A Survey of the Agronomic and End Use Characteristics of Low Phytic Acid Soybeans

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ABSTRACT

Phytic acid (PA) accounts for up to 75% of the P in soybean (*Glycine max* L. Merr.) seeds, but it is indigestible by mono- and agastric animals resulting in economic and environmental detriment. Soybean lines with genetically reduced PA contents have been developed using three distinct mutant alleles at the *MIPS1, LPA1,* and *LPA2* genes resulting in up to a 75% reduction in PA. Low PA (LPA) soymeal-based feeds have been tested on several agricultural species and shown to reduce the P in the animal effluent, but they have not been tested on any aquacultural species. However, LPA soybean lines often exhibit low field emergence making them commercially inviable. The cause of this phenomenon is widely debated with possibilities ranging from increased disease pressure to decreased seedling vigor. The objectives of this research were to 1) enhance field emergence of LPA soybean varieties through pre-planting seed treatments, 2) study the impact of the LPA mutant alleles on agronomic, quality, and seed composition traits, and 3) design a low-error method for studying the effect of LPA soymeal-based feeds on aquatic animals using Pacific White Shrimp (*Litoenaeus vannamei*). These results describe a variety of agronomic and genetic strategies with which the low field emergence of LPA soybeans can be addressed, reveal a heretofore not reported interaction between the *mips1* and *lpa2* alleles to further increase the digestibility of soymeal, and a possible method for studying LPA soymeal based feed on aquacultural animals.

Dedication

I would like to dedicate this thesis to my niece, Kendall Hart. For her, I will continue to tilt at windmills. I hope that this research will contribute in some small way to the preservation of the waterways in our home of Eastern North Carolina which has long been plagued with nutrient pollution, so that Ken can have the same amphibious joys which I was lucky enough to experience. Plus, who doesn't love some good local seafood?

I would also like to dedicate this to the legends Joey Ramone and Joe Strummer who probably never thought they would have a research thesis dedicated to them but taught me from a young age that the only way to make a positive change in this world is to stop waiting around and do it yourself.

"People can change anything they want to, and that means everything in the world."

–Joe Strummer

Hey, ho. Let's go.

"Teenage kicks right through the night."

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Attributions

Below is a brief summary of the roles played by several people that contributed significantly to the completion of the research in and writing of this thesis and the chapters to which they contributed.

2. Employing Seed Treatments to Increase Field Emergence in Low-Phytic Acid Soybeans

Greg Welbaum- Professor in the Department of Horticulture, Virginia Tech. Dr. Welbaum provided expert advice about seed treatments contributing to the design of this experiment. He also provided seed treatment materials as well as lab space and inventory with which to perform pre-trials and seed priming. Finally, he contributed to the editing of the manuscript represented by this chapter.

Jun Qin- Professor at the China Huang-Huai Regional GM-Soybean Testing and Commercialization Center, National Soybean Improvement Center Shijiazhuang Sub-Center, Institute of Food and Oil Crops, Hebei Academy of Agricultural and Forestry Sciences. Dr. Qin contributed to the statistical analysis and editing of the manuscript represented by this chapter.

Mengchen Zhang- Professor at the Hebei Academy of Agricultural and Forestry Sciences. Dr. Zhang contributed to the editing of the manuscript represented by this chapter.

Bo Zhang- Research Assistant Professor in the Department of Crop and Soil Environmental Sciences, Virginia Tech, and committee chair. Dr. Zhang contributed to the experimental design development, statistical analysis, and editing of the manuscript represented by this chapter.

Benjamin Averitt- Master's Degree Candidate in the Department of Crop and Soil Environmental Sciences, Virginia Tech. Mr. Averitt performed pre-trials to determine target seed treatments, prepared, treated, and planted seed, took and analyzed field and lab data, performed statistical analysis, and prepared the manuscript represented in this chapter.

3. Impact of *mips1*, *lpa1* and *lpa2* Alleles for Low Phytic Acid Content on Agronomic, Seed Quality and Seed Composition Traits of Soybean

Chao Shang- Senior Research Associate in the Department of Crop and Soil Environmental Sciences, Virginia Tech. Dr. Shang developed the sugar analysis protocol reported in this chapter and provided a large amount of technical support as we moved to the new protocol and machine. He also edited the sugar analysis protocol for the manuscript represented by this chapter.

Luciana Rosso- Research Associate in the Department of Crop and Soil Environmental Sciences, Virginia Tech. Dr. Rosso assisted with genetic analysis and seed composition data acquisition.

Jun Qin- Dr. Qin contributed to the editing of the manuscript represented by this chapter.

Mengchen Zhang- Dr. Zhang contributed to the editing of the manuscript represented by this chapter.

Bo Zhang- Dr. Zhang contributed to the experimental design, data analysis, and editing of the manuscript represented by this chapter.

Benjamin Averitt- Mr. Averitt prepared and planted seeds, collected field and lab data, performed statistical analysis on all data, and prepared the manuscript represented by this chapter.

4. Developing a Low Error Protocol for Testing Low Phytic Acid Soymeal Based Feed on Pacific White Shrimp

Daniel Taylor- Research Associate in the Department of Food Science and Technology, Virginia Tech. Mr. Taylor contributed to the experimental design, data collection, shrimp upkeep, and statistical analysis for this study.

David Kuhn- Assistant Professor in the Department of Food Science and Technology, Virginia Tech. Dr. Kuhn contributed to the experimental design and statistical analysis for this study as well as providing equipment and shrimp.

Bo Zhang- Dr. Zhang supplied the soybeans used to make both feeds used in this study as well as contributing to the experimental design.

Benjamin Averitt- Mr. Averitt performed the data acquisition and analysis for this study. He also wrote the manuscript represented by this chapter.

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1. Introduction

Soybean (*Glycine max* L. Merr) is a protein and oil rich seed crop adaptable to a wide range of end uses including human and animal consumption. Generally, whole beans are processed by pressing out the oil before grinding the remaining solid to make a protein rich meal. Further, the great diversity of soybean germplasm in growth habits, maturity, and other agronomic traits makes it readily available for production in nearly all environments. Therefore, soybean is one of the most widely planted crops in the world yielding over 241 million metric tons annually (FAO, 2014).

Phytic Acid Overview

Phytic acid (PA), myo-inositol 1,2,3,4,5,6-hexakisphosphate, also known as phytate in its cation salt form, is the primary storage form of phosphorus (P) in soybean seed comprising up to 75% of the total P in mature seeds. PA is also a strong chelator of cationic metal micronutrients including calcium, magnesium, and iron (Raboy et al., 1984).

Though PA is nearly ubiquitous throughout all plant tissues, it is an especially vital component of the seed. As a whole unit, PA works as a signal transductor and osmoprotectant in the cell membrane, so both the phosphate and inositol groups play an important role in seed germination and seedling growth. Since it is a stable storage unit, PA can store phosphorus and chelated minerals without leaching until acted on by natural phytase enzymes when P is needed by the young plant (Erdman, 1979)

Soybean Meal and PA in Animal Production

With a high protein content and relatively low cost, soybean meal is a major component in many feeds for both companion and agricultural animals with ~98% of all soybean meal going to animal feed in 2013, 76% of which went to swine and poultry production (Soystats, 2015). There is also growing interest in using soybean meal as the main protein source in aquacultural feeds to replace the traditional but costly squid or fish meal (Asche et al., 2013).

However, agastric and monogastric animals, including chickens, pigs, and most aquatic animals, lack the activity of a phytase enzyme in their digestive tract. It was found that animals, especially swine, cannot digest PA due to a lack of hydrolysis at the end of the tract (Dilger and Adeola, 2006; Kleinmann et al., 2005; Powers et al., 2006); thus, the vast majority of P in the meal is unavailable thereby lowering the efficiency of the feedstuffs. The chelating function of PA has also been shown to cause nutritional deficiencies in animals since the metals that PA binds are unavailable (Leytem et al., 2008; Pallauf et al., 1998; Plumstead et al., 2007).

Because these animals cannot digest PA, there is a much higher level of P in their manure compared to ruminant animals. For example, a survey of P in various animal manures by Kleinmann et al. (2005) found 28.8 g P/kg in swine manure and 25.6 g P/kg in layer chicken manure vs. 5.1 g P/kg in beef cattle manure. Through runoff or leaching from either waste lagoons or fields fertilized with manure from monogastric animals, these high levels of P often enter into natural bodies of water. PA is digested into a bioavailable form with natural phytases present in the ecosystem such as bacterial β-propeller phytases (Chang and Lim, 2006). P is often the limiting factor for plant and algal growth in aquatic environments, so this eutrophication can lead to widespread blooms which may wreak environmental havoc through hypoxia, a drastic decrease in dissolved oxygen in the water, leading to massive fish kills (Shindler et al., 2008, Sinkko et al.,

2013). These environmental issues, in turn, are economically destructive through both lost fishery production and reduced tourism.

Producers have long used synthetic phytase as an additive to soy-based feeds for mono- and a-gastric animals to compensate for the natural lack of this enzyme and improve the feed's efficiency. However, this method is both expensive and less efficient than natural phytase activity as it relies heavily on various factors including temperature, pH, and mineral concentration (Brejnholt et al., 2011; Hassan et al., 2013)

Low PA Soybeans

Numerous genes have been identified as playing a role in the PA synthetic pathway. Three genetically recessive mutant alleles from two different sources have been recognized as most important in creating a low phytic acid (LPA) phenotype: *lpa1*, *lpa2*, and *mips1*. Though all three genes act on the same general pathway, each has been identified and confirmed to be distinct and separate (Gao et al., 2008; Oltmans et al., 2004).

The first two LPA alleles, *lpa1* and *lpa2*, were discovered on GM19 (LG N) and GM3 (LG L), respectively, of the mutant soybean line CX-1834 and have homologs in several other crop species including corn and barley (Pilu et al., 2009; Wilcox et al., 2000). The mutant allele, *lpa1*, has a greater effect than *lpa2*, the other LPA gene from this source, which codes for a constituent protein in an ATP-binding cassette (ABC) transporter that partitions PA into the seed (Fig. 1). The missense mutation in the mutant allele produces a truncated and non-functioning ABC transporter (Pilu, 2009; Shi et al., 2007). Thus, though PA may be produced in lines with the *lpa1* mutation, that PA will not be efficiently partitioned into the seed. *lpa2* contains a nonsense mutation to a gene also involved in the ABC transporter in the PA production pathway (Gillman et al., 2013). While this mutation decreases the amount of PA produced, other inositol kinases may compensate

for its lack of production leading to this mutation having a much more minor effect on the overall PA content of the seed than *lpa1* (Pilu, 2009). In combination, these two alleles have been shown to lower the PA content to only 25% of the phosphorus in these lines is in the form of PA or phytate while the other 75% is inorganic and, thus, available for animals that cannot digest PA (Bilyeu et al., 2008; Wilcox et al., 2000).

The other major gene that has been shown to be related to LPA in soybeans, *MIPS1*, has been discovered on GM11 (LGB1) in several, distinct soybean germplasms. This gene is one of a family of four myo-inositol phosphate synthase genes responsible for the addition of phosphates to a sugar backbone in the early steps of the PA production pathway (Fig. 1). *MIPS1* codes for the first step in pathway converting Glucose 6-phosphate to Inositol 3-phosphate. The LPA trait in mutant line LR33, which has the *mips1* allele, has been traced to a single nucleotide change in the 10^{th} exon of the gene causing the *MIPS1* protein to be non-functional (Hitz et al., 2002; Saghai Maroof, 2009). Compared to LPA mutants from the CX-1834 source, *MIPS1* mutants have more PA in the seed where it usually accounts for 50% of the total phosphorus. However, *MIPS1* mutants have the added benefit of a modified, beneficial sugar profile with sucrose, an easily digestible sugar, content being high while raffinose and stachyose, both of which are not fully digestible by mono- and a-gastric animals, contents are low (Maroof and Buss, 2008). Therefore, *MIPS1* mutants increase feed efficiency for mono- and a-gastric animals.

Closely linked genetic markers have been identified for each of the three mutant alleles and can be used to screen and identify lines with the LPA phenotype. Satt237 and Satt561 are simple sequence repeat (SSR) markers that are associated with the *lpa1* and *lpa2* mutant alleles, respectively (Scaboo et al., 2009). Two genotyping techniques exist for the *MIPS1* mutant allele.

Satt 453 is an SSR marker and a single nucleotide polymorphism (SNP) marker linked to *MIPS1* has been used to identify *MIPS1* mutants such as in soybean line V99-5089 (Rosso et al., 2011).

LPA Based Animal Feeds

Experimental LPA soybean based animal feeds have been tested in a number of mono-gastric species to confirm their use as both a highly efficient and environmentally friendly alternative to traditional soymeal. The overall consensus shows that the P in LPA soymeal has a much higher bioavailability and bioretention rates than that in normal PA soymeal in mono-gastric animals while the P rate in the waste is significantly lowered. These results account for all the expectations and goals of LPA soybeans thereby confirming the validity of the concept.

Broiler chickens have been one of the most widely studied species with LPA soymeal based feeds. Dilger and Adeola (2006) compared two feeds, one LPA and the other normal phytic acid (NPA), on broilers and found that those broilers fed with the LPA feed retained 17% more of the soymeal P (77%). There was not any significant difference in the P bioavailability between the two feeds as both had a bioavailability of between 79-89%. This, conversely, is well correlated to those found by Scaboo et al. (2009) and Wilcox et al. (2000) that ~75% of the seed P in LPA lines is in the form of P_i.

Similar results have been noted in swine. In a feeding trial comparing LPA or NPA soybean meal based swine feeds with and without the inclusion of a synthetic phytase, Powers et al. (2006) reported a 19% decrease in total P (tP) in the feces of those pigs fed with the LPA diet. Water soluble P (WSP) also decreased in LPA treatments by 17%. In addition, the LPA diets had a statistically significant reduction of both tP and WSP than the NPA diet with phytase (16% and 6%, respectively) suggesting that LPA soybean meal is a valid alternative to synthetic phytase. The addition of phytase to the LPA soybean meal diet, however, saw an even greater reduction of

both tP and WSP in the feces (27% and 23%, respectively). This is to be expected since PA is still present in LPA soybean meal. In total, these results highlight the potential benefits of a LPA based diet in monogastric animals.

However, few such tests have been performed on agastric aquatic animals probably because soy-based feeds are not widely used in aquatic animal production. There is a growing interest in soymeal as a cheaper alternative to traditional protein sources such as fish or squid meal. In fact, many areas of the world, including Europe, still have tight regulation of soy-based fish feeds because of the environmental impacts of the P in soymeal (Asche et al., 2013; Kumar et al., 2012). Therefore, testing LPA soymeal based feeds on agastric aquatic animals could provide a major stepping stone in advancing the development of both LPA soybean varieties and the aquacultural sector. Such experiments could possibly open up new markets around the world for American soybean exports and lift an economical hurdle for the aquacultural sector, one of the fastest growing agricultural industries in the United States.

Decreased Field Emergence in LPA Soybeans

Decreased field emergence in LPA soybeans has been observed in many field experiments, which is the greatest issue that breeders are facing in in the effort to produce a commercially viable LPA soybean variety Consistently, LPA soybean lines show diminished field emergence rates well below the commercial threshold of 85%. However, the reasons causing low field emergence in LPA soybeans is still under study as emergence is a very complex trait. Of the possibilities noted in previous research, reduced germination, weakened seedling vigor, accelerated seed aging and seed source environment have all been implicated in this issue (Anderson and Fehr, 2008. Khaliliaqdam et al., 2013; Maupin and Rainey, 2011; Oltmans et al., 2005). Of these, seed source environment has by far been the most consistently observed.

Several studies with CX-1834 and its derived lines, have indicated that increased phytic acid content in seed does not necessarily account for increased field emergence. It has been indicated that the emergence issues common in LPA soybean lines may not be due to the decreased PA content, but, instead, to other genetic factors in those lines.

Emergence is an extensively complex trait. Genetic factors affect emergence and the environment in which the seeds are grown and harvested would have a high impact on field emergence in the next generation. Several studies have observed significant decreases in emergence rates of LPA soybean lines when grown in tropical or subtropical climates, a common growing area for soybean breeding winter nurseries, which was as low as 8%. Maupin et al. (2011), compared emergence in lines derived from both CX-1834 (*lpa1*/*lpa2*) and V99-5089 (*mips1*) grown in temperate and tropical environments. Most seeds grown in the tropical environment exhibited lower emergence. However, some of the V99-5089 derived lines performed at or above the commercial field emergence threshold of 85%. It suggested breeding high emerging LPA soybean varieties is possible due to natural variation on emergence within *MIPS1* mutants.

Seed Treatments for Field Emergence

Various seed treatments have been employed in a wide variety of agricultural pursuits including soybean production. By far, the most common seed treatment is inoculation with rhizobial species necessary for nodule formation and nitrogen fixation. There has been a growing trend of using a broader swatch of treatments in recent years. These treatments can be any combination of physical (e.g. scarification, etc.), chemical (e.g. insecticide, fungicide, etc.), biological (e.g. bio-priming), physiological (e.g. matric/osmotic priming, etc.). While these treatments act on a number of different factors, most of them are to increase field emergence.

One of the greatest issues affecting soybean field emergence is disease pressure from root rot and damping off pathogens such as *Pythium spp.*, *Phytophthora sojae*, and *Colletotrichum truncatum*. These diseases can attack the young radicle the young seedling causing low field stands. Thus, chemical fungicides are the most widely used seed treatments in order to improve field emergence. However, the fungicide treatment must be highly specialized to account for the specific pathogen species, the races, environmental conditions, and field history. Broad spectrum fungicidal treatments may be used for specialization need, but they may not be as effective or efficient at controlling the diseases (Shultz et al., 2008; Xue et al., 2007). Biological treatments, especially in the form of bio-priming wherein seeds are treated with innocuous fungal species that can compete against pathogens are in some cases, as effective as fungicidal treatments in dealing with these diseases. However, they require a greater knowledge of the exact pathogen in the field for specialization (Begum et al., 2010). Another common chemical treatment is insecticides to control various pests, most notably aphids. Including such treatments prior to planting does not necessarily aid emergence but protects the newly emerged plants at their most vulnerable thus ensuring the stand lasts (Frewin et al., 2014; Horii et al., 2007).

Generally, fertilizers are not readily used as a chemical seed treatment because the concentration of nutrients and salt will chemically burn the seed thus damaging or even killing the germ. However, some weak fertilizers have been adapted and are in common usage especially in horticultural crops (Kepczynska et al., 2003; Mohammed et al., 2014). One such weak fertilizer is crushed diatomaceous earth made from the ground shells of aquatic diatoms. This fine powder is water soluble and melts off the seed during imbibition, creating a weak solution around the seed containing some nitrogen, phosphorus, and other nutrients required by the seed (Murillo-Amador et al., 2007). Synthetic forms of magnesium and calcium silicate, such as Microcel-E (Manville

Filtration and Minerals, Denver, CO) are also used. While this has worked well in horticultural applications, it is widely viewed as uneconomical in agronomic crops due to limited profit.

Physiological seed treatments are a much newer area of interest especially in agronomic crops. These treatments are meant to artificially start germination, sugar hydrolyzation, and other physiological reactions involved in early seedling growth. The popular treatment is matric or osmotic priming that takes advantage of an osmotic potential between the seed and a water solution to partially hydrate the seed. Once partially hydrated, the seeds are then dried to stop the germination process before being planted. This treatment quickens the rate of germination thus not allowing some pathogens to fully attack the growing seedling (Jett et al., 1996; Kepczynska et al., 2003; Mushtaq et al., 2012). As with the diatomaceous earth, these treatments have been successfully used in horticultural production, but the economics of its use in agriculture are debated.

Objectives

LPA soybean meal will be a valuable asset to all facets of agriculture. The increased feed efficiency of LPA varieties will be a benefit to animal producers which, in turn, will benefit soybean producers by providing a new and sought after product. Further, LPA soybean varieties may be able to provide a breakthrough for soymeal based feeds in aquacultural production thereby opening a new, large market for soymeal. Lastly, the use of LPA soymeal based feeds will have a large, positive impact on the environment thus preserving our natural resources and providing benefit to the tourism and fisheries industries. However, the low field emergence continuously observed in LPA soybeans is a serious barrier the realization of these potential advantages.

The main objective of the first two experiments, represented by the second and third chapter of this thesis, was to examine possibilities for producing LPA soybean varieties with

acceptably high field emergence. The main objective of the first experiment was to study the ability of seed treatments to improve field emergence and, thus, determine the possibility of using agronomic means to address this issue. The second experiment was designed to study the effect on field emergence, yield, and seed compositional traits of each LPA mutant allele individually and in combination in a single family population for the first time. Secondarily, this experiment was also aimed at studying the different correlations between various traits with either yield or field emergence to identify traits which may be targeted in breeding LPA soybean varieties which are agronomically and commercially viable.

The objective of the final experiment, represented in the fourth chapter of this thesis, was to establish a high power, low error method for studying the effects of LPA soymeal based feeds on the water quality and growth of Pacific white shrimp (*Litopennaeus vannamei*). This experiment can act as a guide for future studies of this sort to ultimately confirm the concept of LPA soymeal based feeds and open possible markets in the aquacultural sector.

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2. Employing Seed Treatments to Increase Field Emergence in Low- Phytic Acid Soybeans

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Abbreviations: LPA, low phytic acid; *mips1*, D-*myo*-inositol 3-phosphate synthase 1; NPA, normal phytic acid; PA, phytic acid

Abstract

Phytic acid (PA) accounts for the vast majority of phosphorus in soybean (*Glycine max* L. Merr) seeds but is unavailable to mono- and agastric animals. Low-PA soybean varieties have been developed to improve feed efficiency, but they often exhibit low field emergence, an important agronomic trait which aids in nutrient and water efficiency, weed control, and soil preservation. This low field emergence is a major barrier to producing and marketing a commercial low PA soybean variety. The purpose of this study was to study the effect of field treatments on field emergence, growth, and yield of LPA soybean varieties. A total of 12 treatments consisting of two broad spectrum, preplanting fungicides, osmotic priming, MicroCel-E, and all possible combinations except for the combinations of two fungicides were designed to treat four low and two normal PA soybean varieties. A non-treated control for each variety was planted along with the treated plots. The plots were planted in Blacksburg and Orange, VA in 2014 and 2015 under irrigated and non-irrigated conditions. The result indicated that field emergence was significantly affected by the seed treatments. Rancona Summit and ApronMaxx treatments were the fungicide treatment to significantly improve field emergence, increasing by 12.04 to 15.37% in low PA soybeans. Variety MD 03-5453, which had the lowest control field emergence, exhibited significantly increased field emergence with both fungicide treatments. However priming treatments, if significant, were negatively associated with field emergence across all six varieties. The effect of seed treatment on yield, seed weight, seed quality, protein content, and oil content were analyzed, and seed quality was the only trait which was significantly affected by the seed treatments. Correlation analysis was performed between field emergence, yield, and seed composition and quality traits. The strongest correlations with field emergence was seed size (-0.33) and protein content (-0.30). Oil (-0.13) and starch (-0.17) were also significantly correlated

with field emergence. The results showed not only that seed treatments can improve emergence in low PA soybeans but further suggests that reduced phytic acid in soybean seeds may dramatically decrease seedling vigor after germination. The study will provide effective approach for the soybean breeders to increase the low field emergence in low-PA varieties.

Introduction

Soybean (*Glycine max* L. Merr) is one of the most important crops for animal feedstuffs in the United States due to its uniquely beneficial seed composition and adaptability to a wide range of growth environments. It is especially common in swine and poultry production with 76% of US soybean meal being consumed by swine and chickens in 2013 (Soystats, 2015).

This, however, presents a glaring issue. About 75% of the P in soybean seeds is in the form of phytic acid (PA), *myo*-inositol-1,2,3,4,5,6-hexakisphosphate, which is indigestible for a- and monogastric animals such as swine, poultry, and most aquacultural animals (Raboy et al., 1984). Therefore, a majority of the P is unavailable to these animals thus lowering the efficiency of the feed and causing economic loss (Dilger and Adeola, 2006; Kleinmann et al., 2005; Powers et al., 2006). The chelating function of PA has also been shown to cause nutritional deficiencies in animals since the metals that PA binds are unavailable (Leytem et al., 2008; Pallauf et al., 1998; Plumstead et al., 2007). Further, the PA in animal waste may end up through runoff in natural waterways where it can be digested by natural phytase in the environment causing an influx of inorganic P in slower moving bodies of water such as the Chesapeake Bay in Virginia and Maryland and lower Neuse River in North Carolina (Boynton et al., 1995; Burkholder et al., 2004; Chang and Lim, 2006). In turn, this can lead to massive algal blooms and fish death due to hypoxia and disease causing wider economic damage and environmental degradation (Shindler et al., 2008, Sinkko et al., 2013).

Animal producers have long used synthetic phytase as an additive to animal feed to deal with this issue. However, a much more effective method is to use low PA (LPA) seeds which have been developed from mutant lines in various crop species including soybean, corn, and barley (Pilu, 2009). In soybean, three mutant alleles have been especially exploited to create LPA

varieties. The first two, *lpa1* and *lpa2*, were both discovered in a mutant line CX-1834 (Wilcox et al., 2000). Both the *lpa1* and *lpa2* alleles produce a truncated ABC transporter responsible for partitioning PA into the seed thus disallowing PA to enter the seed (Gillman et al., 2013; Shi et al., 2007). The third mutant allele, *mips1*, is responsible for the first step in PA biosynthesis converting glucose-6-P to Inositol-3-P (Fig. 1). The mutant allele *mips1* is non-functioning (Hitz et al., 2002; Saghai Maroof, 2009). This mutant allele also confers a beneficial sugar profile being high in easily digestible sucrose and low in the less digestible raffinose and stachyose (Hitz et al., 2002; Saghai Maroof, 2009). For all of these mutations, the P content of the seed is virtually unchanged, but inorganic P represents the majority of P in the seed (Bilyeu et al., 2008; Wilcox et al., 2000).

However, decreased field emergence in LPA soybeans has been observed in many field experiments, which is the greatest issue that breeders are facing in the effort to produce a commercially viable LPA soybean variety. Consistently, LPA soybean lines show diminished field emergence rates well below the commercial threshold of 85%. However, the reasons causing low field emergence in LPA soybeans is still under study as emergence is a very complex trait. Of the possibilities noted in previous research, reduced germination, weakened seedling vigor, accelerated seed aging and seed source environment have all been implicated in this issue (Anderson and Fehr, 2008. Khaliliaqdam et al., 2013; Maupin and Rainey, 2011; Oltmans et al., 2005). Of these, seed source environment has by far been the most consistently observed factor. Several studies with CX-1834 and its derived lines have indicated that increased phytic acid content in seed does not necessarily account for increased field emergence (Anderson and Fehr, 2008; Maupin et al. 2011). It has been indicated that the emergence issues common in LPA soybean lines may not be due to the decreased PA content, but, instead, to other genetic factors in those lines.

Emergence is an extensively complex trait. Genetic factors affect emergence and the environment in which the seeds are grown and harvested would have a high impact on field emergence in the next generation. Several studies have observed significant decreases in emergence rates of LPA soybean lines when grown in tropical or subtropical climates, a common growing area for soybean breeding winter nurseries, which was as low as 8% (Anderson and Fehr, 2008; Maupin et al., 2011; Meis et al., 2003; Yuan et al., 2007). Maupin et al. (2011) compared emergence in lines derived from both CX-1834 (*lpa1*/*lpa2*) and V99-5089 (*mips1*) grown in temperate and tropical environments. Most seeds grown in the tropical environment exhibited low emergence. However, some of the V99-5089 derived lines performed at or above the commercial field emergence threshold of 85%. It suggested breeding high emerging LPA soybean varieties is possible due to natural variation on emergence within *mips1* mutants.

Various seed treatments have been employed in a wide variety of agricultural pursuits including soybean production. By far, the most common seed treatment is inoculation with rhizobial species necessary for nodule formation and nitrogen fixation (Catroux et al., 2001). There has been a growing trend of using a broader swath of treatments in recent years. These treatments can be any combination of physical (e.g. scarification, etc.), chemical (e.g. insecticide, fungicide, etc.), biological (e.g. bio-priming), and physiological (e.g. matric/osmotic priming, etc.) treatments (Begum et al., 2010; Horii et al., 2007; Jett et al., 1996; Myint et al., 2010; Schulz and Thelan, 2008). While these treatments act on a number of different factors, most of them are to increase field emergence.

One of the greatest issues affecting soybean field emergence is disease pressure from root rot and damping off pathogens such as *Pythium spp.*, *Phytophthora sojae*, *Colletotrichum truncatum* (Blackman et al., 1982; Kato et al., 2013; Schmitthenner, 1985) These diseases can

attack the young radicle, the young seedling, causing low field stands. Thus, chemical fungicides are a widely used seed treatments in order to improve field emergence. However, the fungicide treatment must be highly specialized to account for the specific pathogen species, the races, environmental conditions, and field history. Broad spectrum fungicidal treatments may be used for specialization need, but they may not be as effective or efficient at controlling the diseases (Shultz et al., 2008; Xue et al., 2007). Biological treatments, especially in the form of bio-priming wherein seeds are treated with innocuous fungal species that can compete against pathogens are in some cases, as effective as fungicidal treatments in dealing with these diseases. However, they require a greater knowledge of the exact pathogen in the field for specialization (Begum et al., 2010). Another common chemical treatment is insecticides to control various pests, most notably aphids. Including such treatments prior to planting does not necessarily aid emergence but protects the newly emerged plants at their most vulnerable thus ensuring the stand lasts (Frewin et al., 2014; Horii et al., 2007).

Generally, fertilizers are not readily used as a chemical seed treatment because the concentration of nutrients and salt will chemically burn the seed thus damaging or even killing the germ. However, some weak fertilizers have been adapted and are in common usage especially in horticultural crops (Kepczynska et al., 2003; Mohammed et al., 2014). One such weak fertilizer is crushed diatomaceous earth made from the ground shells of aquatic diatoms. This fine powder is water soluble and melts off the seed during imbibition, creating a weak solution around the seed containing some nitrogen, phosphorus, and other nutrients required by the seed (Murillo-Amador et al., 2007). Synthetic forms of magnesium and calcium silicate, such as MicroCel-E (Manville Filtration and Minerals, Denver, CO) are also used. While this has worked well in horticultural applications, it is widely viewed as uneconomical in agronomic crops due to limited profit.

Physiological seed treatments are a much newer area of interest especially in agronomic crops. These treatments are meant to artificially start germination, sugar hydrolyzation, and other physiological reactions involved in early seedling growth. The popular treatment is matric or osmotic priming that takes advantage of an osmotic potential between the seed and a water solution to partially hydrate the seed. Once partially hydrated, the seeds are then dried to stop the germination process before being planted. This treatment quickens the rate of germination thus not allowing some pathogens to fully attack the growing seedling (Jett et al., 1996; Kepczynska et al., 2003; Mushtaq et al., 2012). As with the diatomaceous earth, these treatments have been successfully used in horticultural production, but the economics of its use in agriculture are debated.

Given the low field emergence of LPA soybeans and wide use of seed treatments in soybean production, there may be a benefit to pairing seed treatments with LPA soybean varieties to improve field emergence of LPA soybeans. The objective of this study was to test the ability of seed treatments to increase field emergence in LPA soybeans.

Materials and Methods

Plant Materials

We used six maturity group V soybean varieties: four LPA and two normal PA (NPA) (Table 1). All seeds used for planting were harvested in Blacksburg, VA the previous year. The four LPA varieties were 56CX-1283, MD 03-5453, V12-4557 and V12-BB144. V12-4557 and V12-BB144 were developed at Virginia Tech and have the *mips1* LPA allele. 56CX-1283 and MD 03-5453 were developed by the USDA-ARS-Purdue Univesity and University of Maryland, respectively, and have both the *lpa1* and *lpa2* LPA alleles. The LPA varieties' PA content ranged from 2131.68 to 4420.50 ppm. AG 5632 (Monsanto, St. Louis, MO) and 5002T (Pantalone et al., 2004) are both NPA commercial varieties. Their PA content ranged from 5886.72 to 6116.10 ppm. MD 03-5453 and V12-4557 have exhibited markedly lower field emergence and were added into the study in 2015.

Field Plot Design and Trait Measurement

The experiment was designed as a triplicated split plot generalized, randomized complete block design (GRCBD) wherein the plots were blocked by the two locations (Blacksburg and Orange, VA) and split into irrigated and non-irrigated plots. Each plot was planted in two 3.05m rows spaced 0.82m with 80 seeds per row. The irrigated plots were irrigated shortly after planting until emergence to provide a harsher growing environment. Stand counts were taken at the V1 stage (Fehr and Caviness, 1977). The plots were harvested in entirety in late October (Orange) and early November (Blacksburg). Grain weight and moisture content were recorded for each plot and converted to yield (kg ha^{-1}) at 13% moisture. Seed weight/100 seeds and seed quality ratings were determined for each plot after harvest. The protein, oil, and carbohydrate composition of each sample was determined using a Foss XDS Near-Infrared Rapid Content Analyzer (Foss, Eden

Prairie, MN). The phytic acid content of each sample was determined using a high-throughput indirect Fe colorimetric method as reported by Burleson et al. (2012). Seed Treatment Design

We used twelve seed treatment combinations, including an untreated control, in 2014: MicroCel-E, a weak mineral earth, matric priming, two fungicides, Apronmaxx and Rancona Summit, all possible two and three way crosses, and a control. The specific treatments were selected based on prior unpublished results. In 2015, all MicroCel-E treatments were dropped due to lack of significant results and operational difficulties (Table 2).

MicroCel-E (Manville Filtration and Minerals, Denver, CO) is a fine, synthetic calcium and magnesium silicate powder which, as a seed treatment, acts as a weak fertilizer. Elmer's glue (Elmer's Products, Westerville, OH) was diluted 10 times with tap water until just tacky to bind the MicroCel-E to the seed. The seeds were spun in the bowl of a seed treater and 2.5 ml/1000 seeds of the diluted glue was added followed shortly by 2.5 mg of MicroCel-E/1000 seeds. The seeds were immediately dried in a 32°C dryer for 24 hours.

Osmotic priming partially hydrates the seeds before returning them to their original moisture. Before treatment, the seeds were soaked in a 30% bleach solution for 4 minutes. The seeds were layered with germination paper, so every seed was in full contact with the paper. Each layer was soaked with 20 ml of a 3% potassium phosphate solution in ddH_20. The seeds were kept in a growth chamber at a constant temperature of 16°C and regularly retreated with the potassium phosphate solution once dry. Once hydrated, the seeds were dried in a 32°C dryer for 24 hours before further treatment.

The two fungicides used were Apronmaxx (Syngenta Crop Protection, Greensboro, NC) and Rancona Summit (Valent USA, Walnut Creek, CA), both of which are labeled for use as a seed treatment against damping off and root rot diseases. To make 100 ml solution, 12 ml

Apronmaxx was mixed with 10 ml red seed treatment dye and 78 ml water, and 26 ml Rancona was mixed with 10 ml dye and 64 ml water, respectively. The rate of fungicide application was 2.25 ml per 1000 seeds in a seed treater, which is consistent with the label suggested rate for each fungicide. Once treated, the seeds were dried in a 32°C dryer for 24 hours. For those treatments with both fungicide and MicroCel-E, the solutions were modified: 8 ml Rancona, 7.5 ml dye, and 34.5 ml water or 6 ml Apronmaxx, 7.5 ml dye, and 36.5 ml water. The rate of 3 ml the fungicide solutions per 1000 seeds was applied to coat the seed. Once treated, the seeds were dried in a 32°C dryer for 24 hours. All untreated controls were also placed in a 32°C dryer for 24 hours.

Statistical Analysis

Analysis of variation and correlation analysis among the lines were calculated using JMP 11 software (SAS Inc, Raleigh, NC). All entries were compared to the appropriate control using the Dunnett's test function.

Results

Effects of Genetic and Environmental Factors on Field Emergence

Across both years, environments, and irrigation levels and all six soybean varieties, field emergence averaged (Table 3). Field emergence was significantly ($p<0.0001$) different between the six varieties used in this study. NPA variety AG 5632 had an average field emergence rate of 81.56% which was significantly higher than all other varieties. The *lpa1/lpa2* variety 56CX-1283 had the next highest field emergence rate (74.55%) which was not significantly different than *mips1* variety V12-4557 (72.79%) or NPA variety 5002T (70.77%). The *mips1* variety V12-BB144 had the next lowest average field emergence rate (68.27%) which was not significantly different from V12-4557 or 5002T. MD 03-5453 had an average field emergence rate of 46.29% which was significantly lower than all the other varieties.

Field emergence across all six soybean varieties was significantly different between several environmental factors (Table 3). Field emergence was significantly different between the two years ($p<0.001$) of this study with field emergence being 4.53% higher in 2014. Irrigation significantly ($p<0.001$) reduced field emergence by 6.62%. Plots grown in Orange, VA had an average field emergence rate 5.98% higher than those grown in Blacksburg, VA, but this was not significant. The interaction between year and location also significantly affected field emergence with 2014 Orange (77.88%) and 2015 Blacksburg (75.39%) not significantly different from each other but significantly greater than 2014 Blacksburg (69.50%), and all three were significantly higher than 2015 Orange (62.93%). The interaction between location and irrigation also significantly affected field emergence ($p=0.0102$) with Orange, non-irrigated (75.85%) and Blacksburg, non-irrigated (74.28%) being significantly higher emerging than Blacksburg, irrigated (69.78%), and all three being significantly higher than Orange, irrigated (67.11%).

General Effects of Seed Treatments on Field Emergence

Analysis of the four soybean varieties used in both years of this study found that seed treatments significantly affected field emergence across all four varieties (Table 4). The control treatment had an average emergence of 80.17%. Both fungicides, Rancona Summit (82.58%) and ApronMaxx (80.71%), as well as the priming + Rancona Summit (82.73%) treatments had higher average field emergence than the control but not significantly so. MicroCel-E with either Apronmaxx (78.39%) or Rancona Summit (76.85%), or on its own (74.45%) emerged at insignificantly lower rates than the control treatment.

The priming treatment had an average field emergence (70.22%) which was significantly lower than the control. All other priming treatments, priming+MicroCel-E+Rancona Summit (69.92%), priming+ApronMaxx (67.19%), priming+MicroCel-E (65.55%), and

priming+MicroCel-E+ApronMaxx (63.68%), also had significantly lower field emergence rates than the control but were insignificantly different from each other.

Effects of Seed Treatments on Field Emergence by PA Phenotype

Analysis of the two NPA and four LPA soybean varieties and six treatments used in the second year of this study showed that the seed treatments significantly affected field emergence (Table 5).

The control treatment average field emergence for the two NPA varieties was 75.95%. No seed treatments significantly improved or reduced field emergence in these varieties by comparison with control, but seeds treated with the field emergence of seed treated with Rancona Summit was significantly higher than that of seeds treated with Priming + Rancona. In addition, the Rancona Summit (82.43%), ApronMaxx (80.92%), and Priming + Rancona (81.78%) treatments had higher average field emergence rates than the control. The priming and priming + ApronMaxx had lower average field emergence rates than the control, 75.09% and 69.90%, respectively, but not significantly.

The control treatment average field emergence for all four LPA varieties was 68.91. Both fungicide treatments significantly improved field emergence for the LPA varieties with ApronMaxx increasing field emergence by 8.3% and Rancona Summitt increasing it by 10.59%. Priming + Rancona Summit also had a higher average (74.29%) than the control, but this also was not significant. Conversely, both priming + ApronMaxx (55.76%) and priming (52.24%) significantly decreased field emergence compared to the control.

The effects of the different seed treatments on the different genotypes were also analyzed (Table 5). The two *lpa1/lpa2* varieties, 56CX-1283 and MD 03-5453, followed the same basic rank trend as the effects on all four LPA varieties except for priming having higher emergence

than priming + Rancona Summit. The control treatment had an average field emergence of 66.79%. Rancona Summit was the only treatment which significantly increased field emergence with an average 80.57%. ApronMaxx (77.69%) and priming + Rancona Summit (69.42%) also had higher average field emergence rates than the control but not significantly. Priming and priming + ApronMaxx, again, both significantly decreased field emergence to 52.93% and 52.86%, respectively.

The effects of the different seed treatments on the two *mips1* varieties, V12-4557 and V12-BB144 followed the same rank pattern (Table 5). However, unlike the other two varieties or the overall effects, no seed treatments significantly increased field emergence for *mips1* lines, although Rancona Summit (79.15%), ApronMaxx (78.42%), and priming + Rancona Summit (76.74%) did have higher average field emergence rates than the control. However, priming was the only treatment to significantly decrease field emergence compared to the control doing so by 7.11%. Priming + ApronMaxx had a lower field emergence rate (58.66%) than the control, but this was not significant. In addition, the average field emergence rate of the control treatment in *mips1* varieties was higher than *lpa1/lpa2* varieties.

Effect of Seed Treatments on Field Emergence of Individual Varieties

The application of seed treatments to the six different soybean varieties significantly affected field emergence, and this effect was specific to each variety (Table 6).

Field emergence between the control groups was significant ($p<0.001$). LPA variety 56CX-1283 had the highest field emergence (82.8%) which was significantly higher than all varieties except NPA variety AG 5632 (80.1%) and LPA variety V12-4557 (72.9%). NPA variety 5002T had the next highest field emergence (71.3%) which was only significantly lower than 56CX-1283. LPA variety V12-BB144 (70.1%) had significantly lower field emergence than 56CX-1283 and

AG 5632 while LPA variety MD 03-5453 had significantly lower field emergence (34.7%) than all other varieties.

The two fungicides, ApronMaxx and Rancona Summit, increased germination from the control for almost every variety except for ApronMaxx for AG 5632 and 56CX-1283. However, this increase was only significant for LPA variety MD 03-5453. ApronMaxx significantly increased field emergence of MD 03-5453 from the control average of 34.7% to 67.7%, and Rancona Summit increased the field emergence of it to 69.5%. ApronMaxx also increased field emergence for LPA varieties V12-BB144 (74.2%) and V12-4557 (81.8%) and NPA variety 5002T (81.1%), but these results were not significant. Rancona Summit, similarly, insignificantly increased field emergence in all other varieties. It increased field emergence for LPA varieties 56CX-1283 (86.1%), V12-BB144 (75.5%), and V12-4557 (80.7%) and both NPA varieties, 5002T (80.0%) and AG 5632 (84.8%).

Priming + Rancona Summit significantly increased field emergence for LPA variety V12-BB144 by 8.3%. This treatment also had increased field emergence for two of the other LPA varieties, V12-4557 by 11.4% and MD 03-5453 by 9.4% and slightly decreased field emergence for the LPA variety, 56CX-1283, by 0.7%, but not significantly so. With NPA varieties 5002T and AG 5632, this treatment had insignificantly increased field emergence rates by 6.1% for both.

Priming when used alone decreased field emergence for all LPA varieties. It significantly decreased field emergence for 56CX-1283, V12-BB144, and V12-4557 by 13.4%, 21.1%, and 12.5%, respectively. The priming treatment on MD 03-5453 had an emergence rate 8.5% lower than the control, but this was not significant. Priming had no effect on the NPA varieties.

Priming + ApronMaxx significantly decreased field emergence for LPA varieties 56CX-1283 and V12-BB144 by 21.3% and 12.3%, respectively. AG 5632 (77.3%), 5002T (62.5%), and

V12-4557 (56.7%), when treated with priming + ApronMaxx, also had lower average field emergence than their respective controls but not significantly so. MD 03-5453 with this treatment had a slight, insignificant increase in field emergence of 0.8%, the only line not to have lower field emergence.

MicroCel-E, which was only applied in 2014, did not significantly affect field emergence for any of the six varieties. The majority of treatments combining MicroCel-E with another treatment were also insignificant for field emergence with a few exceptions. Priming + MicroCel-E significantly decreased field emergence field emergence for NPA variety 5002T (51.5%) but no others. This treatment also decreased field emergence for LPA varieties 56CX-1283 (71.0) and V12-BB144 (59.6%) but not significantly so. Priming + MicroCel-E + ApronMaxx significantly reduced field emergence for both LPA varieties used in 2014, 56CX-1283 (58.3%) and V12-BB144 (68.0%), as well as NPA variety 5002T (50.8%). This treatment also lowered field emergence for AG 5632 (77.6%) but not significantly. Priming + MicroCel-E + Rancona Summit significantly decreased field emergence for 56CX-1283 (61.3%). This treatment also insignificantly decreased field emergence for all other three varieties.

Effect of Seed Treatments on Yield and Quality Traits

Yield, seed size, and seed compositional traits, including protein, oil, carbohydrate, and PA content, were not significantly affected by the application of seed treatments across all four soybean varieties used in this study (Table 7). Seed quality (score out of five where 1= best quality and 5= worst quality) was the only quality trait which was significantly affected by the seed treatments, though no treatments differed significantly from the control treatment (2.30). Priming + MicroCel-E (2.22) had the best seed quality, though it was not significantly different from the control or any other treatment except for priming (2.34). The Rancona Summit (2.25), priming +

ApronMaxx (2.26), MicroCel-E + Rancona Summit (2.26), MicroCel-E + Apronmaxx (2.26), priming + Rancona Summit (2.29), and ApronMaxx (2.29) treatments had better quality than the control but not significantly. The MicroCel-E treatment (2.30) did not differ at all from the control treatment while priming + MicroCel-E + ApronMaxx (2.31) and priming + MicroCel-E + Rancona Summit (2.32) had lower seed quality than the control treatment but not significantly so.

Correlation between Field Emergence, Yield, and Other Traits

Correlation analysis was performed between field emergence, yield, and seed composition and quality traits across the four varieties planted in both Blacksburg and Orange in 2014 (Table 8). The strongest correlations with field emergence was seed size (-0.33) and protein content (-0.30). Oil (-0.13) and starch (-0.17) were also significantly correlated with Field emergence. Field emergence was not significantly correlated with ash, carbohydrate, or PA content nor with seed quality. Yield was significantly correlated with all traits except PA content. Carbohydrate content (-0.63) had the strongest correlation with yield. Ash (-0.42) and oil (-0.13) contents and seed quality (-0.20) were significantly, negatively correlated with yield. Protein (0.59) and starch (0.55) content and seed size (0.43) were significantly, moderately correlated with yield. Correlation analysis between the field emergence and yield across all 1008 plots (Fig. 1) used in this study revealed a significant ($p<0.0001$) moderately positive correlation (0.38) between field emergence and yield.

Effect of Variety and Seed Treatment on Yield

Yield was significantly different ($p<0.001$) among the six soybean varieties used in this study (Table 9). NPA variety AG 5632 had the highest average yield (4788.9 kg ha^{-1}), and 5002T, the other NPA variety, had a significantly lower yield (4359.1 kg ha^{-1}). LPA variety 56CX-1283 had the second highest yield (4616.0 kg ha^{-1}) which was significantly different from all other

varieties except for AG 5632. The other variety MD 03-5353 with the *lpa1/lpa2* alleles had the lowest average yield (1162.8 kg ha^{-1}) in this study. The *mips1* mutant varieties, V12-BB144 and V12-4557, averaged 4174.2 kg ha^{-1} and 3541.4 kg ha^{-1} which was significantly lower than AG 5632 and 5002T but significantly higher than MD 03-5354.

Yield was significantly affected by environmental factors including year ($p<0.001$), location ($p<0.001$), and irrigation level ($p<0.001$) (Table 9). Yields for the four varieties planted in both years were higher in 2014 than 2015. Yields across all six varieties used in this study were higher in Blacksburg (4983.2 kg ha^{-1}) than Orange (3376.6 kg ha^{-1}) and higher with irrigation (4295.9 kg ha^{-1}) than without irrigation (4063.9 kg ha^{-1}).

Several interactions between environmental factors and individual soybean varieties significantly affected yield including variety x year ($p=0.002$), variety x location ($p<0.001$), and variety x irrigation level ($p<0.001$).

Discussion

Field Emergence of LPA Soybean Varieties

Field emergence is a vitally important trait for commercial soybean varieties. However, LPA soybeans, which are of great potential benefit to the environment and efficiency of soy-based animal feeds, exhibit remarkably low field emergence, causing a great challenge to their commercialization.

In this study, the highest emerging control group was *lpa1/lpa2* mutant LPA variety 56CX-1283 which had a significantly higher field emergence rate than the NPA variety 5002T and was not significantly different from the other NPA variety, AG 5632. *mips1* mutant LPA varieties V12-BB144 and V12-4557 both had field emergence rates which were not significantly different from 5002T. In fact, the only variety to have a significantly lower field emergence rate than the other

varieties was *lpa1/lpa2* mutant LPA variety MD 03-5453. The fact that all varieties except MD 03-5453 had average field emergence rates >70% suggests that LPA soybean varieties do not inherently have inhibitively low field emergence. This is in agreement with Maupin and Rainey (2011) who found average emergence rates of between 74-84% for LPA varieties from either genetic source across 12 environments and Anderson and Fehr (2008) who reported up to 81.0% field emergence for *lpa1/lpa2* mutants from various seed sources.

Effect of Seed Treatments on Field Emergence

The study showed that field emergence can be significantly improved using seed treatments. There is no consensus as to the exact cause of the low field emergence exhibited by LPA soybean varieties. Some studies suggest diminished germination as the causal factor while others suggest increased disease pressure due to the lack of PA, an important signaling molecule, on the growing seedling pre-emergence as the main cause of this phenomenon (Anderson and Fehr, 2008; Maupin et al., 2010). These results, especially that both fungicides used significantly increased the field emergence of LPA variety MD 03-5453, supported the proposition that higher pre-emergence disease pressure is the main cause of the low field emergence of LPA soybean varieties. The significant decrease in field emergence for most of the LPA varieties in this trial when treated with matric priming to improve seed germination, which was similar to that observed by Kering and Zhang (2015) in water primed food grade soybeans, also disagrees with the suggestion that diminished germination is the causal factor of the decreased field emergence.

The loss of inorganic P, which is less stable than PA in seeds, has also been implicated as a cause of the low emergence in LPA soybeans. If this were the case, we would expect to see an increase in field emergence in those varieties when they are treated with MicroCel-E as it provides supplemental P to the young seedling. In fact, MicroCel-E treated plots did not have field

emergence rates which were significantly different from the control. Where Microcel-E was combined with any of the other treatments, this combined treatment was not significantly different from the non-MicroCel-E treatment indicating that Microcel-E had no effect whatsoever on field emergence. However, the loss of inorganic P could also help to explain the increased disease pressure experienced by LPA soybeans as evidenced by the significant increase in field emergence when treated with fungicide since P leakage could attract pathogens to the emerging seedling (Veresoglou et al., 2013).

However, as there was no consensus as to a single treatment which will increase field emergence across all LPA varieties or even within the genetic sources of the LPA phenotype, more field-based research is required to identify variety-specific treatments for different varieties. In depth physiological research into the loss of inorganic P from LPA soybean varieties as well as their unique microbial interactions would also be important to understanding the full cause of their decreased field emergence.

Relationship between Field Emergence and Other Traits

Field emergence isn't the only important factor for a variety to be commercially viable. Yield, seed composition, and seed quality are all important traits for crop production. Yield had a moderately positive significant correlation with field emergence. However, yield was not significantly affected by the seed treatments. The lack of any significant effect on yield even with the increased field emergence is neither surprising nor problematic. High field emergence is a desirable trait in agronomic production as it is beneficial for various aspects of production including weed control and soil, nutrient, and water conservation. There is often a point at which higher field emergence rates will not further increase yield due to limitations in nutrients, space, or water. However, the agronomic benefits outweigh this loss of yield increase.

The most notable correlation between any seed composition or quality traits and field emergence is the lack of a significant correlation between PA content and field emergence. Previous studies of populations with either the *mips1* or *lpa1/lpa2* have shown moderate but significant positive correlations between PA content and field emergence or negative correlations between Pi (which has an inverse relationship with PA) and field emergence. For instance, in a study of 153 *mips1* mutant recombinant lines, Maupin et al. (2011) found a significant correlation of -0.59 between Pi and field emergence. As Pi and PA are inversely correlated, this can be taken as a positive correlation between PA and field emergence (Maupin et al., 2011; Scaboo et al., 2009). This lack of significance may be due to the small number varieties used in this study and, thus, a lack of great variation in PA content. Further, it may be due to the effect of the seed treatments on emergence causing dissociation between these two traits. This result could be a sign of just how important is the effect of seed treatments on field emergence for LPA soybean varieties. Conversely, it is unsurprising that PA was not significantly correlated with PA content as this is consistent with several other studies (Oltmans et al., 2005; Scaboo et al., 2009).

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Tables and Figures

Table 1. The PA content, genetic source of the LPA trait, and the years planted for each soybean variety in this trial

Variety	LPA Gene	Years Planted	PA Content (ppm)
5002T	N/A	2014, 2015	6116.10
AG 5632	N/A	2014, 2015	5886.72
56CX-1283	*lpa1/lpa2*	2014, 2015	2486.03
MD 03-5453	*lpa1/lpa2*	2015	2131.68
V12-4557	*mips1*	2015	4060.80
V12-BB144	*mips1*	2014, 2015	4420.50

Table 2. Seed treatments used in this study, the years each was used, and the use of individual treatment

Treatment	Years Used	Use
Control	2014, 2015	Untreated control
ApronMaxx	2014, 2015	Broad spectrum fungicide
MicroCel-E	2014	Weak fertilizer
Priming	2014, 2015	Hydrolyze sugars and start germination pre-planting
Rancona Summit	2014, 2015	Broad spectrum fungicide
Priming + Rancona	2014, 2015	
Priming+ApronMaxx	2014, 2015	
Priming + MicroCel-E	2014	
Priming+MicroCel-E+Rancona	2014	
Priming+MicroCel-E+ApronMaxx	2014	
MicroCel-E + Rancona	2014	
MicroCel-E +ApronMaxx	2014	

Table 3. Field emergence between two NPA and four LPA soybean varieties grown at Blacksburg and Orange in 2014 and 2015 under irrigated or non-irrigated conditions

				Field Emergence (%)								
					Year		Location		Blacksburg		Orange	
Variety	PA	IRR	LPA gene	Total Avg.	2014	2015	BB	O	2014	2015	2014	2015
		B†		70.77^{BC}	68.36	75.58	69.07	72.46	65.36	76.51	71.36	74.65
5002T	N	Y	N/A	66.96	64.59	71.70	66.61	67.32	62.34	71.70	66.84	68.26
		N		74.57	72.13	79.46	71.54	77.60	68.37	79.46	75.89	81.04
		B		81.56^{A}	82.60	79.46	80.12	82.99	76.84	88.37	86.68	72.24
AG 5632	N	Y	N/A	78.90	79.23	78.25	77.13	80.67	71.91	78.25	86.55	68.92
		N		84.21	85.98	80.68	83.11	85.31	81.77	80.68	90.19	75.56
		B		74.55^{B}	72.06	79.54	72.55	76.56	67.65	82.36	76.48	76.72
56CX-1283	L	Y	*lpa1/lpa2*	72.88	69.72	79.20	71.61	74.16	66.20	79.20	73.25	75.97
		N		76.23	74.40	79.88	73.50	78.96	69.10	79.88	79.71	77.47
		B		68.27^{C}	71.75	61.32	70.92	65.63	68.17	76.42	75.33	46.22
V12-BB144	L	Y	*mips1*	63.03	65.99	57.12	66.77	59.29	61.85	57.12	70.12	37.64
		N		73.51	77.51	65.52	75.07	71.96	74.48	65.52	80.54	54.79
		B		46.29^{D}	NA	46.29	53.02	35.57	NA	53.02	NA	39.57
MD 03-5453	L	Y	*lpa1/lpa2*	44.77	NA	44.77	55.31	34.24	NA	55.31	NA	34.24
		N		47.81	NA	47.81	50.73	44.90	NA	50.73	NA	44.90
		B		72.79^{BC}	NA	72.79	77.36	68.21	NA	77.36	NA	68.21
V12-4557	L	Y	*mips1*	68.09	NA	68.09	75.24	60.94	NA	75.24	NA	60.94
		N		77.48	NA	77.48	79.48	75.49	NA	79.48	NA	75.49
		B		71.75	73.69^{a}	69.16^{b}	65.50^{b}	71.48^{a}	69.50	75.39	77.88	62.93
Grand Mean		Y		68.44^{b}	69.88	66.52	69.78	67.11	65.58	75.38	74.19	57.66
		N		75.06^{a}	77.50	71.81	74.28	75.85	74.43	75.41	81.58	68.21

Means followed by the same letter are not significantly different by Tukey's HSD at p=0.05.

†- B= means across both irrigated (Y) and non-irrigated (N) plots

Table 4. Average field emergence and Tukey's separation of means for 12 seed treatment combinations across 4 soybean varieties grown in 2014 and 2015

Treatment	Field Emergence (%)	Range (%-%)
Control	80.17abc	42.50-98.75
Priming + Rancona	82.73a	49.38-98.13
Rancona Summit	82.58a	53.13-98.75
ApronMaxx	80.71ab	58.13-96.25
MicroCel-E +ApronMaxx	78.39abc	53.13-95.63
MicroCel-E + Rancona	76.85abcd	55.63-95.00
MicroCel-E	74.45bcde	53.13-94.38
Priming	70.22def	40.00-96.25
Priming+MicroCel-E+Rancona	69.92def	47.50-93.13
Priming+ApronMaxx	67.19ef	37.50-93.13
Priming + MicroCel-E	65.55ef	35.63-93.5
Priming+MicroCel-E+ApronMaxx	63.68f	36.88-92.50

Treatment means followed by the same letter are not significantly different by Tukey's HSD at p=0.05.

Table 5. Average field emergence and Tukey's separation of means for 6 seed treatments across 4 LPA soybean varieties grown in 2015

Treatment	NPA		LPA					
			Total		*mips1*		*lpa1/lpa2*	
	Emergence	Range	Emergence	Range	Emergence	Range	Emergence	Range
	%	%-%	%	%-%	%	%-%	%	%-%
C†	75.95abc	42.50-96.88	68.91bc	17.50-98.75	71.04ab	25.00-86.88	66.79b	17.50-98.75
R	82.43a	53.13-97.50	79.50a	35.00-99.38	79.15a	35.00-99.38	80.57a	43.13-98.75
A	80.92ab	58.13-96.25	77.21a	38.13-96.25	78.42a	38.13-95.00	77.69ab	46.25-96.25
PR	81.78ab	53.75-98.13	74.29ab	25.63-94.38	76.74a	33.75-94.38	69.42ab	25.63-92.50
PA	69.90c	41.88-93.13	55.76cd	15.00-86.88	58.66bc	23.75-82.50	52.86c	15.00-86.88
P	75.09bc	48.13-96.25	52.24d	6.88-86.25	51.55c	6.88-76.88	52.93c	11.88-86.25

Treatment means followed by the same letter are not significantly different by Tukey's HSD at p=0.05

†C-Control, A-ApronMaxx, M-MicroCel-E, P-Priming, R-Rancona Summit

Table 6 Effect of seed treatments on field emergence in NPA and LPA soybeans and Tukey's separation of means for the control treatments

		Treatment												
		2014 and 2015						2014 only						
Variety	**PA**	**C†**	**A**	**R**	**P**	**PA**	**PR**	**C**	**M**	**MA**	**MR**	**PM**	**PMA**	**PMR**
5002T	N	71.3bc	81.1	80.0	71.2	62.5	77.4	77.6	70.4	72.1	75.0	51.5*	50.8*	67.1
AG 5632	N	80.1ab	80.1	84.8	79.0	77.3	86.2	81.8	82.0	87.2	82.0	80.0	77.6	80.8
56CX-1283	L	82.8a	82.7	86.1	69.4*	61.5*	82.1	85.3	73.9	76.4	77.9	71.0	58.3*	61.3*
V12-BB144	L	70.1c	74.2	75.5	49.0*	57.8*	78.4*	75.9	71.6	77.3	71.9	59.6	68.0*	70.5
V12-4557	L	72.9abc	81.8	80.7	60.4*	56.7	84.3	-	-	-	-	-	-	-
MD 03-5453	L	34.7d	67.7*	69.5*	26.2	35.5	44.1	-	-	-	-	-	-	-

*significantly different from appropriate control according to Dunnett's Test at p=0.05

†C-Control, A-ApronMaxx, M-MicroCel-E, P-Priming, R-Rancona Summit

Table 7. Effects of 12 seed treatments on yield and quality traits and Tukey's separation of means across 4 soybean varieties grown in 2014

Treatment	Yield	Seed Size	Protein	Fat	PA	Seed Quality
	kg ha^{-1}	g 100 $seed^{-1}$	%	%	ppm	(1-5, best-worst)
C†	4726.3	16.00	35.16	17.37	1289.06	2.30ab
A	4862.8	15.99	35.33	17.37	1282.57	2.29ab
M	4858.1	16.17	35.42	17.30	1081.36	2.30ab
P	4690.0	15.94	35.14	17.36	1239.83	2.34a
R	4887.1	16.21	34.50	17.43	1178.83	2.25ab
MA	4821.2	16.08	35.18	17.36	1282.44	2.26ab
MR	4987.3	16.15	35.27	17.68	1172.23	2.26ab
PA	4782.1	16.16	35.32	17.43	1306.14	2.26ab
PM	4665.8	16.00	35.38	17.38	1240.20	2.22b
PR	4903.9	16.14	35.54	17.36	1224.08	2.29ab
PMA	4741.1	16.07	35.54	17.42	1401.78	2.31ab
PMR	4796.9	15.95	34.57	17.01	1201.46	2.32ab
Grand Mean	4810.4	16.07	35.28	17.35	1241.66	2.30

† C= Control, A= ApronMaxx, M= MicroCel-E, P=priming, R= Rancona Summit
Treatment means followed by the same level are not significantly different by Tukey's HSD at p=0.05

Table 8. Correlation coefficients of the relationship between field emergence, yield, seed composition, and quality traits for 4 soybean varieties grown in Blacksburg and Orange, VA in 2014

	Field Emergence	Yield
Ash	ns	-0.42***
Carbohydrate	ns	-0.63***
Oil	-0.13**	-0.13**
PA	ns	ns
Protein	-0.30***	0.59***
Starch	-0.17***	0.55***
Seed Size	-0.33***	0.43***
Quality	ns	-0.20***

**significant at p=0.01
***significant at p=0.001

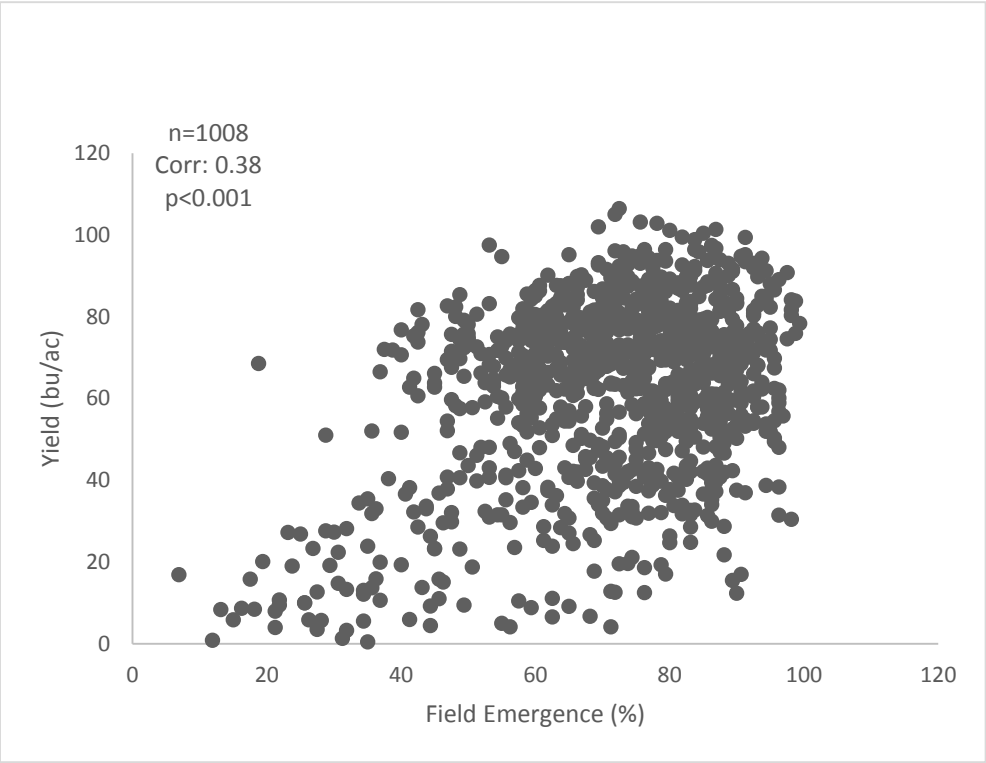
Table 9. The yield of six soybean varieties grown at Blacksburg and Orange in 2014 and 2015

			Yield (kg ha^{-1})								
				Year		Location		Blacksburg		Orange	
Variety	PA	IRR	Total Avg.	2014	2015	BB	O	2014	2015	2014	2015
5002T	N	B†	4359.1^{BC}	4764.7	3546.8	5000.0	3718.3	5246.8	4505.1	4283.2	2587.8
		Y	4519.9	4984.6	3589.1	4964.4	4074.7	5234.1	4423.7	4735.7	2753.9
		N	4198.4	4544.8	3504.4	5035.7	3361.2	5259.6	4586.5	4031.6	2422.3
AG 5632	N	B	4788.9^{A}	5141.9	4084.1	5792.2	3786.2	5976.5	5424.4	4306.7	2743.8
		Y	5000.7	5410.9	4180.3	5713.6	4287.9	5932.1	5275.8	4889.7	3084.8
		N	4577.7	4872.3	3988.6	5871.6	3283.8	6020.9	5573.7	3724.3	2403.5
56CX-1283	L	B	4616.0^{AB}	4851.4	4143.9	5308.0	3923.4	5288.5	5347.7	4414.3	2940.2
		Y	4802.3	4995.3	4415.6	5260.3	4344.4	5189.7	5401.5	4801.7	3430.4
		N	4429.1	4707.5	3872.3	5356.5	3502.4	5387.4	5294.6	4027.6	2450.6
V12-BB144	L	B	4174.2^{C}	4482.9	3557.5	4998.7	3350.4	4933.5	5129.2	4033.0	1985.9
		Y	4200.4	4558.2	3486.2	4855.5	3546.1	4852.1	4861.5	4263.7	2111.0
		N	4148.7	4408.2	3628.8	5141.9	3154.7	5014.8	5396.8	3801.6	1860.8
MD 03-5453	L	B	1162.8^{E}	na	1162.8	1694.7	631.5	na	1694.7	na	631.5
		Y	1053.8	na	1053.8	1466.7	540.9	na	1466.7	na	640.9
		N	1272.4	na	1272.4	1923.4	621.4	na	1923.4	na	621.4
V12-4557	L	B	3541.4^{D}	na	3541.4	4771.4	2312.1	na	4471.4	na	2312.1
		Y	3519.9	na	3519.9	4444.6	2595.9	na	4444.6	na	2595.9
		N	3362.9	na	3562.9	5098.2	2028.3	na	5098.2	na	2028.3
Grand Mean		B	4180.3	4810.4^{a}	3339.6^{b}	4983.2^{a}	3376.6^{b}	5361.2	4478.9	4258.9	2200.4
		Y	4295.9	4987.3	3373.9	4877.6	3714.2	5302.0	4312.1	4672.5	2435.8
		N	4063.9	4633.5	3304.7	5088.1	3039.7	5420.4	4645.6	3846.0	1964.4

Mean yields followed by the same letter are not statistically different by Tukey's HSD at p = 0.05

†- B= means across both irrigated (Y) and non-irrigated (N) plots

Figure 1. Correlation between yield and field emergence for all plots grown in Blacksburg and Orange in 2014 and 2015



3. Impact of *mips1*, *lpa1* and *lpa2* Alleles for Low Phytic Acid Content on Agronomic, Seed Quality and Seed Composition Traits of Soybean

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Abbreviations: ELSD, evaporative light scattering detector; HPLC, high performance liquid chromatography; *lpa*, low phytic acid; *mips1*, D-*myo*-inositol 3-phosphate synthase 1; PA, phytic acid; PCR, polymerase chain reaction; RIL, recombinant inbred line; SNP, single nucleotide polymorphism; wt, wild type allele for phytic acid content

Abstract

Soybean (*Glycine max* L. Merr) is an important agronomic crop around the world used largely for animal feed. However, ~75% of the P in soybean grain is in the form of phytic acid (PA) or phytate, the cation salt form of the same, which cannot be digested by mono- and a-gastric animals including swine, poultry, and aquacultural animals leading to decreased field efficiency and environmental detriment due to P runoff. Soybean varieties have been developed with a reduced PA content using mutant alleles of three genes involved in the PA pathway: *mips1*, *lpa1*, and *lpa2*. In addition to the reduction of PA, *MIPS1* mutants also have an improved sugar profile that is high in easily digestible sucrose and low in the less digestible raffinose and stachyose. Despite these benefits, significant barriers exist to the production of commercial low PA (LPA) soybean varieties, most notably reduced field emergence. In this study, a population 30 recombinant inbred lines (RILs) developed from a cross between V03-5901 (*mips1*) x 04-05N32 (*lpa1/lpa2*) were planted along with the parents at two locations in Virginia in 2014 and 2015. The following findings were obtained from our analysis. 1）Comparison of the various traits amongst the individual alleles and combinations thereof showed that the *lpa1* allele has the highest field emergence and so may be a good trait with which to create a commercially viable LPA soybean variety. 2）It also showed an additive relationship between the three different mutant alleles resulting in lower PA content as more LPA mutant alleles are added. 3）There is a significant, and previously unreported, interaction between the *MIPS1* and *lpa2* mutant alleles resulting in a raffinose content significantly lower than with either allele on its own. Therefore, this combination can be exploited to create LPA soybean varieties with an even more beneficial sugar profile. 4) Seed size was negatively correlated with field emergence across all genotypes and, thus, may be a good target trait for developing a commercially viable LPA soybean variety regardless of the exact

genotype. Correlation analysis between the various traits broken down by the individual mutant alleles and the exact genotype of each revealed differences between the different genotypes suggesting that a unique strategy would be required for each distinct LPA genotype to develop a commercially viable variety.

Introduction

Soybean (*Glycine max* L. Merr) is a protein and oil rich seed crop adaptable to a wide range of end uses including human and animal consumption. With a high protein content and relatively low cost, soybean meal is a major component in many feeds for both companion and agricultural animals with ~98% of all soybean meal going to animal feed in 2013, 76% of which went to swine and poultry production (Soystats, 2015). Phytic acid (PA), myo-inositol 1,2,3,4,5,6-hexakisphosphate, also known as phytate in its cation salt form, is the primary storage form of phosphorus (P) in soybean seed comprising up to 75% of the total P in mature seeds. PA is also a strong chelator of cationic metal micronutrients including calcium, magnesium, and iron (Raboy et al., 1984).

However, agastric and monogastric animals, including chickens, pigs, and most aquatic animals, lack the activity of a phytase enzyme in their digestive tract. It was found that animals, especially swine, cannot digest PA due to a lack of hydrolysis at the end of the tract (Dilger and Adeola, 2006; Kleinmann et al., 2005; Powers et al., 2006); thus, the vast majority of P in the meal is unavailable thereby lowering the efficiency of the feedstuffs. Because these animals cannot digest PA, there is a much higher level of P in their manure compared to ruminant animals. For example, a survey of P in various animal manures by Kleinmann et al. (2005) found 28.8 g P/kg in swine manure and 25.6 g P/kg in layer chicken manure vs. 5.1 g P/kg in beef cattle manure. Through runoff or leaching from either waste lagoons or fields fertilized with manure from monogastric animals, these high levels of P often enter into natural bodies of water.

Producers have long used synthetic phytase as an additive to soy-based feeds for mono- and a-gastric animals to compensate for the natural lack of this enzyme and improve the feed's efficiency. However, this method is both expensive and less efficient than natural phytase activity

as it relies heavily on various factors including temperature, pH, and mineral concentration (Brejnholt et al., 2011; Hassan et al., 2013).

Numerous genes have been identified as playing a role in the PA synthetic pathway. Three genetically recessive mutant alleles from two different sources have been recognized as most important in creating a low phytic acid (LPA) phenotype: *lpa1*, *lpa2*, and *mips1*. Though all three genes act on the same general pathway, each has been identified and confirmed to be distinct and separate (Gao et al., 2008; Oltmans et al., 2004).

The first two LPA alleles, *lpa1* and *lpa2*, were discovered on GM19 (LG N) and GM3 (LG L), respectively, of the mutant soybean line CX-1834 and have homologs in several other crop species including corn and barley (Wilcox et al., 2000). The mutant allele, *lpa1*, has a greater effect than *lpa2*, the other LPA gene from this source, which codes for a constituent protein in an ATP-binding cassette (ABC) transporter that partitions PA into the seed. The missense mutation in the mutant allele produces a truncated and non-functioning ABC transporter (Pilu, 2009; Shi et al., 2007). Thus, though PA may be produced in lines with the *lpa1* mutation, that PA will not be efficiently partitioned into the seed. *lpa2* contains a nonsense mutation to a gene also involved in the ABC transporter in the latter part of the PA production pathway (Gillman et al., 2013). While this mutation decreases the amount of PA produced, other inositol kinases may compensate for its lack of production leading to this mutation having a much more minor effect on the overall PA content of the seed than *lpa1* (Pilu, 2009). In combination, these two alleles have been shown to lower the PA content to only 25% of the phosphorus in these lines is in the form of PA or phytate while the other 75% is inorganic and, thus, available for animals that cannot digest PA (Bilyeu et al., 2008; Wilcox et al., 2000).

The other major gene that has been shown to be related to LPA in soybeans, *MIPS1*, has been discovered on GM11 (LGB1) in several, distinct soybean germplasms. This gene is one of a family of four myo-inositol phosphate synthase genes responsible for the addition of phosphates to a sugar backbone in the early steps of the PA production pathway. *MIPS1* codes for the first step in pathway converting Glucose 6-phosphate to Inositol 3-phosphate. The LPA trait in mutant line LR33, which has the *mips1* allele, has been traced to a single nucleotide change in the 10th exon of the gene causing the *MIPS1* protein to be non-functional (Hitz et al., 2002; Saghai Maroof, 2009). Compared to LPA mutants from the CX-1834 source, *MIPS1* mutants have more PA in the seed where it usually accounts for 50% of the total phosphorus. However, *MIPS1* mutants have the added benefit of a modified, beneficial sugar profile with sucrose, an easily digestible sugar, content being high while raffinose and stachyose, both of which are not fully digestible by mono- and a-gastric animals, contents are low (Saghai Maroof and Buss, 2008). Therefore, *MIPS1* mutants increase feed efficiency for mono- and a-gastric animals.

Closely linked genetic markers have been identified for each of the three mutant alleles and can be used to screen and identify lines with the LPA phenotype. Satt237 and Satt561 are simple sequence repeat (SSR) markers that are associated with the *lpa1* and *lpa2* mutant alleles, respectively (Scaboo et al., 2009). Two genotyping techniques exist for the *MIPS1* mutant allele. Satt 453 is an SSR marker and a single nucleotide polymorphism (SNP) marker linked to *MIPS1* has been used to identify *MIPS1* mutants such as in soybean line V99-5089 (Rosso et al., 2011).

Decreased field emergence in LPA soybeans has been observed in many field experiments, which is the greatest issue that breeders are facing in the effort to produce a commercially viable LPA soybean variety Consistently, LPA soybean lines show diminished field emergence rates well below the commercial threshold of 85%. However, the reasons causing low field emergence in

LPA soybeans is still under study as emergence is a very complex trait. Of the possibilities noted in previous research, reduced germination, weakened seedling vigor, accelerated seed aging and seed source environment have all been implicated in this issue (Anderson and Fehr, 2008; Maupin and Rainey, 2011; Oltmans et al., 2005). Of these, seed source environment has by far been the most consistently observed.

The purpose of this study is to study the effects of and interactions between the three LPA mutant alleles on various agronomic and seed composition traits and compare the differences in correlations between seed composition and agronomic traits.

Materials and Methods

Plant Materials

A recombinant inbred line (RIL) population was developed from a cross V03-5901 x 04-05N32. The hybridization was made at Blacksburg, VA in 2008. The F1 plants were grown in a winter nursery in Costa Rica in the winter of 2008. The population from F2 to the F4 generation was advanced using a modified single-pod descent method. In fall 2011, 30 F_4 single plants were selected based on overall appearance and their genotypes *(mips1, lpa1/lpa2, or mips1/lpa1/lpa2)*. The 30 F4:5 progeny rows were grown in Warsaw, VA in 2012. A total of 30 $F_{4:6}$ RILs as well as their parents (Table 1) were selected based on seed amount as materials in this study.

Allele Identification

Single nucleotide polymorphism (SNP) genetic markers were used to identify the alleles in each F_4 single plant. The *MIPS1* allele was identified using a C/G SNP reported by Saghai Maroof and Buss (2008). The *LPA1* alleles were identified using an A/T SNP while the *LPA2* alleles were identified using a G/A SNP (Gillman et al., 2009). Frozen tissue samples were ground to a powder from which DNA was extracted using a CTAB method (Saghai Maroof et al., 1984),

and the genetic regions were amplified through identical PCR programs before being read and visualized using a BMG Labtech FLUOstar Omega microplate reader (BMG Labtech GmbH, Ortenburg, Germany).

Field Trials

The plots were planted in a triplicated, generalized, randomized complete block design at two locations in Virginia: Blacksburg and Orange. Each plot consisted of four 3.05m rows spaced 0.82m with 80 seeds per row. They were planted in late May and harvested mid-October in 2014 and 2015.

Stand counts were taken at the V1 stage to determine field emergence (Fehr and Caviness, 1977). The middle two rows of each plot were harvested in late October-early November. Grain weight and moisture content were recorded for each plot and converted to yield (kg ha^{-1}) at 13% moisture. Seed weight/100 seeds and seed quality ratings were determined for each plot after harvest.

Seed Composition Analysis

The PA content of each plot was determined using a high-throughput indirect Fe colorimetric method as reported by Burleson et al. (2012). Briefly, samples of soybean powder were extracted with HCL. Starches and proteins were removed with 20% NaCl in ddH_2O before being treated with a ferric iron solution for 2 hours followed by a color reagent. The samples were then analyzed through 510 nm wavelength absorption on a BMG Labtech FLUOstar Omega microplate reader, and PA concentrations were determined from a standard curve taken from 7 known concentration standards.

For sugar composition analysis, seed samples from each plot were ground to a fine powder. A 0.1 g sample of this powder was used to analyze the sucrose, raffinose, and stachyose contents.

Each sample was mixed by vortex with 1 mL double distilled water, and shaken on a back and forth mixer at 400 strokes/min for 15 minutes. The sample was then centrifuged at 17,000x g for 15 minutes. Soluble proteins from 0.5 mL supernatant were precipitated in 0.7 mL 100% HPLC grade acetonitrile for 1 hour before being centrifuged at 17,000x g for 15 min. 100 µL of the clear supernatant was mixed with 900 µL of 65% acetonitrile (35% HPLC-grade water) and filtered through a syringe with an IC Millex-LG 13 mm mounted 0.2 µm low protein binding hydrophilic millipore (PTFE) membrane filter (Millipore Ireland BV, Carrigtwohill, Republic of Ireland). Four calibration standards were prepared containing the three sugars in the following concentrations reported as µg/ml and listed in order of sucrose, raffinose, and stachyose: Standard 1- 50, 5, and 12.5; Standard 2- 150, 15, and 37.5; Standard 3- 500, 50, and 125; Standard 4- 1000, 100, and 250. A reference standard was prepared at a concentration of 490, 70, and 140 µg/ml for sucrose, raffinose, and stachyose, respectively, and was included with each batch of samples run on the HPLC. The calibration was repeated every 30 samples. The sugars in solution samples were separated on an Agilent 1260 Infinity series (Agilent Technologies, Santa Clara, CA), equipped with guard (4.6 × 10 mm) and analytical (4.6 × 250 mm, 5 µm) columns (Supelco apHera NH_2 polymer), and detected using an evaporative light scattering detector (ELSD). The isocratic elution with mobile phase of acetonitrile:water (65:35, v/v) was carried out at a flow rate of 1.0 mL/min. A 10 µL sugar extract was injected. The nebulizer and evaporation temperatures of the ELSD were set at 80º C and dry N_2 at 1.6 lpm.

Statistical Analysis

Analysis of variation among the lines and correlation analysis were calculated using JMP 11 software (SAS Inc, Raleigh, NC). All pairwise comparisons of means were determined using Tukey's multiple means comparison method when possible, and the Student's t-test when not.

Heritability estimates were calculated on a genotypic class basis using R with the lme4 mixed effect package (Bates et al., 2015).

Results and Discussion

Genotypic Analysis of 30 RILs

The 30 RILs used in this study were genotyped with SNP markers for each of the three LPA alleles in the parental lines (Table 1). This analysis determined that there were 5 RILs with each of the following mutant allele genotypes with each being homozygous for the reported allele: *lpa1*, *mips1*, *mips1/lpa1*, *lpa1/lpa2*, *mips1/lpa2*, and *mips1/lpa1/lpa2*. Notably, there were no RILs with either only the *lpa2* mutant allele or no mutant alleles at all.

Environmental Effects on Agronomic Traits

Field emergence was significantly affected by both location ($P = 0.0002$) and year ($P < 0.0001$), but the interaction between the two locations or years was not significant (Table 2). Field emergence was significantly higher in Orange (48.8%) than Blacksburg (54.6%). It was also significantly higher in 2015 (71.1%) than 2014 (32.4%). The drastic differences between the two years may well be due to the fact that the seed planted in 2014 was two years old as opposed to the seeds planted in 2015 which were harvested in 2014.

Yield was significantly affected by location ($p<0.0001$), year ($p=0.0017$), and the interaction between the two locations ($p<0.0001$) (Table 2). Plots in Blacksburg yielded 1654.3 kg ha^{-1} higher than in Orange, and plots in 2014 yielded 302.6 kg ha^{-1} higher than in 2015. The highest

average yield was in 2015 at Blacksburg (4162.8 kg ha^{-1}) which was significantly higher than yield at the same location in 2014 (3597.9 kg ha^{-1}). Plots in Orange had the exact opposite trend with 2014 out yielding 2015 by 1170.2 kg ha^{-1}.

Effect of the Mutant Alleles on Agronomic Traits

Field emergence was significantly affected by both location and year, but the interaction between the location and year was not significant (Table 2). Field emergence was significantly higher in Orange (48.8%) than Blacksburg (54.6%). It was also significantly higher in 2015 (71.1%) than 2014 (32.4%). Yield was significantly affected by location, year, and the interaction between the two years or locations (Table 2).

Field emergence was significantly different between the various LPA genotypes (Fig. 1). *lpa1*-only lines had the highest average field emergence (61.0%) while *mips1*-only lines had a lower field emergence (50.7%), but not significantly. *lpa1/mips1* lines alleles had a field emergence rate (49.3%) which was lower, but not significantly, than either of the single mutant genotypes. The combination of either of these two alleles with the *lpa2* mutant allele resulted in insignificantly lower field emergence than the corresponding single mutant genotype. *lpa1/lpa2* lines had 9.6% lower field emergence than *lpa1*-only lines, but this was not significant. *mips1/lpa2* lines had 5.4% lower field emergence than *mips1*-only lines. Triple mutant *mips1/lpa1/lpa2* lines had a field emergence rate of 50.1% which was lower than that of Genotypes *lpa1*-only, *lpa1/lpa2*, and *mips1*-only, but higher than *mips1/lpa2* and *mips1/lpa1* lines. The only significant difference for field emergence was between *lpa1*-only and *mips1/lpa2* lines. These results are, overall, lower than what would be deemed commercially acceptable which may be due to the dramatically low field emergence in the first year of this study. The lack of a significant difference between the vast

majority of genotypes indicated that any of the three mutant alleles may not necessarily be precluded from producing high emerging, LPA soybean varieties.

Yield was also significantly ($p<0.0001$) affected by the LPA genotypes (Fig. 2), and followed the similar pattern as field emergence. *lpa1*-only lines had the highest yield averaging 3376.0 kg ha^{-1} while the other single mutant genotype, *mips1*-only, had a lower average yield (3201.1 kg ha^{-1}), but this was not a significant difference. *mips1*/*lpa1* lines had an average yield of 3160.75 kg ha^{-1} which was insignificantly lower than either of the single mutant genotypes. The addition of the *lpa2* mutant allele resulted in insignificantly lower yield compared to the appropriate single mutant genotype. *lpa1/lpa2* lines yielded 174.9 kg ha^{-1} less than *lpa1*-only lines while *mips1/lpa2* lines yielded 457.3 kg ha^{-1} less than *mips1*-only lines. The triple mutant *mips1/lpa1/lpa2* lines yielded less (2911.9 kg ha^{-1}) than every other genotype except *mips1*/*lpa2*. The only significant difference in yield was between *lpa1*-only and *mips1/lpa2* lines.

Effect of the Mutant Alleles on Seed Composition Traits

PA content was significantly different ($P< 0.001$) among the different genotypes (Table 3). *lpa1*-only lines had the highest PA content averaging 4602 µg g^{-1}. *mips1*-only lines had an average PA content of 3601 µg g^{-1} which was not significantly different from *lpa1*-only lines. Double mutant lines all had lower PA content than their single mutant counterpart. *mips1/lpa1* lines had an average PA content of 3313 µg g^{-1} which was significantly lower than *lpa1*-only lines but not *mips1*-only lines. *lpa1/lpa2* lines had a significantly lower average PA content (2385 µg g^{-1}) than *lpa1*-only lines, but *mips1/lpa2* lines did not have a significantly lower PA content (3317 µg g^{-1}) than *mips1*-only lines. The triple mutant line *mips1/lpa1/lpa2* lines had the lowest average PA content (1939 µg g^{-1}) which was significantly lower than all other genotypes except *lpa1/lpa2*. These results were not consistent with other studies, especially that *lpa1*-only lines had a higher

PA content than *mips1*-only lines (Bilyeu et al., 2008; Maupin et al., 2011; Wilcox et al., 2000). For example, Maupin et al. (2011), in a study of six LPA soybean lines found that the all three *lpa1/lpa2* lines had significantly lower phytic acid content than all three *mips1* lines with the *lpa1/lpa2* lines ranging in PA content from 878-1269 μg g^{-1} while *mips1* lines ranged from 1935-2071 μg g^{-1}. The results showed the additive nature of each alleles effect on PA content when combined with another allele, which had previously been reported for *lpa1/lpa2* lines, but not for any combination with the *mips1* allele (Bilyeu et al., 2008; Wilcox et al., 2000).

Sucrose (P= 0.0003), raffinose (P=0.0013), and stachyose (P< 0.0001) contents were all significantly affected by the different genotypes in this study (Table 3). Total sugar content, however, was not significantly affected by the genotypes. All lines with the *mips1* allele had higher sucrose contents than those without. *mips1*-only lines had insignificantly higher sucrose content than *lpa1*-only lines, 8.28% and 6.98%, respectively. *mips1/lpa1* lines had an average sucrose content which was slightly lower than *mips1*-only lines at 8.11%. *lpa1/lpa2* lines had virtually the same sucrose content as *lpa1*-only lines (6.95%), and *mips1*/*lpa2* lines had the highest sucrose content (9.12%) which was significantly different than both non-*mips1* genotypes but not significantly different from any of the *mips1* genotypes. The triple mutant genotype, *mips1/lpa1/lpa2*, had a sucrose content of 8.25%, only slightly lower than *mips1*-only lines. The sucrose contents for *mips1* genotypes is in line with results reported by Maupin et al. (2011) who reported sucrose contents of between 9.0% and 9.1%, but the non-*mips1* lines in this study also had higher sucrose contents.

The single mutant genotypes did not have significantly different raffinose contents with *lpa1*-only lines that had an average raffinose content of 0.79% and *mips1*-only line that had an average of 0.71%. *mips1/lpa1* lines had an average raffinose content of 0.74% which was in

between and not significantly different from either of the single mutant genotypes. *lpa1/lpa2* lines had a raffinose content of 0.80% which was virtually identical to *lpa1*-only lines. *mips1/lpa2* lines had the lowest overall raffinose content (0.68) and were the only one which was significantly lower than any of the non-*mips1* lines. The triple mutant *mips1/lpa1/lpa2* lines had the highest overall raffinose content (0.86%), significantly higher than all other *mips1* mutant lines but not significantly different from the non-*mips1* lines. Overall, raffinose contents were higher than previously reported, and none of these genotypes have raffinose contents which would be considered "low" (Maupin et al., 2011; Saghai Maroof and Buss, 2008; Sebastian et al., 2000).

The stachyose contents of the single mutant genotypes were significantly different with *mips1*-only lines (2.80%) being lower than *lpa1*-only lines (4.38%). *mips1/lpa1*-lines had an average stachyose content (3.22%) which was in between and not significantly different from either single mutant genotypes. *lpa1*/*lpa2* lines did not have a significantly different average stachyose content (4.11%) from *lpa1*-only lines. *mips1/lpa2* lines had the lowest average stachyose content (1.59%) which was significantly lower than that of any other genotype. The triple mutant *mips1/lpa1/lpa2* lines had an average stachyose content of 3.69% which was not significantly different from any other genotypes except *mips1*/*lpa2*. The stachyose contents observed in this study are much higher than any reported for *mips1* mutants but on par with those reported for non-*mips1* lines, but the reduction observed is in line with previous reports (Maupin et al., 2011; Saghai Maroof and Buss, 2008; Sebastian et al., 2000). There have been no previous reports of the *lpa2* mutant allele having any effect on sugar content, but this is the first time in which all three mutant alleles were combined in a single population.

Heritability Estimates of PA Content

Heritability analysis was performed for PA content across the 34 LPA soybean lines grown in four environments (2 years x 2 locations). For this population, PA content had a remarkably high h^2 estimate (0.95) which is considerably higher than that found by Maupin et al. (2011) in a *mips1* segregating population (h^2:0.62). This high value could be due to the high level of interrelatedness between the entries in this study since they were all developed from a single biparental cross. As this analysis was done on a genotypic basis and each genotype has multiple entries resulting in a high number of replications (up to 21 replications) for each phenotype within each environment, this high value may also be due to the high number of replications resulting in stronger regressions (off which heritability is based). Finally, the lack of a true wildtype entry (i.e. *MIPS1/LPA1/LPA2*) in this study may skew the data towards higher heritability estimates.

The high h^2 value observed in this analysis would indicate that phenotypic selection is an effective tool for selecting low PA content soybean lines, but this does not take into account more pragmatic considerations including time and cost. The phenotypic analysis for PA content as reported by Burleson et al. (2012) is highly time consuming taking two days to complete the analysis not including the amount of time required to grind samples. Conversely, genetic discrimination using SNPs, as done in this study, is also expensive and requires specialized machines which some may not have access to. Cheaper, less specialized molecular marker alternatives do exist for determining LPA genotypes (Rosso, et al., 2011). Such accurate and user friendly molecular markers may mean that, despite the remarkably high h^2 value here reported, phenotypic selection may not be the most agreeable method for selecting LPA soybean lines.

Correlations between Agronomic, Quality and Seed Composition Traits

Correlation analysis performed on all 30 RILs and their parents regardless of LPA genotype revealed numerous correlations between agronomic and seed composition traits (Table 4).

Field emergence was significantly correlated with all traits included in this analysis except yield. The strongest correlation was with seed size which was moderately negative (-0.52). Seed quality was positively correlated with field emergence (0.29). Of the seed compositional traits, raffinose had the strongest correlation with field emergence which was moderately positive (0.43). PA content was also had a moderately positive correlation with field emergence (0.32). Sucrose and stachyose contents both had weak but significant correlations with field emergence, -0.22 and 0.13, respectively.

Yield was significantly correlated with correlated with all traits except field emergence and PA and raffinose content. The strongest correlation with yield was quality which had a moderately negative (-0.36). Seed size (0.32) had the next strongest correlation with yield and was positively related to it. Sucrose and stachyose both had weak, positive correlations with yield of 0.15 and 0.13, respectively.

Seed quality was significantly correlated with all other traits except PA content. The strongest correlation was with Seed size which was moderately negative (-0.52). Sucrose also had a moderately negative correlation with seed quality (-0.29). Raffinose had a weakly positive correlation with seed quality (0.16) while stachyose had a weakly negative correlation with seed quality (-0.13). Additionally, seed size had a moderately negative correlation with PA content (-0.25) and raffinose content (-0.30) while it had a weakly positive correlation with sucrose (0.13) and stachyose (0.14).

The four seed compositional traits also had several significant correlations between themselves. Notably, PA was only significantly correlated with raffinose with which it had a weakly positive correlation (0.17). The correlation between PA and raffinose is lower than but in broad agreement with Maupin et al. (2011) who reported a strongly negative correlation between Pi and raffinose and Saghai Maroof and Buss (2008) who reported a strongly positive correlation between PA and raffinose. The lack of a significant correlation between PA and sucrose and PA is also in contrast to both of those studies which found that PA is strongly correlated with both raffinose and stachyose. These differences could be due to the inclusion of *lpa1* and *lpa2* mutant alleles which do not have a reported effect on sugar content. Sucrose was negatively correlated with both raffinose (-0.26) and stachyose (-0.46) both of which are weaker but still broadly agree with both Maupin et al. (2011) and Saghai Maroof and Buss (2008) but disagrees with studies of NPA soybeans (Cicek et al., 2006). The correlation between stachyose and raffinose (0.62) also agrees with all three of these studies though it is considerably higher than that reported by Cicek et al. (2006).

Correlations of Field Emergence and Yield with Other Traits by LPA Allele

Some major differences existed for the correlations between field emergence and yield with other traits depending on the LPA allele (Table 5).

Three notable differences were observed for correlations between field emergence and all other traits across the three LPA alleles. First, *mips1* mutants were the only ones who had any significant correlation between field emergence and yield for which it had a weak positive correlation (0.15). The correlation between field emergence and Sucrose content also had some observed differences. *mips1* and *lpa1* mutant lines both had moderately negative correlations with field emergence (-0.15 and -0.22, respectively) while *lpa2* mutants had a moderately positive

correlation (0.35) between these two traits. Further, *lpa1* and *lpa2* mutant lines both had positive correlations of 0.23 between field emergence and stachyose content while *mips1* mutants did not have a significant correlation between those two traits.

Similarly, there were three notable differences in the correlations between yield and other traits for the three LPA mutant alleles. Firstly, both *mips1* and *lpa1* mutants exhibited weakly positive correlations between yield and sucrose content (0.18 and 0.17, respectively) while *lpa2* mutant lines did not have a significant correlation between the two traits. Secondly, raffinose content had a weak positive correlation with yield for *lpa1* mutants (0.16) while *mips1* and *lpa2* mutants did not have a significant correlation between the two, and, finally, stachyose content had a weakly positive correlation (0.18) with yield but was not significantly correlated with that for the other two alleles.

Potential Breeding Lines

ANOVA was performed between the individual RILs for yield, field emergence, and PA content across both years and locations of this study. From this, five lines with high field emergence and low PA content were identified as potential breeding lines for developing high emerging, high yielding, LPA soybean varieties (Table 6). The field emergence rates and yield for the five lines were not significantly different from one another while the PA contents were significantly different ($P = 0.0108$). Due to the high field emergence and satisfactory yield of each line makes them ideal candidates for developing commercially viable LPA varieties. Notably, all three LPA alleles and four different genotypes are represented in this selection including one each *mips1*-only, *lpa1/lpa2*, and *mips1/lpa2* lines and two *mips1/lpa1/lpa2* lines.

Conclusions

This study has provided a unique comparison of the three major LPA mutant alleles, *lpa1*, *lpa2*, and *MIPS1*, and the interactions thereof. It presented a new understanding of the complexity of the agronomic issues and seed compositional possibilities inherent to LPA soybeans.

The correlations between the various agronomic and seed compositional traits vary greatly between the mutant alleles and combinations thereof. Therefore, development of a commercially viable agronomic LPA soybean variety must be specialized to the exact genotype leading to the desired LPA phenotype. Field emergence, the trait most often cited as the main barrier to the production of a commercial LPA soybean variety, was correlated positively with PA, raffinose, and stachyose contents and negatively for sucrose. Since *MIPS1* mutants have higher sucrose and lower raffinose and stachyose contents, this may account for the lower field emergence of *MIPS1* mutant lines. However, they are not so strong of correlations as to preclude from possibility a high emerging *mips1* line. Further, the *mips1/lpa1* mutant had no significant correlation between field emergence and either sucrose or raffinose. This interaction could provide a route for the development of a variety that is both low in PA and has a more digestible sugar profile. Seed size had the strongest correlation, a negative correlation, with field emergence of any traits and was significant for all six genotypes. This suggests that seed size could be used as a selection criterion for developing a high emerging LPA soybean variety.

The *lpa1* lines were the highest emerging and yielding. This may make it a prime target for developing high emerging LPA soybean lines, but they lack the beneficial sugar profile of *MIPS1* mutants and had the highest PA content of any genotype. The majority of *MIPS1* mutant genotypes, though the lowest both germination and yield, were not significantly different from the highest emerging and yielding. Thus, *mips1* lines cannot be precluded from commercial varietal

development. The *lpa2* lines presented a drag to both yield and emergence; though it did significantly lower PA content, the drag on agronomic traits may make it unviable for future usage. However, there was a notable interaction between the *lpa2* and *mips1* lines which resulted in the lowest raffinose and stachyose contents of the entire study, and the *lpa1* lines seemed to counteract this interaction. Finally, the three mutant alleles had an additive effect on the PA content resulting in each double mutant having a lower PA content than their single mutant counterparts, and the triple mutant genotype having the significantly lowest PA content. This presents the possibility of creating extremely low PA soybean varieties.

These results indicate that there is not a single inherent cause of the low field emergence which has been continuously observed in LPA soybean varieties. Therefore, it may be possible to create commercially viable LPA soybean varieties by crossing LPA soybean lines with high emerging and yielding soybean varieties, and thus improving the genetic stock of the variety. Future studies would be required to examine this.

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Tables and Figures

Table 1. Composition of the population, number of entries, and mutant alleles

Name	Entries	Mutant alleles
V03-5901 (Female Parent)	2	*mips1*
04-05N32 (Male Parent)	2	*lpa1, lpa2*
RIL	5	*lpa1*
RIL	5	*mips1*
RIL	5	*mips1, lpa1*
RIL	5	*mips1, lpa2*
RIL	5	*lpa1, lpa2*
RIL	5	*mips1, lpa1, lpa2*

Table 2. Mean field emergence and yield rates for 30 LPA soybean RILs between 2 locations and years

Variables			Field Emergence	Yield
			%	kg ha^{-1}
Location		**BB**	48.8b	3880.3a
		O	54.6a	2226.0b
Year		**2014**	32.4b	3207.8a
		2015	71.1a	2905.2b
Location x Year	**BB**	**2014**	29.1	3597.9b
		2015	68.6	4162.8a
	O	**2014**	35.6	2811.1c
		2015	73.6	1640.9d

Within each environmental factor, trait means followed by the same letter are not significantly different according to Tukey's pairwise comparison at p=0.05.

Table 3. Descriptive statistics and Tukey's separation of means for seed composition traits of RILs grown in Blacksburg and Orange in 2014 and 2015

Genotype	PA (μg g^{-1})		Sucrose (%)		Raffinose (%)		Stachyose (%)		Total Sugar (%)†	
	Mean	range	mean	range	mean	range	mean	range	mean	range
lpa1	4602a	856-8925	6.98b	1.82-13.73	0.79ab	0.18-1.42	4.38a	0.01-7.98	12.16	5.31-17.21
mips1	3601ab	291-10326	8.28ab	2.89-13.96	0.71bc	0.02-1.30	2.80b	0.09-7.32	11.79	6.38-18.33
mips1/lpa1	3313bc	158-8325	8.11ab	1.66-15.43	0.74bc	0.12-1.23	3.22ab	0.09-6.82	12.07	5.18-18.47
lpa1/lpa2	2385cd	88-7351	6.95b	2.78-12.02	0.80ab	0.07-1.47	4.11a	0.08-8.45	11.86	7.13-18.08
mips1/lpa2	3317bc	1044-7034	9.18a	4.24-16.00	0.68c	0.03-1.17	1.59c	0.09-4.93	11.45	5.71-16.92
mips1/lpa1/lpa2	1939d	227-4864	8.25ab	2.43-27.47	0.86a	0.08-2.37	3.69ab	0.02-9.82	12.80	6.26-32.32
Grand Mean	3079	88-10326	8.04	1.66-27.47	0.73	0.02-2.37	3.27	0.01-9.82	12.04	5.18-32.32

Within each trait, genotypic class means followed by the same letter are not significantly different according to Tukey's pairwise comparison at p=0.05.
† Total sugar was not significantly affected by genotype at p=0.05.

Table 4. Correlation coefficients of agronomic and seed composition traits from 34 RILs developed from a cross between V03-5901 x 03-04N32 grown in Blacksburg and Orange, VA in 2014-2015

	Emergence	Yield	Quality	Seed Size	PA	Sucrose	Raffinose	Stachyose
	%	kg ha^{-1}	1-5	g 100 seeds^{-1}	µg g^{-1}	%	%	%
Emergence	-	ns	0.29***	-0.52***	0.32*	-0.22***	0.43***	0.13*
Yield		-	-0.36***	0.32***	ns	0.15**	ns	0.13*
Quality			-	-0.52***	ns	-0.29***	0.16**	-0.13*
Seed Size				-	-0.25***	0.13*	-0.30***	0.14*
PA					-	ns	0.17**	ns
Sucrose						-	-0.26***	-0.46***
Raffinose							-	0.62***
Stachyose								-

*significant at p=0.05
**significant at p=0.01
***significant at p=0.001
ns- not significant at p=0.05

Table 5. Correlation coefficients of agronomic and seed composition traits by LPA mutant allele in a population of 34 RILs developed from a cross between V03-5901 x 03-04N32 grown in Blacksburg and Orange, VA in 2014 and 2015

	Field Emergence			**Yield**		
	mips1	***lpa1***	***lpa2***	***mips1***	***lpa1***	***lpa2***
Emergenc	-	-	-	-	-	-
Yield	0.15*	ns	ns	-	-	-
Quality	0.36***	0.25***	0.37***	0.36***	0.33***	0.45***
Seed Size	-0.55***	-0.53***	-0.62***	0.29***	0.36***	0.28***
PA	0.33***	0.32***	0.26***	ns	ns	ns
Sucrose	-0.15*	-0.22**	0.35***	0.18*	0.17*	ns
Raffinose	0.38***	0.42***	0.53***	ns	0.16*	ns
Stachyose	ns	0.23***	0.23**	ns	ns	0.18*

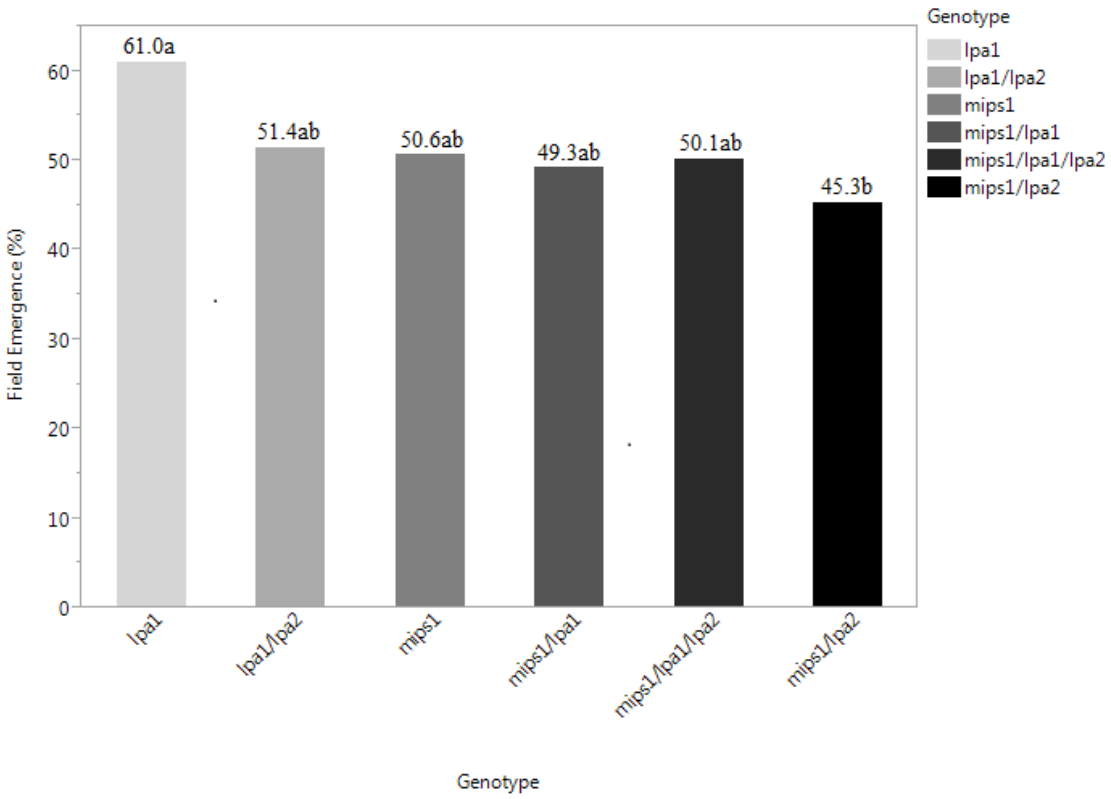
*significant at p=0.05
**significant at p=0.01
***significant at p=0.001
ns- not significant at p=0.05

Table 6. Five potential breeding lines for high field emerging LPA soybeans

Line	LPA Alleles	Field Emergence (%)	Yield (kg ha^{-1})	PA Content (µg g^{-1})
V03-5901	*mips1*	74.0	2949.0	5448.1a
04-05N32	*lpa1, lpa2*	66.7	2845.1	4116.3b
RIL 457	*mips1, lpa1, lpa2*	77.7	2898.5	2412c
RIL 458	*lpa1, lpa2*	70.5	3019.5	1930c
RIL 493	*mips1, lpa1, lpa2*	75.0	2885.0	2170c
RIL 734-333	*mips1*	78.0	3073.3	3889bc
RIL 748	*mips1, lpa2*	77.3	2952.3	4499bc

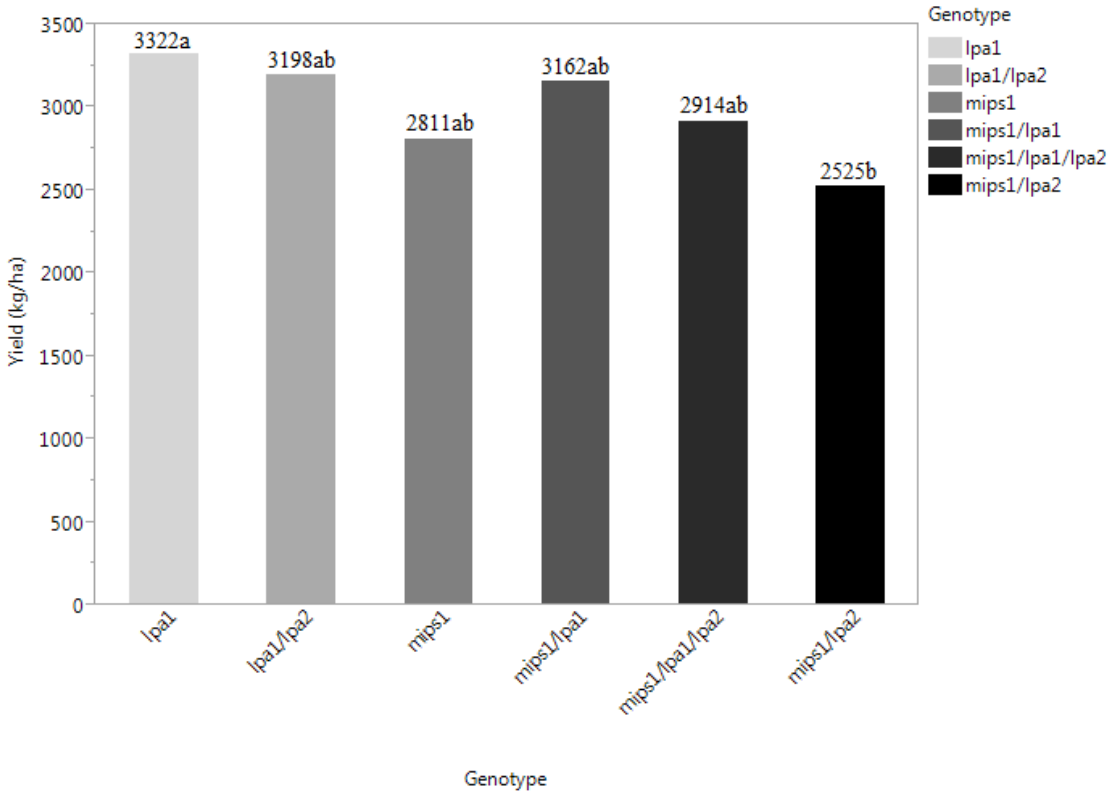
Within each trait, genotypic class means followed by the same letter are not significantly different according to Tukey's pairwise comparison at p=0.05.

Figure 1. Field emergence was significantly different (P= 0.0263) between the six genotypic classes across both years and locations of this study



Means followed by different levels are significantly different by Tukey's HSD at P=0.05
Each genotypic class had n=60

Figure 2. Yield was significantly different (P= 0.0135) between the six genotypic classes across both years and locations of this study



Means followed by different levels are significantly different by Tukey's HSD at P=0.05
Each genotypic class had n=60

4. Developing a Low Error Protocol for Testing Low Phytic Acid Soymeal Based Feed on Pacific White Shrimp

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Abrreviations: LPA, low phytic acid; PA, phytic acid

Abstract

Soymeal is an attractive alternative to more traditional protein sources for shrimp feeds due to its relatively low cost. However, 75% of the P in soybean grains is in the form of phytic acid which (PA) is not digestible by mono- and a-gastric animals such as shrimp. This leads to environmental detriment caused by the excess P in the waste of the animals. For this reason, soymeal is not commonly used in aquacultural animal feeds. Low PA (LPA) soybean varieties have been developed using genetic mutations which have up to 75% lower PA content than conventional varieties. In this study, a low error protocol was developed for studying the effect of LPA soymeal based feeds on the growth and environmental quality of Pacific white shrimp (*Litopenaeus vannamei)*. Three different methods, differing in tank and population size and chemical analysis protocols, were compared to divine a low error testing method. Across the board, using five shrimp over six weeks using a higher capacity ortho-P testing protocol had lower error and should be favored for studying the difference in water quality levels. None of the tested methods were particularly favorable for studying the effect of LPA soymeal based feeds on shrimp growth. It is suggested, then, that this issue can be resolved by either vastly increasing the population size (>100 shrimp/aquarium) or decreased (1 shrimp/aquarium) to account for shrimp death and variation in size between individuals.

Introduction

Soybean (*Glycine max* L. Merr) is an important feedstuff for animal production across the globe due to its unique high protein and oil content and wide geographic adaptability. Upwards of 95% of United States soybeans go into feed for a variety of animals including cattle, swine, poultry, and domestic animals (Soystats, 2015). In recent years, interest has been increasing in the use of soymeal as an economical replacement for the more expensive and traditional fish or squid meal as the main source of protein in feed for aquatic animal production (Asche et al., 2013).

However, there is a significant challenge to the use of soymeal based feeds in aquacultural production. Up to 75% of the P in soybean seeds is in the form of phytic acid (PA), myo-inositol 1,2,3,4,5,6-hexakisphosphate, and phytate, the cation salt form thereof. Mono- and agastric animals, including most aquacultural animals, lack the activity of a phytase enzyme in their gut, and, thus, cannot breakdown PA and utilize the phosphorus. Therefore, up to 75% of the P in soymeal based feeds will be deposited in the animal waste products (Dilger and Adeola, 2006; Kleinmann et al., 2005; Powers et al., 2006). In addition, this P can cause environmental damage through eutrophication leading to algal blooms, hypoxia, and, ultimately, massive fish death (Shindler et al., 2008, Sinkko et al., 2013).

Synthetic phytases can be used as a feed additive to breakdown PA to inorganic P (Chang and Lim, 2006). This process, though, is an added cost for producers, so, to nullify this need, soybean varieties with lower PA contents have been developed using mutant alleles of three different genes involved in the PA pathway. These varieties have up to a 75% decrease in PA content with an equivalent increase in the easily digestible inorganic

P content (Bilyeu et al., 2008; Saghai Maroof and Buss, 2008; Wilcox et al., 2000). Further, LPA soybean varieties with the *mips1* mutation also have a favorable sugar content high in easily digestible sucrose and low in the less digestible raffinose and stachyose (Saghai Maroof and Buss, 2008).

Experimental LPA soybean based animal feeds have been tested in a number of mono-gastric species to confirm their use as both a highly efficient and environmentally friendly alternative to traditional soymeal. The overall consensus shows that the P in LPA soymeal has a much higher bioavailability and bioretention rates than that in normal PA soymeal in mono-gastric animals while the P rate in the waste is significantly lowered. These results account for all the expectations and goals of LPA soybeans thereby confirming the validity of the concept.

Broiler chickens have been one of the most widely studied species with LPA soymeal based feeds. Dilger and Adeola (2006) compared two feeds, one LPA and the other normal phytic acid (NPA), on broilers and found that those broilers fed with the LPA feed retained 17% more of the soymeal P (77%). There was not any significant difference in the P bioavailability between the two feeds as both had a bioavailability of between 79-89%. This, conversely, is well correlated to those found by Scaboo et al. (2009) and Wilcox et al. (2000) that ~75% of the seed P in LPA lines is in the form of P_i.

Similar results have been noted in swine. In a feeding trial comparing LPA or NPA soybean meal based swine feeds with and without the inclusion of a synthetic phytase, Powers et al. (2006) reported a 19% decrease in total P (tP) in the feces of those pigs fed with the LPA diet. Water soluble P (WSP) also decreased in LPA treatments by 17%. In addition, the LPA diets had a statistically significant reduction of both tP and WSP than

the NPA diet with phytase (16% and 6%, respectively) suggesting that LPA soybean meal is a valid alternative to synthetic phytase. The addition of phytase to the LPA soybean meal diet, however, saw an even greater reduction of both tP and WSP in the feces (27% and 23%, respectively). This is to be expected since PA is still present in LPA soybean meal. In total, these results highlight the potential benefits of a LPA based diet in monogastric animals.

However, few such tests have been performed on agastric aquatic animals probably because soy-based feeds are not widely used in aquatic animal production. There is a growing interest in soymeal as a cheaper alternative to traditional protein sources such as fish or squid meal. In fact, many areas of the world, including Europe, still have tight regulation of soy-based fish feeds because of the environmental impacts of the P in soymeal (Asche et al., 2013; Kumar et al., 2012). Therefore, testing LPA soymeal based feeds on agastric aquatic animals could provide a major stepping stone in advancing the development of both LPA soybean varieties and the aquacultural sector. Such experiments could possibly open up new markets around the world for American soybean exports and lift an economical hurdle for the aquacultural sector.

A variety of methods have been used in previously published shrimp nutrition studies each strategically designed to fit unique needs, physical limitations, and parameters to be measured. Aquarium and population sizes are especially sensitive to these factors. For instance, an evaluation of dietary feeding stimulants by Sanchez et al. (2006) included a comparison of population sizes ranging from 50-150 Pacific White Shrimp in 7500 L tanks while Forster et al. (2010) studied the diet optimization on Pacific White Shrimp used five shrimp in 35 L aquariums.

Some parameters, however, are less negotiable. Environmental factors have a large impact on shrimp growth. Growth and digestion is especially temperature sensitive with minor variations in temperature resulting in detectable differences in growth parameters (Wyban et al., 1995). Shrimp are similarly sensitive to salinity (Chen et al., 2014). Thus, these factors must be as constant as possible across both time and different aquariums to assure that observed differences are truly due to the treatment. Commonly used temperature and salinity values are around 29°C and 15%, respectively (Forster et al., 2010; Sanchez et al., 2006; Wyban et al., 1995).

The purpose of this study was to develop a method for studying the effect of LPA soymeal based feeds on Pacific White Shrimp.

Materials and Methods

Feed Formulations

Two feeds were made using recipes designed to be isonitrogenous (equal protein) and near-isocaloric (equal energy). Each feed received the same amount of vitamins, minerals, and other supplemental nutritional compounds (Table 1). The LPA diet was be based on VS07-0094 soybean meal (2347.1 µg g^{-1} phytic acid) which was developed at Virginia Tech and has the *mips1* allele accounting for the low phytic acid content. The NPA diet was based on Glenn soybean meal (3090.8 µg g^{-1} phytic acid). Glenn is a conventional, commercially grown soybean variety also developed at Virginia Tech.

Phytic acid samples of each soybean meal were extracted using an HCl method as described by Maupin et al. (2010) and quantified through high pressure ion chromatography on a Dionex ICS 3000 (Dionex, Sunnyville, Ca) This was used to quantify the total phytic acid being added to each aquarium.

Shrimp

The shrimp used in this experiment were Pacific white shrimp (*Litopenaeus vannamei*) from the same breeding family to control for genetic variation

Method 1

The first method (Table 2) consisted of six 30L aquariums filled with 15% salt water made with ddH_2O and synthetic sea salt. Each aquarium had five shrimp of roughly equal size and was independently filtered and heated at 28°C. Each of the two feeds was randomly applied to three aquariums for the duration of this run.

The total body weight of the shrimp in each aquarium were measured once a week. The feed was applied twice a day to three tanks at a rate equal to 4% of the weekly total body mass of the tank/day. Feed conversion ratios were calculated thus: $\frac{g\ \Delta Body\ Mass}{g\ Feed}$.

Each day, the salinity, temperature, and dissolved O_2 of each tank was measured with a YSI 556 MPS handheld multi-parameter instrument (YSI, Inc. Yellow Springs, OH), and pH of each tank was measured using a VWR Symphony SB70P electrode (VWR International, Radnor, PA). Salinity was adjusted as needed to account for any losses.

The chemical quality of each tank was measured three times a week including alkalinity, NH_4, NO_2^-, NO_3^-, and ortho-P. NH_4 was analyzed using the Hach Nessler reagent method (product #2119449) modified for salt water through the use 10 drops of mineral stabilizer in each sample except for the ddH_2O blank. NO_2^-, NO_3^-, and ortho-P were each treated immediately after collection with the appropriate Hach chemical reagent pillow (product #s 2107169, 2106169, and 2106069, respectively) and read on a Hach DR/2800 Spectrophotometer (Hach US, Loveland, CO). Any samples which had concentrations above the maximum readable value for the appropriate method were diluted

using ddH_2O. For Alkalinity, 100 ml water samples was treated with four drops of Hach Bromcresol Green-Methyl Red Indicator Solution (Cat. 2329232) and titrated with H_2SO_4 to a pH of 4.5. Alkalinity was adjusted with sodium bicarbonate to keep the H_2SO_4 equivalency/100 ml above 100.

The run was terminated after six weeks.

Method 2

The second method (Table 2) consisted of ten 50 L aquariums with 10, roughly equal sized shrimp in each. The salt content and temperatures of the water were unchanged from the first method, and each aquarium was still individually filtrated. The two different feeds were applied randomly to five aquariums, each, for the duration of this run.

The methods for measuring weight, feed efficiency, salinity, dissolved O_2, water temperature, alkalinity, NH_4, NO_2^-, and NO_3^- as well as the amount of feed applied were unchanged from the previous method.

To begin with, ortho-P samples were measured using the same method as the first method. However, once samples had more than 5 µg ortho-P ml^{-1}, the maximum measurable amount for this method, ortho-P samples were analyzed using the Hach Reactive Phosphorus Amino Acid method (method #8178). Total P samples were collected at the end of the run by replacing all solids from the filter sponge into the aquarium water and digesting the solids with a low ascorbic acid method (Hach product #2742745) before being treated with the Hach Reactive Phosphorus Amino Acid method.

This run was terminated after two weeks due to the inability of the filters to treat the larger population.

Method 3

The third method (Table 2) consisted of ten 50 L aquariums with five shrimp in each. Due to lack of stock, the size of the shrimp could not be adequately controlled. The salt content and temperatures of the water were unchanged from the first method, and each aquarium was still individually filtrated. The two different feeds were applied randomly to five aquariums, each, for the duration of this run.

The methods for measuring weight, feed efficiency, salinity, dissolved O_2, water temperature, alkalinity, NH_4, NO_2^-, and NO_3^- as well as the amount of feed applied were unchanged from the previous methods.

The ortho-P samples, unlike the previous 2 runs, were only measured using the Hach Reactive P Amino Acid method. Total P samples were treated in the same way as the previous method.

This run was terminated after six weeks.

Statistical Analysis

All data was transformed to account for the total amount of feed applied to the aquarium and statistical analysis was performed using JMP 11 software (SAS Inc., Raleigh, NC). Analysis of variation (ANOVA) was performed to study the differences in environmental quality and shrimp growth between the two feeds.

To compare the methods, regression analysis was performed the data for each aquarium individually. The R^2 values from these analyses was taken as a measure of the ability of each method to accurately and consistently produce and quantify the data. ANOVA and Tukey's multiple means comparison method were then performed on the R^2 values to compare the three methods.

Finally, power analysis was performed to determine the total number of aquariums required to detect a significant difference between the feeds for each of the methods. The standard deviation of each method as determined by ANOVA was used in this analysis.

Results

The ortho-P and weight measurements were transformed to account for the unique amount of feed applied per tank at the time the samples were taken. Across all three methods, the rate of ortho-P accumulation was not significantly different (P= 0.9747) between the two feeds with the NPA feed averaging 0.358 µg ortho-P L^{-1} /g feed, and the LPA feed averaging 0.354 µg ortho-P L^{-1} /g feed. Feed efficiency was also not significantly different (P= 0.6273) between the two feeds across all three methods with NPA feeds having an average feed efficiency of 0.031 and LPA feeds having an average feed efficiency of 0.054.

Method Comparisons for Ortho-P

Regression analysis was performed individually for each tank on the effect of the different feeds on the ortho-P concentration in the water. The R^2 values for each method were taken as a measure of the veracity of the measurements and compared through ANOVA (Fig 1).

The R^2 values ranged from 0.39-0.9908 across all three methods. Method 2 had the widest range (0.6504-0.9688) followed by Method 1 (0.39-0.6567) and Method 3 (0.7939-0.9908).

The methods were significantly ($p<0.0001$) different for R^2 values. Method 3 had the highest average R^2 values, 0.9 for the NPA feed and 0.945 for the LPA feed. Method 2 had R^2 values for the NPA and LPA feeds of 0.894 and 0.798, respectively, which were

not significantly different from Method 3. Method 1 had the lowest average R^2 values for both the NPA and LPA feeds, 0.587 and 0.415, respectively, which were significantly lower than either of the other two methods.

Power Analysis

Sample size and power estimation was performed to determine the minimum sample size to detect a significant difference in ortho-P concentrations between the two feeds for each method. The α level was set to 0.05, and power was set to 0.7, 0.8, and 0.9. The standard deviations for each method were determined through individual ANOVA (Table 3).

The sample sizes (ss) predicted by JMP varied little between the methods. Method 1 required the fewest samples to detect a significant difference between the feeds: power =0.7, ss=22; power=0.8, ss=25; power=0.9, ss=30. Method 3 required the next fewest samples (power =0.7, ss=24; power=0.8, ss=27; power=0.9, ss=32), and Method 2 required the most samples (power =0.7, ss=25; power=0.8, ss=28; power=0.9, ss=33).

Method comparison for feed efficiency

The methods were compared for feed efficiency measurements in much the same way as the ortho-P methods.

The R^2 values ranged from 0.0285-0.9956. Method 2 had the widest range (0.1574-0.9956) followed by Method 3 (0.0285-0.8659) and Method 2 (0.3718-0.9165).

The methods were not significantly different ($p<0.0508$) for the R^2 values of the regression analysis of feed efficiency (Fig 2). However, method three did have lower R^2 (NPA: 0.298; LPA: 0.56) than the other two methods. Method 1 had a lower NPA R^2 value (0.682) and higher LPA R^2 value (0.714) than Method 2 (0.832 and 0.596, respectively).

Discussion

None of the methods found a significant difference for ortho-P or total P concentrations or feed efficiency. This broad lack of significance could be due to the low difference in PA between the feeds. The LPA soymeal, VS07-0094, was only 24.06% lower in PA than the NPA soymeal, Glenn. Since the LPA feed had a higher proportion of soymeal (560 g/kg vs. 522.6 g/kg), the LPA feed had a PA content of 1314.38 µg g^{-1} while the NPA feed had a PA content of 1615.25 µg g^{-1}, a difference of only 18.63%. Soybean lines with lower PA content, such as S04-053-05 (878 µg P g^{-1}), exist and may be a better candidate for formulating a LPA soymeal based feed to study the effect of LPA soybeans on the growth and waste water quality of Pacific white shrimp (Maupin et al., 2011).

The protocol for measuring ortho-P concentration in the water increased in veracity with each new method. The reagent used seems to be the most important factor. The ortho-P concentrations in Methods 1 and 2 quickly outgrew the maximum readable concentration for the ortho-P powder pillows (5 µg/ml) resulting in high levels of variation (fig 3). However, the measurements became more trustworthy using the Reactive P Amino Acid protocol in Method 3 and the latter parts of Method 2. The increased number of ortho-P samples taken under Method 3 (11/aquarium) compared to Method 2 (6/aquarium) may also have contributed to the higher R^2 values. However, larger population sizes would be nominally better for measurements as it would decrease the variability due to a single individual. Therefore, the larger population size in Method 2 may have been better suited to measuring ortho-P, but this would require hardware capable of filtering and maintaining such a large population over a longer period of time.

For all three methods, a sample size of 30 (15 aquariums/feed) would provide a high enough power (between 0.8 and 0.9). If separated into three runs of 10 aquariums, lab size would not be too constrictive.

None of the methods adequately measured feed efficiency for the two feeds with some aquariums even having a negative relationship between growth and feed. Three factors may account for this phenomenon: low population size, lack of uniformity between individuals, and poor individual health leading to death. The population size was such that any individual death may drastically affect the average weight for that population especially as the size of the individuals within a population varied more than what could be considered ideal. Method 3, which had the lowest R^2 values, was marred by poor health including disease and mineralization which could account for those low values.

There are two possible solutions to this problem. The first is to have a study running concurrent to the water quality experiment consisting of contained units housing a single shrimp. With this method, weight measurements could be taken daily to maximize the power of the inference. This method would compensate for the error due to variation between individuals and individual death. However, this method would require a large number of individual units and, thus, a large lab space. The second possible solution would be to greatly increase the number of individual in each aquarium. The death of an individual in a population of 100 shrimp would not have as large of an effect on the average or total weight of the population. Thus, weight measurements and feed efficiency would be less responsive to individual deaths. However, this solution would require large and powerful equipment as well as a large lab space. In either case, to control for individual size and health, selection of individuals would need to start from a much larger population to allow

for intense selection. For examples, a population of 100 individuals could be selected from a preliminary population of 500 individuals.

Conclusions

The most important step towards creating a high power, low error method for studying the effect of LPA soymeal based feeds on the growth and water quality of shrimp is to have a larger difference in the PA content of the two feeds. For measuring the ortho-P concentrations, the Reactive P Amino Acid protocol is better suited for measuring the high ortho-P concentrations observed in this study with the greatest level of accuracy. A longer run time (~4 weeks) also increase the accuracy of the measurements as the larger number of samples controls for the small levels of variation. The method for studying the effect of LPA soymeal based feeds on shrimp growth requires the most radical change from this study. Measuring the growth of single shrimp in individual units would be highly accurate and easy to implement concurrent with an ortho-P study due to the lack of need for very large lab space, large aquariums, and powerful filters.

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Tables and Figures

Table 1. Feed recipes for both low and normal PA treatments

	LPA (g/kg)	NPA (g/kg)
Fishmeal	100	100
LP Soymeal	560	0
NP Soymeal	0	522.6
Wheat flour	65	65
Fish oil	55	55
Squid meal	50	50
Liquid lecithin	5	5
Starch	114	151.4
CMC	20	20
Vitamins Mix	10	10
Minerals Mix	10	10
KCl	4	4
CaCl	5	5
L-methionine	2	2

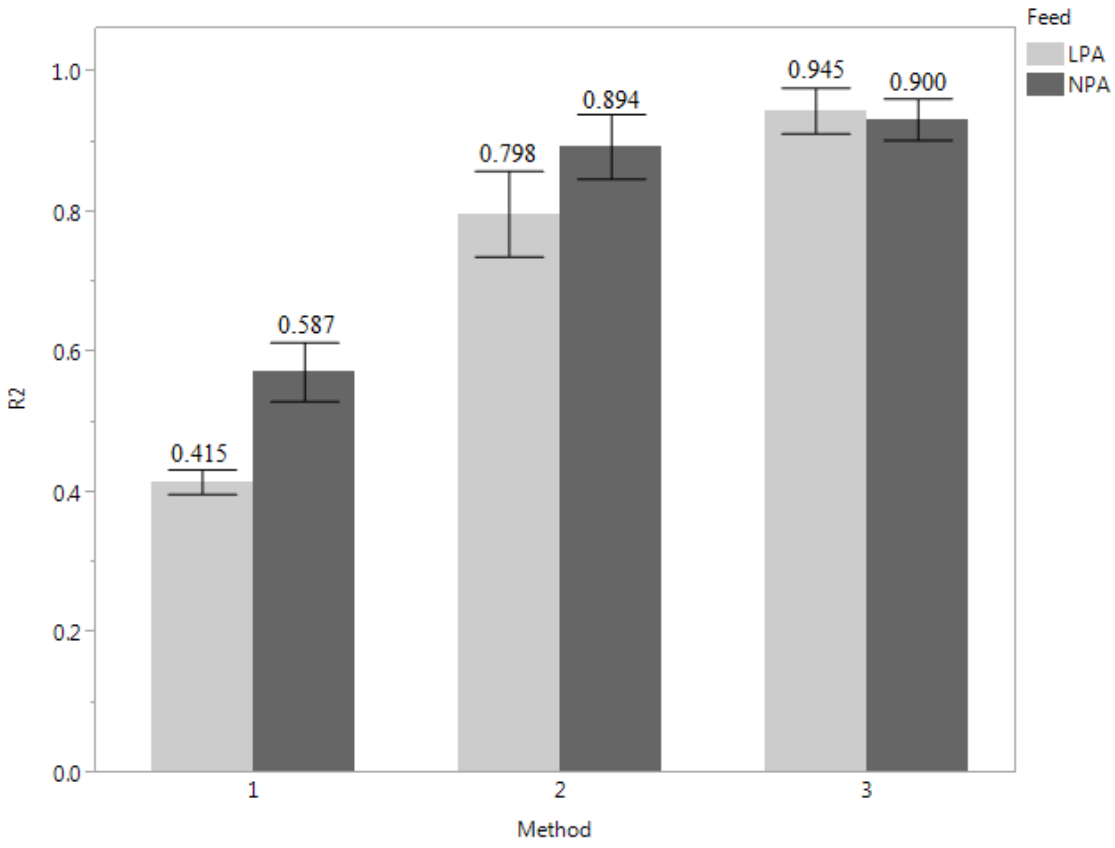
Table 2. Description of the three methods used in this study which differed in population size, aquarium size, length of time, and ortho-P analysis reagent

Method	# of Aquariums	Population Size	Aquarium Size	Time	Ortho-P Reagent
		# of Shrimp	L	weeks	
1	6	5	30	6	Reagent Pillow
2	10	10	50	2	Reagent Pillow, Amino Acid
3	10	5	50	4	Amino Acid

Table 3. Sample size estimates for detecting a significant difference for ortho-P concentration between the two feeds using the standard deviation from each method

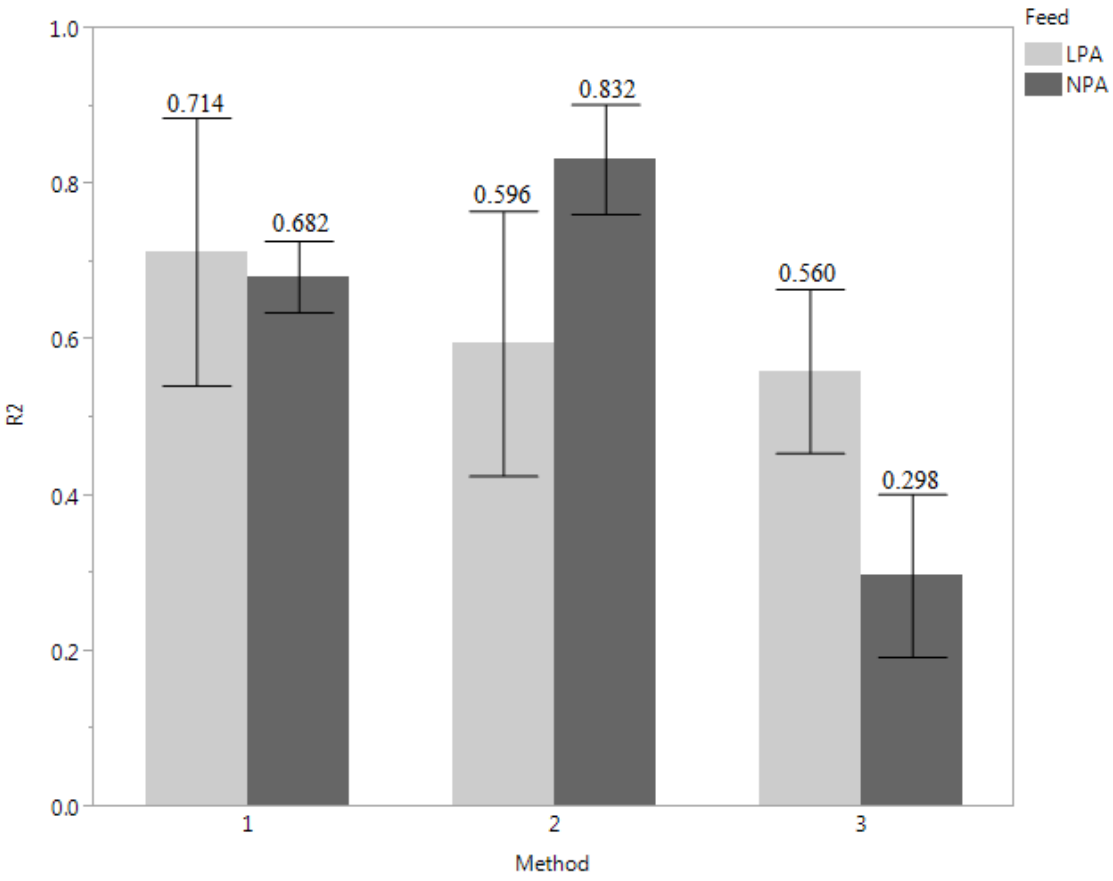
Method	α	Standard Dev.	Power	Predicted Sample Size
1	0.05	0.2309	0.7	22
			0.8	25
			0.9	30
2	0.05	0.0298	0.7	25
			0.8	28
			0.9	33
3	0.04	0.0303	0.7	24
			0.8	27
			0.9	32

Figure 1. Comparison of the R^2 values for regression curves of ortho-P concentration x total amount of feed for both feeds



Each error bar is constructed using 1 standard deviation from the mean.

Figure 2. Comparison of the R^2 values for regression curves of average weight x total amount of feed for both feeds



Each error bar is constructed using 1 standard deviation from the mean.

5. Conclusions

LPA soybean varieties will be a great benefit to producers both of soybeans and animals. Further, they will help to improve and preserve our natural resources, most notably waterways, and the variety of allied industries relying on them including tourism and fishing. This benefit has been shown in a variety of monogastric animals unable to digest PA including swine and poultry but has not been studied on aquacultural animals, the production of which could benefit greatly from a cheaper protein source but is inhibited by the high P content of conventional soymeal. This advancement cannot be fully realized, nevertheless, without out addressing the extraordinarily low field emergence so commonly observed in LPA lines. Previous breeding efforts have uniformly shown that field emergence is not necessarily caused by nor strongly correlated with reduced PA content indicating that developing a LPA soybean variety is not precisely. Regardless, such efforts have not resulted in an LPA variety with consistently high field emergence. Agronomic solutions have not yet been explored though they are used to increase field emergence in conventional soybean varieties.

The LPA phenotype in soybeans has been produced using mutant alleles of three different genes involved at different points in the PA production and sequestration pathways: *MIPS1*, *LPA1*, and *LPA2*. The latter two are derived from the same source while the *MIPS1* mutation was discovered independently. All three mutant alleles have been consistently shown to significantly decrease PA content in soybeans while the *MIPS1* mutation also increases sucrose content while decreasing raffinose and stachyose contents, another desirable trait. Previous studies have examined LPA soybean lines from both sources separately or together with each showing diminished field emergence regardless

of the genetic source of the trait, but no previous study has ever examined the effect on agronomic, quality, and seed composition traits of each allele individually or in combination in a single genetic population.

The research represented in this thesis shows that seed treatments can increase field emergence in soybean lines with genetically reduced PA content. Fungicidal treatments were especially successful in this undertaking. This further suggests that the cause of the observed low field emergence may be increased pathogen pressure which is consistent with electrolyte and P leakage which may be expected from the loss of highly stable PA in the seed. Fungicidal seed treatments are already widely used in the seed industry making this a highly efficient approach, if proved effective. Though the treatments did not affect yield, increased field emergence is an independently important agronomic trait affecting weed control, nutrient management, and soil preservation making these results valuable in their own right.

The research represented in this thesis also provided the first opportunity to compare the three LPA mutant alleles in a genetic population in which any phenotypic differences can be assumed to be due to the individual allele and their interactions because of the interrelatedness of the RILs having been developed from a single biparental cross. These results concur well with previous research that PA content is significantly correlated with field emergence but not strongly enough as to preclude the possibility of LPA soybean varieties with consistently high field emergence. Further, there were differences in the correlations between field emergence and various traits between the different LPA alleles though seed size was the strongest correlated across the board. These traits can be targeted

to develop high emerging LPA soybean varieties in a program specialized to the exact LPA genotype of the lines being used.

Finally, this research developed a protocol designed specifically to test the effect of LPA soymeal based feeds on the environmental quality and growth characteristics of Pacific White Shrimp, an important aquacultural species. This method addresses the rapid increase of ortho-P in aquarium water inherent to soymeal based feeds as well as the error caused in measuring growth in a small population, as is required by limited tank size. This method is also well adapted to limited lab space making it easily applicable. Such a study could be pivotal in opening LPA soybean market to the aquacultural sector which would provide a boost to both industries.

Many possibilities exist to expand upon this research. A larger study of the effect of seed treatments on field emergence on LPA soybeans would be useful in creating a quick and functional solution to the field emergence issues of those lines. Another possibility would be to perform an in depth seed physiology study of LPA soybeans to further identify causes of the decreased field emergence. Such research would provide invaluable insight into the various possible causes allowing for more strategic breeding and agronomic efforts. Finally, a full study of the effect of LPA soymeal based feeds on the water quality and growth habits of Pacific White Shrimp would greatly help to increase waning interest in LPA soybeans allowing for further development and improvement.