roots were scanned and analyzed for root length and area covered. No differences were found in the seedling root area among weedy rice and rice accessions; however, allelopathic weedy rice exhibited longer roots than non-allelopathic weedy rice and were similar in length to the cultivated rice in this study. Root length is, therefore, a potential target trait for weed suppressive varieties, and allelopathic accessions identified in this study can be used for further genetic analysis to determine gene(s) associated with allelopathy.

Introduction

Plant root structures are notoriously difficult to study. Root system architecture (RSA) is defined as the spatial organization of roots and is crucial for normal root functioning. The RSA of a plant is affected by external factors such as water availability or nutrient stress. Roots have an innate ability to respond and adapt to external environmental factors, which determines the plant's RSA and gives insight into the soil environment (Lynch, 1995). Phenotyping allelopathic root structures may provide insight into the plant's ability to thrive and adapt to environmental stresses (Gealy et al., 2013). Allelopathic interactions involve plants detecting neighbors and adjusting the production and excretion of chemicals as a defensive reaction. Detection of competitor plants is presumed to be due to triggering compounds being released into the soil (Li et al., 2019; Kong et al., 2018). For instance, barnyardgrass releases allelochemicals into the rhizosphere that induce increased production of rice allelopathy (Li et al., 2019). Even when plants are segregated belowground and have no physical root contact detection of chemicals still occurs (Kong et al., 2018). Differences in defense pathways and allelochemical regulation between compounds have been found. Still, the mechanisms and genetic origins of allelopathic signaling and reactions need more attention. (Li et al., 2019; Kong et al., 2018) The genetic

factors contributing to allelopathic root activity are still in the early stages of exploration; therefore, phenotyping provides information needed for breeders to select traits associated with the high allelopathic activity (Sanduh et al., 2016). Combined manual measurements, imaging, and computer programs to measure small root parts all contribute to retrieving this hidden information. Allelopathic rice lines exhibit shallow soil depth for the majority of their root mass (Gealy et al., 2013) Soil depth preference may be one characteristic of root structure architecture that contributes to weed suppression (Gealy et al., 2013). Information on root characteristics associated with wild rice types and the plant's vigorousness is limited. It has recently been noted that there are characteristic differences between wild rice and cultivated rice. Weedy rice root systems express deeper, thinner, straighter, and less spread out roots than cultivated rice which are more abundant as found by Wedger et al. (2019). Research efforts to study root structures and how individual aspects relate to the plant prosperity are slow, especially in allelopathic phenotypes, because root systems are difficult to observe without damaging the growing plant (Lynch, 1995).

Phenotyping Methods

Methods for studying root structure architecture are limited. Many of the methods are destructive to the roots systems making data difficult to obtain accurately (Heeraman et al., 1997). Even the smallest root parts as they play a significant role in the plant's productivity. The root hairs, in some cases, compose nearly 77% of the root area comprising most of the coverage of the rhizosphere where the bulk of the plants' nutrients is condensed (Bertin et al., 2003). In addition to viewing difficulties, root architecture is manipulated by the environment and growing conditions. Root architecture data collected from growing plants in controlled environments is not always relatable to field conditions (Dorlodot et al., 2007). Roots growing in an artificial

medium should be trialed in a field setting similar to testing for allelopathy to compare results in different conditions (Nestler et al., 2016). Understanding the complexities of root architecture requires a combination of laboratory, field, and greenhouse methodologies (Kochian and Holley, 2011).

Laboratory phenotyping methods require propagation of the sample plant in non-soil media like gels or on paper given nutrient washes. After growth, root formation features and characteristics are determined by manual measurements or with the aid of 2-dimensional imaging (Walter et al., 2015; Zhu et al., 2011). Soil extractions, imaging systems, or combinations of the two are standard field-root phenotyping techniques. The trench profile method, core break method, and shovelomics all require soil cores to be cut and the soil removed from the roots to be measured (Walter et al., 2016). Soil core samples support root distribution approximation measures (Gealy et al., 2013). X-Ray Computed Tomography (X-RayCT) images roots established in soil and is a nondestructive means of obtaining 3D root system images (Heeraman et al., 1997). Researchers at the University of California Davis performed X-RayCT and noted that the method was not well-developed (Heeraman et al., 1997). Other reviews have highlighted the lack of standard procedures to separate soil and roots in the modeling process (Metzner et al., 2015). Limitations with X-RayCT include the complicated equipment required for the survey and the extensive job of distinguishing roots against soils.

Consequently, the images obtained were not exceptionally clear but did give a broad depiction of the area covered. For example, small roots were not distinguishable from the soil matrix (Heeraman et al., 1997). Similarly, shovelomics will generatea general structure of the root area but the results lack detailed information on individual root parts (Walter et al., 2015). Mathematical models employing fractural geometry creates a reasonable estimate of root

architecture measurements and aid in viewing root systems (Lynch, 1995). Measurements from 2-dimensional, planar models, or images obtained from soil cores may serve as a base for 3D model construction. Although mathematical models can provide insight into root formation patterns, a failure results in accounting for natural recourses that affect a plant's root structure significantly (Lynch, 1995). Field observations of root systems are time-consuming and tedious but do garner useful general information (Walter et al., 2015). There is a vital need for an accurate visual root architecture method.

Three-dimensional imaging software is advancing the prospect of using rice as an experimental subject for studying root structure as well as MRI scanning techniques (Kochian et al., 2011). The complete unique development of RSA can be obtained through 3D imaging techniques; additionally, this digital phenotypic data can be analyzed repeatedly for various traits (Topp et al., 2013; Clark et al., 2011). The limitations with these methods include the equipment needed, finding compatible soil media for these methods, and image processing time and software ability to distinguish soil from root (Pflugfelder et al., 2017; Flavel et al., 2012).

Efforts to create bioassays for herbicide dose responses or observe root growth patterns gave ways to the used of germination pouches. This method is rapid, cost efficient, reproducible, accurate, non-invasive, and easily controlled (Zhang et al., 2015). Germination pouches allow or permit? visualization of seedling growth patterns and have been utilized for a variety of crops and purposes (You et al., 2018). The use of these transparent bags makes it possible to observe and image roots at any growth stage promptly and practically. Screening in a lab setting allows for a uniform and controlled environment as well as sterile conditions. Images obtained can be analyzed and screened though publicly available user-friendly software programs. This phenotyping strategy has the ability to increase noninvasive screenings by saving time and space

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in the seedling stage of development that can lead to the exploration of traits in the accessions screened (Adeleke et al., 2019).

Materials and Methods

Plant sample preparation

From Shrestha et al., 2020, the five most barnyardgrass suppressive (B81, S94, B2, B8, B14) and four least barnyardgrass suppressive (B34, B83, S97, 84) weedy rice accession were selected. Cultivated rice lines Rex, Rondo PI312777, CL163, and PI388046 were also included in this study for comparison. All seeds were surface sterilized by soaking in 70% ethanol for 30 seconds and 5% bleach for 20 minutes and rinsed six times with distilled water. Sterilized seeds were germinated in plastic Petri dishes lined with filter paper and soaked with 5 ml distilled water. Eight seeds from each accession were germinated for sprout selection. After germination, four seedlings of equal height were selected for each accession and placed using forceps into germination pouches (Mega international, Newport MN). Pouches holding the seedlings were placed in the growth chamber in the Weed Physiology Laboratory at Mississippi State University set at 30/21°C day/night for four weeks. Plants were watered with 20 ml half-strength Hoagland's No. 2 basal salts (Caisson Laboratories, INC. Smithfield, UT) every two days to keep the roots damp for four Weeks after germination.

Plant imaging

The bags were cut open, and the roots were removed and placed directly onto an Epson Perfection V370 Photo scanner (Epson America Inc, Long Beach CA). Black paper was placed over the root to provide a contrast for the image. Four images were obtained for each accession serving as individual replications. Root images were measured using Image J® software (Image

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J, University of Wisconsin-Madison, WI), where each image was cropped to encompass the area each root covered and converted into an 8-bit grayscale format. The length of each root was recorded in cm, and in order to obtain the total area covered by each root, the images were first converted to a binary format to remove the background and create a contrast.

Analysis

Accessions were considered as the fixed effect, while replications were considered as the random effects. The experimental design was a completely randomized design with four replications. Data were analyzed using JMP 13 (JMP®, Version 13. SAS Institute Inc., Cary, NC, 1989-2007) to compare the allelopathic and non-allelopathic weedy rice root length and area.

Results and Discussion

Root length and area

The root lengths and areas of weedy rice and cultivated rice were measured. Weedy rice accessions B2 showed the highest mean root length with 22.4 cm of the weedy rice in this study. Rex showed the longest roots of all accessions with a mean of 24.7 cm. It should be noted that Rex and CL163 had only one replication in this study due to germination delays and were, therefore, excluded from the biotype groupings analysis. Non-allelopathic weedy rice, B83, had the shortest mean root length (7.4 cm) (Table 3.1).

Weedy rice was characterized as allelopathic or non-allelopathic based on data from the screening in objective one. Allelopathic weedy rice consisted of accessions B84, S94, B81, and B2 with moderate to high barnyardgrass suppression, while non-allelopathic accessions were S97, B83, B8, B14 and B34 with low barnyardgrass suppression. No differences were found between allelopathic weedy rice and non-allelopathic weedy rice biotypes in terms of root length.

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Allelopathic weedy rice had a mean root length of 17.5 cm, which was similar to cultivated rice (17 cm). Non-allelopathic weedy rice, on the other hand, had a mean of root length of 15.3 cm. It should be noted that rice cultivars CL163 and Rex, and both non-allelopathic cultivars only had one replications, both with and without including these accessions into the cultivated rice group, there was no change in the statistical significance between biotypes. (Table 3.2). Weedy rice accession B34 (2.8 cm^2) covered the greatest area of all accessions. (Table 3.3). Non-allelopathic cultivar Rex showed the greatest coverage (2.6 cm^2), while the allelopathic cultivated rice Rondo showed the least (0.8 cm^2) root area among the cultivated rice. No differences were observed among allelopathic weedy rice, non-allelopathic weedy rice, and cultivated rice based on the root area. Allelopathic rice had a slightly larger root area (2.2 cm^2) than the other two biotypes, cultivated rice (1.9 cm^2) and non-allelopathic weedy rice (2.3 cm^2) (Table 3.2).

Correlations

Correlation analysis was run on root measurements to determine the relationship between allelopathy and root length and area. A positive correlation found between root length and allelopathic suppression. The Pearson correlation statistic was 0.26 indicating a positive correlation. The Spearman's correlation was 2.4 which was consistent with the Pearson correlation results. (Figure 3.1). A negative correlation found between root area and allelopathy. The spearman's correlation was used due to an abnormal distribution of root area. The correlation was found to be - 0.02. (Figure 3.2).

The allelopathic weedy rice, as well as the allelopathic cultivated lines, had longer roots when plants were 4-weeks old. The non-allelopathic weedy rice, on the other hand, had shorter roots compared to cultivated rice. Still, the roots observed had higher numbers of secondary roots

and branched more than the allelopathic biotype resulting in a similar total root area. Allelopathic weedy rice, both black hull and straw hull accessions, have been noted to have deeper rooting than rice crop 13 days after germination (Wedger et al., 2019). The present study assessed seeds 21 days after germination but also included both hull types of weedy rice and found similar results. Three of the cultivated rice accessions used in this study were weed suppressive, and may have been the reason for the similarity between the allelopathic weedy rice groups and the cultivated rice groups. Highly allelopathic weedy rice accession B2 with notably longer root systems, is a candidate for further study for competitive root traits.

Variation in the allelopathic activity is independent of the variation in root mass, although root mass may contribute to the overall competitive advantage (Courtois et al., 2003). As observed in the present study, the root area was not different between allelopathic and non-allelopathic accessions, even though there were differences in the allelopathic potential to suppress barnyardgrass. Non-suppressive rice showed larger root areas and shorter roots than did suppressive rice.

Allelopathic cultivars fall into different groups based on isozymes belonging to different environments suggesting differing evolutionary origins (Jensen et al., 2001: Lafitte et al., 2001). Generally, weed suppressive cultivars fall in the tropical Japonica groups (Jensen et al., 2001). In contrast, other studies have found that in the field, rice Indica lines have more weed suppressive abilities than Japonica (Gealy et al., 2013; Worthington et al., 2013). The cultivars used in this study were a mix of groups based on origin with the weed suppressive accessions, all belonging to Indica with similarly long slender seedling roots to weedy rice. This group is noted for its thin superficial root systems (Lafitte et al., 2001). Weedy rice PI312777 had the longest roots of the cultivars in this study. The results presented are coherent with previous studies regarding root

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phenotypes and genetic backgrounds (Lafitte et al., 2001; Gealy et al., 2013). The cultivated rice in this study in terms of length was similar to allelopathic weedy rice. Weedy rice characteristics include deep, thin, and straight roots like the Indica lines (Wedger et al., 2019). Rex is a japonica variety noted for its deep, thick, and large root system (Lafitte et al., 2001). The present study assessed two characteristics focusing on seedling length in which Rex performed well but was missing data and could not be confidently compared as an individual accession. Deep and long roots during the early plant stages have been associated with stress adaptability such as in drought-tolerant rice (Courtois et al., 2003). Additionally, RSA models from bean roots have found links to vigorous roots and nutrient uptake efficiency.

Conclusion

Laboratory screenings lack natural soil profiles as found under field conditions, but allow for precise measurements. By controlling the environment, morphological characteristics apart from the environment can be expressed and studied. Weedy rice displayed a variety of different lengths and areas. In terms of length, allelopathic weedy rice showed longer seedling roots than non-allelopathic biotypes. Early seedling vigor and the stage at which the accessions were screened could play a role in how the roots expressed in this study. It is important to note that the environment may affect the root architecture, and the genetic potential may effective only under a favorable environment (Courtois et al., 2003). The highly allelopathic weedy rice accessions screened, and phenotyped are ideal candidates for identifying genetic controls of early root length which is a possible trait contributing to underground allelopathic production and competitive advantage. There may be more root architecture traits that contribute to underground allelopathic interaction, but these findings serve as a single piece of a complex puzzle. Long

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seedling roots, as seen in allelopathic and suppressive Indicia varieties, may provide a target for

enhancing the allelopathic potential in rice and providing a competitive advantage to the crop.

Table 3.1   Mean root length (cm) of weedy rice and cultivated rice accessions in ascending order.

Accession	Mean Root Length (cm)
B83	7.4 f
Rondo	8.5 e-f
B81	9.2 c-f
CL163	9.8 d-f
B8	11.0 b-e
B84	15.9 a-e
B34	16.9 a-d
S97	16.9 a-d
S94	17.7 a-c
PI338046	18.9 a-c
PI312777	19.4 ab
B14	21.7 a
B2	22.4 a
Rex	24.8 a

Mean separated with Fisher's protected LSD ($\alpha = 0.05$), means with different letters denote a significant difference.

Table 3.2   Allelopathic weedy rice, non-allelopathic weedy rice, and cultivated rice mean root lengths (cm) and areas (cm^2) 21 DAT.

Biotype Group	Mean Root Length (cm)	Mean Root Area (cm^2)
Allelopathic Weedy rice	17.5 a	2.2 a
Non-Allelopathic weedy rice	15.3 a	2.3 a
Cultivated rice	17.0 a	1.9 a

Mean separated with Fisher's protected LSD ($\alpha = 0.05$), means with different letters denote a significant difference.

Table 3.3   Mean root area (cm^2) of weedy rice and rice accessions in ascending order.

Accession	Mean Root Area (cm^2)
Rondo	0.8 f
CL163	1.1 ef
B84	1.4 ef
B83	1.5 ef
B8	1.7 de
B81	1.9 c-e
PI312777	2.1 b-e
PI338046	2.2 a-d
B2	2.3 a-d
S94	2.4 a-c
B14	2.6 ab
Rex	2.6 a-d
S97	2.6 ab
B34	2.8 a

Mean separated with Fisher's protected LSD ($\alpha = 0.05$), means with different letters denote a significant difference.

Scatterplot Matrix

Pairwise Correlations

Variable	by Variable	Correlation	Count	Lower 95%	Upper 95%	Signif Prob	-.8-.6-.4-.2 0 .2 .4 .6 .8
reduction	Length	0.2590	14	-0.3148	0.6942	0.3713	

Nonparametric: Spearman's ρ

Variable	by Variable	Spearman ρ	Prob>|ρ|	-.8-.6-.4-.2 0 .2 .4 .6 .8
reduction	Length	0.2412	0.4062	
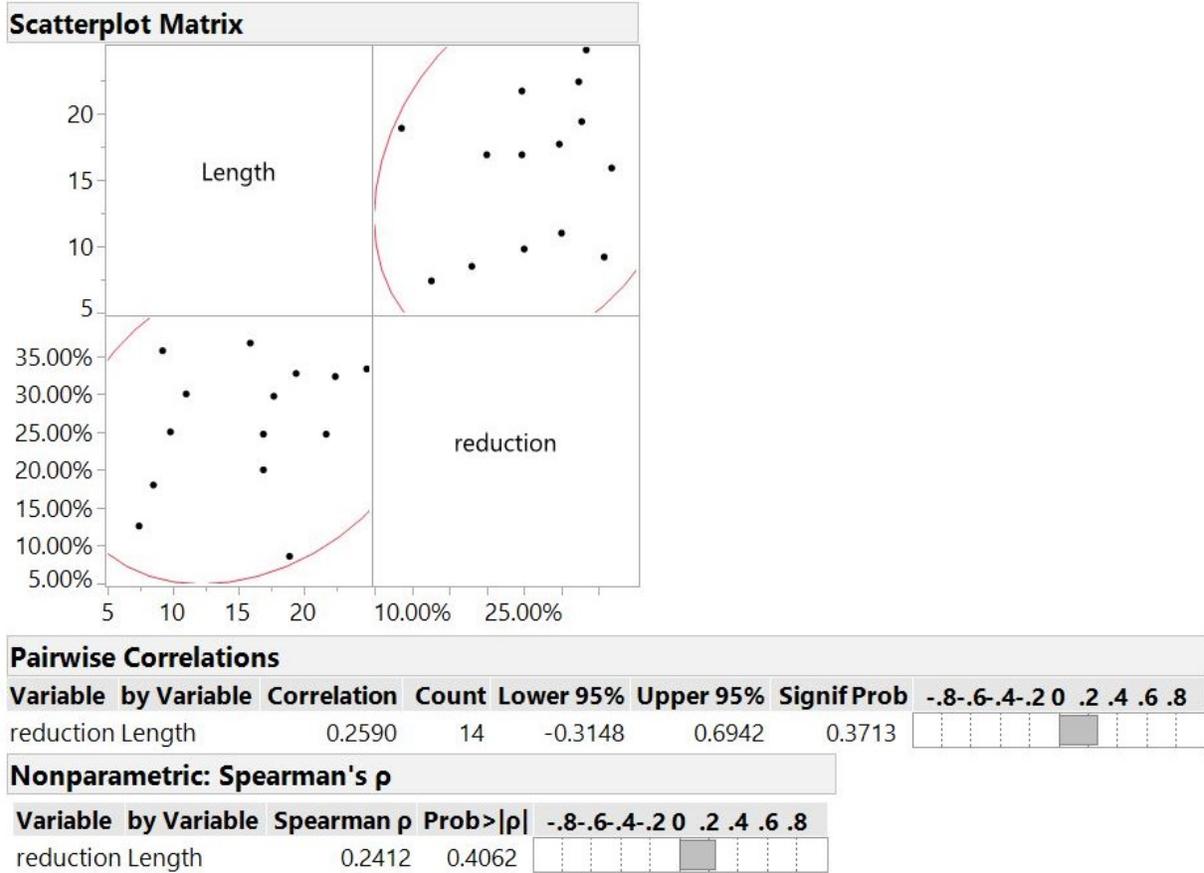
Figure 3.1   Correlation between root length (cm) and allelopathic potential to suppress barnyardgrass of weedy rice and rice.

Scatterplot Matrix

Pairwise Correlations

Variable	by Variable	Correlation	Count	Lower 95%	Upper 95%	Signif Prob	-.8-.6-.4-.2 0 .2 .4 .6 .8
reduction	area	0.1154	14	-0.4423	0.6087	0.6945	

Nonparametric: Spearman's ρ

Variable	by Variable	Spearman ρ	Prob>|ρ|	-.8-.6-.4-.2 0 .2 .4 .6 .8
reduction	area	-0.0243	0.9343	
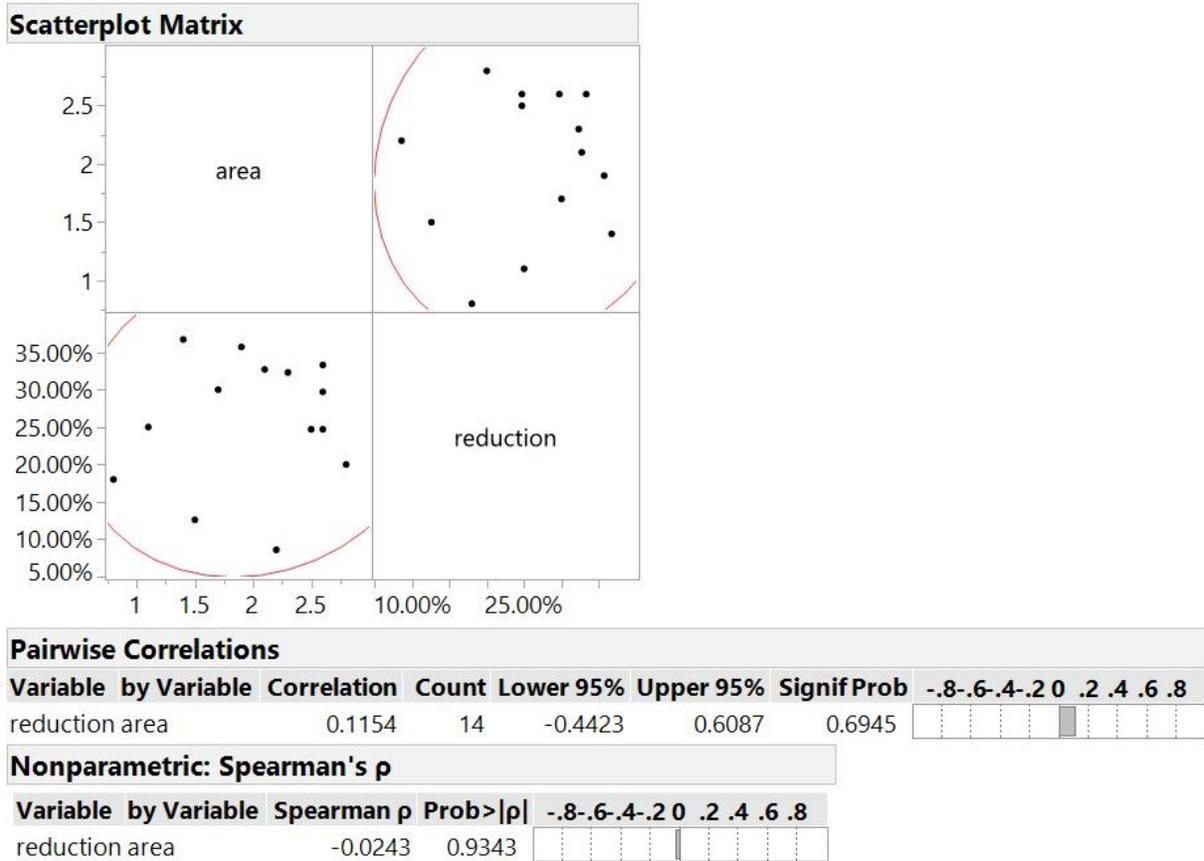
Figure 3.2   Correlation between root area (cm^2) and allelopathic potential to suppress barnyardgrass of weedy rice and rice.

CHAPTER IV

DETERMINING MARKERS ASSOCIATED WITH ROOT SYSTEM ARCHITECTURE IN

ALLELOPATHIC PHENOTYPES OF WEED RICE

Abstract

Rice provides up to 50% of the dietary caloric supply for an estimated 520 million people worldwide. In order to meet the demand of a growing global population increasing productivity of rice production is paramount. The most significant yield limiting factor in rice production is weed competition. The genetic diversity weeds possess allows them to adapt and thrive in various stressful environments. Weedy rice is a wild relative of cultivated rice and one of the most common rice weeds, exhibiting characteristics that suggest a vast germplasm of potential traits for rice crop improvement. One such trait exhibited in weedy rice is allelopathy. Allelopathy is defined as any detrimental effect by one plant on another through the production of chemical compounds that escape into the environment. Incorporation of allelopathic phenotypic traits in root systems into cultivated rice lines may improve growth habits and assist in overcoming yield losses due to weeds. The overall objective of this study is to associate allelopathic root characteristics from weedy rice to the genetic controls. Eleven weedy rice accessions six allelopathic (B14, B2, B8, B81, B84, S94) and five non-allelopathic (B83, B34, S33, S97) and cultivated rice (RONDO, PI312777, PI338047, Rex, CL163) were analyzed using 30 SSR markers. Weedy rice was grouped by biotype and compared to the cultivated rice. Markers used were able to differentiate weedy rice from rice but were weekly associated with

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root length and allopathic ability. Four markers were found to constantly appear in the weedy population (>95%) but were not distinct to allelopathic accessions. Additional markers need to be screened in order to determine the basis of root traits associated with allelopathy in weedy rice.

Introduction

Due to multigenetic factors contributing to the allelopathic chemical process, traditional breeding methods have not selected for allelopathy (Kuijken et al., 2015). The genes in weedy rice typify traits breeder's desire but the genes are difficult to tag. Weedy rice is a species that exhibits allelopathic traits with broad phenotypic variation. Success of weedy rice is attributed, in part, to a diverse germplasm and optimal roots (Shrestha et al, 2020; Green et al., 2001). Rice is a model monocotyledon species for root genetics because it possesses five root architecture types (i.e. embryonic and post-embryonic, radical, embryonic crown, post-embryonic crown, and large and small lateral roots) (Rebouillat et al., 2008). More root characteristic research is needed to understand and identify the genetic basis of weedy rice allelopathic phenotypes.

Molecular markers are ideal for discerning agronomic traits that are otherwise difficult to label, and they may be used to describe a plants genetic make-up (Shrestha 2018; Parker et al., 1998). This includes traits such as resistance to pathogens, insects, and nematodes, and abiotic stresses (Mohan et al., 1997). Molecular markers are small DNA fragments with specific locations in the organism's genome that provide information needed to differentiate varieties within the same species at the genomic level (Shrestha, 2018; Powell et al., 1996). Benefits of employing molecular markers, which only require a small amount of DNA to assess, are reliability, affordability, evenness of distribution throughout the genome, and clear linkage to a specific phenotype (Powell et al., 1996). Environmental conditions such as soil texture and

composition influence root architecture. Moreover, preexisting genetic factors make accurately phenotyping root traits challenging (Rebouillat et al., 2008). Insight into the mechanisms of root development is vital for the progress of developing more adaptable and high yielding rice production varieties.

In most cases, small-effect loci interact with the surrounding environment to regulate root morphology (Dorlodot et al., 2007). Quantitative trail loci analysis revealed genetic effects on biomass, root length, and root number in rice (Rebouillat et al., 2008). In a competitive diversity study of 54 weedy rice accessions, simple sequence repeat (SSR) markers showed allelopathic traits were not strongly associated with the markers used in the study therefore, more research is needed to validate the competitive traits of allelopathy in root morphology (Shrestha et al, 2018).

Traditional phenotyping methods used by breeders lack some advantages that molecular markers compensate for. Molecular markers are more efficient than phenotyping because they specifically map markers linked to the desired trait. The environment does not affect genetic markers whereas environmental conditions manipulate some phenotypes. In addition, the markers are traceable in any plant growth stage (Mohan et al., 1997). As summarized by Mohan et al., (1997), several techniques are useful in selection of desirable characters involving the use of molecular markers. These include: 1) random-amplified polymorphic DNAs, 2) restriction fragment length polymorphisms, 3) microsatellites and PCR-based DNA markers such as sequence characterized amplified regions, 4) sequence-tagged sites, 5) inter-simple sequence repeat amplification, 6) amplified fragment length polymorphic DNAs and 7) amplicon length polymorphisms using F2 and backcross populations, near-isogenic lines, doubled haploids and recombinant inbred lines.

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Genotyping by sequencing (GBS) is ideal for studying the weedy rice germplasm; a genetically diverse species. Genotyping by sequencing is high-throughput next-generation sequencing of genomic subsets targeted by restriction enzymes and can be generalized to any species economically (Elshire et al., 2011). Most capture methods are time-consuming, technologically challenging, and can be cost-prohibitive for assaying large numbers of samples. However, reducing genome complexity with restriction enzymes is efficient, extremely specific, highly reproducible, and may reach essential genomic regions that are inaccessible to other sequence-capture approaches (Elshire et al., 2011). Through genetic association studies, phenotyping the root traits attributing to rice allelopathy is a subject for future crop advancement research.

There is a need to increase the yield and productivity of the rice crop to meet the growing demands of the world's population to which millions depend on. To conserve resources, produce more in less space, reduce health risks, and cost effectively control weeds, the use of allelopathy shows potential to be an efficient biologically based strategy to the rice crisis worldwide. By confirming allelopathic weedy rice accessions, phenotyping root characteristics associated with allelopathy and then mapping their locations these traits may be incorporated into cultivated rice varieties. Markers identified in this study are the precursor to marker assisted selection (MAS) and will provide the missing piece needed to advance rice breeding strategies (Mohan et al., 1997).

Materials and Methods

Plant material and genomic DNA extraction

Seeds from 10 weedy rice accessions and five rice cultivars were selected based their ability to suppress barnyardgrass in terms of height, biomass, and chlorophyll reduction. Weedy

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rice accessions used in this study were selected from a collection of 54 accessions with weed suppressive ability from the Weedy Physiology lab at University of Arkansas; Fayetteville, AR. (Tseng at al., 2013; Shrestha et al., 2020). Six of the weedy rice accessions had confirmed ability to suppress barnyardgrass and five had low barnyardgrass suppression. Rice cultivars CL163 and Rex were supplied by the rice breeding lab at Delta Research and Extension Center, Stoneville MS. Cultivars PI132777, PI338046 and Rondo were acquired from the Dale Bumpers National Rice Research Center in Stuttgart AR. Fresh tissues from all plants were used for DNA extraction using the CTAB method with slight modifications. Quantity of DNA was determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Inc.). Quality of DNA was determined through gel electrophoresis. Extracted DNA was stored at -80 °C until needed for PCR.

Polymerase chain reaction (PCR) using simple sequence repeat markers

DNA samples were diluted to 100ng/ml before PCR. Thirty primers were selected from a publicly available Gramene database with a standard panel of 50 primers developed by McCouch et al., (2002) (https://archive.gramene.org/markers/microsat/50_ssr.html) (Table 4.1). Primers were used to analyze the genetic diversity of the weedy rice accessions. Markers can be used to compare genetic distance and differences between *Oryza* species with AA genome. PCR reactions were conducted using 96 well trays (VWR International, LLC Radnor, PA) with a 25ml total reaction volume. Reaction Mixture for each sample consisted of 12.5ml Taq 2x master mix (Taq polymerase, dATP, dGTP, dCTP, dTTP, MgCL2) (New England biolabs, Inc. Ipswich MA) 9ml micro grade water, 1 ml forward primer and 1ml reverse primer, and 1.5 ml diluted DNA. PCR reactions were carried out with a MJ Research PTC-100 Peltier (Marshall Scientific LLC) with the following profile for DNA amplification: denaturation at 94 °Cfor 5min, 35 cycles of 94

°C for 1 min followed by annealing temperature dependent on primer requirements ranging from 53-67 °C and a final extension for 5min at 72 °C. PCR products were separated in 2% polyacrylamide gels for 30 min at 180 v, and stained with ethidium bromide. Gels were visualized under UV trans illuminator (VWR international LLC) and photographed.

Data analysis

Bands were considered co-dominant markers and scored in Cross Checker 2.91. Bands were scored as binary characters with 1 representing the presence of a band and 0 indicating the absence of bands. Bands sizes were categorized in three groups by size, 300 plus, 189.5 and 110.5. Number of alleles, Nei's genetic diversity, percentage polymorphic loci, and Shannon's index were gathered through POPGEN version 1.32 using data from Cross checker. Dendrograms were developed using Nei's (1972) genetic distance with the UPGMA algorithm to observe the genetic relationship between weedy rice accessions and cultivated rice.

Results and Discussion

Genetic diversity among accession with respect to allelopathic ability

All markers selected were polymorphic for all accessions used. The number of alleles per locus ranged from 2-3 with a mean of 2.833. Genetic diversity of weedy rice and cultivated rice both allelopathic and non-allelopathic was 0.4855. The highest genetic diversity (h) was observed in markers M2, M4, M8, M11, M14, M17, M19 M23, M29, M26 (> 0.6280). The lowest genetic diversity (h) was observed in markers M12, M15, M18 (< 0.2145). Genetic diversity of weedy rice was 0.5133 and cultivated rice was 0.3485 (Table 4.2, Table 4.3).

Dendrogram results based off Nei's genetic distance (1972) identified 4 clusters (Figure 4.1). Cluster one consisting of a black hull weedy rice accession B14. Cluster 2 identified two

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straw hull weedy rice accessions both allelopathic and non-allelopathic. The third cluster consisted of weedy rice and one rice cultivar Rondo. The last cluster grouped all other Cultivated rice and two allelopathic weedy rice accessions together. Cluster 4 encompassed all cultivated rice with the exception of Rondo. Rondo fell independently when clustered with only cultivated rice. Allelopathic cultivated rice PI312777 and PI388046 fell together in a cluster with non-allelopathic rice Rex (Figure 4.2).

To assess allelopathic ability based on genetic diversity, cultivated rice and weedy rice were divided into groups of allelopathic and non-allelopathic. The allelopathic cultivated rice had a mean genetic diversity of .3202 and the non-allelopathic cultivated rice was .3621. Allelopathic weedy rice had a mean genetic diversity of .5011 compared to non-allelopathic with a mean of .4744. (Table 4.3).

Nei's Genetic diversity measures the heterozygosity between and within a population and has a value ranging from 0-1. Higher values indicate higher genetic diversity. The high genetic diversity in weedy rice compared to rice could account for the weed's morphological variation and ability to adapt to a range of environments (Shrestha et al., 2018). Weedy rice populations had a higher genetic diversity (51%) than the cultivated rice (35%). Allelopathic weedy rice was more diverse (50%) than all cultivated rice and non-allelopathic weedy rice. The diverse genetic characteristics exhibited in weedy rice may be a produced of hybridization among itself and with cultivated varieties.

Genetic similarity and root traits

Root characteristics measured in objective 2 of this study were area and length. The markers used did not distinguish similar root lengths into clusters. Mean root lengths of each the clusters formed in the dendrogram based on genetic diversity were calculated (Figure 4.1).

Allelopathic weedy rice accession B14 alone in cluster 1 had the third longest mean root measurement of the weedy rice in this study (21.7 cm). Cluster 2 with two weedy rice accessions S94 and S97 had similar root lengths (17.7, 16.9 cm) Clusters 3 had root lengths ranging from 7.4-22.4 cm and cluster 4 ranged from 9.8-24.8 cm. As the markers in this study were not able to separate allelopathic and non-allelopathic biotypes root characteristics of allelopathic weedy rice were not separated as well. (Table 4.4).

Homogeneity test

A Homogeneity test was run using 30 SSR makers on the weedy rice population to assess markers ability to distinguish allelopathic and non-allelopathic populations. Four markers were found that had over 95% probability of expressing in the weedy rice population, but the markers were the same for both allelopathic and no allelopathic indicating no distinction based on mean barnyardgrass reduction potential of all three parameters measured. (Table 4.5)

The weedy rice population was divided into allelopathic and non-allelopathic based on each component, height, chlorophyll and biomass reduction separately and a homogeneity test was conducted. The five most barnyardgrass height suppressive weedy rice accessions and the five least were grouped together. Four markers were found to be unique in the highly suppressive group, being M4, M17, M26 and M29 all with 96% probability of expression in the height suppressive weedy rice group. The same four markers expressed with 96% probability in the suppressive weedy rice population when groups were determined based on Biomass reduction of barnyardgrass. When accessions were categorized by chlorophyll reduction another set of markers was found to be distinct in the highly suppressive accessions. Markers M10, M12, M15, and M18 all showed 100% probability of expressing among the five most chlorophyll suppressive weedy rice accessions (Table 4.5).

Allopathic accessions were distributed through the dendrogram indicating that there was a weak association with the markers selected and allelopathic ability. Markers used in this study were selected from a standard panel of 30 SSR markers used for distinguishing genetic diversity among individuals of Oryza s. and are not specific to allelopathic production as those markers have yet to be identified. Weedy rice is a product of hybridization with cultivated rice therefore, markers used for rice were able to distinguish the genetic relationship. Allopathic and non-allelopathic accessions were grouped together and distinguished between weedy rice and cultivated rice due to the markers used.

Allelochemicals are secondary metabolites produced though various pathways. Different Allelochemicals have been found to have Multiple physiological effects on plants processes unique to the compound and plant species (Yang et al., 2002). Rice residues in soil contain multiple phenolics and unknown compounds. Compounds found to be excreted through rice roots include momilactone B, Gallic, Protocatechunic, chlorogenic, Ferulic, Vanillic Benzoic acids and Vanillin (Khang et al., 2016) Ferulic acid alone has effects on plant growth, absorption of water, nutrient uptake, leaf water potential and osmotic potential (Yang et al., 2002). Chlorogenic acid effects stomatal aperture in species like tobacco and sunflower. The primary target of phenolic acids is proposed to be on the plasma membrane, thus causing a cascade of effects that contribute to processes in growth reduction (Yang et al., 2002). Photosynthesis is one process that is affected by the compounds excreted from rice which was one perimeter measured. Compounds effect plant height in variable ways for example ferulic and p-coumaric acids inhibited plant height to a similar degree but differently from o-hydroxyphenyl acetic acid (Yang et al., 2002). The differences in levels of chlorophyll reduction, height reduction and biomass

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reduction in the weedy rice suggest multiple compounds effecting multiple plant processes, together causing allelopathic effects in the barnyardgrass.

The genetic diversity of the weedy rice population suggests different genetic backgrounds contributing to the effect. Rice accessions in this study consisted of both Indica and Japonica classifications. Most of the weedy populations observed grouped with Rondo an India rice. Allelopathic weedy rice accessions B2 and B81 cluster with allelopathic rice Rondo in cluster 3 suggesting close relationship. Similarly, allelopathic weedy rice accessions B8 and B84 clustered together with allopathic cultivated rice PI338046 and PI312777 in cluster 4. Allopathy is distributed thought the rice genome and therefore the markers used were not able to differentiate.

The four markers that had the highest genetic diversity were found in both populations of weedy rice. Genetic diversity contributed to adaptability and competitiveness of the plant which is consistent with the weedy rice population seen in comparison to the cultivated rice. This diversity may house traits that contribute to the ability to suppress barnyardgrass better than domestic verities as seen in the weedy rice accessions in this study. Markers that were able to determine higher genetic diversity are more likely to be able to detect allelopathic traits for future studies. A threshold was set at 60% genetic diversity among the markers indicating detection of high diversity. Ten markers were all above the threshold. Markers selected with ability to detect high genetic diversity represented a number of different chromosomes.

Longer root lengths were observed in most of the allelopathic accessions. Root traits, like allelopathy, are controlled by many factors environmental and genetic. The root length seems to be contributing to allopathic ability but genetically was distributed throughout the dendrogram indicating week association with the markers in this study. Some weedy rice root trait studies note that although different accessions may form similar architectures they do not share genetic

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locations (Wedger et al., 2019). QTL on chromosomes 1 and 9 have been linked to maximum root length in rice (Courtois et al., 2009). Markers RM283, RM431, RM215 and RM536 detected high genetic diversity in this study, are associated with chromosomes 1 and 9, and therefore may be candidates for use in targeting competitive root growth in weedy rice. Markers M431, RM455, RM215 and RM536 were unique to the height suppressive and biomass suppressive weedy rice groups. Markers RM124, RM413, RM162 and RM118 were unique to the chlorophyll suppressive weedy rice therefore, are candidates for further studies and may lead to allelopathic gene identification in weedy rice.

Conclusion

Markers detecting high genetic diversity can be used in further studies of allopathic and competitive traits using SSR markers for developing weed suppressive rice varieties. The Markers in this study were able to separate allelopathic and non-allelopathic populations when divided by method of barnyardgrass suppression. These findings indicated multiple mechanisms contributing to barnyardgrass suppression. More markers need to be screened in order to determine the genetic controls contributing to root architecture and allelopathic ability in weedy rice.

Table 4.1   List of markers with chromosome number, Nucleotide sequence, annealing temperature, number of Cycles and allele.

Marker	Chr.	Forward Primer (Labeled)	Reverse Primer	Anneal temp	PCR Cycles	Min Allele	Max Allele
RM495	1	aatccaaggtgcagagatgg	caacgatgacgaacacaacc	55	30	148	160
RM283	1	gtctacatgtacccttgttggg	cggcatgagagtctgtgatg	61	30	130	176
RM237	1	caaatcccgactgctgtcc	tgggaagagagcactacagc	55	30	105	153
RM431	1	tcctgcgaactgaagagttg	agagcaaaaccctggttcac	55	30	233	261
RM154	2	accctctccgcctcgcctcctc	ctcctcctcctgcgaccgctcc	61	30	148	230
RM452	2	ctgatcgagagcgttaaggg	gggatcaaaccacgtttctg	61	30	192	213
OSR13	3	catttgtgcgtcacggagta	agccacagcgcccatctctc	53	40	85	122
RM338	3	cacaggagcaggagaagagc	ggcaaaccgatcactcagtc	55	40	178	184
RM514	3	agattgatctcccattcccc	cacgagcatattactagtgg	55	30	229	278
RM124	4	atcgtctgcgttgcggctgctg	catggatcaccgagctcccccc	67	30	257	289
RM507	5	cttaagctccagccgaaatg	ctcaccctcatcatcgcc	55	30	234	257
RM413	5	ggcgattcttggatgaagag	tccccaccaatcttgtcttc	53	30	71	114
RM161	5	tgcagatgagaagcggcgcctc	tgtgtcatcagacggcgctccg	61	30	154	187
RM133	6	ttggattgttttgctggctcgc	ggaacacggggtcggaagcgac	63	30	226	237
RM162	6	gccagcaaaaccagggatccgg	caaggtcttgtgcggcttgcgg	61	30	191	244
RM125	7	atcagcagccatggcagcgacc	aggggatcatgtgccgaaggcc	63	30	105	147
RM455	7	aacaacccaccacctgtctc	agaaggaaaagggctcgatc	57	30	127	144
RM118	7	ccaatcggagccaccggagagc	cacatcctccagcgacgccgag	67	30	149	165
RM408	8	caacgagctaacttccgtcc	actgctacttgggtagctgacc	55	30	112	128
RM152	8	gaaaccaccacacctcaccg	ccgtagaccttcttgaagtag	53	40	133	157
RM44	8	acgggcaatccgaacaacc	tcgggaaaacctaccctacc	53	30	82	132
RM284	8	atctctgatactccatccatcc	cctgtacgttgatccgaagc	55	30	139	159
RM433	8	tgcgctgaactaaacacagc	agacaaacctggccattcac	53	40	216	248
RM447	8	cccttgtgctgtctcctctc	acgggcttcttctccttctc	55	30	95	146
RM316	9	ctagttgggcatacgatggc	acgcttatatgttacgtcaac	55	30	194	216
RM215	9	caaaatggagcagcaagagc	tgagcacctccttctctgtag	55	30	126	161
RM271	10	tcagatctacaattccatcc	tcggtgagacctagagagcc	55	30	80	120
RM484	10	tctccctcctcaccattgtc	tgctgccctctctctctctc	55	30	286	298
RM536	11	tctctcctcttgtttggctc	acacaccaacacgaccacac	55	30	223	247
RM277	12	cggtcaaatcatcacctgac	caaggcttgcaaggggaag	55	30	104	121

Table 4.2   Genetic variation among the population of all weedy rice and rice used in this study indicated though allele number and Nei's genetic diversity.

Marker	Marker No	Observed alleles	Nei's genetic diversity
RM495	1	3	0.3490
RM283	2*	3	0.6428
RM237	3	3	0.5312
RM431	4*	3	0.6457
RM154	5	2	0.4844
RM452	6	2	0.2778
OSR13	7	2	0.2778
RM338	8*	3	0.6465
RM514	9	2	0.3750
RM124	10	2	0.4764
RM507	11*	3	0.6428
RM413	12	3	0.2145
RM161	13	3	0.5312
RM133	14*	3	0.6428
RM162	15	3	0.2145
RM125	16	3	0.5312
RM455	17*	3	0.6457
RM118	18	3	0.2145
RM408	19*	3	0.6208
RM152	20	3	0.5312
RM44	21	3	0.4050
RM284	22	3	0.4050
RM433	23*	3	0.6428
RM447	24	3	0.2901
RM316	25	3	0.5679
RM215	26*	3	0.6457
RM271	27	3	0.4490
RM484	28	3	0.5679
RM536	29*	3	0.6457
RM277	30	3	0.4490
	Mean	2.8333	0.4855

Markers with * have > 60% genetic diversity based on Nei's (1972)

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Table 4.3   Mean genetic diversity by biotype groups.

Biotype	Mean Genetic Diversity
Cultivated rice (CR)	0.3485
Weedy rice (WR)	0.5133
Allelopathic cultivated rice (ACR)	0.3202
Non allelopathic CR (NCR)	0.3621
Allopathic Weedy rice (AWR)	0.5011
Non allelopathic weedy rice (NWR)	0.4744

Table 4.4   Weedy rice and cultivated rice root measurements by dendrogram cluster based on allelopathic potential.

Cluster	Accession	Biotype	Root Length (cm)	Mean Root Area (cm^2)
1	B14	NWR	21.7	2.6
2	S94	AWR	17.7	2.6
	S97	NWR	16.9	2.6
3	B2	AWR	22.4	2.3
	B81	AWR	9.2	1.9
	B83	NWR	7.4	1.5
	B34	NWR	16.9	2.8
	Rondo	ACR	8.5	0.8
4	B8	AWR	11.0	1.7
	CL163	NCR	9.8	1.1
	PI312777	ACR	19.4	2.1
	Rex	NCR	24.8	2.6
	PI338046	ACR	18.9	2.2
	B84	AWR	15.9	1.4

For rice biotypes (A) indicates allelopathic (N) indicates non allelopathic (WR) indicates weedy rice (CR) indicates cultivated rice

Table 4.5 Markers with > 95% probability of expressing in the weedy rice populations based on height, biomass, chlorophyll and overall barnyardgrass reduction.

Category	Allelopathic		Non-allelopathic	
Mean suppression	**Marker**	**Probability**	**Marker**	**Probability**
	RM431	> 95%	RM431	> 95%
	RM455	> 95%	RM455	> 95%
	RM215	> 95%	RM215	> 95%
	RM536	> 95%	RM536	> 95%
Height suppression	RM431	> 95%	na	na
	RM455	> 95%	na	na
	RM215	> 95%	na	na
	RM536	> 95%	na	na
Biomass suppression	RM431	> 95%	na	na
	RM455	> 95%	na	na
	RM215	> 95%	na	na
	RM536	> 95%	na	na
Chlorophyll suppression	RM124	100%	RM431	> 95%
	RM413	100%	RM455	> 95%
	RM162	100%	RM215	> 95%
	RM118	100%	RM536	> 95%

Not applicable (na) indicates the absence of markers with >95% probability of expressing
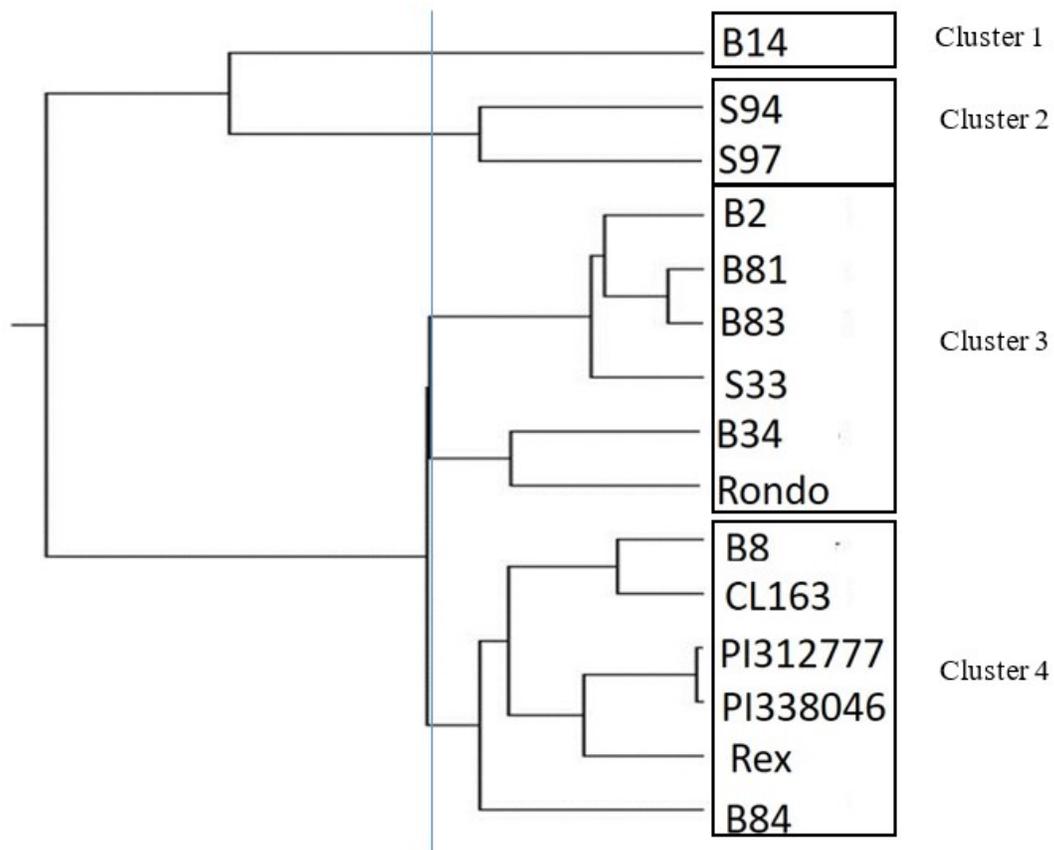
Figure 4.1   Dendrogram of weedy rice and rice based on Nei's genetic distance (1972)
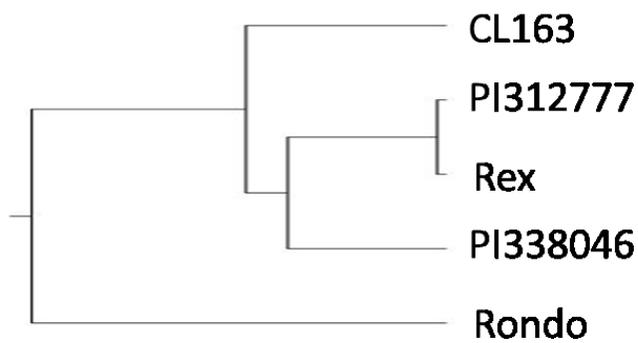

Figure 4.2   Dendrogram of rice cultivars based on Nei's genetic distance (1972).

CHAPTER V

RECOMENDATIONS

The screening method chosen was modified from previous successful experiments exploring allelopathy in species such as turfgrass and barley (Liu and Lovett, 1993; Lickfeldt et al., 2001). The stair-step method proved to be a useful screening technique to determine weedy rice allelopathic potential. Although useful, this method is time consuming in its construction and conduction. The stair-step design was planned to fit the needs of the present study. Modifications could be suggested to allow less manual interruption and longer screening time. The collection tank housing the pump would be suited better for the experiment if it were larger. One initial fill of the tank would allow for measurements of water consumption at the end of the experiment, a steady accumulation of allelochemicals in solution and less labor during the experiment. Additionally, the connecting tubes allowing flow between pots should be larger in inner diameter. The tube coming from the tank on the top bench should remain small as it allowed for mediation of the systems flow. Lastly, filters of another material that could not decompose would lengthen the screening time.

The germination pouches provided a useful tool for screening weedy rice roots. The pouches in this study were the largest size available and allowed for two plants per pouch for one month without any interaction. Allelopathy seems to be an induced response according to some literature (Zhang et al., 2019; Khanh et al., 2018). Future studies could use these pouches with the target species and donor species together. This study looked only at traits the donor possessed

without the presence of a competitor. Additionally, concerning the watering regime, instead of watering each pouch individually, the bottoms of the bags could be cut and then hung above the solution. This would be both efficient and reduce any possibility for error in watering.

Future studies regarding weedy rice allelopathy need to be conducted. The markers found provide a starting point to identify genes associated with allelopathic accessions and the mechanisms of barnyardgrass suppression. Chemicals being produced have yet to be studied in weedy rice and are curtail if development of bioherbicides are to be developed.

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