EFFECT OF FOLIAR FUNGICIDES ON RELATIVE CHLOROPHYLL CONTENT, GREEN LEAF AREA, YIELD AND TEST WEIGHT OF WINTER WHEAT

By

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Abstract: Wheat (*Triticum* spp.) is an important cereal crop susceptible to many foliar diseases that reduce grain production. These diseases can be managed by fungicide application that alleviate grain yield loss from susceptible varieties and can increase grain yield of resistant varieties on occasion. The strobilurins represent a class of fungicides registered as 'plant health' promoters that claim to provide beneficial properties other than disease control such as increased chlorophyll content. Additionally, the strobilurins have been shown to prevent spore germination, which serves to avoid triggering of the hypersensitive response in resistant varieties. As a result, plants maintain green leaf area for greater energy production and increased grain yield. Therefore, the objectives of this research were to determine if fungicide application to resistant varieties i) increased yield and test weight of wheat in the absence of disease in the field by resulting in increased chlorophyll content in flag leaves, and ii) increased chlorophyll content of leaves in the presence of leaf rust in a controlled environment. An irrigated and a dryland field trial planted in fall 2013 with 'Duster' (resistant to foliar diseases) and 'OK Bullet' (susceptible) hard red winter wheat were sprayed with three fungicides (a triazole, a strobilurin, and a combination) at labeled rates. Relative chlorophyll content was determined before and after fungicide application. Fungicides did not increase relative chlorophyll content and did not affect yield or test weight. In controlled environment studies, Duster and OK Bullet plants were sprayed with the same fungicides as used in the field, and inoculated with urediniospores of *P. triticina* (causal fungus of wheat leaf rust). Relative chlorophyll content was determined before and after fungicide application, and after inoculation. Green leaf area was determined at the end of the experiment. In these studies, sprayed-plants of both varieties had the same relative chlorophyll content as the non-sprayed and non-inoculated control plants indicating fungicide did not increase chlorophyll content. However, the early fungicidal action by the fungicides (especially the strobilurin fungicide pyraclostrobin) likely killed spores that prevented the induction of hypersensitive response resulting in greater maintenance of green leaf area. This could explain why resistant varieties occasionally show a yield response to a fungicide applied in the presence of foliar disease.

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CHAPTER I

INTRODUCTION

The United States is among the five top producing countries of wheat grain in the world. Most of the US wheat production is winter wheat (*Triticum aestivum* L.) (FAOSTAT, 2015), which is widely cultivated in the states comprising the Great Plains (National Agricultural Statistics Service [NASS], 2014a). Oklahoma is an important winter wheat producer due to the dual-purpose use of wheat to provide forage for cattle and harvest for grain. Normally, grain production in Oklahoma is approximately 2.7 million tonnes annually (NASS, 2014b).

Foliar fungal diseases pose a constant and severe threat to grain production everywhere wheat is grown. Typically, leaf rust caused by the Basidiomycota fungus *Puccinia triticina* Erikss is considered one of the most important foliar fungal disease in the Great Plains, including Oklahoma, with significant yield losses reported (Kolmer, 2010; United States Department of Agriculture [USDA], 2014). Asexually reproductive spores of *P. triticina*, referred to as urediniospores, constitute the inoculum source for field epidemics from one growing season to the next (Roelfs, 2010). This pathogen grows beneath the epidermal tissue of leaves, which eventually is ruptured. The resulting cavity is called a uredinium (pustule), and is filled with a mass of rust-colored urediniospores. Infected leaves lose water due to tissue disruption directly decreasing photosynthetic capability of infected leaves (Agrios, 2005).

The most economical method to manage fungal foliar diseases, including leaf rust, is planting varieties with genetic resistance. Durable resistance against these foliar diseases often challenges breeding programs due to the pathogen's ability to adapt to resistance genes and render them ineffective (Kolmer, 2013). Another strategy to alleviate yield loss due to foliar fungal diseases is the application of fungicides. The two most important classes of fungicides are the strobilurins and the triazoles (Bartlett et al., 2002). Use of these fungicides results in effective foliar disease control and an accompanying increase in grain yield from susceptible varieties but reports of increased grain yield by resistant varieties following fungicide application also has been observed (Edwards et al., 2012). However, grain yield increases by resistant varieties from one growing season to the next are not consistent (Edwards et al., 2012; Hysing et al., 2012; Kelley, 2001; Ransom & McMullen, 2008).

Recently, improvements in grain productivity have been attributed to fungicidal properties other than disease control, commonly referred to as 'plant health benefits' (PHB). Such claims have been most closely associated with strobilurin fungicides. Studies have shown that strobilurin-sprayed plants demonstrated a shift in hormonal balance that promoted growth, increased greenness, and better tolerance to stresses (Grossmann et al., 1999; Grossmann & Retzlaff, 1997; Köhle et al., 2003).

Although PHB in wheat and other crops has been claimed, these positive benefits are not consistently observed in the field experiments (Edwards et al., 2012; Swoboda & Pedersen, 2009). In contrast to claims of PHB associated with QoI fungicides, others have suggested that these fungicides inhibit spore germination and infection due to early fungicidal activity, which means resistant wheat varieties avoid the triggering of the hypersensitive response against fungal pathogens (Bertelsen et al., 2001; Schöfl & Zinkernagel, 1997). Consequently, these plants retain chlorophyll and green leaf area for more photosynthesis, and energy could be directed to grain production (Bertelsen et al., 2001; Edwards et al., 2012).

The claims of PHB concerned many scientists that fungicides would be applied in the absence of disease, which contradicts the concept of integrated pest management (Brown-Rytlewski & Vincelli, 2009). In addition, some authors indicated that application of fungicide in the absence of disease and/or targeting PHB does not necessarily reflect positive yield increases (Edwards et al., 2012, Ransom & McMullen, 2008; Weisz et al., 2011).

Therefore, the objectives of this thesis research were to determine i) if fungicide application increases yield and/or test weight of winter wheat in the absence of disease in the field by increasing chlorophyll content, and ii) if fungicide application increases chlorophyll content of wheat leaves in the presence of leaf rust in a controlled environment.

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CHAPTER II

REVIEW OF LITERATURE

WHEAT PRODUCTION

Wheat (*Triticum spp*.) is one of the most important cereal crops cultivated in the world. It is considered the most important food crop for human consumption and is a major source of carbohydrates and protein (Gustafson et al., 2009). In 2013, the world produced more than 715 million tonnes (26 billion bushels) of wheat grain. China, India, the United States, and Russia are consistently the biggest wheat producers (FAOSTAT, 2015). The majority of wheat production is represented by common wheat (*T. aestivum* L.), which is utilized primarily in flour, bread and pastry. The second important wheat is durum wheat (*T. durum*), which is used to make pasta (Peña, 2002).

In the United States, wheat is an important crop commodity with approximately 60 million tonnes (2.2 billion bushels) produced annually (FAOSTAT, 2015). Winter wheat (*T. aestivum* L.) accounts for most of the total wheat production in the United States, where it is widely cultivated in the Great Plains. Kansas, Montana, Colorado and Nebraska are currently the biggest winter wheat producers in this region (NASS, 2014a). Oklahoma also is a major winter wheat producer, and is unique in that winter wheat is used both to provide forage for cattle and then harvested for grain. The dual-purpose use of wheat in Oklahoma makes wheat a valuable crop commodity that contributes significantly to Oklahoma's agriculture. Annual grain production in Oklahoma is usually more than 2.7 million tonnes (100 million bushels); however during the last several years, wheat production in Oklahoma has been dramatically impacted by drought and freeze. For example, severe drought during the winter of 2013/spring of 2014 coupled with late freezes in April, 2014, resulted in Oklahoma being ranked 7th in winter wheat production with 1.3 million tonnes (47 million bushels) (NASS, 2014b). This was the lowest grain production in Oklahoma since the 1950s.

FOLIAR DISEASES

A typical annual estimate for total crop yield loss resulting from diseases, insect pests, and abiotic stresses is 30% (Roefls, 2010). For wheat, foliar fungal diseases can cause severe grain yield loss everywhere wheat is grown. Foliar diseases including powdery mildew [*Blumeria graminis* (DC.) Speer f. sp. *tritici* emend. E.J. Marcha], tan spot [*Pyrenophora tritici-repentis* (Died.) Drechsler (*Drechslera tritici-repentis* (Died.) Shoemaker)], Septoria tritici blotch [*Septoria tritici* Roberge in Desmaz. (*Mycosphaerella graminicola* (Fuckel) J. Schrot.)], stripe rust (*Puccinia striiformis* Westernd. f. sp. *tritici* Erikss.), stem rust (*Puccinia graminis* Pers.:Pers. f. sp. *tritici* Erikss. & E.Henn) and leaf rust (*Puccinia triticina* Erikss.) are among the most commonly reported foliar diseases in wheat growing areas. Typically, leaf rust is considered the most important wheat foliar disease because it is widely distributed and adapted to different climates where wheat is grown (Kolmer, 2010). Leaf rust is consistently reported in states comprising the Great Plains, including Oklahoma. Grain yield losses of 50% or more due to leaf rust have been reported (Kolmer, 2010; USDA, 2014).

Classification of *Puccinia triticina* is in the kingdom Dikarya, phylum Basidiomycota, subphylum Pucciniomycotina, Class Pucciniomycetes, Order Pucciniales, and Family

Pucciniaceae (Szabo & Aime, 2008). Rust fungi are obligate parasites, which means they need a living host to obtain nutrients and reproduce. They are also considered specialized parasites because they are capable of infecting limited genera of host species (Agrios, 2005). *P. triticina* is a macrocyclic, heteroecious rust fungus, producing five spore stages and requiring a principal and an alternate host to complete its life cycle (Webster & Weber, 2013). The principal host is common wheat with two alternate hosts listed in the literature, *Thalictrum speciossimum* and *Isopyrum fumarioides*. Neither alternate host is indigenous to North America (Roelfs, 2010). When the alternate host is present, *P. triticina* produces all five spore stages: spermatial, aecial, uredinial, telial and basidial (Webster & Weber, 2013). However, occurrence of all five spore stages is not required for *P. triticina* to produce an epidemic of wheat leaf rust because in the absence of an alternate host, urediniospores serve as the inoculum source from one growing season to the next. Therefore, wheat leaf rust typically relies on clonal reproduction of urediniospores on volunteer and commercial wheat plants to be blown by wind currents from one region to another to start and sustain wheat leaf rust epidemics (Roelfs, 2010).

Urediniospores of *P. triticina* are orange-red to brown colored, one-celled, and dikaryotic with ornamentation on the outer wall. These spores are spread by wind currents and land on the surface of the host (wheat) where they germinate in the presence of a film of water when temperature is between 15^{o} and 25^{o}C (optimum 18^{o}C). When these conditions are fulfilled, urediniospores germinate within 6 to 8 hours (Kolmer, 2010) forming germ tubes that penetrate the host through stomata. Inside the host, hyphae grow intercellularly forming a dense mycelial mass below the leaf epidermis that exerts pressure and eventually ruptures epidermal tissue. A cavity, called a uredinium, is created that contains a mass of rust-colored urediniospores. These structures are referred to as pustules, and are the characteristic symptom produced by rust fungi. With favorable temperature and moisture, *P. triticina* can heavily infect leaves resulting in numerous pustules that increase water loss due to epidermis disruption and that impact photosynthesis. Both of these disruptions result in reduced grain yield and test weight (Agrios, 2005).

GENETIC RESISTANCE

Genetic resistance to manage plant disease is based on growing varieties that have been selected for resistance to various diseases. This is the most economical method for growers to manage a foliar disease such as leaf rust. At least 71 leaf rust genes (*Lr* genes) have been identified, with most of these genes effective in seedling and adult plants (McIntosh et al., 2014). Wheat varieties developed at Oklahoma State University and released by the Oklahoma Agricultural Experiment Station typically have genetic resistance to all or most of these foliar diseases, and are planted widely across Oklahoma every year. 'Duster' and 'Endurance' are the most planted hard red winter wheat varieties released by this program, and they represent 16% and 12% of planted acres in this State, respectively (NASS, 2014c). However, the durability of *Lr* genes often challenges breeding programs due to the pathogen's ability to adapt to resistance genes and render them ineffective. This adaptation can occur in as little as one season (Kolmer, 2013).

The host-parasite interaction in the wheat:leaf rust pathosystem has been extensively studied, and a gene-for-gene interaction in this system has been described (Agrios, 2005). According to Flor (1946), for each gene (*R*) governing reaction in the host there is a corresponding gene in the pathogen governing avirulence (*Avr*). The interaction between the products encoded by these dominant genes confers an incompatible (resistant) reaction. A compatible (susceptible) reaction develops if either of the gene products is absent meaning that one or both of the genes are recessive and hence no product is produced. When both the host and pathogen carry the dominant genes for *R* and *Avr* the hypersensitive response will be triggered in the host (Flor, 1946). The hypersensitive response is a race-specific resistance reaction characterized by death of host cells near the infection site. These cells are programmed to die in order to stop the pathogen's infection and spread since *P. triticina* survives only in living plants. The hypersensitive response produces small chlorotic/necrotic spots in the leaves, called hypersensitive flecks (Agrios, 2005).

CHEMICAL CONTROL

Another strategy to alleviate yield loss and to manage wheat leaf rust and other foliar fungal diseases is the application of fungicides. Synthetic fungicides were first discovered following the expansion of the chemical industry during and after the Second World War. More recently advances during the 1980's and 1990's resulted in development of the triazole and the strobilurin classes of fungicides (Fernandez-Ortuño et al., 2010). These two fungicide classes became important due to their remarkable broad-spectrum activity, and their systemic and eradicant activity against fungal diseases. Strobilurins were first sold in 1996, and in just four years, sales had reached 10% of the market share (Bartlett et al., 2002). In 2005, triazoles and strobilurins held the highest market share of fungicide groups at 20% and 15%, respectively. (McDougall, P., 2006, as cited in Morton & Staub, 2008).

Triazoles belong to the sterol demethylation inhibitors mode of action class of fungicides. Triazole fungicides inhibit the activity of a specific enzyme, C14-demethylase, which is directly involved in the production of sterol. Ergosterol is a type of sterol that is a fundamental compound of fungal membrane structure. Triazole fungicides result in dysfunctional ergosterol that impairs membrane structure and fungal growth leading to death. These fungicides have little effect on spore germination, because spores carry enough ergosterol to initiate germination and penetration. However, if applied preventively or during early stages of infection, these fungicides prevent disease development. Triazole fungicides have limited systemic movement in the xylem of the plant, but are considered to be locally systemic because these compounds move outward through a leaf but are not transported from one leaf to another or through phloem (Mueller et al., 2013).

Strobilurins originally were discovered as natural fungicidal compounds produced by species of Basidiomycota wood-rotting fungi, such as *Strobilurus tenacellus* (Pers. Ex Fr.) Singer. Current synthetic strobilurin fungicides, which represent the class of fungicides known as quinone outside inhibitors, were derived from these natural substances (Bartlett et al., 2002). The strobilurin fungicides inhibit mitochondrial respiration by binding at the quinone-binding site of the cytochrome bc1 complex. As a consequence, electron transfer is blocked, energy production is stopped, and the fungus dies (Becker et al., 1981, as cited in Fernandez-Ortuño et al., 2010, p.206; Mueller et al., 2013). The strobilurins are effective against spore germination and early fungal growth; however, once a fungus has successfully colonized a host these fungicides have limited effect (Bartlett et al., 2002; Mueller et al., 2013). The strobilurin fungicides are considered locally systemic; the chemical can be absorbed into leaf tissue and moves to the other leaf surface by translaminar activity (Mueller et al., 2013).

The success of triazole and strobilurin fungicides has been attributed to: i) broad-spectrum fungal activity, ii) registration for use on many different crops in many countries, and iii) possibility of foliar, seed and in-furrow application. As a result, these two classes of fungicides were highly effective, which was attractive to growers (Bartlett et al., 2002). However, the serious weakness of these modes of action, especially of the strobilurin fungicides, was the rapid development of resistant strains of fungi. Resistance to both classes of fungicides emerged soon after their introduction in the market (Fernandez-Ortuño et al., 2010). Emergence of this resistance was attributed primarily to the highly specific mode of action characteristic of these classes and their widespread and heavy use. The FRAC (Fungicide Resistance Action

Committee) has established a code list to provide guidelines and advice on the use of fungicides in order to reduce the development of resistance (FRAC, 2015). Triazole fungicides are in FRAC code 3, in medium risk; whereas strobilurin are in FRAC code 11, high risk.

USE OF FUNGICIDES IN WHEAT PRODUCTION

Application of fungicide can be used to effectively manage foliar diseases in crops like wheat and barley. For example, Ransom & McMullen (2008) noticed more than an 80% decrease of leaf spots in hard red winter wheat cultivars in the Northern Great Plains, and Hysing et al. (2012) observed up to a 97% reduction of powdery mildew in barley. In addition to disease control, increase in grain yield typically is associated with fungicide application. Ransom & McMullen (2008) reported that fungicides were responsible for yield improvement of up to 44%. Edwards et al. (2012) noticed an average yield gain of between 10 and 24%, and Hysing et al. (2012) reported an 11 to 17% increase. Kelley (2001) reported use of fungicide resulted in a 77% grain yield increase over all varieties used in that study.

Although greatest yield gains typically occur in susceptible varieties, significant yield increases also have been observed in tolerant and resistant varieties. Kelley (2001) showed fungicide treatment resulted in a significant yield increase from a cultivar such as Karl that had resistance to multiple foliar diseases. Edwards et al. (2012) noticed a grain yield improvement for resistant wheat varieties as well. These studies focused on the effect of fungicides on disease control and grain yield. Results indicated highly variable responses among varieties, with increased yield often associated with varieties that were resistant to many foliar diseases. The variable response of resistant varieties was associated with a variety's genetic resistance, environmental conditions and the presence/severity of disease (Edwards et al., 2012; Hysing et al., 2012; Milus, 1994; Ransom & McMullen, 2008).

These studies indicated that positive benefits, especially in yield and economic return to fungicide application, were most likely to occur in environments with high disease pressure (Edwards et al., 2012; Hysing et al., 2012; Kelley, 2001; Milus, 1994; Ransom & McMullen, 2008). It was also reported that varieties with high levels of tolerance to diseases were more responsive to a fungicide application when disease pressure was high (Edwards et al., 2012; Hysing et al., 2012; Ransom & McMullen, 2008).

CONTROVERSIAL PHYSIOLOGICAL EFFECTS OF FUNGICIDES

An additional consideration of foliar disease management with the use fungicides has recently become evident. In 2009, the Environmental Protection Agency (EPA) approved a request by BASF to claim crop benefits other than disease control on its label for Headline® fungicide. Commonly known as "plant health benefits," fungicide-sprayed plants not only yielded more but also showed improvements in growth, overall greenness and tolerance to heat, cold and other stresses. Headline® was the first agrochemical to officially carry the concept of plant on a registration label (BASF, 2009), which concerned many scientists that fungicide would be applied in the absence of disease. Such applications (i.e., in the absence of disease) contradict the basic concept of integrated pest management and would increase the likelihood of fungi developing with resistance to the fungicide (Brown-Rytlewski & Vincelli, 2009).

There are numerous reports describing plant health benefits. For example, fungicide sprayed wheat plants showed an increased chlorophyll content of leaves (Beck et al., 2002; Jones & Bryson, 1998; Zhang et al., 2010) and a higher photosynthetic activity (Beck et al., 2002; Oerke et al., 2001) that were referred to as a 'greening effect'. Treatment with the strobilurins kresoxim-methyl and pyraclostrobin was shown to result in increased activation of the enzyme NADH-nitrate reductase (Glaab & Kaiser, 1999; Köhle et al., 2003). This enzyme is directly involved in nitrogen assimilation in plants. Nitrogen is essential for the formation and maintenance of chlorophyll (Tam & Magistad, 1935) and nitrogen content is directly related to the photosynthetic capacity in leaves (Evans, 1989). Therefore, activation of NADH-nitrate reductase could explain the enhancement of green leaf pigmentation. Besides greening effect NADH-nitrate reductase could also be involved in increased biomass production after fungicide application (Köhle et al., 2003).

Fungicide-sprayed wheat plants were reported to have a longer grain filling period (Dimmock & Gooding, 2002), and delayed of senescence (Berdugo et al., 2013; Bertelsen et al., 2001; Blandino et al., 2009; Jones & Bryson, 1998; Wu & Von Tiedemann, 2001). Authors attributed these differences to phytohormonal changes induced by fungicides. Grossmann & Retzlaff (1997) found that the strobilurin kresoxim-methyl negatively affected ethylene biosynthesis by reducing the activity of the enzyme ACC synthase. Ethylene is the hormone involved in plant senescence, in reaction to stress, and in chlorophyll degradation (Abeles et al., 1992, as cited in Grossmann & Retzlaff, 1997, p. 17). By delaying production of ethylene, fungicide-sprayed wheat plants had delayed leaf senescence, which delays ripening of grain and contributes to prolonged grain filling (Köhle et al. 2003). Along the same line, increased IAA (indole-acetic acid, an auxin growth hormone) levels were observed after application of the strobilurin pyraclostrobin, which could also delay leaf senescence (Köhle et al., 2003).

Claims of PHB also extolled the ability of fungicides to alleviate stresses imposed on plants. Kresoxim-methyl- and pyraclostrobin-sprayed plants showed increases of the phytohormone ABA (abscisic acid) that improved plant tolerance to drought stress (Grossmann et al., 1999; Köhle et al., 2003). In addition, Köhle et al. (2003) found that F 500® promoted the activity of peroxidase, which is an enzyme that alleviates stress by eliminating reactive oxygen species and other radicals formed upon exposure to conditions that induce stress. Furthermore, Wu & von Tiedemann (2001) suggested that triazoles and strobilurins promoted antioxidative potential in wheat plants, which directly reduced reactive oxygen species, and thus could also be connected to delayed senescence.

Improved yield, especially from resistant varieties, due to fungicides are not consistently observed (Kelley, 2001; Ransom & McMullen, 2008; Edwards et al., 2012). Bertelsen et al. (2001) and Edwards et al. (2012) suggested a hypothesis that fungicides applied to those varieties kill spores before the defense reaction (hypersensitive response) is triggered. This was observed by Schöfl & Zinkernagel (1997) who reported that spores of *Septoria tritici* were inhibited by fungicide, and that necrotic flecks would not develop. As a result, there was a higher amount of chlorophyll available for photosynthesis, and consequently, an increase in energy that could be directed toward grain production and filling (Edwards et al., 2012). Greater productivity also was connected to increased green leaf area, which likely is a result of the fungicidal activity of these fungicides (Blandino et al., 2009; Blandino & Reyneri, 2009; McCartney et al., 2007; Ruske et al., 2003a; Ruske et al., 2003b).

Another way PHB may be obtained is by affecting the community of saprophytes present on the phylloplane. Dik et al. (1991) (as cited in Bertelsen et al., 2001, p. 201) reported that fungicides inhibited the growth of saprophytes, which diminished their antagonistic role against necrotrophic leaf pathogens. These saprophytes negatively affected wheat plant development by inducing senescence and decreasing chlorophyll content, which could compromise grain yield (Jachmann & Fehrmann, 1989). Results obtained by Bertelsen et al. (2001) showed that fungicide treatment inhibited the senescence-promoting activity by saprophytes, which resulted in greater green leaf area retention. The mechanism used by these organisms to promote senescence is not well understood (Bertelsen et al., 2001). In addition, saprophytes living on the leaves are in constant interaction with the host cells, and could potentially attempt infection that would cause the host to trigger defense reactions (Knogge, 1997, as cited in Bertelsen et al., 2001, p. 200; Smedegaard-Petersen & Tolstrup, 1985). Taking into consideration these two latter cases, wheat plants may benefit from a fungicide application as a result of activity against saprophytic microorganisms that induce plant senescence or the defense reactions rather than activity directly against foliar pathogens. These studies described mechanisms whereby fungicides affected microorganisms that might not seem readily obvious, and that could also explain why resistant varieties showed increased yield following fungicide application.

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CHAPTER III

FOLIAR FUNGICIDES DO NOT INCREASE RELATIVE CHLOROPHYLL CONTENT, YIELD AND TEST WEIGHT OF WINTER WHEAT IN THE ABSENCE OF FOLIAR DISEASE

ABSTRACT

Fungicides can alleviate losses caused by foliar diseases when applied to susceptible varieties of wheat, and occasionally also improve yield of resistant varieties. Plant health benefits such as prolonged greenness, higher chlorophyll content in leaves and stress tolerance also have been claimed following application of strobilurin fungicides. This study measured changes in relative chlorophyll content, yield and test weight associated with fungicide application in hard red winter wheat in the absence of foliar disease. Two field trials (dryland and irrigated) were planted with 'Duster' (resistant to most foliar diseases) and 'OK Bullet' (susceptible) hard red winter wheat varieties. At Feekes 10.3, the commercial fungicides Headline (pyraclostrobin), Caramba (metconazole), and Twinline (pyraclostrobin plus metconazole) were applied at labeled rates. Relative chlorophyll content of leaves was determined using a SPAD 502 Plus chlorophyll meter before and after fungicide application. Foliar fungal disease incidence in both experiments was absent due to an abnormally dry winter and spring. No significant differences were observed in yield or test weight of sprayed plots compared with the non-sprayed. In the dryland trial, yield ranged from 44 to 53 bu/A, and test weight from 56 to 57 lb/bu. In the irrigated trial, yield ranged

from 53 to 63 bu/A, and test weight from 58 to 60 lb/bu. Fungicide did not increase relative chlorophyll content in flag leaves, grain yield or test weight of these varieties compared to nontreated plants. Hence, results do not support claims of plant health benefits afforded by foliar fungicide application in winter wheat varieties in the absence of disease.

INTRODUCTION

Winter wheat for forage and grain is one of the most important crop commodity in the State of Oklahoma with a production value for grain alone of approximately $727 million dollars, in 2013 (NASS, 2014). Foliar fungal diseases can cause severe grain yield loss wherever wheat is grown. Powdery mildew [*Blumeria graminis* (DC.) Speer f. sp. *tritici* emend. E.J. Marcha], tan spot [*Pyrenophora tritici-repentis* (Died.) Drechsler (*Drechslera tritici-repentis* (Died.) Shoemaker)], Septoria tritici blotch [*Septoria tritici* Roberge in Desmaz. (*Mycosphaerella graminicola* (Fuckel) J. Schrot.)], stripe rust (*Puccinia striiformis* Westernd. f. sp. *tritici* Erikss.) and leaf rust (*Puccinia triticina* Erikss.) are frequently observed in wheat growing areas. Leaf rust typically is considered the most important among the foliar diseases because it is consistently reported in states comprising the Great Plains including Oklahoma. Grain yield losses of 50% or more due to leaf rust have been reported (Kolmer, 2010; USDA, 2014).

Fungicides are effective in controlling foliar diseases and protecting yield of susceptible varieties of wheat (Gooding et al., 2000; Milus, 1994; Ransom & McMullen, 2008). Applying fungicides to varieties with moderate to high resistance to foliar diseases also can have positive benefits, such as increased grain yield (Bertelsen et al., 2001; Edwards et al., 2012; Kelley, 2001; Ransom & McMullen, 2008). Recently, the class of strobilurin fungicides has been associated with plant physiological effects other than disease control. Claimed as ‘plant health benefits’ or‘greening effect’, sprayed wheat plants show an increased chlorophyll content of leaves (Beck et al., 2002; Jones & Bryson, 1998; Zhang et al., 2010), a higher photosynthetic activity (Beck et al., 2002; Oerke et al., 2001), a longer grain filling period (Dimmock & Gooding, 2002), and delayed of senescence (Berdugo et al., 2013; Bertelsen et al., 2001; Blandino et al., 2009; Jones & Bryson, 1998; Wu & Von Tiedemann, 2001), all of which contribute to greater yield (Blandino et al., 2009; Jones & Bryson, 1998; Zhang et al., 2010).

The improvement in crop physiology and performance associated with fungicide application, especially when applied to resistant varieties, is not consistently observed (Edwards et al., 2012; Kelley, 2001; Ransom & McMullen, 2008). The concept of plant health benefits and physiological effects caused concern among many scientists that fungicides would be applied in the absence of disease, which contradicts the concept of integrated pest management, and could increase the likelihood of more fungicide-resistant plant pathogenic fungi (Brown-Rytlewski & Vincelli, 2009; McCartney et al., 2007).

Several field studies have focused on the effect of fungicides on yield and disease control, with only a minority reporting physiological improvements afforded by fungicides in the complete absence of disease (Weisz et al., 2011). In order to evaluate whether fungicides promote the greening effect, the objective of this research was to measure changes in chlorophyll content of flag leaves, yield, and test weight associated with fungicide application to resistant and susceptible hard red winter wheat varieties in the absence of disease.

MATERIAL AND METHODS

This study was conducted during 2013-2014 at the Oklahoma State University Agronomy Research Farm near Stillwater, OK, in a field with a Norge loam soil. The area used was planted to wheat in previous seasons. Based on soil testing and a 50 bu/A (56 kg/ha) yield goal, the area was fertilized 10 September 2013 with 86 lb/A (96 kg/ha) of 46-0-0 in the form of granular urea.

The soil was prepared using conventional-tillage methods. Two field trials were sown on 03 October 2013 at a 90 lb/A (100 kg/ha) seeding rate. In order to avoid aphid infestations and subsequent incidence of barley yellow dwarf, seed was treated with imidacloprid insecticide (formulated as Gaucho® 600, Bayer CropScience LP, Research Triangle Part, NC) at 2.4 fl oz/cwt (71 ml per 45 kg of seeds). Control of weeds was accomplished with one post-emergence application of chlorsulfuron plus flucarbazone-sodium (formulated as Finesse® Broadleaf and Grass, DuPont™, Wilmington, DE) at 0.6 oz/A (42 g/ha), mixed with pinoxaden (formulated as Axial® XL, Syngenta Crop Protection Inc., Greensboro, NC) at 16.4 oz/A (1.1 kg/ha) on 1 November 2013. One trial was a dryland trial, and the other was irrigated from 16 April until 20 May 2014. Irrigation was conducted two or three days/week for 15 to 20 min, which delivered approximately 0.20 in. (5 mm) per application.

The experimental design consisted of a split-plot arrangement in a randomized complete block design with six replications. The hard red winter wheat varieties, 'Duster' (resistant to most foliar diseases) and 'OK Bullet' (susceptible) were sown as the main plots. Plot size was 10 ft (3 m) long with 7 rows spaced at 7 in. (18 cm), and seed was planted approximately 0.75 in. (2 cm) deep. The treatments (subplots) consisted of three foliar fungicide treatment and a nontreated control. Pyraclostrobin (250 g of active ingredient/L of product, formulated as Headline® 250 SC) was applied at 9 fl oz/ac (0.6 L/ha); metconazole (90 g a.i./L of product, formulated as Caramba® 90 SL) was applied at 17 fl oz/acre (1.2 L/ha); pyraclostrobin + metconazole (commercial mixture with 129 g of pyraclostrobin/L of product + 80 g of metconazole/L of product, formulated as Twinline® 210 SC) was applied at 9 fl oz/acre (0.6 L/ha). All fungicides are products of BASF Ag Products, Research Triangle Park, NC. Fungicides were applied on 25 April 2014 between Feekes' growth stages (GS) 10.3 (heading ½ complete) and 10.4 (heading ¾ complete) with a CO_2 wheelbarrow sprayer as a broadcast foliar application using flat fan nozzles (8003EVS) spaced 18 in (45 cm) apart and calibrated to deliver a volume of 20 GPA (187 L/ha).

Starting at Feekes' GS 10.1 (heading started, awns visible), before fungicide application, relative chlorophyll content of leaves was obtained using a Soil and Plant Analyzer Development (SPAD) 502 plus chlorophyll meter (Spectrum Tecnologies, Inc, Aurora, IL). This instrument provides non-invasive and non-destructive analysis of the relative chlorophyll content of leaves. The reading is a ratio calculated according to the intensity of light transmitted through the leaf at wavelengths of 650 and 940 nm and the intensity of light transmitted with no sample (calibration) (Spectrum Technologies Inc., 2009). The SPAD-502 Plus can be used for determining nitrogen content of plants and for facilitating nutrient management (Arregui et al., 2006). This meter was used in previous studies to determine relative chlorophyll content (Jones & Bryson, 1998; Sibley et al., 1996; Swodoba & Peterson, 2009) because the results obtained with this device had a strong correlation with the amount of chlorophyll extracted from the leaf (Markwell et al., 1995; Sibley et al., 1996; Uddling et al., 2007).

A total of five relative chlorophyll content readings were taken. Two readings were taken before fungicide application, and three were taken after fungicide application. There was a five-day interval between each reading, and the last reading was taken when plants reached Feekes' GS 11 (growth stage of ripening). For each reading, a total of 15 SPAD measurements were taken on flag leaves of different plants within the three inner rows of each plot. When taking a measurement, the instrument was positioned between the middle and the tip of a flag leaf. The mean of 15 measurements taken from each plot served as the data for each experimental unit.

Trials were harvested 17 June 2014 using a Hege 140 small-plot combine. Grain moisture, in percentage, was determined using a Mini GAC Plus meter (Dickey-John Corp, Illinois). Grain yield in bushels per acre (bu/A), and test weight in pounds per bushel (lb/bu) were determined for each treatment.

Analysis of variance was performed using mixed procedure in SAS (version 9.4; SAS Institute Inc., Cary, NC). Results were compared within variety and experiment (dryland vs irrigated) in relation to the non-treated control.

RESULTS

Annual total precipitation in Stillwater, OK was below the 1981-2010 average of 37.26 in. (946 mm). During the period of this study (October 2013 to May 2014), precipitation recorded by the Oklahoma Mesonet was approximately 8 in. (203 mm), which was lower than the long-term average of approximately 22 in. (558 mm). Rainfall during August to November 2013 provided sufficient moisture to support emergence and crop establishment through winter. Timely rain during March and April 2014 was sufficient to allow growth and development of plants, but low soil moisture in the dryland trial caused plants of both varieties to mature faster. For example, fungicide application, which occurred on 25 April 2014, was applied at GS 10.3 and 10.4 in the irrigated and dryland trials, respectively. Drought conditions across Texas and Oklahoma prevented the establishment of wheat foliar diseases, including these field trials. Therefore, no disease ratings were taken.

In the dryland trial, relative chlorophyll content of flag leaves in Duster and OK Bullet varied from the lower to upper 40s (Table 1). The lowest relative chlorophyll content readings (average of 44 for Duster and OK Bullet) occurred on the first reading, which was 7 days before fungicide application. Relative chlorophyll content values on this day were not statistically different, which indicated plants of both varieties were initially at the same relative chlorophyll content. As time progressed, a trend for relative chlorophyll content was observed in most of the treatments, with relative chlorophyll content numerically increasing between 7 DBF and 2 DBF, but from 3 DAF to 7 DAF, relative chlorophyll content was stable. A slight decrease was observed on 12 DAF. For both varieties, there was no statistical difference among relative chlorophyll content of treatments when compared with the nontreated control. Average grain moisture was 12%, grain yield ranged from 50 to 54 bu/A for Duster, and from 44 to 47 bu/A for OK Bullet. The test weight for Duster averaged over treatments was 56 lb/bu, and for OK Bullet was approximately 57.5 lb/bu. There was no statistical difference between varieties for test weight.

For the irrigated trial, relative chlorophyll content readings for the varieties ranged from 46 to 55 (Table 2). Similar to the dryland trial, the lowest relative chlorophyll content readings were observed 7 DBF, with averages of 49 and 47 for Duster and OK Bullet, respectively. An increase in relative chlorophyll content at 2 DBF was observed for both varieties. In Duster and OK Bullet, from 3 DAF to 12 DAF at the end of the experiment, relative chlorophyll content remained basically constant.

Despite these trends, no statistical differences were observed in relative chlorophyll content values of fungicide treatments compared with the nontreated control. Average grain moisture was 11.5%, which was similar to the dryland trial. As expected, grain yield and test weight were higher in the irrigated trial than in the dryland trial. Grain yield from Duster ranged from 57 to 64 bu/A, and for OK Bullet from 53 to 57 bu/A. Test weight of grain from both varieties ranged from 59 to 60 lb/bu. No statistical differences in test weight between treatments were observed for OK Bullet. For Duster, test weight associated with pyraclostrobin was significantly greater than for metconazole. However, none of the fungicides significantly increased test weight compared to the nontreated control.

DISCUSSION

Fungicides are used to manage foliar diseases in winter wheat and may contribute to

Table 1 - Effect of foliar fungicide on yield, test weight and relative chlorophyll content of winter wheat flag leaves before and after fungicide application in a dryland field experiment in the complete absence of foliar disease.

Variety	Treatment and rates	Yield (bu/A)	Test Weight (lb/bu)	Relative Chlorophyll Content[x]				
				7 DBF[w]	2 DBF	3 DAF[v]	7 DAF	12 DAF
Duster	Nontreated control	51.4 ± 1.23	56.3 ± 0.18	43.8 ± 0.93	48.8 ± 0.99	48.4 ± 0.87	48.6 ± 0.80	47.2 ± 1.26
	Metconazole, 1.2 L/ha[z]	53.8 ± 1.30	56.4 ± 0.27	44.0 ± 0.62	48.2 ± 0.46	47.8 ± 0.48	48.2 ± 1.17	47.2 ± 0.77
	Pyraclostrobin, 0.6 L/ha	52.0 ± 2.07	56.8 ± 0.22	44.0 ± 0.83	49.7 ± 1.09	48.3 ± 0.87	49.7 ± 1.11	48.3 ± 0.75
	Metconazole + pyraclostrobin, 0.6 L/ha	50.9 ± 1.58	56.5 ± 0.19	43.9 ± 0.56	49.1 ± 0.83	47.4 ± 0.81	47.7 ± 1.03	46.1 ± 1.36
	F[y]	*0.5468*	*0.273*	*0.997*	*0.522*	*0.724*	*0.244*	*0.244*
OK Bullet	Nontreated control	45.1 ± 1.68	57.5 ± 0.31	43.2 ± 1.07	47.9 ± 0.60	47.6 ± 0.53	47.9 ± 1.00	47.1 ± 1.20
	Metconazole, 1.2 L/ha	47.3 ± 1.26	57.3 ± 0.21	43.7 ± 1.12	48.2 ± 0.66	47.5 ± 0.63	47.9 ± 0.70	48.3 ± 0.69
	Pyraclostrobin, 0.6 L/ha	44.9 ± 1.44	57.7 ± 0.15	44.8 ± 0.90	47.4 ± 0.56	49.1 ± 0.75	48.6 ± 1.10	48.6 ± 1.21
	Metconazole + pyraclostrobin, 0.6 L/ha	45.5 ± 1.57	57.5 ± 0.13	43.6 ± 0.63	48.1 ± 0.69	47.0 ± 0.56	47.2 ± 0.86	47.0 ± 1.02
	F	*0.665*	*0.408*	*0.537*	*0.904*	*0.278*	*0.676*	*0.401*

[z] Fungicides metconazole (Caramba® 90SL, 90 g active ingredient/L), pyraclostrobin (Headline® 250 SC, 250 g a.i./L) and pre-mixture of pyraclostrobin and metconazole (Twinline® 210 EC, 80 g metconazole/L and 129 g pyraclostrobin/L) are registered trademarks of BASF Corporation, Research Triangle Park, NC

[y] Probability of a significant treatment at α=0.05. Means followed by the same letter within column and variety are not significantly different

[x] Relative chlorophyll content was determined using a SPAD 502 Plus Chlorophyll Meter (Spectrum Technologies Inc.). Means of relative chlorophyll content are followed by standard error values

[w] Number of days before fungicide application

[v] Number of days after fungicide application

Table 2 - Effect of foliar fungicide on yield, test weight and relative chlorophyll content on winter wheat flag leaves before and after fungicide application in an irrigated field experiment in the complete absence of foliar disease.

Variety	Treatments and rates	Yield (bu/A)	Test Weight (lb/bu)	Relative Chlorophyll Content[x]				
				7 DBF[w]	2 DBF	3 DAF[v]	7 DAF	12 DAF
Duster	Nontreated control	58.77 ± 2.86	58.83 ab ± 0.23	49.3 ± 0.54	54.1 ± 0.61	54.5 ± 0.39	54.2 ± 0.81	52.8 ± 0.45
	Metconazole, 1.2 L/ha[z]	57.76 ± 2.70	58.50 b ± 0.25	49.6 ± 0.65	53.0 ± 1.09	54.9 ± 0.83	54.0 ± 0.71	52.9 ± 0.45
	Pyraclostrobin, 0.6 L/ha	63.74 ± 1.50	59.48 a ± 0.09	49.9 ± 0.72	54.1 ± 1.28	54.4 ± 0.59	53.2 ± 0.71	54.5 ± 0.62
	Metconazole + pyraclostrobin, 0.6 L/ha	60.41 ± 1.49	59.17 ab ± 0.29	49.7 ± 0.54	54.9 ± 0.63	54.7 ± 0.34	53.7 ± 0.47	54.7 ± 0.37
	F[y]	*0.258*	*0.037*	*0.932*	*0.179*	*0.939*	*0.735*	*0.094*
OK Bullet	Nontreated control	54.28 ± 1.58	59.95 ± 0.38	46.9 ± 0.48	49.0 ± 1.01	52.0 ± 0.33	50.2 ± 0.68	51.9 ± 0.52
	Metconazole, 1.2 L/ha[z]	54.36 ± 1.88	60.30 ± 0.24	47.3 ± 0.62	50.4 ± 0.37	51.1 ± 0.22	50.2 ± 0.46	51.9 ± 0.41
	Pyraclostrobin, 0.6 L/ha	57.22 ± 1.67	60.57 ± 0.13	46.9 ± 0.29	49.8 ± 0.65	51.1 ± 0.52	50.5 ± 0.54	51.1 ± 0.89
	Metconazole + pyraclostrobin, 0.6 L/ha	52.85 ± 1.82	59.98 ± 0.50	46.7 ± 0.32	50.4 ± 0.47	51.1 ± 0.48	50.0 ± 0.45	52.1 ± 0.34
	F	*0.179*	*0.508*	*0.886*	*0.154*	*0.475*	*0.914*	*0.176*

[z] Fungicides metconazole (Caramba® 90SL, 90 g active ingredient/L), pyraclostrobin (Headline® 250 SC, 250 g a.i./L) and pre-mixture of pyraclostrobin and metconazole (Twinline® 210 EC, 80 g metconazole/L and 129 g pyraclostrobin/L) are registered trademarks of BASF Corporation, Research Triangle Park, NC

[y] Probability of a significant treatment at α=0.05. Means followed by the same letter within column and variety are not significantly different

[x] Relative chlorophyll content was determined using a SPAD 502 Plus Chlorophyll Meter (Spectrum Technologies Inc.). Means of relative chlorophyll content are followed by standard error values

[w] Number of days before fungicide application

[v] Number of days after fungicide application

significant yield increases (Edwards et al., 2012; Kelley, 2001; Milus, 1999). Such yield gains, which are occasionally observed in resistant varieties, are thought to be related to blocking the resistance response (hypersensitive reaction) of these varieties in environments with high disease pressure (Bertelsen et al., 2001; Edwards et al., 2012; Ransom & McMullen, 2008). Plant physiological effects including increased chlorophyll content, delayed senescence, and tolerance to stresses also have been associated with certain classes of fungicides, particularly the strobilurins (Grossmann et al., 1999; Grossmann & Retzlaff, 1997; Jones & Bryson, 1988; Köhle et al., 2002). However, application of strobilurin fungicides has not always resulted in yield and test weight increases (Edwards et al., 2012; Kelley, 2001; Ransom & McMullen, 2008). Therefore, this study investigated if application of strobilurin or triazole fungicides was associated with increases in relative chlorophyll content of flag leaves, grain yield and test weight in hard red winter wheat varieties in the absence of disease.

Using a susceptible cultivar of durum wheat, Blandino et al. (2009) reported no significant increase in relative chlorophyll content of sprayed plants in a location where disease pressure was low (22% foliar disease incidence). Swoboda & Peterson (2009) also did not see significant effects of fungicide on relative chlorophyll content of soybean plants compared with the non-treated control in an environment not conducive for foliar diseases (15% of foliar disease incidence). Although disease incidence was low, disease was present and could have affected the relative chlorophyll content results obtained in these studies. By contrast, the results obtained from the dryland and irrigated trials in the present study were in the absence of foliar disease. In these trials, relative chlorophyll content values were influenced only by fungicide application, which did not significantly increase relative chlorophyll content of flag leaves of either a variety resistant or susceptible to foliar diseases.

Occasionally strobilurin and triazole application can increase relative chlorophyll content and delay senescence of sprayed plants (Blandino et al., 2009; Zhang et al., 2010) with the greatest increases in relative chlorophyll content observed in plants sprayed with fungicide that also received foliar nitrogen application (Blandino et al., 2009; Blandino & Reyneri, 2009). These studies suggest that physiological effects induced by strobilurin fungicides could explain increases in relative chlorophyll content that results in a "greening effect." Such an effect has been reported to be due to a direct effect in the activation of NADH-nitrate reductase, an enzyme involved in nitrogen assimilation by plants (Glaab & Kaiser, 1999). Köhle et al. (2002) reported that plants treated with a strobilurin fungicide showed a 70% increase in NADH-nitrate reductase activity. Such studies help to explain positive physiological effects by strobilurin fungicides. Since nitrogen is essential for the formation and maintenance of chlorophyll (Tam & Magistad, 1935) and nitrogen content is directly related to the photosynthetic capacity in leaves (Evans, 1989), an increase in nitrogen assimilation could explain the enhancement of green pigmentation on plants sprayed with a strobilurin fungicide.

Köhle et al. (2002) showed that the activation of NADH-nitrate reductase activity resulted in greater biomass production, which was reflected in greater yield. Significant improvements in wheat grain yield were observed after application of strobilurin fungicides as reported by Blandino et al. (2009), Ruske et al. (2003a), and Zhang et al. (2010). However greater yield as a result of physiological effects were questionable since solely application of strobilurin and other fungicides did not always guarantee yield increases, as observed by Blandino & Reyneri (2009), Edwards et al. (2012), Kelley (2001) and Ransom & McMullen (2008). Other studies state that increases in yield and profitability of fungicide use vary within variety and between years with greatest responses observed in years with moderate to high disease severity (Edwards et al., 2012; Kelley, 2001; Ransom & McMullen, 2008; Thompson et al., 2014; Wegulo et al., 2011; Wiik & Rosenqvist, 2010). Two of these studies suggested that positive effects on grain productivity and quality were more closely associated with green leaf area retention as a result of foliar disease control, instead of physiological effects of fungicides (Dimmock & Gooding, 2002; Ruske et al., 2003b).

The results of our study showed that fungicide (whether a strobilurin, a triazole, or a mixture) did not significantly increase relative chlorophyll content, grain yield or test weight in either foliar disease susceptible or resistant wheat varieties in the absence of foliar disease. Finally, our results agree with Weisz et al. (2001), who did not observe significant grain yield increases in the absence of disease in a multi-year study, confirming that calendar-based application or application solely targeting plant health is not necessarily advantageous.

There is evidence that fungicides can cause physiological effects on crops (Grossmann et al., 1999; Grossmann & Retzlaff, 1997; Jones & Bryson, 1988; Köhle et al., 2002), as well as enhance productivity due to non-fungicidal activity (Blandino et al., 2009; Ruske et al., 2003a; Zhang et al., 2010). Such claims led to Headline® (pyraclostrobin; BASF Ag Products, Research Triangle Park, NC) being the first fungicide to receive EPA approval for plant health benefits as part of the label (BASF, 2009). This labeling concerned many scientists who thought that fungicides would be applied excessively to target those physiological effects. Application of fungicide in the absence of disease contradicts accepted integrated pest management strategies, and is likely to select fungicide-resistant pathogen strains (Brown-Rytlewski & Vincelli, 2009; McCartney et al., 2007). Previous studies showed small or non-significant yield increases due to fungicide application in years of low disease incidence in foliar disease resistant varieties (Blandino et al., 2009; Edwards et al., 2012; Kelley, 2001; Ransom & McMullen, 2008; Swodoba & Peterson, 2009; Weisz et al., 2013). The results of this study agree with these previous reports, and demonstrate that a strobilurin fungicide, a triazole fungicide, and a commercial mixture did not increase chlorophyll content of leaves, yield, or test weight of winter wheat foliar disease susceptible and resistant varieties in the absence of disease.

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CHAPTER IV

FOLIAR FUNGICIDES DO NOT INCREASE RELATIVE CHLOROPHYLL CONTENT BUT DO MAINTAIN GREEN LEAF AREA OF WINTER WHEAT INFECTED WITH LEAF RUST (*Puccinia triticina*) IN A CONTROLLED ENVIRONMENT

ABSTRACT

Fungicides can alleviate losses caused by diseases when applied to susceptible varieties of wheat. In resistant varieties, high foliar disease pressure may lower the chlorophyll content of leaves due to the development of severe ‘flecking’ (leaf necrosis) that results from the hypersensitive response. The objective of this study was to evaluate if foliar fungicide application prevents reduction of relative chlorophyll content and green leaf area in hard red winter wheat infected with *Puccinia triticina* (leaf rust). In two growth chamber experiments, young plants of ‘Duster’ (resistant to leaf rust) and ‘OK Bullet’ (susceptible) varieties were sprayed with labeled rates of commercial fungicides containing pyraclostrobin, metconazole, and a pre-mixture of pyraclostrobin plus metconazole. Two days after fungicide application, plants were inoculated with urenidiospores of *P. triticina*. Relative chlorophyll content of leaves was determined before and after fungicide application using a SPAD 502 Plus Chlorophyll Meter. Percent green leaf area was determined at the end of the experiment using Assess digital image analysis. At the last

reading of the experiment, the relative chlorophyll content of sprayed and inoculated OK Bullet plants did not differ from that of non-sprayed and non-inoculated plants. Duster plants sprayed with pyraclostrobin and the pre-mixture had a relative chlorophyll content similar to non-sprayed and non-inoculated plants. Fungicide sprayed and inoculated plants of both cultivars had similar percent green leaf area compared to non-sprayed and non-inoculated plants. By contrast, non-sprayed and inoculated plants had significantly less green leaf area. These results indicate that pyraclostrobin and the pre-mixture of pyraclostrobin and metconazole maintained the relative chlorophyll content in Duster, and may explain why resistant varieties sometimes show a yield response to a fungicide applied in the presence of a foliar disease such as wheat leaf rust.

INTRODUCTION

Winter wheat for forage and grain is the one of most important crop commodity in Oklahoma, with a production value in 2013 of around $727 million dollars (NASS, 2014). Foliar fungal diseases can cause severe grain yield loss wherever wheat is grown. Powdery mildew [*Blumeria graminis* (DC.) Speer f. sp. *tritici* emend. E.J. Marcha], tan spot [*Pyrenophora tritici-repentis* (Died.) Drechsler (*Drechslera tritici-repentis* (Died.) Shoemaker)], Septoria tritici blotch [*Septoria tritici* Roberge in Desmaz. (*Mycosphaerella graminicola* (Fuckel) J. Schrot.)], stripe rust (*Puccinia striiformis* Westernd. f. sp. *tritici* Erikss.) and leaf rust (*Puccinia triticina* Erikss.) are frequently observed in wheat growing areas. Leaf rust typically is considered the most important foliar diseases because it is consistently reported in states comprising the Great Plains including Oklahoma. Grain yield losses of 50% or more due to leaf rust have been reported (Kolmer, 2010; USDA, 2014).

Fungicides are effective in controlling foliar diseases and protecting yield of susceptible varieties of wheat (Gooding et al., 2000; Milus, 1994; Ransom & McMullen, 2008). Applying fungicides to varieties with moderate to high resistance to foliar diseases also can have positive benefits, such as increased grain yield (Bertelsen et al., 2001; Edwards et al., 2012; Kelley, 2001; Ransom & McMullen, 2008). Recently, the class of strobilurin fungicides has been associated with plant physiological effects in addition to disease control. Claimed as ‘plant health benefits’ or ‘greening effect’, sprayed wheat plants show an increased chlorophyll content of leaves (Beck et al., 2002; Jones & Bryson, 1998; Zhang et al., 2010), a higher photosynthetic activity (Beck et al., 2002; Oerke et al., 2001), a longer grain filling period (Dimmock & Gooding, 2002), and a delay of senescence (Berdugo et al., 2013; Bertelsen et al., 2001; Blandino et al., 2009; Jones & Bryson, 1998; Wu & Von Tiedemann, 2001), all contributing to greater yield (Blandino et al., 2009; Jones & Bryson, 1998; Zhang et al., 2010).

An alternative explanation for increased yield of resistant varieties following a fungicide application is related to the effect of the fungicide on fungal pathogens. Under conditions of high disease incidence, strobilurins prevent spore germination (Bertelsen et al., 2001); therefore the energy that would be spent by the host to develop a hypersensitive reaction (i.e. flecking) can be directed to grain production. In addition, preventing development of chlorotic/necrotic flecking on leaves results in more photosynthetic leaf area for energy and grain production (Bertelsen et al., 2001; Edwards et al., 2012).

In Oklahoma, wheat growers typically rely on varieties with genetic resistance to provide protection against most foliar diseases. Application of fungicide is recommended only when a susceptible variety is planted, the field has high yield potential, the price of wheat is favorable, and high disease pressure is imminent. Foliar fungicide application to resistant varieties can occasionally improve grain yield production; however significant increases are not consistently observed (Edwards et al., 2012; Kelley, 2001; Ransom & McMullen, 2008). There is a need to determine if fungicide application affects the chlorophyll content of flag leaves or allows resistant plants to avoid development of the hypersensitive reaction. The objective of this study was to assess the effects of foliar fungicide application prior to foliar pathogen inoculation on development of the HR and reducing chlorophyll content and green leaf area.

MATERIAL AND METHODS

Inoculum maintenance and inoculation

The hard red winter wheat variety TAM 110, which is considered highly susceptible to wheat leaf rust, was used to maintain *Puccinia triticina*. Urediniospores were initially collected in spring of 2012 from wheat plants growing in fields near Lahoma and Stillwater, OK using a cyclone spore collector. Urediniospores were stored in liquid nitrogen until used in inoculations. TAM 110 seedlings were grown in 6.5 in. plastic pots (American Plant Products, Oklahoma City, OK) kept in a growth chamber set at 18°C with a 12 hr photoperiod. Plants were fertilized with a solution containing 1 tbs/gal of 24-8-26 plus micronutrients (Miracle-Gro®, Scotts Miracle-Gro Products, Inc., Marysville, OH) every 15 to 20 days. At the two-leaf stage, plants were inoculated using a small paint brush to transfer spores onto leaves (Browder, 1971). Inoculated plants were kept in a humidity chamber inside of a growth chamber at the conditions described previously. After 24 hours, inoculated plants were placed outside of the moist chamber, inside the growth chamber. Approximately, 7 days after inoculation leaf rust pustules were fully developed and diseases plants were used to inoculate healthy seedlings. Urediniospores were deposited onto healthy leaves by brushing the diseased leaves against the healthy leaves of plants being inoculated (plant-to-plant method) (Browder, 1971).

Plant material

The hard red winter wheat varieties ‘Duster’ and ‘OK Bullet’ were used in this study. Duster was released in 2006 and OK Bullet in 2005 by the Oklahoma Agricultural Experiment

Station. They were chosen based on their contrasting genetic resistance; Duster is resistant to most foliar diseases (including leaf rust), and OK Bullet is susceptible. Seeds were sown in 2.5 in. plastic conetainers (Stuewe and Sons, Inc., Tangent, OR). Two plants were grown in each conetainer, which were kept in a growth chamber receiving 12 hours of light at 18ºC. Plants were fertilized as described previously. There were four replications, and the experiment was repeated twice. Ten containers were accommodated in conetainer racks (20 conetainer capacity) in a randomized complete block design with a 2 x 2 + 1 factorial arrangement of treatments.

Treatments

There were five treatments; a non-sprayed and non-inoculated negative control, a non-sprayed and inoculated positive control, and three foliar fungicides. The fungicides were pyraclostrobin (250 g of active ingredient/L of product, formulated as Headline® 250 SC) applied at 9 fl oz/ac (0.6 L/ha), metconazole (90 g a.i./L of product, formulated as Caramba® 90 SL) applied at 17 fl oz/acre (1.2 L/ha); pyraclostrobin + metconazole (commercial mixture with 129 g of pyraclostrobin/L of product + 80 g of metconazole/L of product, formulated as Twinline® 210 SC) applied at 9 fl oz/acre (0.6 L/ha). A single foliar application was made when plants reached the four leaf stage. Each fungicide rate was adjusted to the volume necessary to spray the area of the conetainers. The comparable rate of each fungicide was diluted in 5 fl oz (150 ml) of distilled water and sprayed onto foliage using a 1.5 liter (50 fl oz) hand-held pressurized spray bottle (Kwazar UK Ltd., United Kingdom). No adjuvant was added to the fungicide solution.

Relative chlorophyll content

Starting at the four leaf stage, relative chlorophyll content, or leaf greenness was measured using a Soil and Plant Analyzer Development (SPAD) 502 plus chlorophyll meter (Spectrum Technologies, Inc, Aurora, IL). This device allows determination of relative chlorophyll content while being non-invasive and non-destructive. Results can be obtained quickly and relative chlorophyll content can be determined at any time. Results are a ratio calculated according to the optical density difference of light transmitted through the leaf at wavelengths of 650 and 940 nm, which is compared to the light transmitted with no sample (calibration). The ratio is unitless and has strong correlation to the amount of chlorophyll in the leaf (Spectrum Technologies Inc., 2009).

The first relative chlorophyll content reading was taken 2 days before inoculation, which was 2 hours before fungicide application. The second relative chlorophyll content reading was taken 48 hours after fungicide application, and before plants were inoculated (day of inoculation). Three subsequent readings were taken 5, 7 and 9 days after inoculation.

All relative chlorophyll content readings were taken on fully expanded fourth leaves on three locations of the leaf (base, middle and tip). The main vein of the leaf was avoided and the window of the SPAD meter (2 mm x 3 mm) was fully occupied by the leaf lamina. A total of three readings were taken on each of the two plants in each conetainer. The average obtained from the six readings taken on the two plants in each conetainer was the experimental unit used in data analysis.

Green leaf area

Two hours after the fifth and final relative chlorophyll content reading, the fourth leaf of each plant was detached and scanned. Percent green leaf area was determined from the digital images using Assess 2.0 disease quantification software (Lamari, 2008, APS Press). Green leaf area was determined as related to the control.

Statistical analysis

Analysis of variance was performed using mixed procedure in SAS (version 9.4; SAS Institute Inc., Cary, NC). Comparison of results was done within variety and was based on comparing means of each fungicide treatment to the positive and negative controls.

RESULTS

The first relative chlorophyll content reading was taken at 2 day before inoculation, which was before any fungicide was applied to foliage. On this day, there was no statistical difference in chlorophyll between treatments that indicates treatments had similar levels of relative chlorophyll content at the beginning of the experiment (Table 1). The relative chlorophyll content averages were 37 and 37.6 for Duster and OK Bullet, respectively. The second reading occurred 48 hours after fungicide application. Relative chlorophyll content had increased in both cultivars to an average of 41. However there was no statistical difference in chlorophyll between treatments on the second day of reading.

After the second relative chlorophyll content readings, plants were inoculated with urediniospores of *P. triticina*. Subsequent readings were taken at 5, 7 and 9 days after inoculation. At 5 days after inoculation, no difference in relative chlorophyll content occurred in OK Bullet plants although positive control (non-sprayed and inoculated) plants were beginning to show the impact of disease initiation with a decrease in relative chlorophyll content (40.6) compared to negative control (non-sprayed and non-inoculated) plants (43.8). Significant differences in relative chlorophyll content of Duster were observed on the third day of reading. Duster plants sprayed with pyraclostrobin and the pre-mixture did not show statistical difference compared with the negative control plants. However metconazole-sprayed Duster plants (41.4) had a significant decrease in relative chlorophyll content compared with the negative control plants (45.0). The decrease was comparable to positive control plants (40.9), indicating metconazole did not

Table 1 - Effect of foliar fungicide on relative chlorophyll content and percent green leaf area of winter wheat plant leaves inoculated with urediniospores of *Puccinia triticina* in a controlled environment.

Variety	Treatment	Relative Chlorophyll Content[w]					Green Leaf Area (%)[s]
		2 DBI[v]	0 DBI[u]	5 DAI[t]	7 DAI	9 DAI	9 DAI
Duster	NSNI control[z]	37.5 ± 0.63	41.7 ± 0.42	45.0 a[r] ± 0.67	43.8 a ± 0.46	42.5 a ± 0.80	100 a
	NSI control[y]	37.1 ± 0.54	41.6 ± 0.40	40.9 b ± 0.56	37.2 c ± 1.06	30.8 c ± 2.10	67.9 b ± 9.19
	Metconazole, 1.2 L/ha[x]	36.4 ± 1.13	41.0 ± 0.73	41.4 b ± 0.49	40.0 b ± 0.25	39.2 b ± 0.48	97.0 a ± 1.43
	Pyraclostrobin, 0.6 L/ha	37.5 ± 0.93	41.2 ± 0.58	42.9 ab ± 0.72	42.0 ab ± 0.48	40.5 ab ± 0.77	99.1 a ± 0.68
	Metconazole + pyraclostrobin, 0.6 L/ha	37.0 ± 0.58	41.2 ± 0.54	42.8 ab ± 0.79	42.1 ab ± 0.62	40.8 ab ± 0.63	99.8 a ± 0.81
	P-value	*0.878*	*0.958*	*0.005*	*<.0001*	*<.0001*	*<.0001*
OK Bullet	NSNI control	37.0 ± 1.21	40.8 ± 0.98	43.8 ± 0.25	44.2 a ± 0.38	40.5 a ± 0.94	100 a
	NSI control	37.6 ± 0.80	42.0 ± 0.80	40.6 ± 0.80	34.6 c ± 1.09	31.1 b ± 0.79	31.7 b ± 4.39
	Metconazole, 1.2 L/ha	37.3 ± 1.00	40.9 ± 0.96	41.8 ± 0.66	40.2 b ± 0.96	39.3 a ± 1.10	94.0 a ± 2.53
	Pyraclostrobin, 0.6 L/ha	38.5 ± 0.78	41.7 ± 0.83	43.4 ± 0.35	42.4 ab ± 0.49	41.0 a ± 0.35	93.7 a ± 3.31
	Metconazole + pyraclostrobin, 0.6 L/ha	37.9 ± 0.58	41.6 ± 0.53	42.4 ± 0.75	40.3 b ± 0.81	39.2 a ± 1.36	95.4 a ± 4.19
	P-value	*0.782*	*0.815*	*0.053*	*<.0001*	*<.0001*	*<.0001*

[z] Not sprayed and not inoculated (negative) control plants
[y] Not sprayed and inoculated (positive) control plants
[x] Fungicides metconazole (Caramba® 90SL, 90 g active ingredient/L), pyraclostrobin (Headline® 250 SC, 250 g a.i./L) and pre-mixture of pyraclostrobin and metconazole (Twinline® 210 EC, 80 g metconazole/L and 129 g pyraclostrobin/L) are registered trademarks of BASF Corporation, Research Triangle Park, NC
[w] Relative chlorophyll content was determined using a SPAD 502 Plus Chlorophyll Meter (Spectrum Technologies Inc.). Means of relative chlorophyll content are followed by standard error values
[v] Reading was taken 2 days before inoculation and before fungicide application
[u] Reading was taken 48 hours after fungicide application, and immediately before inoculation
[t] Readings were taken respectively 5, 7 and 9 days after inoculation
[s] Percent green leaf area was determined using ASSESS 2.0 disease quantification software (Lamari, 2008, APS PRESS)
[r] Probability of a significant treatment at α=0.05. Means followed by the same letter within column and variety are not significantly different

maintain relative chlorophyll content.

The fourth and fifth relative chlorophyll content readings occurred at 7 and 9 days after inoculation, respectively. At 7 days after inoculation, differences were first observed in OK Bullet plants. Positive control plants showed the greatest decrease in relative chlorophyll content at both 7 and 9 days after inoculation (34.6 and 31.1), which was due to the development of pustules. Pyraclostrobin-sprayed OK Bullet plants showed relative chlorophyll content retention comparable to the negative control plants at 7 and 9 days after inoculation. Metconazole- and pre-mixture-sprayed OK Bullet plants did not show relative chlorophyll content maintenance comparable to negative control plants at 7 days after inoculation. However, relative chlorophyll content of these treatments were not statistically different from the negative control at 9 days after inoculation, which indicated relative chlorophyll content maintenance may have resulted from inhibition of initial stages of spore germination. Duster plants showed similar results at 7 and 9 days after inoculation. Pyraclostrobin- and the pre-mixture-sprayed Duster plants maintained relative chlorophyll content comparable to the negative control from 5 through to 9 days after inoculation. Metconazole-sprayed plants did not maintain relative chlorophyll content in Duster leaves because a significant decrease in the relative chlorophyll content compared with the negative control was again observed at 7 and 9 days after inoculation. In comparison to negative control plants, the greatest reduction in relative chlorophyll content was observed on positive control plants because of the hypersensitive response.

At 5 days after inoculation, no statistical differences in relative chlorophyll content were observed for OK Bullet but chlorotic spots had began to appear on leaves of the positive control. These chlorotic spots indicated the start of rust pustule formation, and became lightly sporulating pustules by 7 days after inoculation and heavily sporulating pustules by 9 days after inoculation. Consequently, the relative chlorophyll content of positive control plants (34.6) was significantly affected by the disease compared with negative control plants (44.2) at 7 days after inoculation.

Fungicide-treated plants showed similar relative chlorophyll content levels (average = 41), with pyraclostrobin (42.4) treated plants the only fungicide statistically similar to the negative control (44.2). At 9 days after inoculation, all fungicide-treated plants (avg = 39.8) had an relative chlorophyll content statistically the same as the negative control (40.5) with all being greater than the positive control.

Percent green leaf area of both cultivars showed a trend similar to relative chlorophyll content at 9 days after inoculation. The green leaf area of positive control plants can be considered as an expression of the area available for photosynthesis on the leaves scanned, which was approximately 68% and 32% for Duster and OK Bullet, respectively. Fungicide application to plants of both varieties effectively protected leaves against the disease, and no statistical differences compared to the negative control were observed. Compared to the green leaf area of negative control plants, the average green leaf area was 98% and 94% for fungicide-treated plants of Duster and OK Bullet, respectively.

DISCUSSION

Fungicides offer a means to manage wheat foliar diseases, and when applied to a susceptible variety before disease is established can mean the difference between a good and poor yield. However, yield increases also have been reported when varieties considered resistant to a foliar disease such as wheat leaf rust are sprayed with a fungicide (Edwards et al., 2012; Kelley, 2001; Milus, 1994; Ransom & McMullen, 2008). Such reports could be related to i) avoidance of development of the HR in resistant varieties (Bertelsen et al., 2001; Edwards et al., 2012), or ii) physiological effects closely associated with the class of strobilurin fungicides, which have been demonstrated in wheat (Grossmann et al., 1999; Grossmann & Retzlaff, 1997; Jones & Bryson, 1988; Köhle et al., 2002). Results obtained in this study demonstrated fungicide application affected relative chlorophyll content and green leaf area of a hard red winter wheat varieties resistant (Duster) and susceptible (OK Bullet) to wheat leaf rust.

The relative chlorophyll content of Duster and OK Bullet plants at 2 days before inoculation (which was just before fungicide application) and on the day of inoculation (which was 48 hours after fungicide application) were statistically the same. Hence, application of a fungicide did not increase relative chlorophyll content 48 hours after fungicide application. Immediately following the relative chlorophyll content reading on the day of inoculation, plants were inoculated with urediniospores of *P. triticina*, and subsequent development of disease affected the relative chlorophyll content associated with the three fungicide treatments. At 9 days after inoculation, the last day of reading, fungicide-sprayed plants of both varieties had similiar relative chlorophyll content readings compared with the negative control plants, with the exception of metconazole-sprayed Duster plants. The relative chlorophyll content maintenance observed on pyraclostrobin- and pre-mixture-sprayed Duster plants likely resulted from the killing effect of fungicide on urediniospores of *P. triticina* that prevented the development of hypersensitive response or formation of sporulating pustules on leaves. This observation is supported by Bertelsen et al. (2001) and Godwin et al. (1994) (as cited in Bertelsen et al., 2001, p. 201) who reported that azoxystrobin inhibited spore germination prior to infection. The results observed in our study are also supported by Buck & Williams-Woodward (2002), who reported that urediniospores of *P. hemerocallidis* (causal agent of daylily rust) was inhibited in medium amended with azoxystrobin. A similar mode of action likely occured with the pyraclostrobin and the pyraclostrobin plus metconazole pre-mixture.

Metconazole-sprayed Duster plants had a significantly lower relative chlorophyll content compared to the negative control plants at 5, 7 and 9 days after inoculation. Triazole fungicides, such as metconazole, act after penetration and during pathogen spread in the host (Mueller et al., 2013), which implies there was opportunity for interaction between the host and pathogen. We speculate that the interaction likely was sufficient to trigger the hypersensitive response in metconazole-sprayed Duster plants, which resulted in a significant reduction of relative chlorophyll content values of this treatment at 5 to 9 days after inoculation. By contrast, metconazole- and pre-mixture-sprayed OK Bullet plants had significantly lower relative chlorophyll content in comparison to the negative control only at 7 days after inoculation, but relative chlorophyll content of these treatments were maintained at 9 days after inoculation. Considering the mode of action of this triazole, relative chlorophyll content results observed in metconazole- and pre-mixture-sprayed OK Bullet plants suggest the fungicide was able to block further disease development resulting in maintenance of the relative chlorophyll content at 9 days after inoculation.

The data presented in this paper are insufficient to suggest metconazole-sprayed Duster plants completely avoided induction of the hypersensitive response. A molecular-based study would be necessary in order to confirm whether the hypersensitive response was triggered. However, previous studies have demonstrated that triazole fungicides affect spore germination only slightly, but do inhibit disease spread in the host. Bertelsen et al. (2001) found that epoxiconazole, a fungicide in the triazole family, produced defense responses against the pathogen infection similarly to untreated plants. Godwin et al. (1994) reported that spores of phytopathogenic fungi were able to germinate, penetrate, and initiate internal growth in wheat leaves before inhibition of disease development was initiated by epoxiconazole. Buck & Williams-Woodward (2002) observed that the three triazoles tested in their study did not affect *P. hemerocallidis* urediniospore germination, however these chemicals reduced the development of pustules. This could explain why metconazole-sprayed Duster plants had an relative chlorophyll content significantly less than the negative control, but significantly greater than the positive control even though green leaf area retention did not differ from negative control plants.

The relative chlorophyll content and green leaf area parameters used in combination offers the best estimate of leaf health. It is important that both parameters be considered when analyzing the effects of fungicides on leaves. Green leaf area estimates the area of green tissue in leaves that was not affected by disease, and complimentary relative chlorophyll content readings reflect the leaf greenness. As demonstrated in this study, metconazole-sprayed Duster plants developed reduced flecking compared to positive control plants but greater then the other fungicide treatments. However the green leaf area of metconazole-sprayed Duster plants did not differ from negative control leaves but the relative chlorophyll content was statistically different from the negative and the positive control plants. These results indicate that even though metconazole retained green leaf area, it did not maintain an relative chlorophyll content level in sprayed leaves similarly to negative control plants.

The strobilurin pyraclostrobin in Headline® was the first fungicide to receive Environmental Protection Agency approval for a plant health label in the United States (BASF, 2009). Since its release, claims of plant physiological effects as a means to increase crop yield have been debated because consistent positive results have not been observed over years, especially in years of low disease incidence (Blandino et al., 2009; Edwards et al., 2012; Kelley, 2001; Ransom & McMullen, 2008; Swodoba & Peterson, 2009; Weisz et al., 2013). Further, studies by Bertelsen et al. (2001), Godwin et al. (1994), and Buck & Williams-Woodward (2002) led to the hypothesis that application of fungicides with activity against germinating spores resulted in avoidance of the hypersensitive response. This hypothesis has been proposed to occur specifically for disease resistant wheat varieties (Bertelsen et al., 2001; Edwards et al., 2012).

In the present study, fungicide-sprayed winter wheat plants did not show a significant increase in relative chlorophyll content indicating no plant health benefit as a result of using a strobilurin fungicide. Early fungicidal effect as indicated by the lack of flecking was observed for Duster plants sprayed with pyraclostrobin and a pre-mixture of pyraclostrobin + metconazole.

Metconazole-sprayed Duster plants had a consistent and significantly lower relative chlorophyll content compared to the negative control. This agrees with the mode of action described for triazoles, which act after penetration and during pathogen spread and establishment inside the host. Thus, we speculate this would trigger the hypersensitive response in the host as a result of interaction between host and pathogen during the early stages of disease development and likely result in reduced relative chlorophyll content. In contrast, the fungicides containing pyraclostrobin, which act directly against spore germination, would prevent any triggering of the hypersensitive response and consequently relative chlorophyll content would be maintained. Further molecular-based studies are required to elucidate whether these fungicides triggered or avoided hypersensitive response. As a conclusion, the results of this study do not support claims of increased chlorophyll content associated with fungicide application, and also provide insight explaining why resistant varieties sometimes show a yield response to a fungicide applied in the presence of foliar disease.

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APPENDIX

VARIATION IN THE REACTION OF HARD RED WINTER WHEAT CULTIVARS TO COMMON ROOT ROT AND SPOT BLOTCH

INTRODUCTION

Due to the adoption of no-till systems, the incidence of residue-borne foliar fungal diseases has increased in Oklahoma. Diseases such as tan spot [*Pyrenophora tritici-repentis* (Died.) Drechsler (*Drechslera tritici-repentis* (Died.) Shoemaker)], Septoria leaf blotch [*Septoria tritici* Roberge in Desmaz. (*Mycosphaerella graminicola* (Fuckel) J. Schrot.)], and Stagonospora glume blotch [*Stagonospora nodorum* (Berk.) E. Castell. (*Phaeosphaeria nodorum* (E. Müll.) Hedjaroude)] have had higher incidence in the past few years. The incidence of other diseases has increased as well; these are common root rot and spot blotch both caused by *Bipolaris sorokiniana* (Sacc.) Shoemaker (*Cochiliobolus sativum* (S. Ito & Kurib.) Drechsler ex Dastur) (Stein, 2010). This pathogen is distributed worldwide and the diseases it causes affect common wheat (*Triticum aestivum* L.), other cereal grains such as barley, and species of monocots (Bakonyi et al., 1997, as cited in Kumar et al., 2002, p.187).

B. sorokiniana causes many diseases in many crops, and the severity of the diseases it causes varies greatly with environmental conditions. In wheat, *B. sorokiniana* causes kernel blight (or black point), seedling blight, root rot, and leaf spot (Stein, 2010).

Disease severity is favored under warm and humid growing conditions. In growing areas with these conditions, spot blotch is often considered the most important foliar disease. Moderate to warm temperatures (between 18º to 32ºC) and at least 18 hours of leaf wetness favors infection and growth of this fungus (Reis, 1991, as cited by Acharya et al., 2011, p. 1067). In contrast, common root rot is more severe in drought environments. Wheat growing regions most impacted by spot blotch are South Asia (including India, China and Australia), and Latin America (Argentina and Brazil) (Kumar et al., 2002). In North America, *B. sorokiniana* is frequently observed in Canada, and in the United States (Minnesota, Montana, North Dakota and South Dakota) (Acharya et al., 2011; Farr & Rossman, 2006).

The diseases caused by this pathogen can be very destructive. Grain yield losses of 40% due to spot blotch had been reported in South Asia (Sharma et al. 2006). In contrast, losses of only 3–5 % due to spot blotch have been reported in the US and Canada (Stein, 2010), but losses up to 20% due to common root rot and seedling blight have been reported in regions of Brazil (Murray et al., 1998, as cited in Acharya p. 1065).

The fact two diseases are caused by this pathogen complicate management in the field (Stein, 2010). Cultural practices such as crop rotation, tillage, and use of disease free seeds reduce inoculum of common root rot and decrease incidence of spot blotch. Chemical control using fungicides to protect foliage or seeds also has been recommended (Acharya et al., 2011; Stein, 2010). The use of disease resistant varieties is another alternative. However selection for resistance to spot blotch in wheat has not typically been a high priority in the United States as other diseases such as rusts and powdery mildew demand the greater priority (Sharma et al., 2007). Sources of resistance have been found in wheat lines from Brazil and China (Kohli et al., 1991 as cited in Acharya p. 1069); however, even wheat varieties identified as resistant to SB can have significant yield loss under a severe epidemic (Duveiller et al., 2005).

These two diseases caught the attention of the Oklahoma State University (OSU) wheat improvement program because the incidence of spot blotch appeared to be increasing in years prior to the recent drought. Therefore, this study used protocols previously reported to evaluate wheat varieties for reaction to common root rot and spot blotch with the goal of adapting those protocols to evaluate adapted varieties and breeder lines for reaction to common root rot and spot blotch.

MATERIAL AND METHODS

Inoculum maintenance

Two *B. sorokiniana* isolates were used in this study. BsFL was obtained from foliage collected in 2014 from wheat growing in a field near Stillwater, OK. BsRT was obtained from a wheat sample submitted to the Plant Disease & Insect Diagnostic Laboratory in the Department of Entomology and Plant Pathology at Oklahoma State University. BsRT was isolated from wheat roots collected in 2013 from wheat growing in Jackson County, OK. Infected tissues were surface sterilized, and place on water-agar. In order to obtain a pure culture of each isolate, single-spores were isolated. Isolates were stored long-term on autoclaved wheat seeds amended with 1 ml of autoclaved distilled water at -20^{o}C.

Inoculation

Isolates were grown on 20% clarified V8-agar medium at 22^{o}C in the dark for 10 to 15 days. Spores were harvested by scraping cultures with a glass slide in a solution of distilled water and 0.01% non-ionic surfactant (Hi-yield® spreader sticker). The spore suspension was filtered through two layers of cheese cloth, and the resulting spore suspension was used to inoculate wheat.

For spot blotch, a spore suspension containing 10^5 to 10^6 spores/ml was sprayed uniformly onto foliage of young plants using a hand-held pressurized bottle. Plants were inoculated outside a growth chamber, and allowed to dry for 15 to 30 min before placing the conetainer rack into a plastic bag with wet towels. Plants were kept in this moist chamber inside of a growth chamber at 25°C with a 12 hr photoperiod. After 48 hr, plants were removed from the moist chamber, and kept in a growth chamber at 23°C with a 12 hr photoperiod for 5 to 7 days. Seven to 9 days after inoculation, leaves were detached from plants and evaluated for disease severity.

For common root rot, the spore suspension was adjusted to 10^5 spores/ml. A constant light stirring was provided to avoid settling of spores. Sterilized seeds (procedure is described later) were dipped into this spore solution and then placed on saturated filter papers inside of 7 in. x 8 in. plastic bag. Plastic bags containing inoculated seeds were kept in an incubator at 23°C in the dark. After 3 days, seedlings were removed from the plastic bags and analyzed as described below.

Plant material

The hard red winter wheat varieties tested were Billings, Cedar, Deliver, Doublestop CL Plus, Duster, Endurance, Everest, Gallagher, Garrison, Jagger, Iba, and Ruby Lee. They were tested for reaction to spot blotch and common root rot using both of the isolates described previously.

For spot blotch, two seeds were sown in 2.5 in. plastic conetainers and grown in a growth chamber at 23°C with a 12 hr photoperiod. Conetainers were arranged in a complete randomized block design with seven replications. Plants were grown for 2 wk. or until the 3-leaf stage at before inoculation proceeded. Two reference varieties were used in this experiment. Two soft white spring wheat varieties, Sonalika and Atilla, were used as susceptible and resistant control checks, respectively (Khan & Chowdhury, 2011; Mahto et al., 2011).

For common root rot, seeds were surface sterilized with a solution of 10% bleach (sodium hypochlorite, 8.25%) and 0.01% non-ionic surfactant (Hi-yield® spreader sticker) for 10 min agitated at 5,000 rpm. Seeds were washed with autoclaved distilled water three times for 10 min agitated at 5,000 rpm. Surface sterilized seeds were air dried for 15 min and 5 seeds were inoculated and placed on the filter paper. After inoculation, plastic bags were arranged in trays in a complete randomized block design with three replications. Two spring wheat varieties, Cadet and Rescue, were used as resistant and susceptible checks, respectively (Neal et al., 1973). Sonalika and Atilla were also included in this screening.

Disease assessment

For spot blotch, leaf spot was visually assessed by estimating the percent diseased leaf area and digitally using Assess 2.0 disease quantification software (Lamari, 2008, APS Press). Correlation between visual and digital quantification was performed. Analysis of variance was performed using mixed procedure in SAS (version 9.4; SAS Institute Inc., Cary, NC).

For common root rot, root system length (in millimeters) was determined using Assess 2.0, and percent reduction of root system was determined comparing inoculated treatments to the not-inoculated control. Analysis of variance was performed using mixed procedure in SAS (version 9.4; SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Spot Blotch

A strong linear correlation ($R^2 = 0.87$) was observed between visual and digital rating of percent diseased leaf area (Figure 1). Assessment of foliar diseases can be done using visual rating scales, such as the one developed by Fetch, Jr. and Steffensom (1999) to assess severity of spot blotch in barley. This one designates grades of infection responses from 1 to 9 based on type and size of lesions. The high and significant correlation between visual and digital assessments indicated that visual assessment of foliar disease can be used to estimate a plant's reaction to spot blotch However, this validation was only possible to obtain if disease severity scale was equivalent to Assess software units. This software reads in units percent-infected leaf area from 0 to 100% with increments of one decimal. Visual estimation according to these units is not feasible for human eyes. Instead, we assessed disease severity in increments of 5% leaf area (Figure 2). This disease severity scale can be used as a reference in disease severity assessment of spot blotch and similar foliar diseases such as tan spot and Septoria leaf blotch. Therefore, we recommend the adoption of this scale in the screening procedure of the OSU wheat improvement program for determining spot blotch reaction when testing wheat seedlings.

Statistical analysis revealed no significant differences in disease severity between the isolates used with P values of 0.589 and 0.785 for digital and visual quantification, respectively. Results of digital assessment of percent diseased leaf area ranged from 16 to 44%, and visual assessment from 10 to 52% (Table 1). The control checks, Sonalika and Atilla, showed contrasting reactions to spot blotch, which was expected according to the literature. Sonalika was susceptible with almost 40% percent diseased leaf area, and Atilla resistant with 21%. Garrison and Endurance were the most resistant varieties in the digital and visual analysis, respectively. Duster was the most susceptible among all varieties in both analyses. Although there was variation in the values of percent diseased leaf area of digital and visual assessments, high correlation between these analyses confirm the validity of visual determination of percent diseased leaf area in a screening procedure for disease resistance.

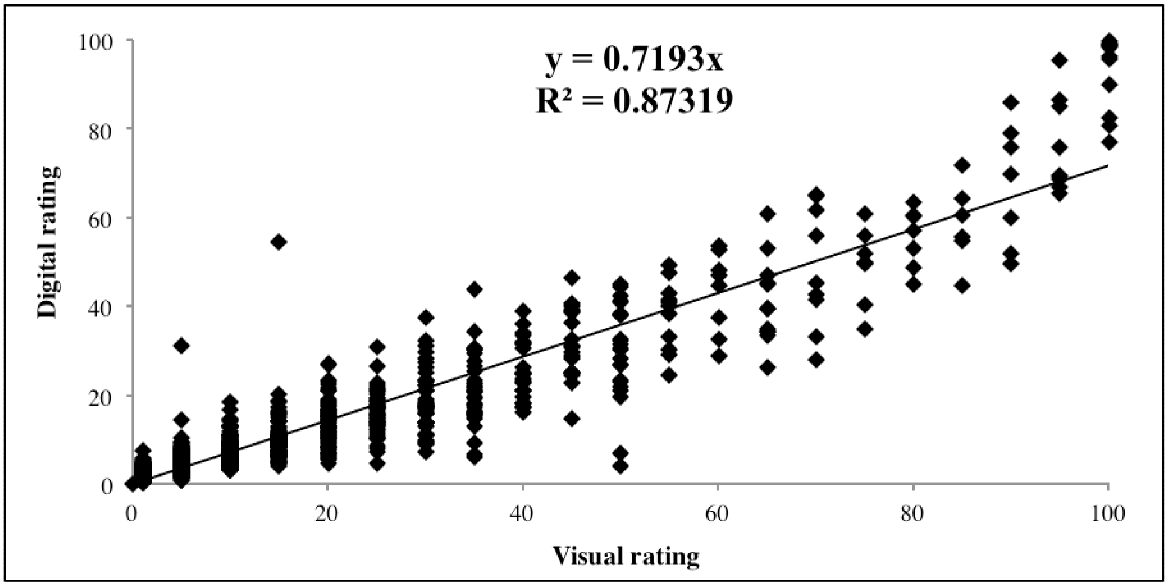

Figure 1 – Linear correlation between visual and digital assessments of spot blotch disease of wheat, caused by *Bipolaris sorokiniana*.

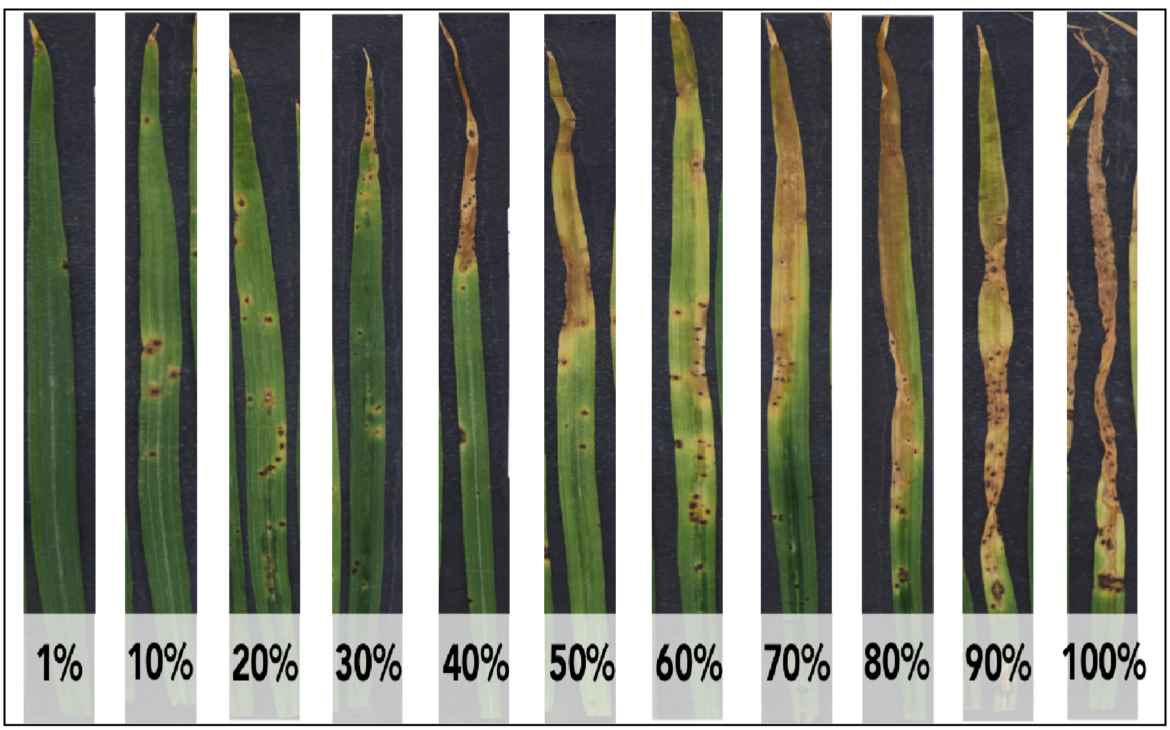

Figure 2 – Percent infected leaf area rating scale for assessing infection responses and disease severity of spot blotch disease of wheat, caused by *Bipolaris sorokiniana*.

Classifying the reaction of varieties to SB was based solely on visual assessment of percent diseased leaf area because there was a high correlation between visual and digital analyses. Classification of varieties by their reaction to spot blotch was more complicated than just using the results of the statistical analysis. The biggest question was to determine the thresholds that best discriminate between resistance and susceptibility. Tentative thresholds were stipulated in order to classify these varieties according to their resistance or susceptibility to SB. Three thresholds were used: from 0 to 18 percent diseased leaf area, varieties were considered resistant; from 19 to 32 percent, intermediate; and greater than 33 percent, susceptible. The three groups of varieties obtained in the digital and visual assessments were similar. Atilla, Endurance, Everest, Deliver, Gallagher, Garrison and Ruby Lee were consistently the most resistant to spot blotch. Billings, Cedar, Doublestop CL Plus, and Jagger were considered intermediate, and Iba, Duster and Sonalika were consistently the most susceptible varieties. This similarity adds to the confirmation that visual determination of percent diseased leaf area can be used in screening procedures for disease resistance.

Common Root Rot

For common root rot, statistical analysis revealed no significant difference between the results obtained with the two isolates (Pr > | t | = 0.1987), but significant differences were observed for varieties (Pr > | t | = 0.0023). The results of percent reduction in root system are shown in Table 2. Rescue showed an increase in root system following inoculation with *B. sorokiniana*, even though Rescue was reported as being susceptible to common root rot. The other control check, Cadet, was reported to be resistant to common root rot, but showed a large percent reduction in root system (45%).

Table 1 – Spot blotch severity, in percentage, determined by digital and visual assessments. Least significant differences were obtained using mixed procedure at p=0.05.

Variety	Digital	Variety	Visual
Garrison	15.8	Endurance	10.5
Endurance	16.7	Ruby Lee	13.4
Ruby Lee	17.2	Garrison	13.5
Gallagher	17.8	Gallagher	14.3
Deliver	19.4	Deliver	15.7
Everest	19.6	Everest	17.5
Billings	20.5	Atilla	18.1
Doublestop CL Plus	21.0	Billings	20.7
Atilla	21.8	Doublestop CL Plus	22.8
Cedar	24.8	Jagger	24.7
Jagger	28.5	Cedar	27.7
Iba	33.5	Iba	38.5
Sonalika	38.4	Sonalika	43.4
Duster	44.3	Duster	51.8
LSD	*9.40*	*LSD*	*7.8*

Table 2 - Percent reduction in root system length due to common root rot of wheat as determined by digital assessment. The least significant difference was obtained using the mixed procedure at p=0.05.

Variety	Root reduction (%)
Sonalika	+58.1
Rescue	+15.0
Garrison	8.6
Cedar	12.4
Billings	17.4
Iba	20.5
Jagger	21.9
Gallagher	23.8
Doubletop CL Plus	26.2
Everest	27.0
Atilla	34.7
Ruby Lee	38.7
Deliver	44.2
Cadet	45.3
Duster	73.7
Endurance	87.9
LSD	*27.0*

The reaction of Sonalika and Atilla to common root rot has not been reported previously. So this study determined their reaction to common root rot in comparison to spot blotch. Sonalika and Atilla showed contrasting reactions to spot blotch and common root rot. For spot blotch, these varieties are considered susceptible and resistant, respectively, which was validated in the spot blotch study. However, Sonalika and Atilla were shown to be resistant and susceptible to common root rot, respectively (Table 2).

The commercial hard red winter wheat varieties tested in this experiment showed percent reduction in root system from 8.6 to 87.9%. Garrison and Cedar showed the lowest reduction, whereas Duster and Endurance had the highest reduction. Interestingly, Garrison and Duster reacted similarly to spot blotch and common root rot, in that these varieties were resistant and susceptible, respectively, to spot blotch and common root rot.

One of the goals of this experiment was to develop an easy and fast protocol based on existing inoculation procedures for common root rot for use in screening for resistance. Due to the large number of varieties tested in this experiment, the number of replications and the number of seeds in each replication was reduced. Consequently, high experimental error was observed in this experiment, which does not allow for a good separation of the reactions to common root rot. Even with the reduced number of replications and seeds per replication, the protocol used in this study was time-consuming and impractical to screen in a breeding program. Handling of seedlings to be scanned is problematic because of damage and breaks to root filaments. Root filaments cannot be clumped to one another; otherwise the software will recognize the clump as one unique filament, which underestimates the root system length. In addition, considering the reaction observed in Rescue and Cadet, the procedure used in this experiment needs to be improved in order to obtain more consistent results.

CONCLUSION

The protocol used in the inoculation procedures for spot blotch was successful and has been extended to testing breeder lines for reaction to spot blotch. Results obtained in those experiments showed promising levels of resistance for spot blotch in the OSU hard red winter wheat varieties Endurance, Gallagher, Garrison and Ruby Lee. These varieties may provide useful resistance to spot blotch in wheat varieties adapted to the southern plains. In contrast, the protocol used to test for reaction to common root rot need further improvement to better estimate the reaction of wheat lines and varieties to this disease.

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