IMPROVING LOW PH AND ALUMINUM TOLERANCE IN ALFALFA

(*MEDICAGO SATIVA L.*)

by

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(Under the Direction of Ali M. Missaoui)

ABSTRACT

Alfalfa (*Medicago sativa L.)* is the most valuable forage crop in the world. Alfalfa production is limited in the southeastern United States due to the prevalence of acidic, aluminum-rich soils. Despite significant efforts, there currently exists no alfalfa cultivar with sufficient agronomic performance under low pH soil conditions. The present research represents a three-pronged approach for improving low pH and high aluminum tolerance in alfalfa. The first characterizes the effect of low pH soils on yield and fall dormancy rating through a replicated, multi-year, multi-location field phenotyping approach in which 138 half-sib families were evaluated in both a natural low pH field soil (4.9-5.2, extractable aluminum=10.41-11.38 mg kg^{-1}) and a field soil amended with lime prior to establishment (pH=6.37-7.07, extractable aluminum=0.01-2.29 mg kg^{-1}). Yield data was used to develop an Acid Soil Adaptation Index (ASAI) for use as a selection criterion for the next round of recurrent phenotypic selection (RPS). The second objective was to develop an efficient greenhouse rhizobox assay to screen acid-soil tolerance, including a high-throughput image analysis procedure. Root system architecture (RSA) traits associated with acid tolerance, as determined by the field evaluation, were identified. Lastly, we performed a genome-wide association study (GWAS) for field and

nutrient-related traits using genotype-by-sequencing (GBS) and DArTag sequence data. Twenty-two genetic markers were significantly associated with these traits and candidate genes were annotated. Genomic prediction models were built for both genotyping sets and compared for accuracy. Through this work, we combine traditional plant breeding approaches and technological advances in image processing and genomic analysis to contribute to the development of locally-adapted, acid-tolerant alfalfa cultivars.

INDEX WORDS: Alfalfa, low pH tolerance, aluminum toxicity, abiotic stress, forage breeding, nutrient quality, root structure architecture, genome-wide association study, genomic prediction

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DEDICATION

I would like to dedicate this work to my husband, Joshua Presley. Your steadfast love and support have carried me through the ups and downs of graduate school and life. Thank you for being my rock and my best friend.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Alfalfa (*Medicago sativa L.*) is one of the most important forage crops worldwide. Known for its high nutritional value and benefits to livestock production, the "Queen of Forages" is the fourth most valuable crop in the United States (Narasimhamoorthy et al., 2007; USDA-NASS, 2025). Alfalfa hay in the United States was valued at 9.6 billion dollars in 2021 and alfalfa grown in mixture valued at a staggering 19.3 billion dollars (USDA-NASS, 2023). The global importance of alfalfa is evidenced by the 32 million hectares dedicated to its production every year (USDA-NASS, 2024a). As a legume, alfalfa roots form a symbiotic relationship with soil rhizobia, which fix atmospheric nitrogen into ammonium, making it available for plant uptake and nutrition. This natural process improves physical, chemical, and biological qualities of soil and makes alfalfa desirable as a mixture component in forage production systems, as a rotation crop in food crop production, or as a cover crop to improve soil properties of marginal lands. The importance of this ability is underscored by the increasing cost of nitrogen fertilizers and as producers look for more ecologically friendly methods to improve soil qualities and crop yields. A major limitation of alfalfa cultivation is sensitivity to low pH and aluminum rich soil conditions, which are characteristic of the highly eroded ultisols in Georgia and much of the southeastern USA (Bouton & Sumner, 1983). Despite decades of research into this problem, there is still no available cultivar displaying sufficiently high yield in low pH and high Al conditions (Bouton, 1996, 2021; Dall'Agnol et al., 1996).

As pH drops, Al is solubilized in the soil solution, increasing its availability for uptake, and stunting root and plant growth. The need for an acid-tolerant alfalfa cultivar is accentuated when considering that 30%-40% of the world's ice-free soil is acidic, as much as 50% of available arable land, and the difficulty of cultivating these acres is a major contributor to deforestation and climate change (Von Uexküll & Mutert, 1995). Aluminum is the most prevalent metal ion in the Earth's crust, adding to the pervasiveness of this problem (E. Delhaize & P. Ryan, 1995). The lush forests found in the northern half of Georgia and much of the southeastern United States are characterized by acidic soils and have been a major barrier to alfalfa production in this region. Soil acidification happens in a variety of ways, including erosion, acid rain, and nitrification, which is exacerbated by additions of fertilizers containing ammonium, pollution, and deforestation. Alfalfa performs best at a pH above 6.5. Below this, root growth, biomass yield, and N-fixation are drastically reduced (Bouton et al., 1981). This diminishes some of the greatest benefits of growing alfalfa, which include high quality and high value hay and perennial pasture forage production and the natural rejuvenation of soil properties. The decreased ability of alfalfa roots to develop and associate with soil bacteria results in greatly reduced stand persistence, as alfalfa requires sufficient energy reserves from the roots to facilitate regrowth after every cut and to break winter dormancy (Rice et al., 1977).

UGA Forage Extension recommends liming of low pH soils to raise the pH to an optimal range (Sumner et al., 1986). Though lime applications have been shown to improve alfalfa establishment and performance, the pH adjustment does not reach the subsoil layer, and as alfalfa can develop root systems greater than six feet in depth, stands are still underdeveloped, drought intolerant, and less persistent (Bouton & Sumner, 1983; Hall et al., 2004). Lime application is

also a costly and impractical long-term solution in many field settings, especially in marginal lands and developing countries.

Alfalfa is an obligate outcrosser and an autotetraploid (2n=4x=32). The complexity of its genome and tetrasomic segregation patterns has been a significant barrier to making genetic gains in breeding.

The proposed research includes GWAS of a diverse panel of plant introductions that have been through two rounds of selection, first at low pH in Tifton, GA and second at a low pH in Athens, GA. Selections from this population were intermated in a greenhouse bee cage and the progeny were planted in both low pH and adjusted pH field conditions at the JPC, where they will be genotyped, phenotyped, and statistically analyzed to associate genetic markers with desirable traits, such as acid tolerance, yield, persistence, and fall dormancy. The goal of this research is to elucidate candidate genes that confer tolerance to low pH and high aluminum conditions with the hope of using genomic selection (GS) to facilitate cultivar development in the future. The second objective of this work is to develop a cost and time-effective protocol to screen alfalfa germplasm for tolerance to low pH and aluminum using rhizoboxes and modified Hoagland solutions in conjunction with high-resolution imaging and computational analysis of root structures.

Mechanisms of Low pH and Aluminum Toxicity

Low pH and aluminum concentrations in soils result in significant yield losses every year for many crops. As much as 50% of the currently arable land, including much of Georgia and the southeastern USA, is characterized by low pH soils and pose a significant barrier to the production of sensitive crop species (Von Uexküll & Mutert, 1995). This problem has been exacerbated by increasing acid rain, pollution, and climate change, and can be a driving factor of

deforestation and species extinction. Though low pH soils can be adjusted by the application of lime and aluminum toxicity reduced by the application of gypsum, these soil amendments only affect the topsoil layer, which limits their effectiveness in deep-rooting crops such as alfalfa. The mechanisms of toxicity and tolerance to these soil conditions have prompted a great deal of research in many crop species, but much remains to be elucidated. However, the negative effect of low soil pH on alfalfa production is clear. Studies have found that alfalfa at a low pH experiences root stunting and decreased nodulation with soil rhizobia (Franco & Munns, 1982; Graham, 1992). Other studies have also shown that only 9.1% of alfalfa survived a trial in a field with pH=3.9 (Liatukiene et al., 2020) and that alfalfa stand persistence in low pH soils is reduced from 9-10 years to a mere two years (Wolf et al., 1994).

Aluminum is the most abundant metal element in the Earth's crust but is usually bound to other ions and sequestered in the soil, preventing uptake by plants via root systems. As pH drops below 6, mineral aluminum deposits primarily in the form of Bauxite ore are solubilized in the soil solution as the trivalent aluminum ion, which is toxic to many plant species (Hajiboland et al., 2023; Riede & Anderson, 1996). This solubilization increases uptake of the toxic ions and results in root stunting (E. Delhaize & P. Ryan, 1995; Delhaize et al., 1993). Since proper root development is critical for the absorption of water and nutrients, root stunting is associated with decreased aboveground growth and nutritive qualities. Aluminum toxicity has been shown to disrupt cell division of root apices and increases the rigidity of the cell wall by cross-linking pectins (Zhang et al., 2007). This increases the polysaccharide content of the cell wall and further reduces water and nutrient uptake, as well as cell wall elasticity, which likely obstructs normal ion exchange, signaling, and cell division (Ahn et al., 2001; Nguyen et al., 2005; J. L. Yang et al., 2011).

When concentrations of solubilized Al^{3+} reach levels higher than 10 mg/kg of soil, many plants exhibit severe toxic phenotypes that ultimately result in decreased yields and crop failures (Kochian, 1995). Other studies have proposed that aluminum toxicity occurs when the ratio of extractable Al to the sum of extractable Al and exchangeable Ca, Mg, and K is greater than 60% within 50 cm of the soil surface because Al competes with these essential nutrients for uptake (Buol & Eswaran, 1993). Aluminum toxicity is thought to be the primary limiting factor for crop production on low pH soils around the world and is estimated to affect more than 50% of the soils in humid tropical regions (Horst, 1995).

This prolific problem has prompted a significant amount of research towards investigating the mechanisms of aluminum toxicity and its exacerbation by low pH conditions. The proposed mechanism of aluminum toxicity involves blocking water and nutrient uptake, inhibiting the ability of roots to absorb N, P, Ca, Mg and Fe, which have important roles in cell functioning, growth, and division (Buol & Eswaran, 1993; Lazarević et al., 2014; Ribeiro et al., 2013). More specifically, aluminum toxicity is thought to disrupt cellular calcium homeostasis, which is critical for stabilizing cell walls, cellular signaling, and cell division (Plieth et al., 1999). Aluminum toxicity has been shown to target the root meristems and inhibit cell elongation and division, decreasing apical and lateral growth in primary roots and root hairs (Inostroza-Blancheteau et al., 2012). Because aluminum toxicity affects many cellular processes, it is difficult to isolate the primary effect from the consequences (Samac & Tesfaye, 2003). Studies investigating the differences in aluminum and acid sensitivity suggest that, though these stressors are often simultaneous and interrelated, the mechanism of phytotoxicity is likely different, but many questions persist as to exactly how they differ (Yokota & Ojima, 1995). For example, Zhang et al. (2007) found that the physiological effects of aluminum concentrations on soybeans

under normal pH conditions vary substantially. At low amounts (100 mg/kg), Al^{3+} improved plant growth, with plants exhibiting greater leaf areas and root surface areas. It was only above the 800 mg/kg Al^{3+} threshold that plants began to show toxic responses, such as depressed chlorophyll content and photosynthesis, increased transpiration, decreased cell membrane stability and water use efficiency (Zhang et al., 2007). However, this "low concentration" of aluminum would likely produce substantial toxic effects under low pH conditions in a more sensitive species.

Low pH and high aluminum concentrations in the soil have been associated with the production of Reactive Oxygen Species (ROS) by the reaction centers of photosystems I and II in the chloroplasts, decreased cell respiration and activity of the mitochondria, and ATP-depletion (Nunes-Nesi et al., 2014). The prolonged upregulation of ROS has been shown to cause oxidative stress, which can disrupt plant cell membranes or damage both DNA and the photosynthetic machinery, induce lipid peroxidation, and cause a plethora of negative downstream effects for the plant (Yamamoto et al., 2002; Yang et al., 2015). Decreased ability of the mitochondria to perform cell respiration and produce ATP naturally leads to the inhibition of root cell growth and division and results in a stunted phenotype. Because low pH stress can also induce ROS production, this adds to the difficulty of teasing apart the mechanism of aluminum toxicity from the aftereffects. Other commonly observed physiological features under high Al-stress include irregular development of leaves and buds and decreased mesophyll cells, both in number and size, which further contribute to decreased photosynthetic abilities and energy depletion (McQuattie & Schier, 1993; M. Yang et al., 2011).

Additionally, it has been shown that low pH and aluminum toxicity result in the slow decrease in chlorophyll content, photosynthetic rates, transpiration rates, and water use

efficiency, and that increasing concentrations of aluminum ions is correlated with more pronounced deficits in photosynthetic parameters (Yang et al., 2015). Even relatively low concentrations of Al^{3+} of less than 0.05 mM has been shown to reduce stomatal apertures, which may contribute to the decreased transpiration and photosynthetic rates observed under Al-stress (Smirnov et al., 2014), though it may be noted that other studies have found that Al-stress increased transpiration (Zhang et al., 2007). While studies have demonstrated decreased water use efficiency (WUE) in mung bean (Ali et al., 2008) and soybean (Ying & Liu, 2005), other studies have shown that WUE decreased at 0.1-, 0.2-, or 0.4-mM Al^{3+}, but increased at 0.5 mM Al^{3+} (Pereira et al., 2000). Similarly, studies using hydroponics and nutrient solutions showed improved root elongation at lower Al-concentrations and inhibition only at high Al-concentrations (Silva et al., 2004). This shows the complexity of the Al-toxicity mechanisms and the variability of responses by different species that are mediated by differing genotypes, environments, and the concentration of aluminum ions.

Alfalfa is particularly susceptible to low pH and aluminum toxicity. Legumes like alfalfa can fix atmospheric nitrogen into ammonium through association with soil rhizobia (*Sinorhizobium meliloti)* through a complex signaling cascade and root nodulation process. In these types of soils, nodule formation has been reported to be inhibited by nearly 90% in low pH soils, reducing nitrogen fixation (Alva et al., 1990; Evans et al., 1990; Ferguson et al., 2013). This reduction is exacerbated by molybdenum deficiency, a key component of the nitrogenase enzyme complex (Adhikari & Missaoui, 2017; Giddens & Perkins, 1972; Kim & Rees, 1992; Marschner, 1991). Though yield losses can be improved with the addition of N fertilizers, this minimizes one of the major environmental and economic benefits of growing alfalfa. The mechanism by which low pH and aluminum disrupt nitrogen fixation has been shown to include

poor rhizobia attachment to root hairs (Caetano-Anollés et al., 1989), root colonization (Taylor et al., 1990), and disruption of the host plant and soil microbiota signal recognition (Hungria & Stacey, 1997).

Mechanisms of Tolerance to Low pH and High Aluminum Soils

Tolerance mechanisms to high aluminum concentrations and low pH conditions are broadly categorized into two groups: exclusion and tolerance. Exclusion mechanisms often involve the excretion of organic acid anions, such as malate, citrate, and oxalate, from soil rhizobia which associate closely with root systems (Che et al., 2023; Inostroza-Blancheteau et al., 2012). These negatively charged particles chelate aluminum ions and create an insoluble compound, effectively sequestering the toxic particle and preventing its uptake by the roots. It has thus been proposed that the upregulation of organic acid synthesis and transport in apical roots may contribute to Al-tolerance (Kinraide et al., 2005; Nunes-Nesi et al., 2014). This exclusion mechanism has been demonstrated by maize, rice, corn, barley, rye, and many tree species (Yang et al., 2015). For example, research in buckwheat has shown that tolerant varieties excrete oxalic acid in response to Al stress, forming a non-toxic compound (Ma et al., 1998). The apical root cell wall is thought to be the primary binding site of Al and is thus implicated as the target of Al toxicity and exclusion in plants (Kopittke et al., 2015; J. L. Yang et al., 2011). Available physiological and molecular evidence suggests that modification of the cell wall composition plays an important role in tolerance to Al (Che et al., 2016). It has been shown that the transgenic addition of a bacterial citrate synthase or the overexpression of a malate dehydrogenase can improve tolerance to aluminum in alfalfa in lab and greenhouse assays (Barone et al., 2008; Tesfaye et al., 2001).

In the tolerance mechanism, organic acids are kept intracellularly to detoxify the Al^{3+} that is absorbed by the roots. Malic acid and citric acid concentrations are increased preferentially at the root tips, with increasing concentrations correlating positively with tolerance (Silva et al., 2004). Additionally, some research has suggested that the enhanced scavenging of ROS may increase tolerance in some species, though this mechanism may confer resistance to low pH conditions rather than aluminum toxicity (Inostroza-Blancheteau et al., 2012). Fluorescent-tagging studies in maize have shown that Al-tolerant cultivars sequester Al in root cell vacuoles (Vázquez et al., 1999), while other studies in wheat linked rapid root epidermal cell turnover with increased Al-tolerance (Delisle et al., 2001), presenting other possible tolerance processes. Despite this vast amount of research, there is much that remains unclear about the physiological underpinnings of tolerance mechanisms utilized by many plant species (Kochian et al., 2004; Silva et al., 2004)

The Genetic Basis of Aluminum Tolerance

The significant decrease in crop productivity in low pH and aluminum-rich soil conditions has led many plant breeders to attempt to develop tolerant cultivars. However, this has often been a difficult task due to the complexity and variety of the phenotypes exhibited by and within different species (Bouton, 1996). While the inheritance pattern of low pH and aluminum tolerance is unclear, research in a variety of crops indicates that it is likely a polygenic trait (Ma et al., 2006b), including alfalfa (Narasimhamoorthy et al., 2007). However, de Oliveira Camargo et al. (2000) and Riede and Anderson (1996) suggest that a single pair of dominant alleles control Al-tolerance in wheat, AltBH, indicating that there is a great deal of variation between species in response to aluminum toxicity is just as diverse as the genetic basis of tolerance. In plant species such as corn, wheat, sunflower, soybean where Al-tolerance has been strongly

associated with production of organic acids from roots, which chelate Al and prevent Al uptake in the roots, genetic engineering to upregulate gene induction of proteins which synthesize and transport these acids, such as the Al-activated malate transporter or ALMT1 protein discovered in *Triticum aestivum* (Inostroza-Blancheteau et al., 2012; Ma et al., 2001) may be an efficient way to breed for tolerance, though lag times may minimize effectiveness. Additionally, the addition of selective anion channels in the plasma membrane by genetic manipulation have shown promise in conveying tolerance to some crop species (Ryan et al., 2001).

As technology advances, the decreased cost of genotyping should facilitate our understanding of tolerance mechanisms and breeding for these characteristics. The identification of genetic markers that are associated with Al-tolerance and the increasing ability for labs to conduct Genome Wide Association Studies (GWAS) should allow plant breeders to select tolerant plants more efficiently, as well as open the door for genetic engineering to enhance tolerance through the introduction of genes found in one species to other agronomically important crops.

Prior Work in Alfalfa

Genetic improvement in alfalfa has historically been a slow process using traditional breeding methods, such as recurrent phenotypic selection, due in part to the long breeding cycle. The autotetraploid genome of alfalfa lends itself to tetrasomic inheritance patterns which further limit genetic gain per cycle of selection and necessitates the use of large populations (Casler & Brummer, 2008). Alfalfa exhibits high heterosis and severe inbreeding depression, making inbred lines infeasible and increasing the difficulty of breeding. Additionally, genetic variation for Al-tolerance in tetraploid alfalfa has been difficult to find (Bouton, 1996; Campbell et al., 1988). Because low pH soils are common and alfalfa is an important agronomic crop, a lot of

research has gone towards investigating the mechanisms of aluminum toxicity in alfalfa and ways to enhance tolerance. Prior work indicates that aluminum ions primarily target the root tips and cell walls of alfalfa and disrupt normal root development, leading to a stunted phenotype and decreased yield and persistence (Bouton et al., 1982; Bouton et al., 1986b; Liatukiene et al., 2020).

It is essential to measure low pH and aluminum tolerance in alfalfa as a ratio of growth comparing the stress condition to a non-stress condition. This is because high heterosis in a genotype may cause it to perform well in a low pH/high Al environment even if it lacks true tolerance alleles, while a genotype that has undergone inbreeding depression may appear intolerant even if it possesses tolerance allele(s). Comparing the same genotypes across both environments helps account for this problem and ensures we are selecting for genetic tolerance. Previous research has utilized methods to evaluate Al tolerance in alfalfa primarily based on greenhouse or lab conditions rather than field conditions, due in part to the increased time, difficulty, and lack of tight control of conducting field evaluation for alfalfa. These methods include callus-based assays in tissue culture (Barone et al., 2008; Parrott & Bouton, 1990), hydroponic solution assays (Pan et al., 2008; Sledge et al., 2002), and whole-plant assays in media (Khu et al., 2012). The callus assay measures relative callus biomass in media with Al^{3+} and without Al^{3+} as an index of Al^{3+} tolerance and was used to elucidate QTL associated with tolerance at a cellular level in diploid alfalfa (Narasimhamoorthy et al., 2007; Parrott & Bouton, 1990; Sledge et al., 2002). Some studies have measured the differences in low pH and aluminum phytotoxicities by monitoring the fluorescein diacetate (FDA) activity by cellular esterases and subsequent accumulation of derived fluorescein within cells and found that some alfalfa varieties tolerate restrictive aluminum concentrations (20 mmol m^{-3} Al at pH 5.0) better than restrictive

pH (pH=4) (Yokota & Ojima, 1995). The soil-based assays evaluate Al-tolerance as the ratio of root growth in limed soil to the root growth in unlimed soil of plants grown in cone-tainers in the greenhouse (Bouton, 1996; Bouton et al., 1982; Dall'Agnol et al., 1996).

Researchers at UGA and the Noble Institute have been evaluating alfalfa germplasm for tolerance to low pH and aluminum for more than 40 years using a variety of methodologies. This work culminated in the development of two populations, GA-AT and Altet 4, which showed promising potential for tolerance to low pH and high Al in greenhouse studies, but were not released commercially as tolerant cultivars because their annual dry matter yield in acid soils was very low (2176 to 2819 kg ha^{-1}) compared to the average yields of over 10,000 kg ha^{-1} in limed plots (https://www.naaic.org/TAG/TAGpapers/Bouton/BoutonAlPaper.htm). The Altet-4 genotype was created through a 4x × 2x hybridization involving a diploid *M. sativa* subsp. *caerulea* (Less. ex Ledeb.) and PI 464724-25 Al-tolerant clone (Al-4). Altet-4 shows a high ratio of root dry weight (DW) in unlimed soil to the root DW in limed soil and has been used to identify QTL associated with Al-tolerance in tetraploid alfalfa (Khu et al., 2012).

Much work has been done in recent years using next-generation sequencing, genotyping by sequencing (GBS), and association mapping (AM) to develop molecular markers and QTL, primarily in the form of SNPS, MNPs, SSRs, and RFLPs (Hawkins & Yu, 2018). Work at the Noble Institute resulted in the development of an Illumina Infinium array containing 9,277 SNPs, which have facilitated mapping and association studies in alfalfa (Li et al., 2014). This chip allows for allelic dosages to be evaluated as well, an important and often limiting criterion for autotetraploids (Han et al., 2014). The effective implementation of marker assisted selection (MAS) and genomic selection (GS) has the potential to greatly improve genetic gains in modern breeding programs, as these methods can be done at the seedling stage, allow for shorter

generation cycles, and decreased need for extensive visual phenotyping in selective environments, which is often error-prone, time-intensive, and expensive (Li & Brummer, 2012). GWAS in alfalfa have successfully identified markers and candidate genes associated with tolerance to other stressors, such as salt tolerance (Liu & Yu, 2017; Liu et al., 2019), drought tolerance (Zhang et al., 2015), and resistance to Verticillium wilt (Yu et al., 2017). The reliability of molecular markers and their ability to facilitate transgenic breeding methods are major advantages contributing to the need for this work. Much of this earlier work was made possible by using the reference genome of *Medicago truncatula*, which is an ideal model organism with a short life cycle and simple diploid inheritance patterns that has high synteny with cultivated alfalfa (Li et al., 2014). However, *M. truncatula* varies from *M. sativa L.* in several important ways, including ploidy, persistence, reproductive nature, and genome complexity. The recent release of assembled autotetraploid alfalfa genome sequence potentiates more accurate GWAS and GS in alfalfa (Chen et al., 2020; Long et al., 2022; Shen et al., 2020).

The Breeding Insight program was initiated at Cornell University in collaboration with the USDA-ARS in 2018 with the goal of developing genomic resources for polyploid crops. Since its inception, they have developed a set of universal SNP markers that can be used for genotyping any alfalfa population quickly and cost-effectively using the DArTag technology, in which regions of DNA containing the SNPs are amplified by PCR and then sequenced to identify the polymorphisms present. These markers have been shown to reliably distinguish alfalfa subspecies, dormancy classes, and drought tolerance and show high correlations with results obtained by more costly and time consuming GBS approaches (Zhao et al., 2023).

Transgenic approaches involving plants overexpressing organic acid synthesis and/or organic acid transporter genes like citrate synthase (CS) and the *Daucus carota* L. plasma

membrane H^+-transporting adenosine triphosphatase (H^+ATPase) (DcPA1) were also assessed in greenhouse conditions and have shown limited enhanced tolerance to Al (Reyno et al., 2013). Similar transgenic studies, which produced plants using nodule-enhanced forms of malate dehydrogenase and phosphoenolpyruvate carboxylase cDNAs and increased root exudation of citrate, oxalate, malate, succinate, and acetate 7.1-fold and showed promise for increasing tolerance in greenhouse conditions, did not translate to natural field soils (Tesfaye et al., 2001). This may be due to metabolic tradeoffs associated with the constitutive regulation of these energetically expensive pathways and again emphasizes the need for field evaluation for Al-tolerance.

A low correlation between the results of cell-based assays and soil-based assays has been reported (Dall'Agnol et al., 1996). These assays are complicated by the genetic variation in alfalfa, in which each seed is a genetically distinct genotype. This prior work reveals the necessity of evaluating alfalfa germplasm in field conditions to ensure that selected genotypes tolerate low pH and aluminum in settings that mimic real-world conditions, while also possessing desirable characteristics, such as high yield and persistence.

Significance of Research

Low pH soils around the world pose a great challenge to the cultivation of alfalfa in many regions, primarily by the interaction with aluminum deposits in the soil solution which facilitates the uptake of toxic Al^{3+} by plant roots. These problems have historically been mitigated by the application of lime, which adjusts the soil pH, or gypsum, which sequesters aluminum cations, but this is economically inefficient and is less effective for alfalfa due to its deep root systems. Developing an alfalfa cultivar with genetic tolerance would go a long way to increasing the number of acres of alfalfa grown and could have enormous benefits for livestock and hay

producers in Georgia and the southeast USA that currently rely on importing alfalfa hay. This is not only good for farmers but would provide an economic benefit for the state and region. Understanding the mechanisms of aluminum and low pH toxicity at both the whole plant and cellular level is critical to achieving this goal, and the use of advanced genetic technologies, such as MAS and GS, will allow breeders to achieve greater genetic gain quickly and efficiently by allowing selection to occur in the seedling stage and in the absence of the selective environment. The goal of this study is to facilitate the breeding of low pH and aluminum-tolerant cultivars that will increase the acreage of alfalfa in these regions to the benefit of producers, seed companies, and the livestock industry and continue adding to the body of knowledge surrounding the genetic basis of low pH and aluminum tolerance in alfalfa.

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CHAPTER 2

IMPROVING FIELD TOLERANCE TO LOW PH AND HIGH ALUMINUM IN ALFALFA (*MEDICAGO SATIVA L.*)[1]

[1]Wright Presley, H. and Missaoui A. Submitted to Crop Science, 08/05/25

Abstract

Alfalfa (Medicago sativa L.) is the fourth most valuable field crop in the United States. Alfalfa production is limited in regions predominated by acidic soils, including the southeastern United States. The purpose of this study was to characterize the effect of low pH soils on yield and fall dormancy ratings and select top-performers for the next breeding cycle. A population of 133 genotypes was selected from 966 diverse accessions from the NPGS collection, evaluated for 3 years in a low pH field environment. Half-sib families resulting from intermating these selections have been evaluated in row plots in a natural low pH soil (4.9-5.2, extractable aluminum=10.41-11.38 mg kg-1) and an adjusted pH soil (pH=6.37-7.07, extractable aluminum=0.01-2.29 mg kg-1) across six environments. The results revealed that acid stress significantly reduced yield by 61 g dry matter per plot ($p < 0.001$) and fall dormancy ratings by 1.47 classes ($p < 0.01$). The adjusted pH field condition did not show significantly increased survivorship compared to the low pH field during the relatively short duration of the study. An Acid Soil Adaptation Index (ASAI) based on yield data across low and adjusted pH conditions identified 56 acid-tolerant half-sib families in at least 4 of the 6 tested environments, indicating high stability. Despite significant variability across environments and genotype x environment interactions, high heritability estimates were observed for yield (0.80-0.90) and acid soil tolerance (0.854), suggesting selections from this germplasm panel can be leveraged to develop regionally adapted, acid and aluminum-tolerant cultivars.

Keywords: alfalfa, tolerance, stress, forage breeding

Abbreviations: ASAI, Acid Soil Adaptation Index; AY, average yield per cut; Cation Exchange Capacity, CEC; DMY, dry matter yield; Fall Dormancy Rating, FDR; NPGS, National Plant

Germplasm System; Organic Matter, OM; PIs, plant introductions; RCBD, randomized complete block design; TY; total yield per year

Introduction

Acidic soils (pH < 5.5) are widespread, comprising as much as 50% of the ice-free soil globally (Von Uexküll & Mutert, 1995). More than 60% of global acidic soils are concentrated in warm tropical and subtropical regions, and present a significant obstacle to crop production in many food-insecure countries (Che et al., 2023; Kopittke et al., 2019; Sanchez et al., 2003; Zhu & Shen, 2024). Soil pH affects the availability of most essential nutrients required by plants, which results in highly variable responses that depend on other soil characteristics, such as texture, cation exchange capacity (CEC), and organic matter (OM) content, as well as the specific crop and cultivar being grown, the extent of the acidity, and the growing environment (E. Delhaize & P. Ryan, 1995; Hajiboland et al., 2023). At soil pH < 5.5, the availability of N, P, K, S, Ca, Mg, and Mo are significantly decreased and the availability of heavy metals, such as Fe, Mn, B, Cu, and Al are greatly increased (Roques et al., 2013). These can lead to a myriad of nutritional deficiency symptoms, as well as Al and Mn toxicity (Riede & Anderson, 1996; Zheng, 2010). Al toxicity and P deficiency are considered the two major constraints to crop productivity on acidic soils, in which P deficiency is exacerbated by fixation by Fe and Al oxides in the soil solution (Che et al., 2023; Zheng, 2010). As these stresses occur simultaneously in the field, it is difficult to tease apart the effects of excess protons, various nutritional deficiencies, and metal toxicity (Zhao et al., 2014). Acid-tolerant cultivars must have improved nutrient acquisition and utilization and improved mechanisms to evade Al and Mn toxicity.

Alfalfa is an economically valuable crop with more than 15.6 million acres (6.3 million hectares) grown in the United States in 2023, with a value of more than 10.6 billion dollars

(USDA-NASS, 2024a, 2024b). Alfalfa is considered highly sensitive to aluminum toxicity in low pH soil conditions, which has been shown to decrease root growth (Delhaize et al., 1993; Khu et al., 2012), biomass yield (Bouton, 1996; Liatukiene et al., 2020), persistence (Wolf et al., 1994), N-fixation (Ferguson et al., 2013), and callus growth in tissue culture (Parrott & Bouton, 1990). High concentrations of free Al-ions in the soil solution disrupt several root cell processes, including cation nutrient uptake, cell wall stability, and calcium signaling (Buol & Eswaran, 1993). Al-ions bind and disrupt proteins and lipids critical to the structure and function of the plasma membrane, leading to the depolarization of the membrane and disturbing cellular homeostasis, especially that of Ca^{2+} (Panda & Matsumoto, 2007). Al-toxicity also perturbs root cell wall dynamics by binding carboxylic groups in the pectin matrix, increasing rigidity, reducing water and mineral nutrient acquisition, and interfering with root apical cell division and elongation (Delhaize et al., 2007; Kochian et al., 2004; Zhang et al., 2014). This obstructs normal ion exchange, signaling, and cell division (Nguyen et al., 2005). Decreased nutrient uptake and cell division result in stunted root systems, which serve as critical energy reserves for regrowth after harvest and winter dormancy in alfalfa stands (Hesterman & Durling, 1991; Rice et al., 1977). Alfalfa stands require significant amounts of P, K, and Ca to remain productive, removing 6 tons of P, 48 tons of K, and 30 tons of Ca per ton of dry matter produced (Undersander et al., 2011). The decreased bioavailability of these nutrients in low pH soils, coupled with stunted root systems caused by Al-stress, likely underpins the poor performance of alfalfa under low pH soil conditions.

This damage ultimately results in stunted alfalfa stands, with reduced persistence and susceptibility to other abiotic and biotic stresses (Hall et al., 2004; Sumner et al., 1986). Though lime and gypsum applications improve alfalfa performance, these soil amendments generally do

not permeate deep enough into the subsoil to accommodate vast root systems that can reach more than 6 m deep (Undersander et al., 2011). Soil amendments can also be costly and impractical long-term solutions in many field settings, especially in marginal lands and developing countries (Bouton, 1996). Therefore, the best approach for cultivating alfalfa in acidic soils in the long term is to develop a low pH and Al-tolerant cultivar.

Understanding the genetic basis of low pH and Al-tolerance in alfalfa has been the target of a great body of research over the last three decades (Barone et al., 2008; Bouton, 1996; Bouton & Sumner, 1983; Bouton et al., 1986b; Dall'Agnol et al., 1996; Khu et al., 2010; Khu et al., 2012; Ma et al., 2018; Narasimhamoorthy et al., 2007; Pan et al., 2008; Parrot & Bouton, 1990; Sledge et al., 2002). However, these efforts have not resulted in a tolerant cultivar release. There are many factors intrinsic to alfalfa breeding that have slowed the improvement of this trait. As a perennial, alfalfa must undergo three to five years of phenotypic evaluation before selections can be made for recurrent phenotypic selection (RPS). This breeding approach, while effective, is limited by the resources required to accommodate large, diverse populations that must be harvested multiple times a year for multiple years, exhibit significant genotype x environment interactions, and have low heritability of many important quantitative traits, such as yield (Li & Brummer, 2012). Continuous genetic segregation, non-additive genetic effects, and environmental influences lead to noisy phenotypes and less efficient selection (Acharya et al., 2020; Annicchiarico et al., 2015; Casler, 1998). Additionally, many other economically important traits must be considered, such as persistence, nutritional value, and fall dormancy ratings (FDR), which may be uncorrelated or negatively correlated with DMY (Casler, 2001). Because of the multifaceted nature of low pH and Al-stress, tolerance is expected to be a highly

quantitative trait, including alleles associated with improved nutrient efficiency and Al-tolerance or exclusion mechanisms (Khu et al., 2010; Ma et al., 2018).

The objective of this research was to evaluate the low pH and Al-tolerance in a selected panel of half-sib families using a multi-year, multi-location field phenotyping system. Dry matter yield (DMY), fall dormancy rating (FDR), and persistence were measured in six environments over the course of four years under both low pH and adjusted pH field conditions. Acid tolerance was assessed using a ratio of genotype performance under low pH stress and under adjusted pH conditions over the average performance of both trials. Heritability of DMY under both conditions and the effect of low pH stress on FDR and persistence were evaluated. The Acid Soil Adaptability Index (ASAI) (Howeler, 1991) was used to select top-performing families, and the best individuals within each family were selected to maximize genetic gain for the next round of recurrent phenotypic selection.

Materials and Methods

Population development and field establishment

A set of 966 Plant Introductions (PIs) from the NPGS collection was planted in a low pH soil (pH = 4.90) at the Black Shank Farm in Tifton, GA (31.5030° N, -83.5450° W), in 2014. After four years of evaluation, the most vigorous surviving plants were dug and planted in Athens, GA, in a field with an equivalent water pH of 5.12 and evaluated for three years. 133 selected PIs were dug from the field and crossed together using bee cages. The resulting half-sib seed was established in the greenhouse, and seedlings were transplanted in two field trials at the West Unit of the J. Phil Campbell Research (JPC) and Education Center in Athens, GA (33.8693° N, -83.4499° W). One field trial was established in a natural low pH field site (pH=4.90, extractable

aluminum=10.41 mg kg^{-1}) and the other trial was in an adjusted pH field site which received 405 kg ha^{-1} of Pennington Fast Acting Lime before planting (pH=6.37, extractable aluminum=2.29 mg kg^{-1}) in Athens, GA in May 2020. Each field site is characterized by highly weathered Cecil sandy clay loam soils (clayey, kaolinitic, thermic, Typic Kanhapludults), which are typical of the Piedmont region. Each trial consists of 138 entries (133 half-sib populations and 5 checks, including 2 commercial cultivars developed for the southeast and 3 populations developed in the lab and greenhouse for Al-tolerance) in a randomized complete block design (RCBD) with two replications. Each plot consisted of eight individual plants from the same family, equally spaced in a 5 ft row with 2.5 ft between rows. Both trials were replicated by stem cuttings and used to establish an identical field experiment in the fall of 2022 at the University of Georgia's Animal Science Farm in Tifton, GA (31.4996 ° N, -83.5312° W). The low pH field site in Tifton has a pH = 4.9 and an available aluminum of 11.38 mg kg^{-1} and the adjusted pH site has a pH = 7.07 and an available aluminum of 0.01 mg kg^{-1}. Both field soils are characterized as Tifton series clay loams (fine-loamy, kaolinitic, thermic Plinthic Kandiudults). The fields were managed according to recommendations by UGA Extension (Hancock et al., 2015), and soil tests were conducted at the University of Georgia Soil, Plant, and Water Laboratory (Athens, GA) to determine pH, exchangeable Al, and fertilization requirements using the 1 M KCl method (Reeve & Sumner, 1971).

Phenotypic data collection

Phenotypic data, including dry matter yield (DMY) in g, plant count per plot, and fall dormancy ratings (FDR), were collected throughout 2021-2024 in Athens and 2023 and 2024 in Tifton. Each row plot was harvested at 10-25% bloom every 25-28 days throughout the growing season (roughly May-October each year). DMY was determined by measuring the fresh weight of each

plot in the field, drying representative samples in a convection dryer for 48 hours, and multiplying fresh weights by the dry weight percentage. A few harvests in 2022 and 2023 in Athens had to be discarded due to significant deer damage (July and September 2022 and August and September 2023). Total yield per year (TY) was calculated by summing the DMY from each harvest over the growing season, and Average yield per cut (AY) was calculated by dividing the TY by the number of cuts included for each year.

Persistence was measured by regularly counting the number of individual plants per plot. Plant counts were taken at least twice a year in all locations and conditions. The plant count taken closest to the first harvest was averaged with the plant count taken closest to the final harvest to give an average plant count per plot for each condition x location x year combination. Average plant count per plot across environments and conditions was assessed to determine the effect of low pH soil conditions on persistence, as well as to identify families with superior persistence under low pH field stress.

To assess FDR of the half-sib population, the height in cm of the tallest, shortest, and intermediate individuals were measured in each family-row plot. Plant heights were recorded following the final harvest on December 20, 2021, and October 24, 2022, in Athens. The same three plant heights were measured for all five replications of the eleven check cultivars of each dormancy class, which was used to create a regression equation that allowed FDR to be assigned to each family based on average height.

In Tifton, it was discovered post-establishment that replication 1 in the adjusted pH section was being affected by nematodes. To account for this pressure while reducing the amount of data removed, the AY for each entry in rep 2 was compared to the DMY in rep 1. Entries that

yielded more than 50% less in replication 1 than they yielded in replication 2 were removed (Supplementary Figure S1). 6 plots were removed from replication 1 in 2023, and 51 were removed in 2024.

Yield data analysis

DMY data for all locations, years, and conditions were compiled in Microsoft Excel (version 2412), and summary statistics were generated in RStudio (version 2025.5.0.496). Variation across conditions and environments was evaluated to assess the effect of low pH field soils on yield performance in this population, as well as to identify superior family genotypes. DMY under the adjusted pH condition was used to predict DMY under the low pH condition to evaluate whether testing in low pH field soils is explicitly necessary to identify acid-tolerant genotypes.

Genotype, environment, and genotype-by-environment (GxE) interactions were evaluated using restricted maximum likelihood (REML) mixed model analysis. Multiple model parameterizations were tested, and the model with the lowest Akaike Information Criterion (AIC) was selected to achieve the best balance of goodness of fit and model complexity. The final model included genotype, environment (year x location), and GxE as random effects, with soil pH condition and average plant count per plot included as fixed effects.

Spatial analysis was conducted using the SpATS (Spatial Analysis of Field Trials with Splines) package in RStudio to account for spatial heterogeneity within each soil pH condition. For each environment (year x location combination), models were fitted with genotype as a fixed effect and spatial trends modeled across row and range positions. Model fit and assumptions

were verified via residual diagnostics, including QQplots, histograms of residuals, and residual vs. fitted plots (Supplementary Figure S2).

2.4 ASAI

Tolerance of alfalfa genotypes to low acid soil was assessed according to the Acid Soil Adaptation Index (ASAI) as described in Howeler (1991): $\boldsymbol{ASAI = \frac{Ys\ x\ Yp}{\mu s\ x\ \mu p}}$, where ASAI = Acid soil adaptation index, Ys = Yield in low pH condition, Yp = Yield in normal pH condition, μs = Average yield in low pH condition, μp = Average yield in normal condition. The index is such that tolerant genotypes have ASAI>1 and non-tolerant genotypes have ASAI<1. ASAI values were assigned to family genotypes separately for each year x location combination and across all environments.

2.5 Heritability

Heritability (H^2) was estimated using three approaches. Heritability was calculated separately for the adjusted and low pH conditions on a family-means basis (H^2_f) as: $\boldsymbol{H^2_f = \frac{V_g}{V_g + \frac{V_{gxe}}{L} + \frac{V_e}{LR}}}$, where V_g is the genotypic variance, V_{gxe} is the genotype by environment interaction, V_e is the residual variance, L is the number of environments, and R is the number of reps (Holland et al., 2002). Variance components were derived from a mixed model with genotype, environment, genotype x environment, and average stand count as random effects. L is equal to 6 (4 years of data in Athens and 2 years in Tifton), and R is equal to 2. In addition to the general model, heritability was estimated using Piepho's model: $\boldsymbol{H^2_{Piepho} = \frac{\sigma^2_g}{\sigma^2_g + v^{BLUE}_{\Delta\ldots}/2}}$ and Cullis' model: $\boldsymbol{H^2_{Cullis} = 1 - \frac{\bar{v}^{BLUP}_{\Delta}}{(2 \cdot \sigma^2_g)}}$, which are robust to unbalanced research designs (Piepho & Mohring, 2007; Schmidt et

al., 2019). The H^2cal function (inti R package) was used to calculate $\boldsymbol{H_f^2}$ across environments, accounting for the number of environments and replicates. $\boldsymbol{H_{Piepho}^2}$ was estimated by fitting the same linear mixed model with lmer (lme4 package) and extracting adjusted means with emmeans. Finally, $\boldsymbol{H_{Cullis}^2}$ was estimated using empirical best linear unbiased predictors (BLUPs) from the model, calculated via the ranef function. Broad-sense heritability estimates were also calculated separately for each location and year and condition combination using the equation: $\mathbf{H^2} = \frac{V_g}{V_g + \frac{V_e}{R}}$.

Results

The effect of low pH stress on DMY

Low pH field conditions reduced DMY by 52.7 g per row plot per cut ($p < 0.001$) compared to the adjusted pH condition, though the magnitude of difference varied substantially by year and location (Figure 1). The trend was reversed in Tifton 2024, with the low pH condition

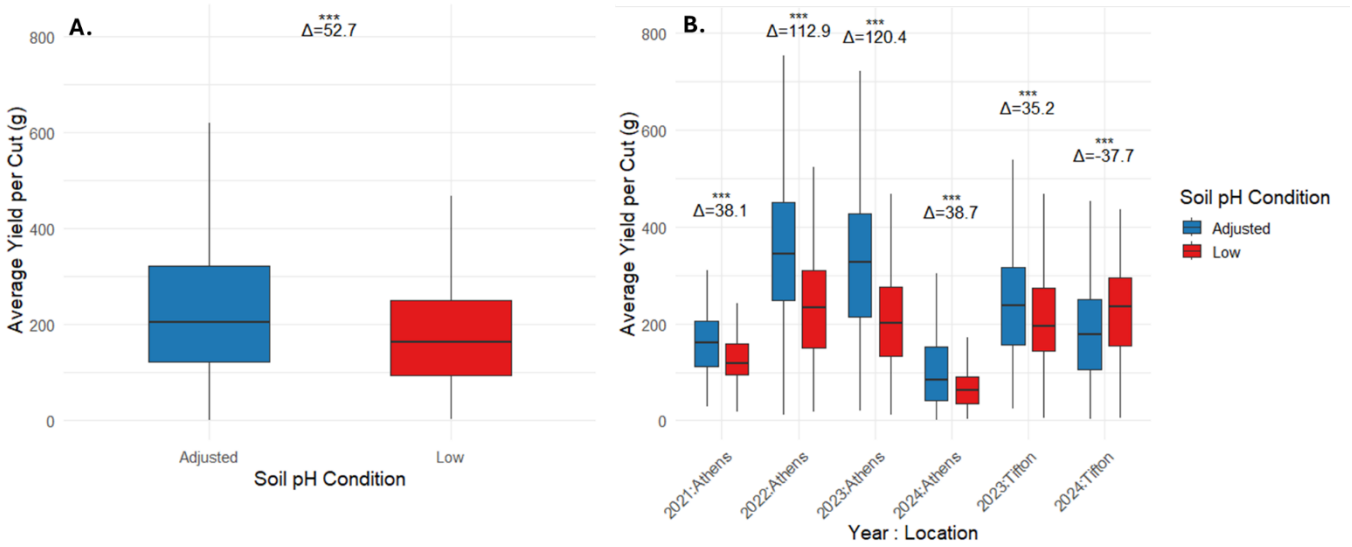


Figure 1. Variation in average dry matter yield (DMY) per cut across pH conditions overall (A) and by environment (Year x Location) (B) under adjusted and low soil pH conditions. Delta values (Δ) indicate the difference in mean yield between adjusted and low pH conditions. Asterisks denote statistically significant differences based on t-tests ($p < 0.001$).

outyielding the adjusted by 37.7 g per cut. Paired student t-tests in all environments showed that the yield difference was statistically significant ($p < 0.001$). Means and standard deviations of AY and TY varied significantly by environment and condition (Table 1).

Table 1. Means, standard deviations, and maximum of average yield per cut (g) and total yield per year (g) by environment (Location x Year) and soil pH condition.

Environment	Condition	Mean_AY	SD_AY	Max_AY	Mean_TY	SD_TY	Max_TY
				g DMY per plot			
Athens 2021	Adjusted	163	68	436	327	136	871
Athens 2021	Low	125	48	263	249	98	525
Athens 2022	Adjusted	348	142	806	1045	427	2419
Athens 2022	Low	236	107	524	707	321	1571
Athens 2023	Adjusted	328	152	793	985	455	2378
Athens 2023	Low	208	101	467	624	304	1402
Athens 2024	Adjusted	108	89	431	538	447	2157
Athens 2024	Low	69	43	260	344	215	1301
Tifton 2023	Adjusted	244	110	646	975	439	2582
Tifton 2023	Low	209	91	468	834	363	1870
Tifton 2024	Adjusted	187	105	548	1121	631	3286
Tifton 2024	Low	224	103	436	1347	619	2617

Low pH and adjusted pH soil conditions were well-correlated with a Pearson's coefficient of 0.597 across all environments. Fitting a simple linear model predicting AY under adjusted pH conditions using AY under low pH shows that the relationship is highly significant with a $p<0.01$ and an adjusted R^2 value of 0.36 (Figure 2). Mixed model analysis predicting performance under low pH conditions using yield performance in the adjusted pH as a fixed effect and average plant count in the low pH condition as a random effect produced a more robust model. The intraclass correlation (ICC) for average plant count showed that average plant count per plot accounts for 22% of the variation in yield under low pH conditions and 32% of the variance was accounted for by average DMY in the adjusted pH condition. This can also be shown by simple regression by plotting AY under low pH by AY under adjusted pH conditions (Figure 2).

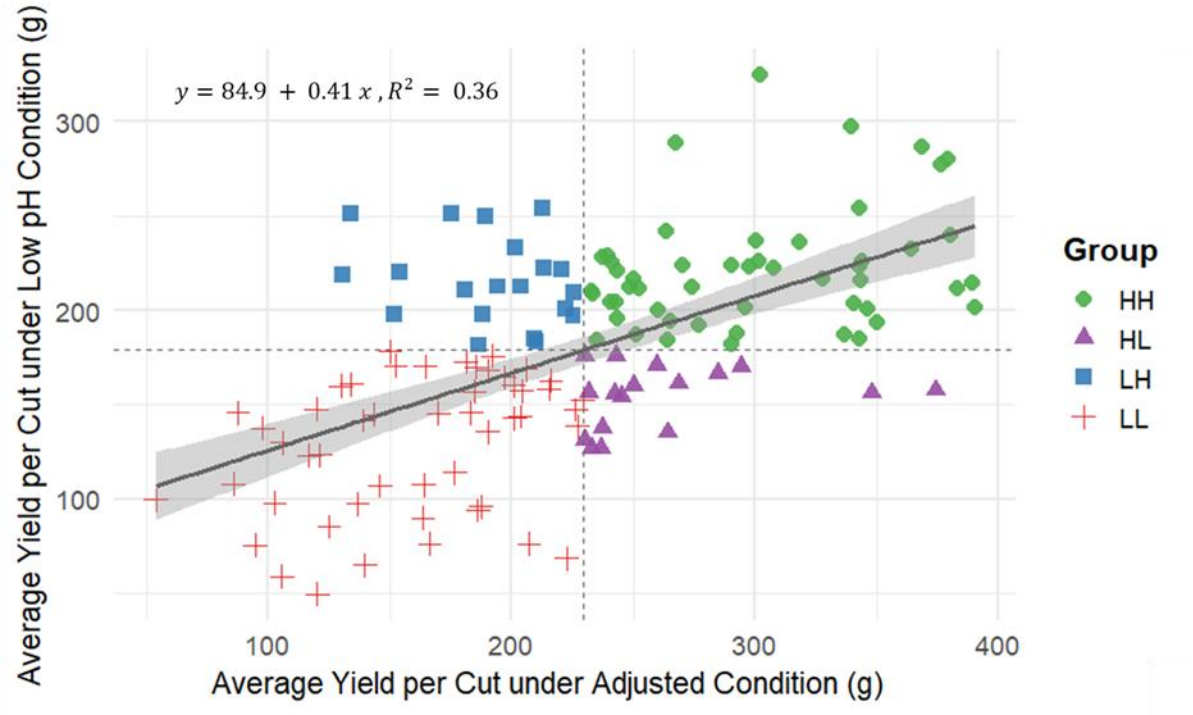


Figure 2. Graph depicting the correlation between DMY under low pH conditions and under adjusted pH conditions. The dotted gray cross corresponds to the average yield in both conditions. 51 genotypes yielded above average in both conditions (green circles, HH), 17 genotypes yielded above average in the adjusted condition and below average in the low pH condition (HL), 20 genotypes yielded above average

in the in the low condition but below average in the adjusted condition (LH), and 50 yielded below average in both conditions (LL).

A linear mixed model predicting AY was fit using restricted maximum likelihood (REML), with soil pH condition and average plant count per plot included as fixed effects and Genotype, Environment (year x location), and GxE as random effects (Table 2). Degrees of freedom and significance for fixed effects were estimated using Satterthwaite's approximation. This parameterization reduced AIC of the initial model that included condition as a fixed effect and genotype as a random effect (AIC = 38988) to an AIC = 36266. A plot in the low pH condition produced 61 g less DMY than a plot in the adjusted pH condition (t = -23.252, df = 2403.574, $p < 0.001$). Similarly, each additional plant per plot results in 33.7 g more DMY (t = 40.03, df = 3024.327, $p < 0.001$). The environment (location x year) explained the largest amount of variation (6589.2), six times more than the genotypic effect (1104.3), and the GxE term explained a modest amount (253.1). A considerable error variance remained (5325.7), indicating residual noise in the data.

Table 2. ANOVA results from mixed model analysis predicting AY with condition, plant count per plot as fixed covariates, and genotype, genotype x environment, and environment as random effects.

Effect	Estimate	Std. Error	t-value	df	p-value	Variance	Std. Dev	Pr(>Chisq)	Effect Type
(Intercept)	69.42	33.56	2.07	5.236	<0.1				Fixed
Condition (Low vs Adjusted)	-61.10	2.63	-23.25	2403.57	<0.001				Fixed
Average Plant Count	33.73	0.84	40.03	3024.32	<0.001				Fixed
Genotype						1104.3	33.23	<0.001	Random
Genotype x Environment						253.1	15.91	<0.01	Random
Environment						6589.2	81.17	<0.001	Random
Error						5325.7	72.98		Residual

Acid Tolerance (ASAI)

ASAI values were used to approximate the acid tolerance of each half-sib family in this population and were calculated using AY and TY for the Athens and Tifton data separately, as well as combined (Figure 3).

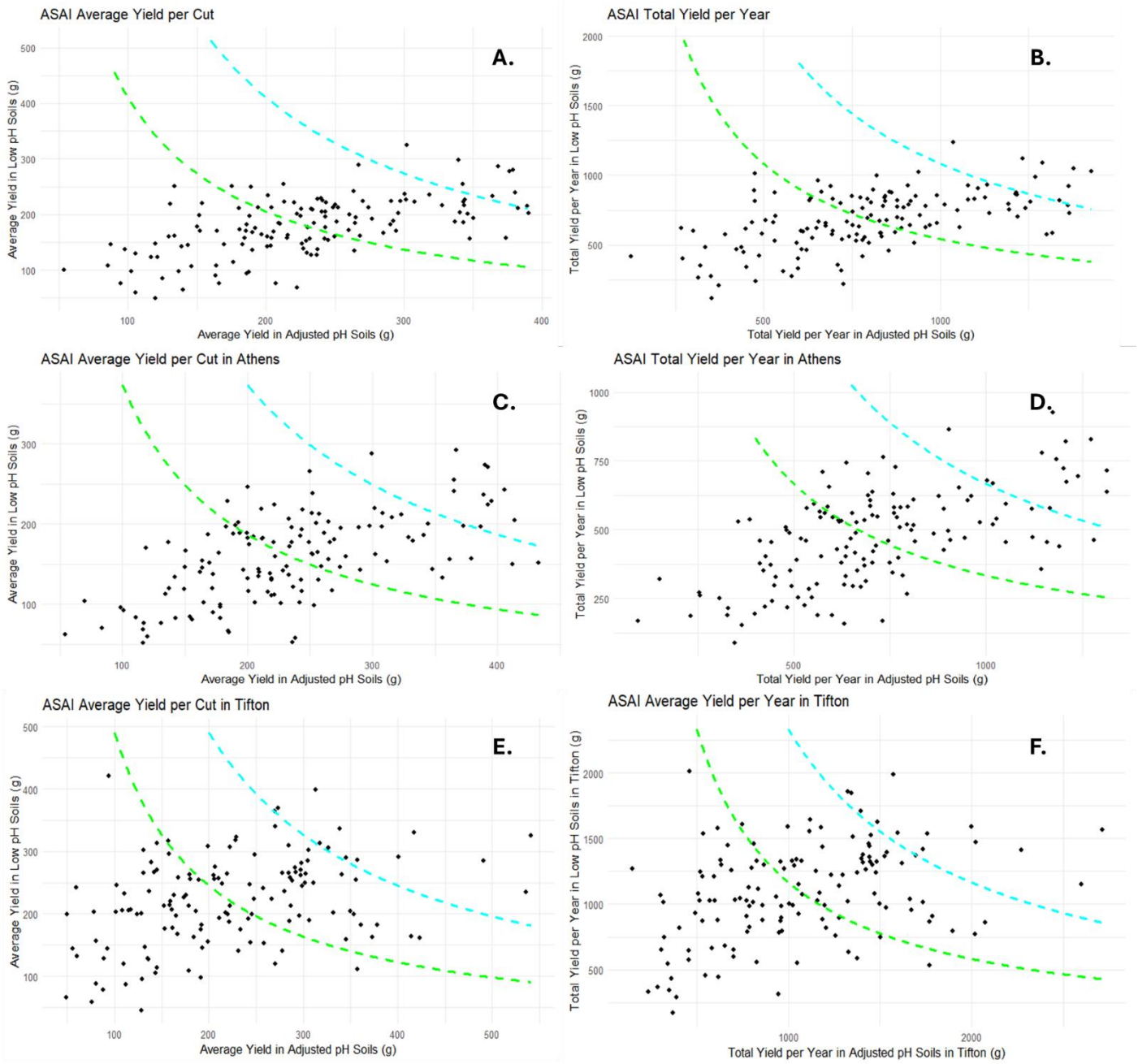


Figure 3. ASAI graphs with yield in the adjusted pH field on the x-axis and yield in low pH field on the y-axis. The green dashed line corresponds to ASAI=1 and the blue dashed line corresponds to ASAI=2. Graphs are shown for the overall harvest data (A, B) each location separately (C-F) using both AY (average yield per cut) (A, C, E) and TY (total yield per year) (B, D, F).

The stability of the ASAI trait across environments was assessed by examining which genotypes were consistently classified as tolerant (ASAI>1) using separate ASAI calculations for each year x location combination. While there was variation in the relative rankings of genotypes, the same

genotypes were consistently identified. 16 genotypes were identified as tolerant in all 6 environments and 56 were identified in at least 4 environments (Figure 4). All ASAI values by environment, as well as overall, can be viewed in Supplementary Table S1.

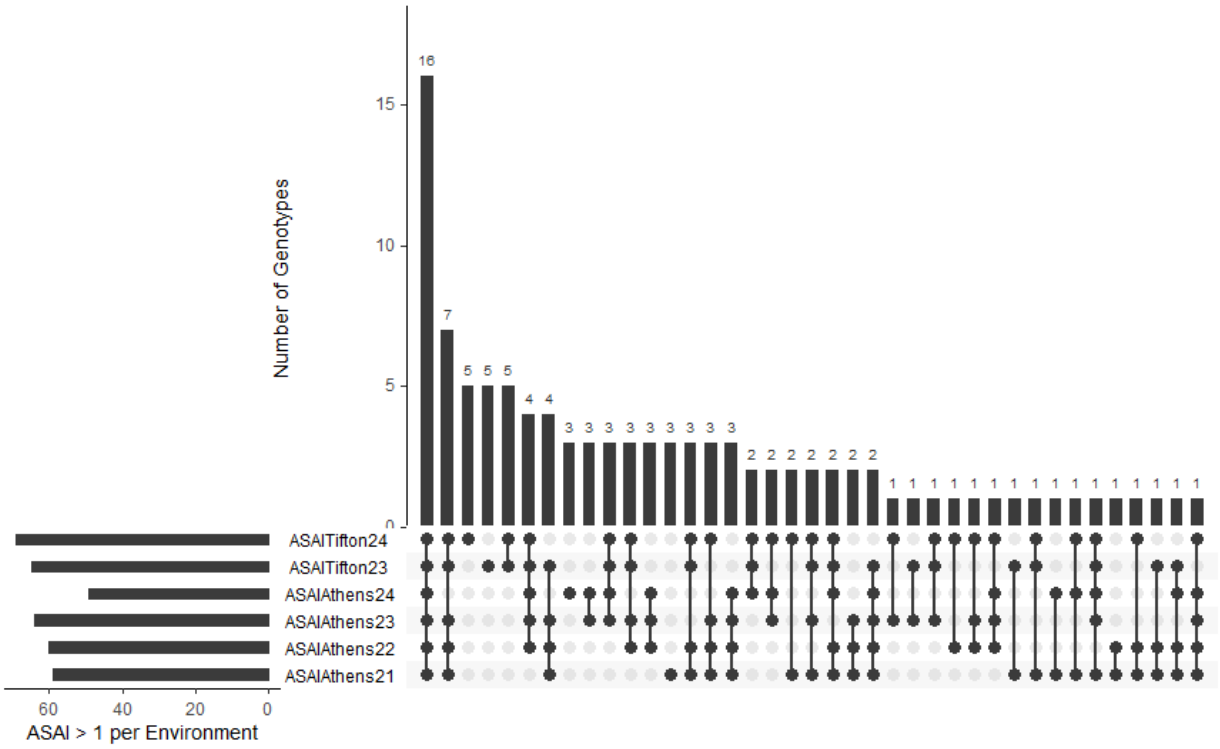


Figure 4. Depicts the number of genotypes identified as tolerant (ASAI>1) across different environments (year x location combinations). The number shown on the bar graph identifies how many genotypes are identified in each combination of environments (black dots below the plot).

Heritability of DMY and ASAI

$H^2_{Family-means}$, H^2_{Piepho}, and H^2_{Cullis} was calculated for the complete dataset and for the low and adjusted pH conditions separately (Table 3). Heritability varied substantially across environments and ranged from 0.344 to 0.815 (Table 4).

Table 3. Heritability estimates for average yield per cut (AY) by pH condition and overall using three models.

Condition	Family-means H^2	Piepho H^2	Cullis H^2
Adjusted	0.780	0.754	0.876
Low	0.751	0.736	0.866
Overall	0.802	0.800	0.898

Table 4. Broad-sense heritability estimates for yield across each unique Condition x Location x Year combination.

Environment	Condition	H^2	Variance (Genotype)	Variance (Residual)
Athens 2021	Adjusted	0.466	1382.7	3219.7
Athens 2021	Low	0.344	482.3	1867.1
Athens 2022	Adjusted	0.550	7610.1	12618.4
Athens 2022	Low	0.571	4432.7	6748.4
Athens 2023	Adjusted	0.553	8719.8	14289.9
Athens 2023	Low	0.478	3228.3	7163.1
Tifton 2023	Adjusted	0.756	7954.5	5033.1
Tifton 2023	Low	0.422	2172.8	6050.5
Athens 2024	Adjusted	0.288	1275.4	6383.2
Athens 2024	Low	0.388	442.8	1420.0
Tifton 2024	Adjusted	0.815	9240.9	3536.3
Tifton 2024	Low	0.447	3023.1	7574.4

The $H^2_{Family-means}$ of ASAI was estimated using a linear mixed-effects model predicting ASAI values calculated separately for each environment (location x year) The model was fit with genotype and environment as random effects and variance components were extracted. The $H^2_{Family-means}$ was estimated at 0.854, slightly higher than the heritability estimated for DMY using the same formula. ASAI showed very similar correlation patterns to DMY across

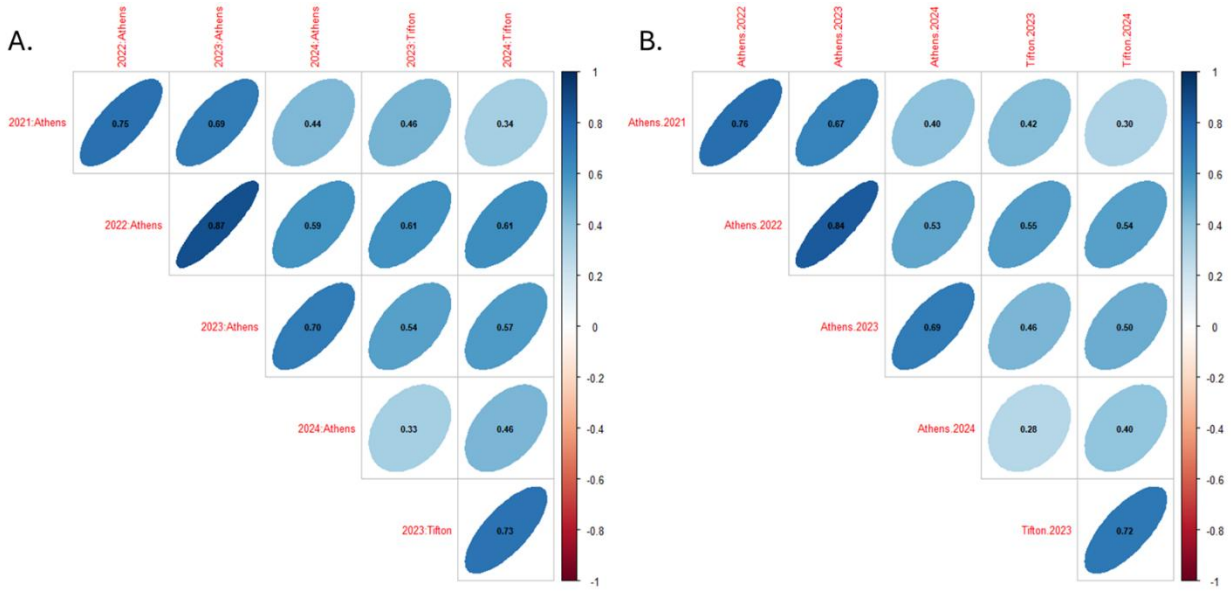


Figure 5. Correlation plots showing the Pearson's pairwise correlation coefficient for AY (A) and ASAI (B) with dark blue indicating strong positive associations, dark red indicating strong negative correlations, and light colors indicating weak associations.

environments, as expected, ranging from 0.28 (Tifton 2023-Athens 2024) to 0.84 (Athens 2023-Athens 2022) (Figure 5). In general, higher correlations were observed within locations with correlations decreasing across time (i.e., data taken in Athens 2021 is more correlated with Athens 2022 than Athens 2024).

Fall Dormancy Ratings (FDR)

Paired t-tests show that FDR was significantly affected by low pH and AL-stress, on average 1.47 classes lower (t = -16.096, df = 137, $p < 0.01$) than the adjusted pH condition. Average plant height was similarly decreased in the low pH condition, on average 4.87 cm shorter (t = -19.134, df = 137, $p < 0.01$).

The overall correlation between FDR and AY is 0.261 and between average plant height and AY is 0.299. Analysis of variance showed that FDR, modeled as the sole fixed effect, was a significant predictor of AY ($F(1, 545) = 34.02$, $p < 0.01$), though the adjusted R^2 was small at

0.057. This indicates that while there is a meaningful correlation between the AY and FDR, the effect size is either small or the signal too noisy to obtain high predictive power. Modeling with FDR and condition as fixed effects showed that for every one increase in fall dormancy class, AY increased an average of 34.06 g ($F(2, 544) = 20.27$, $p < 0.01$). When evaluating the low and adjusted pH conditions separately, FDR is not a significant predictor of AY in the low pH condition ($F(1, 272) = 2.634$, $p = 0.106$) but is significant in the adjusted pH condition ($F(1, 271) = 13.22$, $p = 0.0003$) (Figure 6).

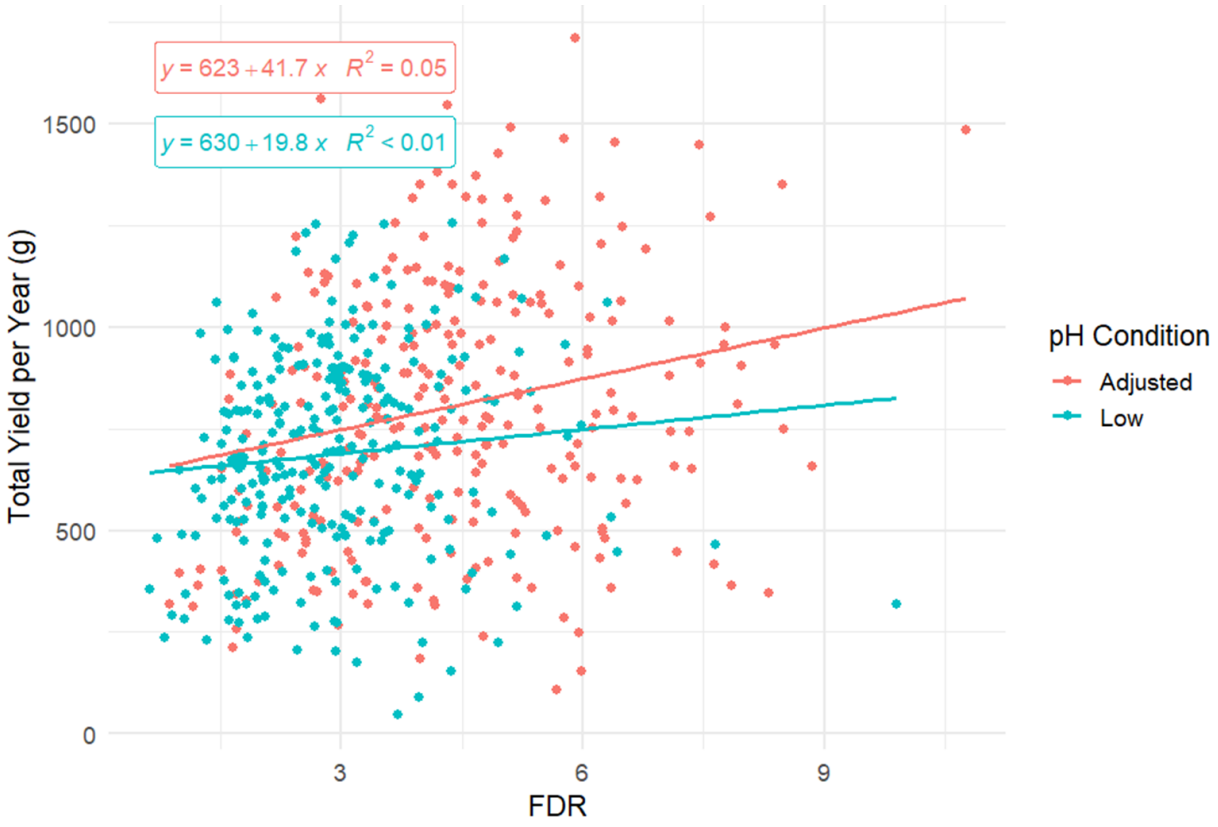


Figure 6. Dot plot that shows the difference in the correlation between TY (g) and FDR across pH conditions.

Overall, higher averages and greater variation were observed in the adjusted pH condition (FDR: mean=4.38, SD=1.68; AY: mean=233 g, SD=145) compared to the low pH condition (FDR: mean=2.90, SD=1.22; AY: mean=182 g, SD=105). This is likely due to genetic potential, which

can be more fully expressed without stress (Figure 7). Under stressful conditions, the distribution

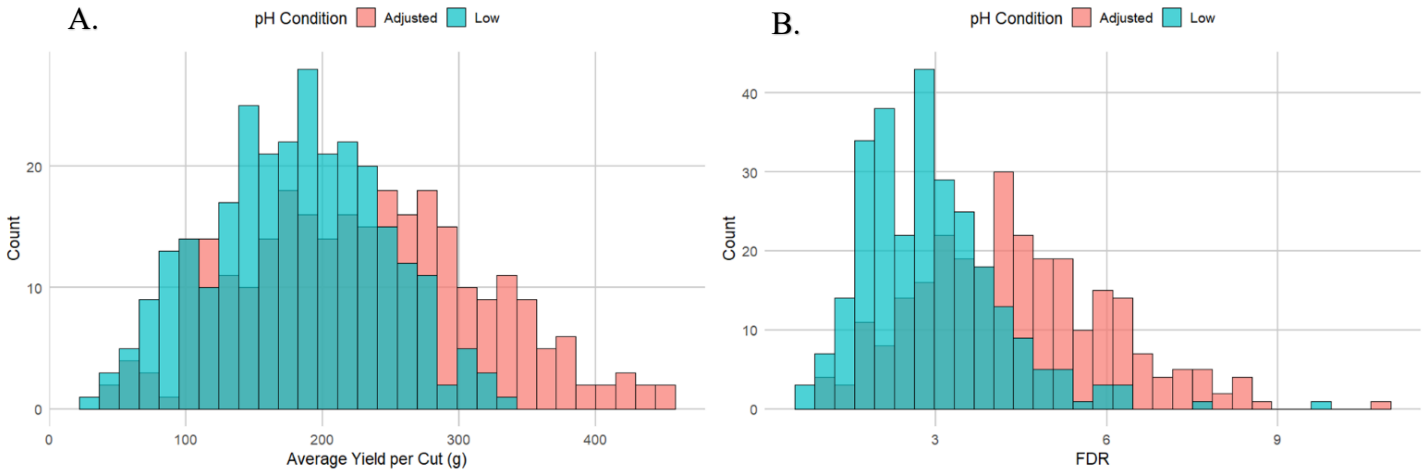


Figure 7. Histograms showing the distributions for AY (g) in box A and FDR in box B across pH conditions with the adjusted shown in salmon and the low shown in teal. The left skew and smaller standard deviation of the low pH compared to the adjusted pH is evident.

becomes more compressed, which may explain why FDR was not significantly correlated with DMY in the low condition but was significant in the adjusted. Because the dormancy checks were grown in adjusted pH field soil, the FDR from the low pH condition should be considered an indication of how low pH and Al-stress affect dormancy timing, but not an accurate measure of dormancy class for the genotype.

To see if this trend differs among tolerant genotypes, the 16 genotypes that were identified as tolerant (ASAI>1) across all environments (year x location combinations) were considered. The average FDR in the adjusted pH condition was 4.57 with an SD=1.15 and in the low pH condition the average FDR is 3.05 with an SD=1.12. This shows that FDR is positively

correlated with DMY and acid tolerance under both low and adjusted pH conditions (R=0.413), which is a much stronger correlation than observed in the overall population (R=0.261).

Persistence

Persistence, measured as the average of the number of plants per plot each year, was significantly different across pH conditions. Across all years in Athens, the low pH condition had, on average, 0.217 more plants per plot than the adjusted ($p = 0.030$) and in Tifton, the low pH condition had 0.398 more plants per plot than the adjusted ($p < 0.01$). These differences varied significantly by year, with the only significant differences occurring in 2024 in both Athens and Tifton (Table 5).

Table 5. Shows the average number of plants per plot by Location x Year and Condition. P-values were determined by t-test to identify significant differences in persistence between conditions. Single asterisk * indicates P<0.05 and double asterisk ** indicates P<0.01.

Location	Year	Average plants per row in Adjusted pH	Average plants per plot in Low pH	p-value
Athens	2021	6.754	6.743	0.934
Athens	2022	4.793	5.016	0.230
Athens	2023	3.959	4.273	0.064
Athens	2024	2.654	2.995	0.022*
Tifton	2023	5.420	5.786	0.051
Tifton	2024	3.888	4.434	0.003**

Viewing persistence instead as the percentage dead per plot by location and condition shows that after two years in Tifton, there is little difference (0.4%) between survivorship, as well as after four years in Athens (0.6%). The direction of the difference is different by location, with the low pH section having marginally greater persistence in Tifton, while the adjusted pH section has the advantage in Athens.

Discussion

Effects of low pH and Al-stress on DMY

As expected, low pH soil conditions significantly reduced DMY overall. The mean and spread of DMY were reduced under low pH and Al-stress (Figure 4). The average difference between yields is seemingly small at 61 g but given the small row-plot size used in this field trial (5 ft x 2.5 ft), that difference corresponds to a decrease of 525 kg ha^{-1} per cut. The correlation of yield in space-planted plots versus densely planted sward plots is variable due to differences in competition among plants, but row-plot evaluations are common in early stages of forage breeding programs to accommodate larger population sizes (Casler et al., 1996). The difference in DMY from adjusted to low pH conditions varied significantly by environment, indicating that year and location are important drivers of DMY. Significant GxE effects are common in alfalfa research, but are not a useful source of variation for breeding (Acharya et al., 2020; Brummer & Casler, 2014). Although environmental and GxE effects were significant, the substantial genotypic variance observed for yield suggests that there is potential for genetic improvement within this population.

Yield in the adjusted pH section and the low pH section were moderately-correlated with an R^2 value of 0.351. This suggests that performance across pH conditions is positively correlated, but significant variation is unaccounted for using only the adjusted pH condition to predict performance under low pH stress. This highlights the benefits of direct field phenotyping in both naturally low pH soils and amended soils. Given the investment of resources required to maintain and phenotype in both conditions across years and locations, it may be more efficient to

select only in the low pH environment at early stages of the breeding process and validate that performance is maintained in adjusted pH soils at later stages.

The yield difference in Tifton 2024 was flipped, with the low pH section outyielding the adjusted pH section even after accounting for nematode damage. A soil test in December of 2023 showed that the low pH section in Tifton had higher K (average 56 mg kg^{-1}) and P (average 80 mg kg^{-1}) than the adjusted pH section (average 40 K mg kg^{-1}, 49 P mg kg^{-1}), despite the highly acidic and Al-rich soil (pH=4.90, bioavailable Al=11.58 mg kg^{-1}) (full soil test results can be viewed in Supplementary Table S2). Fertilization recommendations based on the soil test were followed to restore the low pH and adjusted pH fields to similar fertility. However, this difference in fertility suggests higher OM content in the low pH field site and may explain some of the improved performance of the low pH condition in Tifton. If so, this indicates that soils with high fertility can overcome some of the negative impacts of low pH and Al-stress on alfalfa yield and performance. This is consistent with previous hypotheses indicating the primary effect of low pH and Al-stress is reducing nutrient uptake and availability, especially of P (Kochian et al., 2004; Zhao et al., 2014; Zheng, 2010). Other soil traits, such as CEC and water holding capacity, might also explain this unexpected result, but were not measured in this study.

ASAI and heritability of DMY

The complex nature of the data set allowed acid tolerance to be estimated in several ways, using different subsets of the data to calculate ASAI values. While the relative rankings of the genotypes and the magnitude of difference varied across environments, the same genotypes were consistently identified overall. This is evidenced by the 56 genotypes that were identified in at least four environments (location x year combinations) (Figure 4) and emphasized by the 44

genotypes identified in both Athens and Tifton overall. The high degree of overlap is an indication that acid tolerance is being driven by genetics instead of environmental effects, despite significant GxE effects.

This high degree of overlap is validation that the field evaluation conducted in this study is effective for identifying tolerant genotypes and indicates that acid tolerance is a stable trait. Identifying 56 tolerant genotypes out of 138 total evaluated is a high proportion, especially given the difficulty of improving Al-tolerance in previous studies (Bouton, 1996; Dall'Agnol et al., 1996; Khu et al., 2012). This is likely due to the genetic diversity in the initial population and the round of selection that identified parental genotypes with improved low pH and Al-tolerance.

Overall heritability estimates for DMY (0.800-0.898) and ASAI (0.854) were relatively high for quantitative traits. Heritability estimates for DMY in alfalfa vary widely by study and population from extremely low (0.02) to extremely high (0.97) (Acharya et al., 2020; X. He et al., 2022). Heritability for DMY was a bit higher in the adjusted pH condition compared to the low pH but was generally stable across conditions. This may be due to the greater variation in yield in the adjusted pH which allows for more variation to be attributed to genotype. The high heritability for DMY and ASAI in this population is likely due to the extensive phenotyping effort in which yields by cut were averaged or summed to create 3,302 data points to describe 138 genotypes. This vast amount of data decreases phenotyping errors and helps provide an accurate evaluation of each genotype, despite significant environmental and GxE effects.

Effects of low pH and Al-stress on FDR and Persistence

The mean and standard deviation of FDR were significantly reduced under low pH soil stress. This suggests that low pH and Al-stress perturb dormancy timing, resulting in plants that go

dormant earlier in the season. This may be advantageous to alfalfa stands by allowing plants to retain energy reserves in root systems for more optimal growing conditions in the spring. Because the fall dormancy check cultivars were not grown in a low pH field site, the FDR from the low pH condition are not an estimate of the true dormancy ratings but reflect the physiological response of alfalfa to low pH field stress.

The average FDR observed in the adjusted pH section is somewhat lower than expected at FDR=4.38, with a minimum of 1.02 and a maximum of 9.34. This may be due in part to the check cultivars, which were developed in different climatic conditions (Teuber et al., 1998) or to the time of data collection, which occurred once temperatures dropped sufficiently to visually see reduced growth. For example, the check cultivar Bulldog505 (Entry 133) was given an average FDR of 2.77 when the known dormancy rating is 5, and the check Bulldog805 (Entry 132) was given an FDR of 5.62 when the known dormancy rating is 8. Given this reduction, selecting individuals with stable, high ASAI and an FDR>4 should ensure we are retaining semi- and non-dormant tolerant genotypes. Selections that go on to the next round of breeding will undergo further FDR characterization.

Overall, FDR had a small positive correlation with DMY (r=0.261). We saw an increased correlation of FDR and DMY in the highly stable tolerant genotypes (r=0.413), but this may be due to less noise in the dataset of 16 genotypes compared to all 138 genotypes. In any case, FDR is an important trait to consider when developing a cultivar for the southeastern USA and subtropics, as a non-dormant variety in a warm climate can be harvested up to 10 times a year,

resulting in higher yields for hay/baylage production or nearly year-round pasture-grazing (Acharya et al., 2020).

Persistence was not drastically different between the low pH and adjusted pH conditions. While the overall average plant count per plot was statistically significantly different across conditions, the average difference was less than 0.5 plants in favor of the low pH condition. Across environments, the difference was only significant in 2024 in Athens and Tifton, with the adjusted pH section having the edge in Athens after four years and the low pH section in Tifton having the edge after two. Average plant count per plot is an important factor in minimizing residual errors when conducting linear mixed model analysis, but it does not seem to have a large enough difference to be biologically relevant. This is likely due to the relatively short duration of the study. As alfalfa stands can persist for 10+ years, acid and Al-stress may take longer to reduce persistence significantly. This is evidenced by the increased mortality in the low pH condition after four years in Athens, though slight (Table 5). The slight advantage of the low pH section in Tifton may also be explained by differences in soil fertility, but further investigation is needed to determine survivorship in the long term.

Breeding Implications

The goal of this work is to contribute to the development of high-yielding, low pH and Al-tolerant cultivars that are adapted to the production conditions of the southeastern United States. The multi-year, multi-location, split-condition field phenotyping effort undertaken seems to be highly effective in screening for improved low pH and Al-tolerance in alfalfa. This method of evaluation is more labor and time-intensive than previously described methods involving greenhouse (Dall'Agnol et al., 1996; Khu et al., 2010; Sledge et al., 2002) or lab assays (Barone

et al., 2008; Parrott & Bouton, 1990; Rosellini et al., 2002; Tesfaye et al., 2001), but allows for selection of essential agronomic traits, such as DMY and FDR, in addition to Al-tolerance. Selection in the target environment helps ensure that these cycles of selection translate meaningfully to the development of an improved cultivar. The extensive data collected on this population improves the phenotypic accuracy and should translate to improved genetic gain in the next cycle of selection. Genotypes that had been developed through lab assays and greenhouse screening performed very poorly in the field, further emphasizing the value of field phenotyping (Supplementary Table S1).

Using the ASAI as a selection criterion ensures that selections have a high yield in both the low and adjusted pH conditions. Selecting only genotypes with high ASAI values across environments and FDR that are appropriate for the southeastern USA (semi-dormant: 5-7, non-dormant: 8-11) can maximize the desired genetic gain from this cycle of selection. This further emphasizes the benefits of directly phenotyping in the field environment, as FDR cannot be reliably estimated otherwise and is an important factor in cultivar development.

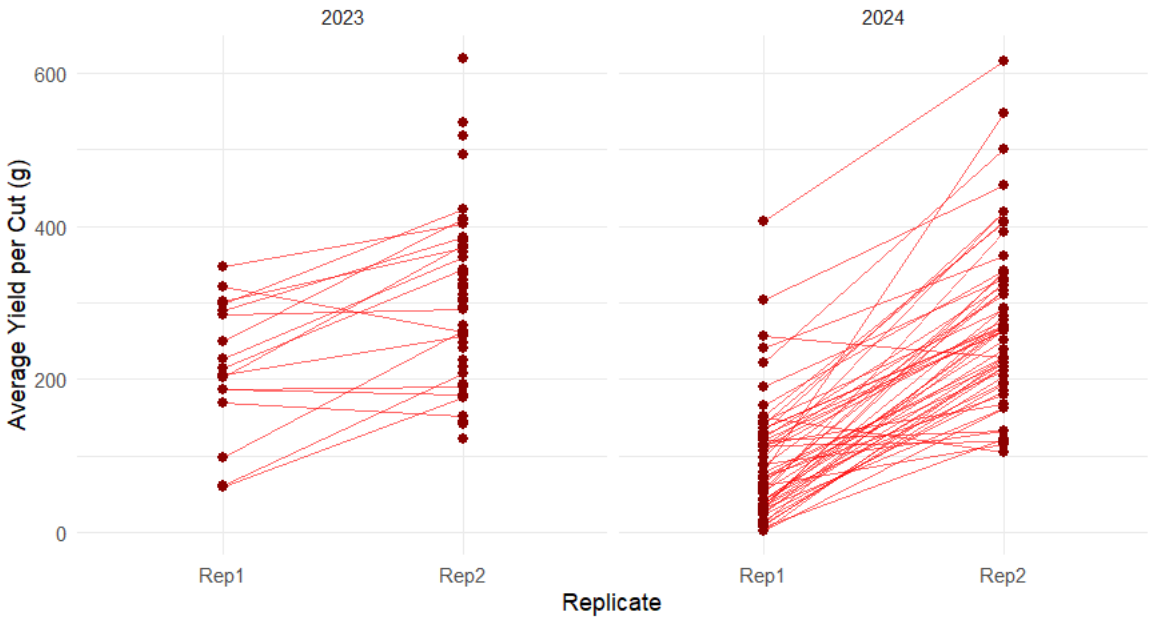
Future directions

This current research has culminated in identifying 56 alfalfa genotypes with improved tolerance to acidic field soils, including 21 with better performance than the best performing available commercial cultivar (Supplementary Table S1). Within-family selection will be conducted by visually selecting the most vigorous plants for each selected family in the low pH environment. These individuals will be intermated in greenhouse bee cages to generate the population for the next cycle of RPS. In addition to high yields under acidic field conditions, these selections will have the appropriate FDR for the production region of interest, as well as high persistence in warm, subtropical environments. These will go on to replicated, multi-location sward plot

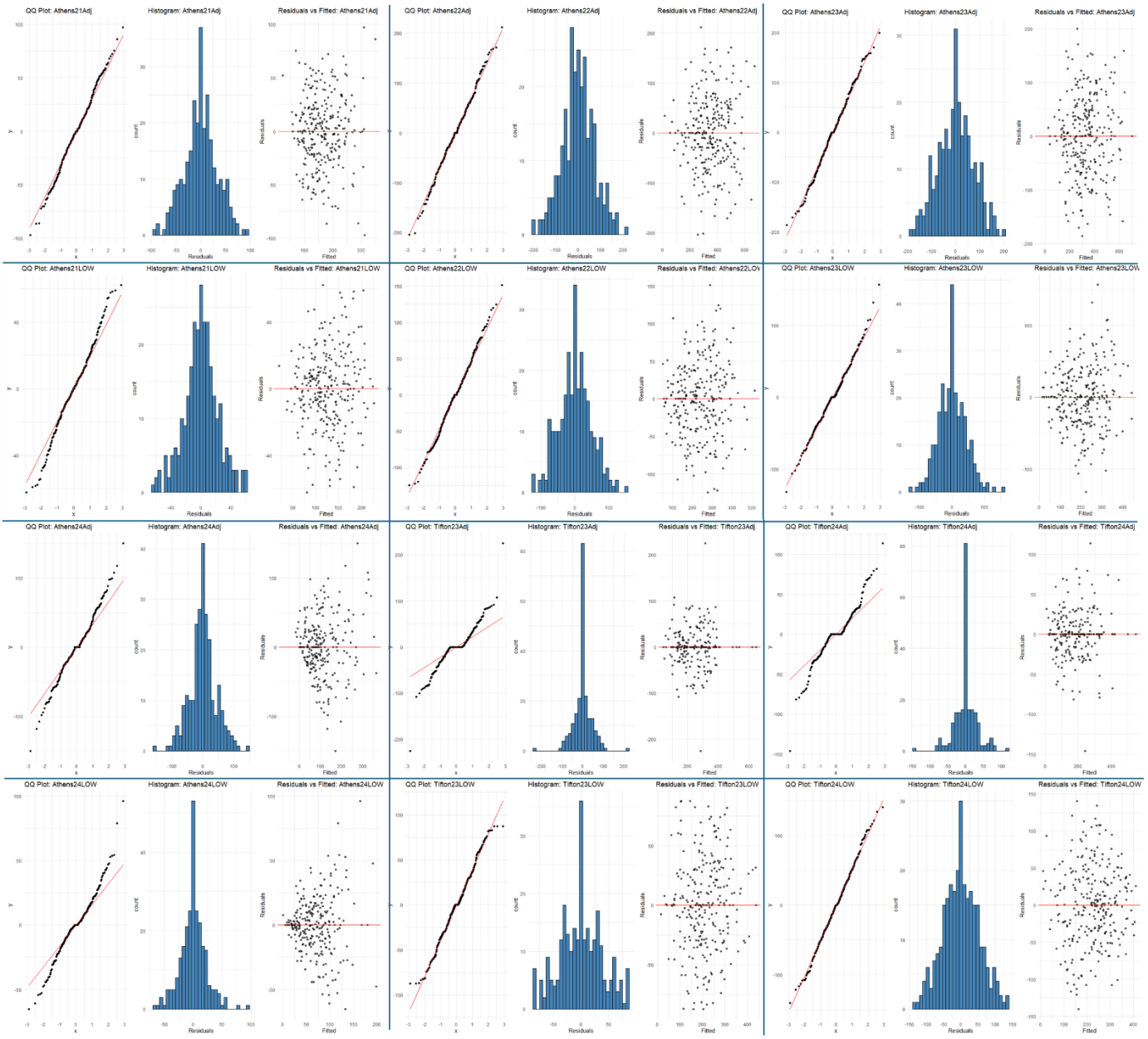
evaluations under low pH field conditions and continue to be cycled until sufficient tolerance and yield are achieved and a synthetic cultivar can be released.

Additional work is needed to decipher the molecular and physiological basis of acid and Al-tolerance in alfalfa. Current research using this panel for GWAS analysis is ongoing to identify molecular markers significantly associated with acid tolerance and high yield under low pH stress. Significant markers linked to genes that have been implicated in acid tolerance in other species may shed light on the genetic mechanism of tolerance utilized by alfalfa and aid in the development of genomic selection models to accelerate genetic gain by decreasing cycle time. In addition, selections from this population are being used in greenhouse rhizobox assays to see which root traits are significantly associated with acid and Al-tolerance as determined by field evaluation. If the findings are significant, this would allow germplasm to be screened much more quickly and cost-effectively in the greenhouse. Further understanding the genetics and root traits that underly the acid tolerance trait can also inform future genetic engineering approaches and add to the body of knowledge regarding low pH and Al-tolerance.

Supplementary Material



Supplementary Figure S1. Depicts the yield drop for the 56 entries that were removed from rep 2 to rep 1 in the adjusted pH section in Tifton, GA for 2023 and 2024. These entries yield more than 115.716 g (1 SD) less than they did in rep 2.



Supplementary Figure S2. QQplots, histograms, and residual vs fitted scatterplots from the modeling genotype as a fixed effect in the SPaTS package by environment and condition.

Supplementary Table S1. ASAI values by environment and overall, including a count of the number of environments where the genotype was identified as tolerant (n_env). AvgFDR is the average fall dormancy rating from the adjusted pH section. Entries with asterisks indicate check cultivars.

Entry	Athens: 2021	Athens: 2022	Athens: 2023	Athens: 2024	Tifton: 2023	Tifton: 2024	Overall	n_env	AvgFDR
Entry 10	2.50	2.76	2.91	3.01	1.73	1.78	2.38	6	4.14
Entry 105	1.69	1.90	2.29	5.71	1.37	2.21	2.08	6	4.71
Entry 123	1.54	2.69	3.12	4.40	1.68	2.56	2.48	6	4.66
Entry 134	1.03	1.59	1.66	4.12	1.30	2.28	1.71	6	7.79
Entry 15	1.45	1.57	1.11	2.23	1.65	1.16	1.39	6	4.77
Entry 2	2.21	2.26	2.46	3.67	2.05	4.11	2.50	6	5.12
Entry 21	2.49	1.76	2.55	3.88	1.55	2.26	2.17	6	4.29
Entry 49	1.15	1.53	1.62	1.87	1.63	1.44	1.48	6	4.09
Entry 50	1.08	1.01	1.24	1.56	1.48	1.49	1.27	6	4.61
Entry 6	1.47	1.65	2.20	1.08	2.16	1.38	1.79	6	5.77
Entry 65	1.42	1.80	1.83	1.56	1.02	1.29	1.49	6	4.75
Entry 74	1.67	3.26	2.02	1.28	2.42	3.01	2.36	6	4.24
Entry 8	1.69	2.40	2.85	2.52	1.30	1.23	2.04	6	4.45
Entry 93	1.71	1.72	1.97	1.07	2.20	2.30	1.76	6	3.65
Entry 94	2.04	2.19	3.03	6.83	1.91	1.64	2.40	6	4.52
Entry 1	1.14	1.38	1.10	0.07	1.41	1.41	1.16	5	3.67
Entry 107	1.19	1.04	1.23	0.73	1.09	1.88	1.27	5	5.65
Entry 108	2.11	2.06	0.72	1.54	3.11	1.67	1.64	5	6.63
Entry 113	0.87	1.82	1.70	1.34	1.29	1.87	1.52	5	3.83
Entry 124	1.07	1.31	1.33	0.53	1.22	1.98	1.31	5	4.74
Entry 130	1.14	2.32	2.07	0.93	2.25	2.20	1.86	5	3.29
Entry 133*	1.89	2.21	3.16	2.60	1.03	0.94	1.89	5	2.77
Entry 137	0.83	1.46	2.24	1.66	2.18	2.72	1.82	5	9.34
Entry 23	1.97	1.20	1.82	0.87	2.02	1.16	1.64	5	2.44
Entry 24	2.45	2.11	1.88	1.40	1.14	1.00	1.70	5	3.64
Entry 29	0.74	2.12	1.93	2.72	1.44	1.97	1.75	5	6.23
Entry 31	0.81	1.29	1.59	1.01	1.80	2.49	1.60	5	5.73
Entry 38	0.86	1.36	1.27	2.18	1.07	1.77	1.30	5	5.52
Entry 4	1.23	1.19	0.80	1.24	2.96	4.37	1.62	5	3.64
Entry 47	0.80	1.00	1.30	1.22	1.20	1.90	1.21	5	6.92
Entry 52	1.37	1.66	1.51	1.17	0.60	1.08	1.25	5	5.13
Entry 57	1.33	1.47	1.12	1.15	1.28	0.59	1.21	5	1.62
Entry 70	1.59	1.67	1.60	0.94	1.57	1.72	1.63	5	6.31
Entry 84	2.35	2.83	1.70	0.65	1.44	1.82	1.84	5	5.13
Entry 9	2.60	1.89	2.55	2.62	1.04	0.97	1.79	5	3.41
Entry 100	0.85	1.12	1.12	0.24	1.75	1.61	1.17	4	3.64
Entry 103	0.90	1.01	1.15	1.80	0.83	1.20	1.06	4	7.24
Entry 11	1.90	1.29	1.22	0.27	1.14	0.84	1.13	4	4.13
Entry 12	1.90	1.82	1.57	0.55	1.27	0.55	1.30	4	2.79
Entry 120	0.53	0.73	1.31	2.94	1.34	2.29	1.25	4	4.43
Entry 128	1.64	1.07	1.00	2.27	1.10	0.75	1.14	4	2.22
Entry 136	0.52	1.10	1.09	0.47	2.11	2.20	1.27	4	5.52
Entry 17	1.18	0.85	1.22	0.30	1.68	1.05	1.09	4	4.84

Entry 25	1.48	1.58	1.39	0.65	0.90	1.05	1.22	4	2.70
Entry 27	1.18	1.32	0.77	0.68	1.39	1.80	1.20	4	4.77
Entry 3	1.25	1.90	2.78	3.66	0.88	0.74	1.66	4	4.95
Entry 36	1.28	1.15	1.35	1.11	0.37	0.15	0.80	4	2.98
Entry 42	1.51	1.64	0.88	0.63	1.15	1.94	1.27	4	4.01
Entry 60	1.65	1.40	1.54	0.88	1.02	0.99	1.26	4	3.21
Entry 61	2.32	1.45	1.65	0.59	0.93	1.38	1.52	4	3.57
Entry 68	1.63	1.31	1.49	0.36	1.16	0.93	1.24	4	3.55
Entry 77	2.07	2.14	1.13	1.77	0.98	0.97	1.46	4	3.04
Entry 78	1.16	1.38	1.38	0.55	0.99	1.73	1.29	4	6.34
Entry 83	1.06	0.83	1.11	0.84	1.35	1.27	1.04	4	3.92
Entry 88	1.61	1.49	0.78	0.79	1.70	1.74	1.40	4	3.78
Entry 99	0.74	0.93	1.05	3.47	2.10	1.41	1.37	4	3.38
Entry 101	0.51	0.74	0.61	1.13	1.45	1.72	1.07	3	5.98
Entry 109	0.80	0.78	1.26	2.04	0.92	1.89	1.15	3	4.01
Entry 111	0.57	0.92	1.02	1.90	0.99	1.57	1.02	3	5.88
Entry 117	0.93	1.21	1.48	1.81	0.59	0.82	1.11	3	4.63
Entry 119	0.64	1.23	1.26	1.41	0.57	0.92	0.98	3	7.05
Entry 132*	0.98	1.47	1.25	1.77	0.79	0.69	1.14	3	5.62
Entry 37	1.05	0.74	0.97	3.25	0.52	1.12	0.96	3	3.78
Entry 44	0.60	0.78	1.07	0.76	1.46	2.63	1.18	3	4.07
Entry 5	1.01	0.82	0.63	0.11	1.85	1.44	1.00	3	5.81
Entry 54	1.58	1.17	0.91	0.68	0.87	1.54	1.11	3	4.42
Entry 63	0.68	1.39	0.72	0.37	1.01	1.34	0.97	3	3.26
Entry 73	0.94	1.35	1.25	0.01	0.92	1.08	1.13	3	3.60
Entry 76	1.11	1.36	0.72	0.53	1.27	0.54	0.95	3	4.33
Entry 79	1.33	1.36	1.40	0.59	0.57	0.72	1.05	3	2.39
Entry 81	1.68	1.50	1.21	0.67	0.89	0.87	1.21	3	4.34
Entry 91	0.86	0.58	0.55	1.21	1.12	1.33	0.91	3	5.14
Entry 110	0.89	0.66	0.70	0.84	1.35	1.49	0.91	2	4.91
Entry 135	0.49	0.99	1.29	0.56	0.98	2.04	1.02	2	5.13
Entry 19	0.90	0.43	0.31	0.11	1.83	1.05	0.64	2	3.18
Entry 28	0.67	0.90	1.01	0.22	1.55	0.51	0.93	2	4.63
Entry 41	1.06	0.92	0.73	1.16	0.72	0.94	0.91	2	5.22
Entry 48	0.65	0.83	1.02	1.97	0.35	0.88	0.83	2	6.21
Entry 55	0.86	0.82	1.33	1.13	0.54	0.29	0.70	2	3.11
Entry 56	0.89	0.70	0.85	0.34	1.23	1.38	0.86	2	4.55
Entry 59	0.56	0.88	0.60	0.07	1.20	1.79	0.81	2	7.73
Entry 64	0.92	0.52	1.24	3.61	0.51	0.73	0.79	2	4.75
Entry 7	1.01	0.40	0.16	NA	0.61	1.52	0.65	2	4.77
Entry 72	0.79	0.69	0.44	0.46	2.10	3.04	1.07	2	6.52
Entry 80	1.02	0.95	0.90	0.21	0.71	1.10	0.84	2	4.45
Entry 86	1.51	0.50	0.97	0.48	1.46	0.19	0.66	2	2.84
Entry 90	1.79	1.27	0.57	0.36	0.60	0.34	0.85	2	2.27
Entry 104	0.95	0.73	0.59	1.07	0.89	0.80	0.76	1	4.34

Entry 106	0.53	0.34	0.20	0.07	1.49	0.29	0.50	1	1.71
Entry 112	0.23	0.52	0.62	0.62	0.90	1.49	0.69	1	4.66
Entry 114	1.22	0.54	0.28	0.33	0.80	0.26	0.54	1	1.87
Entry 118	0.56	0.76	0.67	0.18	1.61	0.33	0.69	1	6.16
Entry 121	0.77	0.73	0.80	1.46	0.63	0.82	0.79	1	6.85
Entry 125	0.54	0.68	0.64	1.32	0.81	0.78	0.76	1	5.85
Entry 22	1.34	0.50	0.41	0.05	0.40	0.66	0.58	1	3.09
Entry 33	0.61	0.56	0.73	0.85	0.98	1.31	0.75	1	3.29
Entry 35	1.21	0.66	0.83	0.38	0.75	0.52	0.77	1	4.06
Entry 45	0.65	0.52	0.71	0.26	0.82	1.21	0.71	1	6.28
Entry 53	0.63	0.70	0.89	0.00	1.58	0.83	0.85	1	4.26
Entry 67	0.85	0.50	0.53	0.06	0.84	1.10	0.76	1	4.17
Entry 75	0.59	0.60	0.43	0.11	0.50	1.09	0.62	1	5.31
Entry 95	0.56	0.72	0.88	0.82	1.31	0.43	0.89	1	6.11
Entry 102	0.81	0.81	0.89	0.74	0.92	0.43	0.79	0	4.53
Entry 115	0.18	0.08	NA	0.09	NA	0.03	0.08	0	3.74
Entry 116	0.21	0.33	0.03	NA	0.15	0.15	0.29	0	2.96
Entry 126	0.23	0.41	0.81	0.18	0.42	0.70	0.52	0	2.57
Entry 127	0.54	0.52	0.55	0.57	0.84	0.70	0.67	0	4.36
Entry 129	0.54	0.51	0.87	0.75	0.44	0.47	0.63	0	2.49
Entry 131	0.17	0.42	0.23	0.29	0.07	0.07	0.22	0	2.56
Entry 138*	0.65	0.04	0.27	0.21	0.31	0.03	0.19	0	1.02
Entry 139*	0.90	0.09	0.22	0.10	0.98	0.14	0.31	0	1.77
Entry 14	0.37	0.36	0.36	0.85	0.70	0.44	0.47	0	3.73
Entry 140*	0.23	0.16	0.34	0.04	0.23	0.16	0.22	0	1.36
Entry 16	0.17	NA	0.07	0.05	NA	0.24	0.09	0	5.67
Entry 18	0.74	0.27	0.16	0.03	0.20	0.23	0.28	0	7.32
Entry 20	0.77	0.09	0.25	0.97	NA	0.41	0.38	0	4.16
Entry 26	0.40	0.36	0.24	0.52	0.28	0.42	0.37	0	3.96
Entry 30	0.62	0.63	0.92	0.70	0.67	0.97	0.75	0	2.00
Entry 32	0.79	0.71	0.52	0.03	0.14	0.11	0.43	0	1.75
Entry 34	0.52	0.65	0.33	0.44	0.64	0.13	0.41	0	3.41
Entry 39	0.49	0.34	0.16	0.94	0.22	0.08	0.29	0	3.85
Entry 40	0.60	0.63	0.69	0.92	0.34	0.77	0.64	0	4.62
Entry 43	0.36	0.13	0.08	0.24	0.04	0.23	0.16	0	6.52
Entry 46	0.50	0.43	0.28	0.27	0.24	0.39	0.38	0	5.72
Entry 51	0.66	0.66	0.44	0.40	0.92	0.30	0.66	0	4.09
Entry 58	0.76	0.94	0.80	0.94	0.59	0.68	0.83	0	2.00
Entry 62	0.35	0.28	0.21	0.04	0.38	0.40	0.32	0	4.52
Entry 66	0.81	0.12	0.22	0.07	0.14	0.20	0.21	0	5.17
Entry 69	0.42	0.41	0.41	0.09	0.24	0.55	0.41	0	7.84
Entry 71	0.38	0.16	0.03	0.11	0.27	0.16	0.17	0	6.81
Entry 82	0.79	0.43	0.88	0.04	0.28	0.25	0.44	0	2.71
Entry 85	0.50	0.36	0.31	0.21	0.53	0.86	0.43	0	6.38
Entry 87	0.59	0.31	0.21	0.25	0.40	0.15	0.30	0	2.47

Entry 89	0.76	0.85	0.60	0.80	0.59	0.39	0.70	0	3.90
Entry 92	0.45	NA	0.09	0.31	NA	0.98	0.26	0	1.25
Entry 96	0.63	0.24	0.33	0.08	0.40	0.38	0.35	0	3.21
Entry 97	0.51	0.51	0.32	0.21	0.54	0.40	0.49	0	4.45
Entry 98	0.39	0.85	0.34	NA	0.60	0.71	0.66	0	3.54

Supplementary Table S2. Soil test results from the low pH and adjusted pH fields in Tifton, GA taken on 12/13/2023.

	ppm $CaCO_3$/pH			**Mehlich 1 mg/kg (ppm)**						**mg/kg**
Sample	**LBC**	**LBCeq**	**pH**	**Ca**	**K**	**Mg**	**Mn**	**P**	**Zn**	**Extractable Al**
Tifton Adjusted pH Q1	239	689	6.96	1293	37.2	195.8	4.80	38.0	1.25	<0.01
Tifton Adjusted pH Q2	253	734	7.06	1685	40.3	228.2	7.46	57.1	1.64	
Tifton Adjusted pH Q3	230	656	7.13	1241	30.3	176.8	4.16	31.2	1.04	
Tifton Adjusted pH Q4	251	728	7.12	1298	54.1	188.2	6.99	67.9	1.73	
Tifton Low pH Q1	308	893	5.00	296	54.6	36.6	7.52	62.4	1.70	11.58
Tifton Low pH Q2	315	914	4.82	148	73.0	28.9	6.44	82.0	0.90	
Tifton Low pH Q3	313	908	4.96	238	49.2	32.3	6.26	64.3	1.43	
Tifton Low pH Q4	207	572	4.61	249	45.6	23.8	7.56	112.3	1.52	

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CHAPTER 3

ROOT SYSTEM ARCHITECTURE TRAITS ASSOCIATED WITH LOW PH AND ALUMINUM TOLERANCE IN ALFALFA

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Abstract

Acidic soils with elevated exchangeable Al limit alfalfa productivity, yet root system architectures (RSA) conferring tolerance remain poorly defined and difficult to phenotype at scale. Building on a multi-location, multi-year field study, we developed a medium-throughput, split-condition rhizobox assay to screen acid-soil tolerance. 51 half-sib families phenotyped in the fields were grown for 6 weeks in greenhouse rhizoboxes filled with a natural low-pH field soil (pH = 5.30, available Al = 6.05 mg kg^{-1}) and lime-adjusted soil (pH= 6.87, exchangeable Al 1.92 mg kg^{-1}). Roots were imaged in situ (RB-in) and after careful excavation (RB-ex), and manual measurements were also recorded (RB-man). 67 field-dug plants from 16 families were similarly imaged and phenotyped (FD). 31 RSA traits were extracted with RhizoVision Explorer, and the effect of low pH stress and associations with a yield-based Acid Soil Adaptation Index (ASAI) were evaluated. Low pH generally induced deeper and thicker roots without increasing root or shoot biomass. In contrast, highly tolerant genotypes (ASAI > 1) exhibited greater total root volume and more fine, laterally branched roots concentrated in the topsoil, relative to low-tolerance genotypes (ASAI < 1). A simple binomial logistic model using a single RB-ex trait (volume of roots with a diameter < 0.7 mm) discriminated tolerance classes with a mean test AUC = 0.741 (SD = 0.113) across 100 entry-grouped cross-validations. Together, these results support an acid-tolerant RSA ideotype with abundant shallow angle fine laterals and robust taproot development. This study characterizes RSA traits linked to ASAI by soil pH condition

and can enable the efficient screening of larger, more diverse breeding populations towards the development of an acid-tolerant alfalfa cultivar.

Keywords: alfalfa, stress, roots, phenotyping, rhizobox

Abbreviations: ASAI, Acid Soil Adaptation Index; Al, aluminum; DMY, dry matter yield; FD, field-dug image and phenotypic data; HT, High tolerance; LT, Low tolerance; NPGS, National Plant Germplasm System; PIs, plant introductions, RB-in; in situ rhizobox image data; RB-ex, excavated rhizobox root image data; RB-man, manually measured rhizobox data

Introduction

Acidic soils (pH < 5.5) predominate in humid and subtropical regions of the world and present a major obstacle to crop production (Che et al., 2023; Kopittke et al., 2019; Von Uexküll & Mutert, 1995). Low pH stress is highly multi-faceted because it involves many essential nutrient deficiencies, such as N, P, K and Ca, and is also associated with increased heavy metal toxicity, primarily Al (Hajiboland et al., 2023; Roques et al., 2013; Zheng, 2010). It is thus difficult to tease apart whether the primary effect of soil acidity is through the increased concentration of available hydrogen ions in the soil solution, nutrient deficiency, or metal toxicity (Riede & Anderson, 1996; Zhao et al., 2014). Al toxicity and P deficiency are often considered the major constraints to crop productivity on acidic soils, in which P deficiency is exacerbated by fixation by Fe and Al oxides (Che et al., 2023{Delhaize, 1995 #143)}. As soil pH drops, trivalent Al cations become increasingly available for uptake and negatively affect numerous cellular processes, including disrupting cell wall and cytoskeletal structures, nutrient and water uptake, DNA replication, and apical root meristem cell division and elongation

(Bhattacharjee et al., 2023; Hajiboland et al., 2023; Ma et al., 2006a). This results in stunted or malformed root systems, increased susceptibility to biotic and abiotic stressors, and decreased yield and persistence (Dall'Agnol et al., 1996; E. Delhaize & P. Ryan, 1995; Silva et al., 2004).

Low pH tolerance thus needs to be multi-faceted as well. Tolerant plants need to have increased nutrient acquisition and utilization, and the ability to tolerate high concentrations of Al-ions. Al-tolerance mechanisms can be broadly broken into two categories, exclusion and tolerance. Exclusion mechanisms involve the sensing of high concentrations of aluminum and increasing the excretion of negatively charged organic acids, such as malate, citrate, and oxalate, into the immediate rhizosphere. These anions chelate aluminum ions, sequestering them in a non-toxic compound. The exclusion mechanism has been demonstrated by maize, rice, corn, barley, rye, and many tree species (de Oliveira Camargo et al., 2000; Yang et al., 2015; Zhang et al., 2007), with some species preferentially producing one of the organic acids and utilizing different genes to upregulate and transport the compounds to root apical meristems (Inostroza-Blancheteau et al., 2012; Nunes-Nesi et al., 2014). Tolerance mechanisms involve the intracellular detoxification of Al-ions. Al-ions move into roots cells through passive transport and are bound to organic acids and sequestered into vacuoles (Hajiboland et al., 2023; Kochian et al., 2015). Some studies have linked rapid root epidermal turnover with increased tolerance, suggesting that Al-ions bind preferentially to carboxylic acid groups attached to cross-linking cell wall pectins and are detoxified by apoptosis (Delisle et al., 2001; Horst, 1995). Hajiboland et al. (2023) provides a recent and thorough review of Al toxicity and tolerance mechanisms.

Alfalfa, “the Queen of Forages,” is the most valuable forage crop globally due to its high yields, impressive nutrient density and digestibility, and soil benefits (Hancock et al., 2015; USDA-NASS, 2024b). Alfalfa production is limited in low pH soil and is sensitive to Al-

toxicity. Alfalfa is a tap-rooting perennial with root systems that can grow up to 6 meters deep, which limits the long-term effectiveness of soil amendments such as lime and gypsum (Sumner et al., 1986; Undersander et al., 2011). Improving the genetic tolerance of aluminum tolerance in alfalfa has been the objective of several projects since the 1970s, but there still exists no cultivar with superior tolerance and agronomic performance (Bouton et al., 1981; Devine et al., 1976). Selection methods have largely focused on greenhouse studies in limed and unlimed soil (Dall'Agnol et al., 1996; Hartel & Bouton, 1989; Khu et al., 2012), in nutrient solution (Campbell et al., 1988; Yokota & Ojima, 1995), in tissue culture (Barone et al., 2008; Parrott & Bouton, 1990) or in naturally acidic field soils (Bouton & Sumner, 1983). The previous work illustrates the difficulty of developing efficient and effective screening methods for improving Al-tolerance in alfalfa, which is often dependent and interrelated to other mineral cation deficiencies (Bouton, 1996; Dall'Agnol et al., 1996; Khu et al., 2012; Pan et al., 2008).

Previous research has shed light on aluminum tolerance mechanisms in alfalfa, though much remains unclear. Campbell et al. (1994) demonstrated that tolerant alfalfa genotypes produce significantly more low molecular weight proteins under low pH stress compared to non-tolerant genotypes and Campbell (1999) showed through MRI imaging that tolerant genotypes accumulate Al throughout the root tissue and epidermis. The results indicate that tolerant genotypes are taking up Al ions and detoxifying intracellularly, as is typical of Al-tolerance mechanisms, but where this occurs and what genes underpin the process is unclear. Significant variation within tolerant genotypes may indicate that other tolerance mechanisms are also at play (Campbell, 1999). Transgenic approaches to improve tolerance in alfalfa have included the addition of a bacterial citrate synthase and the overexpression of a malate dehydrogenase (Barone et al., 2008; Tesfaye et al., 2001). While this improved root and shoot weight in solution

and in greenhouse assays, these results did not successfully translate to improved yields in low pH field soils.

The current work sought to develop a portable, low-cost rhizobox assay to select acid and Al-tolerant alfalfa genotypes by leveraging multi-year and multi-location field phenotypic data on the same germplasm. The study also characterizes which root system architecture (RSA) traits are affected by low pH and aluminum stress and whether these differences provide separation between field-derived acid-tolerance classes with implications on the potential tolerance mechanisms employed in this population.

Materials and Methods

Plant Materials and Field Phenotyping

The population used in this study was developed from a diverse collection of 966 Plant Introductions (PIs) from the National Plant Germplasm System (NPGS) collection. These accessions were evaluated in naturally an acidic field soil (pH = 4.90) in Tifton, Georgia for three years. Selections were dug and established in another low pH field site (pH = 5.12) at the J. Phil Campbell research station in Watkinville, GA. The surviving most vigorous plants were selected after three years and polycrossed in greenhouse bee cages to generate half-sib families. 133 half-sib families originating from different PIs and 5 check cultivars were selected for inclusion in a field phenotyping experiment, in which each half-sib family was evaluated in two replications in both a low pH and adjusted pH field at two locations over multiple years (4 years in Athens, GA and 2 years in Tifton, GA). Acid Soil Adaptation Index (ASAI) scores were assigned to each half-sib family as described in Howeler (1991): $\boldsymbol{ASAI = (Ys\ x\ Yp)/(\mu s\ x\ \mu p)}$, where Ys = Yield in low pH condition, Yp = Yield in adjusted pH condition, μs = Population

average yield in low pH condition, μp = Population average yield in adjusted pH condition. This index is scaled so that scores above 1 indicate tolerant genotypes and scores less than 1 indicate intolerant genotypes.

Of the 138 half-sib families included in the field phenotyping study from 2020-2024, a subset of 51 half-sib families that represent the range of ASAI scores was selected for inclusion in the rhizobox study.

Rhizobox Assay

The rhizoboxes and racks used in this study were designed by Bertelkamp Automation Inc. (4716 Middle Creek Lane, Knoxville, TN 37921), the framing produced by 80/20 Inc. (1701 South 400 East, Columbia City, IN 46725), and the panels produced by the University of Georgia's Instrument Shop (101 Thomas Textile Building, 955 East Whitehall Rd., Athens, GA 30602). Each box consists of two clear 1/4" Lexan panels that are 350 mm L x 425 mm W, secured with aluminum framing with drainage holes on the bottom. Each rack holds 10 boxes at a 45-degree angle. Each box was placed in a metallic bubble mailer to occlude light from the root systems.

Natural low pH field soil (pH = 5.17, exchangeable Al = 10.63 mg kg^{-1}) was collected from Tifton, GA, to be used in the rhizoboxes. The field soil is characterized as Tifton series clay loams (fine-loamy, kaolinitic, thermic Plinthic Kandiudults), which are characteristic of the Coastal Plain region. The soil was mixed with sand to half volume. The soil mix was sterilized with a Pro-Grow Electric Sterilizer at 93°C/200°F for 5 hours and sieved to remove large particles. The soil was then divided in half to amend for the low pH and adjusted pH conditions. The adjusted pH condition was amended with lime to bring the soil pH to 7.5 (93 g of lime per

cubic foot of soil). Hoagland's No.2 Basal Salt Mixture Without Nitrogen was adjusted to a pH of 4 with HCl and a pH of 7.5 with NaOH, and added to saturate each soil condition, respectively, and mixed well before filling the boxes. After amending, the acidic soil condition had a pH of 5.30 and an exchangeable aluminum concentration of 6.05 mg kg^{-1}, while the adjusted soil condition had a pH of 6.87 and an exchangeable aluminum concentration of 1.92 mg kg^{-1}.

10 seeds of each genotype were inoculated with *Sinorhizobium meliloti* and *Rhizobium leguminosarum biovar trifolii* (Exceed Superior Legume Inoculant, Alfalfa and True Clover) and sown evenly spaced in boxes of both conditions. Boxes were watered gently with water daily for the first seven days, after which, the number of germinated seedlings was counted. If more than four germinated, the rest were removed, and the remaining four were transplanted with even spacing in each box. The experiment was conducted in greenhouse conditions (28°C/20°C day/night, 70% relative humidity).

After the first week of water only, a half-strength Hoagland's No.2 Basal Salt Mixture Without Nitrogen with a pH of 4 or a pH of 7.5 was applied to saturation three times a week for the next 6 weeks. Pictures of each box were taken on a Canon Rebel T3i EOS 600D with a focal length of 18 mm, an exposure time of 1/40 sec, and ISO-500. The no-flash setting with automatic light adjustment was used to better account for ambient light variability throughout the day in the greenhouse. A 2 ft x 2 ft (0.6 m x 0.6 m) lightbox was fabricated from PVC pipes and black

corrugated board with one side open to allow ambient light exposure and a hole cut into the top for the camera. High-quality images of each box were taken (Figure 1).

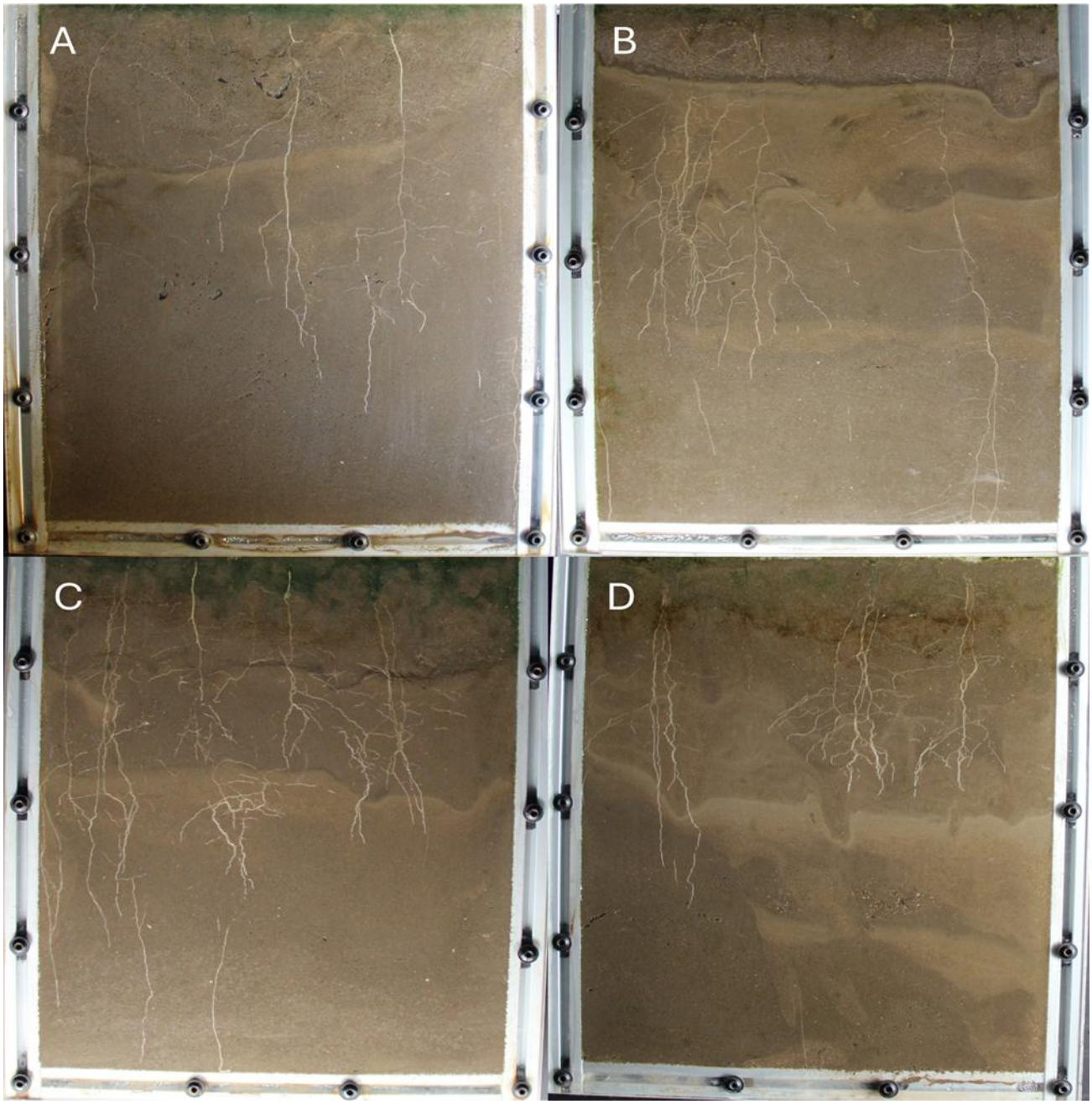

Figure 1. Original rhizobox images. Panels A (adjusted pH condition) and B (low pH condition) show the original images of the rhizoboxes for a genotype with a high ASAI. Panels C (adjusted pH condition) and D (low pH condition) show the rhizoboxes for a genotype with a low ASAI.

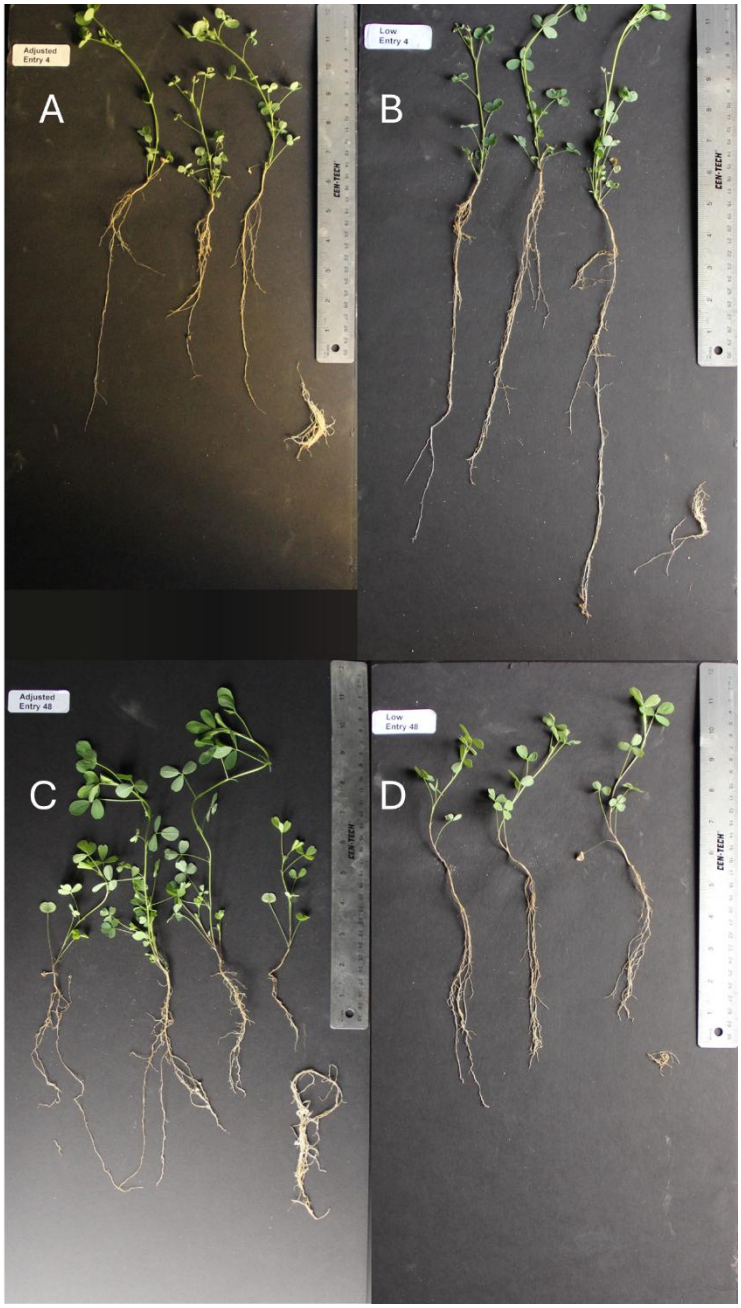

Figure 2. Original excavated root images. Panels A (adjusted pH condition) and B (low pH condition) shows an example genotype with a high ASAI. Panels C (adjusted pH condition) and D (low pH condition) show an example genotype with a low ASAI.

After rhizobox imaging, the roots were carefully separated from the soil, rinsed in water, and then dried on paper towels. The dried roots were spread out on a black posterboard and imaged in the lightbox with the same camera settings (Figure 2). The aboveground and below ground tissues were then measured for maximum root length and maximum plant height with a ruler. Roots and shoots were then separated, weighed fresh, and then dried in a convection dryer at 35°C/95°F for 48 hours for dry weight measurement.

The rhizobox experiment was carried out in two parts to accommodate the population size. The first study included 24 half-sib genotypes and was carried out following the same procedure above, aside from using the lightbox to image the rhizoboxes. The rhizobox images

were thus unable to be analyzed in RhizoVision Explorer accurately. However, the phenotypic measurements and images of the excavated roots were analyzed and included in the analysis.

To observe correlations between greenhouse related RSA and field RSA, 68 survivors from 16 half-sib families were dug from the low pH and adjusted pH Athens field sites in July 2025 using a mini excavator with a 2 ft/61 cm bucket attachment. After excavation, soil was removed from the roots and plants were trimmed down to the crown level. Fresh weight of the root system, crown circumference, and taproot diameter measurements (at the base of the taproot, 10 cm down, and 20 cm down) were collected. Roots were imaged in the same lightbox with the same camera settings described above and analyzed in RhizoVision Explorer.

Figure 3. Root images from field-dug selections. Panels A and C (adjusted pH condition) and B and D (low pH condition) show two example genotypes with a high ASAI. Panels E and G (adjusted pH condition) and F and H (low pH condition) show two example genotypes with a low ASAI.

Image Analysis

Rhizobox root images, excavated root images, and field validation images were preprocessed individually using Microsoft Designer. Rhizobox images were modified to increase sharpness to 100% and cropped to generate a separate image for each plant's root system. Excavated root images were similarly modified to increase sharpness to 100%, contrast to 100%, and decrease brightness by 35-50% to ensure optimal contrast. Field-derived images were modified to increase sharpness to 100%, contrast to 100%, and decrease brightness by 40%. Each preprocessed image was cropped so that each image contained a single plant's root system. If other objects (label, ruler, neighboring plant, etc.) could not be removed by cropping, it was covered with an opaque square. Differences in lighting of the rhizobox images necessitated manual analysis of each picture in RhizoVision Explorer (version 2.0.3) (Seepthepalli & York, 2020). Image threshold levels were systematically decreased by 10 units until no more dirt particles could be removed without losing the resolution of the segmented roots. Removing contaminant dirt particles comes at the cost of removing thinner root elements, so this process was done iteratively to ensure the optimal trade-off was achieved for high fidelity.

Image preprocessing used "Whole root" analysis mode without "Keep largest component" or "Enable edge smoothing" settings. Pixels were converted to physical units using a 150 DPI threshold. Image thresholding levels were adjusted between 20-100 and filter non-root objects were adjusted between 5-20 to achieve the most accurate segmented image from the input. "Fill holes in root objects" was maintained at 1 mmsq. Root pruning was enabled with a threshold of 15 and root diameter ranges were 0-0.7 and 0.7 and above, which was shown to delineate taproots from lateral roots fairly well, despite significant variation. Excavated root images were batch-analyzed using the same settings described above aside from maintaining the

image thresholding level at 150 and the filter non-root objects threshold at 30 mm. The same process was carried out for field-derived image analysis, apart from not converting pixels into physical units and using diameter ranges of 0-5 px, 5-10 px, and 10+ px. All segmented images were manually verified for high-fidelity with the input images and manually redone if necessary.

RhizoVision Explorer analysis measured 31 variables for each plant root system for images taken in the rhizobox and removed from soil (Table 1). A graphical depiction of root angle traits is shown in Figure 4. Phenotypic values measured after imaging the rhizobox-grown plants (maximum plant height, maximum root length, and shoot and root weights) and acid-tolerance level as determined from field-harvest data (ASAI) were added to each of the image datasets.

Table 4. Description of output variables generated by RhizoVision Explorer for the rhizobox images and excavated root images.

Feature Name	Description	Units
Median Number of Roots	Median number of roots detected by horizontal line scans through the segmented image.	Count
Maximum Number of Roots	Maximum number of roots detected in any horizontal scan line.	Count
Number of Root Tips	Count of tip points in the root skeleton.	Count
Total Root Length	Sum of all skeleton pixel lengths (diagonal pixels counted as √2).	Mm
Perimeter	Number of pixels forming the root system's outer boundary.	Mm
Average Diameter	Mean diameter computed from distance map at each skeleton pixel.	Mm
Median Diameter	Median of the diameters across all medial axis pixels.	Mm
Maximum Diameter	Maximum diameter observed in the skeleton.	Mm
Volume	Summed cross-sectional volume across all medial axis pixels.	mm^3
Surface Area	Summed perimeter across all medial axis pixels.	mm^2
Network Area	Total pixel area of the segmented root.	mm^2
Depth	Vertical span (top to bottom) of the root system.	Mm
Maximum Width	Horizontal span (left to right) of the root system.	Mm
Width-to-Depth Ratio	Ratio of width to depth.	Unitless

Feature Name	Description	Units
Convex Area	Area of the smallest convex polygon enclosing the root system.	mm^2
Solidity	Ratio of Network Area to Convex Area.	Unitless
Lower Root Area	Root area located below the widest root segment (max diameter pixel).	mm^2
Number of Holes	Number of enclosed white (background) regions within the root system.	Count
Average Hole Size	Average area of the enclosed holes.	mm^2
Average Root Orientation	Mean direction of medial axis pixels, averaged across local neighborhoods.	Degrees
Shallow Angle Frequency	Fraction of local medial axis orientations < 30°.	Proportion
Medium Angle Frequency	Fraction of local medial axis orientations between 30° and 60°.	Proportion
Steep Angle Frequency	Fraction of local medial axis orientations > 60°.	Proportion
Computation Time	Time taken to analyze and extract features from one image.	Seconds
Root Length Diameter (Range 1)	Sum of root skeleton lengths where diameter is less than 0.7 mm.	Mm
Root Length Diameter (Range 2)	Sum of root skeleton lengths where diameter is greater than 0.7 mm.	Mm
Projected Area Diameter (Range 1)	Total root segmented area where medial axis diameter is less than 0.7 mm.	mm^2
Projected Area Diameter (Range 2)	Total root segmented area where medial axis diameter is greater than 0.7 mm.	mm^2
Surface Area Diameter (Range 1)	Summed root surface area across medial axis pixels with diameter less than 0.7 mm.	mm^2
Surface Area Diameter (Range 2)	Summed root surface area across medial axis pixels with diameter greater than 0.7 mm.	mm^2
Volume Diameter (Range 1)	Summed root volume across medial axis pixels with diameter less than 0.7 mm.	mm^3
Volume Diameter (Range 2)	Summed root volume across medial axis pixels with diameter greater than 0.7 mm.	mm^3

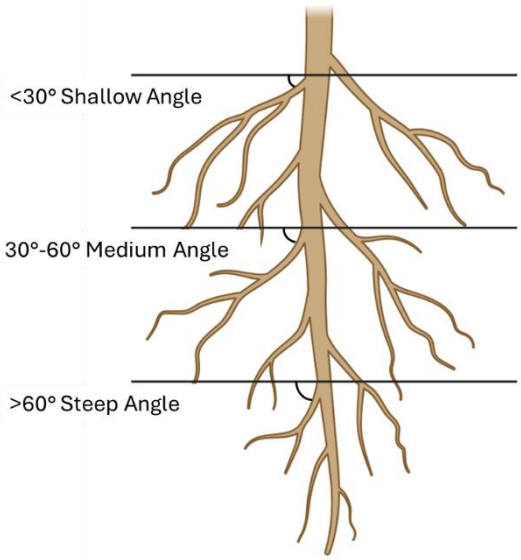

Figure 4. Graphical representation of root branching angles. 0° is a completely horizontal lateral, 90° is a completely vertical lateral.

In total for this study, we collected and analyzed four separate datasets: (i) rhizobox in-situ image traits (RB-in) (families=24, n=105), (ii) image traits from the same plants after excavation and cleaning (RB-ex) (families=51, n=296), (iii) manual phenotypes of rhizobox-grown plants (RB-man) (families=51, n=296), and (iv) field-derived RSA traits (FD) (families=16, n=68).

All analysis was conducted in RStudio (R version 4.1.2 (2021-11-01)). RSA traits that were significantly altered by low pH stress were identified through paired t-tests for all traits in the four datasets (RB-in, RB-ex, RB-man, and FD). For each trait, plant-level replicates were averaged to obtain an entry × condition mean and paired t-tests comparing performance in the adjusted vs low pH within the same entry were conducted using the t.test function. Only entries observed under both conditions were included. To account for the large number of variables being tested and control the false discovery rate across traits, we adjusted p-values using the Benjamini–Hochberg procedure.

We then wanted to see if trait plasticity varied by tolerance class as determined by field phenotyping (ASAI > 1 = high tolerance (HT), ASAI < 1 = low tolerance (LT)). Delta values were computed as Adjusted / Low for each trait and paired t-tests were conducted to see if the change in RSA traits across pH conditions is significantly different by tolerance class.

Additionally, interaction plots were generated to determine if RSA traits change differently depending on the tolerance class for each dataset. We then wanted to explore if any RSA traits are significantly correlated with field tolerance (ASAI). Spearman correlation plots were generated for all four datasets. Finally, we fit a simple binomial logistic regression model to classify tolerance class (HT vs LT) using only data from the low pH condition of the RB-ex dataset, with the volume of roots with a diameter < 0.7 mm (Volume Diameter Range 1 (mm^3)) as the predictor. Performance was estimated using an entry-grouped Monte Carlo cross-validation (100 repeats) with 75/25 train/test splits and stratified by tolerance class. In each repeat, the decision threshold was chosen on the training set by maximizing Youden's J from the ROC curve. Test entries were then evaluated and the average AUC of the training and test sets was determined to evaluate model accuracy.

Results

Effect of low pH stress on RSA traits

The rhizobox image data revealed 24 traits that were significantly different between the low and adjusted pH conditions, including surface area (mm^2), perimeter (mm), and total root length (mm) (Figure 5). The overall trends from this data show that root systems are significantly larger under low pH conditions than under adjusted pH conditions in several aspects, including small (Range 1, <0.7 mm) and large diameter root (Range 2, >0.7mm) growth. This also shows that root angles are significantly affected by low pH stress, namely that roots tend to be shallower, indicating greater horizontal root development under low pH stress and more steep angled roots

in the unstressed condition, indicating vertical root development.

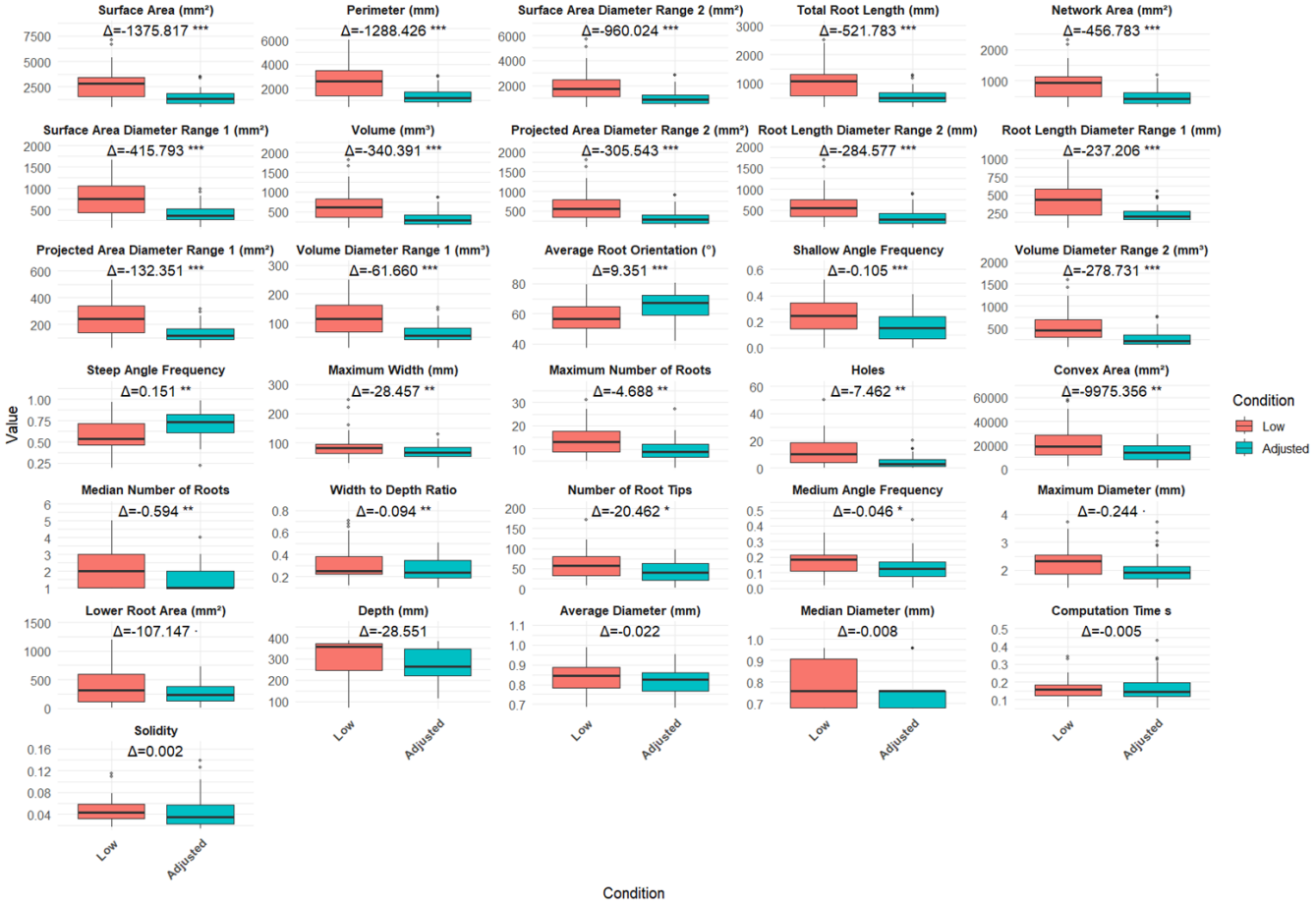

Figure 5. Boxplots showing the difference in RSA traits from RB-in dataset (in situ rhizobox image analysis) under low pH (salmon) and adjusted pH (teal) soil. Δ is the mean paired genotype-level difference (Adjusted – Low) for each trait. Panels are ordered by increasing significance from paired t-tests across entries. Asterisks denote BH-FDR adjusted significance (* $q < 0.05$, ** $q < 0.01$, *** $q < 0.001$). Positive Δ indicates higher values in adjusted condition.

Paired t-tests of the excavated root image dataset showed no traits were significantly different at a $q<0.05$ (Figure 6). While nothing was significantly different across conditions, the trend shows greater root depth and increased proportion of thinner roots (Range 1, <0.7 mm) under low pH stress.

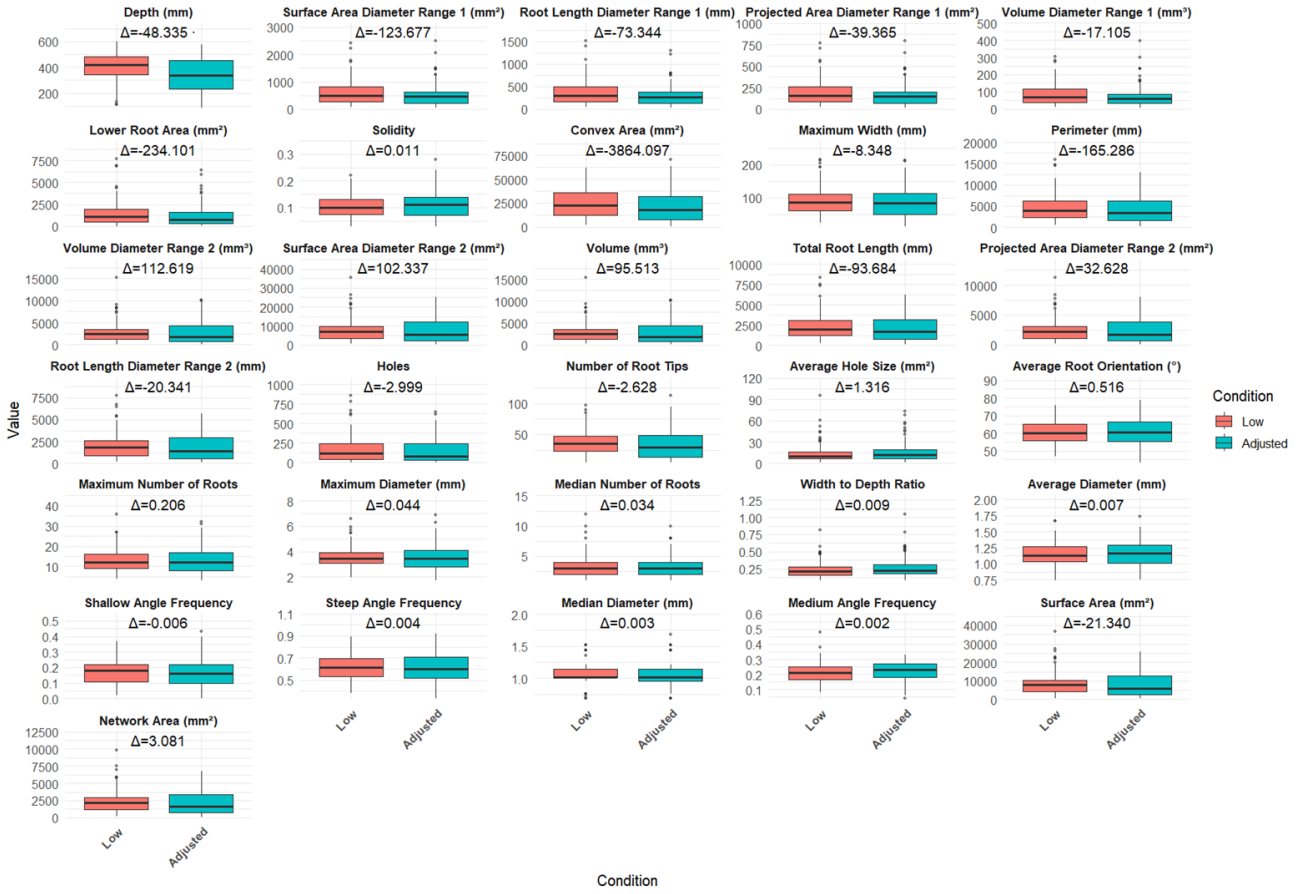

Figure 6. Boxplots showing the difference in RSA traits from the RB-ex (excavated root image-derived data) under low pH (salmon) and adjusted pH (teal) soil. Δ is the average paired entry-level difference (Adjusted – Low) for each trait. Panels are ordered by increasing significance from paired t-tests across entries. Asterisks denote BH-FDR adjusted significance (* $q < 0.05$, ** $q < 0.01$, *** $q < 0.001$). Positive Δ indicates higher values in the adjusted condition.

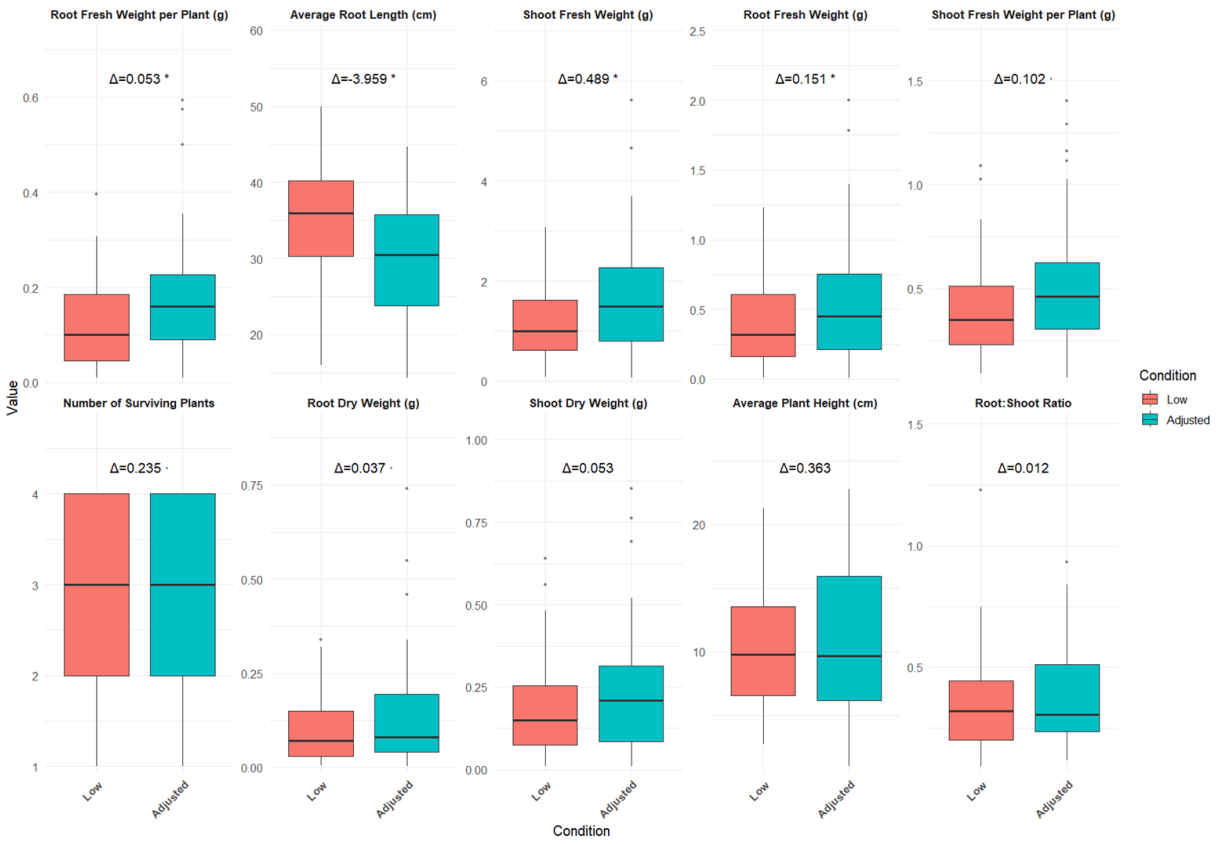

Figure 7. Boxplots showing the difference in RSA traits from the RB-man dataset (manually measured phenotypes from rhizobox study) under low pH (salmon) and adjusted pH (teal) soil. Δ is the mean paired entry-level difference (Adjusted – Low) for each trait. Panels are ordered by increasing significance from paired t-tests across entries and asterisks denote BH-FDR adjusted significance (* $q < 0.05$, ** $q < 0.01$, *** $q < 0.001$). Positive Δ indicates higher values in the adjusted condition.

Four traits were found to be significantly different between the low and adjusted pH conditions in the RB-man dataset, including fresh root weight, average root length, and fresh shoot weight (Figure 7). Despite the longer root systems exhibited by the low pH condition, the adjusted pH condition produced significantly more above and below-ground biomass.

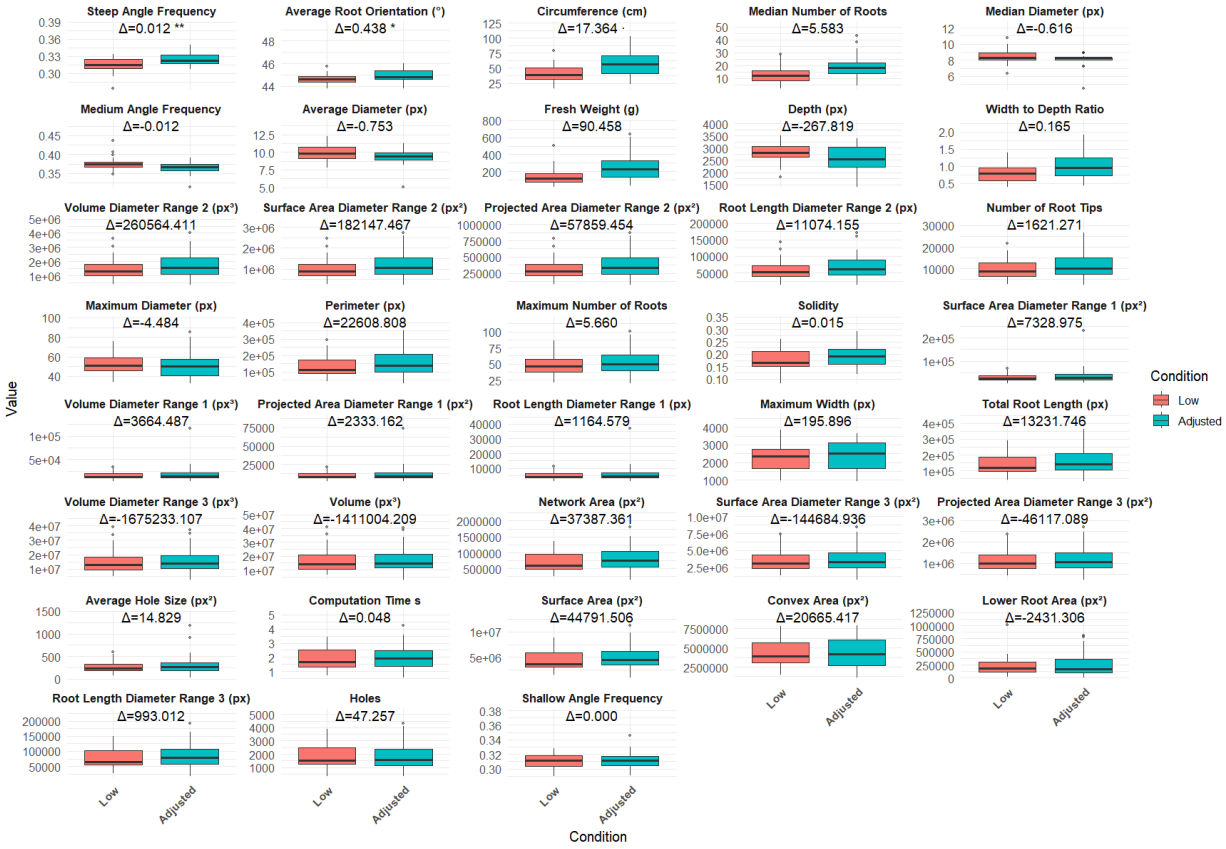

Figure 8. Boxplots showing the difference in RSA traits from the FD dataset (field-dug root image traits and manually measured phenotypes) data under low pH ("Low", salmon) and adjusted pH ("Adjusted", teal) soil. **Δ** is the average paired entry-level difference (Adjusted – Low) for each trait. Panels are ordered by increasing significance from paired t-tests across entries. Asterisks denote BH-FDR adjusted significance (* $q < 0.05$, ** $q < 0.01$, *** $q < 0.001$). Positive Δ indicates higher values in the adjusted condition.

Only two traits were significantly different at $q < 0.05$ in the FD dataset, both related to root angles (Figure 8). Plants in the adjusted pH section had a higher frequency of steep angles and a higher average root angle orientation, indicating a more vertically developed root system compared to plants in the low pH condition.

Associations between RSA traits and acid soil field tolerance (ASAI)

Spearman correlation analysis was conducted to identify RSA traits correlated with ASAI. Analysis was conducted for all four datasets separately for the low pH and adjusted pH conditions (Figure 9).

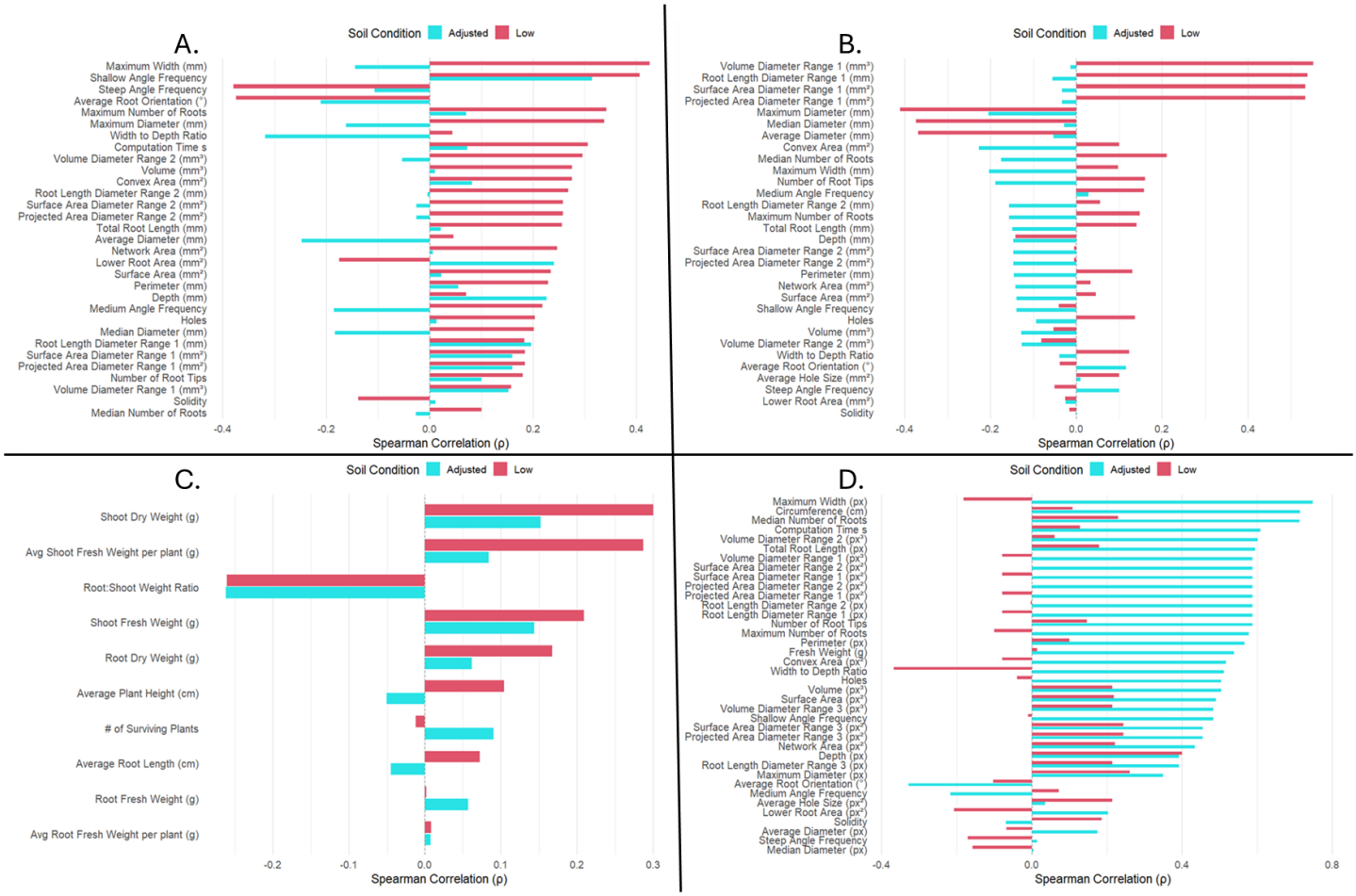

Figure 9. Spearman correlation plots for the RSA traits and ASAI by condition. Panel A shows RB-in (in situ rhizobox image analysis), panel B shows RB-ex (excavated rhizobox root image analysis), panel C shows the RB-man (manually measured rhizobox phenotypes), and panel D shows the FD (field-dug root image and phenotypes).

Several traits showed strong to moderate correlations with ASAI. Correlations were moderate in the rhizobox image-related datasets (RB-in and RB-ex), ranging from -0.4-0.5, and were strongest in the adjusted pH condition of the field-related traits (FD, max=0.75). The RB-in data set shows that having a larger maximum width is positively associated with ASAI in the low pH condition but negatively associated with ASAI in the adjusted condition. Steep root angles were

positively associated with ASAI under both conditions, and shallow root angles were negatively associated with ASAI under both conditions. The RB-ex correlation analysis showed strong positive associations with narrow root diameter traits (<0.7 mm) in the low pH condition, with weaker negative associations in the adjusted condition. Other diameter traits (maximum, median, and average diameter) showed negative correlations with ASAI in both conditions but were stronger in the low pH condition. For the RB-man data set, shoot and root weight showed the strongest positive association with ASAI in both conditions, though correlations in this dataset were smaller than the others, with a max of 0.3.

Variation in RSA plasticity by acid-tolerance class

Trait plasticity was assessed by comparing average performance under low pH and adjusted pH using all four datasets across acid tolerance classes (ASAI > 1 = high tolerance (HT), ASAI < 1 = low tolerance (LT)). Many traits showed separation and/or interaction by tolerance class. The RB-in analysis shows that HT genotypes have consistently longer roots across conditions (depth, width, perimeter) and more branched root systems (number of root tips, maximum number of roots) (Figure 10). LT genotypes have consistently larger average diameters and width-to-depth ratios. Interactions are seen for many traits, including volume, maximum and median diameters, and range 2 diameter traits (>0.7 mm). HT genotypes maintain more roots with a small diameter under both conditions but show an increase in thicker roots (>0.7mm) under low pH stress.

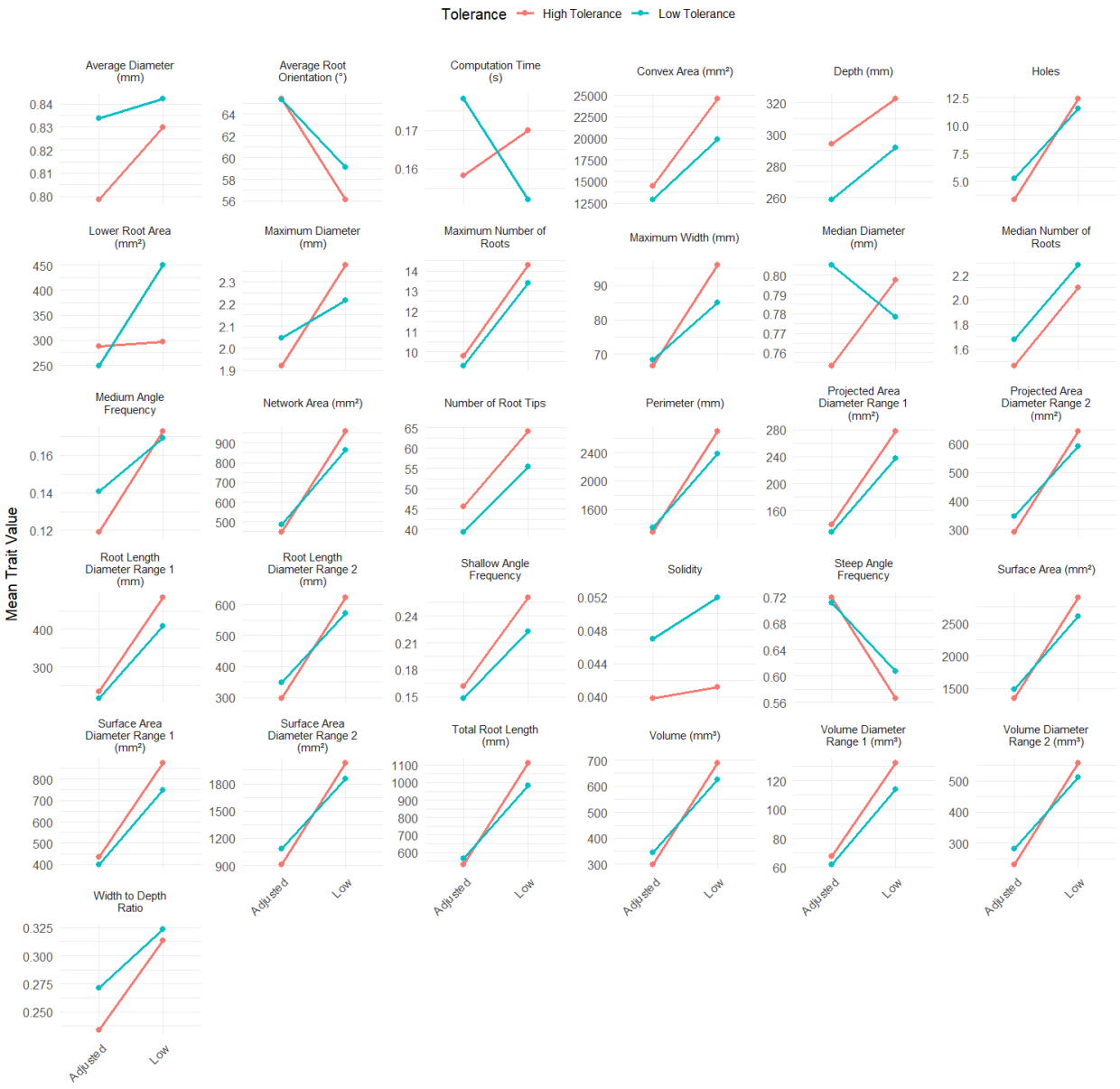

Figure 10. Plasticity graphs showing the average performance of each RSA trait from the RB-in dataset (in situ rhizobox image analysis), in the adjusted pH condition (left) and in the low pH condition (right). High tolerance genotypes (ASAI>1) are shown in salmon and low tolerance genotypes are shown in teal.

The RB-ex data set shows more separation between the tolerance classes, where HT genotypes tend to increase root system traits under low pH stress, while LT genotypes decrease (maximum, median, and total number of roots, network area, width, perimeter, total root length, volume) (Figure 11). This was maintained over both diameter size ranges. Interactions were seen in depth, convex area, and median and average diameters, which showed that HT genotypes tend

to have a longer vertical depth and smaller median and maximum diameters. Since root angles were disrupted during excavation and cleaning, root angle traits derived from the RB-ex data are likely not meaningful.

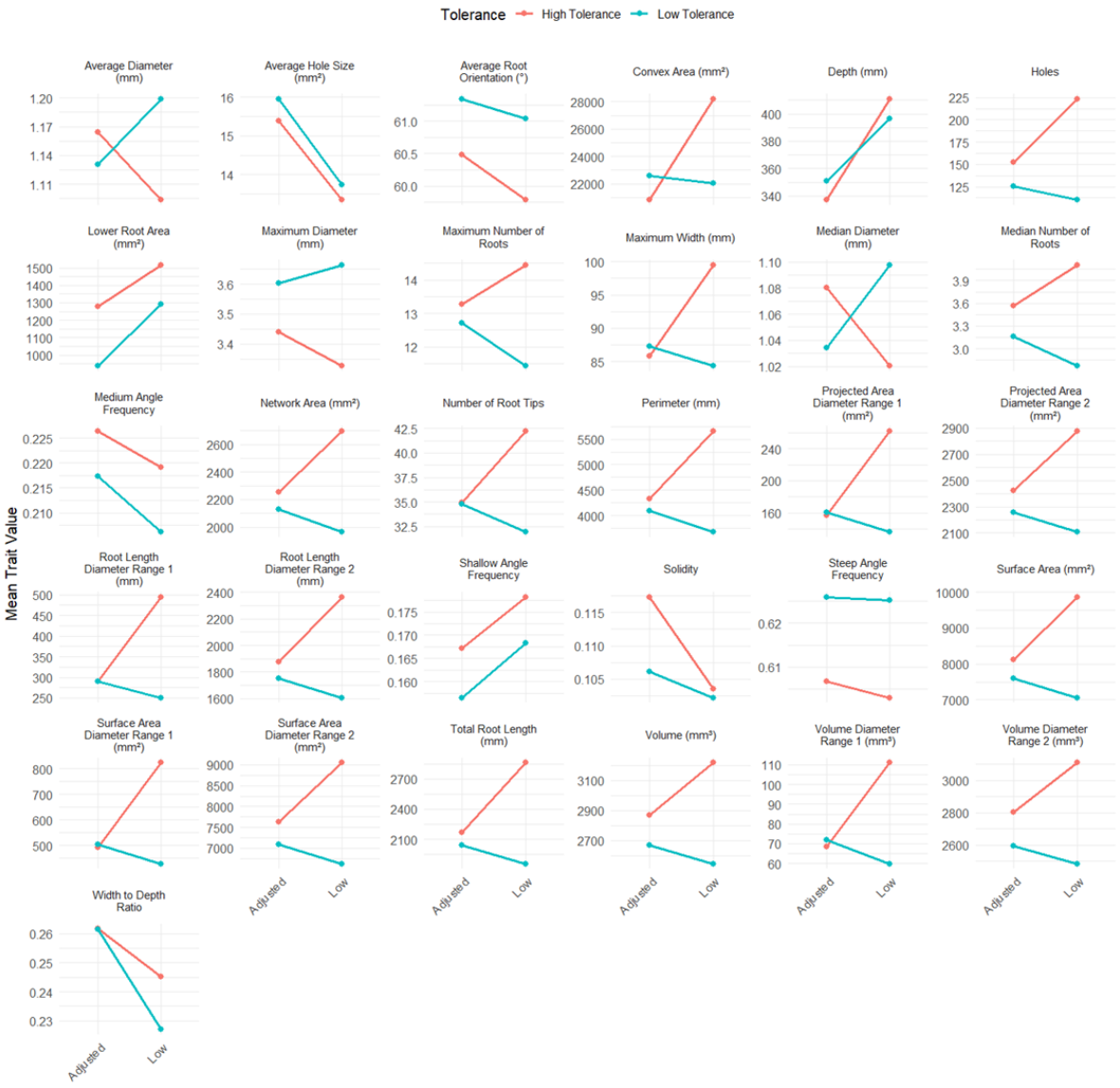

Figure 11. Plasticity graphs showing the average performance of each RSA trait from the RB-ex dataset (excavated rhizobox root image analysis) in the adjusted pH condition (left) and in the low pH condition (right). High tolerance genotypes (ASAI>1) are shown in salmon and low tolerance genotypes are shown in teal.

The RB-man analysis showed that LT is associated with taller plants and greater root fresh weights, though HT genotypes produce consistently greater shoot weights across conditions (Figure 12). Interactions were seen for root dry weight, in which highly tolerant genotypes produce heavier roots than genotypes with low tolerance under low pH soil conditions. These results show that root and shoot weight are decreased under low pH stress, but maximum root length and plant height are increased.

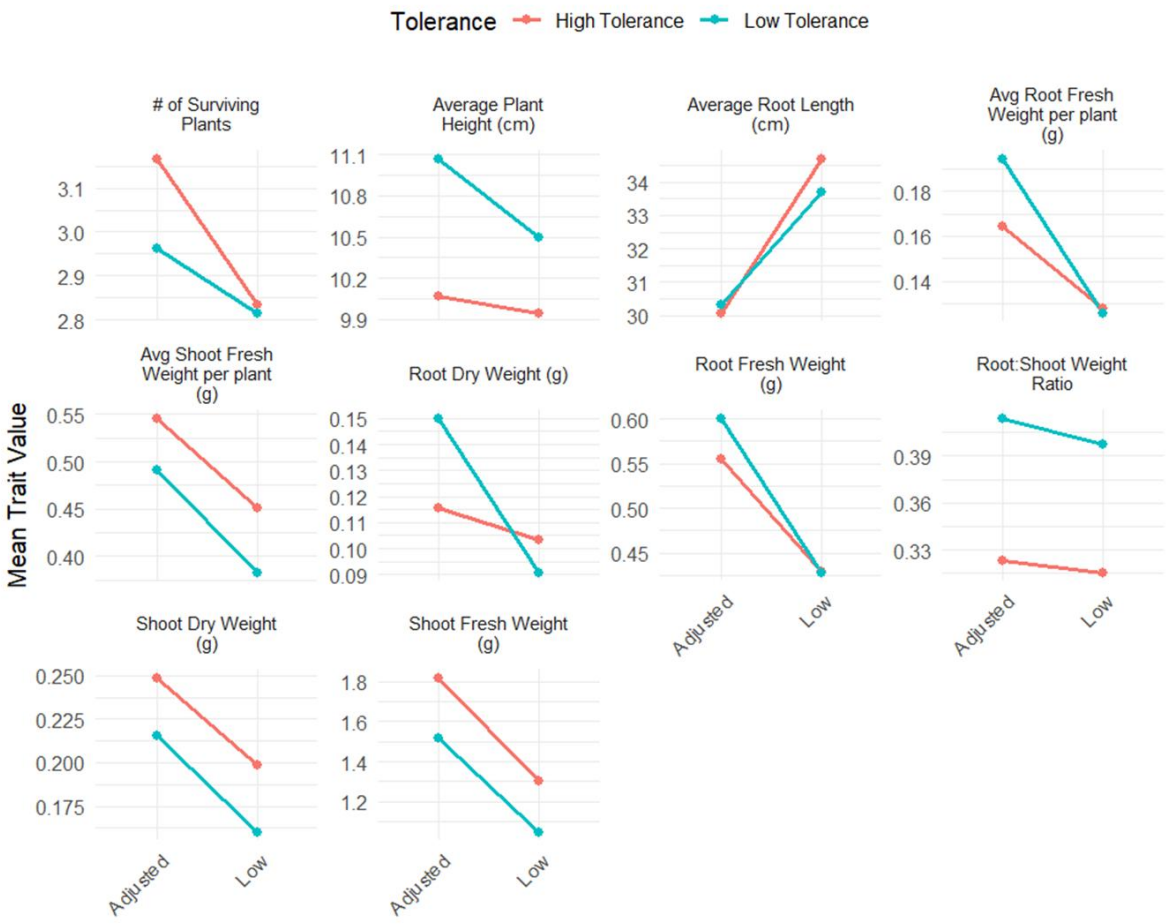

Figure 12. Plasticity graphs showing the average performance of each RSA trait from the RB-man dataset (manually measured rhizobox phenotypes) in the adjusted pH condition (left) and in the low pH condition (right). High tolerance genotypes (ASAI>1) are shown in salmon and low tolerance genotypes are shown in teal.

The FD data similarly shows that highly tolerant genotypes produce larger, heavier root systems compared to genotypes with low tolerance across conditions (average taproot diameter,

depth, crown circumference, fresh weight, maximum number of roots, volume, etc.) (Figure 13). Most root traits were decreased by low pH stress in both tolerance classes, except for depth, medium angle frequency, and average, median, and maximum diameters, which increased under stress. HT genotypes have smaller average and median diameters, while also maintaining larger maximum diameters. This indicates that acid tolerance is likely associated with larger taproot diameters as well as an increase in the number of small-diameter lateral roots, which decrease the median and average variables. Root angle traits are also of interest in this data set, in which steep angle frequency is associated with more vertically developed root systems and shallow angle frequency is associated with more horizontal development. Interactions were seen in lower root area, steep angle frequency, and width to depth ratio, suggesting that HT genotypes tend to grow more laterally under low pH stress compared to LT genotypes (i.e., greater root development near the soil surface rather than deeper in the soil strata).

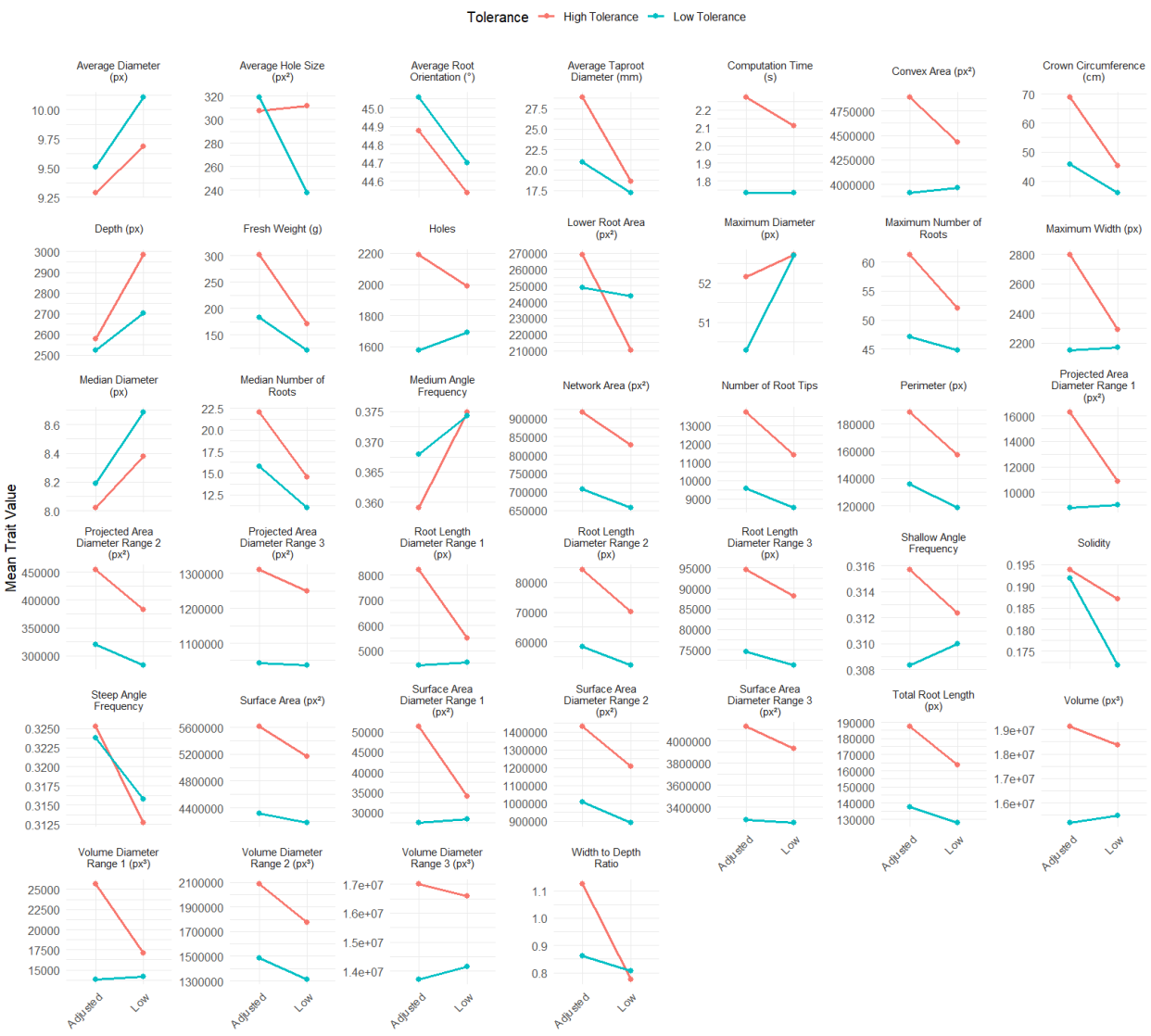

Figure 13. Plasticity graphs showing the average performance of each RSA trait from the FD dataset (field-dug root image and phenotypes) in the adjusted pH condition (left) and in the low pH condition (right). High tolerance genotypes (ASAI>1) are shown in salmon and low tolerance genotypes are shown in teal.

Delta values were computed as the ratio between mean performance in the adjusted pH over performance in the low pH (adjusted/low) for each genotype using all four datasets and compared across acid tolerance classes (ASAI > 1 = HT, ASAI < 1 = LT). The delta value is an estimate of the phenotypic plasticity of the RSA trait for each genotype, which explains how RSA traits vary in response to low pH stress by tolerance class. The results revealed that no RSA deltas were significantly different by tolerance class in the RB-in, RB-man, or FD data sets. In

the RB-ex data set, six traits were significantly different across acid tolerance classes (Figure 14). All traits identified are diameter related and show that HT genotypes increase small diameter (< 0.7 mm) root branches under low pH stress. This likely results in the decreased average and median diameter traits, which are depressed by the greater number of small diameter root branches.

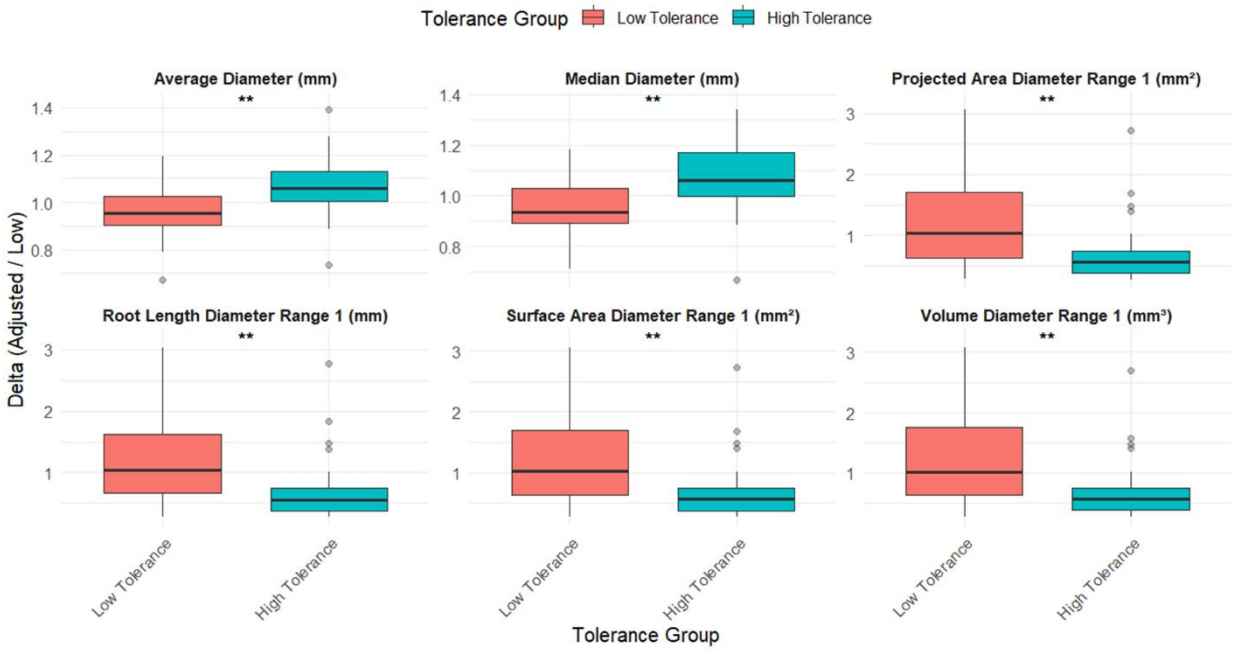

Figure 14. Boxplots of RB-ex trait deltas (Δ = Adjusted / Low) by tolerance class (ASAI > 1, High Tolerance, ASAI < 1, Low Tolerance). Only traits with significant between-class differences (Wilcoxon rank-sum test, $p < 0.05$) are shown. Asterisks denote significance tiers (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Values less than one indicate an increase in the trait under low pH stress.

3.3 Predicting tolerance class from RSA traits

The RB-ex dataset was selected to model tolerance class since it seemed to best differentiate tolerance classes. We fit a binomial logistic regression to classify tolerance class using only data from the low pH condition, with only Volume Diameter Range 1 (mm^3) as the predictor. This was selected because the delta value was significantly different across tolerance classes and is representative of the other diameter traits without being redundant. Performance was estimated by grouped Monte Carlo cross-validation with 100 repeats and a 75/25 train/test

splits at the genotype level to prevent training leakage and stratified by tolerance class. Across the 100 repeats, training AUC averaged 0.766 (SD 0.034; 2.5–97.5th percentiles 0.700–0.824). Test AUC averaged 0.741 (SD 0.113), indicating decent discrimination power despite some variability across splits.

Discussion

Effect of low pH and aluminum toxicity on RSA traits

This study confirms that low pH soil stress significantly affects many RSA traits. Roots tended to grow longer and thicker under low pH stress, but this did not translate to greater root or shoot weight. Many RSA traits were increased under low pH stress, especially in the RB-in data analysis. This is at odds with other studies in alfalfa, which have shown reduced root growth under low pH stress (Bouton et al., 1982; Bouton et al., 1986b; Dall'Agnol et al., 1996; Khu et al., 2012; Liatukiene et al., 2020; Pan et al., 2008; Sledge et al., 2002; Yokota & Ojima, 1995). This may be due to the relatively mild low pH stress utilized in this study (low pH condition: pH 5.30, available Al = 6.05 mg kg^{-1}; adjusted pH condition: pH 6.87, exchangeable Al 1.92 mg kg^{-1}). It has been shown that low concentrations of aluminum can stimulate root growth and lead to larger root surface areas in soybean, another AL-sensitive legume (Zhang et al., 2007). The regular addition of nutrient solution, which was intended to isolate the effects of low pH and aluminum toxicity, may have alleviated some of the nutritional stress that may underly the toxic low pH phenotype and led to increased root growth (Kochian et al., 2004; Muhammad et al., 2019).

A large advantage of the current research was the ability to leverage multi-location, multi-year field phenotyping in both low and adjusted pH field conditions to observe correlations with

RSA traits in the rhizobox context. This approach flips the typical approach of greenhouse or lab screening before moving to field evaluations, which have not been effective in developing high-yielding, low pH-tolerant varieties. The ASAI is scaled such that it accounts for the performance of the entire population in both pH conditions and ensures that genotypes identified as tolerant are not just performing equally across conditions but have a true yield advantage over the average. Using ASAI to assign tolerance status (ASAI > 1, HT; ASAI < 1, LT) allows us to evaluate if and which RSA traits are associated with improved vigor under low pH field conditions. HT genotypes were better able to increase the number of roots and root volume traits under low pH stress compared to LT genotypes, especially in the small diameter range (<0.7 mm). HT genotypes also tended to have a higher proportion of roots with shallow angles and a smaller lower root area, indicating greater horizontal root development near the soil surface. This suggests that fine, laterally branching roots may underlie the acid-soil tolerance displayed in this population. It is interesting to note that this is in conjunction with having roots with greater vertical depth. HT genotypes also had greater maximum diameters, indicating robust taproot development, while having lower median and average diameters, indicating preferential development of thin root structures. This was confirmed with the field-dug selections, in which HT genotypes showed increased number, volume, length, and depth of roots compared to LT genotypes under low pH stress. The FD data showed HT genotypes also have smaller average and median diameters, decreased lower root area, and greater shallow angle frequency, emphasizing the importance of fine lateral root branches in overcoming low pH toxicity. This is consistent with other studies that have shown the benefit of top-soil foraging roots in overcoming aluminum toxicity through increased nutrient uptake (Lynch, 2015; Ofoe et al., 2022; Rao et al., 2016).

That no deltas were significantly different by tolerance class in the RB-man dataset may explain why such little progress has been made in developing a reliable greenhouse assay for assessing low pH and aluminum tolerance in alfalfa to date. Root weight ratios (adjusted/low) have been the primary selection criteria in previous studies (Barone et al., 2008; Bouton et al., 1986a; Dall'Agnol et al., 1996; Khu et al., 2012), but showed no significant correlation with field-derived ASAI values or tolerance class in the present study. Shoot dry weight in the low pH rhizobox assay had a Spearman correlation of 0.30, while root dry weight had a 0.17, and root to shoot ratio was negatively correlated at 0.26. This underscores the difficulty of accurately predicting field performance through simple manual measurements of greenhouse-grown plants. Using image data allows many more RSA traits to be accurately and efficiently collected and reveals significant correlations with field performance traits, such as ASAI.

Using one RB-ex RSA feature (volume diameter range 1 (<0.7 mm)), we trained a binomial logistic classification model to distinguish HT from LT genotypes. Across 100 entry-grouped Monte-Carlo splits (75/25), the model achieved a mean test AUC of 0.741 (SD 0.113), indicating moderate discriminative ability with some sensitivity to the specific train/test partition. Owing to its simplicity and interpretability, this single-trait model is useful as a screening tool to prioritize entries for follow-up evaluation. However, threshold choice should be calibrated on the training data used in each experiment and further optimization may led to a more stable model.

Dataset considerations and study limitations

The different datasets analyzed in this study revealed different aspects of acid tolerance. The RB-in provides an in-situ view of RSA traits and is especially valuable for evaluating root angle traits. The RB-ex provides a more complete view of the root system and especially fine lateral

roots, which were often not visible on the glass of the rhizobox images. This may explain why no delta values were significantly different by tolerance class in the RB-in data, but all small-diameter root traits were significant in RB-ex. Because the rhizobox images from the first study could not be analyzed, differences in sample size (RB-in: genotypes=24, n=105; RB-ex: genotypes=51, n=296) may also explain this result. The RB-man was most important for capturing root and shoot weights and validating the image analysis. The FD dataset provides a real-world view of RSA traits associated with acid soil field tolerance in alfalfa and is a useful comparison for the rhizobox results. This helps provide an idea of RSA traits associated with acid tolerance after five years of field evaluation in the target environment. The results of the FD-analysis may be skewed, as natural selection had acted on the population for 5 years, and significant mortality was observed, especially for LT genotypes in the low pH condition. This means that in some cases the single surviving individual of a family was sampled, which likely possesses better acid-tolerance than the family-mean. The FD data is also limited by the sample size (genotypes=16, n=67) and the depth sampled. As a taprooting plant, alfalfa can grow more than 6 m into the soil, so the 0.6 m dug provides only a fraction of the total root volume (Undersander et al., 2011). However, this still provides a good estimation of field root phenes while being mindful of time and resources.

The study examined RSA traits at only two pH levels. It is possible that different pH and aluminum concentrations would produce different effects and therefore further investigation under more extreme stress may be informative. However, screening sensitive species at moderate aluminum stress (exchangeable Al < 4 mg kg^{-1}) may be more discriminatory because extreme stress depresses trait means and compresses variance, masking genetic differences (Dos Santos Neto et al., 2020). The generalization of the results of the study may also be affected by the

uniformity of the mixed soil within the rhizoboxes, which does not mimic natural field soil layers. Additionally, the rhizobox itself is a constraint to plant growth and may have affected depth (length) measurements specifically, as many roots had reached the bottom of the box by the end of the 6-week experiment. As alfalfa is a long-lived perennial, the 6-week evaluation may be insufficient to capture root traits associated with long-term field tolerance, especially when breaking fall dormancy or experiencing co-morbid abiotic stressors.

From the present research, it is impossible to separate true acid-tolerance from simple vigor. Since HT plants had overall larger root systems with greater fine lateral branches even under adjusted pH conditions, it could be that the HT genotypes have generally improved root growth and nutrient acquisition. HT plants are better able to retain these characteristics under low pH stress than the LT, but it is probable that this is more related to increased nutrient acquisition and subsoil Al avoidance than a cellular Al-tolerance or exclusion mechanism.

Conclusions and future directions

The current work leverages field performance data to identify RSA traits associated with low pH and aluminum tolerance in alfalfa. The results show that tolerant genotypes have greater phenotypic plasticity in RSA traits which respond to low pH stress by developing deeper, wider root systems. Of particular importance are root diameter traits, in which tolerant plants are better able to develop narrow-diameter lateral root branches under low pH stress. Despite significant variation across genotypes for RSA traits, the rhizobox assay and image analysis provides a clear view of the root ideotype of improving low pH and aluminum tolerance in alfalfa (Figure 15).

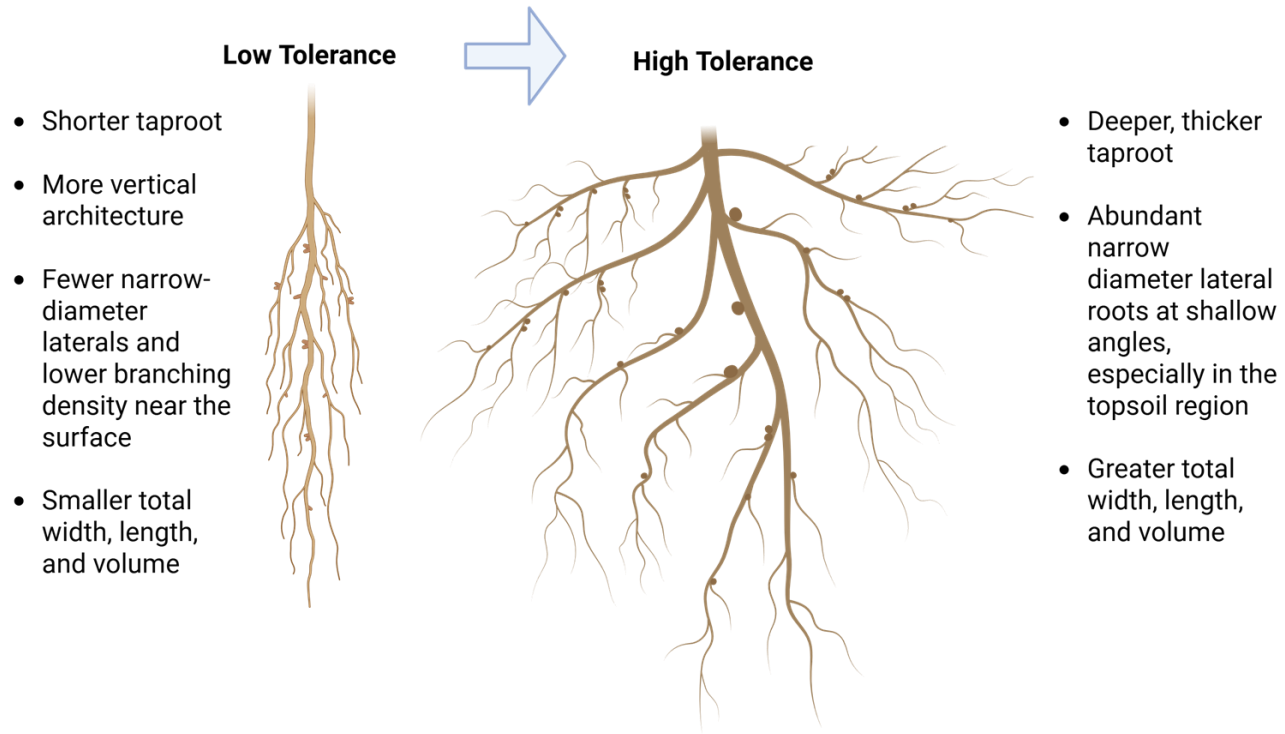

Figure 15. Root ideotype associated with improved field performance in low pH soils. Low-tolerance plants exhibit a more vertical architecture with fewer narrow-diameter laterals near the surface and reduced spatial extent, while high-tolerance plants show a deeper, thicker taproot and numerous shallow-angle, narrow-diameter laterals concentrated in the topsoil, resulting in greater total width, length, and volume.

The results of the present study suggest that indirect selection of acid tolerance through RSA evaluation in rhizobox assays may be a viable option. Selecting genotypes that show increases in total root length, volume, and number under low pH stress, especially in narrow-diameter traits, would allow greater numbers of genotypes to be evaluated for low pH and aluminum tolerance over the course of a few months rather than 4+ years of field phenotyping. The rhizobox screening system developed here will allow the breeding program to accommodate larger populations, increasing the genetic diversity, and decreasing the cycle-time for selection. While field screening for other agronomic traits, such as yield, fall dormancy, disease resistance, and forage quality is essential, the rhizobox assay can help ensure we include only the best possible

genotypes in field trials to maximize resource allocation and improve genetic gain per cycle of selection.

While this study brings new information that may explain the morphological response to low pH and aluminum tolerance in alfalfa, further work is needed to validate the results with a larger population size, more diverse germplasm, and across more pH levels. The genetic underpinnings of RSA phenotypic plasticity, which seem to be critical for low pH tolerance under field conditions, should be further investigated. The current population would be amenable to a Bulk RNA-seq procedure, which would identify differentially expressed genes (DEGs) in the HT versus LT class. An approach like this could provide insight into which genes are preferentially expressed in root tissues to confer tolerance and be used to develop molecular markers to be used in a genomic selection model, which would further improve the accuracy and speed of breeding cycles. Significant DEGs may also inform transgenic approaches to improve low pH tolerance in alfalfa beyond the genetic diversity available and aid in the current understanding of the tolerance mechanism at a cellular level.

Supplementary Material

Supplementary Table S1. T-test results from the RB-in (in situ rhizobox image analysis) dataset comparing performance under low pH conditions to performance under adjusted pH conditions.

Variable	T-Statistic	P-Value	Mean in Low pH Condition	Mean in Adjusted pH Condition	Significance
Perimeter (mm)	5.746	0.000	2,526.082	1,315.065	***
Total Root Length (mm)	5.621	0.000	1,040.417	548.627	***
Network Area (mm²)	5.573	0.000	906.162	469.020	***
Surface Area (mm²)	5.471	0.000	2,737.866	1,420.786	***
Root Length Diameter Range 1 (mm)	5.469	0.000	444.384	224.769	***
Projected Area Diameter Range 1 (mm²)	5.455	0.000	255.974	132.884	***

Variable	T-Statistic	P-Value	Mean in Low pH Condition	Mean in Adjusted pH Condition	Significance
Surface Area Diameter Range 1 (mm^2)	5.455	0.000	804.166	417.469	***
Volume Diameter Range 1 (mm^3)	5.424	0.000	122.158	64.610	***
Volume (mm^3)	5.330	0.000	653.272	322.783	***
Volume Diameter Range 2 (mm^3)	4.948	0.000	531.114	258.172	***
Surface Area Diameter Range 2 (mm^2)	4.891	0.000	1,933.700	1,003.317	***
Projected Area Diameter Range 2 (mm^2)	4.890	0.000	615.464	319.351	***
Root Length Diameter Range 2 (mm)	4.840	0.000	596.033	323.858	***
Holes	4.624	0.000	11.913	4.322	***
Maximum Number of Roots	4.110	0.000	13.804	9.525	***
Average Root Orientation (°)	-4.002	0.000	57.746	65.374	***
Steep Angle Frequency	-3.887	0.000	0.589	0.715	***
Shallow Angle Frequency	3.795	0.000	0.240	0.154	***
Convex Area (mm^2)	3.751	0.000	22,007.073	13,619.964	***
Median Number of Roots	3.416	0.001	2.196	1.576	***
Maximum Width (mm)	3.223	0.002	89.927	67.521	**
Maximum Diameter (mm)	3.043	0.003	2.290	1.985	**
Number of Root Tips	2.958	0.004	59.413	42.356	**
Medium Angle Frequency	2.680	0.009	0.171	0.130	**
Width to Depth Ratio	2.542	0.013	0.319	0.253	*
Lower Root Area (mm^2)	2.066	0.043	380.533	267.294	*

Supplementary Table S2. Paired t-test results from the phenotypic measurements and field-related traits

Variable	T-Statistic	P-Value	Mean in Low pH Condition	Mean in Normal pH Condition	Significance
Average yield per cut (g) in field trials	-4.639	0.000	150.886	193.008	***
Root Length-3	3.161	0.002	38.086	29.900	**
Root Length-4	2.790	0.008	38.350	30.604	**
Shoot Fresh Weight (g)	-2.472	0.015	1.169	1.657	*
Average Fresh Weight Root per Plant (g)	-2.370	0.020	0.127	0.180	*

Variable	T-Statistic	P-Value	Mean in Low pH Condition	Mean in Normal pH Condition	Significance
Average Root Length	2.355	0.020	34.155	30.196	*
Shoot Dry Matter %	2.214	0.029	0.152	0.130	*

Supplementary Table S5. Paired t-test results from the excavated image analysis showing only variables that significantly vary under low pH conditions.

Variable	T-Statistic	P-Value	Mean in Low pH Condition	Mean in Adjusted pH Condition	Significance
Root Depth (mm)	4.165	0.000	402.875	344.105	***
Root Length Diameter Range 1 (mm)	2.616	0.009	363.374	289.763	**
Surface Area Diameter Range 1 (mm^2)	2.356	0.019	610.753	498.931	*
Projected Area Diameter Range 1 (mm^2)	2.356	0.019	194.406	158.814	*
Lower Root Area (mm^2)	2.080	0.038	1,395.016	1,103.045	*
Volume Diameter Range 1 (mm^3)	1.971	0.050	83.503	70.259	*

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CHAPTER 4

GWAS IDENTIFYING MULTIPLE LOCI ASSOCIATED WITH FIELD PERFORMANCE AND FORAGE NUTRITIONAL CONTENT OF ALFALFA UNDER LOW PH AND ALUMINUM STRESS

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Abstract

Alfalfa performance is significantly reduced on acidic, Al^{3+}-toxic soils that predominate subtropical regions globally. The genetic basis for low pH and aluminum tolerance in alfalfa is not well-characterized, and no cultivar with sufficient tolerance is currently available. The present research leverages a population of 138-half-sib families that were phenotyped in row plots in a natural low pH soil (4.9-5.2, extractable aluminum=10.41-11.38 mg kg-1) and an adjusted pH soil (pH=6.37-7.07, extractable aluminum=0.01-2.29 mg kg-1) across six environments (locations x years). Yield traits and fall dormancy ratings (FDR) were assessed on a plot-basis in each pH condition and used to generate an Acid Soil Adaptability Index (ASAI). Near Infrared Reflectance Spectroscopy (NIRS) was also used to assess 12 key nutrient traits for each row-plot. The effect of low pH and aluminum stress on each of these traits showed significant reductions in yield, FDR, and many nutritional parameters. Each row plot was sequenced by genotype-by-sequencing (GBS) and DArTag methods to perform a GWAS and identify QTL associated with these traits. 17 candidate genes associated with nutrient traits and 5 candidate genes associated with field-related traits were annotated. Genomic prediction (GP) models were built using both sequencing datasets and compared for accuracy. The GBS sequencing produced more SNP markers than the DArTag approach (17,991 vs. 2609), leading to an increase in the number of QTL identified and the higher prediction accuracy of GP. Taken together, the results of the study identify genomic regions of interest and biologically coherent candidates for improving alfalfa performance on acidic soils and demonstrate that targeted sequencing panels can provide competitive prediction power while complementing discovery from higher SNP-density GBS.

Keywords: alfalfa, GWAS, stress, genomic prediction, sequencing

Abbreviations: Acid detergent fiber, ADF; ASAI, Acid Soil Adaptation Index; AvgYield, average yield per cut; Best linear unbiased estimates, BLUEs; Best linear unbiased predictors, BLUPs; Dry matter yield, DMY; Fall Dormancy Rating, FDR; Genotype-by-sequencing, GBS; Near Infrared Reflectance Spectroscopy, NIRS; NDF, neutral detergent fiber; NPGS, National Plant Germplasm System; Randomized complete block design, RCBD; Single nucleotide polymorphism, SNP; TotalYield, recurrent phenotypic selection, RPS; total yield per year

Introduction

Alfalfa, the so-called "Queen of Forages," is known for being a high-quality forage and a major source of protein for livestock and dairy production. Alfalfa is a highly adaptable perennial legume and is grown widely around the world, with more than 1.85 million acres planted in the US alone in 2024 (USDA-NASS, 2025). Alfalfa is an exceptionally valuable crop, worth more than 9.5 billion USD in 2024, down from 11.8 billion USD in 2023 (USDA-NASS, 2025). These values do not include the value of alfalfa grown in grass mixtures, pasture-fed, silage, or the value associated with improved animal performance (Popp et al., 2000). Alfalfa production is currently limited in regions with acidic soils largely due to susceptibility to aluminum toxicity, which decreases yield, quality, and persistence of alfalfa stands (Campbell et al., 2011; Dall'Agnol et al., 1996; Khu et al., 2012). Despite significant efforts, there still exists no commercially available cultivar with sufficient tolerance to acidic and aluminum-rich field conditions.

As the final product of alfalfa production is animal performance, nutritive quality is essential. Alfalfa is a nutritionally dense forage, contributing to increased milk and body mass in livestock

production (Higginbotham et al., 2008; Humphreys, 2005). Alfalfa leaves are highly digestible and rich in protein and mineral nutrients, while the stems are higher in fiber and contribute significantly less energy to the animal system. The stem component comprises up to 50-70% of the total biomass, with the portion increasing as maturity increases (Wilman & Altimimi, 1984). High lignin concentration in stem tissues is considered an anti-nutritive quality, as it decreases the digestibility and energy content of the hay or silage. This creates a yield-quality trade-off, where increased maturity increases DMY but decreases hay quality (Casler et al., 1987). Efforts to increase alfalfa quality and digestibility by decreasing lignin content have included traditional recurrent phenotypic selection (Jung & Lamb, 2006; Jung et al., 1997), gene knockdowns of key components in the lignin biosynthetic process (Guo et al., 2001), and increasing the leaf to stem ratio (Luckett & Klopfenstein, 1970). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) both include lignin, but NDF consists of hemicellulose, cellulose, and lignin, while ADF contains only cellulose and lignin, making it the less digestible portion. Hay or silage quality is most accurately assessed through laboratory analysis, through which lignin, NDF, ADF, crude protein, mineral nutrient concentration (P, K, Ca, Mg, etc.) (Zhang & Rocateli, 2017). However, wet-lab chemical analyses for forage quality are costly and time-consuming. Near-infrared reflectance spectroscopy (NIRS) is a cost-effective alternative to approximate these values (Shenk & Westerhaus, 1994). NIRS analysis measures the wavelengths of light reflected and absorbed by a sample and use a calibration model built on thousands of paired spectra and wet chemistry results to estimate key nutritive traits.

It has been shown in previous studies that inadequate soil fertility results in significantly decreased yield and nutritive quality of alfalfa stands (Lissbrant et al., 2009; Wan et al., 2022). As low pH stress decreases nutrient availability in the soil solution and aluminum toxicity

disrupts nutrient uptake, it stands to reason that the nutritive quality of alfalfa stands under low pH stress will also be decreased. To the authors, the effect of low pH soil stress on alfalfa's nutritional quality has not been directly measured. However, the effect of low pH stress in citrus has been shown to significantly decrease concentrations of N, P, Ca, and Mg in leaf tissues, but this effect varied across tissues (stems, leaves, and roots) and by genotype (Long et al., 2017).

Alfalfa is an obligate outcrosser and exhibits significant inbreeding depression, which, when coupled with long generation times associated with perennials, increases the difficulty of recurrent selection efforts (Annicchiarico et al., 2015; Casler & Brummer, 2008). Cultivated alfalfa (*Medicago sativa L*. or lucerne) is an autotetraploid (2n = 4x = 32) with basic chromosome number of 8 and a genome size originally estimated to be 800–1000 Mbp through flow cytometry (Blondon et al., 1994). Alfalfa exhibits tetrasomic inheritance patterns, caused by random pairing of the four homologous chromosomes during meiosis (Muller, 1914). The complexity of the genome and segregation patterns resulted in a scarcity of available genomic resources compared to other agronomic crops. It is only relatively recently that software tools have been developed that are capable of handling polyploidy (Rosyara et al., 2016). Prior to this point, it was common to "diploidize" marker data by collapsing genotypic values to ignore allelic dosage (AAAB, AABB, and ABBB would all be called AB, for example) or by using only dominant markers such as amplified fragment length polymorphisms (AFLPs) or codominant markers such as simple sequence repeats (SSRs), scored as simple presence/absence (Hackett et al., 2017). This allowed software designed for diploid organisms to be employed but is limited to traits displaying simplex dominant (where AAAA, AAAB, AABB, and ABBB all have the same phenotype and BBBB is dominant, for example) and diploidized additive effects (where all heterozygotes have the same intermediate phenotype between AAAA and BBBB). It has been

demonstrated that using these different models of gene action increases the power to detect QTL with the matching mode of action over diploidized models (Rosyara et al., 2016).

Additionally, genetic studies prior to 2020 relied on the genome of its close relative, the diploid M. *truncatula* or barrel medic (2n = 2x =16 = 860 Mb) (Young et al., 2011). M. *truncatula* or is an ideal model species, with simple inheritance patterns, quick generation times, ample seed production, the ability to be transformed, and a relatively recent divergence at 5.29 MYA (Cook, 1999). While there are obvious drawbacks to relying on a genome assembly that is from a different species with a different ploidy, it has been shown that there is high synteny between *M. truncatula* and *M. sativa* (Li et al., 2014) and genetic studies have successfully used this reference to identify marker associations with many traits, such as forage quality traits (Biazzi et al., 2017; Lin et al., 2025), performance under salt stress (Liu et al., 2019; Liu & Yu, 2017), and aluminum tolerance (Khu et al., 2010). An allele-aware chromosome genome assembly of a common autotetraploid Chinese cultivar, XinjiangDaye, was published in 2020 with a combined BUSCO score of 97.2% comprising ~3.15 Gb (Chen et al., 2020). 164,632 protein-encoding genes were identified, of which more than 157,000 have been annotated, providing an excellent resource for researchers and breeders. This research also provided molecular evidence for tetrasomic inheritance patterns with allelic chromosomes consistently displaying ~0.01 sequence divergence and showed that alfalfa seems to be a stable autotetraploid with no overall allelic dominance in gene expression and no signs of diploidization (Chen et al., 2020; Mandáková & Lysak, 2018). This genome has since been leveraged in many GWAS and genomic selection approaches and has resulted in genetic markers associated with root rot (He et al., 2025), leaf size (Xu et al., 2023; Xu et al., 2025), protein and fiber content (Lin et al., 2025;

Yang et al., 2021), salt tolerance (F. He et al., 2022; Medina et al., 2020), and drought tolerance (Medina et al., 2025) in autotetraploid alfalfa.

In the present research, recent developments in genomic resources for alfalfa and decreasing sequencing costs have been leveraged to identify QTL associated with yield, fall dormancy rating (FDR), and 14 nutrient quality traits under low pH field stress on these traits. A partially improved panel of 138 half-sib families has been evaluated for yield in a multi-year, multi-location trial (Athens, GA: 2020-2024, Tifton, GA: 2023-2024) in both low pH and adjusted pH field conditions. Each plot was also assessed for fall dormancy rating and analyzed for nutrient content. Conventional GBS and DArTag sequencing data were obtained for each half-sib family under evaluation and used for GWA analysis.

Materials and Methods

Phenotypic data collection

A partially-improved population of 133 individuals was developed by screening 966 diverse plant introductions (PIs) from the National Plant Germplasm System (NPGS) in a low pH field environment. The 133 selections were crossed in a greenhouse bee cage to develop half-sib families and planted with 5 check cultivars in both a naturally low pH field site (pH=4.90, extractable aluminum=10.41 mg/kg) and an adjusted pH field site which received 60 lbs of Pennington Fast Acting Lime prior to planting (pH=6.37, extractable aluminum=2.29 mg/kg) in Athens, GA in May 2020. Each trial consists of 138 entries (133 experimental and 5 commercial checks, including 2 cultivars developed for the southeast and 3 accessions developed in the lab and greenhouse for AL-tolerance) in a randomized complete block design (RCBD) with two replications. Each plot was established with eight individual plants from the same family equally

spaced in a 1.52 m row with 0.76 m between rows. The trials were replicated by stem cuttings and used to establish an identical trial in the fall of 2022 at the University of Georgia's Animal Science Farm in Tifton, GA. The low pH field site in Tifton has a pH = 4.9 (exchangeable aluminum content of 11.38 mg kg^{-1}) and the adjusted pH site has a pH = 7.07 (exchangeable aluminum content of 0.01 mg kg^{-1}).

Yield data was collected on a per-plot basis every month through the growing season (April-October) in Athens from 2021-2024 and in Tifton from 2023-2024 using a Carter flail harvester. Dry matter yield (DMY) was calculated by drying a representative sample in a convection dryer for 48 hours and multiplying by the fresh weight measured in the field. Harvests in July and September 2022 and August and September 2023 in Athens had to be discarded due to significant deer grazing. Average yield per cut (AvgYield) was calculated by summing the DMY for each plot over the year and dividing by the number of harvests, and total yield per year (TotalYield) was calculated by summing the DMY over year x location combination. Best linear unbiased estimates (BLUEs) of average DMY per cut were obtained from a linear mixed-effect model that included genotype (plot) and soil pH condition as fixed effects, and year, location, and plant count per plot as random effects. Statistical analysis was carried out in Microsoft Excel (version 2412) and RStudio (version 2024.12.0).

Tolerance of alfalfa genotypes to low acid soil was assessed according to the Acid Soil Adaptation Index (ASAI) as described in Howeler (1991): $\boldsymbol{ASAI} = \frac{\boldsymbol{Ys\ x\ Yp}}{\boldsymbol{\mu s\ x\ \mu p}}$, where Ys = Yield in low pH condition, Yp = Yield in normal pH condition, μs = Average yield in low pH condition, μp = Average yield in normal condition. The index is scaled such that tolerant genotypes have ASAI>1 and non-tolerant genotypes have ASAI<1. ASAI values were assigned on a plot basis to align with the genotyping scheme used in this study, using the average yield per cut summarized

over years and locations. Because the ASAI assigns one value per adjusted-low combination, the same ASAI value was assigned to both the low pH and adjusted pH plot entries.

Fall dormancy ratings (FDR) were determined by measuring plant heights for each plot roughly 30 days after the final harvest in Athens in 2021 and 2022 and comparing them to the heights of the 11 check cultivars of each dormancy class. The 11 checks were established in five replications in an RCBD beside the adjusted pH field site and were cut on the same day of the final harvest. Plant heights from the checks and their known dormancy class were used to create a regression equation modeling plant height by dormancy class. This equation was rearranged to estimate FDR from plant height. BLUEs for FDR were calculated using the two years of data.

Nutrient data was obtained by collecting a sample from each plot during the first harvest in April 2024, drying, grinding to 2 mm with a Thomas Wiley Mill, and scanning with a Perkin Elmer DA 7250 Near-infrared spectrophotometer (NIRS). The NIRS measured 12 nutrient traits, including Moisture (%), Ash (Dry basis %), ADF (Dry basis %), NDF (Dry basis %), Energy (MJ kg^{-1}), Fat (Dry basis %), Calcium (As is %), Phosphorus (Dry basis %), Fiber (As is %), Crude protein (Dry basis %), Sugar (Dry basis %), and Lignin (Dry basis %).

Generating GBS data

One leaf per plant in a plot was collected and freeze-dried for 48 hours. This ensured equal contribution of each individual in the half-sib family. The tissue was ground using the Geno/Grinder 2010 (Cole-Parmer) and a modified CTAB DNA extraction protocol was carried out as originally described by Doyle and Doyle (1987). DNA quality was assessed with the Qubit 3.0 fluorometer and NanoDrop 2000 spectrophotometer. High quality DNA was diluted to 50 ng/µL. The population was divided into three roughly equal parts (187, 187, and 186 samples in

Pools 1-3, respectively). An in-house library preparation procedure was followed for each pool largely as described by Qi et al. (2018). Briefly, the high quality, diluted DNA was digested with MspI and PstI and a common Y-adaptor and individual barcoded adaptors were ligated to the MspI and PstI sites, respectively. Samples were then PCR-amplified and equal amounts of each sample bulked. The bulk was then cleaned twice using a QIAquick PCR Purification Kit (Qiagen) to remove fragments <200 bp. The result was quantified on the Qubit 3.0 and verified by agarose gel electrophoresis. The three pooled libraries were submitted separately to the Georgia Genomics and Bioinformatics Core (GGBC) for paired-end sequencing with Illumina NextSeq2000 (Athens, GA, RRID:SCR_010994). Pools 1, 2, and 3 generated 529,159,008, 639,531,520, and 541,720,896 paired-end reads respectively.

GBS data filtering

The UGbS-Flex pipeline was followed as described by Qi et al. (2018) up until variant calling (scripts and descriptions can be accessed: https://devoslab.franklinresearch.uga.edu/scripts-used-gbs-pipeline). Briefly, raw reads were split by barcode and barcode adapter sequences, and low-quality bases were removed using FastX trimmer. Reads were then aligned to the first allelic chromosome of the XinjiangDaye reference genome using BWA mem (Chen et al., 2020). The resulting files were sorted and indexed using SAMtools prior to variant calling with FreeBayes. All samples were then merged using bcftools. The original VCF contained more than 864K SNPs, 115K MNPs, 73K indels, and 24K others across 551 half-sib families. Variants were filtered in a stepwise workflow using bcftools (v1.18), and filtering thresholds were determined empirically by assessing the parameters of the original file to isolate the best-supported portion of loci. We first retained only biallelic SNPs. We required a high site quality by excluding sites with QUAL < 1,000 and site-level depth

INFO/DP between 500-4,238 to ensure high coverage while trimming extreme values. Next, we addressed missingness at both the site and sample levels. Site-level missingness was computed with +fill-tags, and sites with >50% missing genotypes were removed. Genotype-level missingness per sample was calculated from the GT field and samples with >50% missing calls across sites were excluded. Finally, we filtered based on $0.05 \geq MAF \leq 0.95$ to exclude exceedingly rare or near-fixed SNPs. BGZF compression and tabix indexing were applied after each step, yielding the final analysis set. Quality and SNP density plots were evaluated at each stage of the filtering process to ensure a good tradeoff between coverage and quality was achieved.

A dosage file was generated using the VCF2dosage function in the GWASpoly package (Rosyara et al., 2016), which produced 18,130 high-quality SNP markers across 518 half-sib families. SNPs were well-distributed across the genome, and 37% were found in coding regions. An LD plot showed that linkage was low ($R^2 = 0.15$) even at 0 bp distance and decayed slowly to 0.02 at ~2 Mb and 0.01 at ~10 Mb.

DArTag sequencing data

DArTag sequencing data was generated using the 3K genotyping panel by the Breeding Insight at Cornell University (Ithaca, NY, USA) in collaboration with the USDA-ARS (Zhao et al., 2023). This assay uses custom-designed oligos to amplify targeted SNPs and flanking sequences prior to NGS. The sequenced amplicons are demultiplexed and analyzed using a proprietary pipeline developed by Breeding Insight. The amplicon sequences are processed as microhaplotypes (81 bp), producing a Missing Allele Discovery Count (MADC) file. This sequencing approach generates fewer markers than conventional GBS but due to the increased

accuracy and specificity for known regions of interest, it has been shown to be effective for separating populations and making breeding decisions in complex autotetraploid systems (Endelman et al., 2024; Cesar A. Medina et al., 2025; Zhao et al., 2024). The same high-quality, diluted DNA used for GBS library preparation was sent to Breeding Insight for sequencing (~100x per marker per sample) and upstream processing. A final dosage file containing 2,604 high-quality, polymorphic, biallelic SNPs was generated for 551 half-sib families. These markers were well-distributed across the genome, and 43% of the SNPs were in coding regions.

GWAS and gene annotation

Prior to GWA, the R function pcadapt was used to identify and remove SNPs showing significant association with population structure (Luu et al., 2017). Locus p-values were adjusted for multiple testing using the Benjamini–Hochberg procedure, and SNPs with FDR $q<0.10$ were classified as outliers and removed from downstream analyses (18 removed from DArTag, 139 removed from GBS). PCAs were conducted for both the DArTag and GBS data using the prcomp function in R and PCs 1 and 2 were plotted and visually inspected to determine a threshold for removing sample outliers (PC1 or PC2 > 20 for DArTag, PC1 or PC2 > 30 for GBS). The final cleaned DArTag dosage file contained 2,609 polymorphic SNPs for 529 half-sib families, and the GBS dosage file contained 17,991 SNPs for 495 families.

The GWASpoly package in R was used to conduct GWAS for both the GBS and DArTag data. Both Q and K matrices were included in the analysis. The Q matrix was obtained from PCA, and the K matrix was determined by default in GWASpoly after inputting the phenotype and SNP data. The leave-one-chromosome-out (LOCO) option was used to control population structure and the significance threshold was set using “M.eff” parameter and the recommended maximum genotype frequency of 1 – 5/N, where N is the number of individuals with marker and

phenotypic data (Rosyara et al., 2016). This helps control the false-positive rate and remove markers that lack sufficient power to be useful. All five possible models for an autotetraploid were ran (Table 1) and QQplots evaluated to ensure good fit. GWAS was conducted for field-related BLUEs and on raw NIRS derived nutrient data. Scree plots for both datasets indicated the inclusion of three PCs in the model to further control population structure. For the DArTag data, pH condition, and three PCs were included as fixed effects. The GBS data was processed identically, aside from including pool additionally as a fixed effect to account for any residual technical artifacts.

Table 6. Descriptions of the dosage-dependent models available in the GWASpoly R-package.

Model	GWASpoly code	Assumed dosage effect
Additive	Additive	Linear dose-response; each additional B allele adds the same increment to the phenotype
General	General	No constraints; allows any pattern across dosages
Simplex dominant (B>A)	1-dom-alt	1 copy of B produces the dominant effect
Simplex dominant (A>B)	1-dom-ref	1 copy of A produces the dominant effect
Duplex dominant (B>A)	2-dom-alt	2 copies of B produce the dominant effect
Duplex dominant (A>B)	2-dom-ref	2 copies of A produce the dominant effect
Diploidized general	diplo-general	Heterozygotes all share one mean; not constrained to be halfway between homozygous
Diploidized additive	diplo-additive	Heterozygotes assumed to be the average of the two homozygous

The get.QTL function was used to identify QTL in a 5 Mb clumping window around each significant marker. If more than one model supported a marker, the model with the highest score was selected and used to obtain effect estimates and partial R^2 values using the fit.QTL function. Candidate genes were identified by building fixed ±2 kb windows around each lead SNP position (Lin et al., 2021) and intersecting those windows with gene models from the XinjiangDaye reference GFF using the rtracklayer package. The minimal distance from the marker to the gene

was calculated as defined as the bp distance from the SNP to the nearest gene boundary, not to the edge of the ±2 kb window. Therefore, if a SNP lies within a large gene, the distance can be greater than 2 kbp. Identified genes within the region were characterized using the MODMS database, which was released in 2024 using recent genomic and transcriptomic data for alfalfa (access: https://modms.lzu.edu.cn/) (Fang et al., 2024).

Genomic prediction and cross-validation

The BreedWheat Genomic Selection (BWGS) package was used to generate GEBVs, perform genomic prediction (GP), and cross-validate the results (Charmet et al., 2020). Markers were filtered by minor-allele frequency (MAF) ≥ 0.01, and monomorphic markers were removed. BLUPs were generated by fitting a mixed model predicting AvgYield by genotype (Condition x Plot combinations), environment (year x location), and average plant count per plot included as fixed effects. BLUPs were extracted using the ranef function in R and used for both DArTag and GBS GP. Dosages were linearly recoded to BWGS's expected diploid scale and missing values were imputed within BWGS using the Expectation–Maximization Imputation (EMI) algorithm. We used an 80/20 training/test split using ridge regression (RR) to obtain genomic estimated breeding values (GEBVs) for the test set. Predictive ability was summarized by the Pearson correlation between observed BLUPs and GEBVs and the coincidence index for

the top 20% (proportion of individuals jointly ranked in the top quantile by observation and prediction).

Results

Field Traits

BLUEs were generated for all field phenotypic traits, apart from ASAI, as described in the methods section. Because “condition:” was included as a fixed effect in the model, the distribution mirrors that of the raw distribution, with the adjusted pH having a significantly greater yield and higher FDR compared to the low pH conditions. Sufficient normality and variation were present in these phenotypes to use for GWAS, and suggest these traits are controlled by many loci.

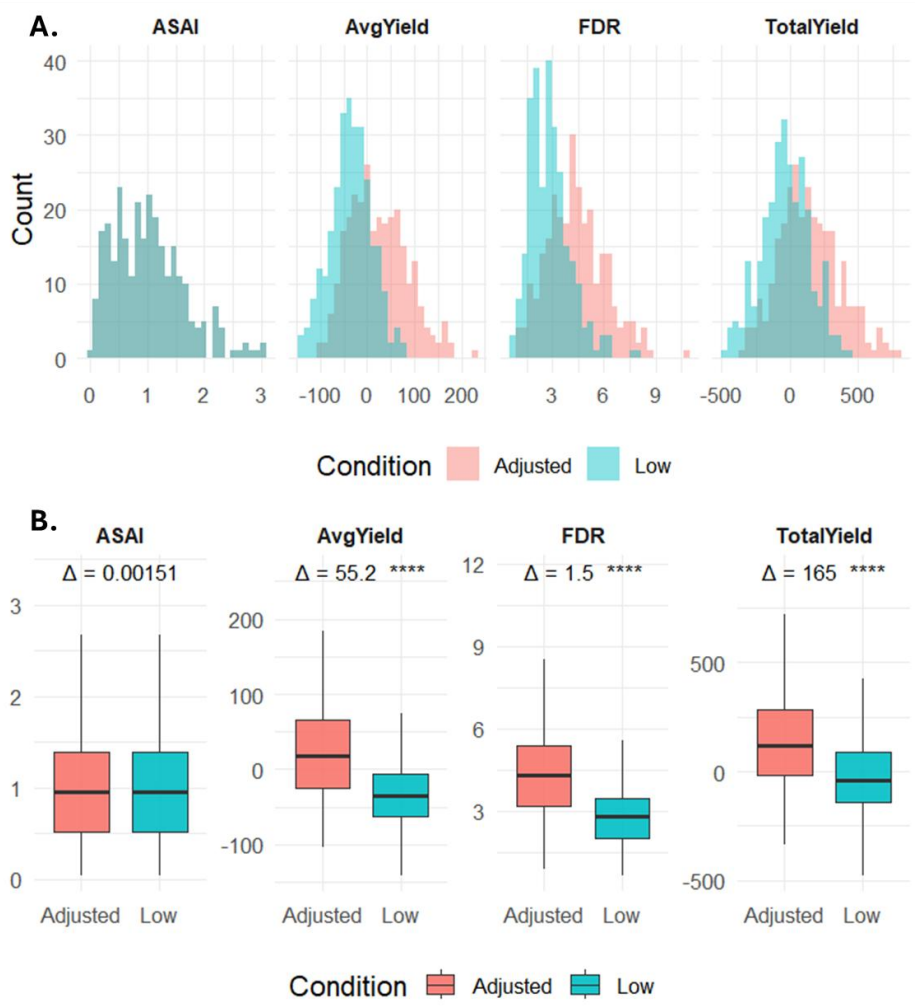

Figure 1. Histograms (A) and boxplots (B) show the distribution of each field-related trait by pH condition. The delta values (Δ) were computed as: (Average in the adjusted pH condition – Average in the low pH condition). A two-sided Wilcoxon rank-sum test was conducted per trait, and the false discovery rate was controlled using Benjamini–Hochberg adjustment. Adjusted p-values were summarized as significance codes (ns = $p>0.05$, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$).

For the GBS data, there were a total of 17,991 SNP markers and 495 half-sib families with both genotypes and phenotypes. For the DArTag data, there were 2,609 SNPs and 529 half-sibs with paired phenotypes and genotypes. The GBS data identified one QTL for ASAI and one QTL for AvgYield, both on Chromosome 7 (Figure 2A). The DArTag data identified three significant markers for ASAI on chromosomes 3, 4, and 8 (Figure 2B). Many markers were just under the threshold for the GBS data, including peaks for AvgYield and TotalYield on chromosomes 3 and

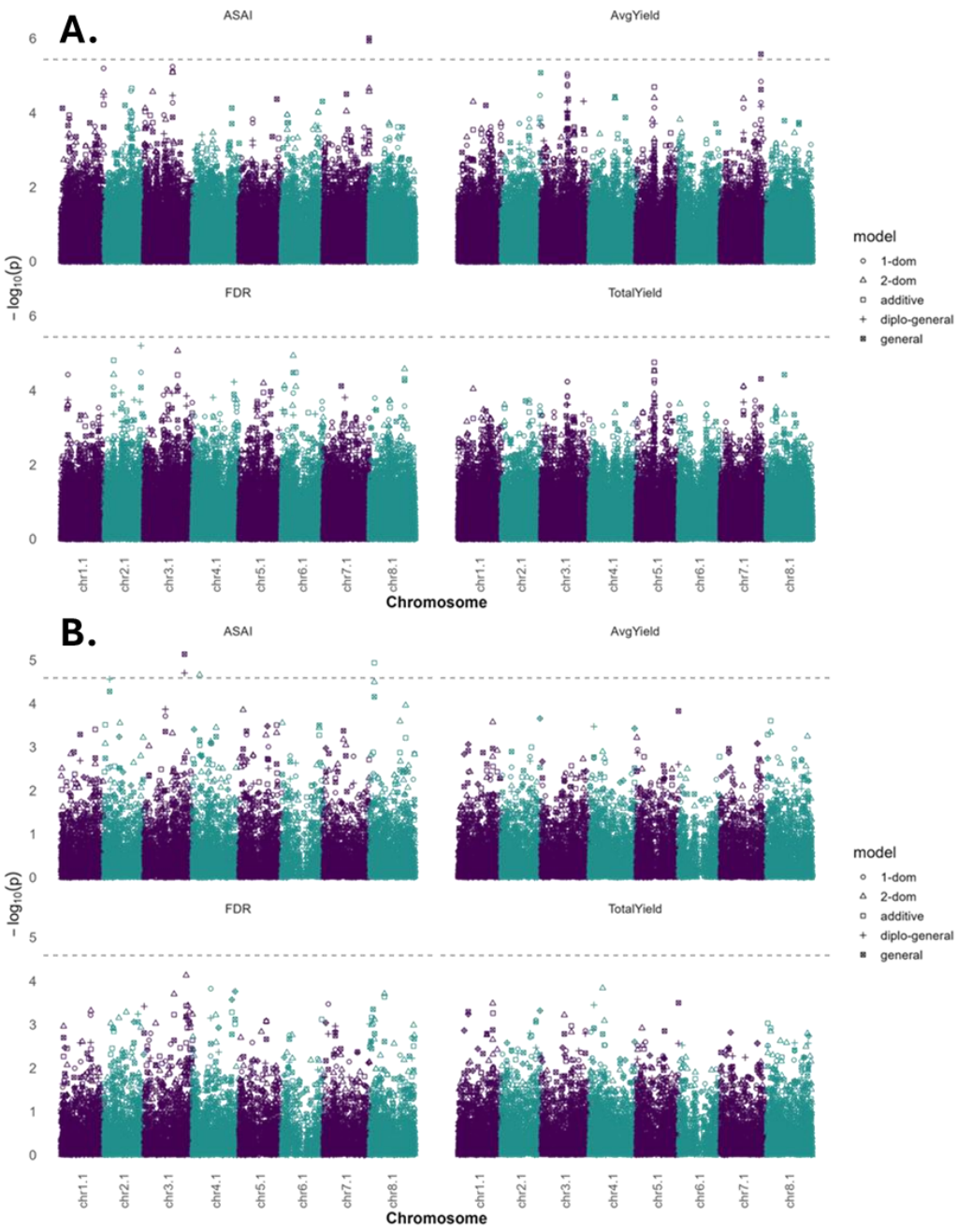

Figure 2. Manhattan plots for field-related traits with GBS genotyping (A) and DArTag genotyping (B).

5, and peaks for FDR on chromosomes 2, 3, and 6. Scores and effects for the significant markers are shown in Table 2. Effects cannot be estimated for general and diplo-general models. DArTag data was able to identify one marker that positively affects ASAI (chromosome 4) and one that negatively affects ASAI (chromosome 8), while the rest could not be estimated. Two markers

less than 30 bps from each other on chromosome 7 (chr7.1_87481703 and chr7.1_87481731) were identified by the GBS data and were therefore associated with the same candidate gene.

Table 7. Significant markers associated with field-related traits by genotyping system. Markers are given as chromosome_postition and if more than one model identified the marker, the one with the higher score is given in max_Score and the higher effect is given in max_Effect.

Genotyping	Trait	Marker	Ref	Alt	Models	max_Score	max_Effect
DArTag	ASAI	chr3.1_77747393	A	T	diplo-general; general	5.13	NA
DArTag	ASAI	chr4.1_13872451	G	A	2-dom-alt	4.66	0.23
DArTag	ASAI	chr8.1_9061291	A	C	additive	4.93	-0.20
GBS	ASAI	chr7.1_87481703	G	A	general	6.01	NA
GBS	ASAI	chr7.1_87481731	TAA	TAG	general	5.94	NA
GBS	AvgYield	chr7.1_77424810	T	A	general	5.58	NA

Each significant marker was within ±2 kb of a gene and was annotated using the MODMS database. Partial R^2 values, which approximate the percentage variance explained (PVE) by the marker, were generated for each (Table 3).

Table 8. Candidate genes within 2 kb flanking window of markers significantly associated with field traits by genotyping system. Gene annotation and R^2 (approximates percent variance explained) are given for each candidate.

Genotyping	Trait	Gene ID	Distance from SNP (bp)	Gene annotation	R^2	p-val
DArTag	ASAI	MS.gene06325	4668	Cation-transporting ATPase	0.05	6.78E-06
DArTag	ASAI	MS.gene53383	2348	Unknown	0.03	2.45E-05
DArTag	ASAI	MS.gene035287	1057	Chromatin-remodeling complex ATPase	0.03	3.74E-05
GBS	ASAI	MS.gene069346	825	Heparan-alpha-glucosaminide N-acetyltransferase-like	0.06	1.13E-06
GBS	AvgYield	MS.gene022104	1624	Protein MEI2-like 4 isoform X1	0.06	6.20E-06

Nutrient Traits

All phenotypic traits were evaluated for normality prior to GWA analysis (Figure 3A). Overall, nutrient traits were normally distributed except for moisture, which showed two peaks. Given the sample size (n=439), there was sufficient data to move forward with each trait for GWA analysis. All traits except for calcium and ADF varied significantly by pH condition. Low pH stress significantly reduced ash, crude protein, energy, fiber, moisture, phosphorus, and sugar, and increased fat and lignin (Figure 3B).

Figure 5. Histograms (A) and boxplots (B) show the distribution of each nutrient trait by pH condition. The delta values (Δ) were computed as: (Average in the adjusted pH condition – Average in the low pH condition). A two-sided Wilcoxon rank-sum test was conducted per trait, and the false discovery rate was controlled using Benjamini–Hochberg adjustment. Adjusted p-values were summarized as significance codes (ns = $p>0.05$, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$).

GWAS using the GBS sequencing data consisted of 17,991 SNP markers and 410 paired nutrient phenotypes and identified significant markers associated with ash (1), ADF (1), calcium (1), crude protein (2), energy (2), fat (1), lignin (3), and phosphorus were identified (Figure 4A). This analysis showed that three markers were associated with two nutrient traits: marker chr8.1_72142036 with ADF and energy, chr1.1_69599234 with calcium and crude protein, and chr2.1_42727915 with crude protein and phosphorus. Marker chr8.1_72142036 was negatively associated with ADF and positively associated with energy, as expected. Marker chr1.1_69599234 was negatively associated with both calcium and crude protein. The effect of marker chr2.1_42727915 could not be estimated on either crude protein or phosphorus because the only model to identify this locus was the general model. GWAS with the DArTag consisted of 2,609 markers and 439 paired phenotypes, and identified markers significantly associated with fat (1) and sugar (3) (Figure 4B). Significant markers with scores and effects are given in Table 4.

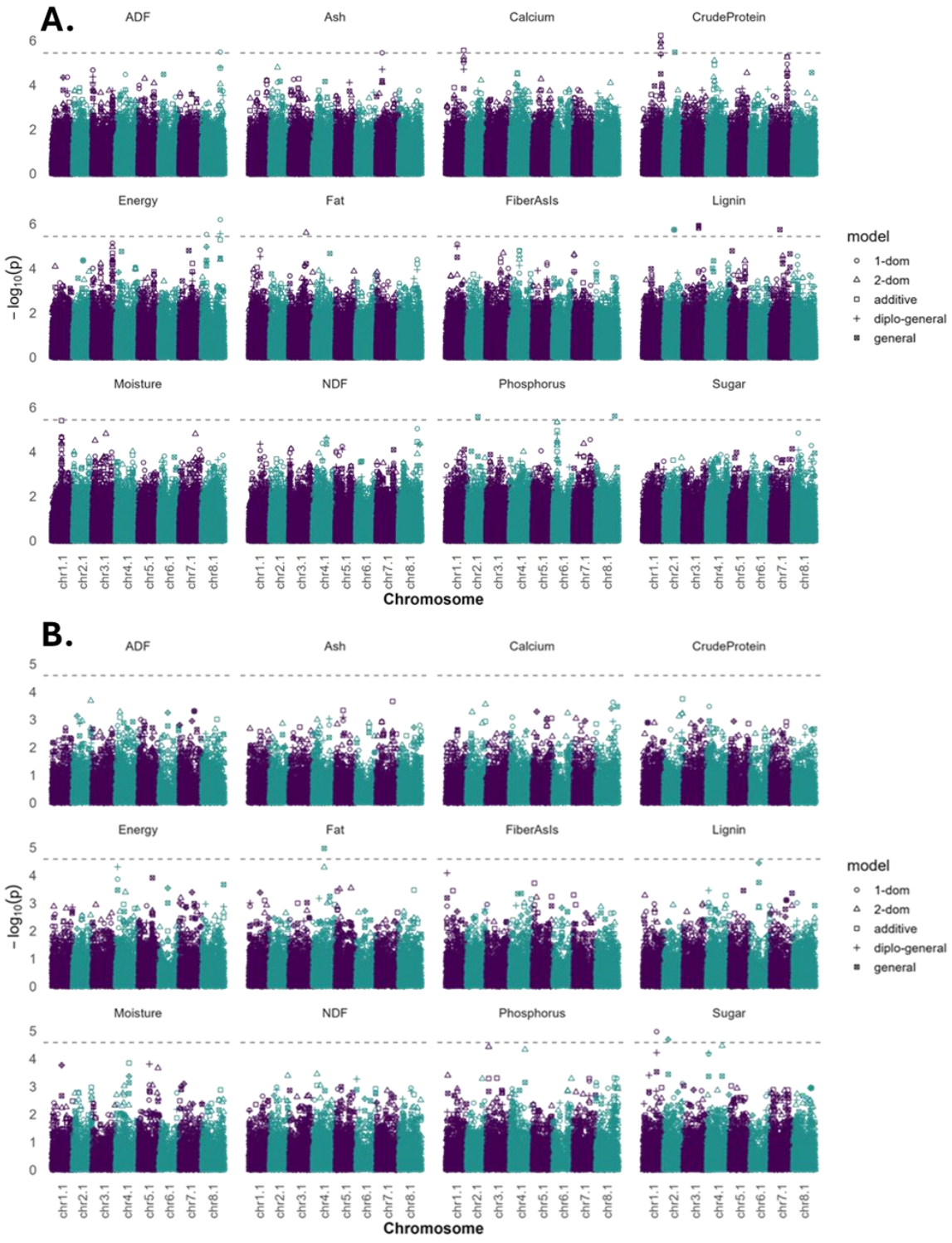

Figure 6. Manhattan plots for nutrient traits with GBS genotyping (A) and DArTag genotyping (B).

Table 9. Markers significantly associated with nutrient traits by genotyping system. Markers are given as chromosome_postition and if more than one model identified the marker, the one with the higher score is given in max_Score and the higher effect is given in max_Effect.

Genotyping	Trait	Marker	Ref	Alt	Models	Max Score	Max Effect
DArTag	Fat	chr4.1_44553513	G	A	General	4.97	NA
DArTag	Sugar	chr1.1_53207743	C	T	1-dom-alt	4.98	-1.14
DArTag	Sugar	chr2.1_16773051	A	G	1-dom-ref; diplo-general	4.70	0.96
DArTag	Sugar	chr4.1_6641999	T	C	1-dom-ref	4.17	1.99
GBS	ADF	chr8.1_72142036	TTAG	TTAC	1-dom-ref	5.48	-1.70
GBS	Ash	chr7.1_21998320	C	A	1-dom-alt	5.44	-0.65
GBS	Calcium	chr1.1_69599234	A	G	Additive	5.55	-0.01
GBS	CrudeProtein	chr1.1_69599234	A	G	1-dom-alt; 2-dom-alt; 2-dom-ref; additive	6.22	-1.15
GBS	CrudeProtein	chr2.1_42727915	C	T	General	5.47	NA
GBS	Energy	chr8.1_17824261	G	A	1-dom-ref	5.52	-0.27
GBS	Energy	chr8.1_72142036	TTAG	TTAC	1-dom-ref; diplo-general	6.19	0.29
GBS	Fat	chr3.1_66443643	A	G	2-dom-alt	5.60	0.19
GBS	Lignin	chr2.1_39856842	GA	AA	diplo-general; general	5.74	NA
GBS	Lignin	chr3.1_60231904	A	G	General	5.92	NA
GBS	Lignin	chr7.1_36583194	C	T	General	5.74	NA
GBS	Phosphorus	chr2.1_42727915	C	T	General	5.58	NA
GBS	Phosphorus	chr8.1_73731891	TAGTA	TAGTC	General	5.61	NA

Genes within a 2 kb flanking region around each significant marker were identified as described in the methods section and annotated with MODMS database (Table 5). Two genes were located in the region surrounding marker chr1.1_53207743, identified by DArTag

sequencing to be associated with sugar content. Otherwise, a single gene was identified for each significant marker.

Table 10. Candidate genes within 2 kb flanking window of markers significantly associated with nutrient traits by genotyping system. Gene annotation and R2 (approximates percent variance explained) are given for each candidate.

Genotyping	Trait	Gene ID(s)	Distance from SNP (bp)	Gene annotation(s)	R^2	p-val
DArTag	Fat	MS.gene070810	95	Unknown	0.06	7.22E-06
DArTag	Sugar	MS.gene91501	2789	SWI SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 3-like	0.04	4.29E-05
DArTag	Sugar	MS.gene49352; MS.gene49351	192; 1924	Protein OSB2, chloroplastic-like; Aux IAA protein, repressor of early auxin response genes at low auxin concentrations	0.03	8.65E-05
DArTag	Sugar	MS.gene70091	953	Belongs to the tRNA nucleotidyltransferase poly(A) polymerase family	0.03	8.05E-05
GBS	ADF	MS.gene066429	1476	Unknown	0.05	2.92E-06
GBS	Ash	MS.gene024221	487	Phenylalanine-tRNA ligase, chloroplastic	0.05	5.82E-06
GBS	Calcium	MS.gene88831	567	Receptor homology region, transmembrane domain- and RING domain-containing protein	0.05	2.75E-06
GBS	Crude Protein	MS.gene88831	567	Receptor homology region, transmembrane domain- and RING domain-containing protein	0.06	6.39E-07
GBS	Crude Protein	MS.gene75840	631	Unknown	0.08	7.38E-07
GBS	Energy	MS.gene56884	9932	Exportin 1-like protein	0.03	1.36E-04
GBS	Energy	MS.gene066429	1476	Unknown	0.06	1.01E-06
GBS	Fat	MS.gene70448	1298	BTB POZ domain-containing protein	0.05	2.20E-06
GBS	Lignin	MS.gene97267	1882	Cyclic nucleotide-gated ion channel	0.01	1.37E-01
GBS	Lignin	MS.gene30742	686	Transcription factor	0.05	5.14E-04
GBS	Lignin	MS.gene94794	1703	Phosphatidylinositol ceramide inositolphosphotransferase	0.03	2.77E-02
GBS	Phosphorus	MS.gene75840	631	Unknown	0.07	6.90E-06
GBS	Phosphorus	MS.gene59599	117	Ferric-chelate reductase	0.07	5.57E-06

Genomic prediction with GBS and DArTag sequencing data

Genomic prediction was carried out using BWGS separately for the GBS and DArTag sequencing data for the AvgYield trait, as described in detail in the methods section. Prediction accuracy was assessed using Pearson correlation between observed (BLUPs) and predicted (GEBVs) values and by calculating a coincidence index (CI), which represents the proportion of entries predicted to be in the top 20% that were also observed to be in the top 20% of performers (Figure 5). After additional MAF filtering and EM imputation, 13,026 markers were retained for the GBS data, and 1,176 markers were retained for the DArTag data.

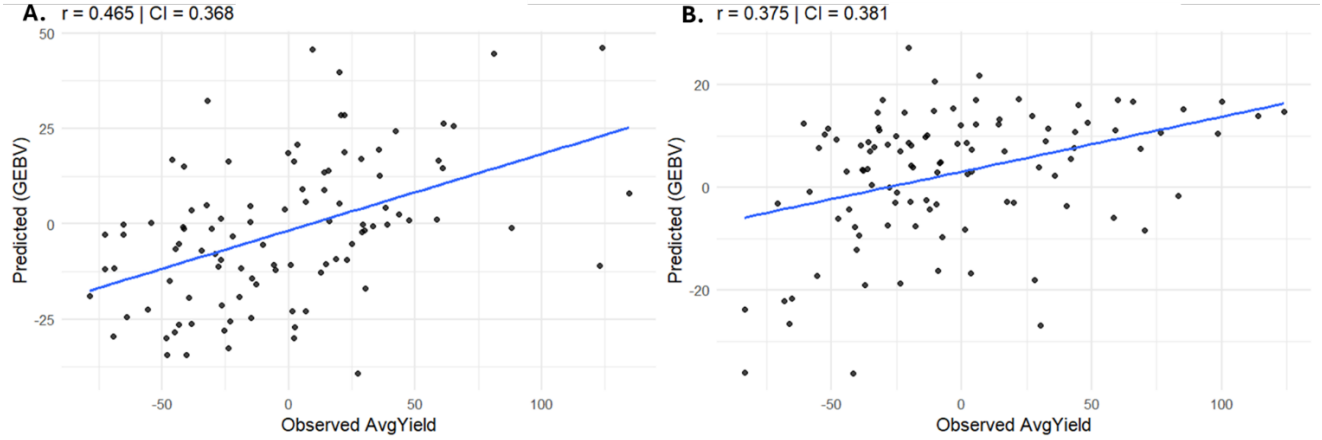

Figure 7. Observed BLUPs for AvgYield versus the predicted (GEBVs) for the 20% training set generated from the GBS sequencing data (A) and the DArTag sequencing data (B). Pearson correlation r and CI are given at the top of each graph.

Discussion

Comparing GBS and DArTag GWAS results

Both GBS and DArTag sequencing data were sufficient to identify significant markers associated with field-related and nutrient traits. The GBS data consisted of many more markers than the DArTag data (17,991 compared to 2,609). However, greater noise, missingness, and association with batch effects in the GBS data resulted in fewer paired entries with both

phenotypes in genotypes (field: 495 vs 529 samples, nutrient: 410 vs 439 samples). The increased SNP density and improved genome coverage of the GBS data increase the chance of capturing LD with causal variants, despite the decreased sample size, and result in identifying more significant markers in both the field and nutrient datasets (Table 6).

Table 11. Number of QTL identified by the genotyping system and trait.

Trait	**GBS**	**DArTag**
ASAI	1	3
AvgYield	1	0
Sugar	0	3
Fat	1	1
Lignin	3	0
CrudeProtein	2	0
Energy	2	0
Phosphorus	2	0
ADF	1	0
Ash	1	0
Calcium	1	0

No QTL were identified by both genotyping systems, despite identical QC and modeling (Figure 6). This was expected rather than surprising, given that the two genotyping platforms interrogate largely nonoverlapping marker sets with different coverage, error, and MAF profiles. Because the two genotyping systems capture different portions of genome, both results are equally valid and identify loci associated with potentially causal variants. The PVE of these loci, as given by the partial R^2 in Tables 3 and 5, ranged from 3% to 7%.

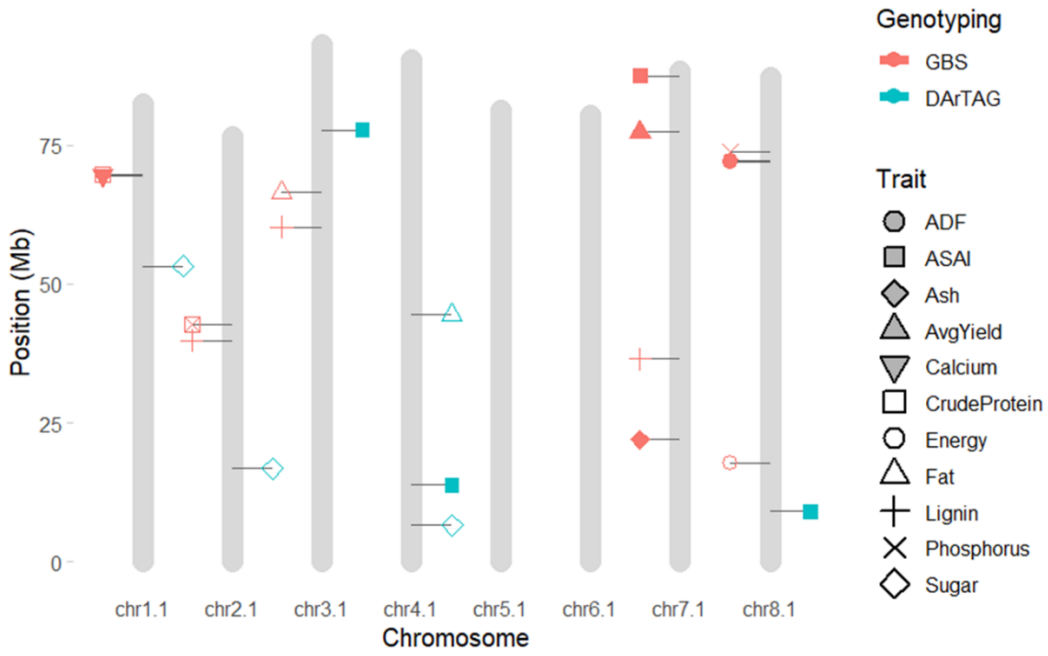

Figure 8. QTL identified by GBS (salmon, shown on left) and by DArTag (teal, shown on right) are mapped to the reference genome chromosomes. Traits are denoted by either filled or outlined shapes.

Genotyping systems capture different portions of the genome; both results are equally valid and identify loci associated with potentially causal variants.

Putative candidate genes associated with field and nutrient traits

A total of five candidate genes were associated with field-related traits. For ASAI, MS.gene06325, a cation-transporting ATPase, may be of particular importance, as these types of proteins have been shown to be important for the uptake and detoxification of toxic Al^{3+} and maintaining membrane potential (Ahn et al., 2001; Matsumoto et al., 1992). The other three genes associated with ASAI have roles in meiosis, signaling, and cell expansion and division with broad implications on plant development and stress responses (Fang et al., 2024). Cell wall stability and structure have been shown to be critical to low pH tolerance in plants, as toxic trivalent aluminum ions can bind carboxylic groups in the pectin matrix, increasing rigidity and interfering with apical cell division and elongation, especially in root tissues (Delhaize et al.,

2007; Kochian et al., 2004; Ma et al., 2001; Ma et al., 2018; Zhang et al., 2014). MS.gene035287, a chromatin-remodeling ATPase, was negatively associated with ASAI and may play a role in reducing cell division under low pH stress. MS.gene069346 is known to acetylate small molecules and polysaccharides and may modulate cell wall dynamics in response to biotic and abiotic stress (Krtenic et al., 2020). MS.gene022104 was associated with AvgYield and is a homolog of AML2 in Arabidopsis, which is known to be important for meiosis and root meristem development (Kaur et al., 2006). As the effect of this locus could not be approximated, it is not clear whether it is a positive or negative regulator of root development.

17 total genes were found to be associated with nutrient traits in this population, including three that were associated with two traits. These genes were associated with cell division (MS.gene91501) and signaling (MS.gene49351, MS.gene88831, MS.gene49351, MS.gene94794, MS.gene97267, MS.gene70448), and protein transcription (MS.gene30742), translation (MS.gene70091, MS.gene024221, MS.gene59599), and trafficking (MS.gene56884). MS.gene066429 is associated with ADF and is located on chromosome 8, which has been shown to be a hotspot for fiber-related traits in diploid alfalfa (Sakiroglu & Brummer, 2017), though the function of this gene is currently unknown. MS.gene49351 was one of two genes neighboring a locus negatively associated with sugar content and is an auxin/IAA repressor. Exogenous auxin has been shown to alleviate aluminum accumulation in cell walls (Wang et al., 2017), indicating increased auxin production may improve aluminum tolerance. The decreased sugar content associated with this gene may be related to root cell wall disruption by aluminum ions, leading to a decrease in root development and nutrient uptake. MS.gene91501 was also negatively associated with sugar and has been implicated in hormone regulation and gene transcription in response to stress (Shani et al., 2017). MS.gene97267 was associated with lignin and encodes a

cyclic nucleotide-gated ion channel (CNGC) which is critical for calcium signaling and homeostasis, and has been implicated in abiotic and biotic stress resistance (Kaplan et al., 2007; Ma, 2011; Panda & Matsumoto, 2007). MS.gene88821 was negatively associated with both calcium and crude protein content and encodes a transmembrane receptor protein that tags soluble proteins for transport to the vacuole (Chen et al., 2025).

Many of these genes have multiple possible functions and participate in a myriad of biosynthetic and signaling processes. Further investigation of these genes into their roles in low pH and aluminum stress tolerance and nutrient accumulation is needed. However, these putative candidate genes provide a starting point for understanding the genetic basis of low pH and aluminum tolerance in alfalfa.

Comparing GBS and DArTag Genomic Prediction

Despite the significantly reduced marker set in the DArTag data, both sequencing types generated similar genomic prediction results. The 5x5-fold cross-validation Pearson correlation was higher in the GBS (0.465 vs. 0.375), while the CI was marginally higher in the DArTag set (0.368 vs. 0.381). The GBS advantage in correlation likely reflects its greater SNP density and genome coverage (13,026 vs. 2,593 SNPs included post-QC), while the similar overall performance underscores the value of DArTag's targeted design focusing on genic regions and improved accuracy through microhaplotype generation. The moderate prediction suggest of both genotyping methods suggests that this model could be effective at accelerating genetic gain for yield in a genomic selection program by significantly decreasing the time of the traditional breeding cycle. One cycle in a typical RPS program for alfalfa takes 4-5 years, whereas up to two cycles of genomic selection could be carried out in greenhouse setting in a single year.

Conclusion

There are many obstacles inherent in alfalfa breeding, including its complex genome and inheritance patterns, long generation times, and continuously segregating phenotypes (Casler, 2001). Improving low pH and aluminum tolerance and nutritional quality in alfalfa has been a slow process (Bouton, 1996; Casler & Vogel, 1999; E. Delhaize & P. R. Ryan, 1995; Kassaw & Tu, 2024). The present research leverages low-cost, high-throughput GBS and DArTag sequencing data, an updated autotetraploid reference genome (Chen et al., 2020), and software capable of handling autopolyploidy (Rosyara et al., 2016) to identify QTL significantly associated with multi-year, multi-location, multi-condition field phenotyping and NIRS-generated nutritional data. While both methods were effective at identifying QTL for both field-related and nutrient traits, the greater genome coverage and SNP density in the GBS data led to the identification of more QTL and a greater GP accuracy.

Increasing marker density and population size generally improve GWAS detection and genomic prediction accuracy (Cericola et al., 2017; Gorjanc et al., 2017). However, gains plateau once marker density exceeds a certain threshold, which is species and trait-specific. In an autotetraploid system such as alfalfa, it is expected that the threshold of diminishing returns for marker number would be higher than that of a diploid, as more markers are needed to capture the increased complexity of allelic dosages and the increased number of possible genotypic states and inheritance patterns (Ferrão et al., 2021). In the autotetraploid blueberry, this threshold was determined to be ~10K markers (de Bem Oliveira et al., 2020), but to the author's knowledge, this threshold has not yet been established in alfalfa. This study investigated the tradeoff between sample size and marker density in a limited way by comparing GBS and DArTag sequencing approaches, and suggests that greater marker density can overcome a small decrease in small size

in GP performance. Further investigation is needed to experimentally determine these thresholds to optimize the GP approach.

In the present study, we show the effect of low pH and aluminum stress on field-related and nutrient traits in alfalfa and identify 22 candidate genes associated with these traits. This adds to the body of knowledge surrounding the genetic basis of low pH and aluminum tolerance and nutrient traits in alfalfa. We also demonstrate the effectiveness of GBS and DArTag sequencing on developing GP models, which can be leveraged to increase the speed of RPS in improving low pH and aluminum tolerance and forage quality traits. For example, the four QTL identified explain 17% of the total phenotypic variance for ASAI. Selecting for these polymorphisms in the seedling stage can facilitate population improvement without the need for costly and time-consuming field phenotyping, which can be further complicated by microclimate variation in the soil and GxE interactions. The improved speed and accuracy of GS breeding programs have the potential to aid breeders in developing improved alfalfa cultivars more efficiently.

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CHAPTER 5

SUMMARY AND CONCLUSIONS

Alfalfa is a valuable forage, known for its high nutritional value in animal production and high return on investment as a cash crop. Alfalfa production is currently limited in subtropical regions of the world, including the southeastern United States, due to decreased performance in acidic, aluminum-rich soils. Alfalfa stands are less productive, less persistent, and less nutritious when grown in acidic field conditions (Bouton, 1996; Bouton & Sumner, 1983; Bouton et al., 1986b; Campbell et al., 1988; Evans et al., 1990; Liatukiene et al., 2020). Improving this trait has been the subject of a great deal of work, but no commercial cultivar with sufficient tolerance has been released (Dall'Agnol et al., 1996; Khu et al., 2012)

The current research utilized a partially improved population originating from a diverse group of plant introductions (PIs) from the National Plant Germplasm Center (NPGS). No progress can be made in a trait without sufficient genetic diversity, so population development was critical to the success of the research. A set of 966 Plant Introductions (PIs) from the NPGS collection was planted in a low pH soil (pH = 4.90) in Tifton, GA in 2014. After four years of evaluation, the most vigorous surviving plants were dug and planted in Athens, GA, in a field with a pH of 5.12 and evaluated for three years. 133 selected PIs were dug from the field and crossed together using bee cages to create the half-sib population utilized in this study. The half-sibs and were planted in row-plots with two reps in a natural low pH field site (pH=4.90, extractable aluminum=10.41 mg kg^{-1}) and an adjusted pH field site which received 405 kg ha^{-1} of

Pennington Fast Acting Lime prior to planting (pH=6.37, extractable aluminum=2.29 mg kg^{-1}) in Athens, GA in May 2020. Each row-plot was replicated by stem cuttings and used to establish an identical field experiment in the fall of 2022 at the Animal Science Farm in Tifton, GA, in a low pH field site (pH = 4.9 and an available aluminum of 11.38 mg kg^{-1}) and an adjusted pH site (pH = 7.07 and an available aluminum of 0.01 mg kg^{-1}). Each row-plot was harvested throughout the growing season (~April-October) in 2021-2024 in Athens and 2023 and 2024 in Tifton (6 total environments with both conditions in each). Plant count per plot was measured at least twice a year in each location. Fall dormancy ratings (FDR) were calculated from plant heights recorded following the final harvest on December 20, 2021 and October 24, 2022 in Athens. Nutrient data was obtained for each row-plot by collecting a representative sample during the first harvest in April 2024, drying, grinding to 2 mm, and scanning with a Near-infrared spectrophotometer (NIRS). To the author's knowledge, this represents the most thorough field phenotyping approach yet undertaken to assess low pH tolerance in alfalfa.

As expected, low pH stress significantly reduced yield, FDR, and nutritive quality in alfalfa. The nested structure of the field phenotyping approach allowed for Acid Soil Adaptation Index (ASAI) values to be calculated for each half-sib family as a ratio of individual yield performance in low and adjusted pH conditions over the average yield performance of the whole population. Using this as a selection criterion ensures selected genotypes have truly superior performance over the population average. Family means heritability estimates for yield and ASAI were high, 0.802 and 0.854, respectively. This indicates that significant genetic gain can be attained from selection within this population. While GxE interactions were significant, many genotypes were consistently among the top-performers, as evidenced by the 56 genotypes that were identified as acid-tolerant (ASAI>1) in at least four environments.

Aluminum toxicity is known to impair root development. Al^{3+} ions bind cell wall components, disrupting cell signaling, division, and ion homeostasis (Hajiboland et al., 2023; Ma et al., 2001). However, these effects range widely between and within species and depend on many interrelated factors, such as the extent of the acid and aluminum stress, soil fertility, the stage of plant development, and the environment (Inostroza-Blancheteau et al., 2012; Zhang et al., 2007). A rhizobox assay evaluated root system architecture (RSA) traits of 51 genotypes included in the field study in a natural low-pH field soil (pH 5.30, available Al = 6.05 mg kg^{-1}) and a lime-adjusted soil (pH 6.87, exchangeable Al 1.92 mg kg^{-1}). Images were taken of the roots in the boxes and after removal and cleaning and analyzed with RhizoVision Explorer to generate data for 31 RSA traits. In this population, low pH stress generally induced deeper and thicker roots without increasing root or shoot biomass. We leveraged field-derived data to identify traits associated with low pH and aluminum tolerance, unlike previous greenhouse assays that relied only on root weight ratios of limed vs unlimed (Bouton et al., 1982; Dall'Agnol et al., 1996; Khu et al., 2012). Acid-tolerant genotypes (ASAI > 1) exhibited greater total root volume and more small-diameter, laterally branched roots concentrated in the topsoil, relative to low-tolerance genotypes (ASAI < 1). The results of the rhizobox assay showed low correlations between ASAI and root weight ratios or any other manually measured trait and may explain why previous greenhouse selection approaches have not translated to improved field performance. A simple binary logistic model was constructed to predict tolerance class from the volume of small-diameter roots (< 0.7 mm) and achieved a mean test AUC of 0.741 (SD 0.113). This emphasizes the value of image analysis, which allowed for many more RSA traits to be assessed quickly and efficiently. These results for validated by digging plants from both the low and adjusted pH fields in Athens and similarly imaging and analyzing RSA traits. The model constructed and key

RSA traits identified can be used to screen larger, more diverse populations and significantly decrease the time and cost of evaluation.

GWAS and GS approaches are increasingly commonplace in many crops, but the application in alfalfa has been impeded by its genomic complexity and lack of resources (Li & Brummer, 2012). However, recent advancements in sequencing, modeling, and software development have resulted in chromosome-level reference genomes for autotetraploid *Medicago sativa L.* and facilitated many GWAS (Chen et al., 2020; F. He et al., 2025; C. A. Medina et al., 2025; Medina et al., 2020; Rosyara et al., 2016; Shen et al., 2020; Xu et al., 2023). This research utilized these new resources to identify SNP markers associated with yield, FDR, and nutrient traits using genotype-by-sequencing (GBS) and DArTag sequencing data. No loci were identified by both sequencing approaches, likely due to probes capturing non-redundant portions of the genome. Significantly associated markers were annotated using the XinjiangDaye reference genome and the newly curated MODMS database. Both genotyping systems were used to develop a genomic prediction (GP) model and compared for accuracy. The greater genome coverage and SNP density in the GBS data led to the identification of more significantly associated SNPs (16 vs. 7) and a greater GP accuracy (0.465 vs. 0.375). Taken together, the results of the GWAS revealed 22 candidate genes associated with yield performance and nutrient quality of alfalfa under low pH stress. Of note were the 4 QTL identified for ASAI, which collectively explain 17% of the phenotypic variation. These could be used to develop a genomic selection (GS) approach and allow selections to be made in the seedling stage and in the absence of the target environment, allowing cycles of breeding to move much faster and more efficiently.

In sum, this research provides a thorough assessment of the effects of low pH stress and aluminum toxicity on alfalfa performance. Sufficient genetic variation is present in the studied

population to make accurate progress in the next round of recurrent phenotypic selection (RPS). Selections from this population can be intermated to form the next generation and advanced to replicated sward plot evaluations in the target environment(s) in the forage breeding pipeline. Rhizobox screening of progenies can facilitate faster cycling through generations while maintaining selection accuracy based on the key root phenes described in this work. A GS approach using the markers identified in this study can complement the field and greenhouse screening and further increase the genetic gain possible per unit time. Though these resources will require additional validation, they present a marked improvement over previous methods and will facilitate the breeding and release of a locally-adapted, acid-tolerant cultivar.

Limitations and Future Directions

This work has a variety of limitations. In the field study, we evaluated a relatively small population and used only 2 replications in each environment x condition. We also evaluated the field trials for a maximum of 4 years, which may have been insufficient to adequately assess survivorship and long-term persistence. Field heterogeneity, such as was seen in the Tifton trials, may also have inadvertently affected the results. Despite these limitations, this work demonstrates the genetic potential of this population for improving low pH and aluminum tolerance in alfalfa. Selection and crossing of top performers can be used to generate the next cycle of RPS. Multi-replicated, multi-location sward plot evaluation is recommended for this cycle. Given the enormous investment of resources required to phenotype large field trials in multiple locations, further field testing may be done only in a low pH field soil environment. However, this work demonstrates the benefits of evaluation in both a low and adjusted field sites, and if resources are available, continuing to select based on ASAI may be more accelerate genetic gain greater than selecting on yield alone.

The rhizobox assay was similarly limited by the population size and scope of the experiment. RSA traits identified in the first six-weeks of growth may not have significant correlations with RSA traits over the course of years in the field. The homogenization of the field soil for the rhizobox assay also does not mimic normal field conditions, where the O and A horizons at the top of the soil are higher quality which typically decreases with soil depth. However, the present research makes a compelling case that lateral roots which branch at shallow angles are driving the improved performance of alfalfa under low pH soil stress. This was seen across the rhizobox and field-dug validations and indicates that roots with superior soil-foraging ability can better acquire nutrients and water, limiting the effects of toxicity. These results need to be validated using a greater number of genotypes and in different germplasm, and potentially over longer time-scales, to evaluate how well these rhizobox-derived traits translate to field performance. Additional testing over a range of pH values, especially under more intense low pH soil stress, may shed light on the thresholds of tolerance and identify more genotypes and RSA traits underlying the tolerance mechanism in alfalfa.

The GWAS conducted here was similarly limited by the number of genotypes included. Because each half-sib family was sequenced up to 4 times for inclusion, significant population structure had to be controlled using principal components. This is necessary to control false-positives but may have decreased the statistical power of the analysis to uncover more significant associations. Annotating markers with suggestive associations (just below the significance threshold) may reveal additional candidate genes associated with field and nutritive traits. Further validation of the markers identified here is necessary before utilization for MAS/GS. This can be done by testing these markers across other populations to see if the association remains significant. Functional validation of candidate genes could be assessed through

CRISPR-cas9 or RNAi to knock out the gene(s) of interest and observe changes in phenotype. The limited number of markers identified in this study are likely only a few of the genomic regions associated with yield and ASAI, as these are quantitative traits. Long-read sequencing, such as PacBio, of selections from this population may also help validate the markers found here and find novel associations. As more genomic resources become available, such as an alfalfa pan-genome published in April of this year (Fei He et al., 2025), the genotypic and phenotypic data used here could be re-analyzed for validation and novel characterization. This work represents a starting point for building a robust genomic selection model which can be used to make selections without field or greenhouse screening and could accelerate the development of novel cultivars.

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