Evaluating the effects of fungicides for the control of *Exserohilum turcicum* on sorghum in South Africa

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It all starts here ™

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ABSTRACT

Sorghum (*Sorghum bicolor* (L.) Moench) is one of the five most widely cultivated grasses in Sub-Saharan Africa. *Exserohilum turcicum* (Pass.) Leonard and Suggs is a fungal foliar pathogen that causes leaf blight (LB) on both maize and sorghum, with losses of up to 50% recorded on sorghum. Certain fungicides used to control the disease have been shown to have plant growth-regulating properties leading to increased yield and delayed leaf senescence. The merit of these growth-regulating properties of fungicides when applied prophylactically has not been evaluated under South African climatic conditions. Accordingly, field trials were conducted in three major sorghum cultivation sites (Greytown, Potchefstroom and Standerton) during the 2013/14 and 2014/15 growing seasons. A glasshouse trial to evaluate certain biochemical and physiological parameter was also conducted during 2015. Two fungicides (azoxystrobin/difenoconazole and epoxiconazole/pyraclostrobin) were applied to four sorghum cultivars at 6, 8, 10, 6 & 8 and 8 & 10 weeks after planting to study agronomic, physiological and biochemical responses of the plants in response to such application. Significant differences in yield were only shown in response to one treatment at Standerton during the 2013/14 planting season. Clear delays in senescence were observed with fungicide application at Greytown for the entire duration of the 2013/14 season, as well as the first senescence observation (S1) during 2014/15, but this effect was not observed at any of the other sites. In the glasshouse, no response to fungicide application was obtained with photosynthetic capacity parameters measured. Enzyme responses were visible with anti-oxidant enzymes showing increased activity with fungicide application. Delays in senescence may be associated with delays in development, as black layer formation was delayed in some cases. These delays in senescence did however not result in increased sugar acculmilation in the seed. To conclude, both senescence observations and enzyme function resulted in supporting evidence that fungicides affect plant function, but with no clear indication that prophylactic application will provide financial return in the form of yield increases which will support increased production cost to the producer.

Key words: black layer, fungicide, leaf blight, photosynthetic efficiency, reactive oxygen species, senescence, sorghum, yield.

OPSOMMING

Sorghum bicolor (L.) Moench) is een van die vyf mees verboude gewasse in Sub-Sahara Afrika. *Exserohilum turcicum* (Pass.) Leonard & Suggs is 'n blaarpatogeen wat blaarskroei (LB) op beide mielies en sorghum veroorsaak, en kan lei tot opbrengsverliese van tot 50% in sorghum. Sekere swamdoders toon tekens van plant-groeiregulerende eienskappe wat kan lei tot verhoogde opbrengs en vertraagde veroudering van blare. Die meriete van hierdie groeiregulerende eienskappe as gevolg van die die toediening van hierdie swamdoders in die afwesigheid van siekte is nog nie geëvalueer onder Suid-Afrikaanse klimaatstoestande nie. Gevolglik is veldproewe uitgevoer by verskeie hoof sorghumproduksie-areas (Greytown, Potchefstroom en Standerton) tydens die 2013/14 en 2014/15 plantseisoen, asook ‘n glashuisproef met twee verskillende swamdoders (asoksiestrobien/difenkonasool en piraklostrobien/epoksikonasool) by verskillende toedieningsdatums (6, 8, 10, 6 & 8 en 8 & 10 weke na plant). Die doel van die studie was om die agronomiese, fisiologiese en biochemiese reaksies van swamdodertoediening op twee sorghumkultivars te ondersoek. Noemenswaardige opbrengsverskille was beperk tot een toediening by Standerton tydens die 2013/14 plantseisoen. Duidelike vertragings in veroudering was waarneembaar met swamdoder toediening veral op Greytown tydens die 2013/14 plantseisoen, asook tydens die eerste observasie (S2) in 2014/15. In die glashuis was geen noemenswaardige reaksie t.o.v. swamdoderbehandeling waargeneem waar die fotosintetiese kapasiteit van die plante bestudeer is nie. Noemenswaardige ensiemreaksies was sigbaar met anti-oksidant ensieme soos SOD wat ’n positiewe reaksie toon met swamdoder toediening. Vertragings in tempo van veroudering mag gekorreleer word met vertragings in die ontwikkeling van die plant, d.w.s. swartlaag vorming is vertraag in sommige gevalle. Hierdie vertraging in veroudering het egter geen effek gehad op die akkumulasie van suiker in die saad nie. Om af te sluit, in sommige gevalle is daar bewyse dat swamdoders wel ’n effek het op sekere fisiologiese parameters, maar met geen duidelike aanduiding dat profilaktiese toediening ’n finansiële voordeel in die vorm van verhoodge opbrengs tot gevolg sal hê vir die produsent nie.

Sleutel woorde: Sorghum, blaarskroei, veroudering, fotosintetiese doeltreffendheid, opbrengs, swamdoder, reaktiewe suurstof spesies, swartlaag

LIST OF ABBREVIATIONS

Abbreviation	Explanation
AT	Amistar Top®
AB	Abacus®
DAP	Days After Planting
ETC	Electron Transport Chain
GOD	Glucose Oxidase
LB	Leaf Blight
ROS	Reactive Oxygen Species
POD	Peroxidase
PSII	Photosystem II
OEC	Oxygen Evolving Complex
S1-3	Senescence observations 1-3
SSA	Sub-Saharan Africa
SOD	Superoxide Dismutase
XOX	Xanthine Oxidase

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1 Introduction

The primary use of fungicides is to protect the crop from fungal attack. The application of fungicide does not increase yield *per se*, but is designed to protect an inherent yield potential. The existence of fungicides with growth-regulating properties has been the focus of various studies since the late 1960's. These fungicides have been selectively used to increase the yield of various grain crops (Morgounov *et al*., 2015; Lopez *et al*., 2015; Jay, 2008; Byerlee and Moya, 1993). However, the merit of such application has not been clearly established as varying results have been obtained.

Triazoles and strobilurins are xamples of these fungicides which according to international literature have been successfull in controlling fungal infections in plants (Rouabhi, 2010). Triazole and strobilurin based fungicides are often mixed together in disease management programmes and are used in combination with genetic resistance to obtain an optimum potential yield. Triazoles inhibit the biosynthesis of ergosterol, which results in the inhibition of much needed fungal steroids (Fera *et al.,* 2009). Triazoles are also known to interfere in gibberellic acid synthesis resulting in morphological changes in the plants (Shani *et al.*, 2013). Triazoles have also been associated with increased lodging resistance, and improved rooting (Berry and Spink 2009).

Strobilurin based fungicides are attracting interest from farmers due to reports that these chemicals improve yield through improving crop biomass and the harvest index, without the presence of disease (Wegulo *et al.*, 2011). This phenomenon has been pinned as the strobilurin "greening effect" because the effect is associated with longer periods of leaf greenness and thereby maximizing the grain-filling period (Kane and Smiley, 1983). The anti-fungal activity of strobilurins is based on their ability to inhibit mitochondrial respiration by binding to the quinol oxidation (Q_1) site of cytochrome b (Grossman and Retzlaff, 1997). Strobilurins also influence physiological processes such as the CO_2 compensation point, leaf senescence, ethylene biosynthesis, chlorophyll content, photosynthetic activity, stomatal

conductance, water consumption, plant anti-oxidant enzyme activity, the endogenous levels of certain phyto-hormones and nitrate reductase activity in the plant (Petit *et al.*, 2012).

As these fungicidal compounds affect the hormonal pathways associated with senescence, it is expected that fungicide application will influence the rate of plant senescence (Vanancio *et al.*, 2003). As a plant develops, hormonal changes lead to the increased production of damaging ROS in the cell which will start the senescence process. ROS scavenging enzymes, such as POD, SOD and CAT are present to combat oxidative damage. Distefano *et al.* (1999) reported on the subsequent upregulation of ROS scavenging enzymes after fungicide application, which could be associated with the decreases observed in the rate of senescence.

The information available regarding the prophylactic (preventative, application before symptoms arise) use of fungicides on sorghum is extremely limited and therefore this dissertation investigates the merit of the use of fungicides with growth-regulating properties, in the absence of disease. Therefore, the objective of the dissertation is to (1) examine the effect of prophylactic fungicide application on selected sorghum cultivars on various agronomic parameters in the glasshouse and different field conditions on different sampling dates within South Africa and (2) how these applications will affect selected physiological and biochemical parameters on selected sorghum cultivars.

It is expected that the prophylactic application will delay the onset of senescence adequately to allow for increased grain fill and higher yield, to provide financial return in correlation with increased production costs. Furthermore, the application of fungicide is expected to upregulate the production of ROS scavenging enzymes as a mechanism to combat the onset of senescence and increase the assimilation of sugar to the seed.

2

Literature review

2.1 Introduction

In South Africa sorghum is primarily used in the food and fodder industries as an alternative food crop. The first cultivation of sorghum dates to ± 1000 B.C. in India and China. Sorghum is mainly milled to produce products like beer, porridges, bread, couscous, soups and batter for cakes in the food industry. In the fodder industry, it is used to produce feed for cattle and poultry.

South Africa is accustomed to dry temperate climates, which makes sorghum an ideal crop, due to its drought tolerance as well as its capability to survive extended periods of waterlogging (Ramathani *et al.*, 2011).



Figure 2-1: *Sorghum bicolor*.

Common threats to sorghum include insect pests, and diseases such as leaf spots, stripes, smuts, root rots, leaf blights and grain moulds (Thomas, 1992).

2.2 Sorghum

2.2.1 Growth and physiological development

Sorghum bicolor ((L.) Moench), is a self-pollinating, diploid C4 grass, with high adaptivity to the semi-arid tropics (ICRISAT, 2015). Sorghum was domesticated in Sub-Saharan Africa (SSA) in 1000 B.C, via trading routes from India and China, and is along with *Zea mays*, one of the most important food sources in Africa, although it is largely classified as a subsistence crop. It ranks number five in the world's most important cereals after wheat, maize, rice and barley (Taylor, 2002). It is a key component of more than 50% of Africa's rural livelihood strategies.

According to de Wet (1978) the African complex of *S. bicolor* is extremely diverse, comprising of cultivated, wild, and weedy types. These "types" are referred to as subspecies. Although the genus sorghum is very diverse, all the cultivated species belong to the species *Sorghum bicolor* ssp. *bicolor*. This can further be divided into five basic races i.e. *bicolor, caudatum, guinea, durra* and *kafir* (de Wet and Huckabay, 1967; Ramathani *et al.*, 2011) and ten hybrid races that combine characteristics of two or more of the basic races. These races differ in morphology, for example *bicolor* will have open inflorescence and elliptic grains, while *durra* have more compact inflorescence. These races contribute to the genetic diversity in cultivated sorghum (de Wet, 1978).

Internationally 242 cultivars of sorghum have been released since 1970 (ICRISAT, 2015). As of 2007 sorghum cultivation has varied quite remarkably in South Africa due to interest in the crop's potential for biofuel application (Department of Agriculture Forestry and Fisheries, 2013). During 2014 a draft position paper on the South African biofuels regulatory framework was released by the Department of Energy in this regard (Anonymous, 2014). As per this paper, sorghum together with soybean are recommended as reference feedstock for the manufacturing of bio-ethanol and biodiesel respectively. This recommendation is based on

sorghum having a greater potential benefit for job creation, whilst the crop requires less water to cultivate - which makes it ideal for a water-stressed country like South Africa.

According to Vanderlip (1993) sorghum development scales (Table 2-1) are assigned much like that of maize, ranging from zero (0) to nine (9). The development time for the sorghum plant to get from one stage to the next will be dependent on the hybrid of the plant in question and the environmental conditions (temperature, soil fertility, rainfall, planting date, pests and disease occurrence) in which it is cultivated.

Table 2-1: Development stages of sorghum plants (Vanderlip, 1993).

Developmental Stage	± Days after emergence	Distinguishing features
0	0	Emergence of plant
1	10	3 – Leaf stage
2	20	5 – Leaf stage
3	30	Growing point differentiation (8 – leaf stage)
4	40	Final leaf visible in whorl
5	50	Boot (head in flag leaf)
6	60	Half-bloom
7	70	Soft-dough
8	85	Hard-dough
9	95	Physiological maturity (black-layer formation)

At harvest (10-13% moisture) the plants have reached physiological maturity (stage 9) and the black layer becomes clearly visible. The black layer, also referred to as the hilum of the seed, forms when the seed detaches itself from the funiculus (Vanderlip, 1993). Giles *et al.* (1975) again states that the black layer can be visible as early as 15 days after pollination and is due to phenolic deposits in the phloem parenchyma cells. Because carbohydrates are transported by the phloem in plants (Turgeon and Wolf, 2009), this blockage prevents the continuous assimilation of carbohydrates and other compounds to the seeds and thus the term black layer and physiological maturity has become somewhat synonymous in sorghum development.

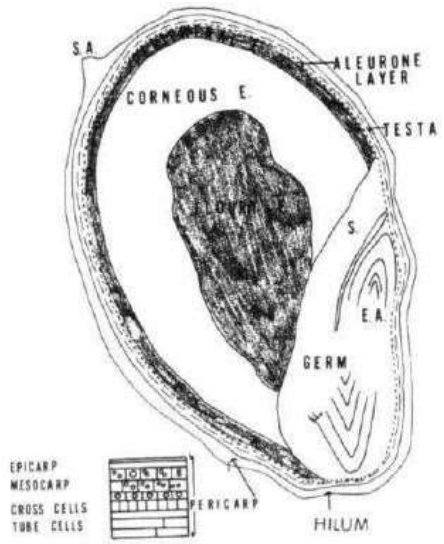


Figure 2-2: Schematic representation of a sorghum seed (Vanderlip, 1993).

2.2.2 Sorghum production in South Africa

Sorghum is ideally cultivated in shallow, neutral (pH 5.5-8.5) soil with a clay content of between 10 and 30% (du Plessis, 2008). Soil temperature should not fall below 5°C or rise above 15°C. Ideal temperatures for optimum yield are between 27° and 30°C during the day; extremely high temperatures will decrease yield. Temperatures below 18°C will cause lateral shoot development and plants exposed to sub-zero temperatures will die. Sorghum production areas vary from wet areas that have upwards of 800 mm of precipitation per annum to areas with approximately 400 mm per annum (du Plessis, 2008).

With the current unpredictable rainfall and declining water resources, attention is shifting towards increased cultivation of sorghum as one of the climate change adaptation strategies. According to ICRISAT (2015) the production of sorghum has increased significantly in East and Southern Africa over the past 40 years, as can be seen in Figure 2-3.

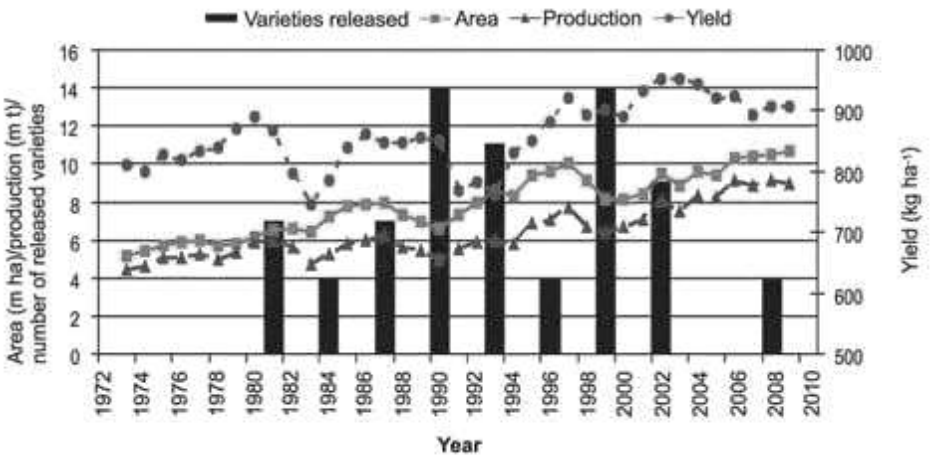


Figure 2-3: Increases in worldwide production area and yield, as well as varieties releases by ICRISAT over the past ± 40 years (ICRISAT, 2015).

During the year 2000, 60 million tonnes of sorghum were produced internationally, with Africa accounting for 20 million tonnes and South Africa approximately 210 000 tonnes (Taylor, 2002). These numbers increased to 25.6 million tonnes in Africa and decreased to 150 000 tonnes in South Africa in 2013 (FAO, 2013). During 2014, sorghum production in South Africa again increased to 265 000 tonnes (Table 2-2).

Table 2-2: Sorghum production statistics in South Africa from the Department of Agriculture forestry and Fisheries (2016).

Season	2009/10	2010/11	2011/12	2012/13	2013/14	2014/15	2015/16
Plantings (ha)	86675	69 200	48 550	62 620	78 850	70 500	48 500
Production (t)	196 500	155 000	135 500	147 200	265 000	120 500	88 500
Yield (t.ha^{-1})	2.27	2.24	2.79	2.35	3.36	1.71	1.83

According to the South African Department of Agriculture, Forestry and Fisheries an estimated 88 500 tons of sorghum will be produced during the 2015/16 season, with the Free State, Limpopo and Mpumalanga being the largest contributors to the area planted to sorghum (Anonymous, 2016). Sorghum is, however, also cultivated on a smaller scale in the Gauteng, North West, and KwaZulu-Natal provinces.

Within SSA, sorghum is mainly cultivated in the dryer temperate regions. More focussed attempts are accordingly made towards successful cultivation and increased yield due to the

high adaptivity of *S. bicolor* (Ramathani *et al.*, 2011), as it is the staple food of many rural people in Africa. However, the current average cultivation rate is at a mere 1 $t.ha^{-1}$ (Ramathani *et al.*, 2011), mostly due to poor or traditional farming practices on unimproved land areas. Where improved farming practises and integrated management strategies are incorporated, such as on commercial land in South Africa, average yields of 2.75 $t.ha^{-1}$ have been recorded (FAO, 2015).

Although sorghum production worldwide has shown steady increases, it may be merely due to increased cultivation areas. This becomes clear by statements from Taylor (2002) showing that yield and hectares planted increases linearly from 1975 to 2002. This continuous increase in production area is both damaging to natural ecosystems and the sustainability of the agricultural sector.

2.2.3 Qualities and attributes

Sorghum is of crucial importance to food security as it serves as the staple food for 500 million people in 30 countries (ICRISAT, 2015). Sorghum's unique tolerance to both drought and waterlogged conditions can be attributed to its unique adaptations such as deep penetrating roots, leaf rolling and stomatal closure to prevent water loss, high levels of epicuticular wax, high capacity for osmotic adjustment, and stay-green genes for prolonged photosynthesis (Taylor, 2002).

In Africa, the use of sorghum to produce food and beverages have become common practice in some countries, either because it is the only grain available or because it is the preferred grain. According to Sorghum Checkoff (2012) sorghum grain consists of 75% carbohydrates, making it an excellent energy source. At 3.3%, sorghum contains more fat than wheat and rice but less than corn. Sorghum has 11-12% protein, which is more than corn and rice, but similar to wheat, however, sorghum is naturally gluten free.

Sorghum products include injera (a pancake-like bread and staple food of Ethiopia), lagers, stouts, cloudy – and opaque beer, alcohol free fermented drinks, malted beverages such as “Milo” substitutes, cereal such as “Morvite” and “Maltabela” as well as a variety of novelty products. The initial boom in sorghum products came in 1988 when Nigeria banned the import of barley to save foreign exchange, which led to the dire need for a substitute crop to fulfil daily nutritional needs of the people (Taylor, 2002).

As sorghum plays a crucial role in the agricultural sector, it is important to continue to study and understand the functioning of the crop and threats to obtaining optimum yield. Therefore, this study focusses on the prophylactic application of fungicides for the control of sorghum leaf blight and the effect of such application on the sorghum plant’s growth and physiology in the absence of disease development.

2.3 Sorghum Leaf Blight

2.3.1 Casual organism

Sorghum Leaf blight (LB) is caused by a fungus *Exserohilum turcicum* (Pass.) Leonard & Suggs (teleomorph = *Setospaeria turcica* (Luttrell) Leonard and Suggs; synonym *Helminthosporium turcicum* Pass.) (Carson, 1995). It is a heterothallic ascomycete that causes leaf blight on grasses such as sorghum, maize and Johnson grass (Ramathani *et al.*, 2011). Conidia of this organism are in general 20 x 105 μm in size and have between three to eight septa. One of the important characteristics of the conidia that sets it aside from that of other similar organisms is the presence of a strongly protruding hilum that is clearly visible in Figure 2-4 (White, 1999).



Figure 2-4: *Exserohilum turcicum* spore (picture courtesy of Dr M. Craven).

The fungus reproduces asexually with conidia under natural conditions, with the sexual stage only reported under laboratory environments. High genetic diversity has been implied with RAPD analysis in isolates obtained from tropical and moderate regions. The cause of this diversity may be attributed to a sexual stage in the environment, but no such experiments have been conducted (Ramathani *et al.*, 2011).

2.3.2 Infection process and subsequent symptoms

Exserohilum turcicum infection occurs when conidia reach the leaf surface and conditions are suitable for infection (moderate temperatures 20 – 25°C and high humidity (Levy and Pataky, 1992). A germ tube forms with an appressorium that penetrates the leaf through the cuticle and form hyphae within the host cells. This causes the formation of a chlorotic fleck that represents the first visible sign of infection (Frederiksen, 1978). These hyphae penetrate living cells and absorb nutrients, causing damage to vessels, due to plugging. The result of infection is an effect described as a “localized wilting” of leaf tissue (Frederiksen, 1978). Some evidence has, however, shown that the observed wilting may be due to tyloses (polysaccharide release due to digestion of the lumen) or due to toxins (Boora *et al.*, 1999).

Symptoms of leaf blight on sorghum are characterized by long, narrow lesions (Figure 2-5) that are brownish at the centre and have a deep red margin (Boora *et al.*, 1999). Initial infection is observed as small flecks with a hand lens after three to four days which develop into larger necrotic lesions two weeks after infection. These lesions are normally 1 – 3 cm long and tend to darken during sporulation of *E. turcicum* (Frederickson, 1978).



Figure 2-5: Leaf blight lesions on sorghum leaf.

After the development of a lesion, fruiting of the pathogen will ensue. Fruiting occurs from the stoma, and conidia will form at the apex of the conidiophore. When moisture levels decrease, the conidia will snap to an upright position and disperse (Frederiksen, 1978).

2.3.3 Leaf blight epidemiology

Leaf blight is a polycyclic disease, (can cause more than one subsequent infection in a single season; Figure 2-6) that is favoured by high rainfall >600mm, moderate temperatures (±25°C), high humidity, and the presence of large amounts of inoculum (Levy and Pataky, 1992; Ngugi, 2000). This disease is accordingly common in regions with moderate climates and high humidity during growing seasons. The occurrence of epidemics can also be favoured in less than optimal conditions if the strain of *E. turcicum* is highly pathogenic. Previous studies on epidemiology have shown that LB may have a higher incidence in younger plants but can

cause more damage in older plants because of greater susceptibility in post-anthesis foliage and inoculum increases (Odvody and Hepperly, 1992). Lesion development is less restricted in older plants, resulting in larger lesions, increased sporulation and higher availability of nutrients (Ngugi, 2000).

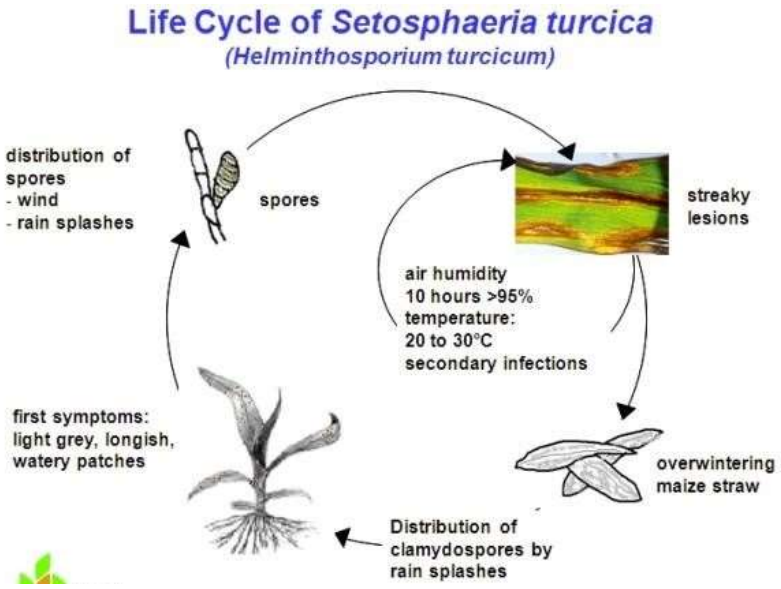


Figure 2-6: The life cycle of *E. turcicum* (Pflanzenschutz, 2014).

2.3.4 Economic impact

Foliar pathogens and their associated economic losses are a topic of constant debate, as these losses will vary with growing season and environment. According to Barrera and Frederiksen (1994), losses due to LB can be severe in susceptible sorghum cultivars if the disease is established before flowering. Losses attributed to this disease of 50% and higher have been reported on susceptible cultivars (Carson, 1995; Ngugi *et al.*, 2000; Ramathani *et al.*, 2011). These losses suffered can be attributed to the nature of the symptoms, causing lesions on the leaves and damaging the photosynthetic apparatus of the plant, causing decreases in total vitality and yield (Ramathani *et al.*, 2011; Smith *et al.*, 2013).

2.4 Leaf blight control

2.4.1 Resistance

According to Rosenow and Frederiksen (1981), breeding for resistance is the most important method of controlling sorghum diseases, and this mechanism has been very successful over past decades. Resistance is, however, harder to establish than other forms of control. Breeding for pathogen resistance is of crucial importance in traditional farming practices where indigenous varieties of sorghum are used (Fredericksen, 1978; Rosenow and Frederiksen, 1981).

Sorghum has a diverse genetic composition. Sources of high resistance levels for most pathogens has accordingly been identified through screening and breeding for these genetic traits should be one of the centre point of each breeding program. Screening for the presence of LB resistance genes in sorghum is done visually by inoculating and observing reactions (Rosenow and Frederiksen, 1981).

In a trial that studied the inheritance pattern of genes that conferred resistance to leaf blight it was found that the resistance genes investigated are dominantly inherited, and could be verified by field and laboratory screening techniques. Resistance does, however, remain race specific, meaning that one sorghum line will be resistant to one race of *E. turcicum*, but not necessarily to the next (Frederiksen, 1978).

Table 2-3: Sorghum lines showing resistance to leaf blight infection (Rosenow and Frederiksen, 1981).

Sorghum lines showing resistance to leaf blight	
SC 326-6 (IS 3758 der)	IS 8337C (SC 574)
IS 12658C (SC 167)	IS 1335C (SC418)
IS 6882C (SC 320)	IS 7254C ISC 566 141
SC 325-12 (IS 2462 der)	R 6956 (326x103)

A typical resistant reaction to *E. turcicum* will look similar to an *Ht* reaction observed in maize, being a small chlorotic halo around the infection site and reduced lesion size (Frederiksen, 1978). According to Odvody and Hepperly (1992), sorghum that possess the stay-green or non-senescent character may be more resilient to foliar diseases.

Resistance genes that have been identified by Ramathani (2010) have similar designations to that of resistance genes found in maize i.e. *Ht1, Ht2, Ht3* and *HtN*, with isolated pathogenic races of *E. turcicum* being identified by the ability of the pathogen to overcome identified genes (Table 2-4).

Table 2-4: Various pathogenic races of *E. turcicum* as identified with visual disease screening (Ramathani, 2010).

	Maize Race differential with Ht resistance genes					
Tester isolates	**A619/0**	**A619/ Ht1**	**A619/ Ht2**	**A619/ Ht3**	**Race designation**	**Race name**
Kaberamaidio	S	S	S	S	Ht1, Ht2, Ht3	123
Soroti	S	S	S	S	Ht1, Ht2, Ht3	123
Usuku	S	S	S	S	Ht1, Ht2, Ht3	123
Kumi	S	S	S	S	Ht1, Ht2, Ht3	123
Soroti-Atiira	S	S	S	S	Ht1, Ht2, Ht3	123
Kumi 2	S	S	S	S	Ht1/ Ht2, Ht3	23
Kaberamaido2	S	R	R	S	Ht1, Ht2/ Ht3	3
Kaberamaido3	S	S	S	S	Ht1, Ht2, Ht3	123
Kaberamaido	R	R	R	R		X
Kabermaido4	S	S	S	S	Ht1, Ht2, Ht3	123
Serere	S	S	S	S	Ht1, Ht2, Ht3	123
Pallisa	S	S	S	S	Ht1, Ht2, Ht3	123
Serere	S	S	R	S	Ht2/ Ht1, Ht3	13
Lira	S	S	S	S	Ht1, Ht2, Ht3	123
Amuria	S	S	S	S	Ht1, Ht2, Ht3	123
Kaberamaido	S	R	R	R	Ht1, Ht2, Ht3	0
C-Usuku	S	S	S	S	Ht1, Ht2, Ht3	123
Apac	S	R	R	R	Ht1, Ht2, Ht3	0

2.4.2 Cultural control

Odvody and Hepperly (1992) stated that the ecology of a pathogen is of utter importance when implementing cultural control methods. *Exserohilum turcicum* conidia are most commonly dispersed by wind and will thus be favoured by tillage as this will disturb debris on the soil surface and aid in dispersal of conidia (Odvody and Hepperly, 1992). The disturbance and

transportation of diseased plant debris will aid in overcoming the distance limitations of conidial dispersal, as under natural conditions conidia are dispearsed by wind. Infected host tissues thus will need to be carefully removed and destroyed to prevent dispersal and subsequent infection. Subsequent seasonal tillage will also cause buried or dormant spores to rise to the surface and re-infect host tissue in future seasons.

Krupinsky *et al.* (2002) reported that disease might be favoured by reduced tillage practices. Reduced tillage practices were incorporated in dry land areas to retain moisture in soil to allow subsequent planting, but increased moisture and the presence of diseased plant debris will greatly increase the risk of soil borne diseases (Sumner *et al.*, 1981; Krupinsky *et al.*, 2002)

Crop rotation, an alternative cultural control practice, involves the planting of non-host plants in subsequent seasons to reduce inoculum pressure, and is common practise with foliar diseases such as leaf blight, as it cannot infect in subsequent seasons (Curl, 1963). This has long been used as a measure to control disease and is based on the principal of planting a host and a non-host plant in subsequent seasons to prevent inoculum build up in the system (Curl, 1963).

2.4.3 Chemical control

Leaf blight can be controlled with fungicidal application whether it be prophylactic or curative (Bingham *et al.* 2012). Amistar Top®, a systemic fungicide consisting of 200 g/L azoxystrobin and 125 g/L difenconazole (Syngenta) is one of several restered fungicides for the control of LB on sorghum within South Africa (Croplife, 2017). Personal communication with local farmers also brought to light that Abacus®, a systemic fungicide consisting of 62.5 g/L epoxiconazole and 62.5 g/L pyraclostrobin (BASF) is also used to control the disease, even though it is not registered on sorghum. Both the above-mentioned fungicides consist of a blend of fungicides belonging to the triazole and strobilurin families of fungicides. The responsible use of fungicides is, however, of dire concern in cropping systems as the unnecessary use of

fungicides or the fact that only one registered fungicide is used may lead to the pathogen developing resistance to such chemical treatment and thus rendering the treatment ineffective (Kemerait, 2015). Integrated control measures based on the inclusion of resistant cultivars in the production together with suitable crop rotation programmes would ultimately result in greater success in controlling leaf blight and obtaining optimal yields

2.5 Fungicides

2.5.1 Fungicide industry

The Food and Agriculture Organisation of the United Nations reported that South Africa was under the top 10 countries importing hazardous pesticides from 2007 to 2009, amounting to an approximate average expenditure of US$ 52 378 000 or R 703 964 000 (Figure 2-7; FAO, 2015).

In the period between 1994 and 2000 there was a steady increase in the use of chemical fungicides for the control of fungal pathogens, ranging from 3 352 000 t to 8 929 000 t of active ingredients used as input on agricultural land in South Africa (Figure 2-8). With increases in cultivated land and yield (DAFF, 2015), in can only be assumed that these numbers have continued to rise since the final values have been updated in 2000.

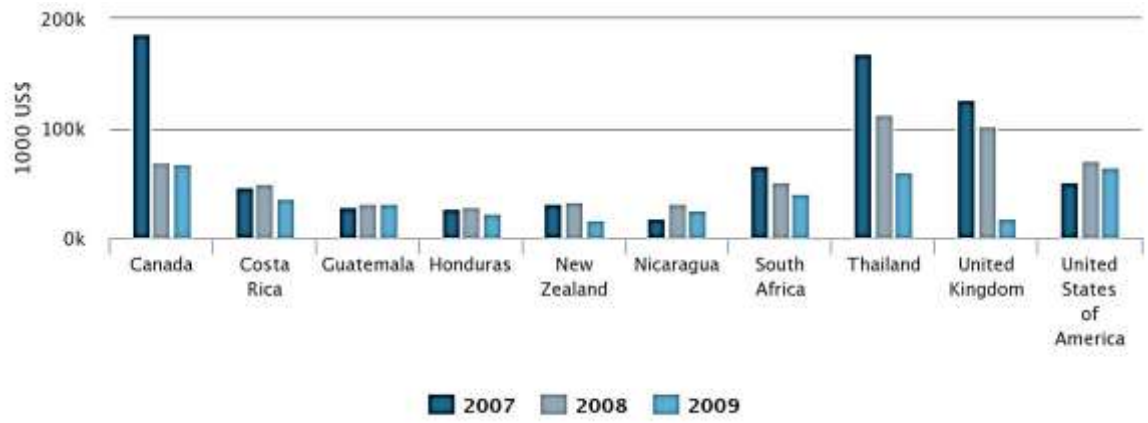


Figure 2-7: The Top 10 importers of hazardous pesticides, showing South Africa during 2007-2009 (FAO, 2015).

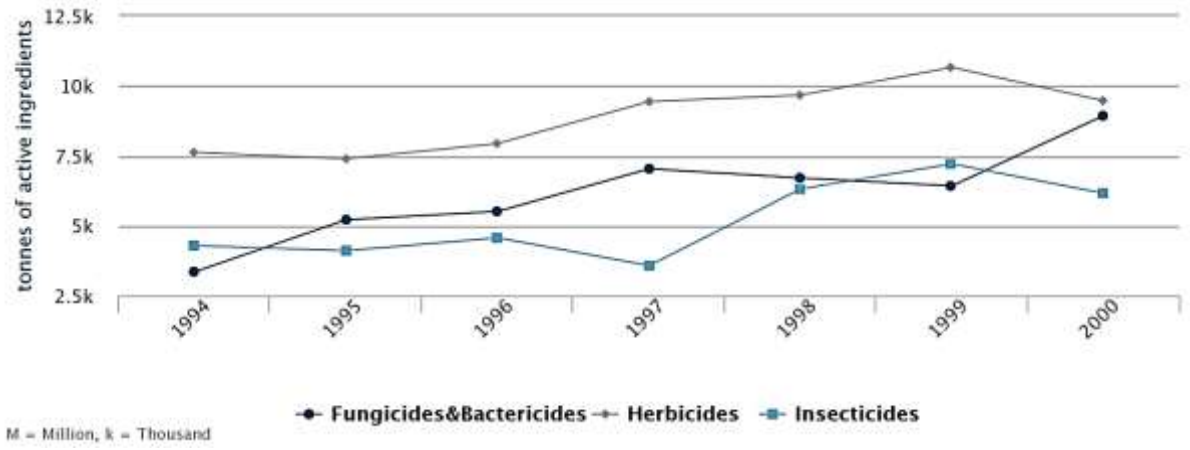


Figure 2-8: Fungicide application trends from 1994 to 2000 in South Africa (FAO, 2015).

2.5.2 Fungicides: Mode of action

Fungicides are commonly classified based on the specific chemical structure of the active ingredient, which will determine the specific mode of action the fungicide will have (Rouabhi, 2010). According to The Environmental Protection Agency of America (2012) common compounds used as fungicides, include copper, organomercury, cadmium, organotin, thiocarbamates, benzenes, triazoles and strobilurins. Some fungicides may also contain living

organisms such as *Bacillus* or *Trichoderma* spp. and are biological in nature, acting as antagonists to the plant pathogen (Rouabhi, 2010).

Fungicides can be effective in two ways, either as a site-specific inhibitor (sterol inhibitors, respirations inhibitors or DNA synthesis inhibitors), or they can be effective in more than one of these area as a multi-site inhibitor (Agrios, 2005; Rouabhi, 2010). These chemicals disrupt normal cell function, leading to impeded development, and will ultimately lead to the death of the cell (Willey *et al.*, 2008).

Fungicides can have one of three modes of action namely contact, translaminar, and systemic (Rouabhi, 2010).

- Contact fungicides are adsorbed to the plant surface and only protect the application surface.
- Translaminar fungicides will move from the cuticle, through the adaxial epidermis cells to the abaxial side of the leaf and offer protection to the entire leaf surface.
- Systemic fungicides are taken up by the plant and translocated through the xylem to upper parts of the plants, and granting protection to new leaf growth for a period of up to 21 days.

Common systemic fungicides in this group are triazoles, some strobilurins, carboximides, and benzimidazoles. Systemic fungicides are more selective in their mode of action, although still effective on wide ranges of pathogens. For example, triazoles may be used on cereals, nuts, ornamentals, turf, fruit and vegetables to treat numerous fungal pathogens (EPA, 2012).

Triazoles and strobilurins are examples of fungicide groups that have shown to have certain plant growth-regulating properties (Zhang *et al.*, 2010, Wegulo *et al.*, 2011). These two fungicides are widely used and effective against numerous fungal plant pathogens (Rouabhi, 2010). As previously stated the only registered fungicide against LB on sorghum is Amistar Top®, which is a blend of a triazole and strobilurin.

Triazoles, along with pyrimidines, belong to the organophosphate fungicides and are very effective in controlling root, foliar, and seedling diseases caused by ascomycetes. Triazoles are applied as foliar sprays and show a long lasting protective effect on a broad spectrum of plant diseases (Agrios, 2005). Triazoles are also known as sterol inhibitors, which means that these fungicides inhibit ergosterol production at the C14-demethylase sterol biosynthesis site, which is essential in the membranes of most fungi.

The triazole group of chemical fungicides do not prevent spore germination, but prevents the formation of membranes used for continued growth and host penetration, thus successful infection (Dow Agrosciences, 2007; Farm Chemicals International, 2014).

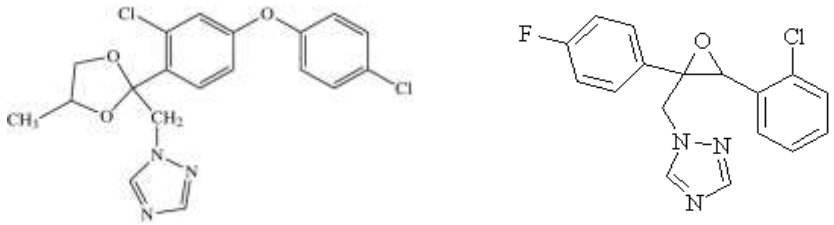


Figure 2-9: Structural formulas of difenoconazole (right) and epoxiconazole (left) (Farm Chemicals International, 2014).

Strobilurins are a group of fungicides also known as Q_oI fungicides that inhibit respiration by binding to the C3: complex III cytochrome bc1 (ubiquinol oxidase) at the Q_oI site and preventing ATP generation (Agrios, 2005; Farm Chemicals International, 2014). This group of fungicides are applied as a foliar spray and is transported translaminarly though leaves and into the vascular tissue, these are not systemic like triazoles (Agrios, 2005) and thus have a shorter effective time range.

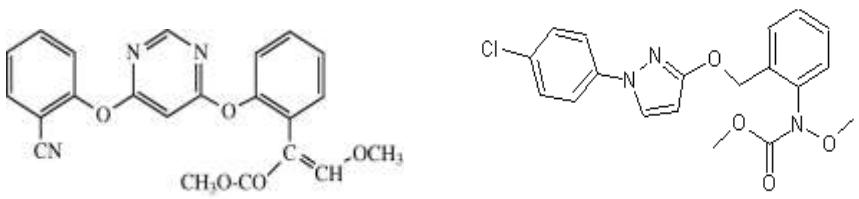


Figure 2-10: Structural formulas of azoxystrobin (right) and pyraclostrobin (left) (Farm Chemicals International, 2014).

2.5.3 The physiological effect

A study conducted by Köehle *et al.* (2001) named the effect of fungicides on plant physiology the "physiological effect" and this has long been a subject of debate. Do fungicides affect plant function and if so, how?

These fungicides include triazoles, strobilurins, and benzimidazoles which all have different modes of action (Zhang *et al.*, 2010). Recent studies have shown that fungicides have anti-oxidant properties as well as the ability to effectively control and/or prevent diseases. Additionally, the leaf "greening" effect and anti-gibberellin activity have been associated with triazoles. Yield enhancement has been associated with strobilurins by way of the auxin and ethylene metabolism, and it has also been implicated in delayed leaf senescence (Shani *et al.*, 2013; George, 2008; Hedden and Phillips, 2000. Researchers have recently been studying the effects of these fungicides on the plants' total yield (Wegulo *et al.*, 2011).

Gibberellins, auxin and ethylene are growth-regulating hormones, with gibberellin and auxin being involved in cell development and differentiation and ethylene being involved in senescence onset in plants. Additionally, it has long been known that fungicides possess anti-oxidant effects and can promote the scavenging of reactive oxygen species (ROS) in plants, further delaying the natural oxidation of plant organs by senescence (Shani *et al.*, 2013).

Gibberellins are a group of plant hormones associated with growth and development, specifically seed germination, root and shoot elongation, flowering and fruit patterning (Shani *et al.*, 2013; Hedden and Phillips, 2000). Triazoles can affect gibberellin biosynthesis because sterol, the active inhibition site of triazoles, involves similar metabolic pathways (Kane and Smiley, 1983). The use of triazoles as growth regulator in this instance may, according to Berry and Spink (2009) lead to improve lodging resistance by shortening plants, increasing seeds/m² by preventing plants from growing too large, and improved rooting by altering the partitioning between shoots and roots.

Auxin is a hormone that is, along with cytokinins, associated with the growth and development of calli, cell suspensions and organs and the regulation of morphogenesis, more simply put, cell division and elongation and the maintenance of dominance and mediations of tropisms (George, 2008). Various secondary metabolites belonging to the strobilurin family of fungicides have shown to interact similarly to auxin, ethylene, cytokinkin, abscisic acid and indol-3-acetic acid with the metabolic pathways associated with their functions in the plants (Grossman and Retzlaff, 1997). Some studies have shown that treatment of wheat with strobilurin has a positive effect on yield, with increases in chlorophyll content, darker leaves tissue, delayed senescence, increased biomass and suppression of the respiration tempo of the plants (Grossman and Retzlaff, 1997).

Strobilurin is also associated with a reduction in the production of ethylene in the plant, though inhibition of 1-aminocycolpropane-1-carboxylic acid (ACC) synthase, an enzyme narrowly associated with plant senescence which is upregulated during stress conditions, fruiting and abscission. Plants treated with pyraclostrobin showed 63% decreases in ACC-synthase in a study conducted by Grossman and Retzlaff (1997).

2.6 Senescence

Plant senescence is an irreversible genetically regulated event in response to changing environmental conditions such as shorter day length and cooler temperatures. This involves the general degradation of cell organs and re-mobilization of the products to other parts of the plants (Tewari *et al.*, 2009). It is an oxidative process characterized by various biochemical and physiological processes such as protein degradation, decrease in membrane permeability, nucleic acid degradation, loss of proteins and chlorophyll, increased lipid peroxidation, and pigment degradation which leads to extensive cell damage, decrease in normal plant function and death of the plant (Distefano *et al.*, 1999; Tewari *et al.*, 2009; Zhang *et al.*, 2010).

2.7 Fungicides and anti-oxidant activity in plants

Since the growth-regulating hormones gibberellin, auxin and ethylene are affected by triazoles and strobilurins, it can only be assumed that the onset of senescence will also be affected by these compounds (Grossmann and Retzlaff, 1997; Vanancio *et al.*, 2003; Berry and Spink, 2009, and Shani *et al.*, 2013). In various studies reviewed by Venancio *et al.* (2003), delays in senescence had close correlation with decreases in levels of ACC-synthase, a precursor of ethylene, and increases in IAA (indol-3-acetic acid), a natural occurring auxin.

Reactive oxygen species (ROS) such as hydroxyl, superoxides and singlet oxygen are mainly responsible for oxidative damage in the plants' cells and membranes (Zhang *et al.*, 2010). According to Distefano *et al.* (1999) and Tewari *et al.* (2009) previous studies have shown an increase in the activity of superoxide producing xanthine oxidase and hydrogen peroxide producing superoxide dismutase along with the down regulation of scavenging enzymes such as peroxidase during senescence.



Figure 2-11: Senescing *Sorghum bicolor* in field trials.

As the oxidative stress of senescence ensues in the plant, the generation of ROS from various sources, like the chloroplast, mitochondria, peroxisome, membranes and endoplasmic reticulum within the cell will increase. Species of ROS include superoxide, hydroxyl radical, hydroperoxyl, hydrogen peroxide, singlet oxygen, and excited carbonyl, all of which are toxic to plants (Karuppanapandian *et al.*, 2011).

2.7.1 Generation and scavenging of ROS in plants

ROS are the by-products of various natural metabolic processes within plants. At senescence, the production of ROS will increase as the oxidation of lipids and proteins in the cells progress (Gill and Tuteja, 2010).

2.7.1.1 ROS generation

In the peroxisomes of cells xanthine oxidase (XOX) catalyses the oxidation of xanthine and hypoxanthine to uric acid. During this process, superoxide is released (Gill and Tuteja, 2010). Superoxide is part of a group of compounds known as reactive oxygen species (ROS). In most cases the production of these radicals lead to cell death, but recent studies have shown that small concentrations of hydrogen peroxide and superoxide released in the peroxisomes are

involved in signalling mechanisms which mediate pathogen induced programmed cell death (PCD) in plants, which may play a role in preventing the spread of such infections (Gill and Tuteja, 2010).

ROS are constantly produced in the form of superoxide within the chloroplast, through normal light excitation, and all excited forms of chlorophyll are transported through the electron transport chain. These molecules are normally directed to the formation of NADPH, but when it is overloaded, more superoxide is formed via the Mehler reaction, leading to photoinhibition in plants (Gill and Tuteja, 2010; Das and Roychoudhury, 2014).

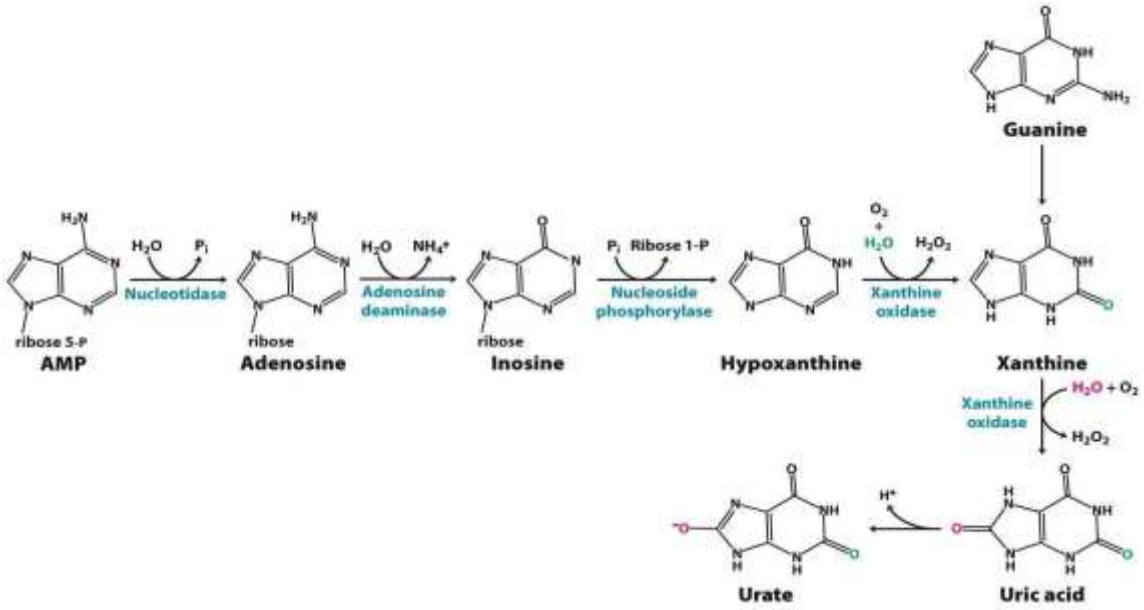


Figure 2-12: Production of superoxide by xanthine oxidase in the peroxisomes of plants (Tymoczko *et al.*, 2009).

During senescence, the normal degradation of cell organelles leads to the increased generation of ROS and thus increase oxidative stress in the plant. Different mechanisms exist within the plant to scavenge these ROS and delay senescence (Zimmermann and Zentgraf, 2005)

2.7.1.2 ROS scavenging

Plant senescence is an oxidative process that involves the denaturing of proteins, lipids, DNA, cell membranes and ultimately leads to cell death and the death of the plant. Because of this, and the known anti-oxidative properties of fungicides, it is important to give attention to the methods of ROS scavenging.

Plants have developed both enzymatic and non-enzymatic methods of scavenging ROS produced during stress and senescence. The production of superoxide during oxidative stress is a key factor in the peroxidation of lipids in cell membranes, these radicals are broken down as shown in Formula 1 by superoxide dismutase to form hydrogen peroxide (Dhindsa *et al.*, 1981).

$$2H^+ + 2O_2^- \rightarrow H_2O_2 + O_2 \quad (1)$$

Hydrogen peroxide is then converted to less harmful compounds by catalase (CAT) (Formula 2) and peroxidase (POD) (Formula 3) (Hopkins and Hüner, 2009).

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad (2)$$

$$2\mathrm{GSH} + H_2O_2 \rightarrow \mathrm{GS{-}SG} + 2H_2O \quad (3)$$

Because senescence is an oxidative process (Tewari *et al.*, 2009), the antioxidant mechanisms in plants should show fluctuating activity, due to more ROS being produced at senescence. Delays in senescence can then be quantified by assays done on these enzymes such as in studies by Kar and Mirsha (1976), Dhindsa *et al.* (1981) Srivalli and Khanna-Chopra (2009), and Chen *et al.* (2012).

These studies showed varying results regarding the activity of catalase and peroxidase as reported in Kar and Mirsha (1976). Their study stated that CAT activity decreased in detached leaves of tobacco, but increased in that of wheat and barley. They also stated that contradicting results were obtained for POD. Increased activity due to senescence was observed, but POD activity also increased when senescence was delayed.

As the plant develops, the metabolic traits will also change, antioxidant enzyme activity will fluctuate and as the plants approach senescence, ROS producing enzymes' activity will increase. To get an indication of the vitality of the plant, and if there is a level of stress due to fungicide treatment over the course of plant development, a non-intrusive method is needed.

2.8 Chlorophyll *a* fluorescence as a technique to investigate plant vitality

2.8.1 Defining photosynthesis and its mechanisms

Nabors (2004) defined photosynthesis as "the process by which plants and certain other organisms use solar energy to make their own food by transforming carbon dioxide and water into sugars that store chemical energy." Plants can convert H_2O and CO_2 into simple sugar compounds. This is done by using H_2O and light to generate chemical energy in the form of ATP and NADPH, and absorbing CO_2 from the atmosphere and fixating it with the chemical energy into three or four carbon sugars by ways of the Calvin cycle.

The link between the photosynthetic and anti-oxidative enzyme responses of plants can only be understood by studying photoinhibition and the flow of electrons during the light dependant reactions of photosynthesis. From Hopkins and Hüner (2009) photoinhibition can be defined as "the light-dependent decrease in photosynthetic rate that may occur whenever the irradiance is more than that required either for the photosynthetic evolution of O_2 or the photosynthetic assimilation of CO_2."

During photosynthesis H_2O is oxidised to O_2 and the electrons released from water is used to produce ATP and NADPH through the electron transport mechanism (Hopkins and Hüner, 2009). The ATP and the NADPH produced are used to reduce CO_2 to sugars. Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) is one of the key enzymes that is responsible for the reduction of CO_2.

The linear increase in CO_2 assimilation, with increases in electron transport, is seen with a linear increase in irradiance. When irradiance further increases and the chloroplasts are light-saturated, ATP and NADPH will be at saturation point and the photosynthetic efficiency of the plant is at an optimum level (Hopkins and Hüner, 2009). These conditions are only suitable for short periods as excess exposure will lead to decreases in photosynthesis, and in turn cause photoinhibition.

Photoinhibition is, however, not the only form of stress that will affect the plant negatively. Lichtenthaler (1995) defined stress responses as “all agents can act as stressors, producing both stress and specific action; and there exist stressor specific responses and non-specific general responses”. Levitt (1980) defined stress as “any environmental factor potentially unfavourable to living organisms”.

These responses may induce both temporary and permanent changes in the plants, ranging from metabolic and physiological changes to cells or plant death (Lichtenthaler, 1995).

Factors that induce plant stress include temperature, soil conditions, abiotic stressors, such as nutrient levels or toxins, or pathogen and insect attack.

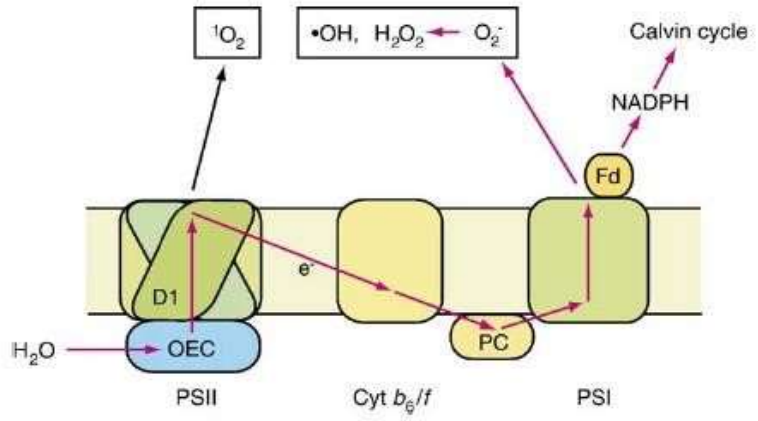


Figure 2-13: Schematic representation of ROS generation under excess light conditions (photoinhibition) (Nishiyama *et al.*, 2006).

Excess light, pathogen and insect attack and pesticide application may increase the rate of generation of ROS in the photosynthetic apparatus of the cell. Oxygen reduction on the acceptor side of PSI leads to the formation of superoxide and in turn hydroxyl radicals (Figure 2-14), and the excitation energy transfer from chlorophyll lead to the formation of singlet oxygen (Nishiyama *et al*., 2006). Under normal conditions these ROS are scavenged by SOD and POD, as well as non-enzymatic responses. Unfortunately, these mechanisms are ineffective under excessive light conditions, due to increases in ROS production and consequent oxidative stress (Nishiyama *et al*., 2006).

2.8.2 Kautsky and chlorophyll *a* fluorescence

When a leaf is dark adapted, PSII is in the open configuration due to the drainage of all electrons from the photosynthetic ETC by PSI. As the leaf is drained of all light, the minimal fluorescence F_0 can be measured. By emitting a subsequent high-intensity white light, an estimation of the maximum fluorescence (F_M) can be made. This is called the Kautsky effect (Hopkins and Hüner, 2009) and allows for a non-invasive tool to quantify the vitality of a plant by estimating the potential photosynthetic efficiency of the plant as a function of the Kautsky transient. The Chlorophyll *a* fluorescence transient, also known as the Kautsky transient, is named so after the Kautsky effect (Strasser *et al.*, 2007).

Chlorophyll *a* fluorescence is the emission of light energy from the chloroplasts with a wavelength <690 nm when a photon of energy returns from the excited state to the ground state during photosynthesis (Hopkins and Hüner, 2009).

As light is absorbed by the photosynthetic apparatus, in this case PSII, the flux of photons is transformed to a flux of excited particles or excitons. However, part of the excitation is not conserved as not all the energy is used to produce O_2. Energy is lost in the form of heat and fluorescence, indicated by (F) in Figure 2-14 (Strasser, *et al.*, 2007). According to Öquist and Wass (1988) the chlorophyll *a* fluorescence signal originates from the chlorophyll *a* pigments

that are present in the PSII complex, and are closely correlated to the availability of acceptor quinone molecules. To summarize the process in PSII, Hopkins and Hüner (2009) simply stated that PSII oxidizes water to produce oxygen. To better understand the results, it is necessary to understand the flow of electrons in PSII.

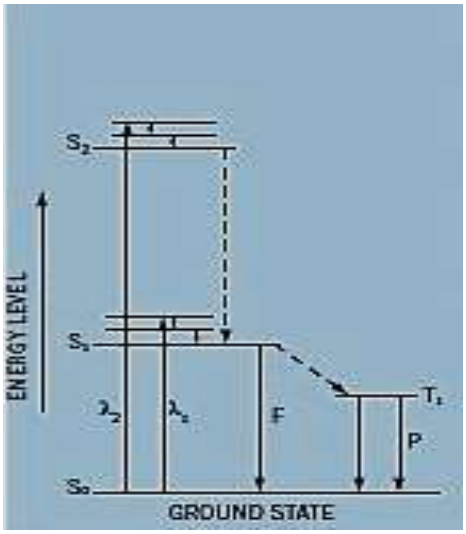


Figure 2-14: Light energy as absorbed in the chloroplast. Fluorescence is released as F indicated in the figure (Hopkins and Hüner, 2009).

As light hits the reaction centre P680 it becomes excited and can rapidly transfer electrons to pheophytin, the primary electron acceptor in PSII. This process results in the release of a single electron, available as stored redox potential. Within the PSII reaction centre there are two complexes, D1 and D2 that are responsible for the orientation of the redox carriers to the inside of the lumen, as well as preventing electron recombination with P680. This happens when pheophytin passes one electron to an electron acceptor in the complex known as a quinone, specifically quinone A (Q_A) and rendering PSII to be in the “closed” state. Q_A then transfers the electron to PQ (plastoquinone), which binds to Q_B (quinone B). PQ is the reduced to plastoquinol and becomes part of the PQ pool. From here PSII is “open” and the process can be repeated (Krause and Weis, 1991).

From the PQ pool the electrons diffuse laterally trough the chloroplast membrane until it encounters the cytochrome *bf* complex. This is a redox protein complex associated with PSI, which is responsible for the oxidation of plastoquinol.

This technique allows the researcher to quantify the differences in fluorescence intensity among different treatments, the absorbance of light (ABS= (y/(1-y))), the trapping of excitation energy (φ= (φ/(1-φ))), the conversion of this excitation energy to the electron transport chain (Ψ= (ψ/1-ψ)), as well as the efficiency of reduction of the end electron acceptors (∆=(P/1-P))) (Strasser *et al.*, 2007). These measurements are done using the following equation:

$$PI_{total} = \frac{RC}{ABS} \bullet \frac{\varphi P0}{1-\varphi P0} \bullet \frac{\Psi 0}{1-\Psi 0} \bullet \frac{\rho}{1-\rho}$$

In the past, chlorophyll *a* fluorescence has been used to quantify the effect of *E. turcicum* infection on the vitality of maize plants (Smith *et al.*, 2012). Using this technique, the difference in photosynthetic efficiency, if any, can be quantified in a non-invasive manner.

These measurements are obtained using a Plant Efficiency Analyser (PEA meter), which induces transients by red light (650nm) for one second, and within these transients various time marks are defined and associated with complexes within the photosynthetic apparatus (Strasser *et al.*, 2004).

This method has been used in various studies to quantify chlorophyll *a* fluorescence as a non-invasive tool to measure stress responses in both plants and algae (Lichtenthaler, 1995).

Table 2-5: List of publications using chlorophyll a fluorescence as a tool to measure stress responses in plants.

Authors	Publication
Kiefer, 1973	Chlorophyll *a* fluorescence in marine centric diatoms: Responses of chloroplasts to light and nutrient stress
Miranda *et al.,* 1981	Limitations of photosynthesis in different regions of the *Zea mays* leaf.
Conroy *et al.,* 1986	Chlorophyll *a* fluorescence and photosynthetic and growth responses of *Pinus radiata* to phosphorus deficiency, drought stress, and high CO_2

Laach, 1987	Non-photochemical quenching of chlorophyll *a* fluorescence in isolated chloroplasts under conditions of stressed photosynthesis
Ralph and Burchett, 1995	Photosynthetic responses of the seagrass *Halophila ovalis* (R. Br.) Hook. f. to high irradiance stress, using chlorophyll *a* fluorescence
Kappen *et al.,* 1998	Chlorophyll *a* fluorescence and CO2 exchange of *Umbilicaria aprina* under extreme light stress in the cold
Mallick and Mohn, 2003	Use of chlorophyll fluorescence in metal-stress research: a case study with the green microalga *Scenedesmus*
Souza *et al.,* 2004	Photosynthetic gas exchange, chlorophyll fluorescence and some associated metabolic changes in cowpea (*Vigna unguiculata*) during water stress and recovery
Oukarroum *et al.*, 2007	Probing the responses of barley cultivars (*Hordeum vulgare* L.) by chlorophyll *a* fluorescence OLKJIP under drought stress and re-watering
Mehta *et a*l., 2010	Chlorophyll *a* fluorescence study revealing effects of high salt stress on photosystem II in wheat leaves
Pompeiano *et al.*, 2016	Allocation pattern, ion partitioning, and chlorophyll *a* fluorescence in *Arundo donax* L. in responses to salinity stress

From Table 2.5 chlorophyll *a* fluorescence has, over the past 50+ years, left its mark as being a usable tool in the detection of stress responses of plants and has contributed to Botany in a huge capacity.

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3

Evaluation of fungicides for potential growth-regulating properties on selected sorghum cultivars under various climatic conditions.

3.1 Introduction

Sorghum (*Sorghum bicolor* (L.) Moench and Suggs) is one of the five most widely cultivated crops in Sub-Saharan Africa (Anonymous, 2015a). As new-generation ethanol processes are studied and improved, it is foreseen that sorghum's role within the agricultural sector may continue to expand (Radebe, 2013). During 2014 a draft position paper on the South African biofuels regulatory framework was released by the Department of Energy (Anonymous, 2014) which recommended sorghum and soybean as reference crop for the manufacturing of bio-ethanol and biodiesel respectively. Based on its potential role in renewable energy, it is foreseen that the sorghum industry could soon expand within Southern Africa with a greater focus on optimizing yield.

According to Chandrashekar and Satyanarayana (2006) annual yield losses in sorghum due to pest and pathogen attacks have been estimated to be 30%. Sorghum leaf blight (LB), is one of the prominent sorghum diseases in South Africa (personal communication; Dr Craven, Agricultural Research Council - Grain Crops Institute). The disease is caused by *Exserohilum turcicum* (Pass.) Leonard and Suggs and has resulted in yield losses of up to 50% (Carson, 1995; Ngugi *et al.*, 2002; Ramathani *et al.*, 2011). Generally, LB has a higher incidence in the more humid eastern production areas of South Africa, but becomes a problem in all sorghum production areas during higher rainfall seasons. Leaf blight is characterized by oval lesions on foliage, leading to reduced leaf area and thus photosynthesis.

Fungicides consisting of combinations of active ingredients such as azoxystrobin/difenconazole, epoxiconazole/pyraclostrobin and carbendazim/difenconazole are used for the control of LB by producers although not all are registered locally for such use (Croplife, 2017). In certain sorghum production areas, such as the Standerton area, some producers apply fungicides prophylactically due to severe infections experienced in previous planting seasons (personal communication, Dr Craven, Agricultural Research Council - Grain Crops Institute). The prophylactic use of fungicides is, however, sometimes propagated with the promise of financial return on expenditure in the form of increased biomass or yield whether disease is present or not.

Certain compounds used as fungicides, such as triazoles, have been shown to have plant growth-regulating properties (Fletcher and Arnold, 1986). It has been demonstrated that the inhibition of gibberellin biosynthesis which is involved in plant growth and development, is the main mechanism behind the plant growth-regulating properties of triazoles, while growth substances including gibberellic acid, cytokinins, abscisic acid and ethylene are also affected resulting in altered cell development as well as delayed senescence or the “stay green” effect (Shanmugam *et al.,* 2012). Berry and Spink (2009) speculated that the application of triazoles could promote lodging resistance as plants are shorter and rooting is improved because of the partitioning between shoots and roots being altered.

Yield enhancement has also been associated with strobilurins by way of auxin and ethylene metabolism, and these fungicides have also been implicated in delayed leaf senescence (Grossman and Retzlaff, 1997). Ethylene production in the plant has been shown to be reduced by strobilurin. This is achieved though the inhibition of 1-aminocycolpropane-1-carboxylic acid (ACC) synthase activity, an enzyme closely associated with plant senescence, fruiting and abscission (Venancio *et al*, 2003). During stress conditions ACC synthase is upregulated. Plants treated with pyraclostrobin showed 63% decreases in ACC synthase activity in a study conducted by Venancio *et al.* (2003). Delayed senescence could mean an extended period of photosynthesis with more carbon fixation and increased yield.

3.2 Aim and Objectives

The objective of the current study was to evaluate the growth-regulating properties of two fungicides containing a mixture of active ingredients belonging to the triazole and strobilurin chemical groups under field trial conditions.

Various agronomic (yield, plant mass and senescence) parameters were investigated using four selected sorghum cultivars in the absence of disease development under various South African field conditions during the 2013/14 and 2014/15 seasons.

3.3 Materials and methods

3.3.1 Field trial layout

Sorghum field trials to evaluate the effect of epoxiconazole/pyraclostrobin and azoxystrobin/difenconazole fungicides on yield gain, plant mass and senescence were conducted at Greytown (Kwa-Zulu Natal; S29º 05' 01.5" EO30º 36' 12.9"), Potchefstroom (North Wes Province; S26° 43' 56.0" EO27° 03' 53.9"), and Standerton (Mpumalanga; S27^0 09' 50.5" EO29^0 14' 54.8") during 2013/14 and 2014/15. The trials were planted as split-plot designs with fungicide as the main plot and cultivar as the sub-plot factors respectively. All treatments were repeated in triplicate. Cultivars were planted as single rows within the main plots and included PAN8816, PAN8906, PAN8611 and NS5511 (2013/14). PAN8611 was replaced with PAN8625 during the following season (2014/15), due to unavailability of seed of the former. Each main plot was flanked by a single border row (PAN8816). Plots at Potchefstroom and Standerton consisted of six rows, 5 m in length spaced 0.9 m apart. Plots at Greytown were smaller due to limited space and consisted of six rows planted at 0.9 m and 4.5 m in length.

The Potchefstroom trial received supplementary water via an overhead sprinkler system, whereas Standerton and Greytown were planted under dry land conditions. To ensure optimum growth, soil analyses were conducted at each locality and fertilizer applied accordingly. Frontier® Optima (dimethenamid – P 75 g/L) (BASF) was applied pre-emergence and Bassagran® (bendioxide 480 g/L) (BASF) post-emergence to prevent weed encroachment. Mid-season stem-borer control was done with Karate® (lambda-cyhalothrin 50g/L) (Syngenta).

3.3.2 Fungicide treatments

The effect of two foliar fungicide formulations i.e. azoxystrobin / difenconazole (Amistar Top®, Syngenta) and epoxiconazole / pyraclostrobin (Abacus®, BASF), were evaluated at five application times (treatments) as indicated in Table 3-1. These two fungicides were selected as Amistar Top® was the only registered fungicide for the control of LB when the trials commenced during 2013, while Abacus® is a product commonly used by producers even though it was not registered for applications in South Africa. A control treatment was included which did not receive fungicide.

Fungicides were applied with a CO_2 gas operated knapsack sprayer and a four nozzle (flat fan; 0.9m spaced) boom. The calibrated spray volume was 78 $L.ha^{-1}$.

Table 3-1: Formulation, application rate and time of application of fungicides investigated for their potential growth-regulating properties on sorghum.

Treatment	Formulation	Rate	Weeks after planting	Treatment reference
Control (no fungicide applied)	*	*	*	Control
Azoxystrobin/difenoconazole (Amistar Top®)	200/125g/L	500 $mL.ha^{-1}$	6	AT 6
			8	AT 8
			10	AT 10
			6 & 8	AT 6 & 8
			8 & 10	AT 8 & 10
Epoxiconazole/pyraclostrobin (Abacus®)	62.5/62.5g/L	$1L.ha^{-1}$	6	AB 6
			8	AB 8
			10	AB 10
			6 & 8	AB 6 & 8
			8 & 10	AB 8 &10

3.3.3 Parameters evaluated

3.3.3.1 Disease Severity

Ten randomly selected plants within each row were accordingly screened at soft dough stage for disease severity. Disease was quantified as the percentage infected leaf material per plant per row using a modified scale of 0.0, 0.5, 1.0, 5.0 10.0, 25.0, 50, 70 and ≥85% (Adipala *et al.*, 1993; Elliot and Jenkins, 1946).

This was done to ensure that infection was low enough to test the growth-regulating properties of the fungicides in the absence of disease.

3.3.3.2 Senescence

Senescence is the natural physiological aging of the plant and was observed as yellowing of the plants. The effect of fungicide application on the so-called “stay-green” effect was quantified by using a combined scale from Verma *et al.*, 2004 and Cromey *et al.*, 2004. A scale of 0 to 100% was used to rate the level of senescence with 0 being an unaffected leaf and 100 being a severely yellowed leaf. To obtain accurate representations of each treatment, only the upper three to four leaves were considered, with five random plants per cultivar per plot used for quantification. These determinations were done at 150 days after planting (S1) (d.a.p), upon harvest (S2) (12.5% moisture), and two weeks post-harvest (S3).

3.3.3.3 Plant mass

In accordance with speculation that the entire plant may be of use for biofuel production the aerial biomass of the plants, henceforth referred to as ‘plant mass’, were determined at harvest. Two weeks’ post-harvest, ten plants per cultivar per plot were randomly selected and weighed, after being severed at the crown.

3.3.3.4 Yield

All panicles were removed at physiological maturity and threshed after which the grain was weighed. The grain moisture content was determined using a Steinlite moisture meter and yield (t ha^{-1}) was adjusted to a 12.5% moisture level using the following formula:

$$Y = L\,x\,\frac{(100 - m\,)}{(100 - 12.5)}\,x\,(\frac{10}{A})$$

Where L = weight, m = moisture, A= plot area and Y= yield (t.ha^{-1}).

3.3.4 Statistical analysis

Analysis of variance (ANOVA) for a split plot experimental design was applied over the three localities for each season separately. The data of the three localities for each season were tested for homogeneity of variances using Levene's test. In cases were the variability in the observations of the three localities were of comparable magnitude an analysis of the three localities' observations together could be validly carried out. In cases where there was strong evidence against homogeneity a weighted analysis of the three localities' observations together were carried out using the inverse of the pooled variances of each locality as weight (John, 1977). The Shapiro-Wilk test was performed to test for normality (Shapiro, 1965). Student's t-Least Significant Difference (LSD) were calculated at the 5% level to compare treatment means for significant effects.

Pearson's correlation analysis was conducted between yield, plant mass, S1, S2, S3 and disease severity for each of the localities over the two seasons separately. All analyses were conducted using Genstat (Version 14, Payne *et al.,* 2011).

3.4 Results

The average rainfall and temperatures of each locality over the two-year planting season are given below.

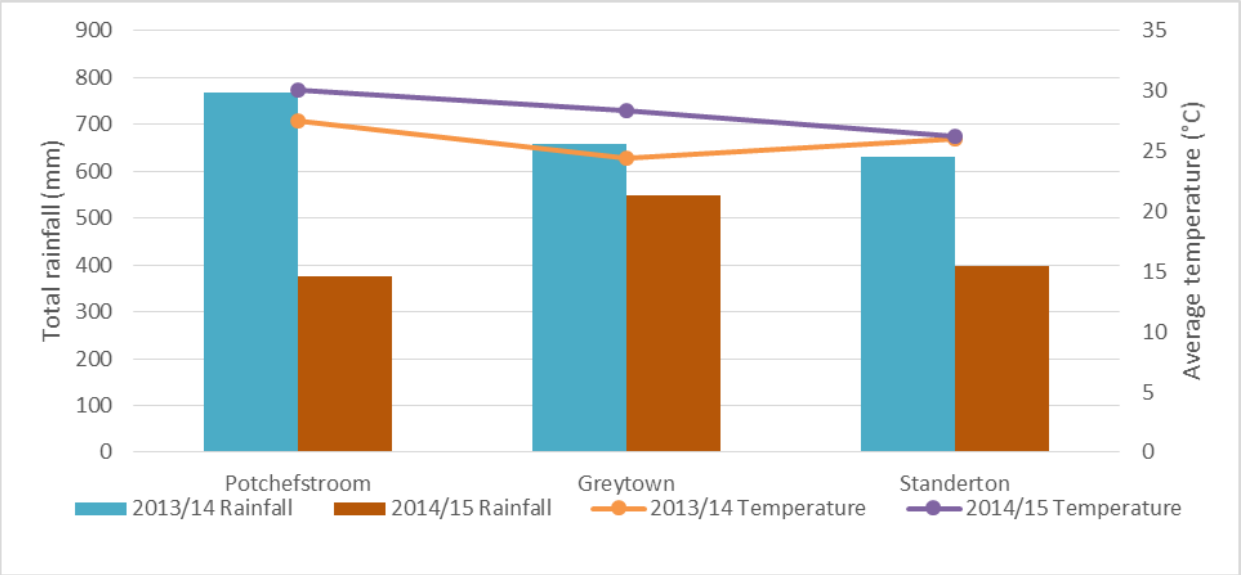


Figure 3-1: Total rainfall and average maximum temperatures obtained for growing seasons 2013/14 and 2014/15 at Potchefstroom, Greytown and Standerton respectively (Courtesy of the Agricultural Research Council's Institute for soil, climate and water).

Figure 3-1 shows the total rainfall and average temperatures for the 2013/14 and 2014/15 growing seasons. The average maximum temperatures over the three localities did not vary significantly, with temperatures fluctuating between 24.4°C in Greytown during the 2013/14 season to 30.08°C in Potchefstroom during the 2014/15 season. Extreme variation in rainfall was observed when comparing the 2013/14 and 2014/15 rainfall seasons in both Potchefstroom and Standerton; both these localities showed rainfall upwards of 650mm during the 2013/14 season and much lower (<400mm) during 2014/15. Greytown showed to have more consistent rainfall over the two planting seasons.

As prophylactic fungicide application and its effects on the various above mentioned agronomic and physiologic parameters were the focus of this study, these are the only interactions that will be discussed.

3.4.1 Disease severity

The average disease severity obtained at each locality is summarized in Table 3-2. Sufficiently low disease severities were present at the various sites during both seasons to evaluate the growth-regulating properties of the fungicides without the confounding effect of disease on growth parameters.

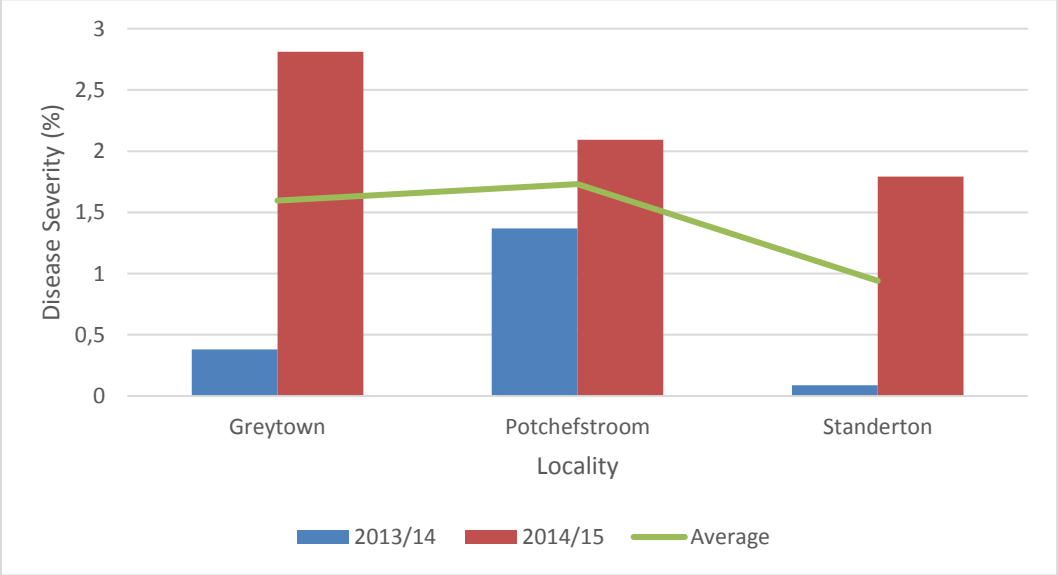


Figure 3-2: Average disease severity over two growing seasons at Greytown, Potchefstroom and Standerton.

Table 3-2: ANOVA for leaf senescence (%) obtained during the 2013/14 and 2014/15 growing seasons.

Source	S1					S2					S3				
	DF	S,S	M,S	F VALUE	PR > F	DF	S,S	M,S	F VALUE	PR > F	DF	S,S	M,S	F VALUE	PR > F
2013/14															
Locality (Loc)	2	1199.20	599.60	14.95	<.0001	2	403203.16	201601.58	3501.50	<.0001	2	128290.91	64145.45	1278.39	<.0001
Rep (Loc)	6	42.67	7.11	0.18	0.98	6	241.29	40.21	0.70	0.65	6	672.73	112.12	2.23	0.05
Treatment (Tmt)	10	1198.71	119.87	2.99	0.00	10	2843.56	284.36	4.94	<.0001	10	1676.77	167.68	3.34	0.00
Loc x Treatment	20	3508.64	175.43	4.38	<.0001	20	5687.12	284.36	4.94	<.0001	20	3353.54	167.68	3.34	0.00
Error (A)	60	2405.83	40.10			60	3454.55	57.58			60	3010.61	50.18		
Cultivar (Cult)	3	3722.03	1240.68	32.13	<.0001	3	9647.66	3215.89	97.65	<.0001	3	6474.75	2158.25	39.15	<.0001
Locality x Cultivar	6	4473.45	745.57	19.31	<.0001	6	19295.33	3215.89	97.65	<.0001	6	12949.49	2158.25	39.15	<.0001
Treatment x Cultivar	30	2084.72	69.49	1.80	0.01	30	1209.97	40.33	1.22	0.21	30	1103.03	36.77	0.67	0.91
Loc x Tmt x Cult	60	3900.05	65.00	1.68	<.0001	60	2419.95	40.33	1.22	0.15	60	2206.06	36.77	0.67	0.97
Error (B)	198	7645.50	38.61			198	6520.83	32.93			198	10916.67	55.13		
Corrected Total	395	30180.80				395	454523.42				395	170654.55			
2014/15															
Locality (Loc)	2	60.91	30.46	12.48	<.0001	2.00	603873.34	301936.67	2262.34	<.0001	1	56848.02	56848.02	292.02	<.0001
Rep (Loc)	6	20.14	3.36	1.38	0.24	6.00	2329.76	388.29	2.91	0.01	4	721.15	180.29	0.93	0.46
Treatment (Tmt)	10	44.32	4.43	1.82	0.08	10.00	2545.30	254.53	1.91	0.06	10	2151.48	215.15	1.11	0.38
Loc x Treatment	20	62.85	3.14	1.29	0.22	20.00	2859.77	142.99	1.07	0.40	10	2151.48	215.15	1.11	0.38
Error (A)	60	146.47	2.44			60.00	8007.74	133.46			40	7786.85	194.67		
Cultivar (Cult)	3	53.00	17.67	17.67	<.0001	3.00	9824.27	3274.76	19.42	<.0001	3	18110.14	6036.71	44.61	<.0001
Locality x Cultivar	6	70.32	11.72	11.72	<.0001	6.00	11675.93	1945.99	11.54	<.0001	3	18110.14	6036.71	44.61	<.0001
Treatment x Cultivar	30	58.61	1.95	1.95	0.00	30.00	5485.20	182.84	1.08	0.36	30	5333.36	177.78	1.31	0.15
Loc x Tmt x Cult	60	69.38	1.16	1.16	0.23	60.00	7969.84	132.83	0.79	0.86	30	5333.36	177.78	1.31	0.15
Error (B)	198	198.00	1.00			198.00	33390.50	168.64			132	17864.00	135.33		
Corrected Total	395	784.00				395.00	687961.66				263	134409.98			

3.4.2 Senescence

3.4.2.1 2013/14

Levels of senescence were significantly affected 150 d.a.p. by the locality x treatment x cultivar interaction during the 2013/14 season (Table 3-2). Close observation of Figure 3-3A-C indicates that the interaction effect was mostly limited to Greytown (Figure 3-3A) as all treatments at Potchefstroom and Standerton gave similar degrees of senescence to that of the respective controls. At Greytown, the control treatments of PAN8911 and NS5511 had significant higher degrees of senescence than what was observed at Potchefstroom and Standerton at 150 d.a.p. The application of fungicides accordingly resulted in significantly lower levels of senescence at Greytown for most of the treatments in both these cultivars (PAN8911 and NS5511) as opposed to their respective controls. No treatment was shown to have a significant reaction when applied to PAN8816 and PAN8906 when these cultivars were compared to the respective control of each cultivar over all three localities.

Analysis of variance indicated that the locality x treatment interaction significantly affected the eventual senescence measured at harvest (S2) (Table 3-2). As per Figure 3-4 the effect observed was similar to the 150 d.a.p, but limited to Greytown. Plants at both Potchefstroom and Standerton were fully senesced at harvest, whilst the material at Greytown were less than 60% senesced. Based on the analysis, the application of fungicides significantly reduced the degree of senescence observed at harvest.

Similar to what was observed with S2, the locality x treatment interaction significantly affected the degree of senescence observed two weeks post-harvest (S3) (Figure 3-5), with all fungicide applications resulting in reduced degrees of senescence compared to the control at the Greytown trial.

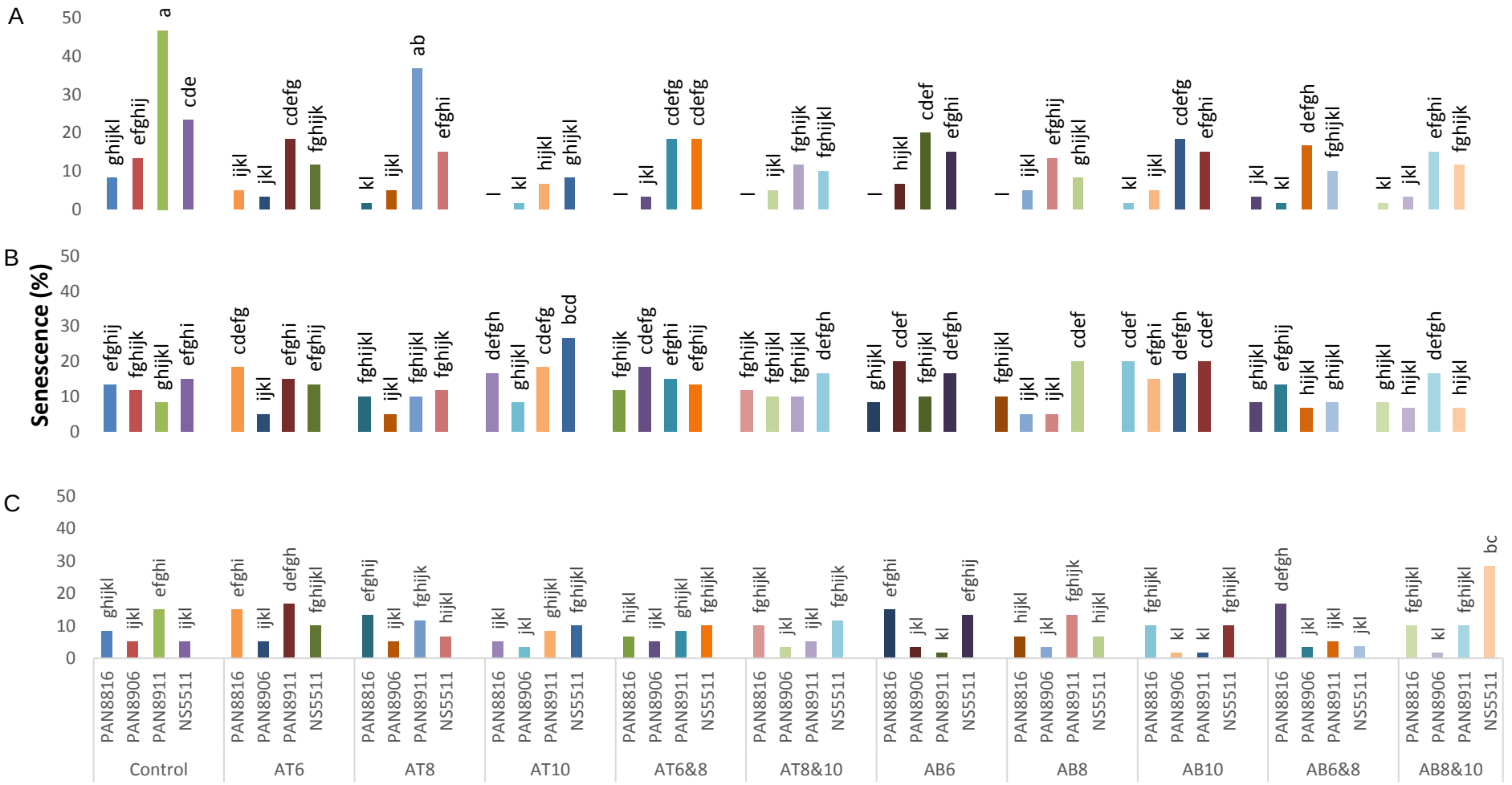


Figure 3-3: A-C The effect Amistar Top® (AT) and Abacus® (AB) applied at five application dates on the percentage senescence observed in four sorghum cultivars as evaluated at 150 days after planted over three localities (A-Greytown; B-Potchefstroom; and C-Standerton) during the 2013/14 season.

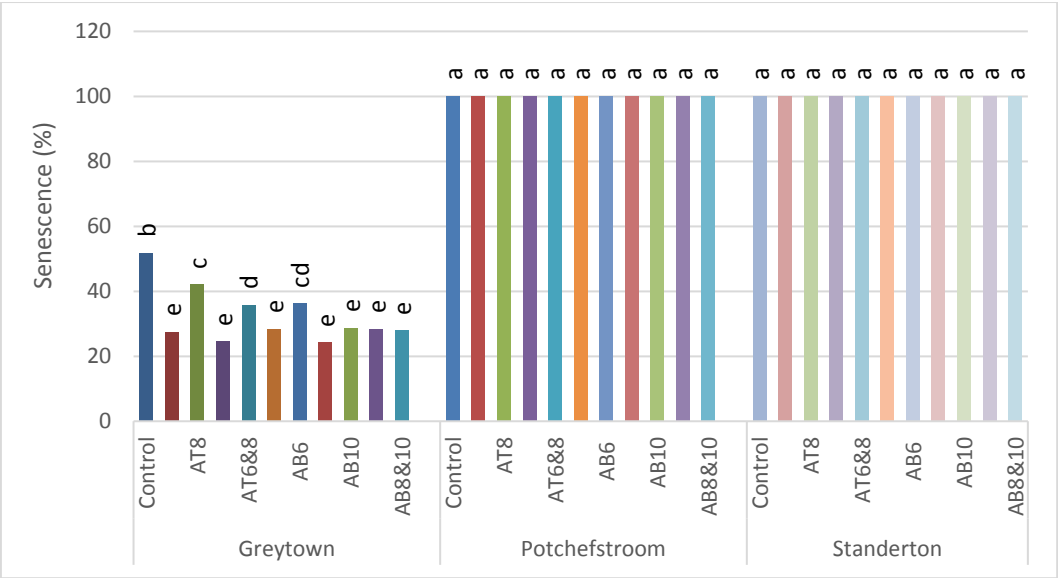


Figure 3-4: The effect Amistar Top® (AT) and Abacus® (AB) applied at five application dates on percentage leaf senescence at harvest of sorghum as evaluated over three localities during the 2013/14 season.

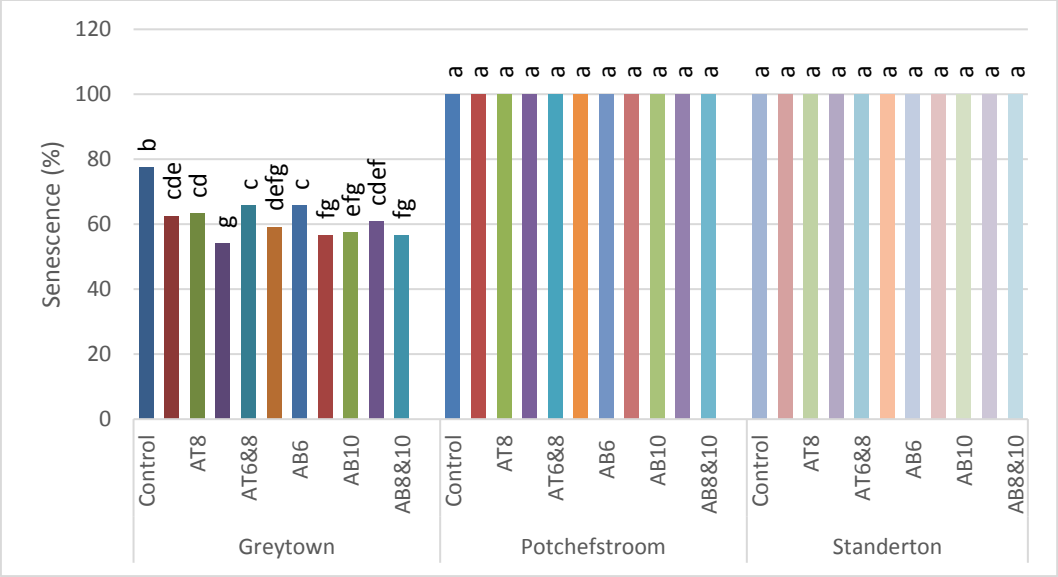


Figure 3-5: The effect Amistar Top® (AT) and Abacus® (AB) applied at five application dates on percentage leaf senescence on sorghum two weeks post-harvest as evaluated over three localities during the 2013/14 season.

3.4.2.2 2014/15

Analysis of variance indicated that the treatment x cultivar interaction significantly affected the degree of senescence at 150 d.a.p during the 2014/15 season (Figure 3-6). Like results obtained during the 2013/14 season, PAN8816 and NS5511 seemed to senesce at a slower rate than the other two cultivars (PAN8906 and PAN8625). With exception of AT 8 on PAN8906 all treatments on PAN8906 and PAN8625 lead to a delay in senescence in Greytown.

Analysis of variance (Table 3-3) indicated that neither treatment as main effect nor any of the treatment associated interactions resulted in significant differences in senescence at harvest.

Similar to S2, analysis of variance for the degree of senescence measured two weeks post-harvest was again not significantly affected by treatment (as main effect) or any of the treatment associated interactions (Table 3-2).

3.4.3 Plant mass and yield

The following ANOVA was obtained for yield and plant mass as observed during the 2013/14 and the 2014/15 seasons.

Table 3-3: ANOVA indicating significant differences between plant mass and yield during the 2013/14 ad 2014/15 growing seasons.

SOURCE	DF	PLANT MASS				YIELD			
		S.S.	M.S.	F VALUE	PR > F	S.S.	M.S.	F VALUE	PR > F
2013/14									
Locality (Loc)	2	9.8	4.9	3.5	0.0365	40.1	20.1	16.5	<.0001
Rep (Loc)	6	39.6	6.6	4.7	0.0005	75.7	12.6	10.4	<.0001
Treatment (Tmt)	10	19.3	1.9	1.4	0.2114	13.4	1.3	1.1	0.3796
Loc x Treatment	20	52.6	2.6	1.9	0.0318	49.0	2.5	2.0	0.0199
Error (A)	60	84.0	1.4			73.2	1.2		
Cultivar (Cult)	3	146.4	48.8	48.8	<.0001	119.7	39.9	39.9	<.0001
Locality x Cultivar	6	61.1	10.2	10.2	<.0001	56.8	9.5	9.5	<.0001
Treatment x Cultivar	30	21.6	0.7	0.7	0.8578	22.8	0.8	0.8	0.8139
Loc x Tmt x Cult	60	51.0	0.9	0.9	0.7691	74.7	1.2	1.2	0.1349
Error (B)	198	198.0	1.0			198.0	1.0		
Corrected Total	395	683.4				723.4			
2014/15									
Locality (Loc) L Oc)	2	611.5	305.7	78.6	<.0001	110.2	55.1	25.2	<.0001
Rep (Loc)	6	266.6	44.4	11.4	<.0001	9.6	1.6	0.7	0.6244
Treatment (Tmt)	10	52.7	5.3	1.4	0.2235	35.5	3.6	1.6	0.1220
Loc x Treatment	20	58.4	2.9	0.8	0.7574	19.1	1.0	0.4	0.9789
Error (A)	60	233.4	3.9			131.1	2.2		
Cultivar (Cult)	3	149.2	49.7	49.7	<.0001	136.0	45.3	45.3	<.0001
Locality x Cultivar	6	147.0	24.5	24.5	<.0001	108.4	18.1	18.1	<.0001
Treatment x Cultivar	30	33.2	1.1	1.1	0.3314	40.3	1.3	1.3	0.1221
Loc x Tmt x Cult	60	65.2	1.1	1.1	0.3305	51.8	0.9	0.7	0.7436
Error (B)	198	198.0	1.0			198.0	1.0		
Corrected Total	395	1815.1				839.9			

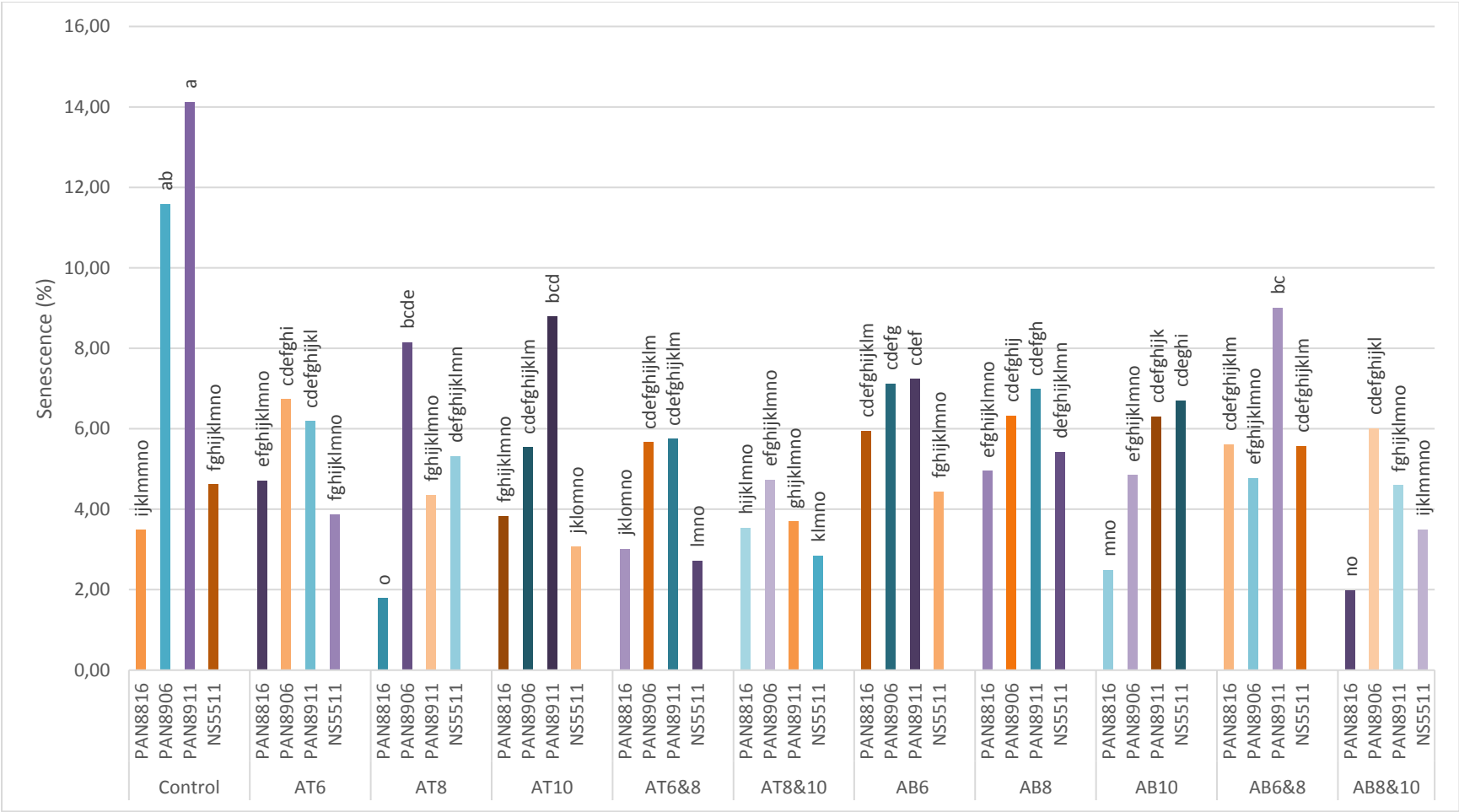


Figure 3-6: Senescence 150 d.a.p (S3) as observed with the treatment x cultivar interaction at three localities during the 2014/15 season.

3.4.3.1 Plant mass

During 2013/14, the locality x treatment interaction significantly affected plant mass. According to the analysis, plant mass was affected differently by the various treatments over the three localities (Figure 3-7). At the Potchefstroom trial, the AT 10 and AB 6 & 8 treatment yielded lower plant mass compared to that of the control. Greytown and Standerton showed no significant differences with respect to various treatments and the respective control plots.

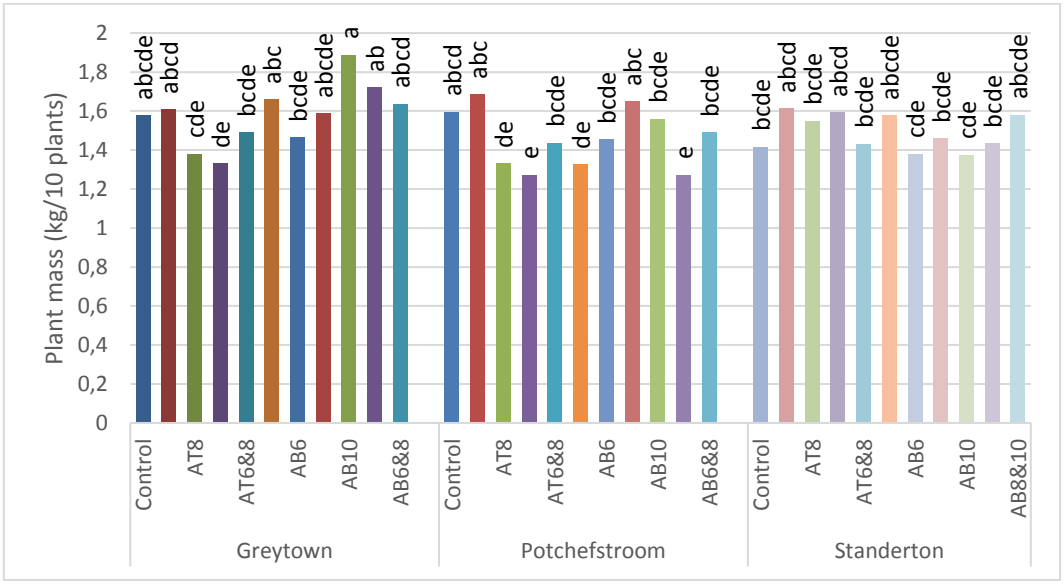


Figure 3-7: The effect of two fungicides applied at five application dates on plant mass of sorghum as evaluated over three localities (Greytown, Potchefstroom and Standerton) during the 2013/14 season.

3.4.3.2 Yield

Analysis of variance conducted on the results obtained during the 2013/14 and 2014/15 seasons indicated that very limited significance occurred which can be attributed to the application of fungicides (Table 3-3). The locality x treatment interaction observed during 2013/14 was the only treatment associated interaction that had a significant effect on yield. According to Figure 3-8, the AT 8 & 10 treatment at Standerton (2013/14) yielded significantly higher yield compared to the respective control at the same locality.

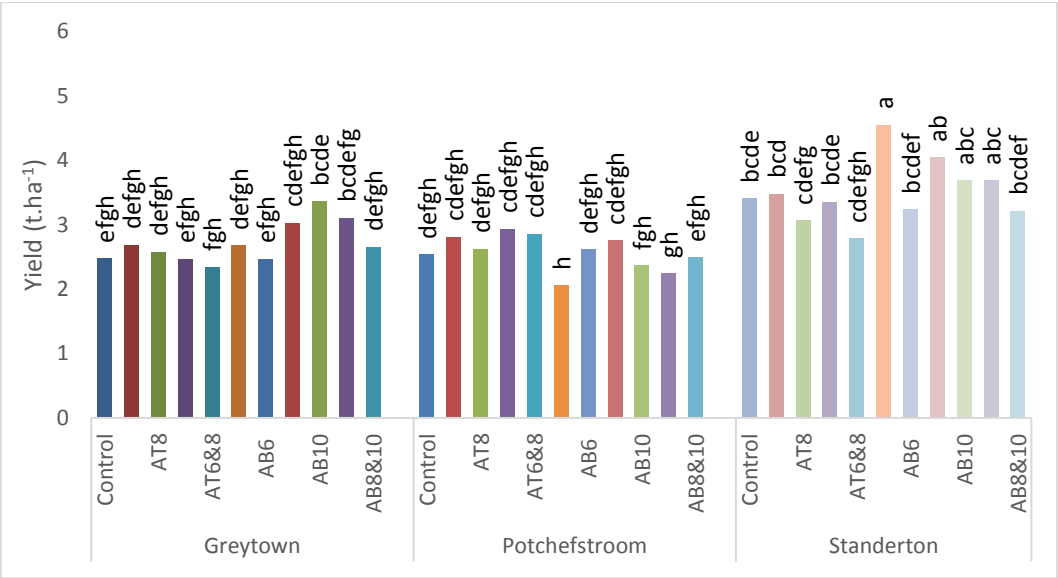


Figure 3-8: The effect of two fungicides applied at five application dates on sorghum yield as evaluated over three localities (Greytown, Potcherstroom and Standerton) during the 2013/14 season.

3.4.4 Correlation analysis

Yield did not correlate well with S1, S2, S3 or plant mass measured at any of the trials in both seasons as most significant correlations obtained were low to moderate (Rayner, 1969) (Table 3.4). Of the three sites, Greytown had the most significant correlations in both seasons. At Greytown, moderate correlations were obtained between yield and plant mass during both growing seasons (r= 0.568; p=<0.0001 – 2013/14 and r=0.407; p=<0.0001). At the same locality yield correlated negatively with S1, S2 and S3 during 2013/14 and S2 and S3 during the following season (Table 3-4). Although significant, the correlations obtained between yield and percentage leaf senescence were weak. Plant mass showed similar effects to that of yield regarding the correlations obtained with senescence (S1, S2 and S3).

Table 3-4: Correlation analysis results between percentage leaf senescence as measured at 150 days after planting, with harvest as well as two weeks post-harvest, plant mass, yield at three localities over two seasons.

	2013/14					2014/15				
Greytown	**Yield**	**PM**	**S1**	**S2**	**S3**	**Yield**	**PM**	**S1**	**S2**	**S3**
PM	0.586 (<.0001)					0.407 (<.0001)				
S1	-0.322 (0.0002)	-0.278 (0.001)				0.146 (0.095)	0.103 (0.240)			
S2	-0.311 (0.0003)	-0.283 (0.001)	0.886 (<.0001)			-0.122 (0.163)	-0.483 (<.0001)	0.274 (0.002)		
S3	-0.384 (<.0001)	-0.351 (<.0001)	0.726 (<.0001)	0.813 (<.0001)		-0.211 (0.015)	-0.580 (<.0001)	0.084 (0.339)	0.589 (<.0001)	
Potch										
PM	0.204 (0.019)					0.083 (0.343)				
S1	0.055 (0.533)	0.162 (0.063)				0.083 (0.344)	-0.083 (0.343)			
S2	-	-	-			0.098 (0.263)	-0.105 (0.231)	0.984 (<.0001)		
S3	-	-	-	-		-	-	-	-	
Standerton										
PM	0.131 (0.133)					-0.280 (0.001)				
S1	0.032 (0.713)	0.077 (0.383)				0.296 (0.001)	-0.236 (0.006)			
S2	-	-	-			-	-	-		
S3	-	-	-	-		-	-	-	-	

3.5 Discussion and conclusions

Inconsistent effects of fungicide application were observed in senescence measured at 150 d.a.p (S1), at harvest (S2) as well as two weeks post-harvest (S3). During 2013/14, all fungicide treatments significantly delayed senescence at Greytown at all three screening times (S1, S2 and S3). Grossmann *et al.* (1999) reported delays in senescence of wheat treated with triazole, and concluded that such delays may be due hormone and enzymatic changes, specifically ethylene and its precursor ACC-synthase. Berdugo *et al.* (2012) reported delays in senesces in wheat treated with triazoles and strobilurins respectively, with effects being described as delayed flag leaf senescence, delayed ear emergence and senescence.

According to Blandino and Reyneri (2009) the use of triazoles in a combined spray i.e. triazole and strobilurin programme was tested to be ineffective in delaying senescence to a significant level as opposed to a triazole only application programme on wheat. These findings were contradicted by a review by Dimmock and Gooding (2002) in which they explained that green flag leaf area duration and grain protein concentration were not affected by the choice of fungicide applied. In this study, however, commercially available and registered fungicides were selected for evaluation. The merit of repeating the study using single fungicide application may be of use to establish the difference in these instances. Dimmock and Gooding (2002) also found that the relationship between the use of fungicides and the effects on different cultivars are dependent on the environment and growing season. These results are similar to data shown in this study, and constitutes further study into the climatic conditions surrounding the data.

The effects of fungicide application on plant mass were inconsistent over season and locality, and did not necessarily influence yield. In a similar study on spring barley, Bingham *et al.* (2012) reported small or non-significant differences regarding plant mass due to fungicide application whilst a paper by Yang *et al.* (2000) stated that some fungicide treatments may in some seasons increase the yield and plant mass of spring barley. Jorgensen and Olesen (2002) reported varying results, and indicated that yield may be more subject to increase due to fungicidal treatment than plant mass in the case of winter wheat. Their results showed significant differences in yield of winter wheat in most trials from 1992 to 1997, but less significant increases and in some cases, decreases in green leaf material, as seen in the current study with plant mass. McCartney *et al.* (2006) showed no increase in green leaf area and mass over two growing seasons with the application of strobilurin and triazoles on wheat. They also stated that these increases are season dependant and cannot be predicted. The latter findings correspond to findings of the current study in that the effect that fungicide applications had on yield and plant mass was inconsistent over season and locality.

Blandino and Reyneri (2009) indicated that delays in senescence did not result in increased yield in wheat. Dimmock and Gooding (2002) displayed no increase in green leaf area but significant increases in TGW (thousand grain weight) of wheat. During 2005 McCartney *et al.* (2006), conducted trials on wheat in Ireland and indicated that the combination of a triazole and a strobilurin did not have a significant effect on yield or green leaf area, but azoxystrobin alone, although unable to control disease, was able to significantly increase yield. Henry *et al.* (2011) reported similar results in which only pyraclostrobin alone showed to increase yield in soybeans. Bertelsen *et al.* (2001) showed that plants display different senescence reactions to fungicide treatments under different conditions, which could explain the inconsistencies observed in the current study. They demonstrated that senescence is more likely to be delayed due to prophylactic fungicide application (in the absence of disease), but such delays do not necessarily imply yield gains in wheat.

Correlation analysis indicated that although a significant negative correlation was observed between % senescence and both yield and plant mass at all three screening times, it was weak correlations, implying that the delay in senescence did not contribute to such a great extend to the eventual yield and plant mass observed.

It can therefore be concluded that with the variable results obtained at the three localities over two growing seasons, that the relationship between fungicide application and growth-regulating effects are dependent on various external factors that will determine whether prophylactic application of fungicides will be cost-effective or not. The responses obtained were, accordingly inconsistent over treatment, locality and season for senescence, plant mass and yield. Results indicated that fungicide application could not be relied upon to reduce the rate of senescence or stimulate yield sufficiently to warrant prophylactic fungicide applications with the expectation of financial gain in the absence of foliar diseases.

4

Biochemical and physiological responses of sorghum to fungicide application under glasshouse conditions.

4.1 Introduction

Results obtained from field trials conducted during 2013/14 and 2014/15 indicated that the prophylactic use of fungicide was not able to increase yield in the absence, or with low levels of LB development. A delay in plant senescence was, however, observed at some localities, which warranted an in-depth study into the plant's response because of fungicide application. Senescence is known to be an oxidative process, characterized by denaturing of proteins, DNA and lipids (Chapter 1), due to increased generation of reactive oxygen species in chloroplasts, mitochondria, endoplasmic reticulum and nuclear cell organelles (Hopkins and Hüner, 2009). The denaturing of proteins will then cause decreases in active photosynthetic pigments, thus decreases in total vitality of the plant can be expected. Another factor to consider, is plant development; if senescence is delayed some morphological changes may be expected.

A glasshouse trial was conducted to examine the effects of fungicide application on sorghum plants' photosynthetic efficiency, anti-oxidative enzyme mechanisms, and sugar accumulation and black layer formation in the seeds.

4.1.1 Chlorophyll *a* fluorescence

Chlorophyll *a* fluorescence induction is a non-invasive tool to measure the photosynthetic efficiency of plants. When plants are subjected to stress conditions the photosynthetic

efficiency of the plant is affected by stress (Lichtenthaler, 1995), and the ability of the plant to photosynthesize is reduced (van Kooten and Snel, 1990).

According to Dias (2012), triazole reportedly lead to a decrease in the quantum yield of PSII due to decreases in photochemical quenching of the quinone pools. Strobilurins, on the other hand, block the electron transport between PSII and PSI by binding to the Q_1 site of the cytochrome *bf* complex. Both triazoles and strobilurins have therefore the ability to reduce photosynthesis. Cordon *et al.* (2016) studied the progression of senescence and the corresponding influences on chlorophyll a fluorescence and found that PSII function decreased as senescence progressed. As only limited significant delays in senescence were observed during the 2013/14 and 2014/15 field trials, the possibility of phytotoxicity of fungicides required investigation under glasshouse conditions.

4.1.2 Reactive oxygen species

As senescence progresses it can be expected that the generation of reactive oxygen species such as superoxide, hydroxyl radical, hydroperoxyl, hydrogen peroxide, singlet oxygen, and excited carbonyl, will increase from sources such as the peroxisomes, chloroplasts, peroxisomes, membranes and endoplasmic reticulum (Karuppanapandian *et al.*, 2011). Oxidative stress in plants can lead to the peroxidation of lipids, protein oxidation, nucleic acid damage, enzyme inhibition and programmed cell death (Sharma *et al.*, 2012). ROS scavenging enzymes such as POD and SOD are present in the plant to metabolically scavenge these damage-causing compounds. Wegulo *et al.* (2011) reported on the anti-oxidative action associated with fungicide application, and found that in some cases ROS scavenging enzymes were upregulated with prophylactic application.

4.1.3 Grain morphology and black layer formation

Because literature commonly links black layer formation to maturity, it can be assumed that if senescence is delayed there may be fluctuations in the development time of the black layer. The specific mechanism behind the formation of the black layer of the sorghum seed is, however, still debatable. According to Giles *et al.* (1975) the black layer is a physical barrier formed by phenolic compounds accumulating in the parenchyma cells of the phloem cells, while Vanderlip, (1993) speculated that it is purely scar tissue formed by the detachment of the hilum from the finiculus. Thus, the specific mechanism behind the formation of the black layer of the sorghum seed, as well as the function thereof is still uncertain. However, a blockage, be it by scar tissue or due to accumulation of phenolic compounds in cells, will disrupt the accumulation of carbohydrates and other assimilates to the seeds (Turgeon and Wolf, 2009) and in turn prevent further increases in mass of the seeds. If delays in senescence are evident to occur due to prophylactic fungicide application, and this process may be delayed, leading to increased sugar accumulation and higher grain yield.

4.2 Aim and Objectives

The aim of this study was to investigate the biochemical and physiological responses that occur due to the prophylactic fungicide application. This will be achieved by examining the following parameters:

- Photosynthetic efficiency,
- Anti-oxidative enzymes (peroxidase, superoxide dismutase, and xanthine oxidase), and
- Grain morphology (black layer size and formation) and sugar accumulation to seeds.

4.3 Materials and methods

Limited differences between cultivars regarding their response to funigicide application could, in genereral, be observed in the field trials conducted druing 2013/14. PAN8816 and NS5511 were accordingly selected for the pot trial experiment as they represent a LB susceptible (PAN 8816) and resistant (NS5511) sorghum cultivar (Personal communication – Dr M. Craven).

The two fungicides, Amistar Top® (azoxystrobin/difenoconazole) and Abacus® (epoxiconazole/pyraclostrobin), were applied at three different application dates (8, 10 and 8 & 10 weeks after planting) with an unsprayed control included (Table 4-1). The application dates were selected as they yielded somewhat more significant results during the 2013/14 field trial with respect to delays in senescence and yield. Both fungicides have active ingredients accordingly belonging to the triazole and strobilurin groups.

Applications were done in the same manner as the field trial; with a four-nozzle spray boom and a CO_2 gas operated knap sack. Once the knap sack spray was calibrated all applications were applied as per recommended application rates. Pots were arranged in the glasshouse in a randomized block design with five replicates, with a single pot representing a replicate.

Table 4-1: Selected treatment used in the 2015 field trial.

	Treatment	Formula	Application time (after planting)	Application rate
1	Control			
2	Azoxystrobin/difenoconazole	200 / 125 g.ha^{-1}	8 weeks	500 mL.ha^{-1}
3	Azoxystrobin/difenoconazole		10 weeks	
4	Azoxystrobin/difenoconazole		8 & 10 weeks	
5	Epoxiconazole/pyraclostrobin	62.5 / 62.5 g.ha^{-1}	8 weeks	1L.ha^{-1}
6	Epoxiconazole/pyraclostrobin		10 weeks	
7	Epoxiconazole/pyraclostrobin		8 & 10 weeks	

Ten sorghum seeds were planted in 5 L pots filled with sterilized soil. Upon emergence seedlings were thinned out to one plant per plot. Fertilizer was applied at 42 and 70 days after planting (3:2:1 dissolved in H_2O – 20 g/L; 100 mL per pot to prevent burning). Aphids were controlled with Metasystox (2.5 mL/L) applied fortnightly from 42 days after planting. Glasshouse conditions were kept constant at 25°C day/17°C night temperatures with a 15-hour day light length. Pots were watered every second to third day to water holding capacity.

4.3.1 Statistical analysis

The treatment design was a 7x2 factorial consisting of 7 treatments (Table 4-1) and 2 cultivars (C1 – PAN8816 and C2 – NS5511) (Snedecor and Cochran, 1967). Chlorophyll *a* fluorescence data was subjected to an analysis of variance (ANOVA) using the repeated measurements (sampling) as a subplot factor (Little and Hills, 1972). Fishers protected t-LSD (least significant difference) were calculated at a 5% significance level to compare means of significant source effects analysis was conducted using Genstat software.

Within the difference in relative fluorescence intensity values obtained various bands may be identified. To determine statistical significance with respect to the control plants at these intervals a paired t-test was additionally conducted with SIGMAPlot 10 software at p=0.05 confidence.

4.3.2 Chlorophyll *a* fluorescence measurements and related parameter calculations

Chlorophyll *a* fluorescence measurements were obtained with a HandyPEA plant efficiency analyser as described by Strasser *et al.*, 2007 and Stirbet and Govindjee, 2011. Measurements were taken at 70 (flowering), 110 (soft dough) and 150 (maturity) days after planting on dark adapted leaves. Three measurements were taken per plant, on the flag leaves, and the average used for further analysis.

Based on a model described by Strasser *et al.* (2007), fluorescence transients are defined within the created graphs which are associated with different components in the photosynthetic electron transport chain (ETC). The curve obtained is known as the OJIP curve and is constructed according to the progression of electron transport in the photosynthetic apparatus of the plant. The name OJIP is so name after a model by Strasser et al. 2007, were O is the chlorophyll intensity at 0.03 ms of a dark-adapted leaf, the J inflection point can be associated with the electron transfer from pheophytin to plastoquinone at 2 ms, the I inflection point is associated with the transfer of electrons between PSII and PSI via the cytochrome *bf* complex at 30 ms and the P inflection point is associated with the final reduction of NADP+ to NADPH. To be able to understand the flow of electrons from absorption to the reduction of the end electron acceptors the OJIP curve is normalized between inflection points O and P and the difference in relative variable fluorescence is calculated.

From the Handy PEA instrument software, such as PEAplus one can then calculate values in order to obtain what is known as the performance index which is an indication of the overall health status of the plant.

Equation 4-1: Calculation of the PI_{total} value; By arranging these parameters into more quantifiable groups the density of reaction centres ($\gamma_{RC}/(1-\gamma_{RC})$), trapping of electrons ($\varphi_{P0}/(1-\varphi_{P0})$), dissipation of electrons ($\psi_0/(1\psi_0)$), and reduction of end electron acceptors ($\delta_{R0}/(1-\delta_{R0})$) respectively over time was calculated as per the above formula.

$$PI_{total} = \frac{\gamma RC}{1-\gamma RC} \bullet \frac{\varphi P0}{1-\varphi P0} \bullet \frac{\Psi 0}{1-\Psi 0} \bullet \frac{\delta R0}{1-\delta RO}$$

To quantify the effect of prophylactic application of fungicide on the photosynthetic efficiency of the sorghum plants these curves are double normalized and processed to give the difference in relative variable fluorescence, with respect to the control plants. This is done with use of the following formula:

Equation 4-2: Calculation of the difference is variable chlorophyll a fluorescence intensity; Where V is the relative variable fluorescence and F is the fluorescence intensity as a given time

interval (a.u.), O is the fluorescence signal at step O (0.05 ms), J (0.2) and P is the fluorescence signal at step P (300 ms).

$$Relative\ variable\ fluorescence\ (V_{OP}) = \frac{(F_t - F_O)}{(F_P - F_{O)}}$$

$$Relative\ variable\ fluorescence\ (V_{OJ}) = \frac{(F_T - F_O)}{(F_J - F_{O)}}$$

$$Relative\ variable\ fluorescence\ (V_{JP}) = \frac{(F_t - F_J)}{(F_P - F_{J)}}$$

$$\text{Difference in relative variable fluorescence } (\Delta VX) = V_{treatment} - V_{control}$$

4.3.3 Analysis of enzyme activity

4.3.3.1 Sampling of leaf material and enzyme extraction

Leaf material was harvested at three sampling dates, i.e. 70 days after planting (flowering), 110 days after planting (soft dough), and 150 days after planting (maturity). Approximately 1 g of leaf material was mechanically removed from each plant and immediately placed in liquid N_2 as to preserve all enzyme activity. After initial sampling, the samples were ground to a powder in additional liquid N_2 until fine and placed in 6 mL scintillation tubes and stored at -80°C until further use.

Extraction of all POD, SOD and XOX extracts was done according to protocols described by Slusarenko and Milosevic (1996) by using a 50 mM KH_2PO_4 buffer solution (PBS) at pH7. Shortly before extraction, 0.0093 g / 25 mL EDTA was added to the buffer.

Frozen leaf material was homogenized in 5 mL of 50 mM PBS (pH 7) containing 1% (w/v) polyvinylpyrrolidone (PVP). The homogenate was centrifuged for 15 minutes at 15 000 rpm and 4°C. The supernatant was accordingly used as the crude enzyme extract.

4.3.3.2 Protein concentration

The protein concentration of the enzyme extracts were determined with the Bradford assay method (Kruger, 2009) using a Berthold Microspectrometer. The following reagents were prepaired:

1. Assay reagent consisting of 100 mg Coomassie Blue dissolved in 50 mL 95% ethanol and added to 100 mL 85% phosphoric acid and top up to 1L with distilled H_2O.
2. A standar solution of 100 µg/mL bovine γ-globulin in distilled H_20 stored at -20°C – precise protein concentration was determined at 280 nm (ABS = 1.35)
3. A calibration curve was constructed by the following method
    a. Volumes of 10-100 µL of the standard solution was pipetted into a 96 well ELISA plate and the volume adjusted to 100 µL with distilled H_2O; distilled H_2O used as the blank standard.
4. Samples of the extact were added in triplicate – no dilution was necessary.
5. 1 mL protein reagent was added to each slot and mixed.
6. Absorbace was measured after 30 min at 595 nm.
7. From the calibration curve the protein concentration was the determined

4.3.3.3 Peroxidase activity

Peroxidase (POD) activity was determined according to Zieslin and Ben-Zaken (1991). Leaf material was extracted as described above and kept on ice. The assay mixture (2 mL) consisted of:

1. 1 mL of 40 mM KH_2PO_4 pH 5.5,
2. 680 µL d H_2O,
3. 200µL 5 mM guaiacol,
4. 20 µL enzyme extract, and
5. 100 µL 8.2 mM H_2O_2.

A spectrophotometric kinetic assay was obtained at 470 nm for 3 minutes. The extraction buffer was used as a blank reference. Protein concentration was also determined according to the method described by Bradford (1976) in which a standard curve was compiled in order to quantify the protein concentration of each sample using the Berthold spectrophotometer at 595nm. From here specific anzyme activity wase xpressed as nmol guaiacol.mg^{-1}protein.min^{-1}.

4.3.3.4 Superoxide dismutase activity

Superoxide dismutase (SOD) activity was determined according to Keppler and Novacky (1987). Leaf material was extracted as described above and the activity was determined right after extraction. The assay mixture consisted of:

* 50mM KH_2PO_4 pH 7.5 (PBS),
* A stock solution (13 mL) to be added to 87 mL PBS to make up a total volume of 100 mL was prepared consisting of the following:

    1. 1 mL of 130 mM methionine,
    2. 10mL of 200 µM riboflavin,
    3. 1 mL of 10 mM EDTA, and
    4. 1 mL of 7.5 mM nitro blue tetrazolium chloride (NBT).

Reaction mixture (1.7 mL) and enzyme extract (30 µL) was added in duplicate. One set was placed in the dark, and the other under UV light for 20 minutes. After incubation absorbance was measured at 560 nm with the dark incubated reference used as the blank. The protein concentration of each sample was also determined as described for POD activity. SOD activity could then be expressed as nmol NBT.mg^{-1}protein.min^{-1}.

4.3.3.5 Xanthine oxidase activity

Xanthine oxidase activity (XOX) was determined spectrophotometrically with an adapted protocol described by Marcocci *et al.* (1994). The crude enzyme extract was prepared as described above. The assay mixture consisted of:

1. 150 mM xanthine was dissolved in a PBS pH 7.5 (substrate),
2. 1.45 mL PBS, and
3. 1 mL 10% w/v xanthine.

The cuvettes were incubated for 15 minutes after which 50 µL of enzyme extract was added and incubated for an additional 30 minutes. Absorbance was measured at 295 nm for 3 minutes. Activity expressed as $mU.mL^{-1}$ XOX.min^{-1}.

4.3.4 Sucrose concentration and black layer morphology

4.3.4.1 Sucrose concentration

Carbohydrate quantification was done by the GOD/invertase method described by Teixeira *et al.* (2012). Sorghum seeds were harvested at 150 days after planting (stage nine) from the panicles and dried in an oven at 100°C for 48 hours. Samples were then ground in liquid nitrogen and 20 mg of each sample was transferred to 2 mL centrifuge tubes, to which 1 mL 80% ethanol was added. Each tube was homogenised for 1 minute in a vortex and placed in a water bath at 70°C for 90 minutes. The tubes were then centrifuged for 10 minutes at 16 000rpm. The supernatant was used as the extract (volume filled to 1 mL with 80% ethanol).

In a 96-well ELISA plate 85 µL dH_2O, 5 µL of sucrose extract, and 10 µL invertase (10 mg/mL) were place in each well, sealed and placed in a water bath at 55°C for 10 minutes, after which 200 µL GOD was added and placed in a water bath at 37°C for 15 minutes, and then allowed to cool for 5 minutes. Standard sucrose solutions from 0% to 1% (0; 0.1; 0.25; 0.5; 0.75 and 1) were also added to the plates to allow a calibration curve to be

constructed. Each sample was analysed in quadruplicate to ensure accuracy. A Berthold spectrophotometer was used at 490 nm to measure the absorbance of the mixture after which the sucrose concentration was expressed as g/100 g.

4.3.4.2 Observation of black layer

Seeds were harvested from the plants at 150 days after planting (physiological maturity) and removed from the panicles. The pericarp (outer layer of the seed) were removed following the method described by Brits *et al.*, (1993), in which dried, intact seeds were soaked in 40°C water for two hours to loosen the pericarp. A scalpel was used to scrape off the loosened pericarp, specifically the hilum (black layer region).

Five seeds for each treatment were observed under the stereomicroscope and the perimeter and area calculated with the microscopic Motic Images Plus 2.0 software.

4.3.4.3 Correlation analysis

Correlation analysis was in addition conducted between the black layer area measured and the eventual sucrose concentration observer at 150 days after planting for each of the two cultivars respectively.

4.4 Results

4.4.1 The use of chlorophyll a fluorescence to quantify the effects of fungicidal treatment on Sorghum bicolor under glasshouse conditions.

4.4.1.1 OJIP and variable fluorescence transients obtained for the various treatments with PAN8816 at 70 days after planting.

The OJIP curves obtained for chlorophyll *a* fluorescence of sorghum leaves (PAN8816) when treated with two commercial fungicides at 70 d.a.p.

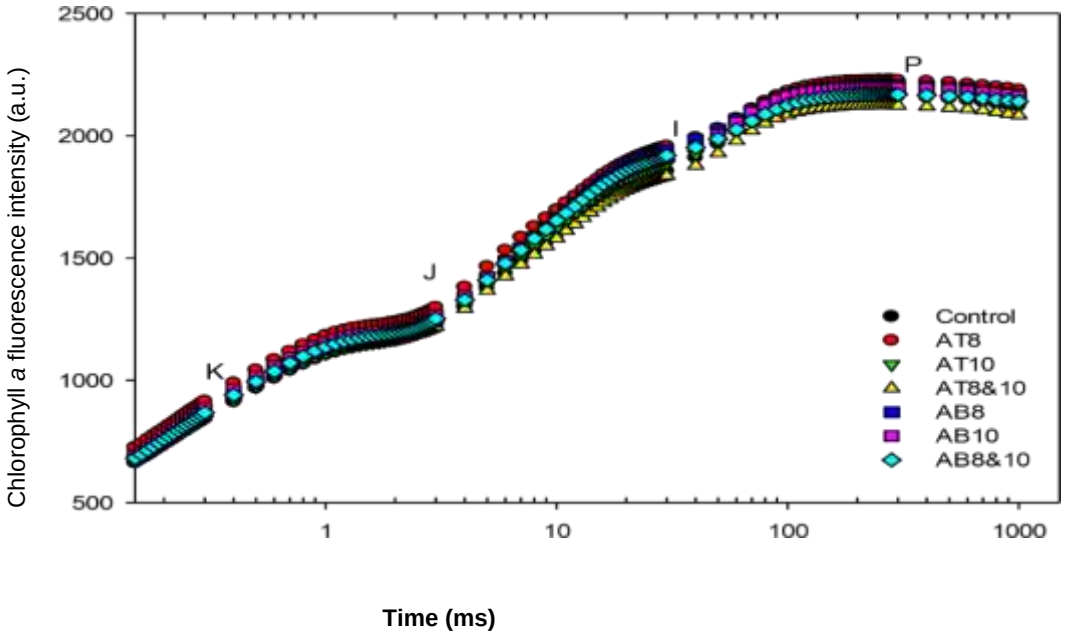


Figure 4-1: OJIP curve for PAN8816 70 days after planting. Treatments are as indicated AT and AB implying treatment with Amistar TOP® and Abacus® respectively and 8, 10 or 8 & 10 the application date in weeks after planting.

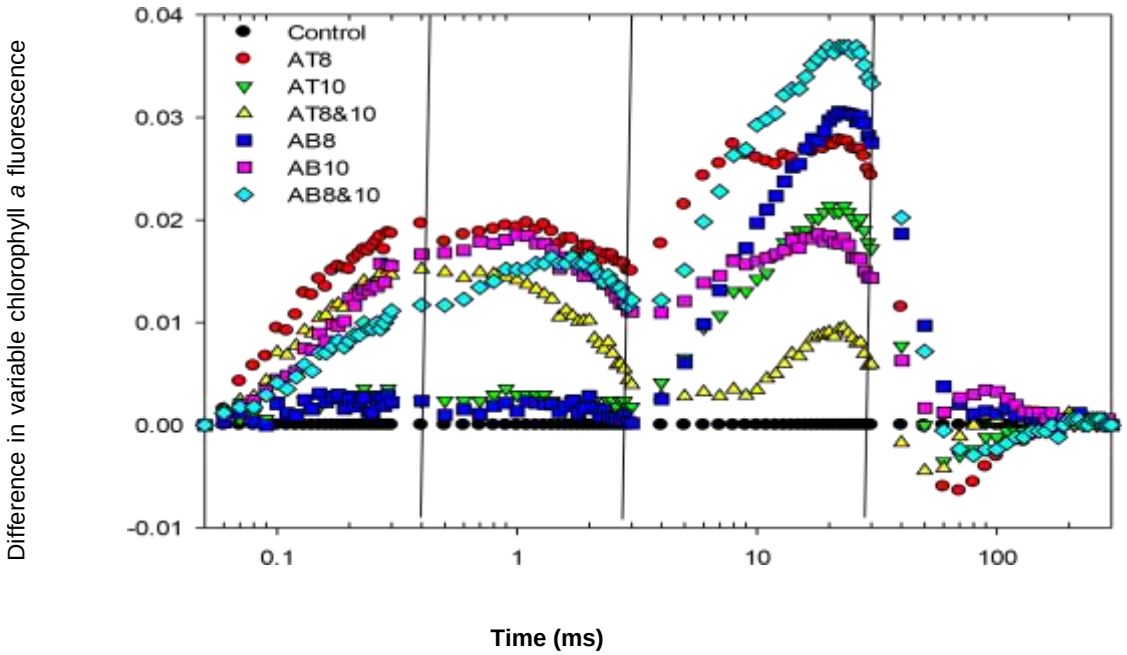


Figure 4-2: Difference in relative variable fluorescence for PAN8816 70 days after planting normalized between 0.05 ms and 300 ms. Treatments are as indicated AT and AB implying

treatment with Amistar Top® and Abacus® respectively and 8, 10 or 8 & 10 the application date in weeks after planting.

Shapiro-Wilks' paired t-test analysis (p=0.05) showed no significant differences relative to the control obtained at time intervals 0.03, 0.2 and 30 ms with the different fungicide treatments (Figure 4-1).

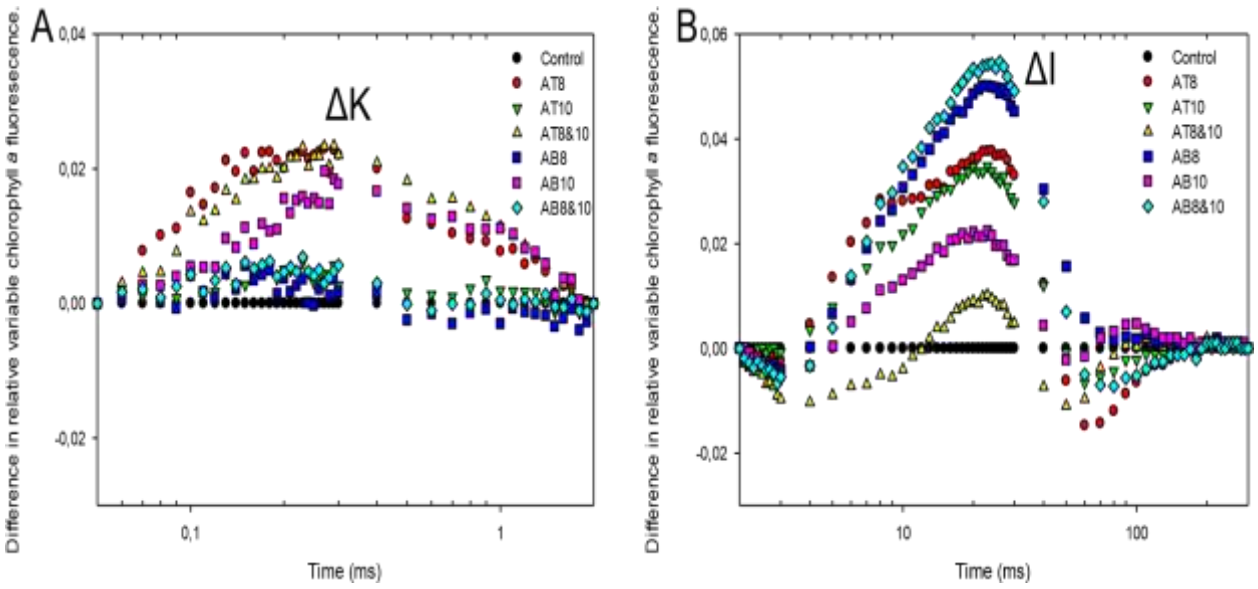


Figure 4-3: A-B: Difference is relative variable fluorescence for PAN8816 70 days after planting normalized between 0.05 ms and 2 ms, and 2 ms and 300 ms respecively. Treatments are as indicated AT and AB implying treatment with Amistar Top® and Abacus® respectively and 8, 10 or 8 & 10 the application date in weeks after planting.

The appearance of the positive ΔK-band at 0.3 ms (Figure 4-3A) and ΔI-band at 30 ms (Figure 4-3B) indicates that there is a reduction in the photosynthetic efficiency in comparison to the untreated control. The positive ΔK-bands indicates a less effective splitting of water at the oxygen evolving complex and a positive ΔI-bands indicates that electron transport between photosystem II and I (Strasser *et al.*, 2000) is less effective in comparison to the control. Though all the applications resulted in a decline in electron transport, AT 8 had the most inhibiting effect on the oxygen evolving complex while AT 10 weeks had a lesser influence on the oxygen evolving complex.

Treatment AT 8 & 10 had the least effect on the transfer of electrons between PSII and PSI (Figure 4-3B). The formation of a positive ΔI-band (Figure 4-3B) located at 20 ms is indicating towards an ineffective secondary reduction of the quinone complex (Strasser *et al*., 2007). This implies ineffective binding of protons end electrons to Q_B to form the electron carrier plastoquinol (PQH_2). AT 8 and AT 8 & 10 had the most inhibiting effect on the ability of the plant to transport electrons between photosystem II and I (Figure 4-3B).

4.4.1.2 OJIP and variable fluorescence transients obtained for the various treatments with PAN8816 at 110 days after planting.

The OJIP curves obtained for chlorophyll *a* fluorescence of sorghum leaves (PAN8816) when treated with two commercial fungicides at 110 d.a.p.

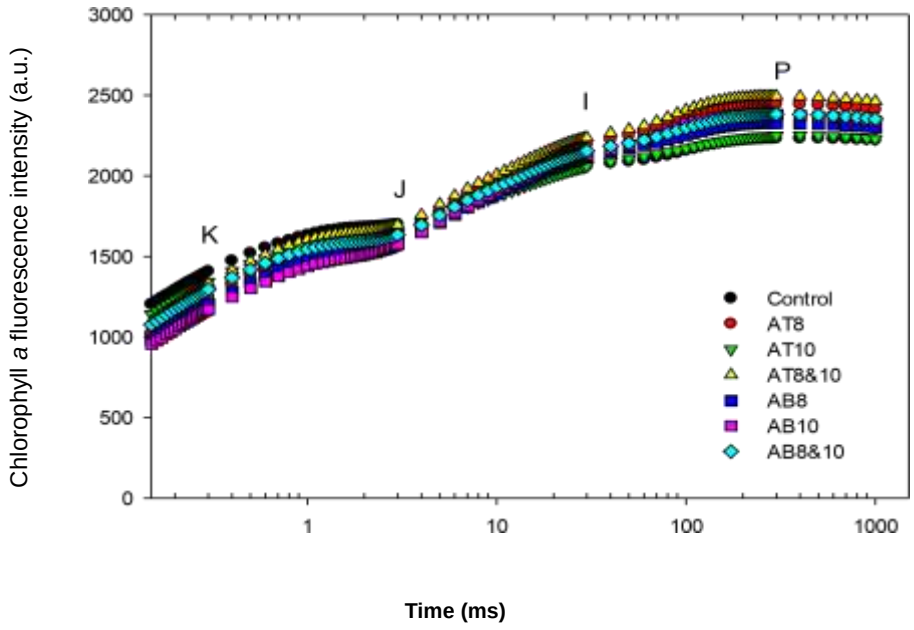


Figure 4-4: OJIP curve obtained 110 days after planting for PAN8816. Treatments are as indicated AT and AB implying treatment with Amistar Top® and Abacus® respectively at 8, 10 or 8 &10 the application date in weeks after planting.

Shapiro-Wilk's paired t-test indicated that no treatment had a significant effect on the difference in relative fluorescence (Figure 4-4).

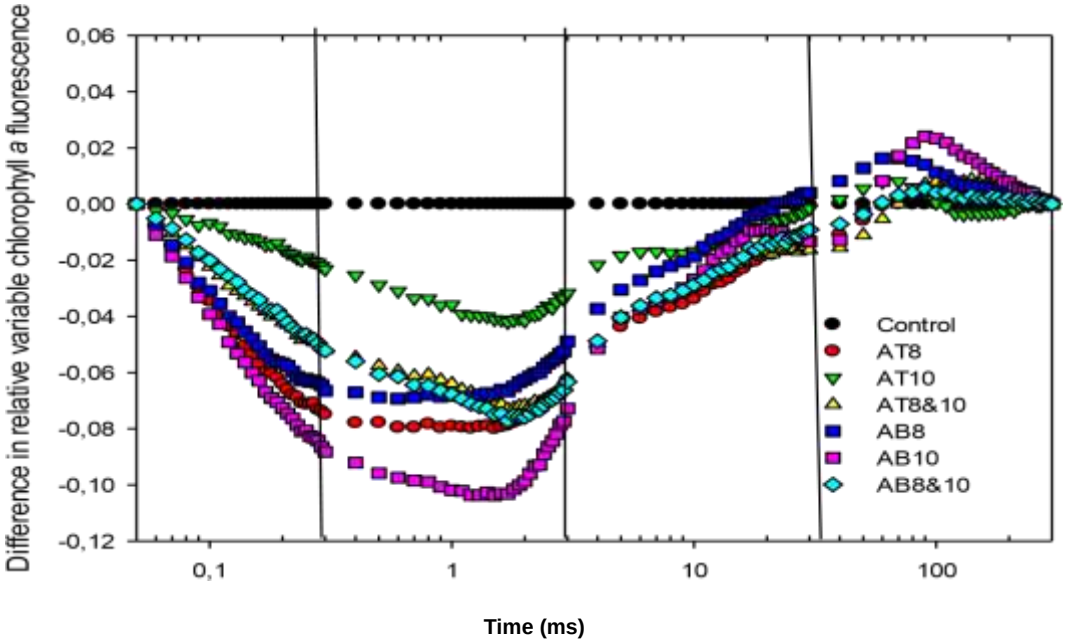


Figure 4-5: Difference in relative variable chlorophyll a fluorescence between 0.05 ms and 300 ms. Treatments are as indicated AT and AB implying treatment with Amistar Top® and Abacus® respectively and 8, 10 or 8 & 10 the application date in weeks after planting.

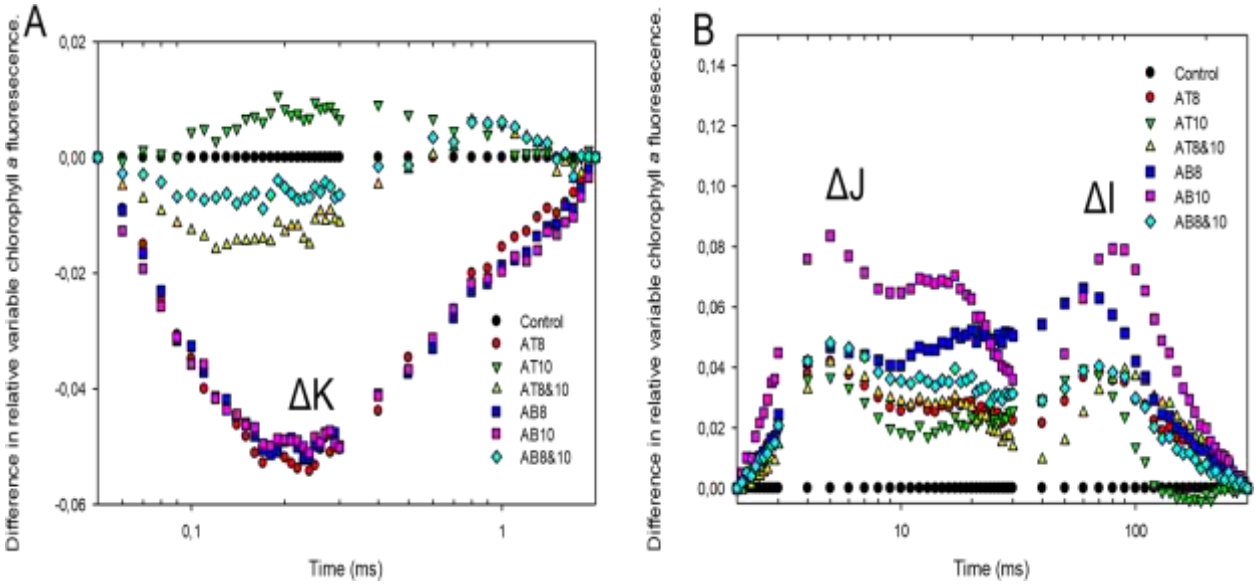


Figure 4-6: A-B: Difference in relative variable chlorophyll a fluorescence between 0.05 ms and 2 ms, and 2 ms and 300 ms respectively 110 day after planting. Treatments are as indicated AT and AB implying treatment with Amistar Top® and Abacus® respectively and 8, 10 or 8 & 10 the application date in weeks after planting.

One hundred and ten days after planting (Figure 4-6) all treatments revealed negative variable fluorescence bands when compared to the control. This indicates a positive change in the photosynthetic efficiency of the plants. Contradictory to 70 days after planting, positive responses are observed for the oxygen evolving complex (∆K-band) with both AT 8, AB 8, and AB 10 showing the most stimulation. The AT 10 treatment resulted in a positive ΔK-band, indicating an inhibitory effect on the oxygen evolving complex. Applying AT 8 &10 and AB 8 & 10 weeks on resulted in a slight stimulation of the oxygen evolving complex (Figure 4-6A). This is an indication that fungicide application may stimulate the OEC to effectively H_2O split into O_2 and H^+ for the electron transport chain and protonation of NADP to $NADPH^+$.

The appearance of a ∆J-band is associated with the transfer of electrons from the OEC to the primary quinone acceptor (Q_A). As soon as the primary quinone acceptor receives an electron it becomes oxidized to form Q_A^-. The ∆J-band refers to the ability of the plant to produce Q_A^- (Strasser et al., 2009). The AB 10 treatment resulted in the ability of the plant to produce more Q_A, thus more potential energy is now available for the plant to use (Figure 4-6B).

The formation of positive ∆I-bands at 5 and 100 ms indicates the accumulation of various electron carriers between photosystem II and I. The accumulation of electron carriers can be the result of the oversupply of energy or that the plant is not effectively converting the energy that is captured by the photosynthetic complexes. Positive ΔI-bands are observable for all the treatments, with AB 10 showing the most inhibiting effect on electron transport (Figure 4-6B).

4.4.1.3 OJIP and variable fluorescence transients obtained for the various treatments with PAN8816 at 150 days after planting.

The OJIP curves obtained for chlorophyll *a* fluorescence of sorghum leaves (PAN8816) when treated with two commercial fungicides at 150 d.a.p.

Sharipo-Wilks pair t-test analysis indicated no statistical significances between treated plants relative to the control (Figure 4-7).

The results obtained 150 days after planting (Figure 4-9) indicate negative photosynthetic responses to fungicide application as the fluorescence is higher for treatment when compared to the respective control for all treatments.

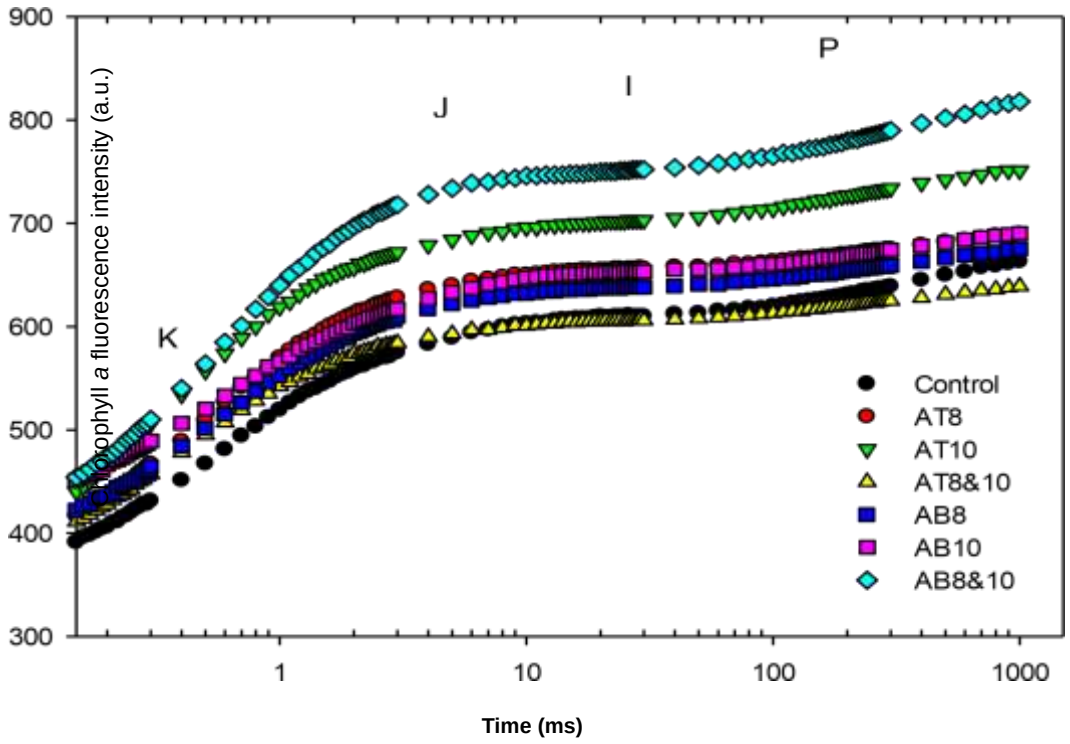


Figure 4-7: OJIP PAN8816 at 150 days after planting. Treatments are as indicated AT and AB implying treatment with Amistar Top® and Abacus® respectively and 8, 10 or 8 & 10 the application date in weeks after planting.

As with 70 days after planting, similar responses (ΔK-bands) are observed at maturity for all the treatments, indicating damage to the OEC (Strasser *et al.*, 2007). However, here the AT

10 and AT 8 & 10 treatments had more apparent responses, for AT 8 there is a ΔI-peak visible, indicating ineffective transfer of electron to the primary quinone acceptors.

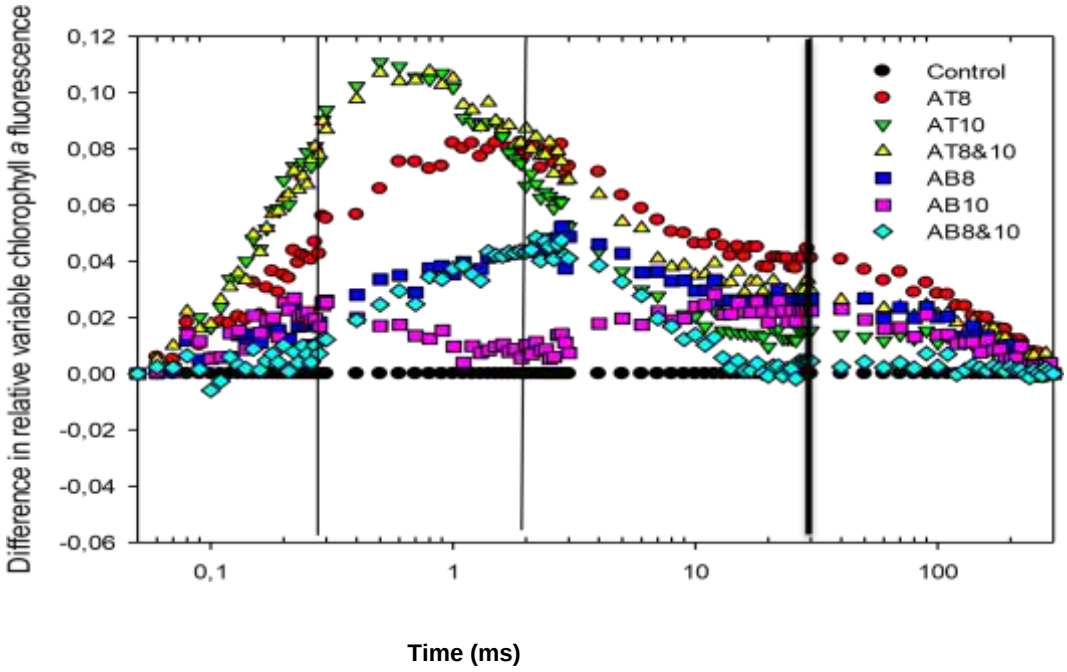


Figure 4-8: Difference in relative variable chlorophyll a fluorescence between 0.05 ms and 300 ms 150 days after planting. Treatments are as indicated AT and AB implying treatment with Amistar Top® and Abacus® respectively and 8, 10 or 8 & 10 the application d date in weeks after planting.

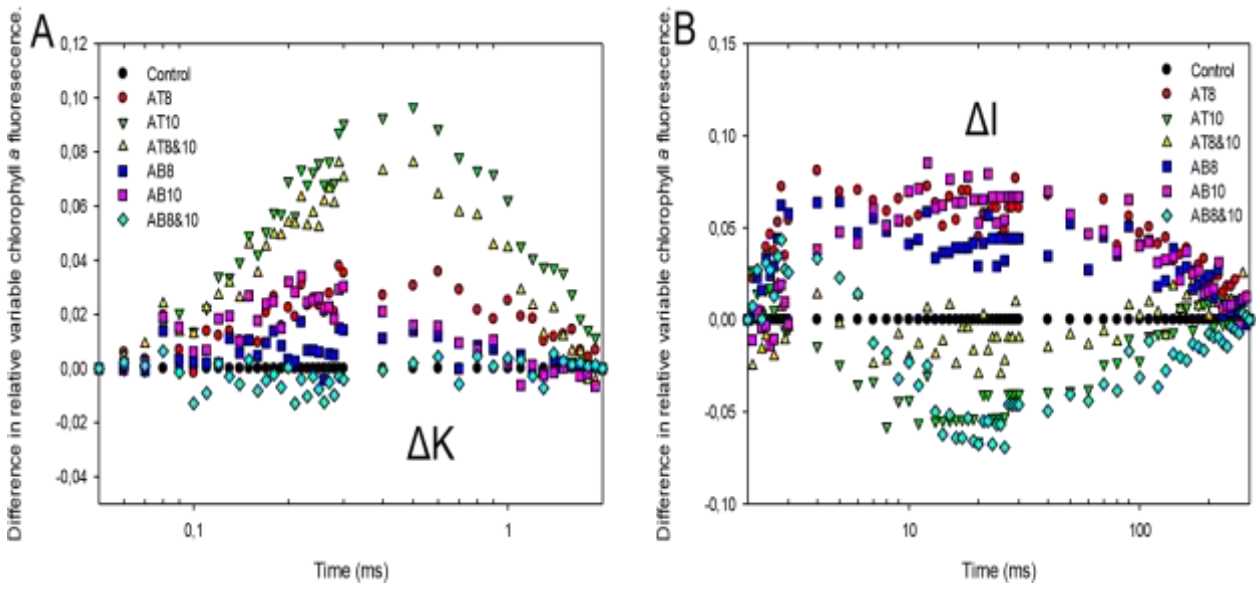


Figure 4-9: A-B: Difference in relative variable chlorophyll a fluorescence between 0.05 ms and 2 ms, and 2 ms and 300 ms 150 days after planting. Treatments are as indicated AT and AB implying treatment with Amistar Top® and Abacus® respectively and 8, 10 or 8 & 10 the application d date in weeks after planting.

4.4.1.4 The performance index and its partial parameters for PAN8816

By arranging the above transients into more quantifiable groups the following results are obtained. Figure 3-10 accordingly indicates the changes in the density of the reaction centres (A), trapping of electrons (B), dissipation of electrons (C) and reduction of end electron acceptors (D) over time. The density of the reaction centres remains constant over time for the control and other treatments. This implies that the ability of the plant to absorb light energy did not change. The effectiveness of trapping and dissipation of electrons decreases rapidly from 70 to 150 days after planting for all treatments. Over time the density of the reaction centres remained constant, no significant differences were observed. However, at 150 days after planting it becomes clear, that the number of active reaction centres are the higher in the control plants (Figure 4-10A). Decreases in trapping and dissipation of electron over time was observed, with increases in reactive oxygen species and coupled protein denaturing

(decreases enzyme complex function) by the photosystems (Figure 4-10B-C). In Figure 4-10D the results obtained for reduction probability of end electron acceptor showed to increase over time. This could be due to unregulated flow of electrons due to oxidative damage by senescence.

The general decline in the performance index over time (Figure 4-11) is expected as the photosynthetic efficiency of the plant will decline due to senescence. Although some treatments resulted in higher photosynthetic efficiency at 110 days after planting, the overall trend was a decline towards physiological maturity (having lower efficiency) at 150 days after planting. This is an indication that although some positive responses to fungicide application is observed at 110 days after planting for some of the treatments the effects are short lived and not quantifiable, as they are not observed at 150 days after planting, and thus will not be of aid during grain fill (±130 d.a.p).

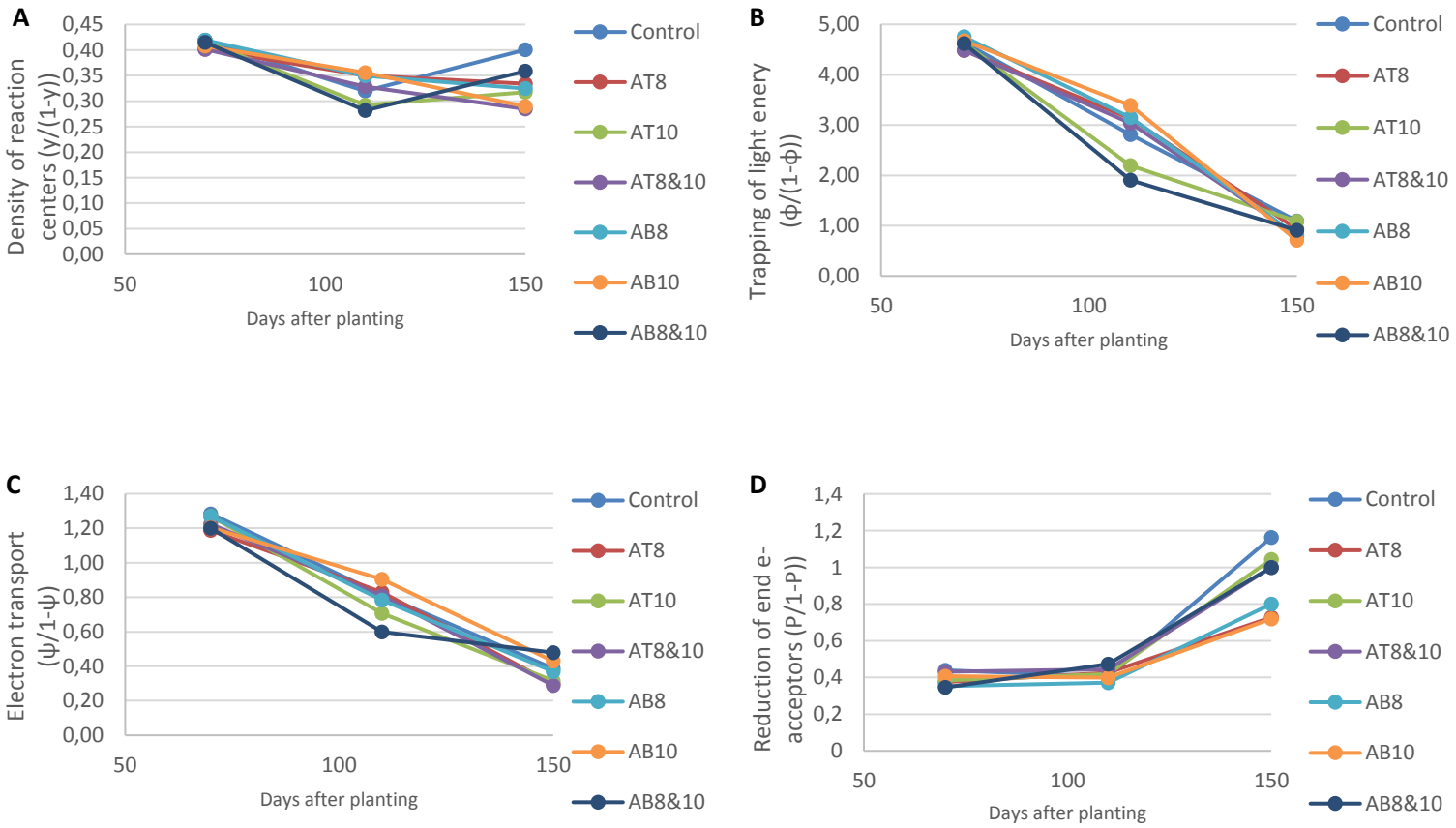


Figure 4-10: A) Absorbance, B) Trapping, C) Dissipation, and D) Probability of reduction of end electron acceptors as function of the total photosynthetic capacity of plants treated en untreated with fungicide as obtained for PAN8816.

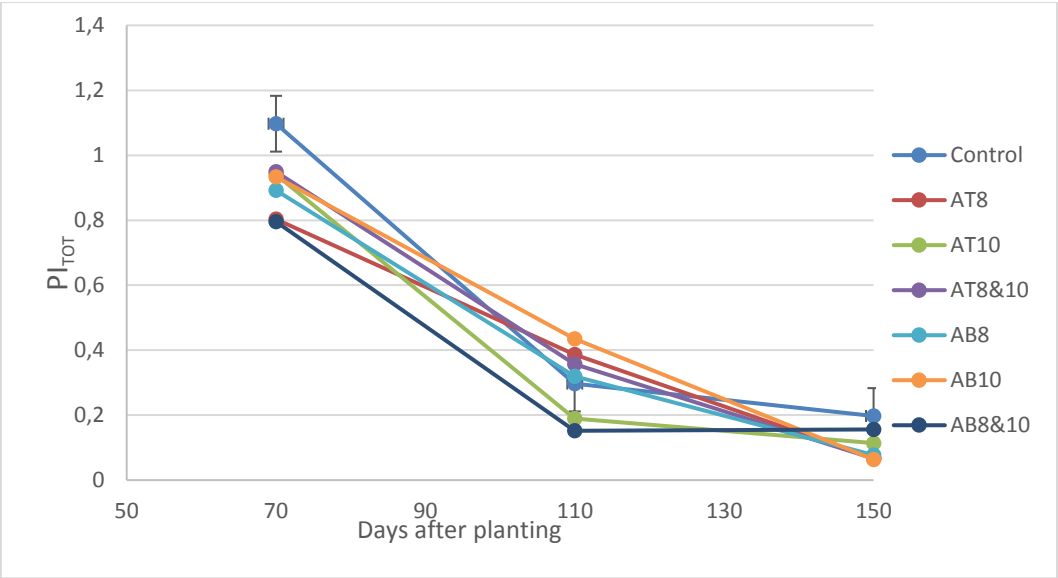


Figure 4-11: Performance index for PAN8816, obtained in the glasshouse trial from 70 to 150 days after planting.

Initially the control plant has the highest performance index at 70 days after planting, but hereafter a decline in the photosynthetic performance index is observed. At 110 days after planting AB 10, AT 8, and AT 8 & 10 shows a higher PI_{tot} and at 150 days the control plants had on average the best performance index.

4.4.1.5 OJIP and variable fluorescence transients obtained for the various treatments with NS5511 70 days after planting.

The OJIP curves obtained for chlorophyll *a* fluorescence of sorghum leaves (NS5511) when treated with two commercial fungicides at 70 d.a.p.

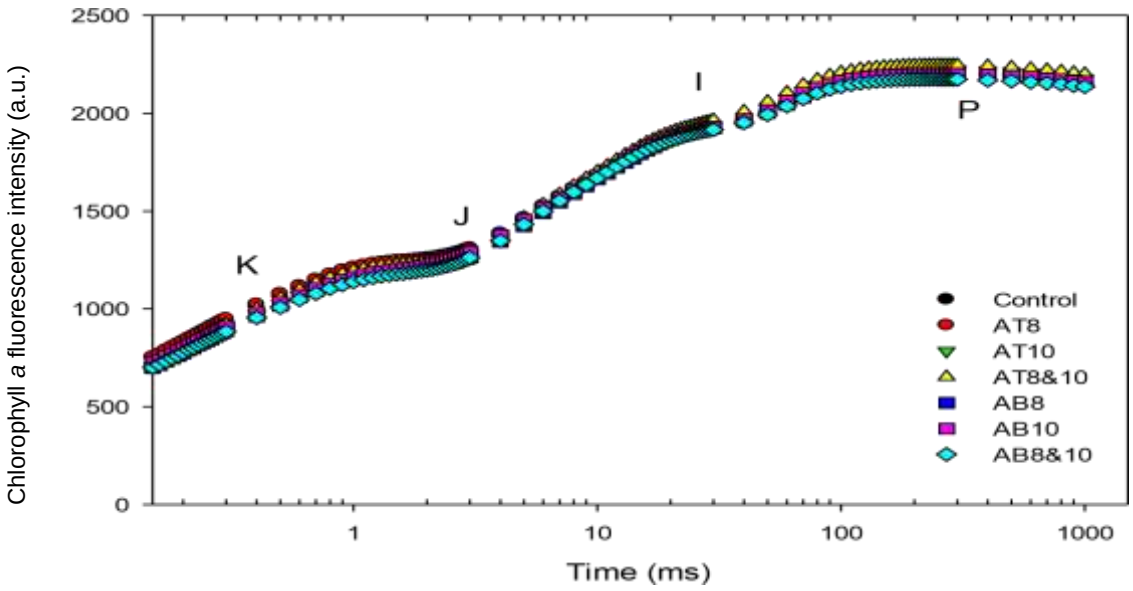


Figure 4-12: OJIP curve obtained for NS5511 70 days after planting. Treatments are as indicated AT and AB implying treatment with Amistar Top® and Abacus® respectively and 8, 10 or 8 & 10 the application date in weeks after planting

No significant differences were obtained after applying Shapiro-Wilks' paired t-test at each of the indicated time intervals (t=0.3, 2, 30, 300).

In Figure 4-14 an initial positive reaction is observed followed by a decrease in the photosynthetic efficiency of the plant at approximately 5 ms for all treatments. Only AT 8 seems to stray from the visible tendency of all other treatments. Stimulating effects for the splitting of water were obtained 70 days after planting for NS5511, except for AT 8 which showed a negative reaction on photosynthesis, specifically the oxygen evolving complex. ΔI-bands are observable for the Abacus® treatments, indicating ineffective reduction of the primary quinone acceptors, more specifically to 10 ms the reduction of Q_B with a second electron. However, a pair t-test analysis of the relative variable fluorescence for treatment vs control showed that no treatments had a significant effect compared to the control.

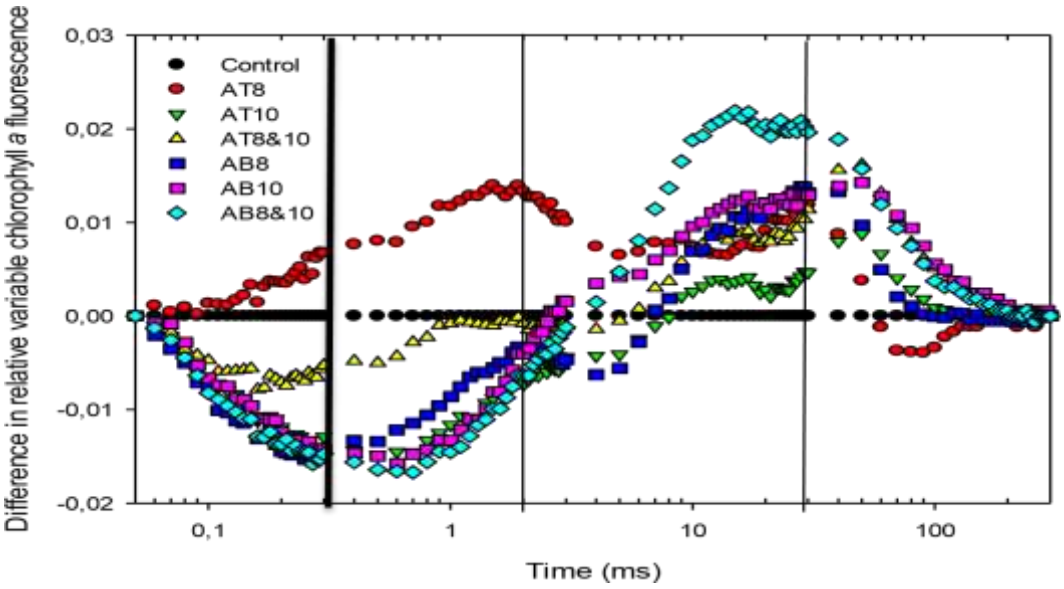


Figure 4-13: OJIP and variable fluorescence curves for NS5511 at 70 days after planting. Treatments are as indicated AT and AB implying treatment with Amistar Top® and Abacus® respectively and 8, 10 or 8 & 10 the application date in weeks after planting.

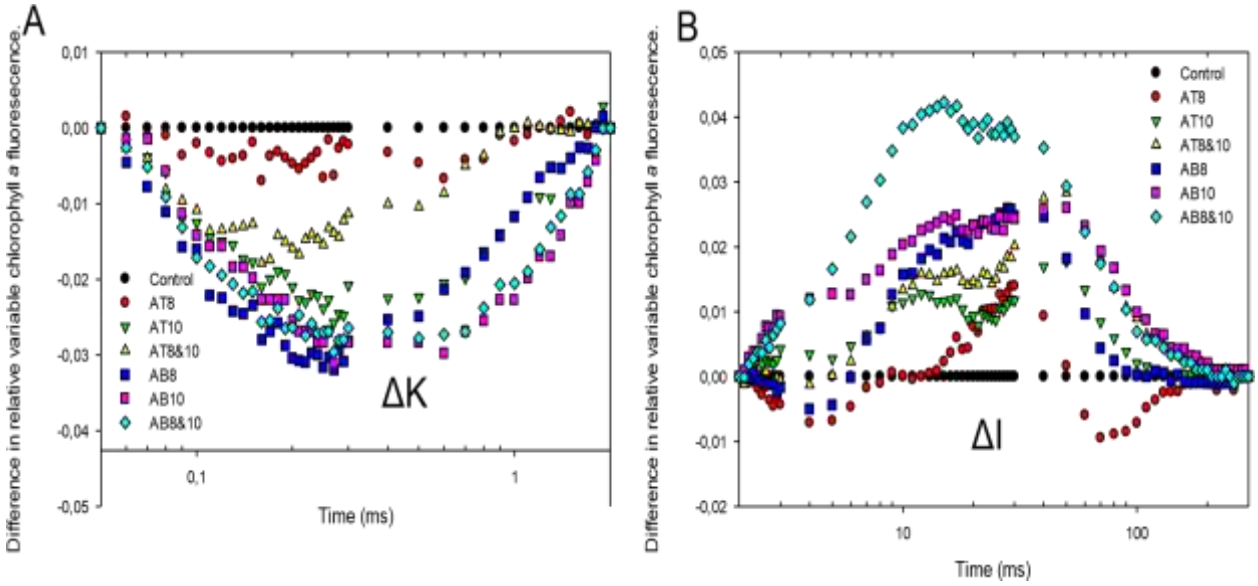


Figure 4-14: A-B: Difference is relative variable fluorescence for NS5511 70 days after planting normalized between 0.05 ms and 2 ms, and 2 ms and 300 ms respectively. Treatments are as indicated AT and AB implying treatment with Amistar Top® and Abacus® respectively and 8, 10 or 8 & 10 the application date in weeks after planting

4.4.1.6 OJIP and variable fluorescence transients obtained for the various treatments with NS5511 at 110 days after planting.

The OJIP curves obtained for chlorophyll *a* fluorescence of sorghum leaves (NS5511) when treated with two commercial fungicides at 110 d.a.p.

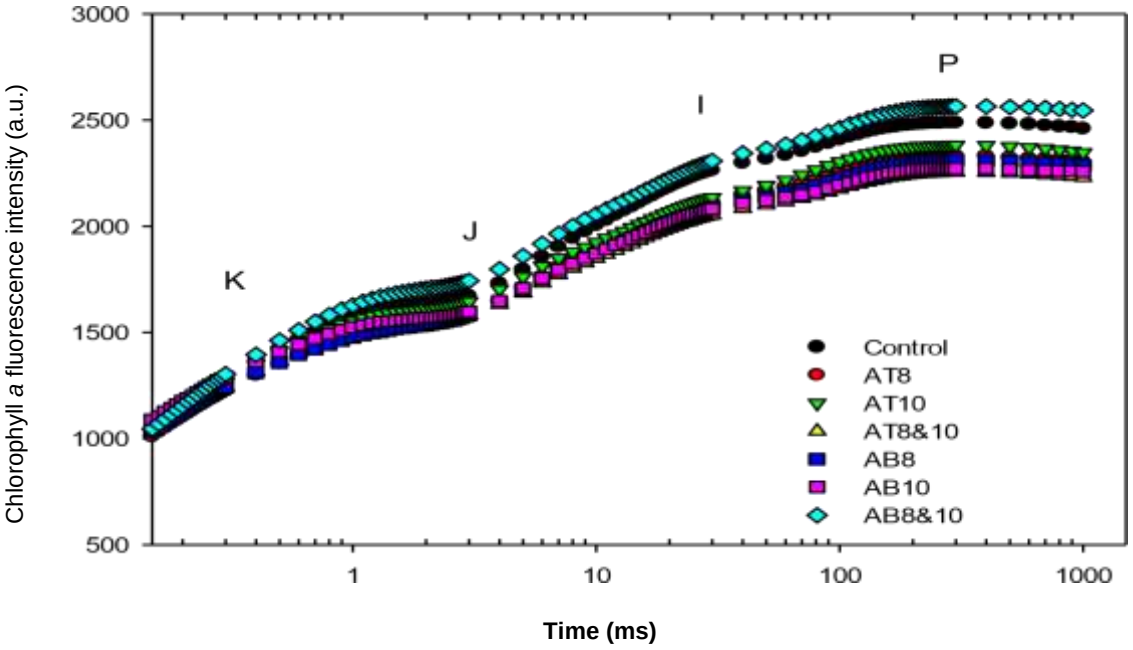


Figure 4-15: OJIP and variable fluorescence curves for NS5511 at 110 days after planting. Treatments are as indicated AT and AB implying treatment with Amistar Top® and Abacus® respectively and 8, 10 or 8 & 10 the application date in weeks after planting.

The bands visible in Figure 4-15 above are not of statistical significance according to Shapiro-Wilks' paired t-test; p values all larger than 0.05 were obtained.

At soft dough (110 d.a.p.) an initial negative effect due to fungicide application was observed, changing to a positive effect at 5 ms (Figure 4-17A). Here all treatments showed to have decreased effectivity of water splitting (ΔK-band) and damage to the OEC, with exception of AB 8 &10, which had a stimulating effect on the cleavage of water. Two of the treatments, AT 10 and AB 8 & 10 showed very effective oxidation and coupled reduction secondary quinone pools (ΔI) (Figure 4-17B). AT 10 and AB 8 & 10 also showed decreased electron transport

from the oxygen evolving complex to the Q_A acceptor site, with AT 10 showing reduced reduction of the secondary (Q_B) electron acceptors as well.

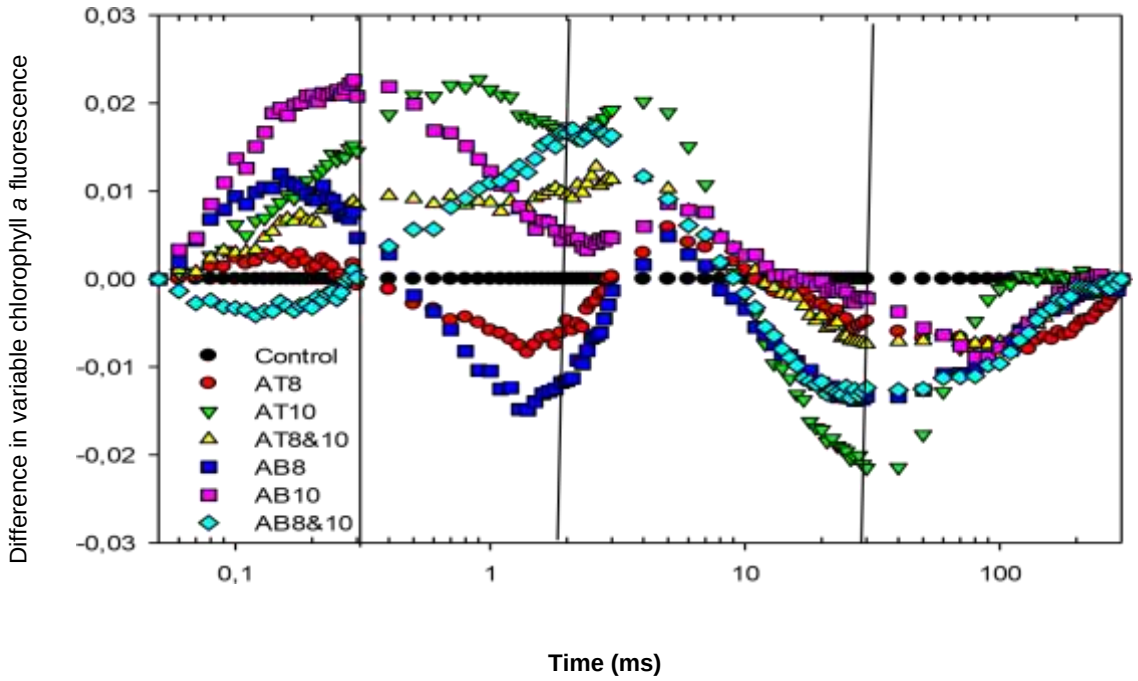


Figure 4-16: Relative variable fluorescence curves for NS5511 at 110 days after planting. Treatments are as indicated AT and AB implying treatment with Amistar Top® and Abacus® respectively and 8, 10 or 8 & 10 the application date in weeks after planting.

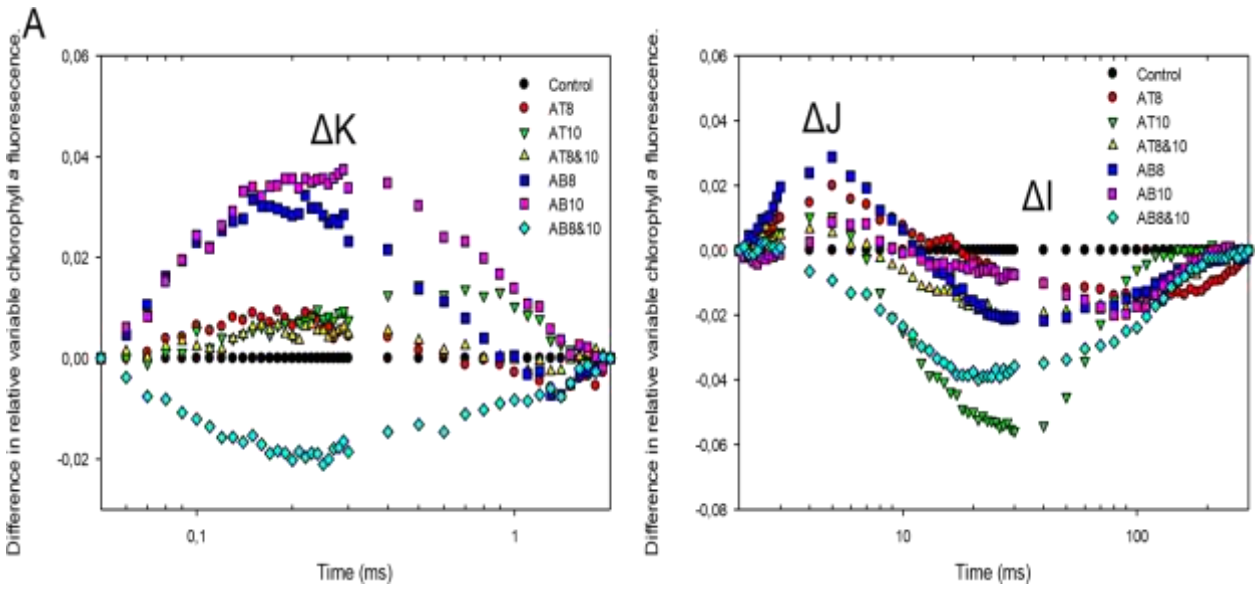


Figure 4-17: A-B: Difference is relative variable fluorescence for NS5511 110 days after planting normalized between 0.05 ms and 2 ms, and 2 ms and 300 ms respectively over the course of plant development.

4.4.1.7 OJIP and variable fluorescence transients obtained for the various treatments with NS5511 at 150 days after planting.

The OJIP curves obtained for chlorophyll *a* fluorescence of sorghum leaves (NS5511) when treated with two commercial fungicides at 150 d.a.p.

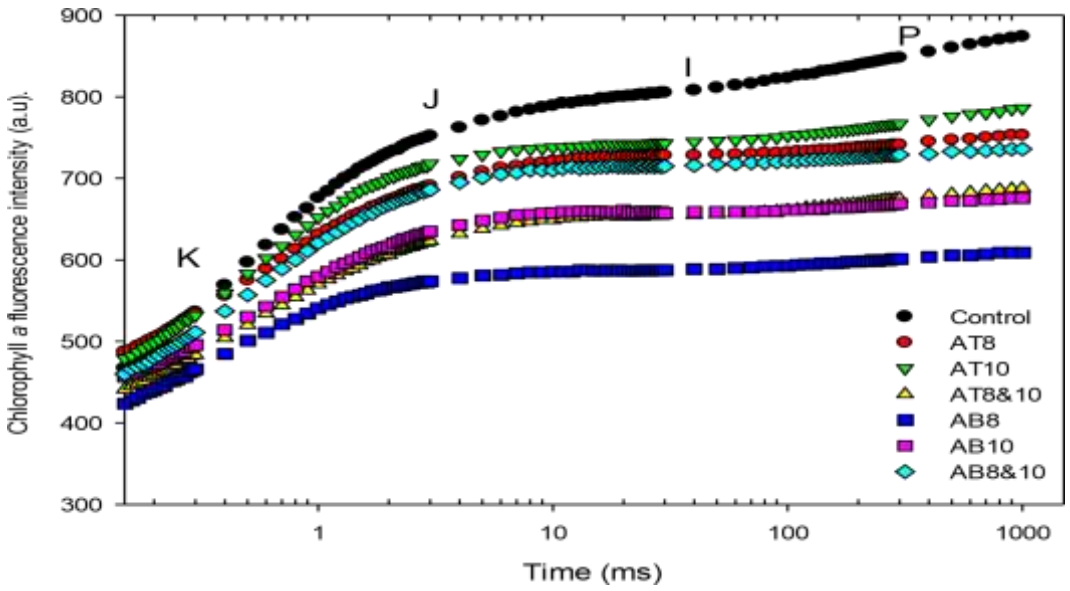


Figure 4-18: OJIP and variable fluorescence curves for NS5511 at 150 days after planting.

Positive ΔK and ΔJ bands observed in Figure 4-19 for relative variable fluorescence of NS5511 treatment vs. control showed to be statistically insignificant with p values all greater than 0.05. Amistar Top® treatment at 8, 10, 8 & 10 week after planting gave p=0.101, 0.243, and 0.202 respectively, with Abacus® resulting in p=0.107, 0.120, and 0.130 in the same order when compared to the control plants. These results obtained with the Shapiro-Wilks t-test indicate insignificant difference at the time intervals as indicated.

At physiological maturity ΔK bands (Figure 4-20A) are visible that indicate stimulatory effects of all treatments on the OEC. This is of course with exception of AB 8 that can be seen showing a decrease in the efficiency of H_2O splitting.

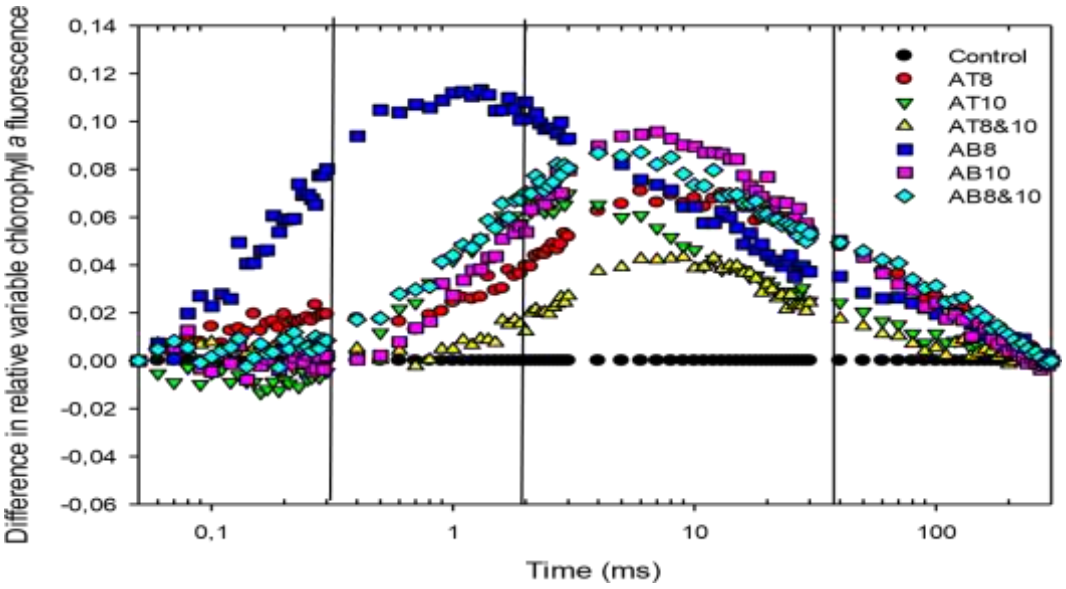


Figure 4-19: Relative variable fluorescence curves for NS5511 at 150 days after planting.

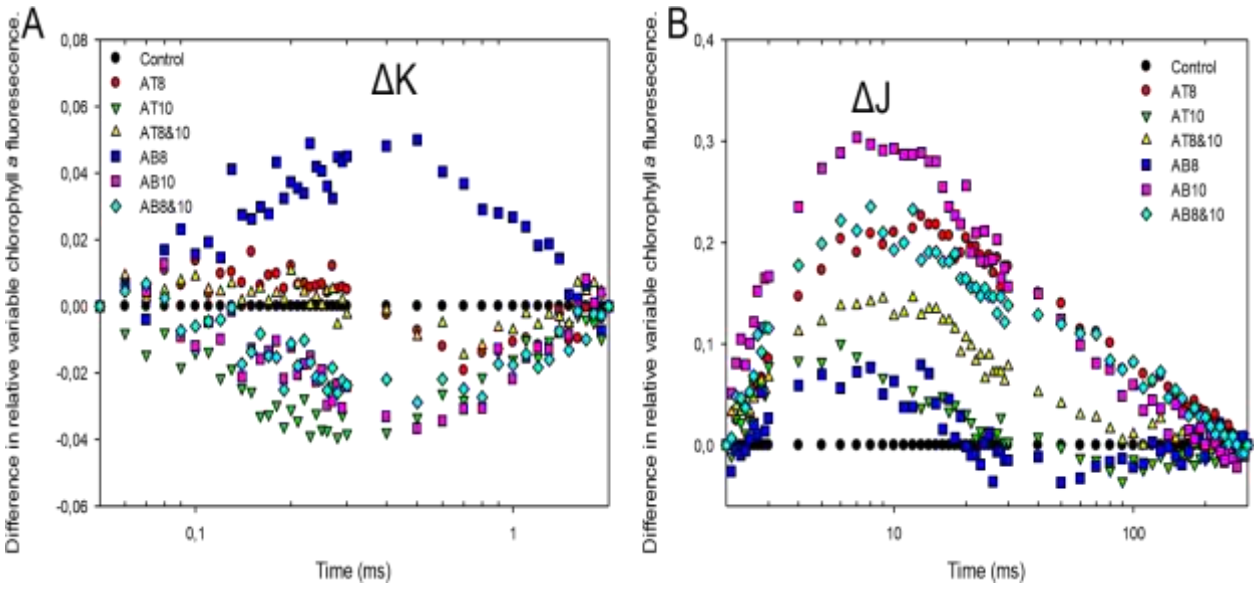


Figure 4-20: A-B: Difference is relative variable fluorescence for NS5511 150 days after planting normalized between 0.05 ms and 2 ms, and 2 ms and 300 ms respectively. Treatments are as indicated AT and AB implying treatment with Amistar Top® and Abacus® respectively and 8, 10 or 8 & 10 the application date in weeks after planting.

All treatments showed decreased the photosynthetic efficiency of the plants when compared to the control plant (Figure 4-20). These decreases indicate that fungicidal application adversely affected the plants ability to effectively transport electrons through the photosystems. Here (Figure 4-20B) ΔJ -bands (5 ms) are visible indicating ineffective primary reduction of the secondary quinone acceptors.

4.4.1.8 The performance index and its partial parameters for NS5511

Similar tendencies are observed for the partial parameters for NS5511 than in the case of PAN8816, indicating that these observations might be due to normal plant function, or decreases thereof over time. Reaction centres density, here, also remained constant, with the control being more effective than most treatments. Decreases in trapping and dissipation of electron are also observes with increases in reduction of the end acceptors (figure 22A-D).

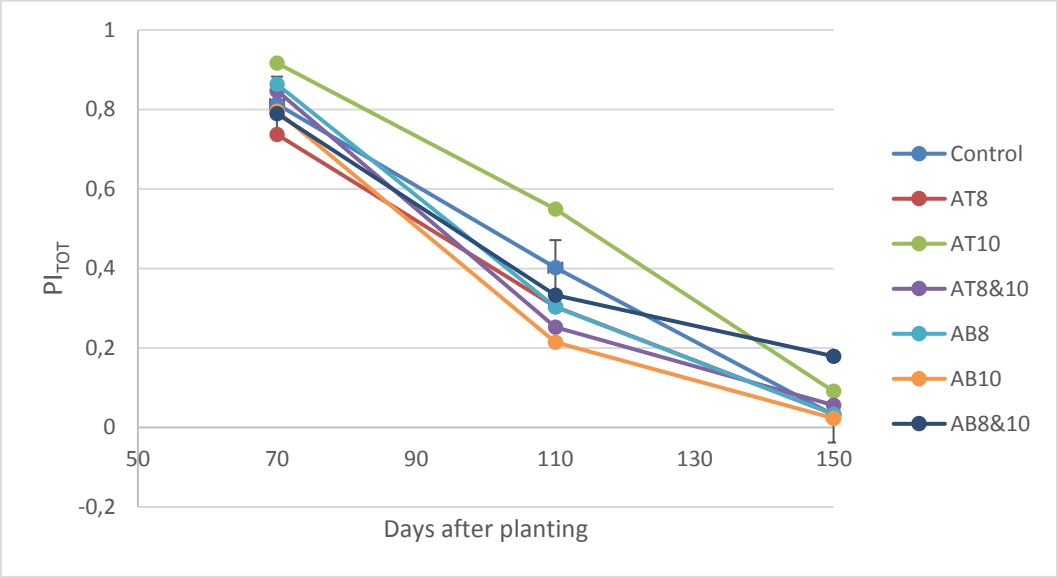


Figure 4-21: PI_{tot} summary for NS5511 as obtained by chlorophyll *a* fluorescence quantification.

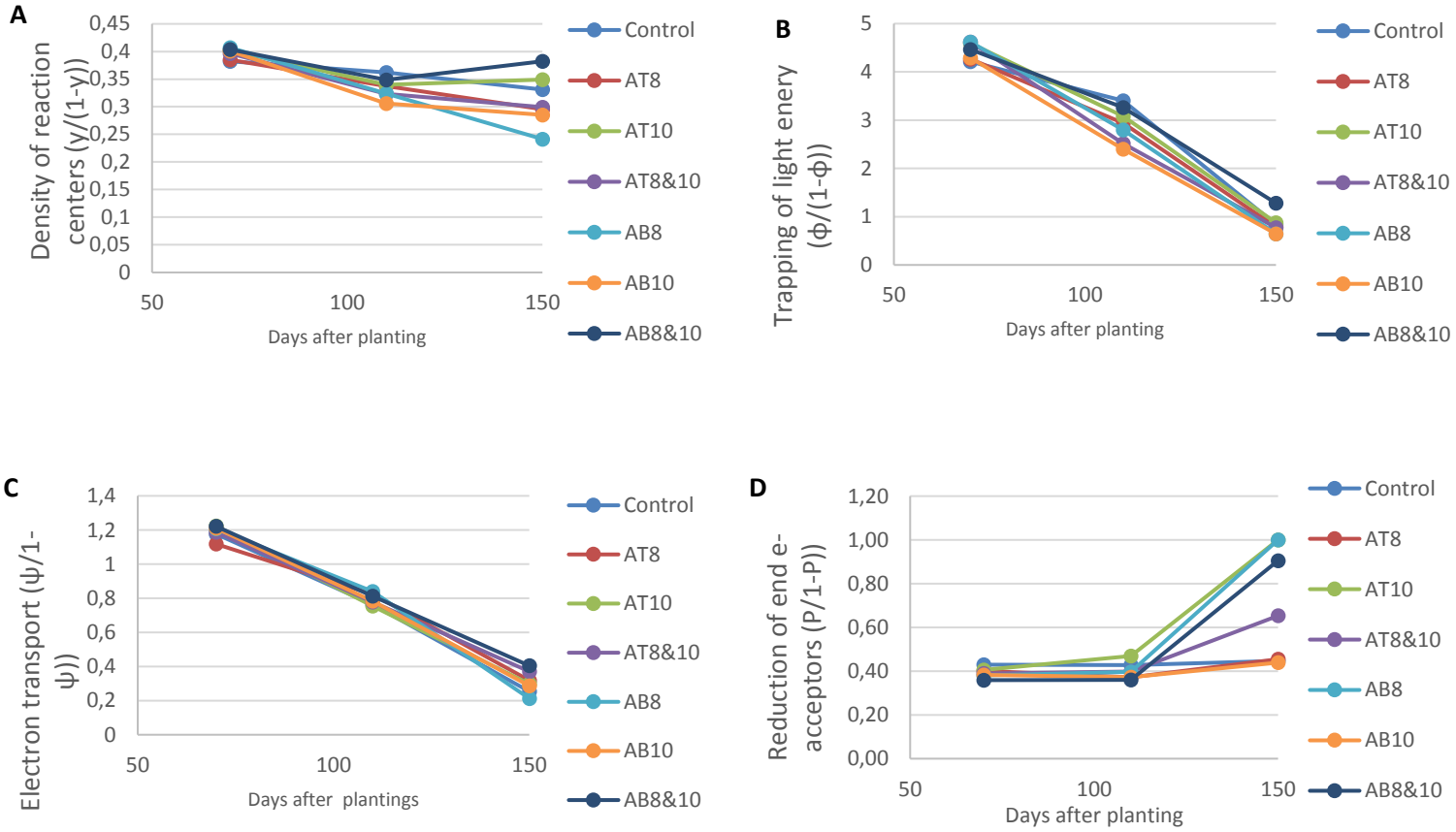


Figure 4-22: A) Absorbance, B) Trapping, C) Dissipation, and D) Probability of reduction of end electron acceptors as function of the total photosynthetic capacity of plants treated en untreated with fungicide as obtained for NS5511.

NS5511 showed to have clustered results for PI_{tot}, (Figure 4-21) AT 10 had the highest 70 days after planting, with AT 8 the lowest. Treatment with AB 8 & 10 had the best photosynthetic efficiency 110 days after planting and AB 10 the lowest, and the control plant showed the highest performance index 150 days after planting indicating, like PAN8816.

4.4.1.9 The performance index and photosynthetic capacity

Results obtained with the analysis of variance conducted performance index (PI_{TOT}) are shown in Table 4-2. Based on the analysis, only the sampling date significantly affected chlorophyll *a* fluorescence.

Table 4-2: Analysis of variance of the impact of selected fungicide treatments on the chlorophyll a fluorescence of two sorghum cultivars at various sampling dates (p=0.05).

Source	DF	S. S	M.S	F Value	Pr > F
Treatment	6	0.08499	0.01417	0.45	0.842
Cultivar	1	0.02278	0.02278	0.72	0.399
Sampling	2	21.3521	0.67608	83.30	**0.0001**
Treatment x Cultivar	6	0.04436	0.00739	0.23	0.963
Treatment x Sampling	12	0.35262	0.02939	0.78	0.670
Cultivar x Sampling	2	0.03349	0.01675	0.44	0.642
Treatment x Cultivar x Sampling	12	0.47058	0.03922	1.04	0.417

The highest performance index (PI_{tot}) is observed at 70 days after planting. As time lapses, the photosynthetic efficiency significantly reduces to 110 days after planting and again to the last sampling date, 150 days after planting (Figure 3-23). The performance index has long shown to be very well correlated with the photosynthetic capacity of a plant (Zivcak *et al*., 2014)

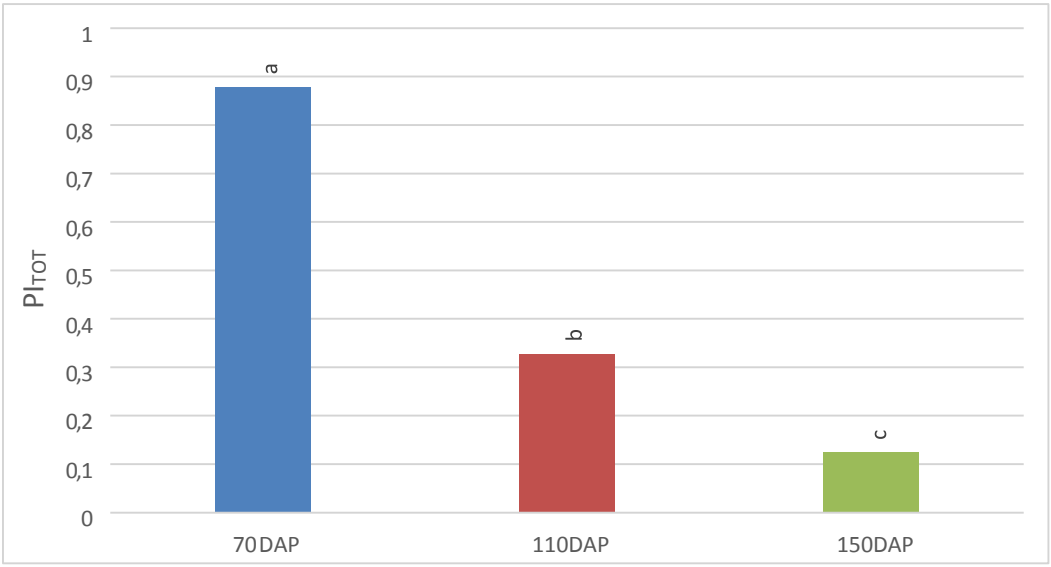


Figure 4-23: The PI_{TOT} values for two sorghum cultivars obtained at various sampling dates under glasshouse conditions.

The application of fungicide did not have any effect under controlled glasshouse conditions on the performance index of the plants. Based on the findings of the current study, only sampling time (i.e. as the plant develops) had a significant effect on the performance index of the plant. This is to be expected as the normal senescence progression will most likely cause damage to the photosynthetic apparatus of the plants.

4.4.2 Enzymatic responses of two sorghum cultivars to fungicide application at various time intervals under glasshouse conditions

According to Zhang *et al.* (2010) fungicides have anti-oxidant properties. To evaluate this statement some enzymatic responses were studied to determine the effect of prophylactic fungicide application on anti-oxidant mechanisms of the plants. Table 4-3 shows the results obtained from the ANOVA conducted for enyme responses to fungicide application

Table 4-3: Analysis of variance of the impact on selected fungicide treatments on POD, SOD and XOX activity of two sorghum cultivars at various sampling dates (p=0.05).

Source	DF	POD				SOD				XOX			
		SS	MS	F	F pr.	SS	MS	F	F pr.	SS	MS	F	F pr.
Treatment	6	6.3E-11	1.1E-11	17.07	<.001	804.97	134.16	79.71	<.001	3.3E-03	5.4E-04	8.91	<.001
Cultivar	1	5.2E-12	5.2E-12	8.35	0.006	13.52	13.515	8.03	0.007	1.0E-04	1.0E-04	1.72	0.196
Treatment x Cultivar	6	3.4E-11	5.7E-12	9.21	<.001	458.44	76.407	45.40	<.001	1.8E-03	3.0E-04	4.97	<.001
Residual	52	3.2E-11	6.2E-13	1.04		87.52	1.683	0.69		3.2E-03	6.1E-05	7.64	
Sampling	2	2.5E-10	1.3E-10	213.3	<.001	234.35	117.18	48.19	<.001	3.9E-04	1.9E-04	24.91	<.001
Treatment x Sampling	12	1.9E-10	1.7E-11	28.14	<.001	299.03	24.919	10.25	<.001	2.6E-04	2.1E-05	2.72	0.003
Cultivar x Sampling	2	2.2E-13	1.1E-13	0.19	0.828	16.09	8.045	3.31	0.040	3.4E-06	1.7E-06	0.21	0.808
Treatment x Cultivar x Sampling	12	3.7E-11	3.2E-12	5.18	<.001	421.86	35.155	14.46	<.001	2.0E-04	1.7E-05	2.12	0.021
Residual	112	6.6E-11	5.9E-13			272.3	2.432			8.9E-04	7.9E-06		
Total	209	6.9E-10				2622.5				1.0E-02			

4.4.2.1 POD activity

Seventy days after planting significantly lower POD activity was observed for treatments AB 8 & 10-PAN8816/NS5511, AT 10-PAN8816/NS5511 AB 10-NS5511, AB 8-NS5511, and AT 8 & 10-NS5511when compared to the corresponding control (Figure 4-24). This could indicate a level of phytotoxicity, as discussed by Zhang *et al.* (2010) decreases in POD activity in cucumber after fungicide application are possible, as fungicides were applied earlier that same day. AT 8-PAN8816 is the only treatment which resulted in significantly higher POD activity as measured at 70 d.a.p. compared to the corresponding control, all remaining treatment showed similar POD activity compared to the control plants.

No significant differences were obtained between treatments and the respective controls for POD activity 110 d.a.p. There were also no differences observed between the two respective cultivars. POD activity was significantly lower 110 d.a.p compared to the other two sampling dates.

At physiological maturity (150 d.a.p) the increase in POD activity was much more noticeable. POD activity significantly increased when the respective cultivars were treated with PAN8816-AT 8, AB 8, AB 10 and AB 8 & 10. Treatment with AB resulted in significant

increases across the board with NS5511 as well as AT 8 & 10. Abacus® was more effective in increasing POD activity for both cultivars.

4.4.2.2 SOD activity

SOD activity was significantly higher 70 d.a.p. compared to 110 and 150 d.a.p (Figure 4-25). AT 8 application resulted in significant increases in SOD activity in both cultivars compared to the controls for the 70 d.a.p sampling. Abacus® similarly resulted in increased activity, but in only one of the cultivars (NS5511). NS5511 was similarly also affected by Abacus® and Amistar Top® applications at both the 10 weeks after planting and 8 & 10 weeks after planting. PAN8816 was less affected with only the AB 10 weeks increasing SOD activity.

SOD activity at 110 days after planting for NS5511 was not significantly (Table 4-3) affected by any of the treatments at this sampling. PAN8816 was, however, highly responsive to the various treatments, as all resulted in significant higher SOD activity compared to the control.

Similar to what was observed at 110 days after planting, PAN8816 was again significantly affected by all the treatments. The application of Amistar Top® also resulted in significant higher SOD in NS5511 while only AB 8 & 10 increased SOD. Most of the treatments resulted in higher SOD activity compared to the respective controls.

SOD activity of treated plants remained significantly higher in most cases, at all sampling dates except for certain outliers, these results are in accordance with studies by Venancio *et al.* (2003), Zhang *et al.* (2000, 2007 and 2010) and Jaleel *et al.* (2006). These studies were, however, only focused on single sampling dates and not coupled to developmental stages of the plants.

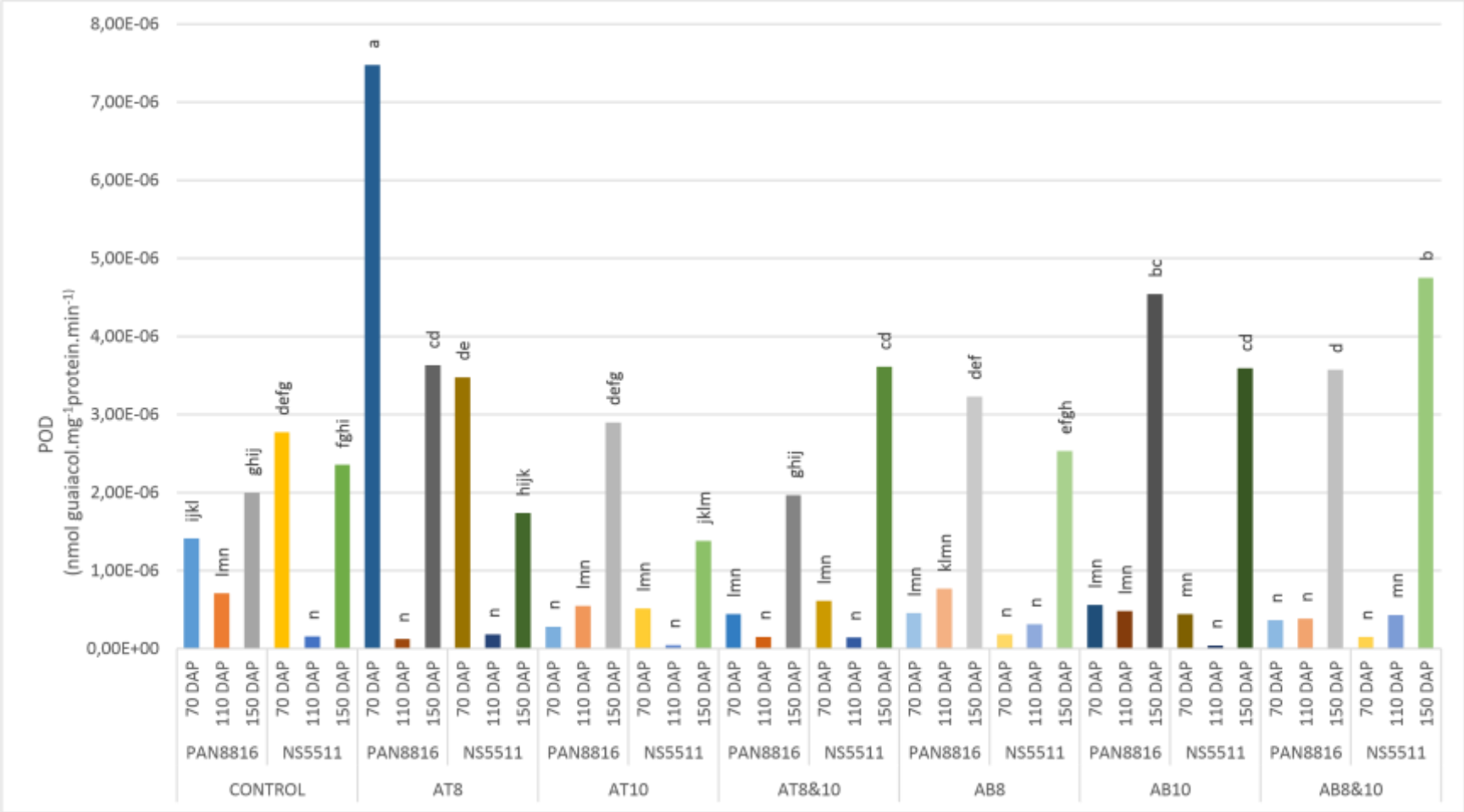


Figure 4-24: The effect of fungicides application on POD activity of two sorghum cultivars as measured at three different sampling dates.

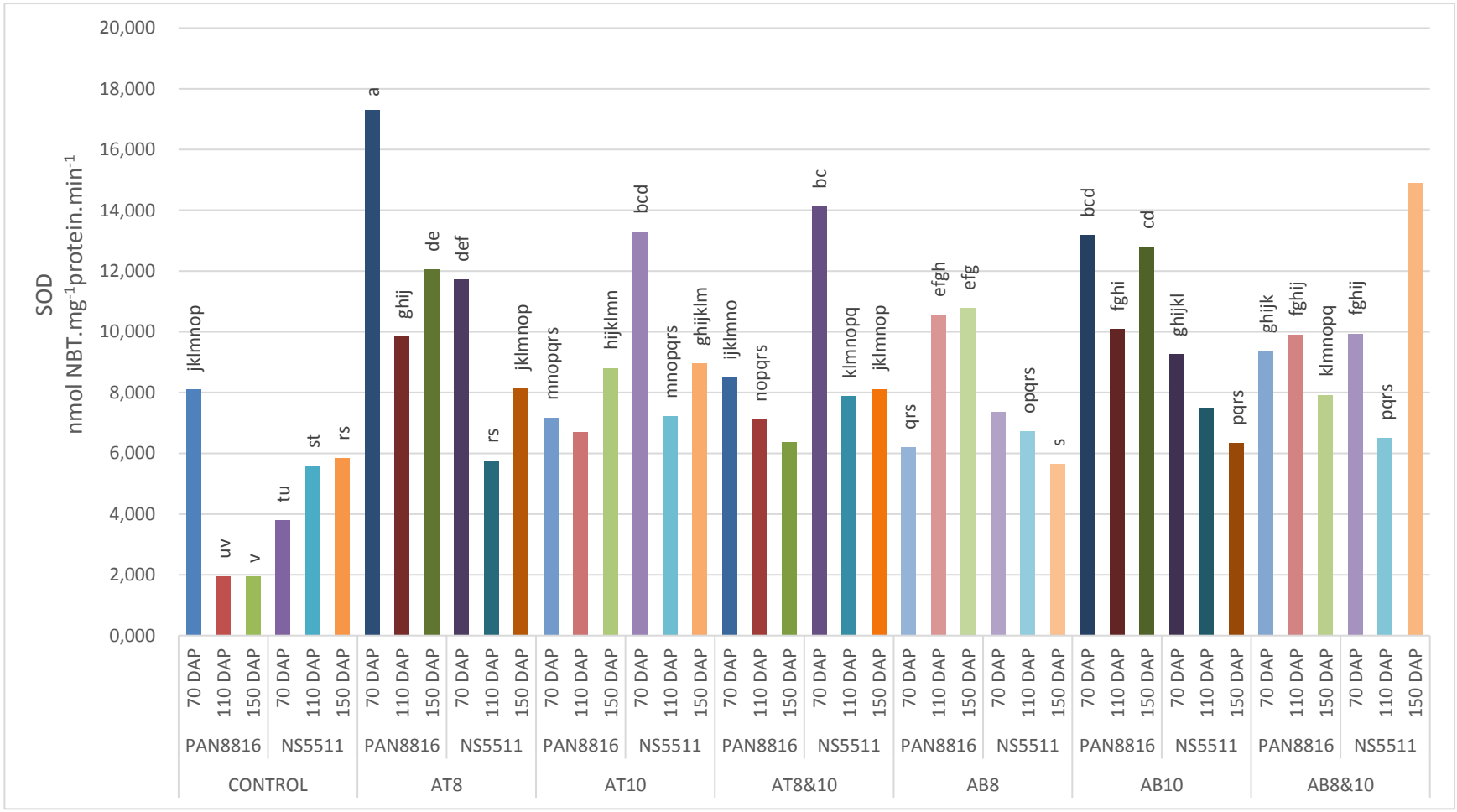


Figure 4-25: The effect of fungicides application on SOD activity of two sorghum cultivars as measured at three different sampling dates.

In general, AB 10-PAN8816 demonstrated to have a stimulating effect over all three sampling dates, indicating that it may be most effective in combating oxidative stress in the plants, but this is a broad setting, as this study was done in a controlled environment and various sampling dates were chosen in accordance with sorghum development.

4.4.2.3 XOX activity

XOX activity was significantly reduced with AT 10 and AB 10 application on PAN8816 compared to the control. Abacus® had a similar effect on NS5511 at this application date, whereas Amistar Top® significantly increased XOX at 10 weeks after planting. AB 8 & 10 significantly reduced XOX in both cultivars. The least responsive reaction was obtained with the 8 weeks after planting for both fungicides and cultivars (Figures 4-27).

A similar plot of data was obtained for the 110 days after planting sampling compared to the 70 days after planting plot. AB 8 increased XOX activity in, while AB1 0 reduced XOX activity in NS5511. The 10 weeks after planting application of both Abacus® and Amistar Top® did not show significant differences in PAN8816. Only AT 8 & 10 gave a significant response, in that it increased the XOX activity in PAN8816.

A similar plot of data was again obtained for the 150 d.a.p sampling compared to the 70 and 110 d.a.p plot (Figure 4-26). Amistar Top® and Abacus® both gave similar responses in XOX activity in PAN8816 with all three application times, whilst the two fungicides gave opposite reactions with NS5511 at especially the 10 weeks after planting application. The AT 8 and AB 8 application XOX compared to the control in PAN8816. When applied at 10 and 8 & 10 weeks after planting the same chemicals had no effect on PAN8816. AB 10 was the only treatment yielding significant lower XOX activities in NS5511.

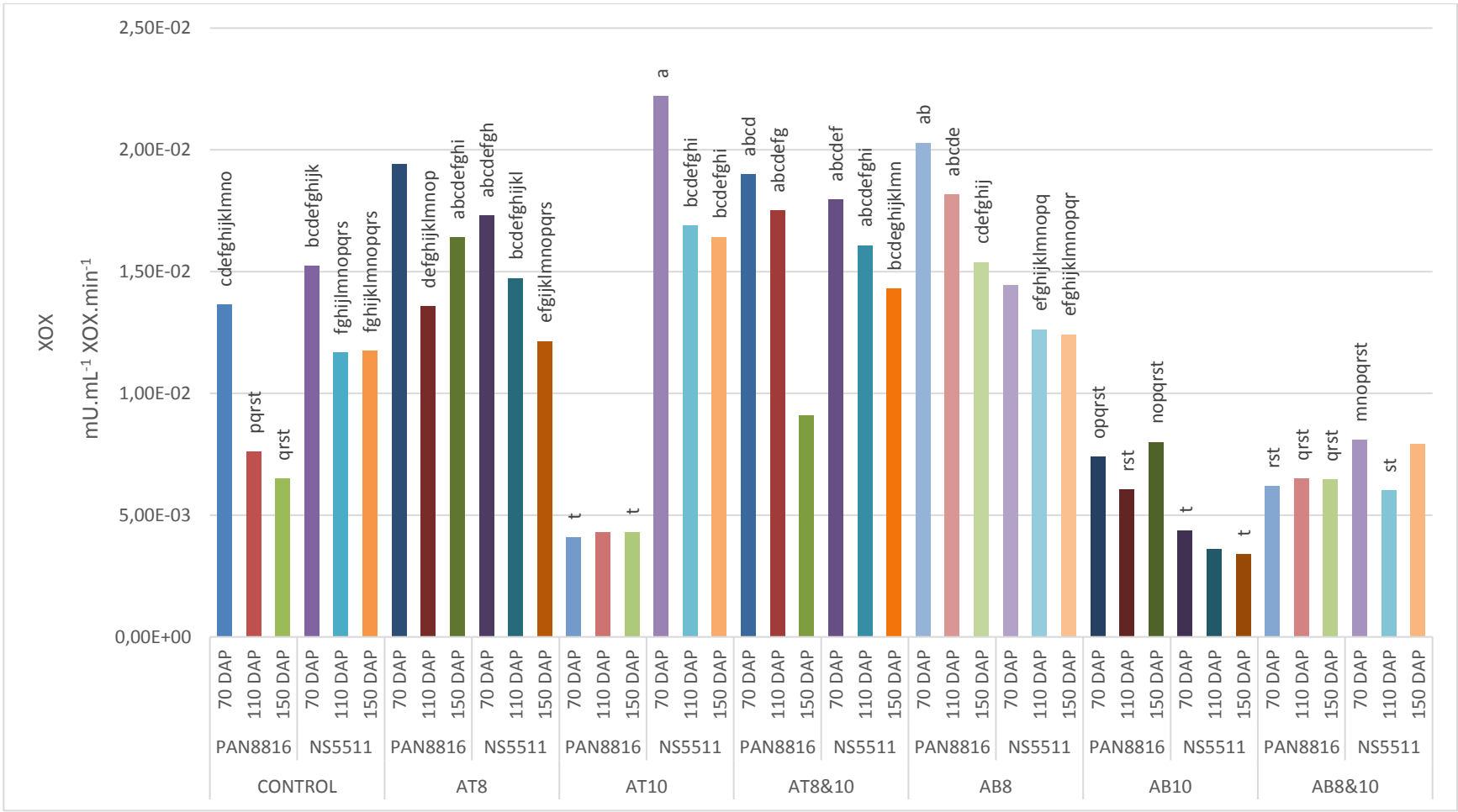


Figure 4-26: The effect of fungicides application on XOX activity of two sorghum cultivars as measured at three different sampling dates.

XOX activity was lowest at all three sampling dates for AB 10-NS5511 (Figure 4-26), indicating it may be most effective in preventing the generation of ROS. Single sampling and analysis by Zhang *et al.* (2010) showed that fungicides should decrease XOX activity. However, this was only a pure fungicide, and not blends as used in this study.

4.4.3 Changes in sucrose concentration and black layer formation in seeds of *Sorghum bicolor* due to fungicidal treatment under glasshouse conditions

Table 4-4: Analysis of variance for the effect of fungicide application on sucrose content and black layer size of seeds of two sorghum cultivars (p=0.05).

Source	DF	SUCROSE				AREA			
		SS	MS	F	F pr.	SS	MS	F	F pr.
Treatment	6	1,595	0,266	1348,71	0.001	7,97E+11	1,33E+11	2.17	0.060
Cultivar	1	0,001	0,001	5,77	0.003	7,46E+08	7,46E+08	0.01	0.912
Treatment x Cultivar	6	0,285	0,048	241,29	0.001	1,80E+12	2,99E+11	4.90	0.001

4.4.3.1 Sucrose Concentration

Analysis of variation for sugar concentration (Table 4-4) indicated that the sucrose concentration was significantly ($P<0.05$) affected by the fungicide application-by-cultivar interaction. All PAN8816 plants treated with fungicide showed a higher sucrose concentration as opposed to the control (Figure 4-27). Three of the six treatments showed increases in sucrose concentration with NS5511. AB 8 & 10 resulted in a reduction in sucrose concentration, whilst AT 8 & 10 as well as AB 10 had sucrose concentrations similar to the control with NS5511.

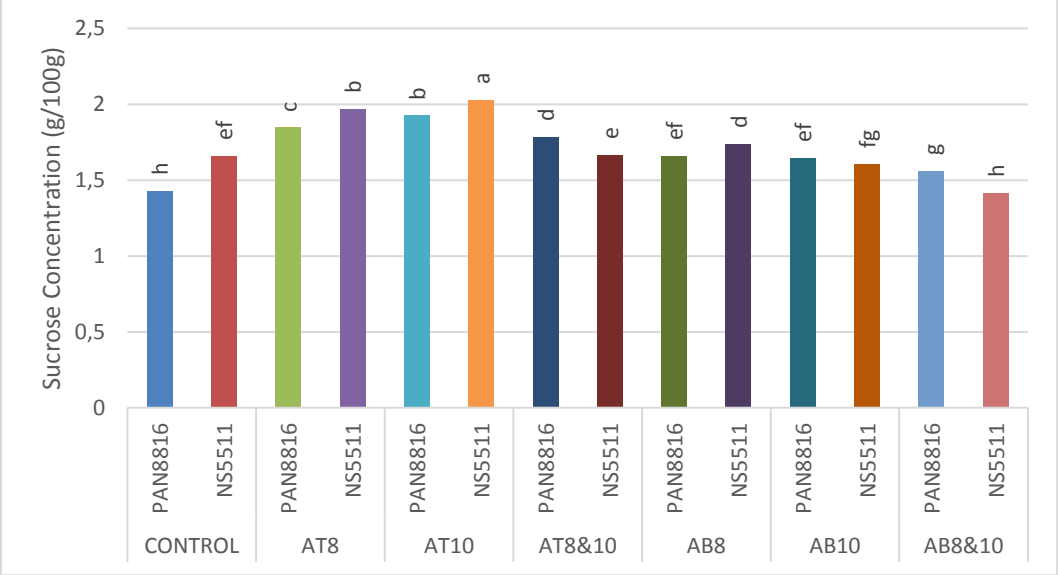


Figure 4-27: Sucrose concentration of sorghum seeds 150 days after planting.

4.4.3.2 Black layer observation

As the black layer observations were done at physiological maturity (stage 9), all the seeds are classified as being at stage 1 as described by Stichler and Livingston (2012) (Figure 4-28).

Some examples of the black layer (hilum) of the sorghum seeds under a stereomicroscope can be seen in Figure 4-29.



Figure 4-28: Method of describing the formation of the black layer (indicated with the red arrow) in sorghum seeds 1 being fully formed and 5 being a young seed with no clear black layer (Pioneer, 2015).



Figure 4-29: Examples of black layer in sorghum seed as seen under a stereomicroscope.

The impact of the selected fungicide treatments on the observed black layer are indicated in Figure 4-30.

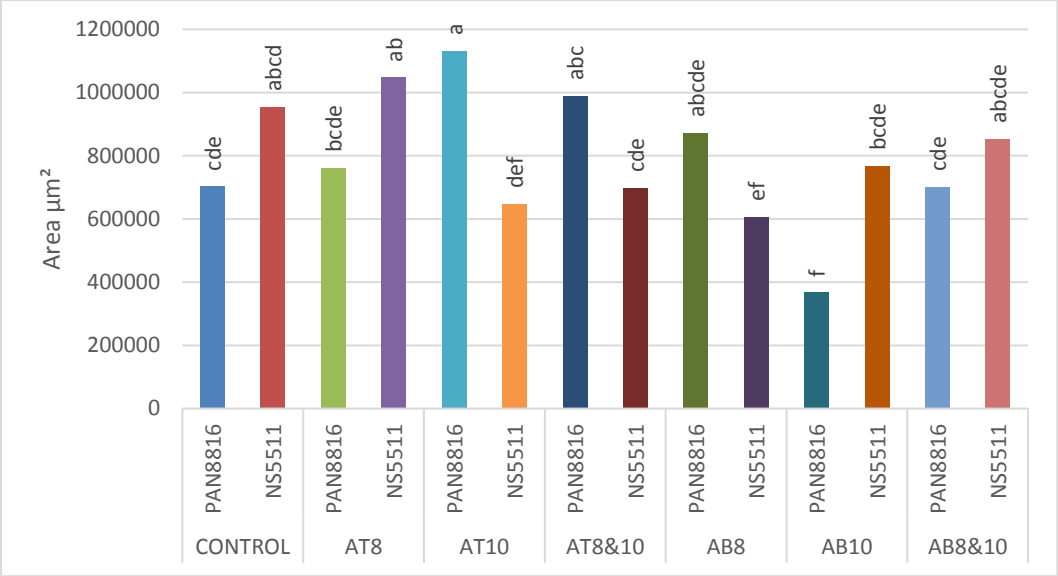


Figure 4-30: Significant differences measure with the area of the black layers in the seeds associated with different fungicide application as obtained from the glasshouse trial.

Significant differences were obtained with AB 10-PAN8816 and AB 8-NS5511 showing significantly smaller black areas in the seed (Figure 4-30). AT 10–PAN8816 showed a significantly larger black area when compared to the respective control. The remainder of the treatments all had had similar black layer areas to that of the respective controls.

4.4.3.3 Correlations analysis

If the assumption is made that the black layer functions as a sort of barrier which eventually ends up blocking the transportation of sugars to the kernel, it stands to reason that should the formation of such a layer be delayed, larger amounts of sugars will eventually end up in such kernels. To test this theory, correlation analysis was conducted in order to establish to what extend the black layer area which was measured at 150 days after planting correlated with the sucrose concentration observed at this date.

Correlation analysis indicated a significant but weak to moderate correlation between the black layer area and the sucrose concentration observed in PAN8816 (r=0.416; p=0.128) (Rayner, 1969). No correlation was observed for NS5511 (r=0.0074; p=0.967) between the two parameters measured.

4.5 Discussion and Conclusions

Swoboda and Pedersen (2009), reported a decrease in photosynthesis with strobilurin application in wheat. According to Bertelsen *et al.* (2001) the entire principal behind increased yield due to fungicide application is because of the delayed senescence. In the field study, a delay in senescence was observed, specifically at Greytown during for the duration of the 2013/14, as well as the first observation (s1) during 2014/15, but this was not accompanied by significant increases in yield over the same growing season. There was also no correlation between delayed senescence and yield. Theoretically, delayed senescence could lead to longer periods of greenness, increased green leaf material, increased time of photosynthesis, thus, theoretically, higher carbon fixation and energy generation through photosynthesis and increased sugar accumulation and thus yield.

Both the fungicides selected with the current study comprised of blends of active ingredients belonging to the triazole and strobilurin groups. A literature review by Petit *et al.* (2012) on the effects of fungicides on crop found that strobilurin delayed the increase of reactive oxygen species, but had no effect on chlorophyll content or photosynthesis. Triazoles, however, did increase cytokinin levels which will theoretically aid in increasing the following: chlorophyll content, accelerated chloroplast differentiation, and protect the integrity of chlorophyll. Negative effects reported include that triazoles retards the growth of clevers and clearly reduces the evolution of oxygen (Petit *et al.* 2012).

Literature around the subject indicated both positive and negative effects for both the fungicides used and the crop involved. It can thus be concluded that the blend of fungicides and crop will determine the effect of fungicide on chlorophyll *a* fluorescence on plants as discussed. The only clear conclusion to be drawn from this study is that the photosynthetic efficiency of plants deteriorates as plants senesce as no significant differences in chlorphyll a fluorescence were observed with regard to fungicide application.

POD levels showed to significantly decrease with AB 8, 10, and 8 & 10 application compared to the corresponding control. No significant differences for observed for 110 days after planting sampling, which is again contradictory to what was expected.

A study conducted by Zhang *et al.* (2007) showed that decreases in the POD activity of leaf tissue is possible after fungicide application on cucumber. These results are uncommon and contradict the increases in POD activity found in the study on the effects of fungicide application on *Catharanthus roseus.* The latter study however, used a pure triazole fungicide and not a blended fungicide (triazole and strobilurin) (Jaleel *et al.*, 2006). Significant increases, similar to what has been described by Jaleel *et al.* (2006) and Zhang *et al.* (2010) could be observed at physiological maturity with PAN8816 and some treatments, indicating that the stay green effect may only be of an anti-oxidative nature at a later developmental stage. A generally lower POD activity is observed at this sampling period, compared to the 70 and 110 days after planting periods. This observation is contradictory as the anti-oxidant effect of fungicides was hypothesized to increase POD activity (Zhang *et al.* 2010). Sudden changes in physiology due to fungicide application may lead to temporary decreases, as the samples were taken immediately after application. Triazoles have been associated with levels of phytotoxicity as discussed in a review by Petit *et al.* (2012), which may explain why the 8 week treatments were not affected but 10 weeks were, as plants had time to recover from the initial shock of the treatment.

In most cases, the POD activity decreases from 70 to 110 days after planting and increases again at maturity at 150 days after planting. This could be due to differentiation of different cell functions for reproduction and the subsequent increase due to natural senescence at 150 days after planting.

The application of AT- and AB 8 weeks after planting on PAN8816 had significantly higher POD activity compared to the respective controls. A similar effect could not be observed for NS5511 at 8 weeks after planting. The application of AB resulted in significantly increase POD activity in PAN8816 when applied at 10 weeks after planting as well as 8 & 10 weeks after planting. With NS5511, the application of AB 10 after planting and 8 & 10 weeks after planting resulted in increased activity. AT only resulted in significantly higher POD activity in this cultivar at 150 days after planting when applied at 8 & 10 weeks after planting.

POD activity was significantly higher in PAN8816 plants treated with AT 8, AB 10 and AB 8 & 10 compared to the control. The only response observed for NS5511 was an increase observed with AB 8 & 10. At physiological maturity, significant increases in POD activity is in correlation with the study by Zhang *et al.* (2010) which indicates that fungicide application will increase the POD activity, but the responses are only limited to one cultivar.

The results obtained suggest that the effects of fungicide application on POD can only clearly be observed in cultivars with known “stay-green” genes. The is in correspondence with results obtained in the field trial were NS5511 was one of the cultivars with the most significant decreases in the rate of senescence; and may be contributed to increases in POD activity at maturity.

According to studies by Venancio *et al.* (2003), Zhang *et al.* (2000, 2007 and 2010) and Jaleel *et al.* (2006) regarding the anti-oxidative nature of the fungicides, such applications should result in an increase in antioxidative enzymes’ activity. Zhang and Schmidt (2000) reported significant increases in SOD activity when bentgrass was treated with propiconazole.

Similarly, Zhang *et al.* 2007 and 2010 reported increases in SOD activity when cucumber and wheat was treated with triazoles and strobilurins respectively. Jaleel *et al.* (2006), likewise reported increases in the anti-oxidative mechanisms of the plants for various varieties of rose plants.

The results obtained with PAN8816 is again contradictory with studies by Venancio *et al.* (2003), Zhang *et al.* (2000, 2007 and 2010) and Jaleel *et al.* (2006). These studies focussed on a single sampling date after fungicide application and observed increases in SOD activity. NS5511 showed to be seemingly unaffected by fungicide application when SOD activity was studied.

The same is true for SOD, however, here the increased activity was immediately noticeable. Significant increases in the activity of the superoxide scavenging enzyme leads to the conclusion that oxidative damage is reduced from the moment of fungicide application and it does in fact serves as a mechanism to delay senescence. In this study the effects of a combination spray was tested and found that in most cases it did increase SOD activity at later developmental stages for PAN8816, but immediate increases visible for NS5511.

According to Pastori and del Rio (1993) XOX activity increases as senescence progresses. This supports the theory that if fungicide application delays senescence it should decrease XOX activity. Distefano *et al.* (1996) reported similar results when studying the proteolytic cleavage of senescent pea leaves.

The mechanism for stay-green must however be linked to increases in anti-oxidative enzymes as well as decreases in ROS producing enzymes as clear decreases in XOX activity is observable. The results varied over the different harvest times and showed significantly lower XOX activity at maturity.

The mechanisms by which fungicides can delay senescence in plants remain complex but it can now with certainty be said that there is a link between the anti-oxidative enzymes SOD and POD in leaf material and systemic fungicide application.

Very little international research is available which can be compared directly to the results of the current study. It seems that Amistar Top® may be more inclined to increase sucrose concentration in sorghum seed than Abacus® application as all the Amistar Top® treatments gave higher sucrose concentrations compared to the control, as opposed to only some Abacus® treatments having higher concentrations. The method used, however, was an experimental method, developed by Teixeira *et al.* (2012) and used on soybeans. Although it appeared to yield sufficient results, duplication of the trial is advisable due to the nature of the results obtained. In a study conducted by Saladin *et al.* (2003) on the treatment of grape with fungicides showed varying results, ranging from 15% increases in sugar concentrations to 9% decreases, to no significant differences. A similar study showed no significant increases in sucrose concentrations in white yams, while significant increases in total starches were observed (Jaleel *et al.*, 2007).

As very little research on the effects of pesticides on black layer morphology is available the only assumptions to be made is that these decreases could be due to decreases in senescence onset as discussed in Chapter 2. Thus, a delay in the accumulation of phenolic compounds to form the black layer in the parenchyma cells of the seeds as an indication of senescence Giles (1975). As there is no clear correlation between the accumulation of sugar to the seed and the size if the black layer, it will also not likely affect the grain sucrose content, if fungicide is applied prophylactically.

Sorghum black layer formation does occur before 150 days after planting (90 days post emergence), and the study did show that in most cases the size (and thus rate of accumulation of phenolic compounds) is decreased or held at a constant due to fungicidal treatment. However, there is no correlation between sucrose concentration and black layer

size, thus it cannot with certainty be said that the black layer is in any way associated with the translocation of carbohydrates via the phloem to the seeds.

5

Concluding remarks

In Chapter 3 of this study the merit of prophylactic fungicide application was evaluated by observing changes in senescence, plant mass and yield. Results obtained for senescence indicated that fungicide application could not be relied upon to definitively delay senescence in all cultivars at all localities. Greytown was the only locality to shown significant delays in senescence over the 2013/14 planting season, however these findings were not supported by increases in yield over the same seasons at the same localities. No significant differences in above ground plant mass was obtained regarding the treatment effect. The only significant effect observed with regards to yield was during the 2013/14 seasons at Standerton with the AT 8 & 10 application. These results reject the hypodissertation in that the delay in senescence will lead to subsequent increases in yield and plant mass

To better understand the prophylactic characteristics of fungicide application, laboratory/greenhouse experiments (Chapter 4) were performed. These experiments investigated the various biochemical and physiological responses of *Sorghum bicolor* to fungicide application under greenhouse conditions. Fungicide application had no effect on the overall photosynthetic capacity of the plants, although the energetic connectivity associated with the oxygen evolving complex was impaired with most application rates. As for enzyme activity; ROS scavenging enzyme activity did increase combined with the decrease of ROS producing enzyme (XOX) activity at maturity. This decrease in the antioxidant mechanism correlates with the decreased senescence onset that were observed in Chapter 3. Sucrose concentration in the seed did increase in almost all cases, indicating higher nutritional value, as measured by the carbohydrate content. However, this was not associated with decreases in the size of the black layer, thus it can be assumed that the seeds still reach maturity at the same time.

From Chapter 3 and 4 the hypodissertation that the prophylactic application of fungicide will decrease the rate of senescence and oxidative damage may be conditionally accepted. This is because the delays in senescence were not visible over all localities, but clear anti-oxidative enzyme responses were visible.

There is no evidence in this study to conclude that yield or plant mass is increased by prophylactic fungicide application. Therefore, the hypodissertation stating there is a promise of financial return in the form of increased yield of plant mass is rejected. The increased sugar content does not merit this hypodissertation to be partially accepted, this would only increase carbohydrate content, but not weight as shown in Chapter 2.

6

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