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Effects of Minor Elements on Cercospora kikuchii, Cercospora Leaf Blight and Rust on Soybeans

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EFFECTS OF MINOR ELEMENTS ON *CERCOSPORA KIKUCHII*, CERCOSPORA LEAF BLIGHT AND RUST ON SOYBEANS

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Abstract

Soybean (*Glycine max*) is one of the most widely grown crops in the world. Many pathogens attack soybeans, but of particular importance to tropical and subtropical areas such as Louisiana is Cercospora leaf blight (CLB). This disease is caused by the fungus *Cercospora kikuchii* and favored by high temperatures. This fungus utilizes a toxin, cercosporin, as its primary pathogenicity factor. Soybean rust (SBR), caused by *Phakopsora pachyrhizi*, is another common disease that occurs in Louisiana under cooler environmental conditions of spring and fall. Both diseases occur regularly in Louisiana and may result in severe yield losses. *Cercospora kikuchii* has recently developed fungicide resistance, and there are few if any resistant soybean cultivars. For these reasons, a search for new management strategies is underway.

This study analyzed the efficacy of using plant-essential micronutrients applied foliarly to suppress CLB and SBR. This was tested in field experiments on these diseases as they occurred, as well as on isolates of *C. kikuchii* on amended agar media; both in randomized complete block design. Disease severity and yield were measured in field experiments, while fungal growth and toxin production were measured *in vitro* for *C. kikuchii*.

Results showed that copper (150 – 300 g ha^{-1}) and high rates of aluminum (160 – 190 g ha^{-1}) and iron (200 – 400 g ha^{-1}) applied foliarly to soybeans were effective in suppressing CLB severity. Iron; in the forms of iron nitrate and iron sulfate, as well as low rates of aluminum (20 – 100 g ha^{-1}) were effective in reducing SBR severity. Agar media amended with iron (> 250 mg L^{-1}), manganese (> 250 mg L^{-1}) and zinc (> 125 mg L^{-1}) inhibited fungal growth at high physiological concentrations found in soybean leaf tissue. Additions of aluminum (up to 150 mg L^{-1}) and copper (up to 35 mg L^{-1}) resulted in increased fungal growth. Aluminum, manganese, and zinc treatments caused higher toxin production by *C. kikuchii* than unamended controls. Iron at lower concentrations (up to 80 mg L^{-1}) resulted in higher fungal growth rates than unamended controls, but this effect was reversed as concentrations exceeded this value. Conversely, toxin production was enhanced as fungal growth began to decline up to the point of complete fungal inhibition. Finally, some metals applied *in vitro* affected pigmentation of *C. kikuchii*. The normally purple pigmentation of cercosporin shifted to yellow when grown on aluminum, black when grown on iron, and green when grown on zinc-amended agar media. Boron (50 g ha^{-1}) and iron (50 g ha^{-1}) applied as iron sulfate were effective at reducing frogeye leaf spot (*Cercospora sojina*) in the one year the disease occurred. While many treatments were effective in suppressing disease severity in the field and reduced fungal growth in culture, there were no significant effects on soybean yield by treatment for either CLB or SBR.

Future work identifying the mechanisms of disease suppression as well as more pronounced effects from commercial formulations and technologies surrounding minor element nutrition could yield sustainable strategies for managing soybean diseases. Research is planned on the siderophore-like properties of the toxin, cercosporin.

Chapter 1: Introduction

Soybeans (*Glycine max* (L.) Merr.) are dicotyledonous plants that are native to eastern Asia; however, the vast majority of soybean production is now situated in the western hemisphere (Brazil, United States of America (U.S.A.) and Argentina constitute 80% cumulative, listed in descending order) (Production Estimates and Crop Assessment Division, FAS, USDA 2013, Food and Agriculture Organization of the United Nations). This species was cultivated from its wild counterpart, *G. max* subsp. *soja* (Siebold and Zucc.); otherwise known as *G. soja*, approximately 5,000 years ago (Carter 2004). The plant grows either as a shrub or as a vine depending on environmental and genetic conditions. Soybeans are legumes, thus requiring less N fertilization than many crops. Their flowers are inconspicuous, white to purple, and appear along the nodes on stems. Leaves are trifoliolate, consisting of three leaflets with many trichomes. The plant produces one to five seeds in each pod, which will dry as the plant matures. These seeds can then remain viable for up to 4 years (2 years before significant germination rate decreases) if stored in cold, dry conditions, or they can germinate quickly under appropriate conditions (Acasio 2010, Priestley 1986). Soybeans are highly sensitive to day length with regard to reproductive development (Cregan and Hartwig 1984) which has led to many "maturity groups" being developed. These groups range from "00" (grown as far north as Canada) to "8" (grown in the tropics) and are bred to perform at specific latitudes (Zhang et al. 2007). Furthermore, each of these groups can be either determinate or indeterminate in growth habit. Determinate cultivars cease main stem growth shortly after first flowering while indeterminate cultivars continue growing vegetatively indefinitely (Bernard 1927).

Soybeans are an important crop partly because of the wide range of products and uses people have conceived from them. Much of the grain goes directly into food products (Erickson 1995), or the oil is extracted to be used in numerous edible products (Gunstone 2011), such as mayonnaise and margarine. Often much of the protein is used for animal feeds, with the animals often being intended for human consumption (Kerley and Allee 2003). The oil can be further refined into hundreds of industrial uses including caulking compounds, diesel fuel, epoxies, herbicides, inks, metal casting, paints, pesticides, and hundreds more (Cahoon 2003).

Soybeans are the third largest row crop in Louisiana behind sugarcane and various feed crops, bringing in a gross value of $805 million in 2011 on roughly 445,154 hectares (1.1 million acres) (USDA Oil Crops Yearbook 2013). In the United States, soybeans hold second place for most important crop at 29.8 million hectares (73.8 million acres) adding up to $37.6 billion at the farm gate in 2011 (USDA Oil Crops Yearbook 2013). Soybean seeds are used in a wide variety of foods, principally as soybean oil, which accounts for 65% of all edible oil in the U.S.A. and 90% of all oilseed production (Production Estimates and Crop Assessment Division, FAS, USDA 2013). Furthermore, the U.S.A. is the largest exporter of soybeans in the world, and soybean oil is the most used oilseed in the world (FAO, Trade and Markets Division 2013).

While many diseases affect soybeans, the most costly in the southern U.S.A. are arguably Cercospora leaf blight (CLB) and purple seed stain (PSS) caused by *Cerocspora kikuchii*, which reduce both yields and quality of product, respectively (Wilcox and Abney 1973, Roy and Abney 1976). Reduced quality is caused by the purple pigment; a toxin produced by the fungus, that can occur on infected seeds (Kuyama and Tamura 1957). Should the percentage of seeds showing these symptoms exceed a small threshold (1, 2, 5, or 10% depending on grade) (U.S.A. Standards for Soybeans), the price paid for the seed is reduced, or the load is denied altogether. These purple seeds can also serve as primary inoculum should they be replanted (Velicheti and Sinclair 1994).

The fungal genus *Cercospora* contains an exceptionally large number of species (Crous and Braun 2003, Pollack 1987, Chupp 1954) that are economically important as plant pathogens. Almost every major agronomic crop in the U.S.A. has a *Cercospora* pathogen associated with it (Supplemental Table 1). Furthermore, there are possibly thousands of species that infect an extraordinarily large range of hosts (Pollack 1987), many of which are horticultural crops or ornamental plants. The genus is situated in the phylum Ascomycota (order Capnodiales) and is generally believed to consist of facultative pathogens, capable of living saprophytically when a suitable host is not available. Of particular interest is the species *Cercospora kikuchii* (Matsumoto and Tomoyasu 1925), which is a pathogen of soybean and a purported pathogen of other legumes such as common bean (*Phaseolus spp*.), cowpea (*Vigna spp*.), and guar (*Cyamopsis tetragonoloba*)(Mulder and Holliday 1975). While the pathogen was formally named in 1925, the disease was first reported in Korea (Suzuki 1921) and secondarily in the U.S.A. (Gardner 1924). *Cercospora kikuchii* is capable of surviving on soybean debris for at least six months under field conditions (Baird et al. 1997) and up to four years under optimal environmental conditions (Kilpatrick 1956) or on various nutritional media. Several weed species common in the southern U.S.A. (McLean 1988), as well as contaminated soybean debris, can serve as inoculum for subsequent years.

Infections by *C. kikuchii* result in two different disease complexes on soybean: CLB and PSS (Murakishi 1951). These two diseases can occur at the same time or individually (Orth and Schuh 1994), and severity of one is not correlated with severity of the other. Cercospora leaf blight is identified by a bronzing or purpling of upper leaves or any leaves that have high exposure to sunlight (Schuh 1999). These leaves also often take on a very thick, leathery texture. Under dry and/or extremely hot conditions, blighting can occur in which the leaves turn gray to black and take on a consistency closer to ash. Following this, defoliation often occurs, which places the plant under high stress and can result in substantial yield losses (Schuh 1999). Petioles also often develop sunken purple or red blotches on them from the infection and circular red spots that turn black can occur on seed pods in susceptible cultivars (Walters 1980). Purple seed stain is, as its name suggests, a staining of the seed coat. A pink to dark purple blotch will manifest on the seed and results in a mostly aesthetic injury; however, some reports indicate that lower oil content is associated with diseased seeds (Giesler, Nebraska Cooperative Extension,

2013). While the symptoms appear on the coat, it has been reported that mycelium of *C. kikuchii* can penetrate the cotyledonary and embryonic tissues (Ilyas et al. 1975).

Furthermore, seed infection by *C. kikuchii* has been linked to lower linoleic, linolenic, stearic and palmitic acids, lower protein content, lower rates of healthy seedlings, lower seed weights and growth speed; and when grown out, resulted in yield losses ranging from 36-80% (Gupta 2014). Strangely, while many fatty acids decreased, seed production of oleic acid and oils increased. This is generally considered to be beneficial for seeds to have higher oleic acid to linoleic acid ratios (Wilson 2004) because this confers better nutritional and anti-oxidative effects. These same effects are not observed when inoculated with other pathogens such as *Diaporthe phaseolorum* var. *sojae* (Xue 2008) and appear unique to infection by *C. kikuchii* and perhaps cercosporin (an oxidizer) production. One interesting fact to note is that *C. kikuchii* also appears to be able to displace both *Diaporthe* spp. (specifically *phaseolorum* var. *sojae*) and *Alternaria* spp. for space on soybean seeds (Roy and Abney 1977). This same study also found that infection by *C. kikuchii* increased seed germination rates in some cultivars, but this work has yet to be reproduced and most studies do not confirm the results (ex. Wilcox 1973).

Primary inoculum of *C. kikuchii* comes from sowing infected seeds (Imazaki 2006) and from existing structures in the soil or debris (Kilpatrick 1956); although one study found that burying the infested debris reduced inoculum levels (Almeida 1980, 2001). Because *C. kikuchii* is able to live facultatively as a saprophyte, it can survive on soybean debris left over from previous seasons. Once inside the soybean plant, it can live for extended periods of time as an endophyte without causing any symptoms (Chanda 2012, Chanda et al. 2014). Under the right conditions, it begins producing higher levels of the toxin cercosporin, leading to the common visual symptoms of the disease. After blight symptoms are produced, the fungus produces conidia on the resulting necrotic tissue (Schuh 1999). Producing inoculum for the purpose of scientific studies has proven very arduous. Some researchers were never able to get the fungus to sporulate except on soybean tissue (Matsumoto 1928, Murakishi 1951), while others were successful with carrot-leaf decoction agar (Kilpatrick 1956), V-8 agar (El-Gholl 1982), potato dextrose agar (Roy and Abney 1976), and soybean agars (Vathakos and Walters 1979). Many of these studies were not able to confirm the results of the others; hence leading to all of these media being tested, but one study had success with all of the above listed media (Yeh and Sinclair 1980).

Currently, control of this disease complex is achieved through early planting (Moore 2009), use of clean seed (Sinclair 1993), and fungicide sprays (Walters 1980, Chanda et al. 2014,). Early planting avoids the hot, dry weather of late summer that can exacerbate CLB symptoms. There are few resistant cultivars of soybean to the pathogen, and current research indicates that many *Cercospora* species, including *C. kikuchii*, are developing resistance to commonly used fungicides, (Imazaki 2006, Zhang 2012, Price 2013).

Infection begins with the conidium producing a haustorium to enter the leaf cells (Orth and Schuh 1992). After this, the primary means of pathogenesis of *C. kikuchii* is a toxin aptly named cercosporin (Kuyama and Tamura 1957). *Cercospora kikuchii* requires light to produce the toxin, and cercosporin also requires light to cause symptoms (Yamazaki et al. 1975, Hartman et al. 1988). This is because of the toxin's ability to produce reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$) and superoxide (O_2^-), which in turn destroy cellular components such as the lipid bilayer and causes cell death (Daub et al. 1982-1, 2005, 2013). *Cercospora* species appear to have a mechanism through which they inhibit the toxin's effects when inside the mycelial cells, and this process is related to proteins encoded by the *crg* (Chung 1999, 2003-1) and *sor* (Ehrenshaft 1999) genes. Another method of self-resistance appears to be keeping cercosporin in a chemically reduced and inactive state while inside the mycelium (Daub et al. 2000). Cercosporin resistance in non-*Cercospora* organisms seems to be based on reductases that degrade cercosporin such as those encoded by *cpd* and *snq* in *Saccharomyces* spp. (Ververidis 2001) and unnamed genes in *Xanthomonas* spp. (Taylor 2006). One study by Daub (1987) found that fungal resistance to cercosporin may be more related to general fungal physiology than any specific resistance, but in separate studies they found that some fungi do reduce and inactivate cercosporin (Daub et al. 1992, Leisman and Daub 1992). Cercosporin can be easily visualized as red, orange, or purple pigments in media and as a purple discoloration in plant tissues and seeds. While not all *Cercospora* species produce cercosporin or require it for pathogenicity, it appears that toxin production and export from mycelial cells is required by *C. kikuchii* to cause infection (Callahan et al. 1999). And while not all *Cercospora* species produce cercosporin, roughly half appear to do so (Assante 1977), and the toxin is required for their pathogenicity (Lynch and Geoghegan 1977, Upchurch et al. 1991). Other species of *Cercospora* have been shown to produce various other toxic compounds, such as the cebetins (alternatively known as beticolins) produced by *C. beticola* (Prange 1995), dothistromins (Stoessl 1984), averufins (Stroessl 1985), and unknown toxins (Fore et al. 1988) produced by *C. arachidicola*. One report claimed that an isolate of *C. kikuchii* produced cebetins (Robeson and Jalal 1993); however this has yet to be corroborated.

Another common pathogen of soybean is *Cercospora sojina* (Hara 1918), which causes frogeye leaf spot. *Cercospora sojina* does not produce cercosporin, unlike *C. kikuchii*, and instead causes a spot on the leaf that resembles a frog's eye. This is described as a tan blotch surrounded by a dark brown ring. These can be irregular or circular shaped. Infection and symptoms are favored by hot, humid conditions; often present in mid to late summer in the southern U.S.A. (Akem 1992, Phillips 1999). Inoculum can survive on debris for up to 2 years and burying the debris seems to have no effect on reducing inoculum (Zhang and Bradley 2014). This disease is not as rampant or as destructive as CLB and PSS; however, it can be severe under certain conditions and has also developed resistance to some fungicides (Zhang et al. 2012). Frogeye leaf spot also is becoming more problematic in northern parts of the U.S.A. (Mengistu 2002) and even in parts of Canada (Wrather et al. 2006).

One of the newest diseases to emerge on soybeans in the U.S.A. is soybean rust (SBR) (Schneider et al. 2005). The causal organism, *Phakopsora pachyrhizi* (Sydow and Sydow 1914), was previously described in parts of Asia; it then spread to Africa (Akinsanmi et al. 2001) and South America (Yorinori et al. 2005), and in 2004 it first appeared in the southern U.S.A. Spores are believed to have been disseminated with hurricanes tracking from South America before making landfall in North America. The disease may be extremely severe being capable of causing yield losses above 50% and, in extreme cases, total crop failure (Yang et al. 1991, Bonde et al. 1976).

Like all rust pathogens, *P. pachyrhizi* is an obligate parasite, unable to complete its life cycle naturally without a compatible host. *Phakopsora pachyrhizi* produces both urediniospores and teliospores within pustules, the former of which are much more prevalent (Posada-Buitrago and Frederick 2005). These pustules can appear to the naked eye as red, brown, or orange spots about the size of a pin head on undersides of leaves. The pustules also can easily be mistaken for the bacterial pustule disease (*Xanthomonas axonopodis* pv. *glycines*) but can be differentiated with a magnifying lens, as bacterial pustules do not contain conidia. The urediniospores appear as small tan ovate objects, and there are many inside each pustule. Severely infected leaves take on a bright yellow hue and will often drop from the plant within a short time (Godoy et al. 2006). Yield loss from this disease stems from fewer pods with less weight per seed (Bromfield 1984).

The pathogen cannot survive in the absence of a suitable living host, one of which is the common vine kudzu (*Pueraria lobata*). If the Gulf South experiences extended periods of freezing temperatures, kudzu shoots may be killed thus depriving the pathogen of an overwintering host (Park et al. 2008). This prevents the pathogen from moving farther north in the U.S.A. and requires that inoculum must be brought in each year from areas where the pathogen survived, probably Mexico and the Caribbean (Isard et al. 2005).

Unlike *Cercospora* species, infection by *P. pachyrhizi* is favored by cooler temperatures of spring and early summer or occasionally in the autumn months when temperatures once again decline into the mid 20's °C range (Kochman 1979). This can be explained as the optimal temperatures for *P. pachyrhizi* germination ranges from 8 – 33°C (Keogh 1974) and infection is favored by 26 – 29°C (Levy, C. 2005), which have remained consistent over 25 years (Bonde et al. 2007). One study by Pivonia and Yang in 2004 found that conidia could only survive a month at 9°C and less than a week at 5°C. A major factor in the severity of this disease is the rapidity with which epidemic levels can occur. Under favorable conditions and lax disease management, a few infected plants can erupt to epidemic conditions in as little as 2 weeks (Yorinori 2005). Currently, control of SBR is achieved through fungicide sprays of strobilurins and triazoles (Yorinori 2005).

Plants require 17 nutrients that are considered to be essential (Arnon and Stout 1939, Epstein 1994). To be classified as an essential nutrient, three conditions outlined by Arnon and Stout in 1939 must be met: 1) A deficiency of it makes it impossible for the plant to complete the

vegetative or reproductive stage of its life cycle; 2) such deficiency is specific to the element in question and can be prevented or corrected only by supplying this element; and 3) the element is directly involved in the nutrition of the plant. There are numerous ways to categorize these elements (function, mobility, etc.), but for this study they are categorized based upon the amount required by plants.

There are six primary nutrients in this classification. Carbon (C), hydrogen (H), and oxygen (O) are readily derived from the atmosphere (oxygen and carbon dioxide) and water and are thus referred to as the non-mineral primary nutrients. These three make up the bulk of plant mass. Nitrogen (N), phosphorus (P), and potassium (K) are considered the primary mineral nutrients because they are taken up from the soil and are required in relatively high amounts, and their role in and requirement by plants has been exhaustively studied (Xu et al. 2012, Plaxton and Lambers 2015, Wang and Wu 2013, respectively). Furthermore, these three elements are the ones most abundantly applied as fertilizers. As a legume, soybeans require much less N input as they can form symbioses with nitrogen-fixing bacteria (Wacek and Brill 1976). The secondary elements required by plants consist of calcium (Ca), magnesium (Mg), and sulfur (S). These are more commonly derived from fertilizers and organic matter, and are often not limiting. Micronutrients are those that are required in smaller amounts than those previously listed. They are most often available in soil in sufficient concentrations, but fertilizers are sometimes necessary. These minerals consist of boron (B), chlorine (Cl), copper (Cu), iron (Fe), molybdenum (Mo), manganese (Mn), nickel (Ni), and zinc (Zn) (Lakanen and Ervio 1971, Barker and Pillbeam 2006). Additionally, there are some elements classified as beneficial, being required only by some plants, or their application may have positive effects on plants. Examples of these include aluminum (Al), cobalt (Co), selenium (Se), silicon (Si), sodium (Na), and vanadium (V) (Barker and Pillbeam 2006). For example, Co is required by legumes for N fixation to occur in roots (Lowe et al. 1962, Blaylock et al. 1986), and Si has been shown to strengthen cell structure in grass species and aid in plant defense (Ma and Yamaji 2006, Fauteux et al. 2005, respectively). Silicon also has been shown to reduce disease severity in some systems, such as brown spot of rice (Datnoff et al. 1992, Prabhu et al. 2012).

Micronutrient deficiencies are becoming more common in agricultural systems, especially in the U.S.A. because of two primary occurrences. The first is the breeding of high-yield crops, which grow rapidly and produce more biomass than previous cultivars, and strip the soil of micronutrients (Fageria et al. 2002). Coupled with the higher demand of these crops is the increasing analysis and purity of fertilizers (Martens and Westermann 1991). In the past, fertilizers had many impurities in them, and these impurities often included many of the micronutrients. As the purity of these fertilizers increases, fewer micronutrients are applied in tandem with the primary fertilizer elements (usually N, P, and/or K), which are the major elements farmers apply. These two factors result in plants that grow faster and yield much more than would occur naturally all while receiving less of the micronutrients required for such levels

of production. Long-term cropping also has a tendency to remove micronutrients from soil, as they are not replaced when the crop is harvested or high purity fertilizers are applied.

Micronutrients are mostly immobile within the plant, with the exceptions being chlorine and molybdenum (Barker and Pillbeam 2006). In soil, chlorine, boron, and molybdenum are mobile, while the rest are immobile. Uptake from the soil solution is favored generally by a lower pH, in the 5 to 6.5 range, with the exception being molybdenum, which is taken up more readily at pH greater than 7 (Lucas and Davis 1961).

While not micronutrients, Ca (Elmer and Datnoff 2014) and magnesium (Jones and Huber 2007) have been shown to reduce disease, although magnesium also has the potential to exacerbate many plant diseases as well. The primary role of magnesium in plant defense appears to be increasing tissue resistance to breakdown by enzymes (Jones and Huber 2007). Calcium applied to foliage as a salt can be directly toxic to plant pathogens (Rahman and Punja 2007). Calcium is also a major constituent of plant cell walls, making them more resistant to maceration (Conway et al. 1991) and is important in plant defense signal transduction (Ishihara et al. 1996).

Iron is taken up by plants in the form Fe^{2+} (preferred) or Fe^{3+}. Plants utilize two strategies to acquire Fe from the soil solution (Brown 1978). Aptly named Strategy I and Strategy II, the first is utilized by dicots and non-grass monocots, while the second is primarily by grasses. Strategy I involves acidifying the rhizosphere via pumping H^+ out of root cells by ATP hydrolysis (Jeong 2009). This aids in releasing Fe bound to soil colloids. A chelator produced by plants binds to Fe^{3+} via nicotinamide adenine dinucleotide phosphate (NADPH) oxidation to $NADP^+$ to form Fe^{2+}. High-affinity Fe^{2+} transporters in the cellular membrane then take up Fe. This is the method soybeans use for Fe acquisition. In Strategy II, phytosiderophores are produced from methionine, and a transporter that is induced by Fe deficiency excretes them. Once in the rhizosphere, they bind to Fe^{3+}, and the complex is taken up by a transporter inside the plasma membrane. Once in the cytoplasm, the siderophore releases the Fe, and it is excreted again for further Fe acquisition.

Iron is not mobile in the plant as is evidenced by the symptomatology of deficiency. Younger leaves show interveinal chlorosis, or total chlorosis in severe cases, and growth can cease entirely if prolonged (Koenig and Juhns 2010). Within the plant, Fe is bound mostly in the chloroplasts (80%) of actively growing leaves (Terry and Low 1982). Under heavy Fe deficiency, stromal Fe concentrations decrease, while lamellar concentrations increase. The roles of Fe in the plant are diverse and very important. They range from being a cofactor in such cycles as nitrogen fixation (Tang et al. 1990) to being a constituent of heme proteins such as nitrate and nitrite reductases (Smith 1984). Iron also plays a large role in photosynthesis, as it is a component of the cytochrome complex (Kurisu et al. 2003) and excess Fe can result in increased lignification of plant cells (Fang and Kao 2000). Furthermore, Fe is important in oxygen quenching as a possible component of super oxide dismutases (SODs) and is involved in intra- and inter-cellular defense pathways (Greenshields et al. 2007). It reduces reactive oxygen

damage in wheat through quenching and turning off pathogen linked pathogenicity-siderophore genes (Greenshields 2007). Plants can also utilize Fe ions for oxidative bursts (usually through H_2O_2 production) in similar ways that *Cercospora* species utilize oxidative species (Wojtaszek 1997). This oxidative burst can be lethal to invading pathogens, help illicit a hypersensitive response within plant tissues (Doke 1983), or signal plant defense pathways (Torres et al. 2006). While Fe uptake is normally via the roots, it can be taken up via mesophyll cells but at a much reduced rate (Bruggerman et al. 1993).

Zinc is taken up by plants almost exclusively as Zn^{2+}, but under high pH it can be taken up as $ZnOH^+$ (Reid et al. 1996, Tiller et al. 1972). Zinc is taken up via similar H^+ pumping and chelation as Fe, but unlike Fe, uptake of Zn is expedited by mycorrhizae (Faber et al. 1990). It has been demonstrated that Zn is readily taken up foliarly (Haslett et al. 2001, Bowen 1969) by plants, including soybeans (Rose et al. 1981), which leads to growth and yield increases. Zinc plays important roles as a constituent of many enzymes such as carbonic anhydrase (Randall and Bouma 1973), an activator or catalyzer of enzymes such as pyrophosphatases (Zyryanov et al. 2004), as well as increasing protein content (Johnson and Simons 1979). Zinc can also bind to phospholipids and sulfhydryl groups of membranes to protect from oxidative damage (Powell 2000) and bind to copper with SOD to scavenge oxygen radicals (Alscher et al. 2002) such as those produced by electron transport or cercosporin (Williamson 1992). Zinc is highly accumulated in meristems of plants, where it is needed for rapid growth (Kitagishi and Obata 1986). Zinc deficiency presents itself as chlorosis of newer leaves (Viets et al. 1954), as Zn is not mobile in the plant. Broad bands are commonly visible on leaves, and deformed and stunted leaves are not rare; depending on the plant a variety of deficiency symptoms can occur such as purpling, chlorosis, or bleaching (Duffy 2007). Zinc application has been shown to reduce severity of a wide range of plant pathogens (Duffy 2007) and can interfere with toxin production in some, such as *Aspergillus* spp. (Cuero et al. 2003).

Copper is taken up by plants as Cu^{2+}, but it is readily bound in the soil as Cu^+ (Song et al. 2004). It can be absorbed by leaves (Bowen 1969), but it appears to be competitive with Zn for foliar uptake. In plants, Cu functions mostly in proteins, the most important of which are arguably plastocyanin of the electron transport chain and cytochrome c oxidase in mitochondria (Yamasaki et al. 2007). Copper plays an important role in cell wall lignification (Chaoui and El Ferjani 2005, Lin et al. 2005, Fang and Kao 2000), the deficiency of which could lead to easier infection by pathogens. Copper deficiency usually manifests as a paling necrosis from the newer leaf tips of the plant (Marschner 1995). Rarely, yellowing and paling of lower and older leaves (Hill et al. 1978) may be attributed to Cu deficiency, but this is more common in regularly senescing leaves as the plant draws nutrients out before abscising the leaf. Deficiencies also often appear in fruit or seeds as pale or damaged product.

Boron is primarily taken up as boric acid (H_3BO_3) and to a much lesser extent, $B(OH)_4^-$ (Hu and Brown 1997). Inside the plant, B participates in sugar translocation (Gauch and Dugger 1953), protein formation (Blevins and Lukaszewski 1998), lignification (Dutta and McIlrath

1964), cell integrity (Cakmak et al 1995), and pollen germination (Wang et al. 2002). Foliar applications of B decreased disease severity in some systems (Simoglou 2006), and the absence of B was associated with marked increases in diseases in other systems (Eaton 1930).

Molybdenum is taken up in the form MoO_4^{2-} by a specific transporter (MOT1) and a low affinity transporter (MOT2) (Tomatsu et al. 2007, Gasber et al. 2011, respectively), but cannot be used as such and is bound into a cofactor dubbed MoCo (Bittner 2014). Within the plant, Mo is essential for nitrogenase and nitrate reductases (Campbell 2001), the latter of which appears to function in protection against ROS, in pathogen resistance and phytoalexin accumulation (Rockel et al. 2002). Molybdenum also is needed for abscisic acid (ABA) synthesis (Nambara and Marion-Poll 2005). It is one of the scarcest nutrients in plants (Kaiser et al. 2005) and its metabolism is tightly linked with iron metabolism; an increase in one results in an increase in the other (Berry and Reisenauer 1967, Kannan and Ramani 1978, Baxter 2009). Molybdenum also functions in nitrogen fixation by the bacteria in legume nodules (Kneip et al. 2007).

Manganese is taken up as Mn^{2+}, and unlike many nutrients, mycorrhizae actually decrease the uptake of Mn because of a lack of uptake systems in mycelia and an alteration in microbial fauna around roots that leads to Mn oxidation (Lambert and Weidensaul 1991, Nogueira et al. 2007). Like Cu and Zn, Mn can be absorbed by leaves at reduced rates (Bowen 1969) with a unique carrier. Manganese plays a key role in Photosystem II, specifically the water-splitting complex (Watkinson et al. 1994) and also in the citric acid cycle as a requirement of NAD^+ malic enzyme (Marschner 1995) and isocitrate dehydrogenase (Coultate and Dennis 1969). Manganese is a common component of SOD (Alscher et al. 2002), is required by phosphoenolpyruvate (PEP) carboxykinase (Burnell 1988), and various peroxidases (involved in lignin biosynthesis) (Brown et al. 1984), among other enzymes. Manganese nutrition was shown to alleviate some diseases such as take-all of wheat (Rengel et al. 1993).

Aluminum exists in many states in the soil and is more available below pH 5.5. Some plants, such as *Camellia sinensis*, activate antioxidant defense when subjected to Al treatments (Hajiboland et al. 2013). Other beneficial effects reported consist of Fe toxicity prevention (Hajiboland et al. 2013) and pathogen deterrent (Poschenrieder et al. 2005). Most all work on plant pathogens and Al have looked exclusively at soil amendments and their effect on pH (Shew et al. 2007). Cobalt is essential for growth of rhizobia (Lowe et al. 1962) and is a component of vitamin B12, which is required for enzymes in N-fixing bacteria (ex. methionine synthase) (Evans and Kliewer 1964). As a result, Co deficiency can cause N deficiency in legumes. Cobalt is hyper-accumulated in certain members of Asteraceae, Fabaceae (includes soybeans), Lamiaceae, and Scrophulariaceae (Brooks et al. 1980). Cobalt also inhibits leaf senescence by inhibiting ethylene production (Gepstein and Thimann 1981).

In relating these elements to pathogen attack and infection, many play pivotal roles. Copper, Mn, and B bolster lignification, making cell walls more impermeable to attack and invasion (Marschner 1995, Brown et al. 1984). Iron and Zn play roles in radical oxygen

quenching. Copper, Fe, Mn, Ni, and Zn, can all serve as cofactors in SODs. Copper and Zn are more prevalent cofactors in eukaryotes while Fe, Mn, and Ni, occur more commonly in prokaryotes; however Cu, Fe, Mn, and Zn have been found in plant SODs (Alscher et al. 2002), and the cofactor required depends on the type of SOD and location. In plant cell peroxisomes, the cytosol, and chloroplasts, Cu/Zn SODs are prevalent. Manganese SODs exist in peroxisomes and are the only type in mitochondria. Iron SODs only exist in the chloroplast. Superoxide dismutase in plants protects cellular components from ROS produced by a wide array of reactions including regular plant metabolism (specifically electron transport), pathogen attack, drought, high light, and nutrient deficiencies (Bowler et al. 1994). Plants also utilize ROS when under attack by producing an oxidative burst, wherein a large amount of ROS are produced in a localized area, which is a detriment to the pathogen (Lamb and Dixon 1997).

It has been reported in the literature that many environmental signals, such as temperature (Jenns et al. 1989), nutritional status (You et al. 2008, Daub and Ehrenshaft 2000), light (Ehrenshaft and Upchurch 1991), and soybean seed proteins (Ehrenshaft and Upchurch 1993), among others can affect cercosporin production by *Cercospora* species. Of special interest are the reports pertaining to elements and *C. kikuchii in vitro*. Studies done by Yamazaki and Ogawa (1972) described a colorimetric change in cercosporin when mixed with various other chemicals. The normally red coloration shifted to dark red with $FeCl_3$ and green with $Mg(OAc)_2$. Furthermore, Cavallini et al. (1979) reported that cercosporin is inhibited in lipoperoxidation when it chelates iron. In the same study, it was found that toxicity of cercosporin to mammalian cells was severely reduced when bound to iron. In a study by Lynch and Geoghegan (1979), numerous trace elements were tested for their effects on growth and cercosporin production of *C. beticola*. They found that only Cu ($CuSO_4 \cdot 5H_2O$) and iron ($FeSO_4$ and $FeCl_3$) increased cercosporin production, while none affected growth habit. However, all of these effects were demonstrated *in vitro*, while the primary purpose of the present study was to determine their effectiveness under commercial agronomic conditions. Furthermore, the concentrations of elements used in these previous studies did not approach what has been reported *in planta*. Lastly, many inorganic salts were recently shown to have ameliorative effects in relation to fungal disease, as summarized in Deliopoulos et al. (2010). Since most micronutrients exist as inorganic salts, this information is highly relevant.

In summary, the following rationales and lines of evidence lead us to pursue the experiments outlined in this thesis:

1. There are no effective and efficient management practices with regard to CLB and PSS caused by *C. kikuchii*, and the fungus continues to develop resistance to the fungicides that are used.
2. Minor element nutrition has been shown to exacerbate disease when lacking and in some cases inhibit disease when supplemented, whether through specific interactions or general plant health, vigor, and defense.

3. Many previous reports list beneficial effects of inorganic salts on a wide array of fungal diseases. Many of the elements noted for their ability to alter cercosporin production are most easily employed in an inorganic salt complex.
4. A review of the literature suggests many ways that cercosporin production and binding habit can be affected by nutritional and elemental status *in vitro*. This study set out to confirm these results for *C. kikuchii* and to answer whether these could be exploited in a field scenario.
5. Because of the opposing optimal conditions for CLB and SBR development, at least one of the two diseases usually presents itself in a given year. Thus the opportunity was taken to observe effects on rust as well.

Objectives:

1. Measure disease severity when minor elements are applied foliarly to soybeans and compare these results with untreated controls under field conditions.
2. Confirm *in vitro* results of previous studies and inferences in relation to minor element presence and cercosporin production.

Chapter 2: Effects of Foliar Applications of Minor Elements on Soybean Diseases

2.1. Materials and Methods of Field Experiments

For the purposes of this chapter, times of application and rating will be described partially by the growth stage of the plant (Fehr et al. 1971). These consisted of only the reproductive stages and are defined in Table 2.1. Field experiments were conducted in the summers of 2011, 2012, 2013, and 2014. In each year, elements were applied as foliar sprays to soybeans, and their effects on disease severity were recorded. Treatments varied slightly from year to year as methods were refined and new information was gleaned (Tables 2.2 – 2.5). Chemicals were mixed in 3 L plastic bottles using deionized water, 2.5 ml L^{-1} Top Surf nonionic 80/20 surfactant (Winfield Solutions, LLC), and the compounds to be tested. Controls consisted of water (and surfactant when used) and in some cases unsprayed blank plots as well.

Commercial soybean cultivars were planted late in the season as described below to increase our chances of obtaining Cercospora leaf blight (CLB). All field experiments were conducted in a randomized block design and planted on 76.2 cm (30") centers. For all experiments alleyways were cut into the fields within and across rows between plots to allow for easy access and observation.

Plots were four rows wide and were trimmed to various lengths (described below) prior to harvest to eliminate edge effects. Plants were planted on flat rows and sprayed with either Baythroid (cyfluthrin, Bayer CropScience) or Orthene (acephate, AMVAC) for stinkbug control and Intrepid (methoxyfenozide, DOW) for lepidoptera control as recommended by the LSU Ag Center (Control Soybean Insect Pests 2011, Willrich et al. 2002). All soybean cultivars used were 'Roundup Ready,' and fields were sprayed with Roundup (glyphosate) as weed pressure dictated according to the manufacturer's label. All formulations and treatments were applied foliarly with a 10–boom plot sprayer (R&D Sprayers, Opelousas, LA. nozzle type TeeJet 8002). All treatments detailed in the following experiments were applied in water at 187 L ha^{-1} (20 US gallons A^{-1}). Finally, all measurements, including yield, were done such that only the middle two rows of each plot were rated and harvested. This was done to reduce the variability of spray near the outside rows of each plot and to reduce border effects. Moisture content of the harvested grain was measured, and yield data were converted to 13% moisture content.

During the summer of 2011, soybean cultivar "Croplan 5007" was planted June 30th at the LSU Ag Center's Central Research Station in Baton Rouge, LA. Plots were trimmed so that plot length was 12.2 m (40 ft). Formulations of minor elements were applied to the foliage on September 30th at the R3 growth stage, with four replications per treatment (Table 2.2). Cercospora leaf blight disease severity was then rated on October 18th. The rating scale was based on the percentage of petioles that showed the characteristic CLB symptoms as depicted in Fig. 2.1.

Table 2.1: Soybean growth stages as used in the present study.

Growth Stage	Description
VE	Emergence (cotyledons through soil surface)
VC	Unrolled unifoliolate leaves
V(n)	(n) sets of unfolded trifoliate leaves
R1	First flower anywhere on plant
R2	Flowers in upper (youngest) 2 nodes
R3	4.7mm (3/16") pods in upper 4 nodes
R4	19mm (3/4") pods in upper 4 nodes
R5	Visible seeds in pods of upper 4 nodes
R6	Beans touching inside pods of upper 4 nodes
R7	Pod mature in color anywhere on plant
R8	50% pods mature in color and containing mature seed

During the summer of 2012, soybean cultivar "Pioneer 95Y20" was planted July 18th at the LSU AgCenter's Central Research Station in Baton Rouge, LA. Plots were trimmed to 6.1 m (20 ft) lengths by 4 rows wide. The treatments were sprayed onto the foliage August 27th (R2) and October 8th (R6), with four replications per treatment (Table 2.3). All treatments including controls were mixed with Top Surf (Winfield Solutions, LLC) nonionic surfactant at 0.25%. Disease severity was then rated on October 10th (late R6) for soybean rust (SBR). The rating scale was published by Bayer Crop Science and is shown in Fig. 2.2. Rating was done such that severity was recorded for leaves in the upper canopy. Yield also was recorded for this experiment as well as seed weight per 100 seeds.

Three separate experiments were conducted during the summer of 2013 in order to increase our chances of obtaining CLB development in our plots, one of which was successful. The first experiment was planted on April 26th at the LSU AgCenter's Central Research Station in Baton Rouge, LA. The cultivar was "Progeny RR4710." The plots were 10.66 m (35 ft) long by 4 rows wide on 76.2 cm (30 in) centers. Each treatment had four replications (Table 2.4). This experiment was sprayed on June 27th (R3) and on August 1st (R5). The plots were then rated for CLB (Fig. 2.1) on August 21st while the beans were at the R6 growth stage. Plots were harvested on October 8th, and the yield and moisture percentage were recorded.

The second experiment was planted on June 20th at the LSU AgCenter's Central Research Station in Baton Rouge, LA. The cultivar was "Asgrow 5322." Plots were 6.1 m (20 ft) long by 4 rows wide planted on 76.2 cm (30 in) centers with three replications per treatment (Table 2.4). They were sprayed on August 7th (R3) and September 6th (R5) and were rated on October 1st (R6) for SBR. The same rating scale used in 2012 was used for this experiment (Fig. 2.2). Plots were harvested on November 15th, and the yield and moisture percentage were recorded.

Soil tests (taken from the Central Research Station, Baton Rouge) were conducted during the 2013 growing season by the LSU AgCenter Soil Testing and Plant Analysis Lab (STPAL) to ensure that there were no nutrient deficiencies. Neither deficiency nor toxicity symptoms have

been observed in the past at this field. The results of those analyses are given in Supplemental Table 2.

During the summer of 2014, soybean cultivar "Pioneer 95Y61" was planted on July 3rd at the LSU AgCenter's Central Research Station in Baton Rouge, LA. There were four replications for each treatment, which were applied on September 29th when the plants were at early R6 growth stage. Plots were rated for frogeye symptoms on October 10th. Frogeye symptoms were so severe that the rust rating scale was used for estimating disease severity (Fig. 2.2).

The initial purpose of this study was to document the effects of nutritional treatments on CLB on soybean. However, because of the unpredictable nature of field experiments and the occurrence of one or more diseases each year, ratings were made for all major diseases that occurred in the field plots. As such, results are presented for ratings and relevant data for CLB, SBR, and frogeye leaf spot. Minitab version 14 was used for all statistical analyses. For disease ratings, outliers (> ±2 standard deviations) were removed (Wilcox 2001) except in the 2013 rust experiments due to having only 3 replications, and one-way ANOVAs were performed to determine if statistical significance ($P = 0.05$) existed. Dunnett's test was used to compare treaments to nontreated controls. Dunnett's test (Dunnett and Tamhane 1992) functions as multiple t-tests between treatments and the control, and better controls for both error and outliers than comparative tests designed to compare all treatments. Where least significant differences are given, the values were calculated using Fisher's Least Significant Differences Test.

Cercospora leaf blight petiole symptoms are defined by sunken purplish-brown lesions that appear on the petiole and ranged from 0 – 40% (Fig. 2.1). Soybean rust symptoms are defined as orange to tan lesions approximately 1-2 mm in diameter that appear on the upper leaf surface, and in severe cases (ranking 7-8) a yellowing of leaf tissue (Fig. 2.2). These symptoms ranged from 0 – 100%.

2.2. Results of Field Experiments

For purposes of this section, CLB will is referred to as Cercospora petiole blight to stress that the petioles were the part of the plant used in assessing and quantifying disease. In 2011, foliar applications of Al (114 g ha^{-1}), Fe (117 ml ha^{-1}), and Mo (63 g ha^{-1}) suppressed CLB severity compared to the experimental controls (Fig. 2.3). However, Mg (110 g ha^{-1}), the high rate of Fe (234ml ha^{-1}), Mn (80 and 161 ml ha^{-1}), and the Zn-Mn combination (Zn: 149, Mn: 79 ml ha^{-1}) enhanced disease severity relative to the controls (Fig. 2.3). The remaining elements had no effects on disease severity at $P = 0.07$. These results were not repeated with identical rates in subsequent experiments as new rates based on these results were devised (Tables 2.3 –2.5); however, similar rates were used in conjunction with CLB and these are given in Table 2.4 and the results shown in Fig. 2.7.

Figure 2.1: Cercospora leaf blight rating scale based on the percentage of the petiole (10 - 40% coverage) showing symptoms.

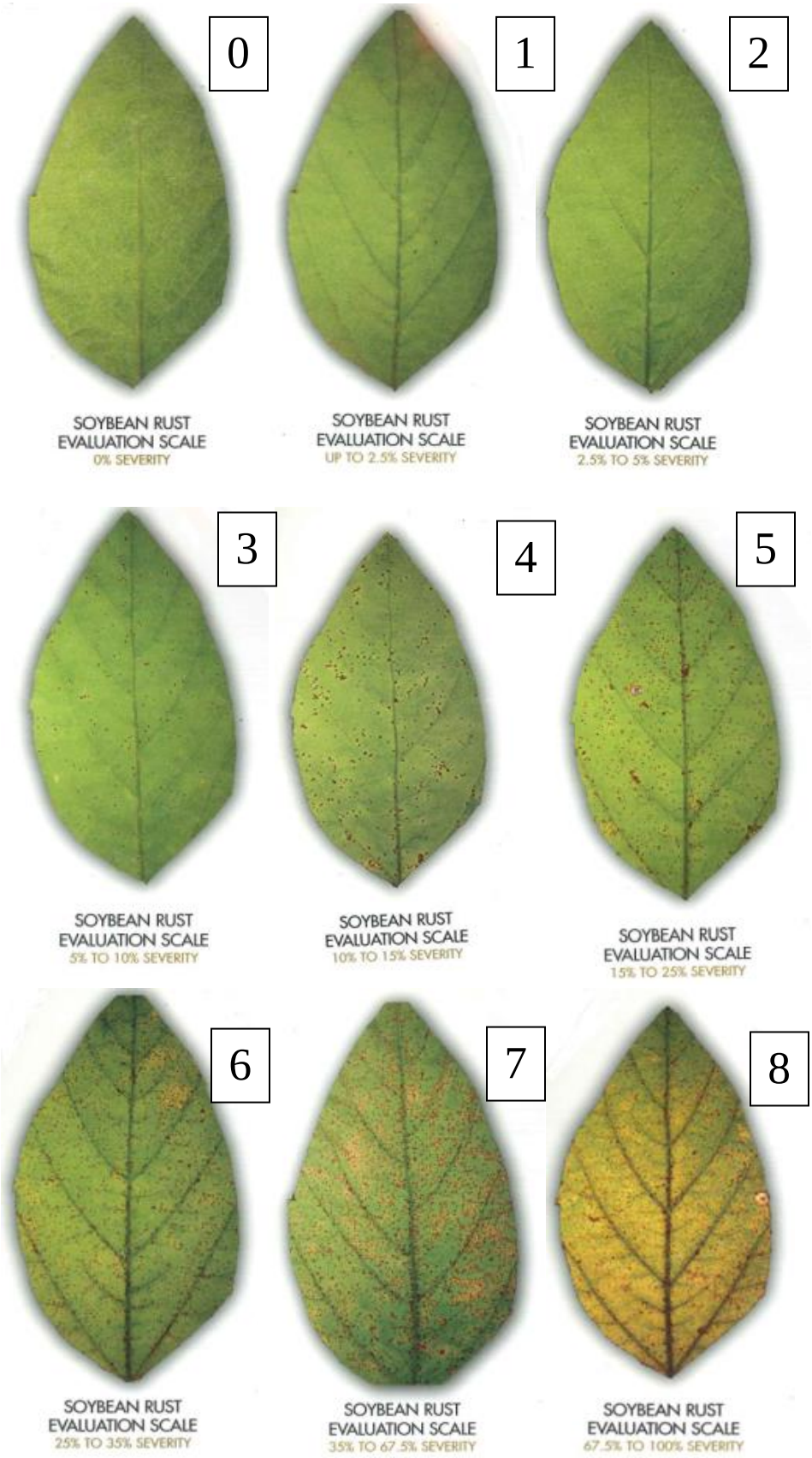


Figure 2.2: Soybean rust rating scale published by Bayer Crop Science.

Table 2.2: All treatments used in field experiments for the 2011 growing season at Central Research Station. Numbers listed indicate whether a solid or liquid form were mixed with water and are designated in either grams or milliliters accordingly. No pH adjustments were made except for aluminum sulfate, which was raised with 1N NaOH to prevent leaf burning. AI = active ingredient in the compound. All 'Max' formulations acquired from NutriAg Ltd.

2011 Field Treatments					
Compound	**%AI**	**AI (ml ha^{-1} or g ha^{-1})**	**Compound (ml ha^{-1} or g ha^{-1})**	**Compound (ml L^{-1} or g L^{-1})**	**pH**
$AlK(SO_4)_2 \cdot 12H_2O$	5.6 (Al)	114 g	2041 g	10.9 g	4.4
BoronMax	8.1 (B)	59 ml	731 ml	3.9 ml	8.7
		118 ml	1461 ml	7.8 ml	8.7
$CaSO_4 \cdot 2H_2O$	23 (Ca)	157 g	685 g	3.6 g	6.4
		315 g	1371 g	7.3 g	6.9
CopperMax	4.2 (Cu)	31 ml	731 ml	3.9 ml	9.2
		92 ml	2194 ml	11.7 ml	9.2
IronMax	4 (Fe)	117 ml	2926 ml	15.6 ml	5.5
		234 ml	5851 ml	31.3 ml	5.6
$MgSO_4 \cdot 7H_2O$	9.9 (Mg)	55 g	556 g	3 g	7.0
		110 g	1111 g	5.9 g	7.3
MangaMax	5.5 (Mn)	80 ml	1461 ml	7.8 ml	4.6
		161 ml	2926 ml	15.6 ml	4.4
Na_2MoO_4	46.5 (Mo)	63 g	137 g	0.7 g	7.1
		127 g	273 g	1.5 g	7.3
ZinManMax	5.1 (Zn), 2.7 (Mn)	149, 79 ml	2926 ml	15.6 ml	5.9
		298, 158 ml	5851 ml	31.3 ml	5.6
$ZnSO_4 \cdot 7H_2O$	22.7 (Zn)	155 g	685 g	3.7 g	5.9
		311 g	1371 g	7.3 g	6.1

Table 2.3: Reagent grade chemicals used in 2012 field experiments at the Central Research Station. No pH adjustments were made. AI = active ingredient in the compound.

2012 Field Treatments					
Compound	**%AI**	**AI ($g\ ha^{-1}$)**	**Compound ($g\ ha^{-1}$)**	**Compound ($g\ L^{-1}$)**	**pH**
$FeSO_4 \cdot 7H_2O$	20 (Fe)	101	504.56	2.65	4.00
		202	1,009	5.31	3.75
		336	1,682	8.85	3.60
		448	2,237	11.77	3.55
$FeCl_3 \cdot 6H_2O$	20.6 (Fe)	58	279	1.47	2.35
		115	558	2.94	2.13
		231	1,117	5.88	1.90
		462	2,237	11.77	1.70
Na_2MoO_4	46.5 (Mo)	52	111	0.58	6.22
		92	197	1.04	6.46
		184	395	2.08	6.70
		368	790	4.16	7.00
MoO_3	66.6 (Mo)	49	74	0.39	4.23
		99	148	0.78	4.35
		197	296	1.56	4.50
		395	592	3.12	4.70
$CaSO_4 \cdot 2H_2O$	23 (Ca)	258	1,120	5.89	5.40
		516	2,237	11.77	5.10
$CaHPO_4 \cdot H_2O$	31 (Ca)	323	1,043	5.49	8.00
		1303	4,205	22.12	7.74
$CoSO_4$	38 (Co)	0.94	2.47	0.013	5.50
		4.69	12.36	0.065	5.52

Table 2.3 (continued): Reagent grade chemicals used in 2012 field experiments at the Central Research Station. No pH adjustments were made. AI = active ingredient in the compound.

2012 Field Treatments					
Compound	**%AI**	**AI ($g\ ha^{-1}$)**	**Compound ($g\ ha^{-1}$)**	**Compound ($g\ L^{-1}$)**	**pH**
$AlK(SO_4)_2 \cdot 12H_2O$	5.6 (Al)	63	1,120	5. 9	3.36
		126	2,237	11.8	3.00
		188	3,364	17.7	2.87
		251	4,474	23.5	2.77
$MnSO_4$	38 (Mn)	111	291	1.5	5.00
		554	1,456	7.7	4.17
		1107	2,910	15.3	4.00
		2215	5,826	30.7	3.77
$ZnSO_4 \cdot 7H_2O$	22.7 (Zn)	99	437	2.3	5.15
		197	870	4.6	5.15
		558	2,456	12.92	5.10
$CuSO_4 \cdot 5H_2O$	25.5 (Cu)	286	1,120	5.9	4.33
		572	2,237	11.8	4.15
H_3BO_3	17.5 (B)	112	639	3.4	5.35
		224	1,278	6.7	4.80
		549	3,135	16.5	4.30
$MgSO_4 \cdot 7H_2O$	9.9 (Mg)	83	841	4.4	5.60
		166	1,682	8.9	5.55

Table 2.4: Reagent grade chemicals used in field experiments conducted at the Central Stations research farm in 2013. The first two columns identify which compounds were applied to each of the two plantings. Plantings were done on April 26 and June 20. AI = active ingredient in the compound.

2013 Field Treatments							
Early	Late	**Compound**	**%AI**	**AI (g ha^{-1})**	**Compound (g ha^{-1})**	**Compound (g L^{-1})**	**pH**
X	**X**	$FeSO_4 \cdot 7H_2O$	20 (Fe)	48	240	1.25	5.69
X	**X**			96	481	2.50	5.50
X	**X**			192	961	5.00	5.42
X				384	1,922	10.00	5.13
X	**X**	$FeCl_3 \cdot 6H_2O$	20.6 (Fe)	49	240	1.25	2.36
X	**X**			99	481	2.50	2.17
X	**X**			198	961	5.00	1.96
X				396	1,922	10.00	1.73
X	**X**	$AlK(SO_4)_2 \cdot 12H_2O$	5.6 (Al)	27	481	2.50	3.14
X	**X**			54	961	5.00	2.85
X	**X**			108	1,922	10.00	2.81
X				161	2,883	15.00	2.85
X	**X**	$Al_2(SO_4)_3$	14.9 (Al)	24	160	0.83	3.74
X	**X**			46	308	1.60	3.66
X	**X**			95	634	3.30	3.50
X				189	1,269	6.60	3.36
X	**X**	Na_2MoO_4	46.5 (Mo)	34	72	0.38	6.38
X	**X**			54	115	0.60	6.99
X				107	231	1.20	6.62
X	**X**	MoO_3	66.6 (Mo)	32	48	0.25	4.36
X	**X**			70	106	0.55	4.46
X				141	211	1.10	4.50
X	**X**	$ZnSO_4 \cdot 7H_2O$	22.7 (Zn)	49	216	1.13	5.80
X	**X**			98	432	2.25	5.78
X				147	649	3.38	5.69
X				196	865	4.50	5.70

Table 2.4 (continued): Reagent grade chemicals used in field experiments conducted at the Central Stations research farm in 2013. The first two columns identify which compounds were applied to each of the two plantings. Plantings were done on April 26 and June 20. AI = active ingredient in the compound.

2013 Field Treatments							
Early	Late	**Compound**	**%AI**	**AI (g ha^{-1})**	**Compound (g ha^{-1})**	**Compound (g L^{-1})**	**pH**
X	**X**	$ZnCl_2$	48 (Zn)	51	106	0.55	5.72
X	**X**			101	211	1.1	5.73
X				148	308	1.6	5.60
X				203	423	2.2	5.50
X	**X**	H_3BO_3	17.5 (B)	50	288	1.5	5.80
X	**X**			101	577	3	5.41
X				202	1,153	6	5.80
		$CuSO_4 \cdot 5H_2O$	25.5 (Cu)	49	192	1	4.82
X				147	577	3	4.63
X				294	1,153	6	4.46
X		$CuCl_2 \cdot 2H_2O$	37 (Cu)	146	394	2.05	4.45
X				292	788	4.1	4.35
X		$MnSO_4$	38 (Mn)	128	336	1.77	5.80
X				650	1,711	8.9	5.67
	X	$MnSO_4 \cdot H_2O$	59 (Mn)	128	217	1.13	5.87
X	**X**	$MnCl_2 \cdot 4H_2O$	27 (Mn)	39	144	0.75	5.92
X				195	721	3.75	5.83
X		$CoSO_4$	38 (Co)	11	29	0.15	6.43
X				22	58	0.3	6.15
X	**X**	$Zn(NO_3)_2$	17 (Zn)	49	288	1.5	4.99
X	**X**			98	577	3	4.07
X	**X**	$Fe(NO_3)_3$	10 (Fe)	48	481	2.5	2.33
X	**X**			96	961	5	2.02

Table 2.5: Reagent grade chemicals used in field experiments conducted at the Central Stations research farm in 2014. AI = active ingredient in the compound.

2014 Field Treatments			
Compound	**%AI**	**Compound (g ha^{-1})**	**Compound (g L^{-1})**
$FeSO_4 \cdot 7H_2O$	20 (Fe)	238	1.25
$FeCl_3 \cdot 6H_2O$	20.6 (Fe)	237	1.25
MoO_3	66.6 (Mo)	104	0.55
$Al_2(SO_4)_3 \cdot H_2O$	15 (Al)	160	0.83
$AlCl_3 \cdot 6H_2O$	11.2 (Al)	215	1.12
$ZnSO_4 \cdot 7H_2O$	22.7 (Zn)	217	1.13
H_3BO_3	17.5 (B)	288	1.5
$CuSO_4 \cdot 5H_2O$	25.5 (Cu)	192	1
$MnSO_4 \cdot H_2O$	59 (Mn)	192	1
$CaSO_4 \cdot 2H_2O$	23.3 (Ca)	374	2

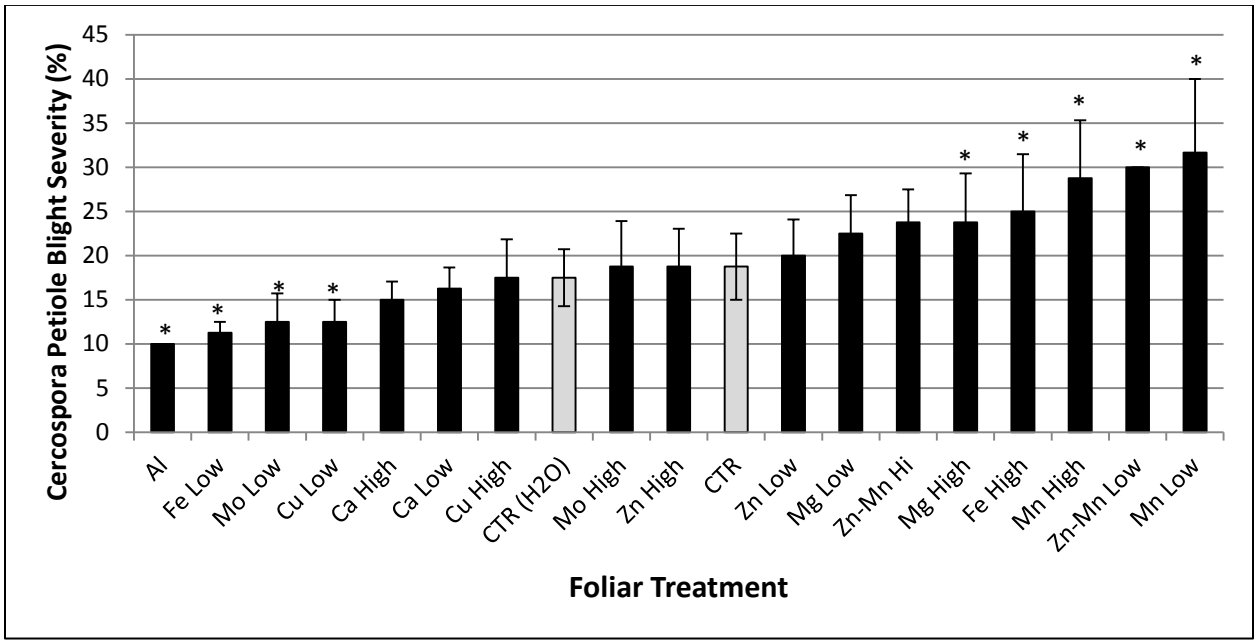


Figure 2.3: Severity of Cercospora leaf blight as rated on petioles (Fig. 2.1) on soybean for the 2011 growing season. Table 2.2 contains descriptions of the treatments. Error bars represent standard error. Treatments denoted with an asterisk are statistically different from the control at $P = 0.07$ as calculated with Dunnett's test (n = 4).

The lowest two rates of zinc sulfate (98 and 197 g ha^{-1} of Zn) and the lowest rate of sodium molybdate (51 g ha^{-1} Mo) reduced rust severity as compared to the control in 2012 (Fig. 2.4). The highest rate of aluminum potassium sulfate (251 g ha^{-1} Al) exacerbated disease. There were no significant effects of these nutritional treatments on plot yields or seed weights (Figs. 2.5 and 2.6, respectively). This experiment was repeated in 2013 (Figs. 2.9 and 2.10).

All rates of both formulations of copper (146 and 292 g ha^{-1} of Cu) reduced Cercospora leaf blight severity compared to the control (Fig. 2.7) in 2013. The high rate of zinc (196 g ha^{-1}) also reduced disease along with both formulations of the high rate of aluminum (161 and 189 g ha^{-1}) and the low rate of aluminum potassium sulfate (27 g ha^{-1}). See Table 2.4 for treatments. As with the rust results (Fig. 2.5) in 2012, there were no discernible yield differences between treatments at $P = 0.714$ (Fig. 2.8). For the sake of clarity, results for iron, copper and aluminum along with the control were extracted from Fig. 2.7 and presented in Figs. 2.12 – 2.14. All copper treatments but only the high rates of aluminum (161 and 189 g ha^{-1} Al) were effective (Figs. 2.13 and 2.14). The higher rates of iron suppressed disease more than the lower rates (Fig. 2.12).

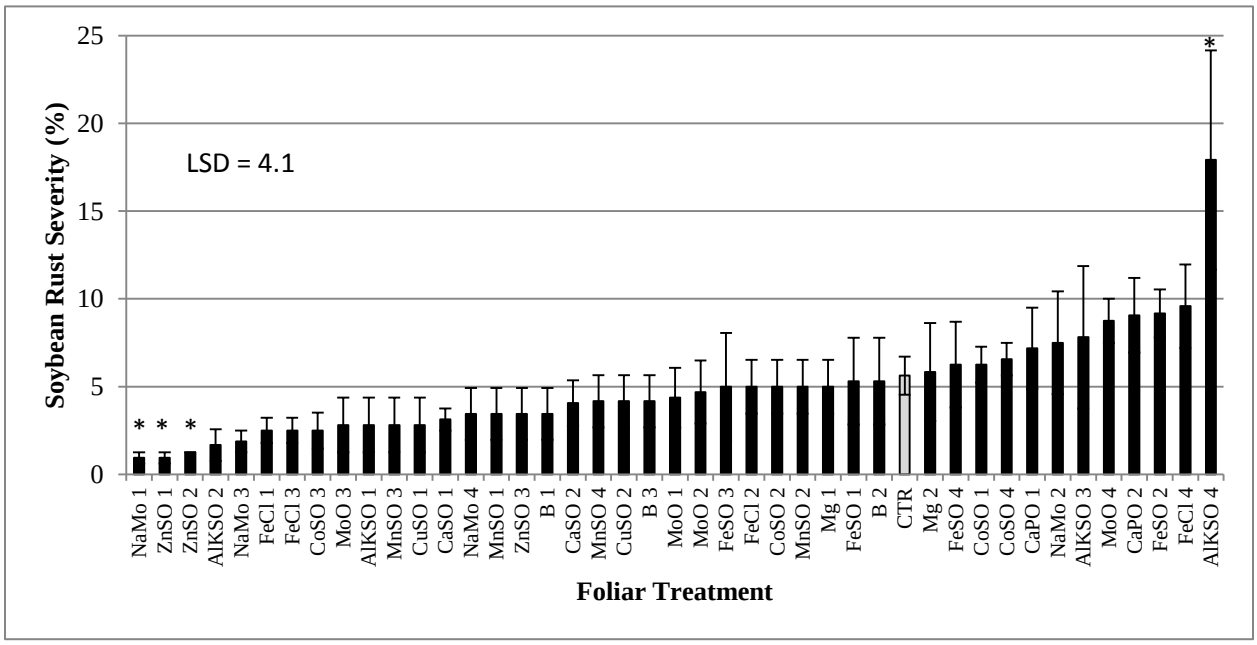


Figure 2.4: Severity of soybean rust in the upper canopy as affected by foliar applications of minor elements in 2012. See Fig. 2.2 for the rating scale and Table 2.3 for treatment details. Treatments denoted with an asterisk are statistically different from controls as calculated with Dunnett's test, Fisher's LSD given for comparative purposes. Error bars represent standard error at $P = 0.05$ (n = 4).

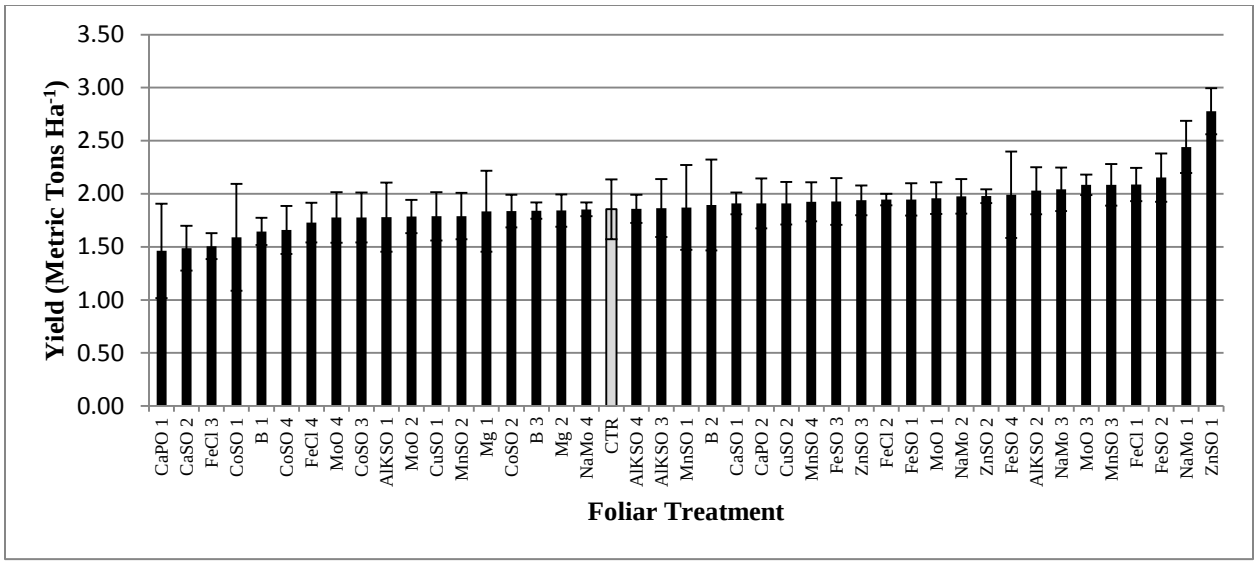


Figure 2.5: Effects of foliar applications of minor elements on soybean yield with regard to soybean rust in 2012 as calculated with Dunnett's test. Descriptions of treatments are provided in Table 2.3 (treatment codes 1-4 correlate to low – high, respectively). The yields in this figure correlate to the ratings in Fig. 2.4. Error bars represent standard error. $P = 0.281$ (n = 4).

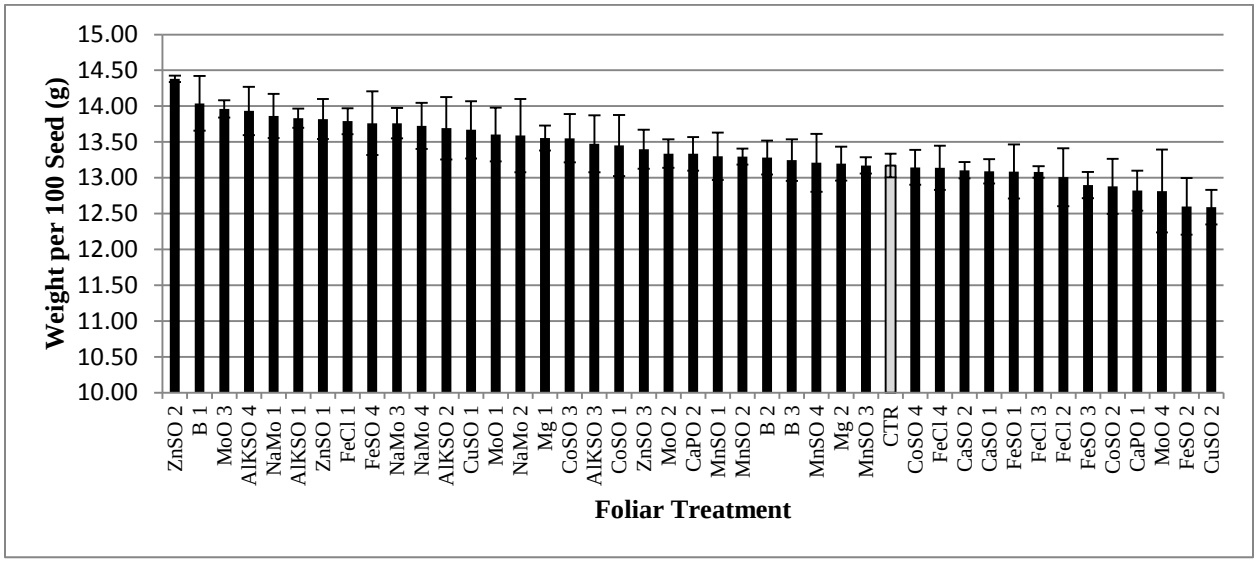


Figure 2.6: Effects of foliar applications of minor elements on weight of 100 soybean seeds as affected by soybean rust in 2012 as calculated with Dunnett's test. Treatments for this figure are given in Table 2.3 (treatment codes 1-4 correlate to low – high, respectively). This figure complements Figures 2.4 and 2.5. Error bars represent standard error. $P = 0.179$ (n = 4).

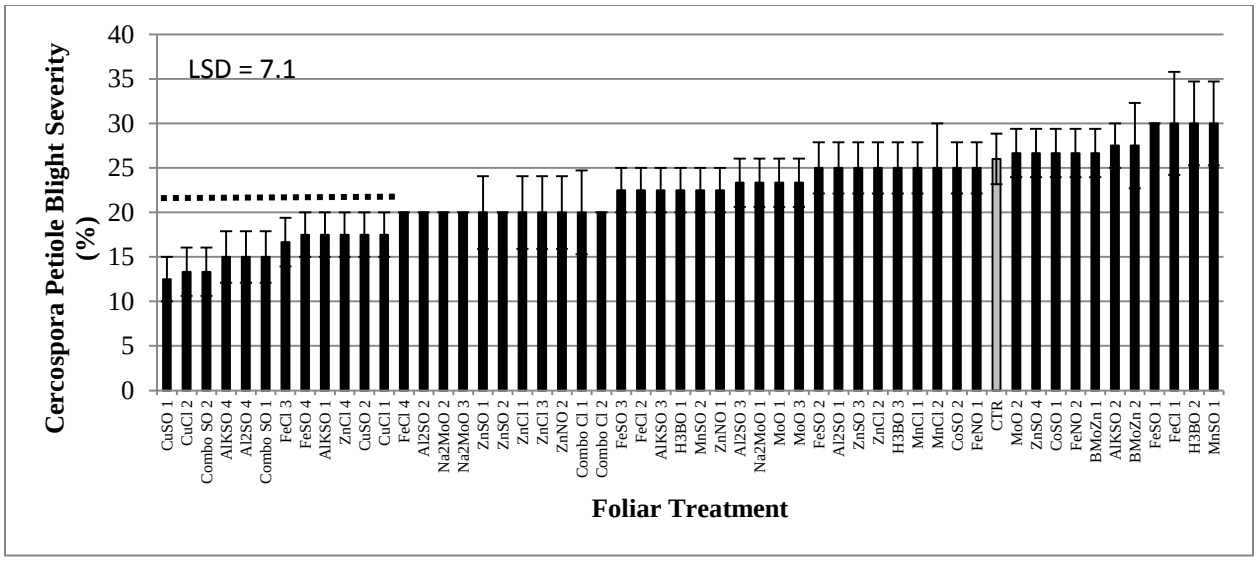


Figure 2.7: Effects of foliar applications of minor elements on Cercospora petiole blight severity. Experiment conducted at the Central Research Station in Baton Rouge in 2013. This figure complements Fig. 2.8. The rating scale is shown in Fig. 2.1, and treatments are described in Table 2.4 in the "early planting" column (treatment codes 1-4 correlate to low – high, respectively). All treatments under the dotted line are statistically different than the control at *P* = 0.05 as calculated with Dunnett's test, Fisher's LSD given for comparative purposes. Error bars represent standard error (n = 4).

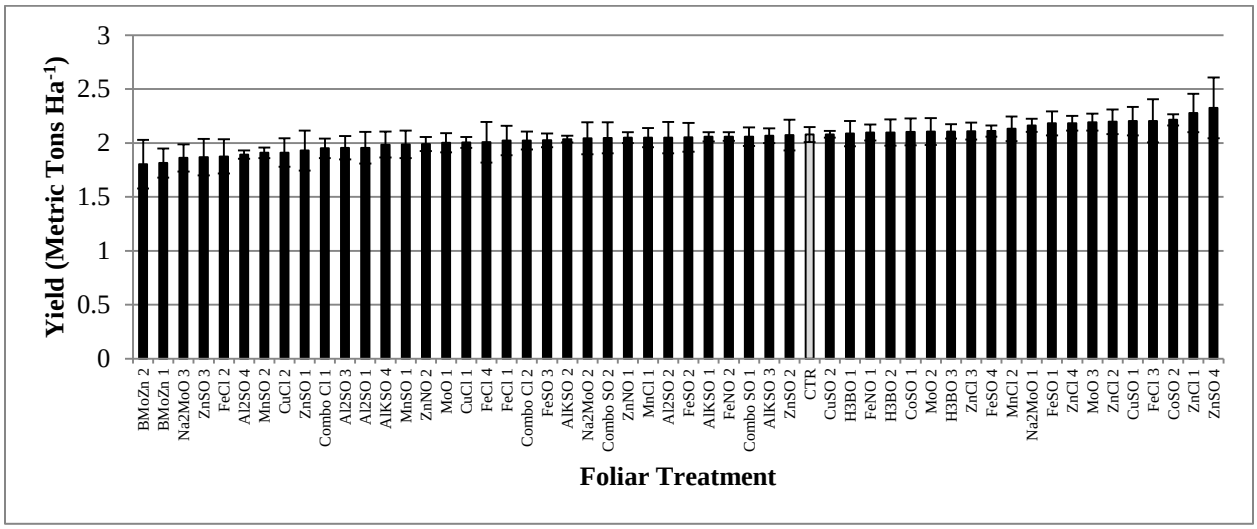


Figure 2.8: Effect of selected minor element applications on soybean yield as affected by Cercospora petiole blight as calculated with Dunnett's test. Yield was corrected to 13% moisture content. Experiment conducted at the Central Research Station in Baton Rouge in 2013. This figure complements Fig. 2.7, and treatments are described in Table 2.4 in the "early planting" column (treatment codes 1-4 correlate to low – high, respectively). Error bars represent standard error. *P* = 0.714 (n = 4).

As in 2012 with rust (Fig. 2.4), zinc (49 and 98 g ha^{-1} Zn from zinc nitrate, 49 g ha^{-1} Zn from zinc sulfate, and 101 g ha^{-1} Zn from zinc chloride) and low sodium molybdate (33 g ha^{-1} Mo) suppressed rust disease severity in 2013 (Fig. 2.9). Furthermore, most of the iron and aluminum formulations and rates were effective. As depicted in Fig. 2.10, yield was not affected by any of the treatments, which was consistent with the 2012 results (Fig. 2.5). Figures 2.15 and 2.16 show certain elements when presented alongside the control; no data are altered from Fig. 2.9.

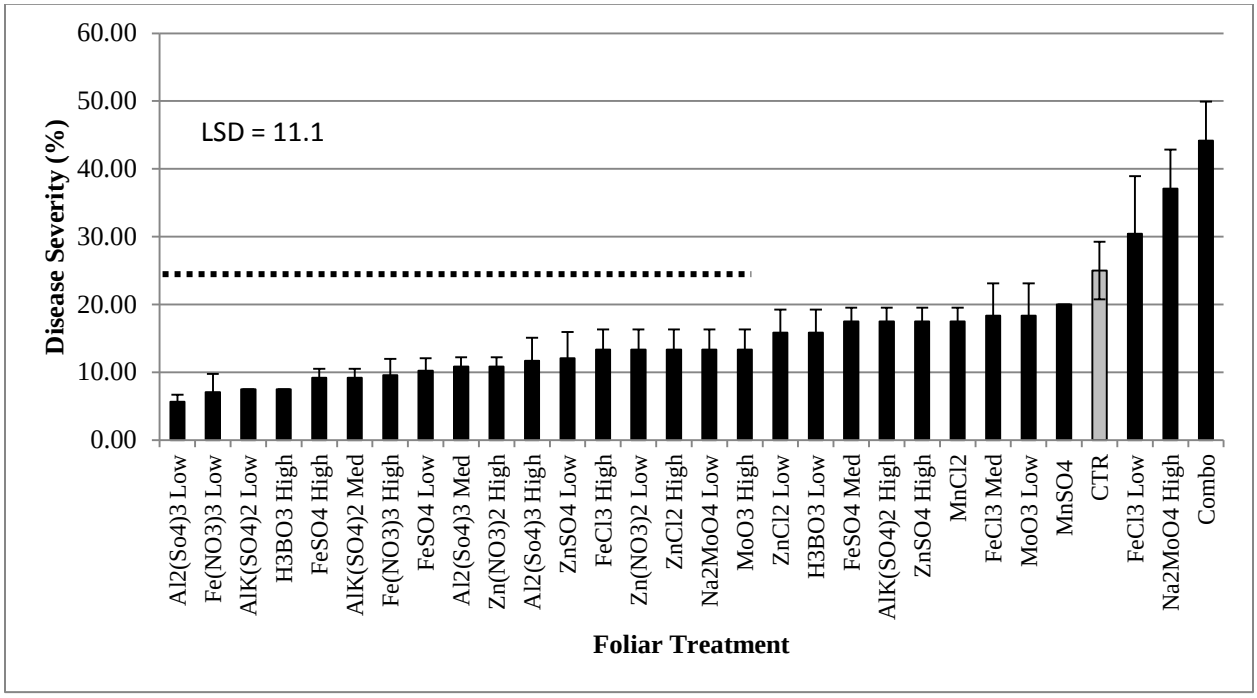


Figure 2.9: Severity of soybean rust in 2013 rated as the average percentage of leaf area affected. The rating scale for these measurements is presented in Fig. 2.2. The treatments can be found in Table 2.4 in the "late planting" column. All treatments underneath the dotted line are statistically different from the control as calculated with Dunnett's test, Fisher's LSD given for comparative purposes. Error bars represent standard error. $P = 0.05$ (n = 3).

Both boron (288 g ha^{-1} boric acid) and iron (238 g ha^{-1} iron sulfate) reduced frogeye severity as compared to the control (Fig. 2.11). However, iron chloride was not effective. While not significant, both chloride treatments had higher average disease than the control.

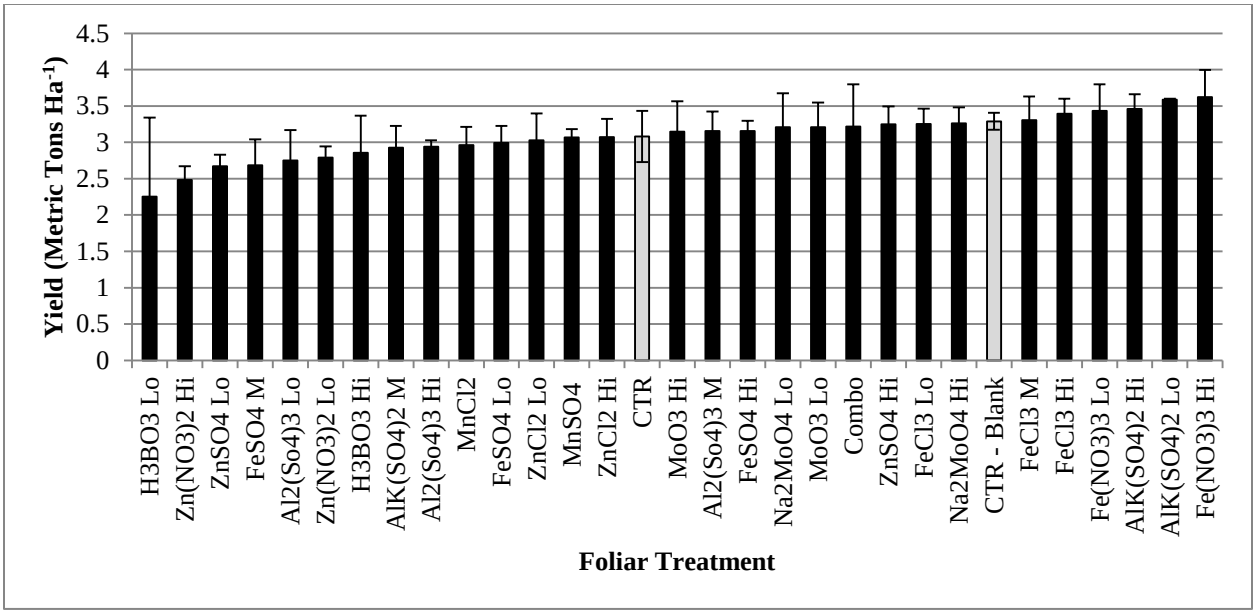


Figure 2.10: Effects of foliar treatments on yield as affected by soybean rust during 2013 as calculated with Dunnett's test, Fisher's LSD given for comparative purposes. Yield data were adjusted to 13% moisture content. Treatments can be found in Table 2.4 in the "late planting" column. This graph relates to Fig. 2.9. Error bars represent standard error. $P = 0.736$ (n = 3).

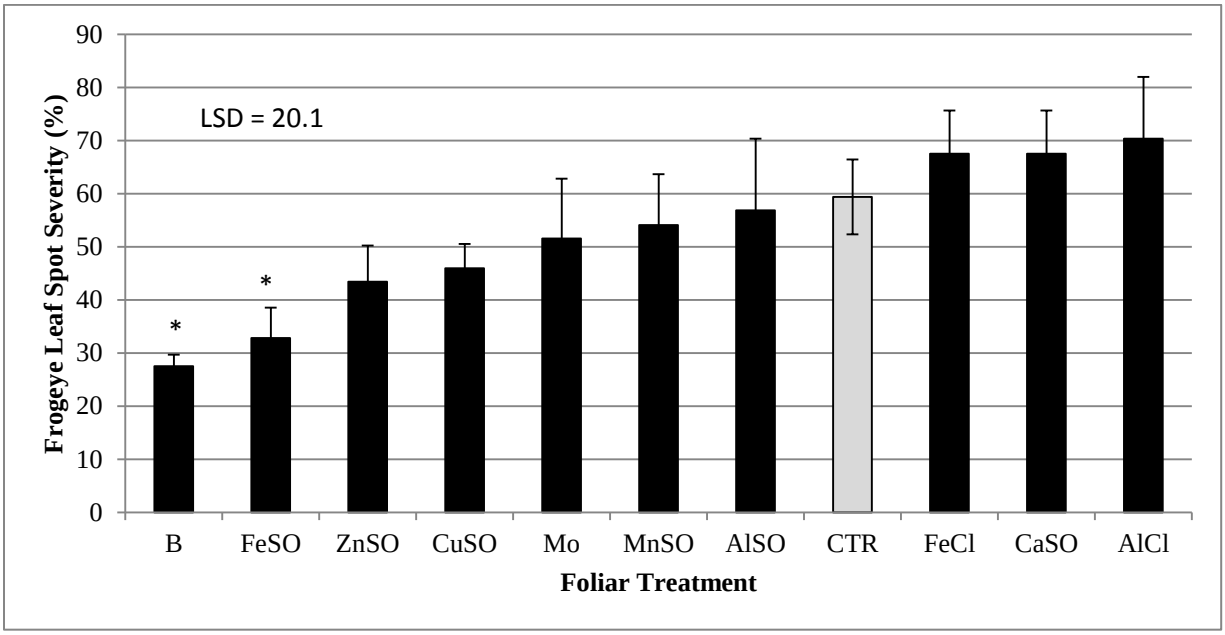


Figure 2.11: Frogeye leafspot severity ratings from 2014 growing season at the Central Research Station. The rating scale used was the soybean rust rating scale and is shown in Fig. 2.2, and treatments are listed in Table 2.5. Error bars represent standard error. Treatments denoted with an asterisk are statistically different from the control at $P = 0.068$ as calculated with Dunnett's test, Fisher's LSD given for comparative purposes (n = 4).

Figures 2.12 – 2.14 depict the effects of iron, copper, and aluminum treatments on CLB compared to the control, respectively. These values were extracted from Fig. 2.7 for clarity, and statistical values were not changed from the original graph (Fig. 2.7). Iron treatments (high rate of iron sulfate and second highest rate of iron chloride) significantly reduced CLB by 8% compared to the control (Fig. 2.12). Copper treatments reduced CLB by 8% to 13% (Fig. 2.13). The two high rates of aluminum reduced CLB by 11% and the low rate of aluminum potassium sulfate reduced CLB by 8% (Fig. 2.14).

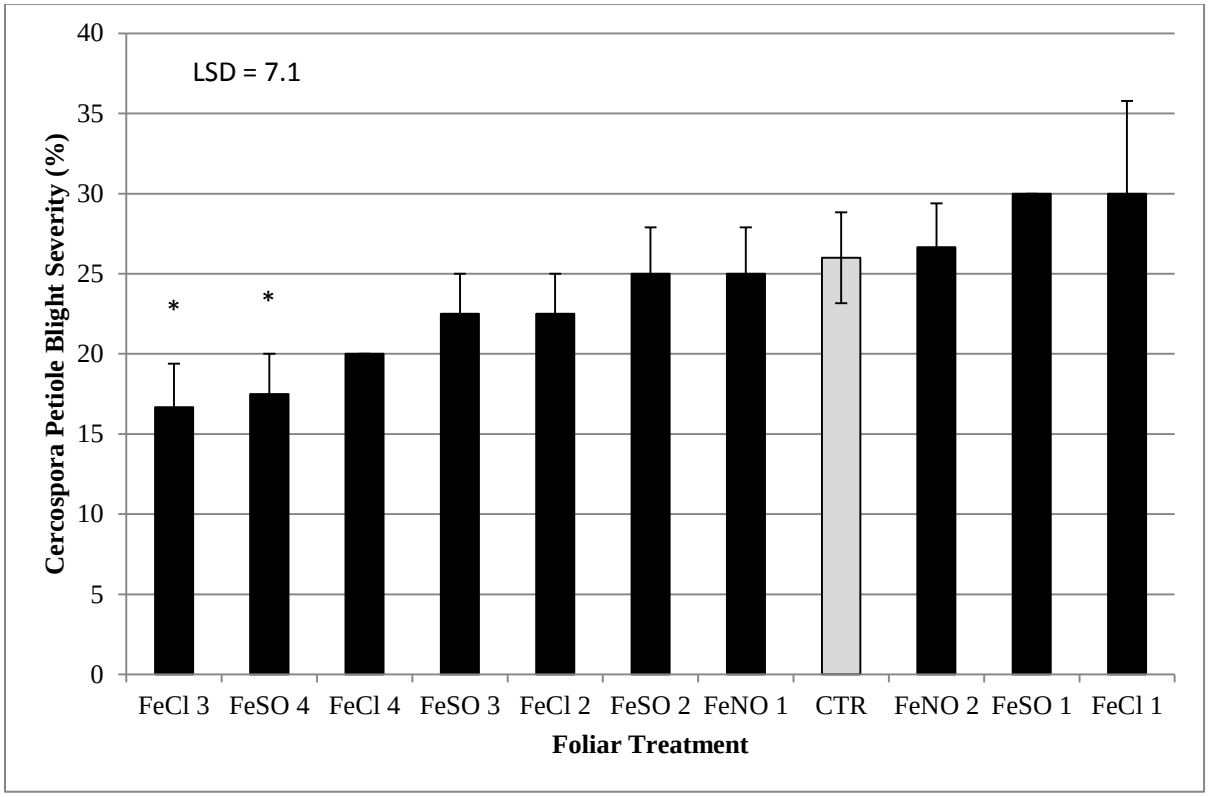


Figure 2.12: Comparison of all iron treatments as compared to the control for effects on severity of Cercospora petiole blight (rated on petioles). Experiment was conducted at the Central Research Station, Baton Rouge, LA, in 2013. Refer to Fig. 2.7 for the full graph and Table 2.4 for a list of treatments (treatment codes 1-4 correlate to low – high, respectively). Treatments denoted with an asterisk are statistically different from the control at $P = 0.05$ as calculated with Dunnett's test, Fisher's LSD given for comparative purposes (n = 4).

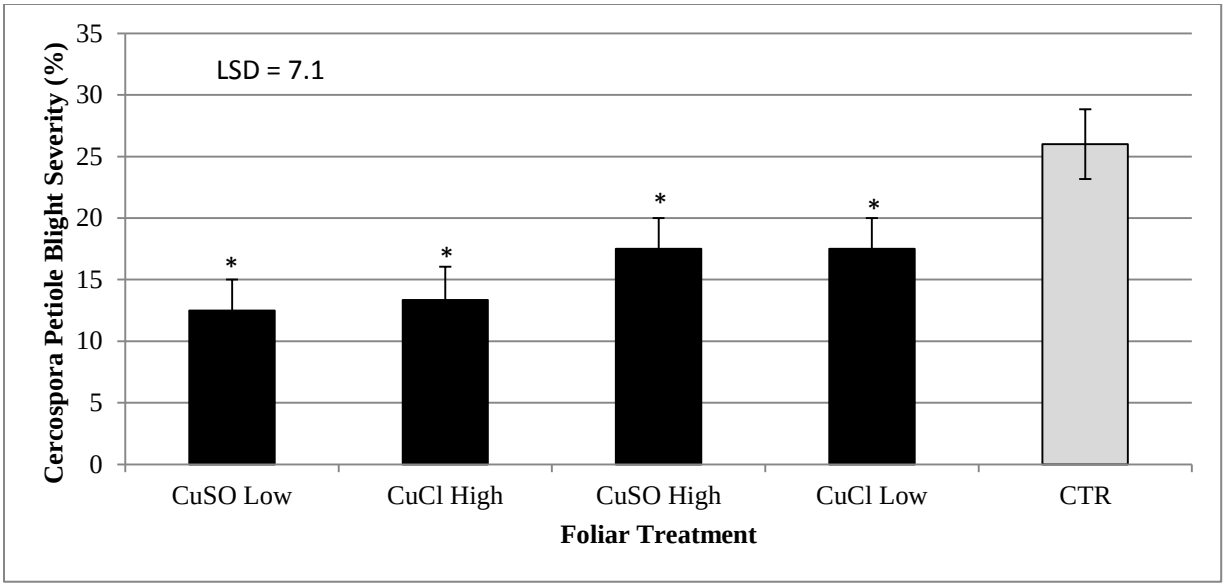


Figure 2.13: Effects of copper treatments compared to control with regard to disease severity from Cercospora petiole blight (rated on petioles) trial at Central Research Station, 2013 (Fig. 2.7). List of treatments given in Table 2.4. Treatments denoted with an asterisk are statistically different from the control at $P = 0.05$ as calculated with Dunnett's test, Fisher's LSD given for comparative purposes (n = 4).

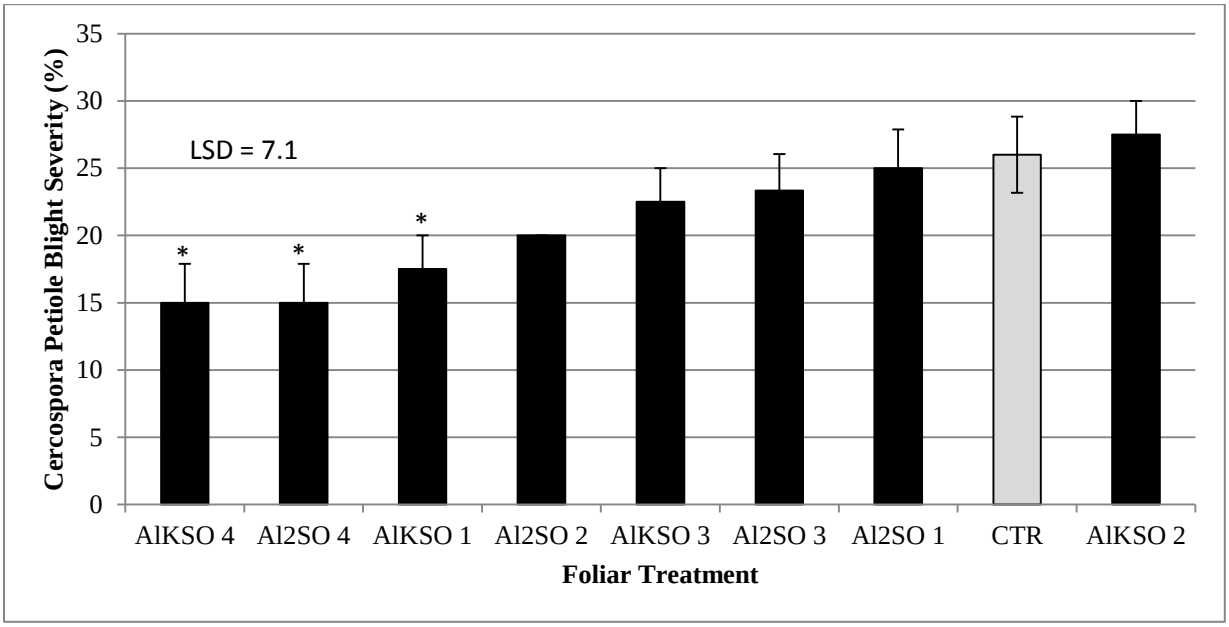


Figure 2.14: Effects of aluminum treatments compared to control in regard to disease severity from Cercospora petiole blight (rated on petioles) trial at Central Research Station, 2013 (Fig. 2.7). List of treatments given in Table 2.4 (treatment codes 1-4 correlate to low – high, respectively). Treatments denoted with an asterisk are statistically different from the control at $P = 0.05$ as calculated with Dunnett's test, Fisher's LSD given for comparative purposes (n = 4).

Figures 2.15 and 2.16 depict the effects of iron and aluminum applications on soybean rust severity originally shown in Fig. 2.9. These values were extracted from Fig. 2.9 for clarity, and statistical values were not changed from the original graph (Fig. 2.9). Both iron nitrate treatments (48 and 96 g ha^{-1} iron) were effective at significantly reducing disease severity (Fig. 2.15). The aluminum treatments specifically show a trend of decreasing disease severity with decreasing aluminum content (Fig. 2.16). All but the highest aluminum potassium sulfate rate (161 g ha^{-1} Al) were statistically significant at $P = 0.05$ (rates found in Table 2.4).

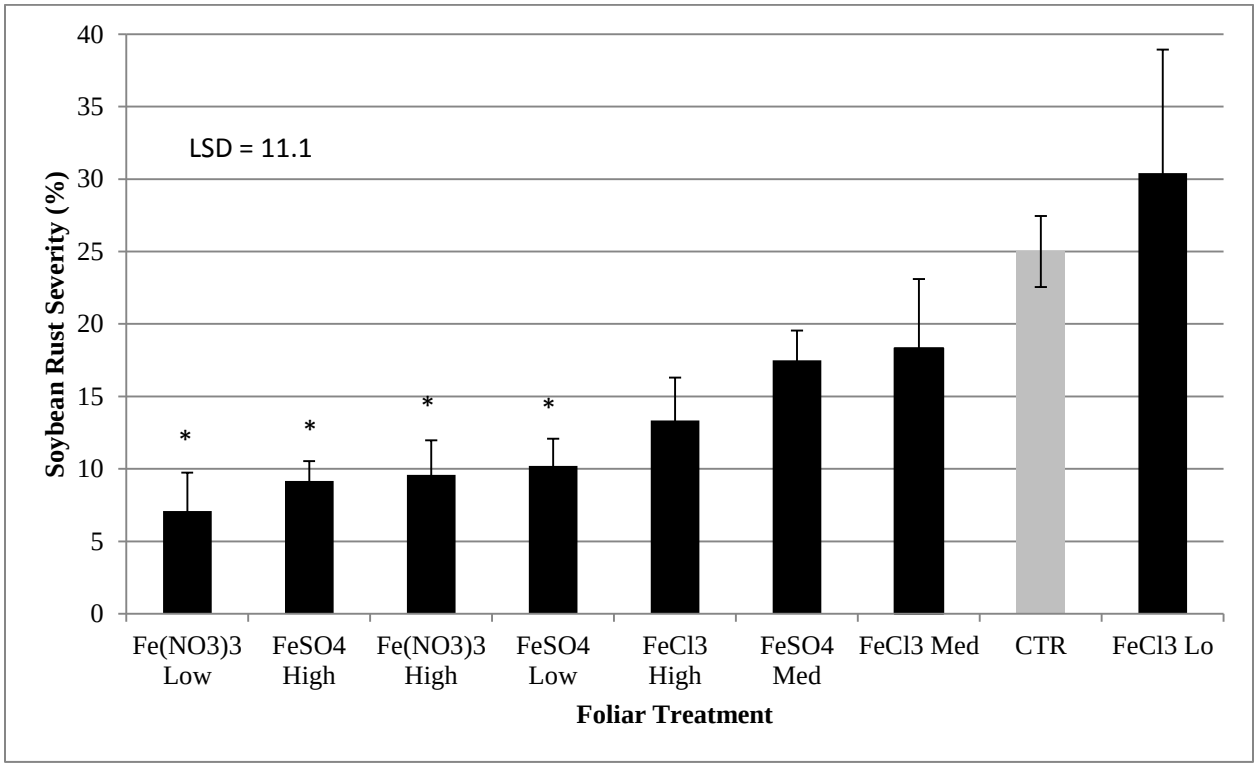


Figure 2.15: Effects of iron treatments on severity of soybean rust in a field experiment conducted at the Central Research Station in 2013. Values were extracted from Fig. 2.9 for comparative purposes. Treatment details are presented in Table 2.4 and the rating scale is given in Fig. 2.2. Treatments denoted with an asterisk are statistically different from the control at $P = 0.05$ as calculated with Dunnett's test, Fisher's LSD given for comparative purposes (n = 3).

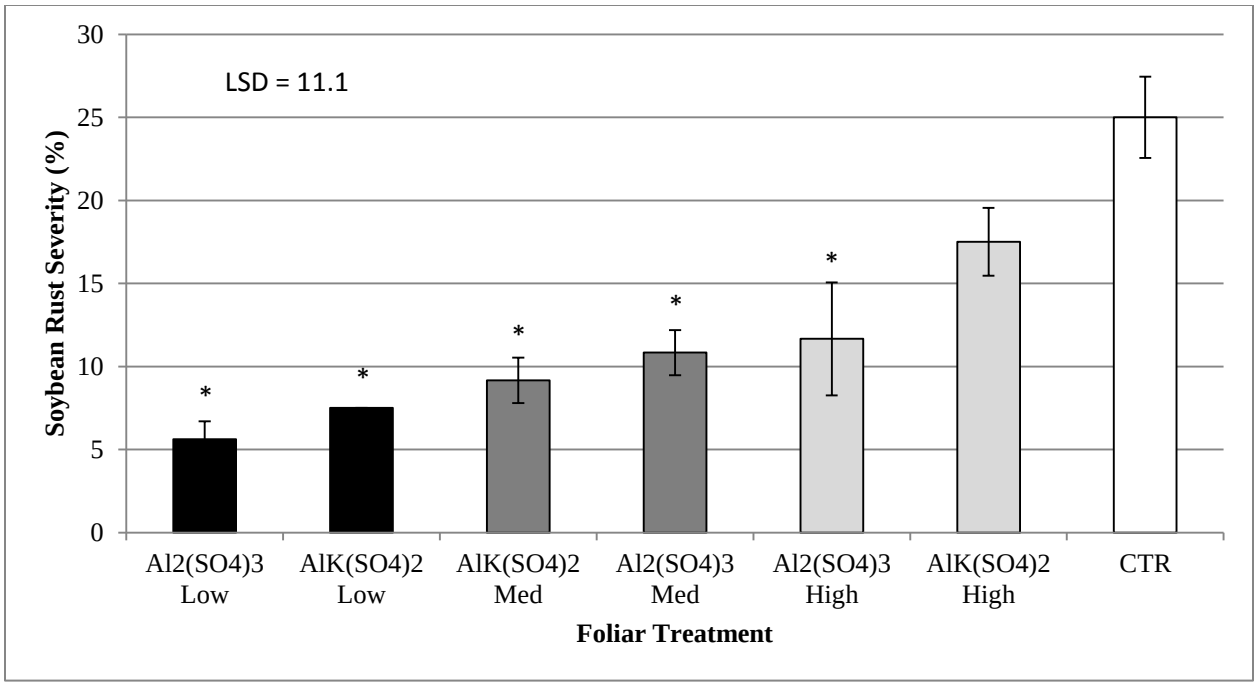


Figure 2.16: Effects of aluminum treatments on severity of soybean rust in a field experiment conducted at the Central Research Station in 2013. These values are extracted from Fig 2.9 for clarity. Treatment details are presented in Table 2.4 and the rating scale is given in Fig. 2.2. Treatments denoted with an asterisk are statistically different from the control at $P = 0.05$ as calculated with Dunnett's test, Fisher's LSD given for comparative purposes (n = 3).

Chapter 3: Effects of Minor Elements on Growth and Cercosporin Production by *Cercospora kikuchii*

3.1. Materials and Methods of Amended Media Experiments

Isolates of *C. kikuchii* were grown both in liquid culture and on solid media amended with different concentrations of minor elements. Concentrations used in these *in vitro* tests were chosen on the basis of soybean leaf tissue analyses from various publications so that they would be comparable to physiological values. A similar study was conducted by Lynch and Geoghegan in 1979; however, it was done with *C. beticola*, and the concentrations of the elements tested in their work were not as high as those found *in planta*.

Potato dextrose agar (PDA, Becton, Dickson (BD) Company) was chosen as the basal medium because previous work showed that the fungus produced luxuriant colonies, and there was ample toxin production (personal observations, Chen et al. 1979, Crane and Crittenden 1967). The following reagent grade chemicals were tested in PDA: Al_2SO_4, $AlCl_3 \cdot 6H_2O$, $AlK(SO_4)_2 \cdot 12H_2O$, H_3BO_3, $CaHPO_4$, $CaSO_4$, $CoSO_4$, $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$, $FeCl_3 \cdot 6H_2O$, Na_2MoO_4, MoO_3, $MnCl_2 \cdot 4H_2O$, $MnSO_4 \cdot H_2O$, $ZnCl_2$, and $ZnSO_4 \cdot 7H_2O$.

Complete medium also was tested (without agar) for experiments conducted in liquid medium because potato dextrose broth was not conducive for cercosporin production. In addition, powdered commercial PDA (lot no. 4258800) was analyzed for elemental composition because there were no published values for minor element composition for this commonly used agar medium (Table 3.2). Samples were digested and analyzed via the nitric acid- hydrogen peroxide method (Wu et al. 1997). Briefly, samples were digested with HNO_3 at 155°C for 5 minutes followed by H_2O_2 at 125°C for 2 hours and 45 minutes and analyzed with a TruSpec CN (LECO) and ICP (make SPECTRO, model ARCOS).

Radial growth of *C. kikuchii* as affected by each of the elements was measured, and visual effects on cercosporin production were noted. Results from preliminary experiments allowed us to eliminate many elements from further testing for various reasons including lack of response at physiological concentrations or solubility problems. Details of these tests are provided below. For promising elements where either *in vitro* or field results indicated some possible effect on disease, the experiments were refined. In instances where pH needed to be lowered, a 25% lactic acid solution was used, and where pH needed to be raised, a 1M NaOH solution was used. Furthermore, PDA was analyzed to account for basal levels of micronutrients present.

Complete medium (CM) also was tested as an alternative solid basal growth medium to PDA because of the possible unknown growth factors from potatoes in the latter medium. The recipe for complete medium (Jenns 1989) was initially selected for use over other media because

of the growth rate and cercosporin production reported in previous studies (Jenns et al. 1989, Chen et al. 2007) as well as the defined elemental ingredients. The recipe is as follows:

1 g $Ca(NO_3)_2 \cdot 4H_2O$
10 ml of:
    2g KH_2PO_4
    2.5g $MgSO_4 \cdot 7H_2O$
    1.5g NaCl
    100ml ddH_2O
    1M NaOH to bring pH up to 5.3
15 g Bacto agar
10 g glucose
1 g yeast extract
1 g casein hydrolysate (Bacto™ Casamino acids, technical)
Bring to 1 L with ddH_2O

It should be noted that minimal medium (MM) is the same recipe but without yeast extract and casein hydrolysate.

While initial growth and cercosporin production on CM appeared to be more robust than on PDA, additions of many of the salts proved problematic. For some metals, such as iron, the compounds precipitated. Furthermore, cercosporin production on CM was inconsistent among repetitions of experiments and even among replications within experiments. For these reasons, PDA was selected as the medium for all subsequent experiments.

Finally, for the last set of experiments, isolates DLL 6013 1B, 2000-4, and 2013-35 were selected over the previous four. The previously used isolates were all recovered from the same year and showed various growth habits. For example, isolate MRS 5012 1A did not produce typical cercosporin pigmentation. The three isolates were chosen based upon typical growth habits for *C. kikuchii*, and all produced cercosporin and encompassed a variety of years in which they were isolated. Each treatment and isolate contained four replications and data points given in the figures are the mean of these four measurements.

Table 3.1: List of isolates of *Cercospora kikuchii* used in amended media trials.

Isolate	Year isolated	Location isolated	Tissue isolated from	Cercosporin production
DLL 6013-1B	2001	Alexandria, LA	leaf	Y
MRS 5012-1A	2001	Winnsboro, LA	seed	N
MRL 6020-1A	2001	Alexandria, LA	leaf	Y
DLS 6013-4B	2001	Alexandria, LA	seed	Y
2000-4	2000	Winnsboro, LA	seed	Y
2013-35	2013	Winnsboro, LA	leaf	Y

Agar plates were inoculated with a 3 mm diameter plug cut from the margin of a 10-day-old PDA culture. For the solid media trials, a randomized block design was used. All plates were placed at 27°C under continuous fluorescent light at 3,550 lux (Traceable® dual range light meter (product # 1235C43), Thomas Scientific). Plates were sealed with parafilm to prevent contamination. Colony diameter was measured on two axes at 10 days after plating, and the mean was calculated using these two measurements.

To measure cercosporin, a modified version of the protocol given by Chung (2003-2) was used. Briefly, five 5 mm plugs were cut from the actively growing margins of colonies and placed in a test tube to which was added 3 ml of 5M KOH. The tubes were then allowed to incubate in the dark for 4 hours before a sample was taken for an absorbance reading at 480 nm (Milton Roy Spectronic 601, item # 335104, serial # 3612051004, Rochester, NY). Concentration was then calculated using the molar extinction coefficient of 23,300 using the formula:

Concentration (M) = Absorbance / 23,300 (Chung 2003-2)

The molar extinction coefficient should also be multiplied by the length of the cuvette in centimeters; however, the length of the cuvettes used was 1 cm, so it was omitted from the above equation. To ensure the above protocol was accurate, cercosporin production was initially measured in the inner colony, the margin, and surrounding diffused pigmentation (data not shown) of test plates. It was determined that the highest concentration of cercosporin existed in the actively growing margin, and these measurements were most uniform within a treatment.

For liquid cultures, a 3 mm diameter plug from a 10-day-old culture was placed into a small sterile tube with ddH_2O and ground with a pestle for 10 sec so that the inoculum was suspended in the water. One hundred microliters of this suspension were then added to 250 ml Erlenmeyer flasks containing 75 ml potato dextrose broth (PDB) or complete medium (CM). The cultures were placed on a rotary shaker set to 200 rpm in a windowless room under continuous fluorescent lights at 27°C.

3.2. Results of Amended Media Experiments

The composition of potato dextrose agar (PDA) was analyzed to ascertain what nutrients were present and in what quantities. These quantities were then used to calculate the amount present in full strength agar after water was added. These results are listed in Table 3.2.

Because many of the supplements that were added to PDA lowered pH, the effect of pH was assessed on growth of *C. kikuchii* (Fig. 3.1). Lactic acid (25%) was added to PDA until the desired pH was attained. In order to equalize pH across all element treatments, 1 M NaOH was

added to those treatments that reduced pH to obtain a constant 5.45 pH (pH of unamended PDA) for all treatments. Error bars are not shown for the following graphs as there was very little variability within replications. The average standard error is given for each graph, calculated from averaging the standard errors of each data point (n = 4).

Table 3.2: Concentrations (mg L^{-1}) of nutrients in Difco potato dextrose agar, assuming 39 g powder per L.

Element	mg L^{-1}
Aluminum	0.2
Boron	2.7
Calcium	24.9
Copper	0.1
Iron	2.7
Magnesium	18.6
Manganese	0.1
Molybdenum	0
Nitrogen	359
Phosphorous	35
Potassium	159.4
Sodium	243
Sulfur	129.6
Zinc	0.27

The effect of lowering pH on radial growth of *C. kikuchii* was detrimental with growth declining as pH was lowered from 5.45 to 2.6 (Fig. 3.1). For this reason, substrate pH in all subsequent experiments was adjusted to pH 5.45. Cobalt and calcium showed negligible effects on fungal growth and no discernable effects on cercosporin production (Figs. 3.2 – 3.4), and these elements were not investigated further. Molybdenum, iron and aluminum were investigated further and are shown here because of the concentrations tested, and, in the case of $AlK(SO_4)_3 \bullet 12H_2O$, because of the formulation used (Figs. 3.5 – 3.7). Further tests with aluminum utilized either aluminum chloride or aluminum sulfate.

Calcium and cobalt had no effects on growth or toxin production *in vitro* within physiological ranges (Figs. 3.2 – 3.4). While molybdenum had a slight effect on growth, the rates required to achieve this inhibition were well beyond physiological ranges in soybean (about 10 mg L^{-1}, data not shown), and this element was therefore discontinued from subsequent trials.

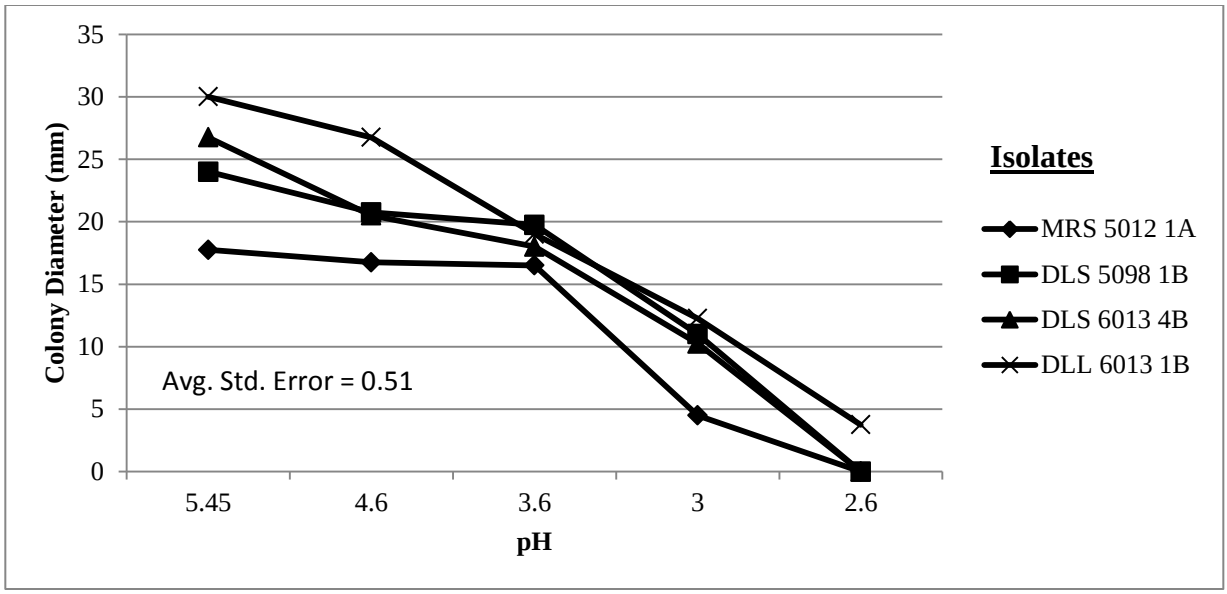


Figure 3.1: Effects of pH of potato dextrose agar on radial growth of *Cercospora kikuchii* after 10 days. Lactic acid (25%) was used to adjust pH. Data points represent mean of four replicates for each of four isolates.

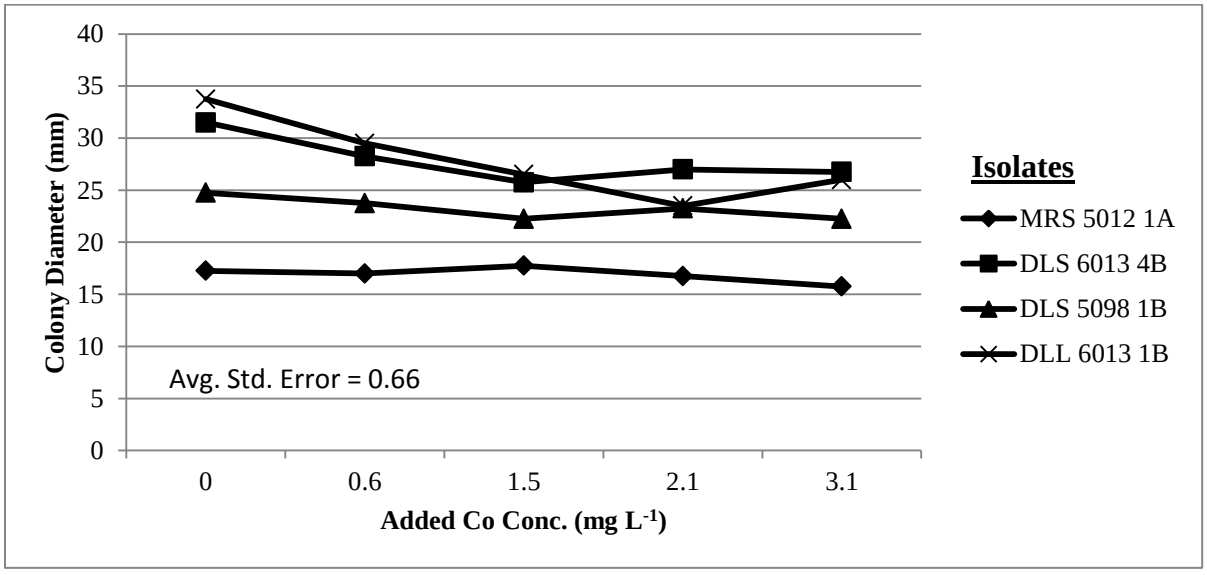


Figure 3.2: Growth of *Cercospora kikuchii* measured at 10 days after inoculation on potato dextrose agar amended with increasing cobalt sulfate heptahydrate ($CoSO_4 \cdot 7H_2O$) concentrations. Data points represent mean of four replicates for each of four isolates.

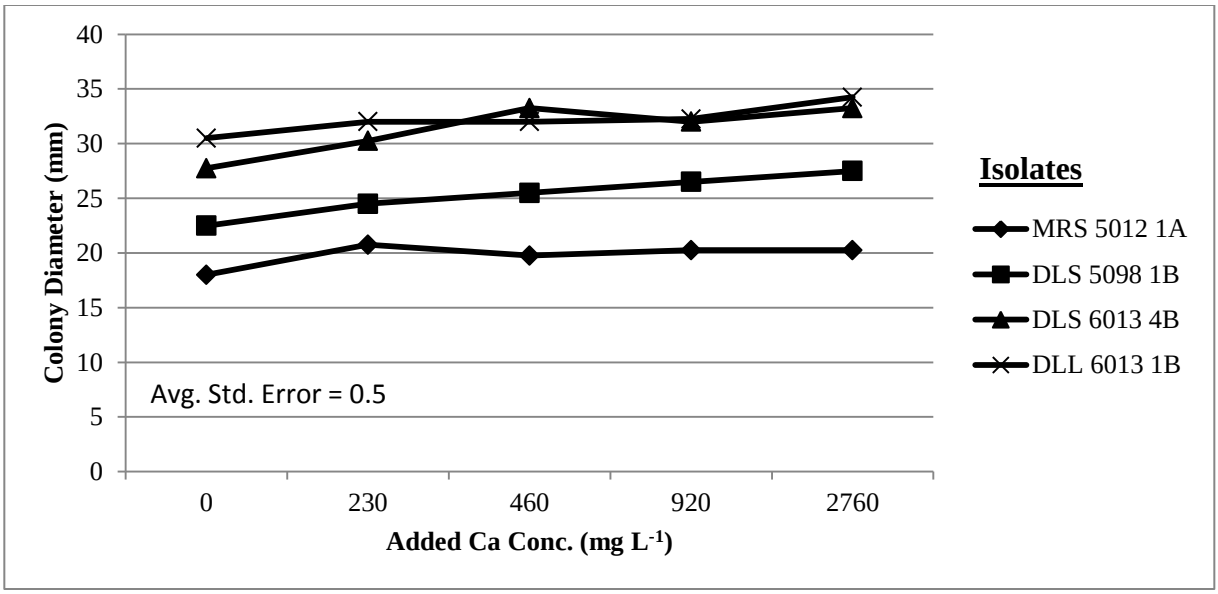


Figure 3.3: Growth of *Cercospora kikuchii* measured at 10 days after inoculation on potato dextrose agar amended with increasing calcium phosphate ($CaHPO_4$) concentrations. Data points represent mean of four replicates for each of four isolates.

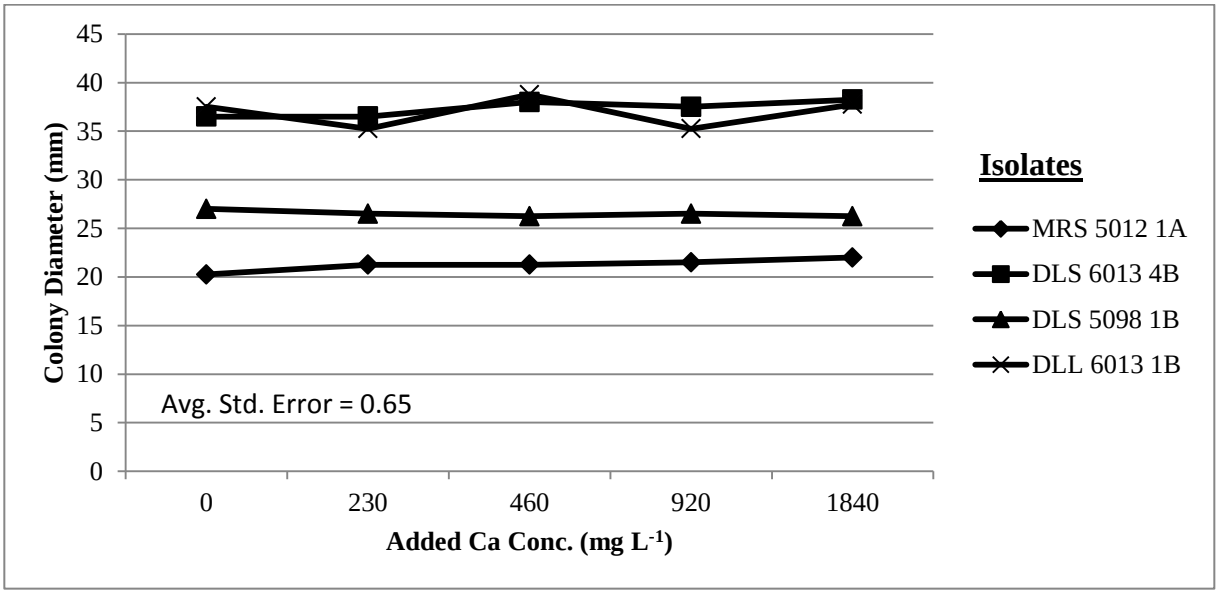


Figure 3.4: Growth of *Cercospora kikuchii* measured at 10 days after inoculation on potato dextrose agar amended with increasing calcium sulfate anhydrous ($CaSO_4$) concentrations. Data points represent mean of four replicates for each of four isolates.

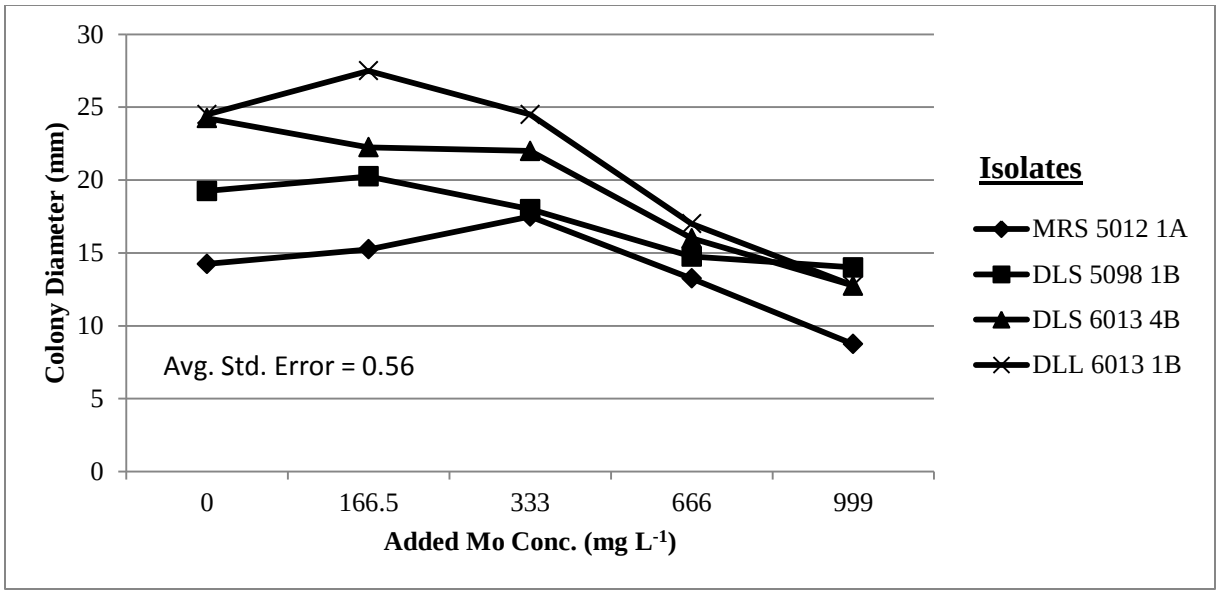


Figure 3.5: Growth of *Cercospora kikuchii* measured at 10 days after inoculation on potato dextrose agar amended with increasing molybdic acid (MoO_3) concentrations. Data points represent mean of four replicates for each of four isolates.

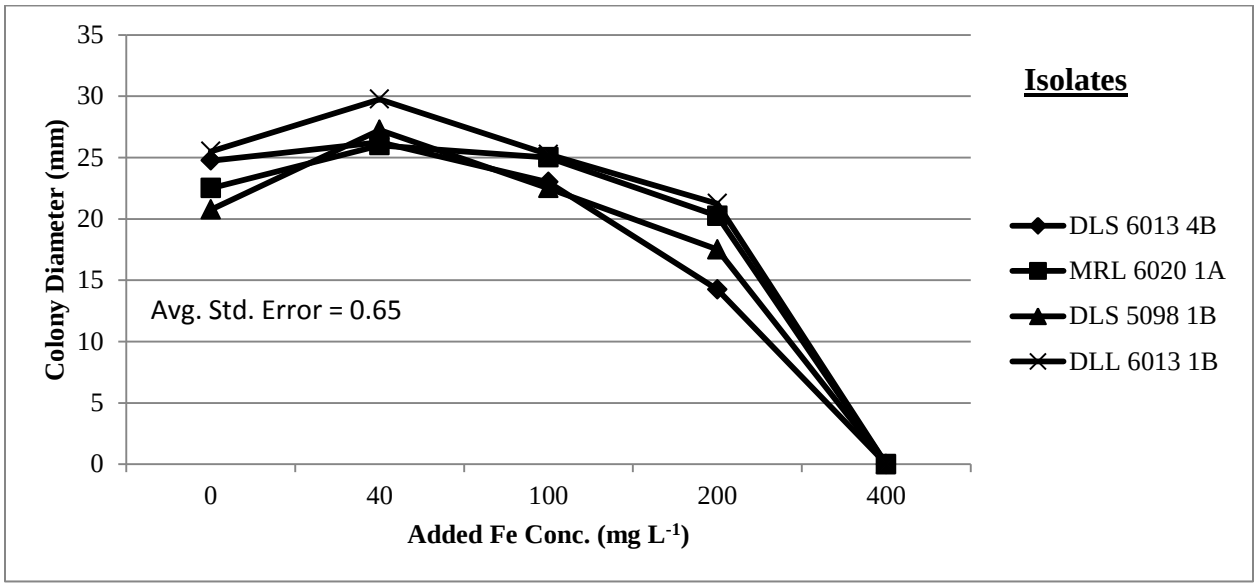


Figure 3.6: Growth of *Cercospora kikuchii* measured at 10 days after inoculation on potato dextrose agar amended with increasing iron sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) concentrations while pH was kept at 5.45 via additions of 1 M NaOH. Data points represent mean of four replicates for each of four isolates.

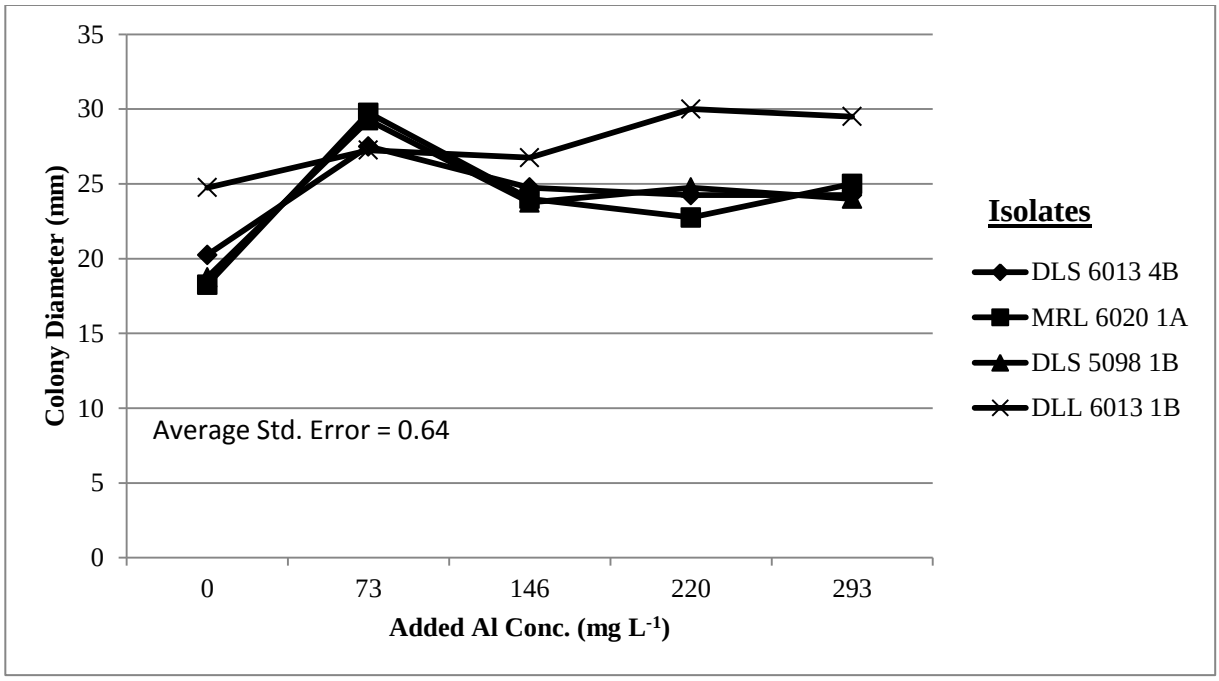
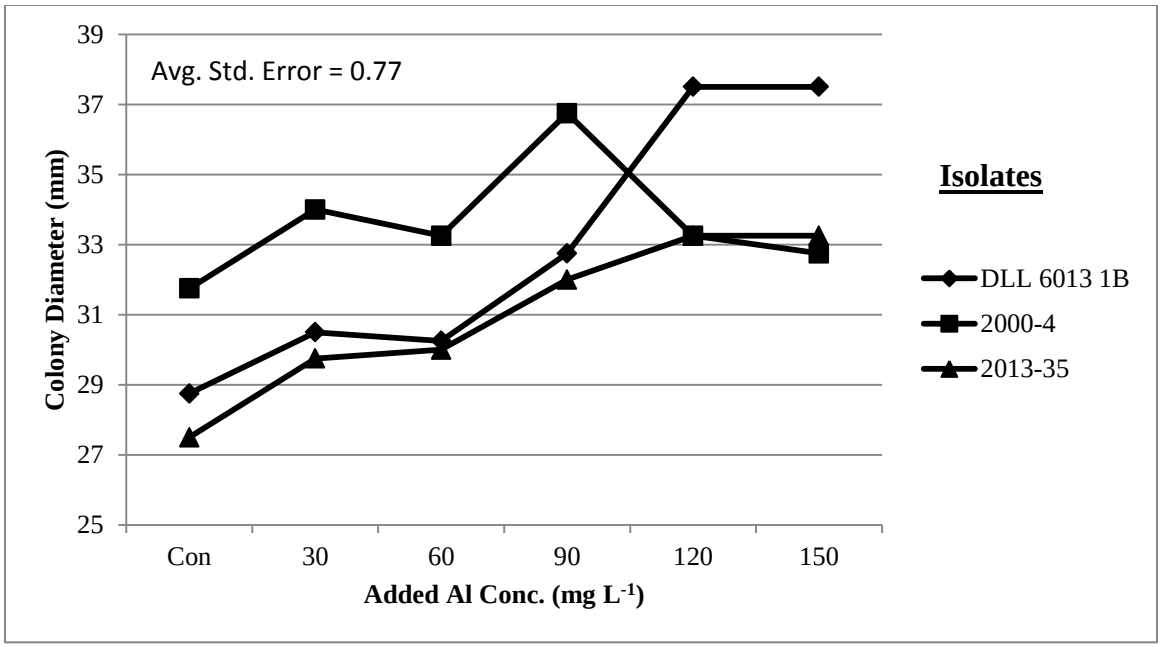


Figure 3.7: Growth of *Cercospora kikuchii* measured at 10 days after inoculation on potato dextrose agar amended with increasing aluminum potassium sulfate dodecahydrate ($AlK(SO_4)_3 \cdot 12H_2O$) concentrations while pH was kept at 5.45 via additions of 1 M NaOH. Data points represent mean of four replicates for each of four isolates.

Iron sulfate, zinc sulfate and zinc chloride treatments inhibited fungal growth at high concentrations. Because of this, a cercosporin measurement could not be made for some rates. This was in part due to these elements lowering pH to a point that *C. kikuchii* did not grow optimally. For all of the following figures (3.8 - 3.18), pH was held constant at 5.45; the pH of nonamended PDA. Furthermore, for these figures three isolates exhibiting classic C. kikuchii growth and cercosporin production were used over the previous four isolates.

High rates of aluminum increased cercosporin production as compared to basal levels in most of the isolates, especially in aluminum sulfate monohydrate trials (Figs. 3.8 and 3.9). With regard to growth, the highest rate of aluminum sulfate monohydrate increased growth of *C. kikuchii* from 1 - 8 mm depending on isolate. Aluminum chloride hexahydrate showed minimal increases in growth, except that isolate DLL 6013 1B showed a parabolic growth curve (initially increasing then decreasing) as aluminum rates were increased (Fig. 3.9).



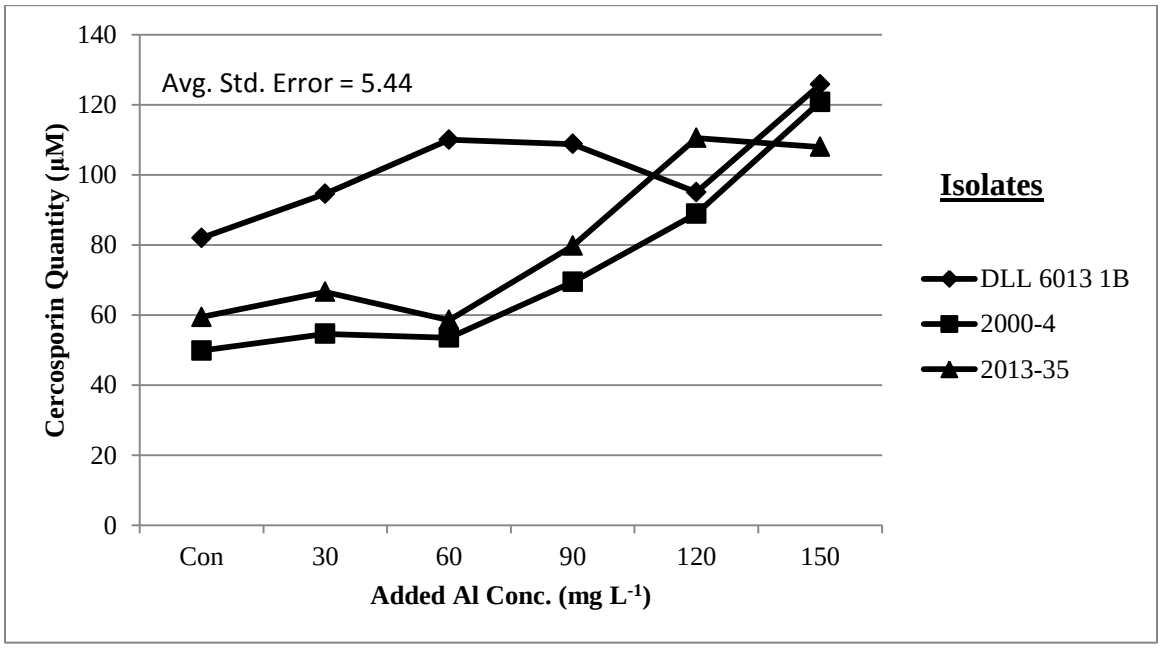
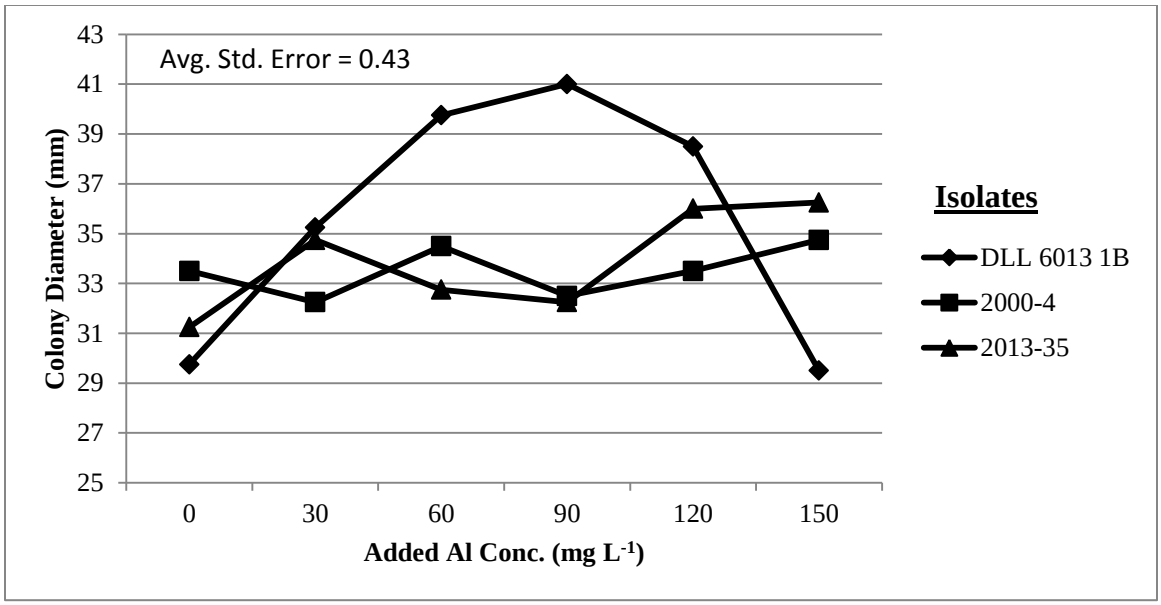


Figure 3.8: Growth of *Cercospora kikuchii* and cercosporin concentration measured at 10 days after inoculation on potato dextrose agar amended with increasing aluminum sulfate monohydrate ($Al_2(SO_4)_3 \bullet H_2O$) concentrations while pH was kept at 5.45 via additions of 1 M NaOH. Data points represent mean of four replicates for each of three isolates. Basal levels of aluminum in control plates were about 0.24 mg L^{-1}.



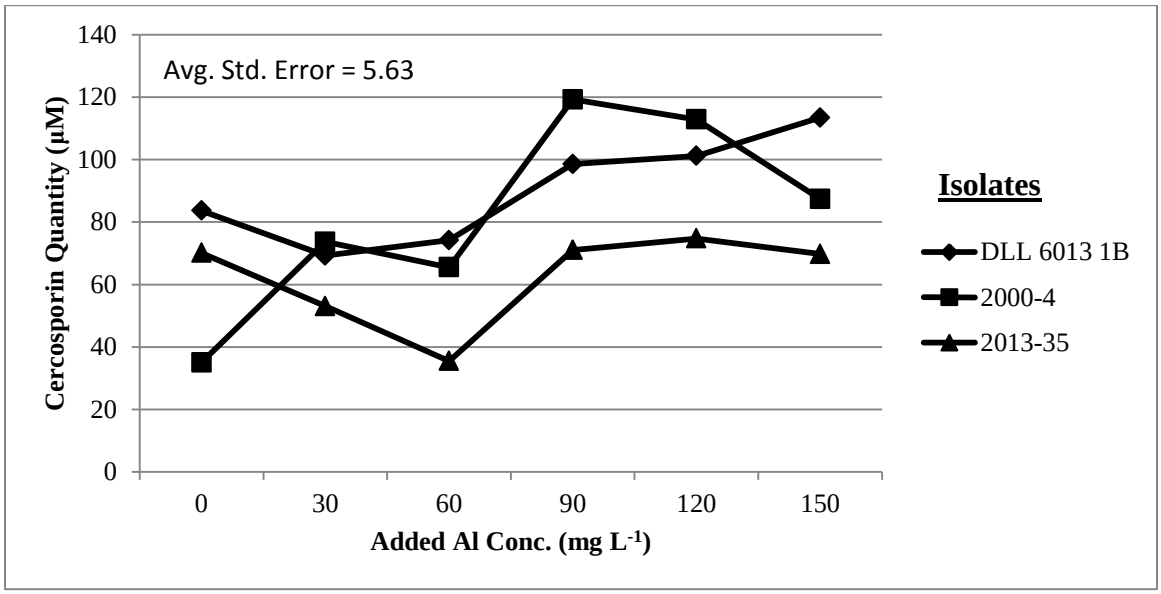
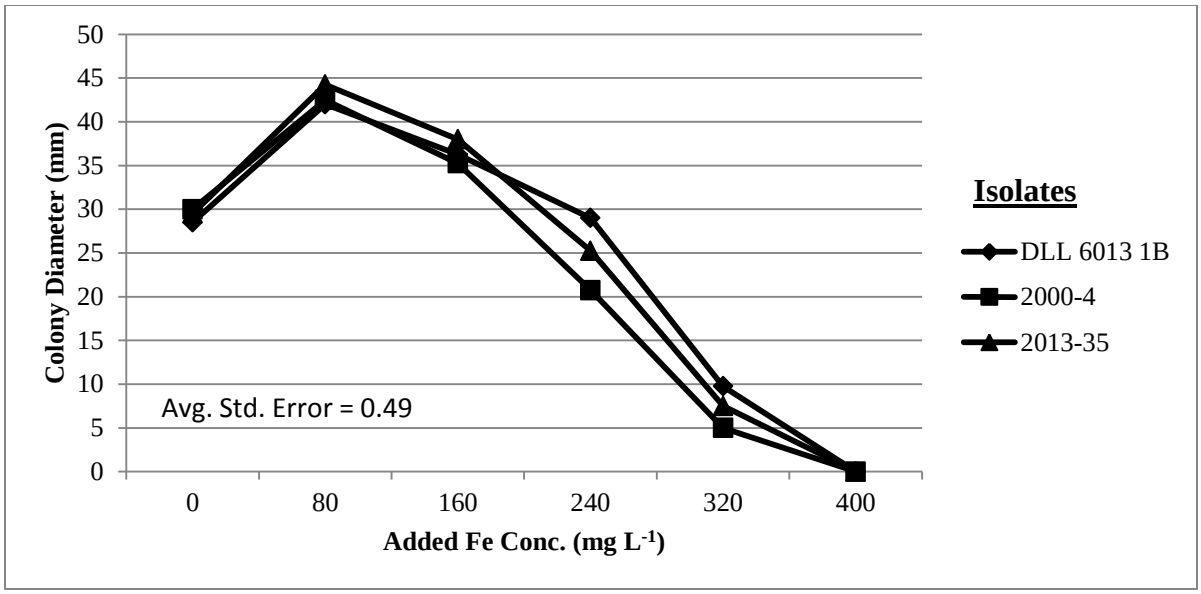


Figure 3.9: Growth of *Cercospora kikuchii* and cercosporin concentration measured at 10 days after inoculation on potato dextrose agar amended with increasing aluminum chloride hexahydrate ($AlCl_3 \bullet 6H_2O$) concentrations while pH was kept at 5.45 via additions of 1 M NaOH. Data points represent mean of four replicates for each of three isolates. Basal levels of aluminum in control plates were about 0.24 mg L^{-1}.



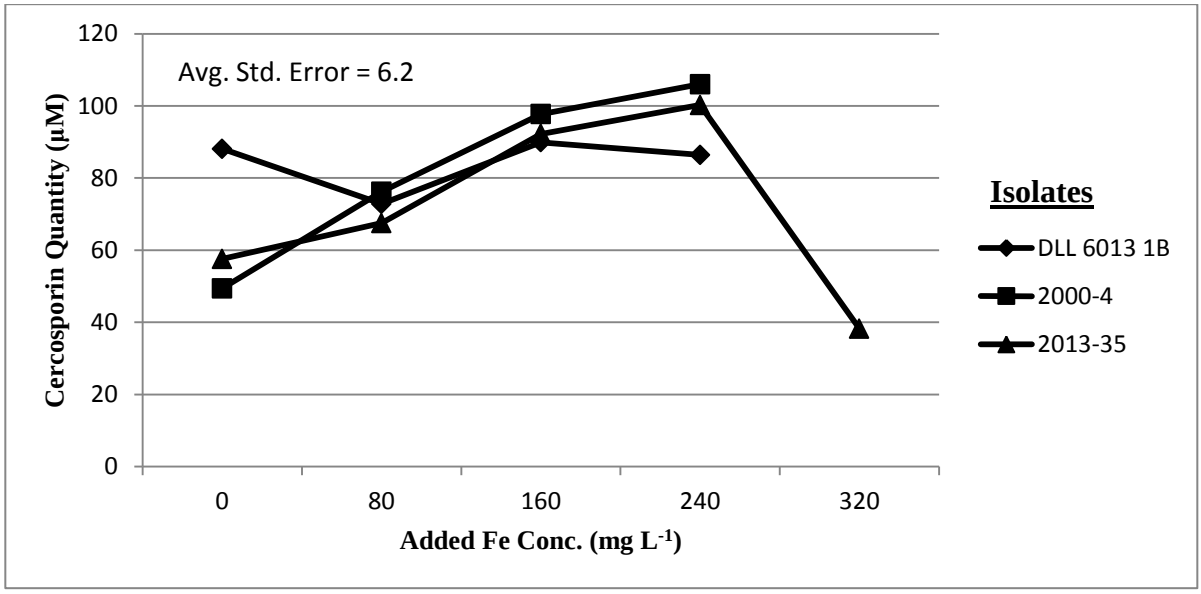


Figure 3.10: Growth of *Cercospora kikuchii* and cercosporin concentration measured at 10 days after inoculation on potato dextrose agar amended with increasing iron sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) concentrations while pH was kept at 5.45 via additions of 1 M NaOH. Data points represent mean of four replicates for each of three isolates. Basal iron levels in control plates were about 2.7 mg L^{-1}.

Regardless of iron formulation, fungal growth increased by about 15 mm (approximately 50% more than control) at about 80 mg L^{-1} (Figs. 3.10 and 3.11). Fungal growth then declined at iron concentrations above 80 mg $L^{-1}$and reached basal levels at about 200 mg $L^{-1}$for iron sulfate heptahydrate and 260 mg $L^{-1}$for iron chloride hexahydrate. Growth ceased at 400 mg $L^{-1}$with iron sulfate (Fig 3.10) and was inhibited by about 40% by iron chloride (Fig. 3.11). Cercosporin production increased with iron chloride in two of the three isolates as compared to

controls. Cercosporin production with iron sulfate followed the same trend before declining precipitously at about 320 mg L^{-1}iron. Figure 3.18 depicts a separate experiment with iron sulfate with smaller increments. The trends were repeated with both growth and cercosporin production initially increasing before decreasing.

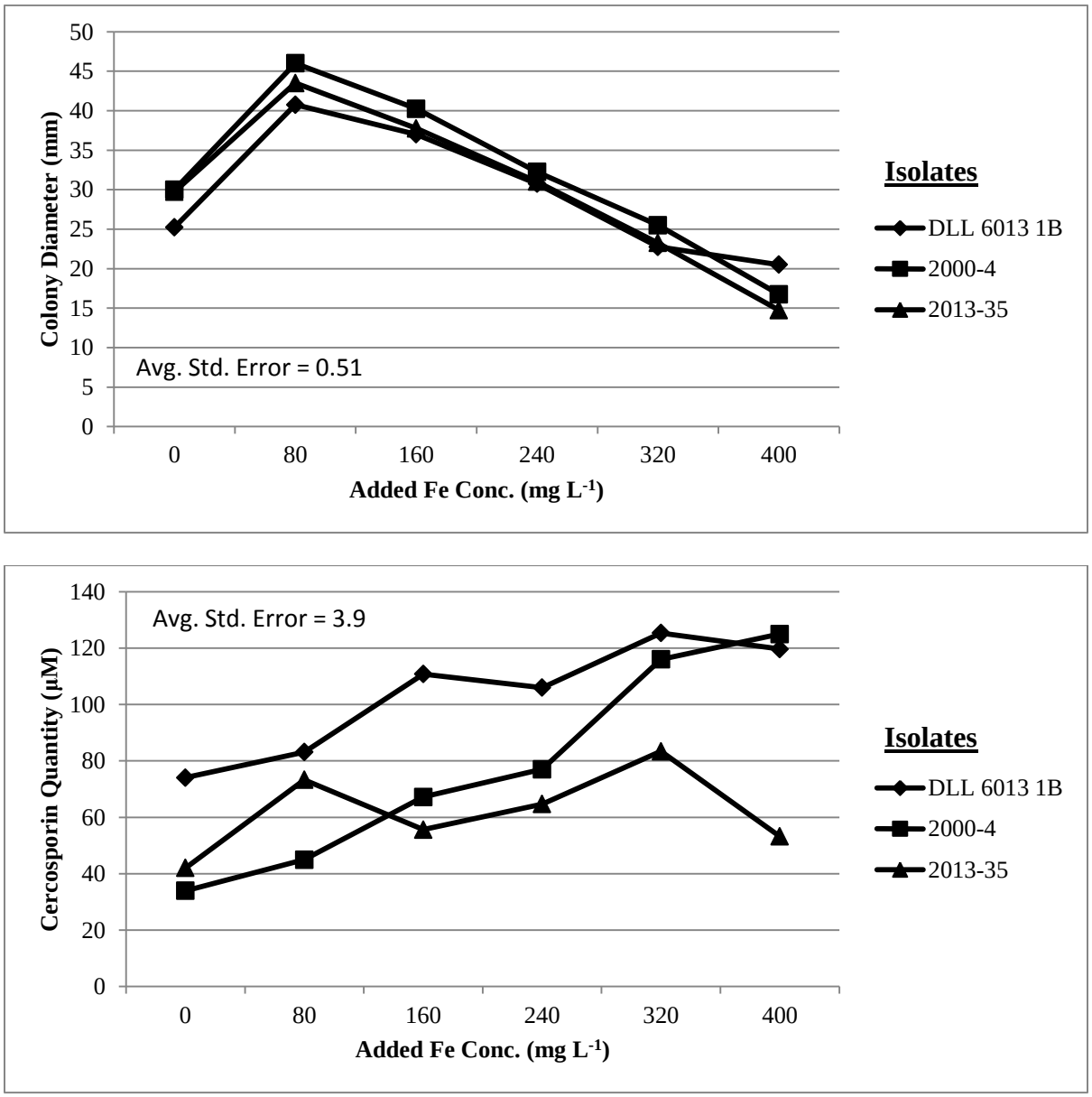
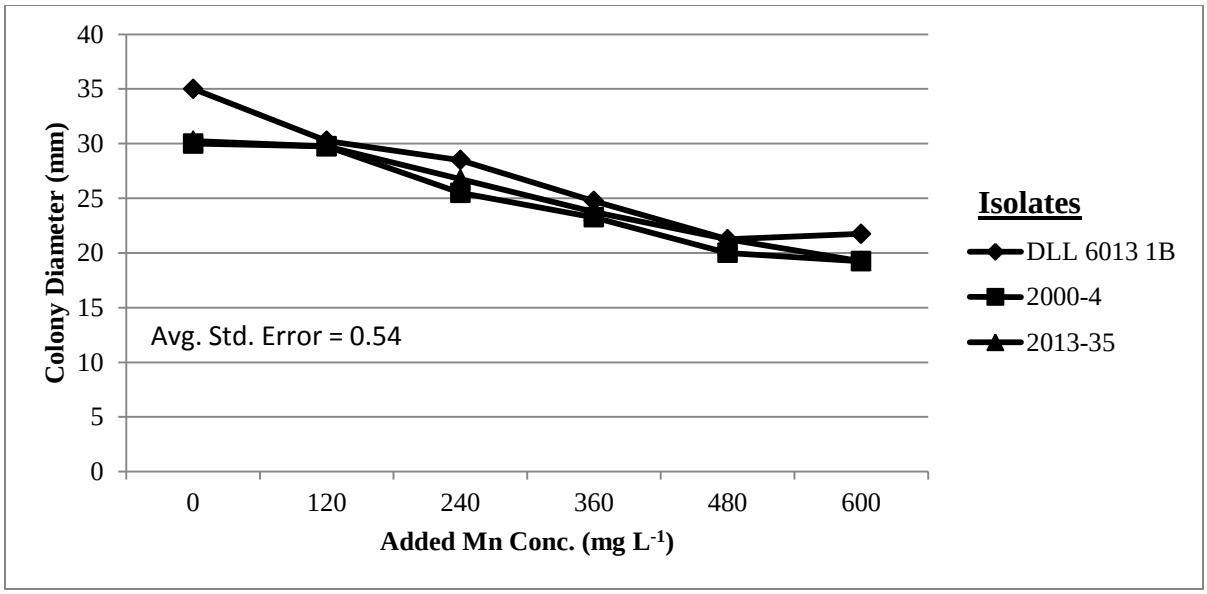


Figure 3.11: Growth of *Cercospora kikuchii* and cercosporin concentration measured at 10 days after inoculation on potato dextrose agar amended with increasing iron chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) concentrations while pH was kept at 5.45 via additions of 1 M NaOH. Data points represent mean of four replicates for each of three isolates. Basal iron levels in control plates were about 2.7 mg L^{-1}.



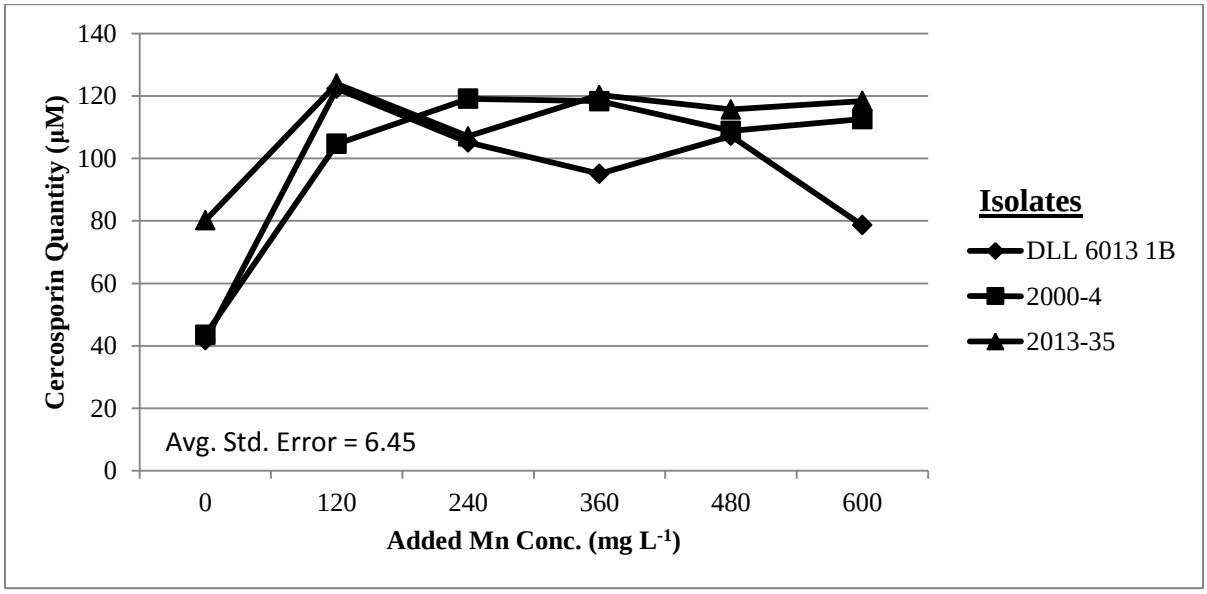
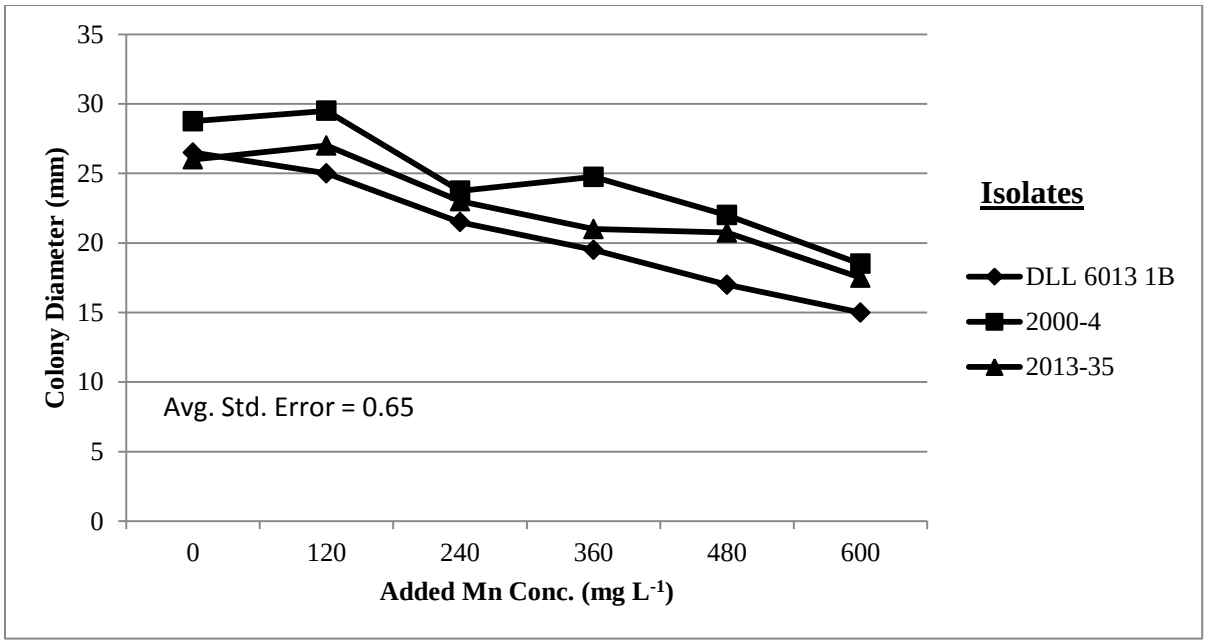


Figure 3.12: Growth of *Cercospora kikuchii* and cercosporin concentration measured at 10 days after inoculation on potato dextrose agar amended with increasing manganese sulfate monohydrate ($MnSO_4 \bullet H_2O$ concentrations while pH was kept at 5.45 via additions of 1 M NaOH. Data points represent mean of four replicates for each of three isolates. Basal manganese levels in control plates were about 0.1 mg L^{-1}.

Both formulations of manganese resulted in slight inhibition (about 30%) of fungal growth at the highest rates (Figs. 3.12 and 3.13), while initially boosting toxin production by 30% to 200% depending on isolate and formulation. The toxin increase plateaued at about 120 mg $L^{-1}$for most isolates and did not change drastically from this elevated amount.



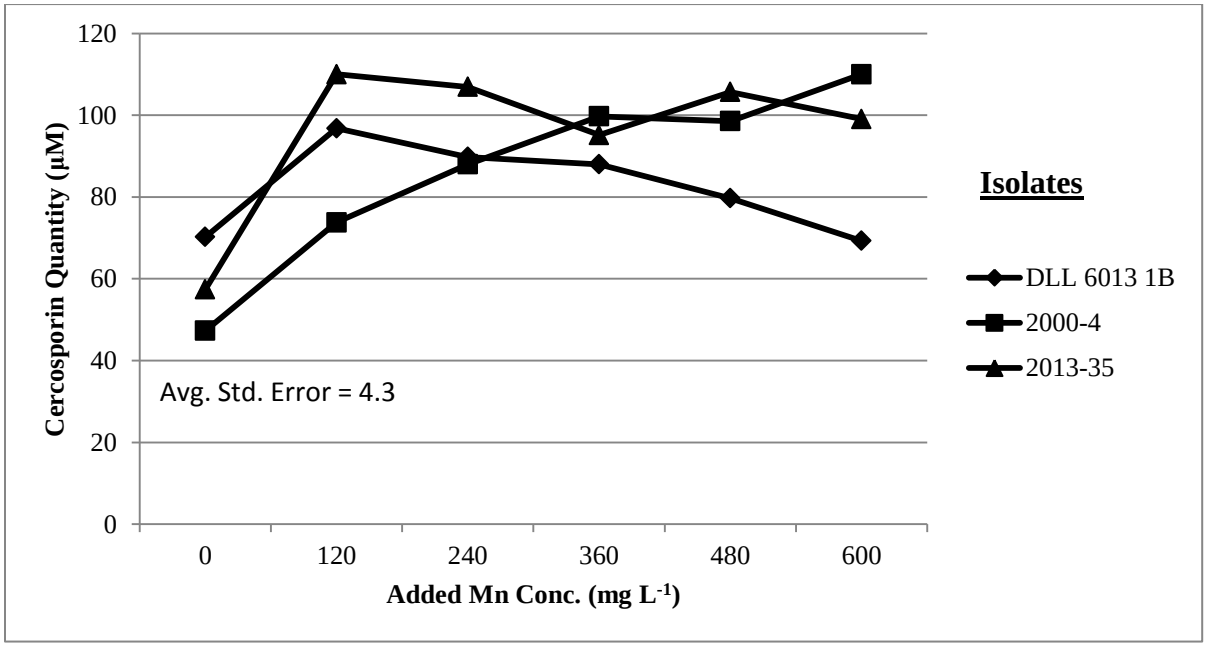


Figure 3.13: Growth of *Cercospora kikuchii* and cercosporin concentration measured at 10 days after inoculation on potato dextrose agar amended with increasing manganese chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$) concentrations while pH was kept at 5.45 via additions of 1 M NaOH. Data points represent mean of four replicates for each of three isolates. Basal manganese levels in control plates were about 0.1 mg L^{-1}.

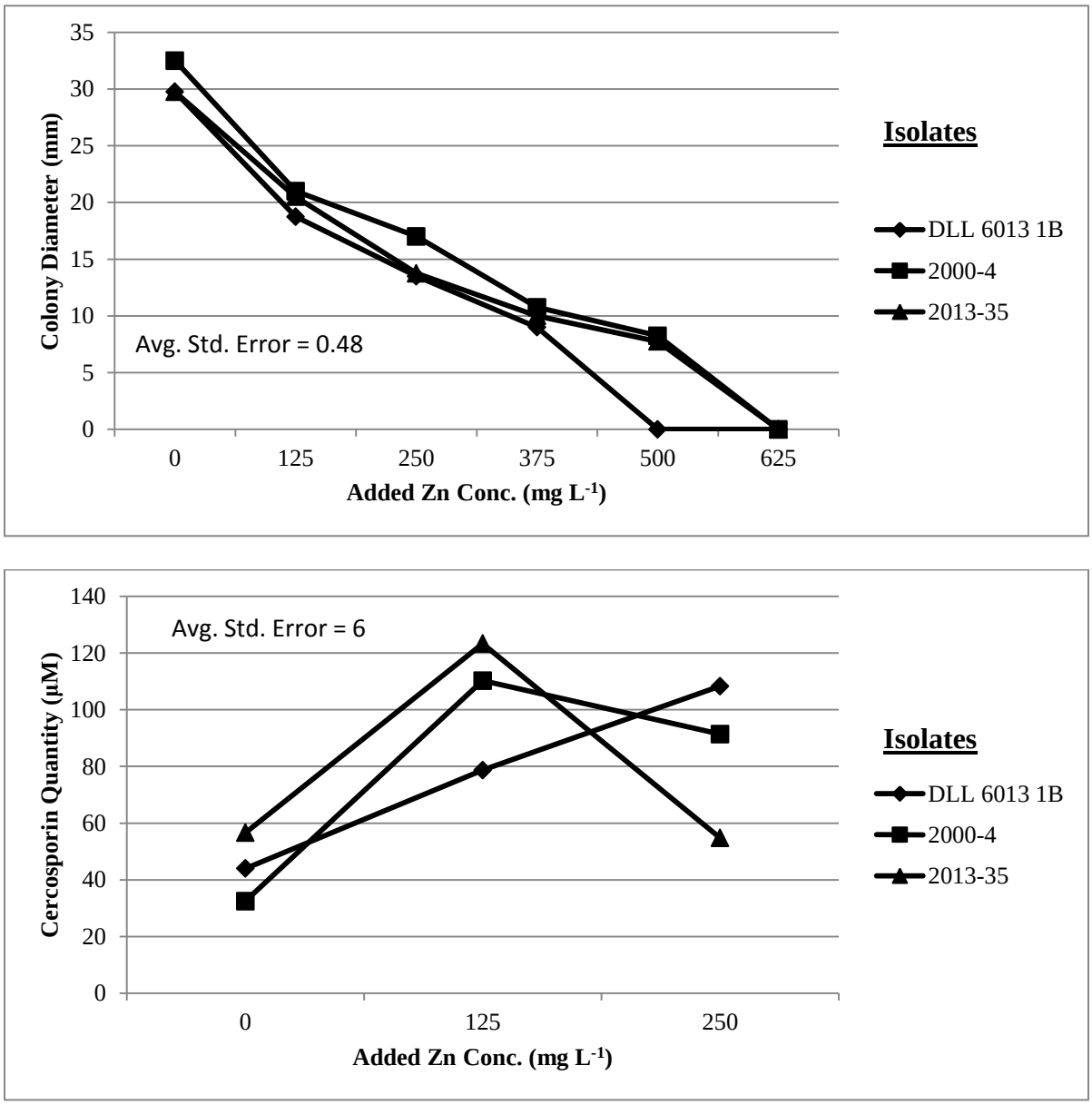
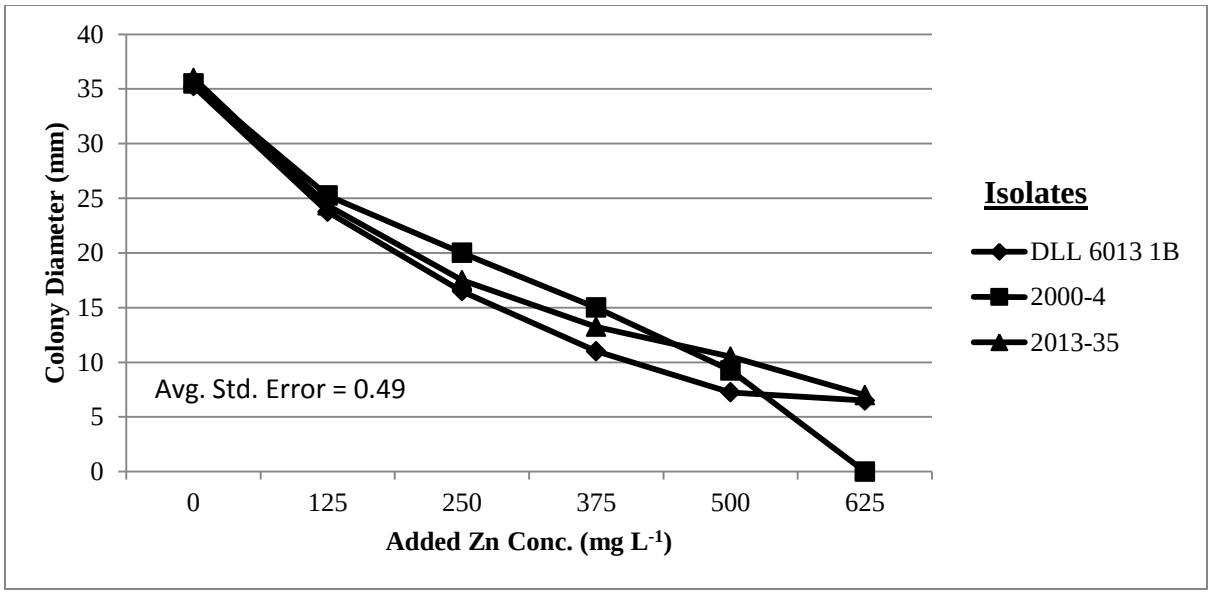


Figure 3.14: Growth of *Cercospora kikuchii* and cercosporin concentration measured at 10 days after inoculation on potato dextrose agar amended with increasing zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) concentrations while pH was kept at 5.45 via additions of 1 M NaOH. Data points represent mean of four replicates for each of three isolates. Basal zinc levels in control plates were about 0.27 mg L^{-1}.

Both formulations of zinc inhibited fungal growth approximately equally (Figs. 3.14 and 3.15). While measuring cercosporin production proved difficult because of the smaller colony size, there was a trend similar to manganese in that toxin production initially plateaued (Figs. 3.12 and 3.13). Unlike manganese, toxin production declined above 250 mg L^{-1}.



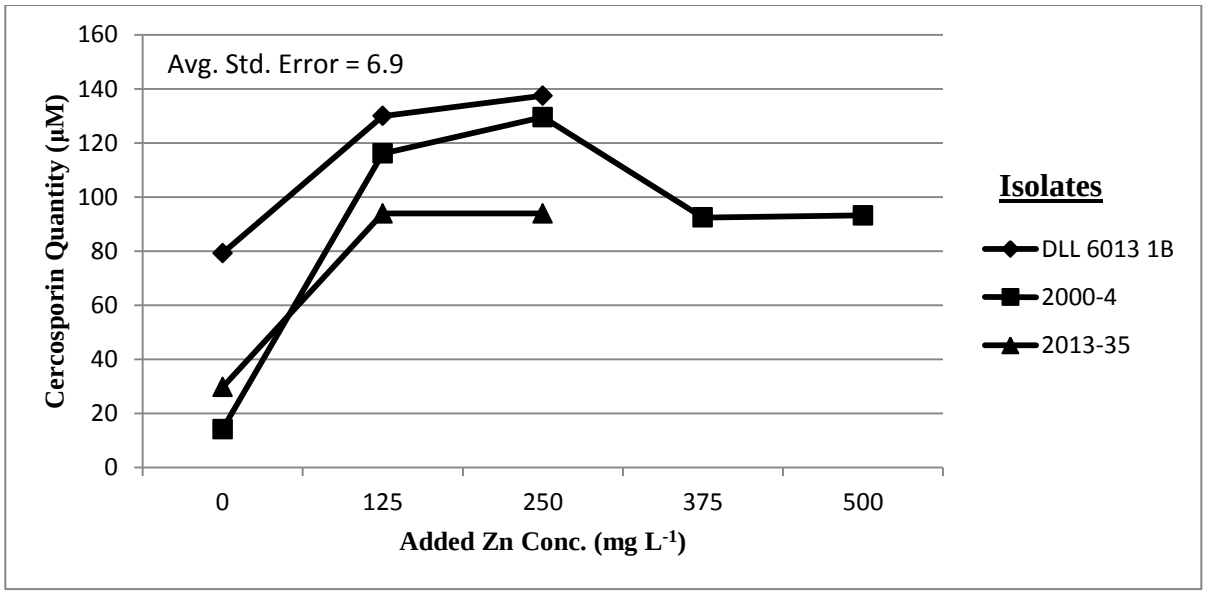
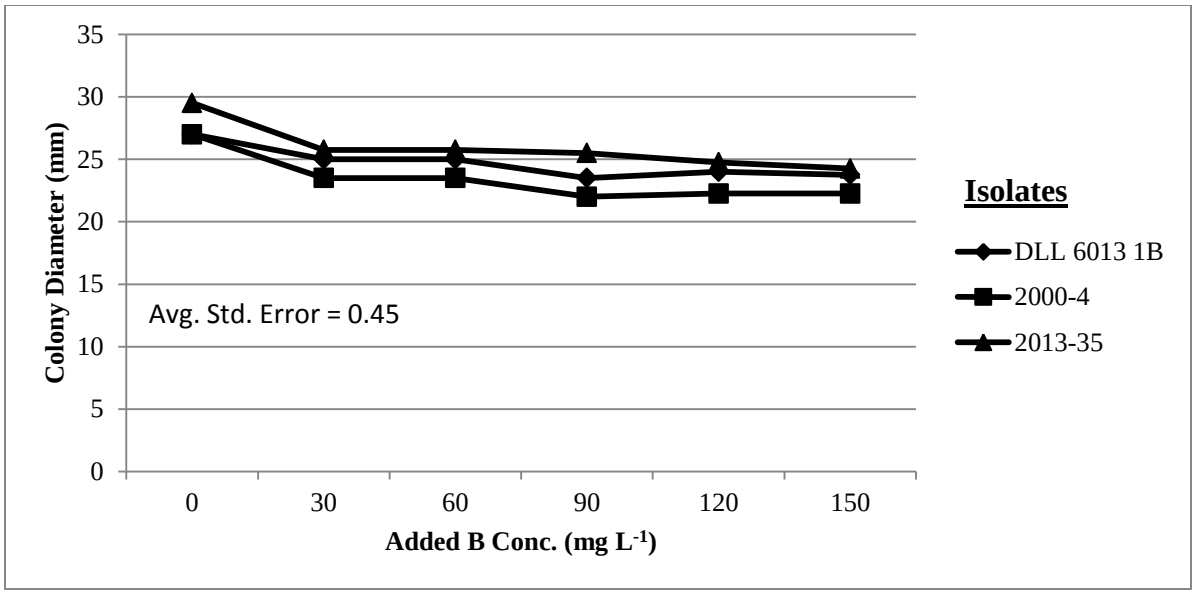


Figure 3.15: Growth of *Cercospora kikuchii* and cercosporin concentration measured at 10 days after inoculation on potato dextrose agar amended with increasing zinc chloride ($ZnCl_2$) concentrations while pH was kept at 5.45 via additions of 1 M NaOH. Data points represent mean of four replicates for each of three isolates. Basal zinc levels in control plates were about 0.27 mg L^{-1}.

Boron was selected for further testing because of early field results, but *in vitro* results did not appear to show any effect on growth or toxin production except for an initial 16% decrease in growth as concentrations approach 30 mg L^{-1}boron (Fig. 3.16). Toxin production declined slightly in two isolates with increasing boron concentration, but with one isolate there was a slight increase in cercosporin production with increasing concentrations of boron.



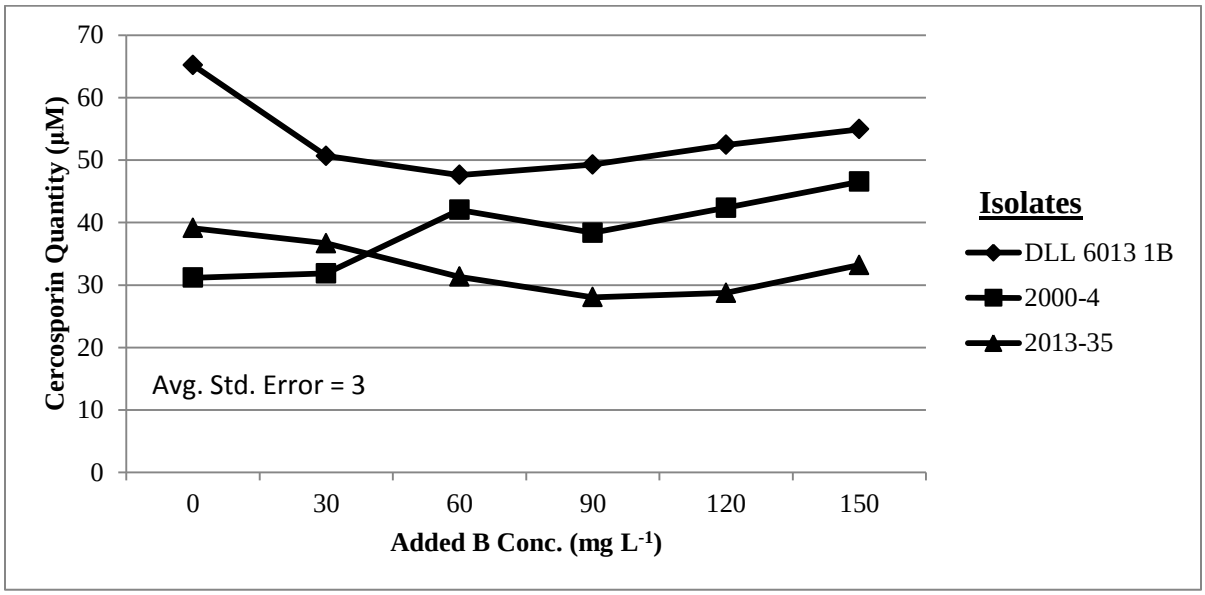


Figure 3.16: Growth of *Cercospora kikuchii* and cercosporin concentration measured at 10 days after inoculation on potato dextrose agar amended with increasing boric acid (H_3BO_3) concentrations while pH was kept at 5.45 via additions of 1 M NaOH. Data points represent mean of four replicates for each of three isolates. Basal boron levels in control plates were about 2.75 mg L^{-1}.

Additions of copper to media resulted in a growth increase for the fungus of 16 to 60%, depending on the isolate, but cercosporin production was not affected until copper concentration reached 35 mg L^{-1} (Fig. 3.17). It should be noted that, with two of the three isolates, toxin production increased at 28 mg L^{-1}. However, all three isolates showed a marked decrease in production at 35 mg L^{-1}.

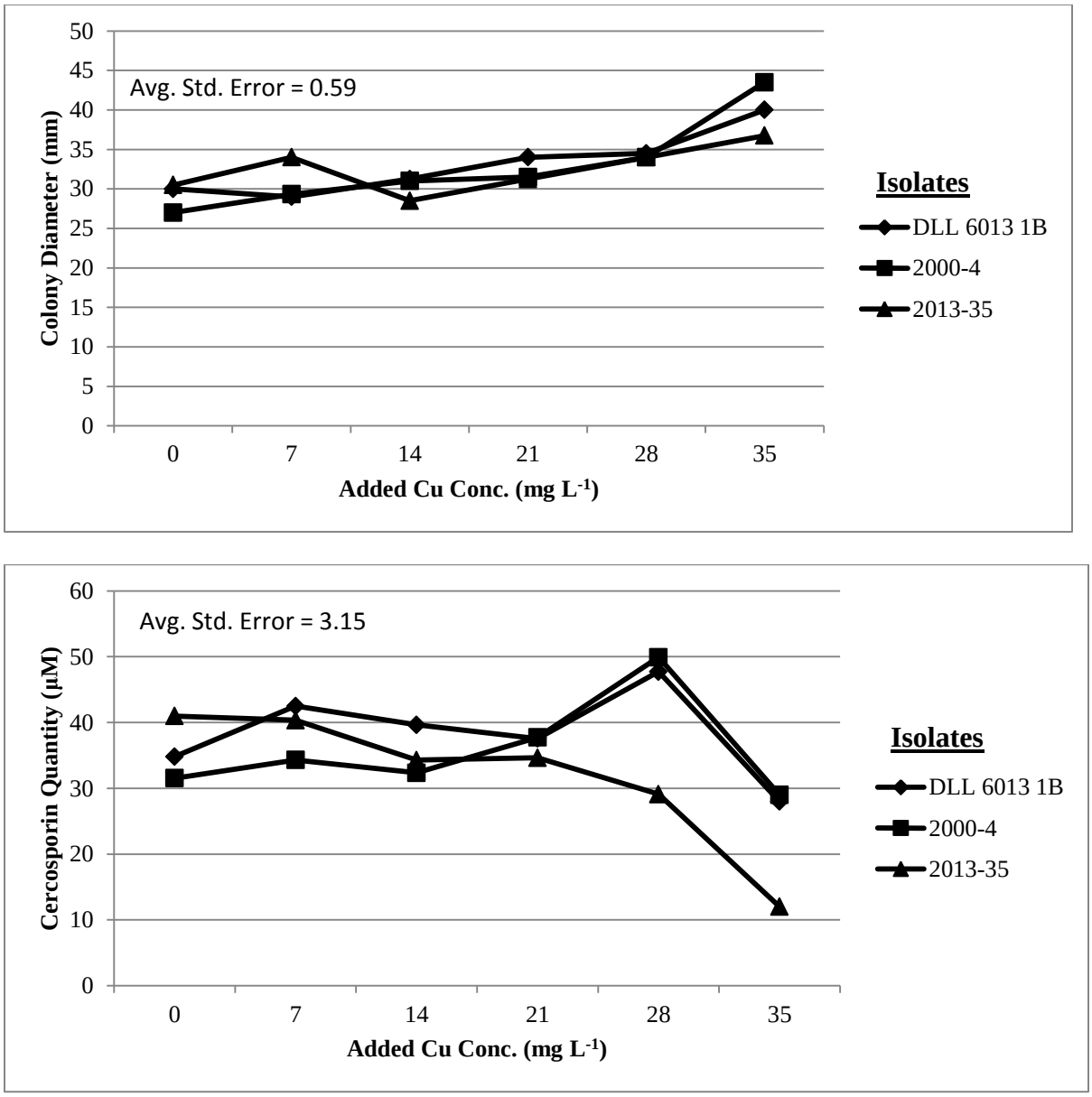


Figure 3.17: Growth of *Cercospora kikuchii* and cercosporin concentration measured at 10 days after inoculation on potato dextrose agar amended with increasing copper sulfate pentahydrate ($CuSO_4 \bullet 5H_2O$) concentrations while pH was kept at 5.45 via additions of 1 M NaOH. Data points represent mean of four replicates for each of three isolates. Basal copper levels in control plates were about 0.09 mg L^{-1}.

A subsequent experiment was conducted with iron sulfate with more emphasis on the 0 – 200 mg L^{-1}range to better document the curve of facilitation and inhibition as shown in Figs. 3.10 and 3.11. Results are shown in Figure 3.18.

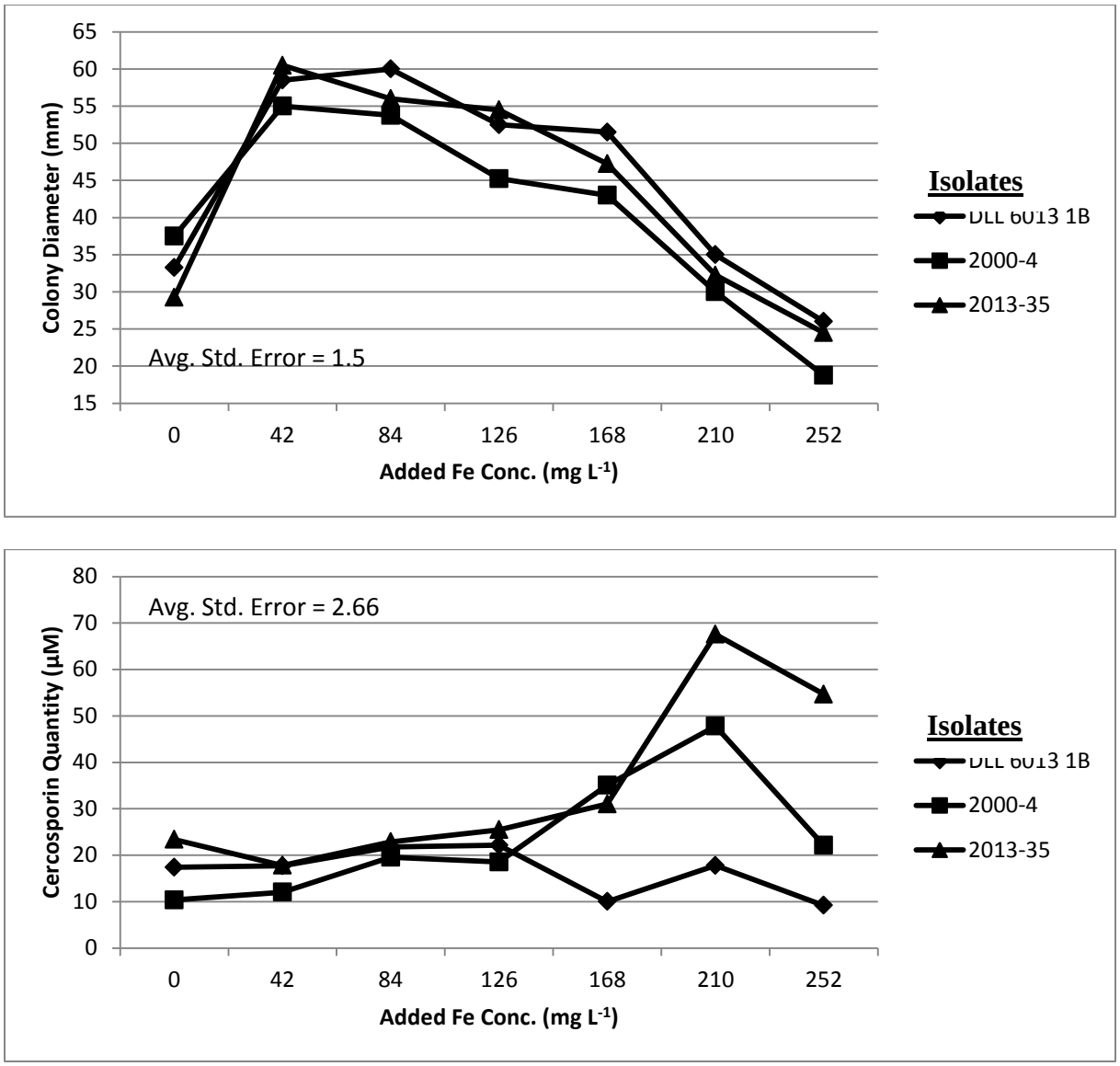


Figure 3.18: Growth of *Cercospora kikuchii* and cercosporin concentration measured at 10 days after inoculation on potato dextrose agar amended with increasing iron sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) concentrations while pH was kept at 5.45 via additions of 1 M NaOH. Data points represent mean of four replicates for each of three isolates. Basal iron levels in control plates were about 2.7 mg L^{-1}.

At roughly 170 mg L^{-1} of iron, *C. kikuchii* growth began to decline as compared to the optimal iron treatments at 42 – 84 mg L^{-1} (Fig. 3.18). Simultaneously, in two of the three isolates, cercosporin production more than doubled. With the third isolate, the increase was less pronounced and did not appear to take effect until closer to the 210 mg L^{-1} range.

With increasing concentrations of iron, a black pigment replaced the normal purple pigmentation of the fungus (Figs. 3.19 and 3.20). Cercosporin was still present as it could be

measured via spectrophotometry (Figs. 3.10 and 3.11); however it did not appear to diffuse as far into the media.

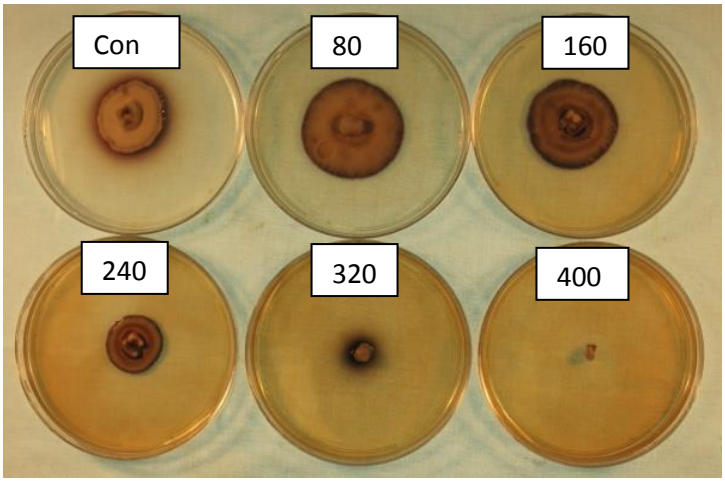


Figure 3.19: Effects of iron sulfate heptahydrate (mg L^{-1} of iron given) on radial growth of *Cercospora kikuchii* and on color of colonies. Control plate is upper left with increasing mg L^{-1} from left to right. Corresponds to isolate DLL 6013 1B in Fig. 3.10.

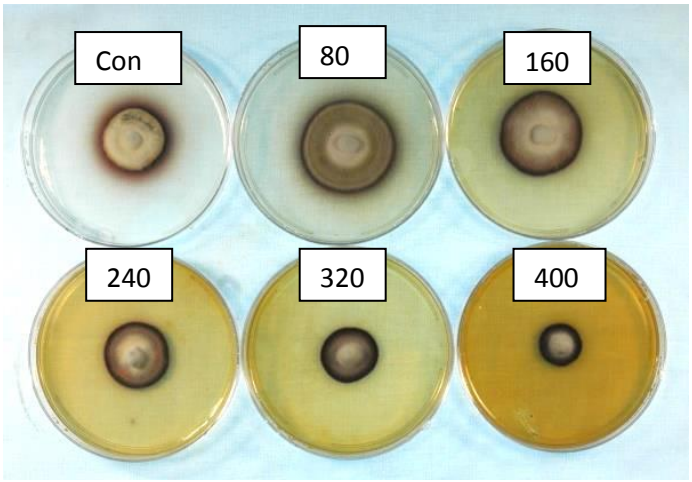


Figure 3.20: Effects of varying rates of iron chloride hexahydrate (mg L^{-1} of iron given) on radial growth of *Cercospora kikuchii* and on color of colonies. Control plate is upper left with increasing mg L^{-1} from left to right. Corresponds to isolate DLL 6013 1B in Fig. 3.11.

At concentrations above 90 mg L^{-1} aluminum, a yellow pigment was produced in lieu of normal purple pigmentation (Fig. 3.21). Not all isolates produced this response and different isolates produced this response with either aluminum sulfate or aluminum potassium sulfate. Figure 3.22 shows three representative examples of the pigment shifts between normal growth

(top), black pigment produced in iron treamtments (bottom left), and aluminum treatments (bottom right). These plates should not be used to compare growth habit among treatments.

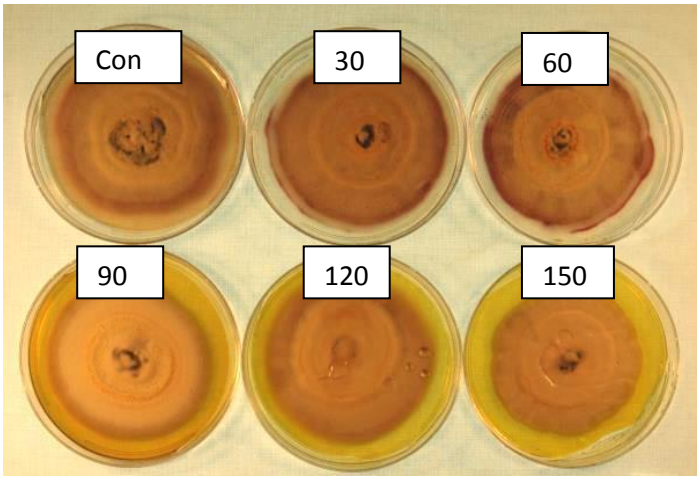


Figure 3.21: Effects of varying rates of aluminum sulfate (mg L^{-1} of aluminum given) on radial growth of *Cercospora kikuchii* and on color of colonies. Control plate is upper left with increasing mg L^{-1} from left to right. This figure corresponds to isolate 2013-35 in the Fig. 3.8.

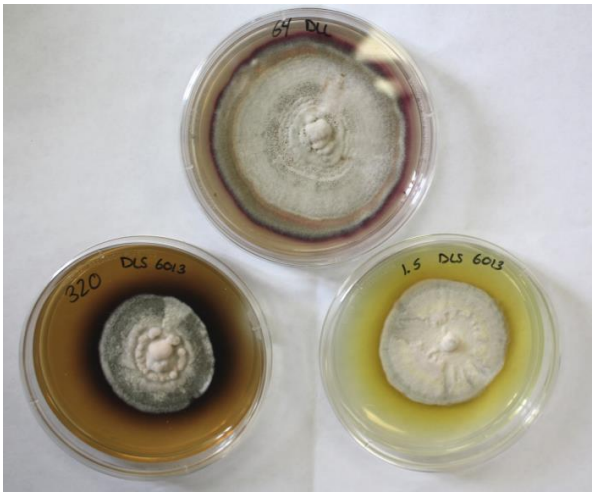


Figure 3.22: Different isolates of *Cercospora kikuchii* on potato dextrose agar (PDA) (top), PDA amended with iron sulfate at 160 mg L^{-1} Fe (left) and aluminum sulfate at 150 mg L^{-1} Al (right). The most exemplary isolate of each treatment was selected for this figure; it is only for reference of color production.

Chapter 4: Conclusions and Discussion

There are several possibilities that could be causing the sometimes sporadic field results seen throughout this study. One possibility is that the most effective elements applied are simply compensating for a deficiency wherever the experiment was located that year. The soil analysis conducted by STPAL of the Central Research Station (Supplemental Fig. 2) did not indicate any deficiencies in the field that was used most years, so at least for these it is unlikely that deficiencies occurred. Another possibility is variable foliar uptake of the elements, which were applied with a boom sprayer. Whether the elements were transported into the leaves in sufficient concentrations would ultimately affect the observable results. One area being explored is using carriers to aid in transport of these nutrients into foliar tissue. Soybean cultivars also were limited to what was commercially available each year. Different cultivars may have different nutritional needs and susceptibilities.

Disease ratings were often highly variable, and in many instances differences were not sufficient to designate them as significantly different with a 95% level of confidence. However, some trends can be observed from the disease ratings. Soybeans treated with manganese formulations had a tendency to have exacerbated CLB symptoms (Figs. 2.3 and 2.7). This trend was not present for frogeye leaf spot (Fig. 2.11). One possible explanation for this discrepancy is that *C. kikuchii* produces cercosporin whereas *C. sojina* does not. Manganese can be oxidized and become very toxic. Some pathogens, such as *Gaeumanomyces graminis*, utilize this phenomenon for pathogenicity (Thompson et al., 2006). Perhaps, cercosporin is able to oxidize manganese to achieve a similar effect. It is also possible that *C. kikuchii* is capable of oxidizing manganese without the aid of cercosporin. High rates of iron decreased CLB severity, but as the rate decreased, the severity increased up to control levels. This effect also was observed *in vitro* (Figures 3.6 and 3.10). From these results, we may conclude that iron sulfate is either more toxic or more available to the fungus than iron chloride as the same trends are apparent for both salts (Figs. 3.10 and 3.11). The sulfate formulation also followed this trend with regards to zinc (Figs. 3.14 and 3.15). Aluminum also suppressed CLB at high levels (Figs. 2.3 and 2.7). Much like iron, as rates decreased the severity increased (Fig. 2.7). Finally, all rates of copper reduced CLB severity. In the first year of the study, manganese was consistently shown to increase CLB severity (Fig. 2.3), but this was not repeated in the second year (Fig. 2.7).

Soybean rust also was reduced with applications of iron and aluminum (Figs. 2.4 and 2.9). Low rates of aluminum decreased soybean rust (Fig. 2.16). This finding is reversed from what was observed for CLB (Fig. 2.14). High rates of iron appeared to reduce rust severity, but the gradient effect from varying rates was not as clearly pronounced (Fig. 2.15). In 2012 (Fig. 2.4), zinc was effective at reducing SBR, but was not observed to do so in 2013 (Fig. 2.9). Alternatively, in 2013 boron was effective in reducing rust severity but was not observed to do so in 2012. The aluminum gradient is also present in 2012; the highest rate of aluminum exacerbated disease, while the lower rates were better at reducing disease.

Frogeye leafspot severity was suppressed by the application of iron and boron (Fig. 2.11). Other elements such as copper and zinc also showed beneficial results but were not significant at $P = 0.068$. One possible explanation for observed of differences is that, since *C. sojina* primarily infects via spores on the leaf surface, making the surface inhospitable by coating with trace metals could prevent initial conidial germination and infection. This line of investigation deserves further attention. *Cercospora kikuchii* would not be hindered by this mechanism as it has an endophytic lifestyle and could escape possibly toxic materials on the leaf surface by either infecting the plant very early or being present since seed germination (Chanda et al. 2014).

The varying effects seen from copper, zinc and iron in these disease systems may be attributed to the different forms of superoxide dismutase (SOD) in plants that require different metal cofactors for functionality (Alscher et al. 2002). Indeed, while copper, iron, manganese, and zinc are known to function differently with different SODs in plants, the phenomenon has not been thoroughly investigated in fungi.

There does appear to be some basis for minor element nutrition affecting not only CLB, but also SBR and frogeye leaf spot. However, there were no significant changes in yield for any of the years where it was measured. The primary requirement for disease management practices is that when used, the practice should return at least as much in value that the practice cost. This trend of disease reduction but similar yield returns was also prevalent in other studies such as those by Simoglou and Dordas, 2006, who, despite finding that foliar elements applied to wheat reduced disease severity for tan spot, there were no effects on yield. Ultimately, this is unfortunate as the primary end goal of disease management is yield increases. Since there were no measurable differences in yield through use of these minor element foliar sprays, these management practices may not be practical for controlling disease on their own. It is of course possible that the relatively small plot sizes that were used in these studies precluded the detection of yield differences. For example, a 10% increase in yield, equivalent to 200 to 350 kg ha^{-1} (3 to 5 bu A^{-1}) in these studies, would be considered a major technological achievement. However, this study did not test the effects of these elements when applied in tandem with fungicides, nor did it test commercial formulations of these minor elements which often are applied with carriers, nitrogen sources, or sugars.

Results from the amended media experiments were clearer. Potato dextrose agar supplemented with both iron and zinc drastically reduced fungal growth as concentrations reached the higher end of possible physiological concentrations (Figs 3.10, 3.11, 3.14, and 3.15). Furthermore, this was consistent across both formulations of iron and zinc, although the sulfate forms appeared more available and/or toxic. Inversely, as concentrations of both of these elements increased, there was a peak in cercosporin production followed by a decline. This peak coincided with the onset of a decrease in colony growth, and this reduced growth may have been responsible for the reduced production of cercosporin.

Both formulations of manganese slightly impaired fungal growth (Figs. 3.13 and 3.14); however, there was a marked increase in cercosporin production. This increase in cercosporin production may be the cause for the exacerbation of CLB severity when manganese was applied to the plant (Fig. 2.3); although this was not significant in 2013 (Fig. 2.7). While aluminum, iron, and zinc elicited an increase in cercosporin production, the color of cercosporin in the medium was different (Fig. 3.22). For iron, cercosporin shifted to a dark red to black (Fig. 3.19 and 3.20). Under aluminum treatments, a yellow pigment appeared (Fig. 3.21), while under zinc treatments either a yellow or green hue was observed. These color shifts could indicate cercosporin binding to the metal in question, as has been previously reported for iron (Cavallini et al. 1979), or a chemical reduction similar to that reported with zinc (Yamazaki and Ogawa 1972). The color changes also could be an intermediary of cercosporin production or another isomer of the toxin as reported in the same paper. Finally, the observed color changes could be a mixture of two or more compounds. The increase in cercosporin under manganese treatments is not accompanied by a color change. As a side note, when cercosporin is extracted with 95% ethanol (Mumma et al. 1973), regardless of the color produced by the fungus or element in the agar, the solution appears red.

Zinc was toxic to the fungus starting as low as 125 mg L^{-1} in agar. Cercosporin production at this concentration increased, but the colony becomes too stunted to accurately measure cercosporin as the zinc concentrations rise. Along with the reduced growth, colonies on zinc amended media tended to take on a green hue along with the agar turning green around the colony. This may be a reduced form of cercosporin as other organisms that biodegrade cercosporin also elicited a green hue (Mitchell et al. 2002). Aluminum had the opposite trend. Fungal growth and cercosporin production slowly rose as aluminum sulfate concentration was increased (Fig. 3.8). Aluminum chloride did not bolster fungal growth but still increased cercosporin production in some isolates (Fig. 3.9). These results were substantiated in the field tests of CLB (Figs. 2.3 and 2.14). Further work should be done to determine effects of aluminum interacting with iron uptake in fungi (Illmer and Buttinger 2006), especially if siderophores are produced by *C. kikuchii*. The aluminum treatments also tended to turn media yellow (Figures 3.20 and 3.21), which begs further investigation.

The only formulation of boron tested in the *in vitro* study, H_3BO_3, slightly inhibited fungal growth (Fig. 3.16); however, it appeared to have no effect on cercosporin production. Molybdenum formulations had a slight inhibitory effect as well (Fig.3.5). However, the concentrations required to induce this response were much higher than can be practically achieved in soybean tissue. Because of this, molybdenum was not carried forward in further experiments.

Copper was reported to drastically increase cercosporin yield at 2 – 5 mg L^{-1} with *C. beticola* (Lynch and Geoghegan 1979). Their study was conducted using a defined medium containing many salts and amino acids. However, results from the present work indicated this is not the case with *C. kikuchii*. Only two of the isolates showed an increase in cercosporin

production, and this only occurred at about 28 mg L^{-1}. Furthermore, cercosporin concentration rapidly decreased for all three isolates as copper concentrations approached 35 mg L^{-1}. This reduction in cercosporin production could explain the effects observed in the field studies where disease severity was much less in copper-sprayed plots. Copper has been proven to have biocidal effects in a large number of microorganisms and has been shown to decrease plant disease in a wide array of disease systems (Evans et al. 2007); the most common mechanism of which is the direct toxicity to the pathogen. It should be noted that even at these concentrations, fungal growth was not hindered *in vitro*. Lynch and Geoghegan (1979) also found that 20 mg L^{-1} additions of ferrous (and to a lesser extent ferric) iron drastically increased cercosporin production, which was validated in this study up to 80 mg L^{-1} additions.

Reagent grade elements are difficult to obtain and utilize when not in an inorganic salt form, such as a sulfate or chloride. However, both sulfur (Haneklaus et al. 2007) and chloride (Elmer 2007) have been shown to affect (and alleviate) some plant diseases. It was for this reason that many elements of interest were applied in more than one form: to attempt to deduce the contributing element. Aluminum, copper, and iron treatments on rust and CLB did not appear to favor one of the formulations over another in regard to disease severity. This hints that it is the metal component of the salts which is responsible for the observed results. In the one year of frogeye leaf spot, it did appear that the sulfate forms were much more efficacious than the chloride versions (Fig. 2.11).

One possible explanation for the phenomenon observed in this study of decreased growth and increased cercosporin production is siderophore-like activity. Siderophores (Neilands 1995) are compounds produced by microorganisms and some plants to acquire iron from the environment (Crowley et al. 1991, Haas et al. 2008) or from a host (Konetschy-Rapp et al. 1988, Expert 2007). It is possible that the fungus utilized cercosporin to either assist in or exclude iron uptake. Many siderophores also bind to other metals such as copper and zinc (Chaturvedi et al. 2012) and this could also help explain the varying effects observed in this study relating *Cercospora* and metal availability. Siderophores can be used in this way to exclude metals such as aluminum (Roy and Chakrabartty 2000) or cope with toxicities such as copper (Chaturvedi et al. 2012). In the Chaturvedi study, it was determined that this binding ability can be utilized for pathogenicity. Further experiments should be done to determine the metal binding properties of cercosporin; and whether they function in pathogenesis (Lamont et al. 2002, Litwin et al. 1996, Budde and Leong 1989), defense (Leong 1986), antimicrobial (Shah et al. 1992) (especially for biocontrol purposes (Hu and Xu 2011)), nutrient uptake, iron storage (Winkelmann 1992), or exclusionary roles (Roy and Chakrabartty 2000). Moreover, whether the fungus is surviving as an endophyte or on the plant surface could also affect possible iron-siderophore interactions (Loper and Buyer 1990). Results from such studies could lead to a practical method for inhibiting cercosporin, and in doing so, CLB development. Finally, one further line of evidence that supports the siderophore-activity theory of *C. kikuchii* is highlighted in Fig. 3.18. At iron concentrations just before growth begins dropping off, there is a marked and substantial increase

in cercosporin production. This could be a coping mechanism of the fungus trying to either exclude or bind the excess iron to protect itself.

In summation, iron and aluminum were found suppress CLB and SBR, which was corroborated by the *in vitro* trials of *C. kikuchii*. All rates of copper were effective in suppressing CLB development, while low rates of zinc and the high rate of boron were only effective in reducing soybean rust one year each. Zinc showed inhibition of fungal growth *in vitro*, but copper only had an effect on growth at high concentrations, and boron did not have any effect on either growth or cercosporin production. There is promise for these practices to be commercialized to both increase yields through minor element fertilization and simultaneously through disease suppression. The mechanisms by which these elements interact with the fungus and toxin should be further investigated, especially any siderophore-like activity. The changes in cercosporin production, cercosporin appearance, and growth habit of *C. kikuchii* when subjected to these minor elements indicate that there is real promise for more sustainable management strategies in dealing with CLB and other diseases.

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Supplemental Figures and Tables

Supplemental Table 1: List of many economically important *Cercospora* species worldwide, their economically important host, and relevant literature. Not all-inclusive.

Host	Pathogen	Reference
Apples/ Pears	*Mycosphaerella pyri*	Cunningham, H. S. (1928)
Banana	*Cercospora hayi*	Calponzos, L. (1955)
Banana	*Cercospora musae* *Mycosphaerella musicola**	Leach, R. (1941)
Carrot	*Cercospora carotae*	Thomas, H. R. (1943)
Celery	*Cercospora apii*	Fuckel, K. W. G. L. (1863)
Citrus	*Cercospora angolensis***	Brun, J. (1972)
Coffee	*Cercospora coffeicola*	Cooke, M. C. (1880)
Coles	*Cercospora longissima*	Savery, S. (1983)
Corn	*Cercospora zeae-maydis*	Tehon, L. R. and E. Y. Daniels (1925)
Corn	*Cercospora zeina*	Meisel, B. J. (2009)
Cotton	*Cercospora gossypina*	Cooke, M. C. (1883)
Cucurbits	*Cercospora citrullina*	Cooke, M. C. (1883)
Fig	*Cercospora bolleana*	Spegazzini, C. (1879)
Papaya	*Cercospora papayae*	Chupp, C. (1954)
Peanut	*Cercospora arachidicola*	Hori (1917)
Pepper	*Cercospora capsici*	Kirk, P.M. (1982)
Potato	*Cercospora solani*	Feuilleaubois (1880)
Rice	*Cercospora jansaneana* *Sphaerulina oryzina**	Hara, K. (1918)
Sorghum	*Cercospora sorghi*	Ellis, J. B. and B. M. Everhart (1887)
Soybean	*Cercospora kikuchii*	Matsumoto, T. and R. Tomoyasu (1925)
Soybean	*Cercospora sojina*	Hara, K. (1918)
Strawberry	*Cercospora fragariae*	Lobik, A. I. (1928)
Sugar beets	*Cercospora beticola*	Saccardo, P. A. (1876)
Sugar cane	*Cercospora atrofiliformis*	Yen, W. Y., T. C. Lo, et al. (1953)
Sweet Potato	*Cercospora bataticola* *Cercospora ipomoeae**	Harter, L. L. (1929)
Tobacco	*Cercospora nicotianae*	Ellis, J. B. and B. M. Everhart (1893)
Tomato	*Pseudocercospora fuligena***	Roldan, E. F. (1938)

* Sexual stage, anamorph, or synonym

** May not be a true *Cercospora*

Supplemental Table 2: Mean and standard deviation of soil analysis for 2013 field at the Central Research Station. Calculated from four measurements, each comprised of 10 samples from four sections of the field. Concentrations of elements are given in mg kg^{-1}.

	Mean	St. dev. +/-
% Organic Matter	2.0	0.1
pH (1:1 Water)	6.4	0.1
Aluminum	1.2	0.5
Boron	0.5	0.0
Calcium	3,569.5	109.0
Copper	2.4	0.2
Iron	118.4	12.9
Magnesium	582.9	28.7
Manganese	10.8	1.4
Phosphorus	58.6	8.2
Potassium	264.4	6.7
Sodium	29.2	1.6
Sulfur	19.4	1.1
Zinc	2.0	0.1

Vita

Brian Michael Ward was born the first of two sons in July of 1989 in Jefferson, Louisiana, to Keith Brian Ward and Michele Mary Mills Ward followed by his younger brother Kevin Ryan Ward in October of 1992. During their childhood they lived in Albany, Georgia before moving to Jackson, Mississippi in middle school. Brian graduated from St. Joseph Catholic High School in Madison, Mississippi in 2007 and followed in both his parents' footsteps in attending Louisiana State University, graduating with a Bachelor degree in Horticultural Science in 2011. He is currently pursuing his Doctorate Degree in Plant Pathology at Louisiana State University under the continuing guidance of Dr. Raymond Schneider. Among his many hobbies are video gaming, cooking, ballroom dancing, and numerous martial arts.