FINAL REPORT

DETAILS

Project number		M141/07 (000409)
Project title		Evaluation of fungicides for the control of Exserohilum leaf blight, grain mould toxin production and associated potential growth regulating properties on sorghum
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Project status		Complete
Duration		01/10/2013 to 31/03/2017
Funder(s)		ARC/Sorghum Trust

Final abstract

Leaf blight (LB) is a common sorghum disease in southern African countries and considered of economic importance in the high potential areas of Zimbabwe and South Africa. A fungus, *Exserohilum turcicum* ((Pass.) Leonard & Suggs.) causes the disease, and yield losses associated with early infection of up to 50% have been recorded. Fungicidal compounds such as triazoles and strobilurins have been shown to have plant growth regulating properties leading to, amongst others, delayed senescence or increased yield. The aim of the study presented in Section 1 of the current report, was to evaluate the merit of the growth regulating properties of fungicides under South African production conditions on sorghum yield. Three split-plot trials were planted during 2013/14 and 2014/15 at Greytown, Potchefstroom and Standerton. Eleven fungicide treatments (main plots) consisted of two fungicides (azoxystrobin/difenoconazole and epoxiconazole/pyraclostrobin) applied at 6 weeks, 6 & 8 weeks, 8 weeks, 8 & 10 weeks and 10 weeks after planting and an untreated control, replicated three times. Four cultivars were included as sub-plots. Leaf blight severity, percentage leaf senescence, plant biomass at harvest as well as yield were determined. Leaf blight severity in all trials was sufficiently low (<16 %) to evaluate the growth regulating properties of the fungicides tested. During 2013/14, fungicide application resulted in delayed senescence at Greytown, but this effect was not observed at Potchefstroom or Standerton. Biomass was reduced by 20.3 and 24.25 % with 10-week azoxystrobin/difenoconazole and 6 & 8-week epoxiconazole/pyraclostrobin applications, respectively, in Potchefstroom relative to the control. Biomass was not affected at the other localities. Yield response to the various treatments during 2013/14 differed between localities, with azoxystrobin/difenoconazole treatment applied at 8 & 10

weeks resulting in a significant yield increase of 33.6 % compared to the control at Standerton. During 2014/15, fungicide applications had no effect on senescence, plant mass or 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.

Unlike *E. turcicum*, which is caused by a single fungus, the term “grain mould” refers to diseased appearance of sorghum grains because of infection by one or more pathogenic/saprophytic fungi. The disease tends to be important on short and medium sorghum cultivars that mature during the rainy season in humid, tropical and subtropical climates. Grain mould, however, also becomes a problem in the semi-arid production areas of South Africa, where the majority of sorghum is produced (i.e. northern Free State, Standerton, Lichtenburg, etc.) when wet conditions are prominent during flowering and grain fill. Some grain mould fungi are prevalent pre-harvest whilst others become problematic post-harvest. *Fusarium graminearum* is a pathogen that is shared between maize and sorghum. Not only does it cause root and crown rot of maize, but it can also cause head mould on sorghum. The question has been raised by the industry as to the effect of fungicide application on grain mould fungi. The aim of the study presented in Section 2 was to determine whether a spray regime developed for the control of leaf blight of sorghum could reduce root rots, control grain moulds, and concomitant mycotoxin production. The interest in root rot lies in the potential translocation of mycotoxin from roots to grains. This aspect was investigated in collaboration with the University of the Free State. Grain mould ratings, ergosterol content and HLC-MS-MS analysis were accordingly conducted on grain samples collected from the field trials presented in Section 1 of the current report. Although significant levels of infection were only recorded during 2013/14 and hence only one season’s data could be used, it would appear that the fungicide regimes used for leaf blight have a limited effect on root rots, grain moulds and mycotoxin accumulation. Final analyses and conclusions regarding this study will be provided in the PhD thesis of MS Danelle van Rooyen to be submitted during 2017.

During 2014/15, a field trial was conducted at Potchefstroom to evaluate four sorghum cultivars for their susceptibility to Leaf blight and to establish potential yield loss associated with infection (Section 3). Eleven fungicide treatments (main plots) were included which consisted of two fungicides (azoxystrobin/difenoconazole and epoxiconazole/pyraclostrobin) applied at 6 weeks, 6 & 8 weeks, 8 weeks, 8 & 10 weeks and 10 weeks after planting and an untreated control, replicated three times. Four cultivars were included as sub-plots (PAN8816, PAN8906, PAN8625 and NS5511). At five as well as 7-leaf stage, all the plots were inoculated with *Exserohilum turcicum.* Leaf blight severity was scored at flowering, soft dough and hard dough stage. Of the four cultivars screened. PAN8906 as

well as PAN8625 were the most susceptible cultivars measuring LB disease severity of 65.33 and 59.78% respectively. PAN8816 had 52% diseased leaf area, whilst NS5511 demonstrated a high level of disease resistance with only 8.9% disease leaf area measured at hard dough stage. The majority of the models fitted to the disease severity data of the various cultivars as measured at flowering, soft dough and hard dough all had very low R^2 values, suggesting that no clear pattern as to how the cultivars reacted regarding yield loss in response to leaf blight severity could be obtained. An interesting observation was that the response obtained by the two fungicides were different, with the best fit in general being where Amistar Top ® was applied. This observation warrants an in-depth study. An R^2 value of 0.7877 and R^2=0.8417, were, however, respectively obtained with PAN8625 when the disease severity observed at flowering and soft dough stages were plotted against the eventual yield obtained (linear fit being achieved). Based on the regression slope, a yield decline of 4.39 kg ha^{-1} was experienced per 1% increase in disease severity at flowering. Expressed differently, a yield loss of 7.9% was experienced for every 10 % increase in disease severity at flowering. A 5.4% yield loss was similarly experienced by this cultivar for every 10% increase observed in leaf blight severity at soft dough stage. This finding is similar to what has been reported internationally on yield losses associated with Northern corn leaf blight (also caused by *E turcicum)* on maize (i.e. 2-8% yield loss for every 10 % increase in disease severity). The findings of our study also reiterated the impact of early infections, with the disease severity measured at flowering resulting bigger yield losses as to what is measured at soft dough stage.

The biochemical and physiological responses of sorghum to fungicide application were investigated under greenhouse conditions in Section 4 of this report. PAN8816 and NS5511 were included in the study, together with two fungicides, Amistar Top® (azoxystrobin/difenoconazole) and Abacus® (epoxiconazole/pyraclostrobin), applied at three different application dates (8, 10 and 8 & 10 weeks after planting) with an unsprayed control included. Pots were arranged in the glasshouse in a randomized block design with five replicates, with a single pot representing a replicate (one plant per plot). Chlorophyll *a* fluorescence and enzyme activity (i.e. peroxidase, superoxide dismutase and xanthine oxidase) were determined at 70, 110 and 150 days after planting. Sucrose concentration as well as black layer morphology were investigated at 150 days after planting. 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 dates. ROS (Reactive oxygen species) scavenging enzyme activity did increase combined with the decrease of the ROS producing enzyme, xanthine oxidase, activity at maturity. As black layer formation is linked 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. With the current study

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.

Keywords: growth regulating properties, plant biomass, senescence, strobilurins, triazoles, yield, grain mould, reactive oxygen species

Section 1: Evaluation of fungicides for potential growth regulating properties on sorghum

Scientific article submitted to *Crop Protection (Ms. Ref. No.: CROPRO-D-16-01098R1* - Resubmitted manuscript with corrections to journal - February 2017*)*

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Abstract

Fungicidal compounds such as triazoles and strobilurins have been shown to have plant growth regulating properties leading to, amongst others, delayed senescence or increased yield. The aim of the current study was to evaluate the merit of the growth regulating properties of fungicides under South African production conditions on sorghum yield. Three split-plot trials were planted during 2013/14 and 2014/15 respectively (Greytown, Potchefstroom and Standerton). Eleven fungicide treatments (main plots) consisted of two fungicides (azoxystrobin/difenoconazole and epoxiconazole/pyraclostrobin) applied at 6 weeks, 6 & 8 weeks, 8 weeks, 8 & 10 weeks and 10 weeks after planting and an untreated control, replicated three times. Four cultivars were included as sub-plots. Leaf blight severity, percentage leaf senescence, plant biomass at harvest as well as yield were determined. Leaf blight severity in all trials was sufficiently low (<16 %) to evaluate the growth regulating properties of the fungicides tested. During 2013/14, fungicide application resulted in delayed senescence at Greytown, but this effect was not observed at Potchefstroom or Standerton. 20.3 and 24.25 % with 10-week azoxystrobin/difenoconazole and 6 & 8-week epoxiconazole/pyraclostrobin applications respectively reduced biomass, in Potchefstroom relative to the control. Biomass was not affected at the other localities. Yield response to the various treatments during 2013/14 differed between localities, with azoxystrobin/difenoconazole treatment

applied at 8 & 10 weeks resulting in a significant yield increase of 33.6 % compared to the control at Standerton. During 2014/15, fungicide applications had no effect on senescence, plant mass or 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.

KEYWORDS

Growth-regulating properties, plant biomass, senescence, strobilurins, triazoles, yield

1. Introduction

Sorghum (*Sorghum bicolor* (L.) Moench & Suggs) is the fourth most cultivated summer grain crop in South Africa. When compared to maize, sunflower and soybean, sorghum is cultivated on a very small scale with only 78 850 ha being documented to be under sorghum production during the 2013/14 season (Anonymous, 2015a). 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 crops 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, in the near future, expand within southern Africa with a greater focus on optimizing yield.

Leaf blight (LB) is a common sorghum disease in southern African countries. In the high potential areas of Zimbabwe and South Africa, the disease is considered of economic importance (Mtisi and McLaren, 2002). Leaf blight is caused by *Exserohilum turcicum* ((Pass.) Leonard & Suggs.) and yield losses associated with early infection of up to 50% have been recorded (Panguluri and Kumar, 2013). Generally, LB is more severe in the humid eastern production areas of South Africa, but becomes a widespread problem during higher rainfall seasons. Fungicides consisting of combinations of active ingredients such as azoxystrobin/difenconazole, epoxiconazole/ pyraclostrobin and carbendazim /difenoconozole are used by producers although not all are registered for LB control (Anonymous, 2015b). In some sorghum production areas, such as Standerton (Mpumalanga, South Africa), producers apply fungicides prophylactically due to severe infections experienced in the past (D Booyens, PANNAR Seed Co. **-** *Personal communication*). 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 or not disease actually developed.

Compounds such as triazoles and strobilurins used as fungicides, have been shown to have plant growth regulating properties (Fletcher and Arnold, 1986). Triazoles have been shown to inhibit gibberellin biosynthesis (Fletcher et al., 2000; Rademacher, 2000). Gibberellins are a group of plant hormones associated with growth and development, specifically seed germination, root and shoot elongation, flowering and fruit patterning (Hedden and Phillips 2000; Fleet and Sun, 2005; Yamaguchi, 2008). Aside from influencing hormonal balances, triazoles have been shown to affect photosynthetic rate, enzyme activities, lipid peroxidation and yield components in carrots (*Daucus carota*) and herbs such as Madagascar periwinkle (*Catharanthus roseus)* and W*ithania somnifera* (Gopi et al., 2007; Jaleel et al., 2008a, 2008b). Observed effects subsequent to triazole applications included shorter plants, reduced leaf area and improved rooting as the partition between shoots and roots is altered in crops such as Chinese potato (*Solenostemo rotumdifolius)* and oilseed rap

(Brassica napus) (Berry and Spink, 2009; Fletcher et al., 2000; Kishorekumar et al., 2007). Triazoles are able to protect plants from injury due to abiotic stresses such as drought, heat and air pollutants (Davis et al., 1988; Fletcher and Hofstra, 1988).

Wu and von Tiedeman (2001) reported improved stalk strength and delayed senescence in wheat as a result of the application of azoxystrobin (strobilurin) and epoxiconazole (triazole), with azoxystrobin being more efficient when applied at earlier growth stages (GS 31/32). Greater yield response as a result of strobilurin application has also been documented (Gooding et al., 2000; Bertelsen et al., 2001; Ruske et al., 2003). Such physiological responses have been attributed to a reduction in either ethylene synthesis or oxidative stress (Grossman and Retzlaff, 1997; Zang et al., 2010) due to increased photosynthetic capacity and translocation (Gooding et al., 2000) or improved regulation of stomatal opening and water-use efficiency (Grossman et al., 1999). Görtz et al. (2008) found in barley seedlings that the intensity of plant growth regulation by fungicides used in seed dressing was modified by early season environmental conditions. In addition, they reported that varying plant growth regulating activities of triazoles in composite fungicides were dependant on the mixing partner.

The merits of the growth regulating properties of fungicides on sorghum have not been evaluated under the various climatic conditions within South Africa. This is of importance as international research has demonstrated that environmental conditions can dictate the degree of response observed to the application of fungicide (Görtz et al., 2008). Producers intending to apply fungicide for the sole purpose of capitalizing on their growth regulating properties must have a guarantee that such applications will work equally well over all seasons and sorghum production areas of South Africa. In addition, few studies report on the evaluation of growth regulating properties of commercially available fungicides which in general consists of a combination of both triazole and strobliurin such as azoxystrobin/difenconazole or epoxiconazole/pyraclostrobin. Research into the growth regulating properties of such dual compounds is of importance as the mixing partner (Görtz et al., 2008) can affect the effectiveness of the growth regulating properties. The absence of consistent response to fungicides in grain crops as well as a dearth of information in the growth regulating effects on sorghum accordingly makes this study essential if fungicide usage is to be optimized.

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 for their effect on yield, plant mass and senescence of selected sorghum cultivars in the absence of, or low LB disease severity levels under field conditions.

2. Materials and Methods

2.1. Field trials

Field trials to evaluate the effect of fungicides azoxystrobin/difenconazole (formulation - 200/125 g l^{-1}; Amistar Top®, Syngenta) and epoxiconazole/pyraclostrobin (formulation - 62.5/62.5 g l^{-1}; Abacus®, BASF) on plant mass, senescence and yield in four sorghum cultivars were conducted at Potchefstroom (North West Province; S26° 43' 56.0" EO27° 03' 53.9"), Standerton (Mpumalanga; S27º 09' 50.5" EO29º 14' 54.8") and Greytown (Kwa-Zulu Natal; S29º 05' 01.5" EO30º 36' 12.9"), during 2013/14 and 2014/15 (Photo 1). The trials were planted as randomised block designs with three block replicates. The treatment design was a split-plot with fungicide as main plot and cultivar as sub-plot factor. Cultivars were planted as single rows within the main plots and included PAN8816, PAN8906, PAN8611 and NS5511 during 2013/14. PAN8611 was replaced with PAN8625 during the subsequent 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. Plant density at all localities was 88 000 plants ha^{-1}.

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 was applied accordingly. Frontier® Optima (dimethenamid – 75 g l^{-1}; BASF) was applied pre-emergence and Basagran® (bendioxide 480 g l^{-1}; BASF) post-emergence to prevent weed encroachment. Mid-season stem-borer control was done with Karate® (ambda-cyhalothrin 5.5%, 100 ml ha^{-1}; Syngenta). Weather data was obtained from automatic weathers stations within 50 km of the trials sites.

2.2. Fungicide treatments

Two foliar fungicide formulations i.e. azoxystrobin/difenconazole (application rate - 500 ml ha^{-1}) and epoxiconazole/pyraclostrobin (application rate - 1 l ha^{-1}), were evaluated at five application times (treatments) i.e. 6 weeks, 6 & 8 weeks, 8 weeks, 8 & 10 weeks and 10 weeks after planting (WAP) and compared to an untreated control (Photo 2). The fungicides were Amistar Top®, the only registered fungicide for the control of LB in South Africa when the trials commenced during 2013, while Abacus® is a product commonly used by producers even though not registered for the control of LB. Fungicides were applied using a CO_2 gas operated knapsack sprayer and a four nozzle (flat fan; 0.9 m spaced) boom. The knapsack sprayer was calibrated to a spray volume of 78 l ha^{-1}.

2.3. Crop measurements and analysis

Leaf blight severity, percentage leaf senescence, plant mass as well as yield were measured in the current study. Ten randomly selected plants within each row were 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% (Elliot and Jenkins, 1946; Adipala et al., 1993).

Panicles were harvested when the kernel moisture was <15%, even though the plant material was, in some cases, not fully senesced. The upper four leaves of five randomly selected plants per cultivar per plot were selected at harvest and used for senescence quantification. A combined scale from Verma et al. (2004) and Cromey et al. (2004) was used where the level of senescence was quantified according to the percentage leaf material showing yellowing (0% - no senescence; 100% - fully senesced).

After the panicles were removed, the remaining plant tissues (stalk and leaves) were used for plant mass determination. Ten plants per cultivar per plot were randomly selected and weighed using a tripod scale. The combined weight (kg) of the ten plants was documented.

Harvested panicles were threshed and grain weight obtained. Grain moisture was determined with a TwistGrain moisture meter (Draminski Elektronics). Yield (t ha^{-1}) was calculated at 12.5% moisture. For each season, the data of the three localities were tested for homogeneity of variances using Levene's test. In cases where the variability in the observations of the three localities were of comparable magnitude an analysis of variance of the observations of the three localities combined could be validly carried out (John, 1977). In cases where there were strong evidence against homogeneity a weighted analysis of the observations of the three localities combined were done using the inverse of the pooled variance of each locality as weight (John, 1977). The Shapiro-Wilk test was used to test the standardised residuals for deviations from normality (Shapiro, 1965). Student's t-Least Significant Differences were calculated at the 5% level to compare treatment means of significant effects. All the analyses were done using SAS v9.2 statistical software (SAS, 1999).

Pearson’s correlation coefficients were calculated to determine the linear relationships between senescence, plant mass and yield for each of the localities over the two seasons separately. All the analyses were done using SAS v9.2 statistical software (SAS, 1999).

3. Results

As the aim was to establish whether the application of fungicide will have an impact on senescence, plant mass and yield, only the highest significant order interaction associated with treatment (i.e. locality x treatment, cultivar x treatment or locality x treatment x cultivar) was reported in the current study.

Weather data observed within 50 km of the various trial sites, indicated that in 2013/14, both Standerton and Greytown were 5 or more degrees cooler during the day than Potchefstroom at the time of treatment applications (i.e. 6, 8 and 10 WAP). Although a similar effect was observed during 2014/15, maximum temperatures for both Greytown and Standerton were 3-5ºC higher than the previous year at these critical periods. In addition, more rainfall was experienced during 2013/14 compared with 2014/15, with Greytown, Potchefstroom and Standerton receiving 56 mm, 199 mm and 362 mm respectively more rainfall during the former season for the period November to July.

3.1. Disease severity

During 2013/14 very low levels of LB severity were observed at soft dough stage at all three localities, with average disease severity measured over all the plots ranging from 0.0 - 0.9 %, 0.01 - 2 % and 0 - 0.2 % for Greytown, Potchefstroom and Standerton respectively (data not shown). Slightly higher disease severities were observed during the 2014/15 season (Greytown: 0.3 - 6 %; Potchefstroom: 0.1 - 16 %; Standerton: 0.3 - 5 % - data not shown). Sufficiently low disease severities were present at the various sites during both seasons in order to evaluate the growth regulating properties of the fungicides without the confounding effect of disease on growth parameters.

3.2. Senescence, plant mass and yield

Analysis of variance (Table 1) indicated that leaf senescence, plant mass as well as yield were all significantly influenced by the locality x treatment interaction during 2013/14, indicating that the effect of the various fungicide applications on the parameters observed were dependant on the locality. None of the parameters was significantly affected by treatment as main effect or as part of an interaction effect during the subsequent 2014/15 season (Table 1). Both Potchefstroom and Standerton leaf material were fully senesced at harvest, while the Greytown control was less than 60% senesced (Figure 1). In addition, all the fungicide treatments at Greytown significantly reduced the percentage leaf senescence measured at harvest by between 15 and 30%, compared to the control.

Plant mass was significantly affected by two fungicide treatments compared to the control at Potchefstroom (Figure 2) during 2013/14. Azoxystrobin/difenoconazole application at 10 WAP as

well as epoxiconazole/pyraclostrobin application at 6 & 8 WAP significantly reduced plant mass relative to the control at this locality. None of the treatments at Standerton or Greytown had any effect on plant mass compared to the control during this season.

During 2013/14, the application of azoxystrobin/difenoconazole at 8 & 10 WAP at Standerton was the only treatment that gave significantly higher yield compared to the untreated control (Figure 3). None of the treatments resulted in a significant increase in yield compared to the control at either Potchefstroom or Greytown. At Greytown, the 8, 10, 6 & 8 as well as 8 & 10 WAP application of epoxiconazole/pyraclostrobin tended to have higher yield than the control although the effect was not significant. A similar effect was observed at Standerton with certain epoxiconazole/pyraclostrobin treatments having higher (but not significant) yield compared to the control.

3.3. Correlation analysis

The Greytown trial gave the most significant correlations between plant mass, senescence and yield (Table 3). The correlations were, however, generally low to moderate. Positive, but moderate correlations were observed between yield and plant mass during both seasons (2013/14: $r = 0.586$, $p = <0.0001$; 2014/15: $r = 0.407$, $p = <0.0001$) at Greytown. During 2013/14, yield, as well as plant mass had significant negative, but low correlation with senescence (Table 3), while only plant mass had a negative correlation with senescence during 2014/15 ($r = -0.483$, $p = <0.0001$).

4. Discussion

Although the advantage of fungicide application lies in the control of disease-causal organisms, numerous international literature studies report additional benefits in the form of delayed senescence and extended green leaf area duration (Wu and Tiedemann, 2001; Cromey et al., 2004). A longer grain fill period associated with such delayed senescence was reported to result in yield enhancements in wheat (Spiertz, 1977) and maize (Byakmukama et al., 2013). In the current study, delayed senescence was recorded only at Greytown during 2013/14 and although a significant negative albeit low correlation was observed between yield and senescence, analysis of variance indicated that the yield response observed was not significant. Differences in plant mass (Potchefstroom 2013/14) as well as yield (Standerton 2013/14) brought about by one or two treatments in this study could also not be repeated over all localities and seasons.

There remains a dearth of information on the potential growth regulating properties of fungicides with combined active ingredients as included in the current study. In most cases, the active compounds have been evaluated individually (Zang et al., 2010). Dimmock and Gooding (2002) and Blandino and Reyneri (2009) indicated that the addition of strobilurins to a triazole programme did not significantly delay leaf senescence or increased yield compared to triazoles applications alone. In contrast, Ijaz et al. (2015), found that combinations with triazoles induced photosynthetic ability and delayed senescence which could improve the morphological and yield components of oil seed rape plants than either alone.

There is in literature, however, a clear apparent debate over the growth regulating properties of fungicides in general. On maize, Byamukama et al. (2013) reported that pyraclostrobin delayed senescence and contributed to a stay green effect. They were unable to show a grain fill extension while Zhang et al. (2010) recorded significant yield increases associated with delayed senescence on wheat. On barley, Görtz et al. (2008) reported a reduction in shoot growth with triazole-containing fungicides applied as seed dressings with no marked plant growth activity in strobilurin-containing seed dressings. Furthermore, they also recorded differences in the activity of triazoles depending on strobilurin mixing partner as well as differences in growth stimulating properties depending on growth conditions, including soil water capacity and ambient temperatures. They recorded growth suppression that was greater under optimal growth conditions while increasing stress tolerance under stress conditions. Balodis and Gaile (2011) recorded similar variation with environmental conditions on oilseed rape while host genotype was also an apparent source of variation. Zhang et al. (2010) also recorded similar variation in growth stimulating response with host genotype on wheat. Marshall (2014) in a MSc study in Illinois, showed that fungicides in a basic crop production system did little to enhance yield in maize, but recorded significantly enhanced yields in intensive

cropping systems and concluded that the response to fungicide applications increased as the yield potential of an environment increased, especially in the intensive management systems. In soybean, Kyveryga et al. (2013) recorded an extended greening effect and greater yield responses where more than 300 mm of early season rain were recorded compared with less precipitation. They proposed that site-specific observations of spring rainfall could be used to identify fields that could potentially produce above-even or break-even responses and avoid unnecessary fungicide applications. Wise and Mueller (2011) questioned the use of fungicides when foliar disease severity in maize was less than 5% while Shah and Dillard (2010) place this figure at 20% for the use of strobilurin fungicides in sweetcorn. It is evident that numerous variables interact to determine the merits of fungicide applications for growth regulating properties and no blanket recommendation is evident.

Lorenz and Cothren (1989) indicated that no leaf greening or yield response was observed on wheat in response to the application of a tank mix consisting of triadimefon and zinc+manganese ethylene bisthiocarbamate over a number of seasons. They concluded that studies demonstrating a yield increase in response to fungicide application in the absence of disease, possibly fail to account for the control of unnoticed secondary pathogens or pathogens of minor significance that may otherwise contributed to an overall reduction in crop yield. Similar conclusions have been drawn by Dickinson (1981). Bertelsen et al. (2001) demonstrated under greenhouse conditions that fungi without the ability to infect plants, but sufficient to stimulate papilla formation and a hypersensitive response, accelerated senescence and reduced grain yield of barley. This was attributed to the energy cost associated with the plant's defence response as well as the reduction in chlorophyll. Bertelsen et al. (2001) recorded fewer defence reactions with azoxystrobin than epoxiconazole which delayed senescence although the extended green period was not associated with aerial plant biomass or yield. This observation could account for the significant reduction in senescence observed with the application of fungicides at Greytown where higher moisture conditions prevail while at the dryer Standerton and Potchefstroom locations plants may have been under less infection pressure and hence no response to fungicide application was observed. The effect was similar for all four cultivars included during 2013/14, but failed to manifest during the warmer and drier 2014/15 season. This hypothesis does, however, not fully explain the general delay in senescence observed in the control plots of Greytown compared to that of the other localities at harvest. Our findings confirm that weather conditions might dictate the response to fungicide application observed, but also suggest that a phyllosphere x fungicide interaction could be at play. This hypothesis would require validation.

In conclusion, the current study was not able to verify the internationally reported growth regulating properties of triazoles and strobilurins on sorghum. The responses obtained were inconsistent over

treatment, locality and season for senescence, plant mass and yield. These inconsistencies still require elucidation and the role of seasonal variation and yield potential still needs to be assessed on a scale that accommodates larger plot sizes. The application of such chemicals for the purpose to delay senescence or increase yield in the absence of the foliar disease is accordingly not an economically viable option for South African sorghum producers.

Acknowledgements
We hereby acknowledge the Sorghum Trust for financial support.

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Photo 1. Field trails were planted at Potchefstroom, Standerton and Greytown during 2013/14 and 2014/15 to evaluate the growth regulating properties of fungicides on sorghum.

Photo 2. Fungicides were applied using a CO_2 gas operated knapsack sprayer and a four nozzle (flat fan; 0.9 m spaced) boom.

Table 1. Analysis of variance of the effect of eleven fungicide treatments on senescence, plant mass and yield of four sorghum cultivars at three localities (Potchefstroom, Standerton and Greytown) over two seasons (2013/14 and 2014/15) respectively.

	Senescence[a]					**Plant mass[b]**				**Yield**			
2013/14	df	S.s.[c]	M.s.[d]	F Value	Pr > F	S.s.	M.s.	F Value	Pr > F	S.s.	M.s.	F Value	Pr > F
Locality (Loc)	2	4.0E+05	2.0E+05	3501.5	<.0001	9.8	4.9	3.5	0.0365	40.1	20.1	16.5	<.0001
Rep (Loc)	6	2.4E+02	4.0E+01	0.7	0.6518	39.6	6.6	4.7	0.0005	75.7	12.6	10.4	<.0001
Treatment (Tmt)	10	2.8E+03	2.8E+02	4.9	<.0001	19.3	1.9	1.4	0.2114	13.4	1.3	1.1	0.3796
Loc*TMT	20	5.6E+03	2.8E+02	4.9	**<.0001**	52.6	2.6	1.9	**0.0318**	49.0	2.5	2.0	**0.0199**
Error (a)	60	3.4E+03	5.7E+01			84.0	1.4			73.2	1.2		
Cultivar (Cult)	3	9.6E+03	3.2E+03	97.7	<.0001	146.4	48.8	48.8	<.0001	119.7	39.9	39.9	<.0001
Loc*Cult	6	1.9E+04	3.2E+03	97.7	<.0001	61.1	10.2	10.2	<.0001	56.8	9.5	9.5	<.0001
Tmt*Cult	30	1.2E+03	4.0E+01	1.2	0.2074	21.6	0.7	0.7	0.8578	22.8	0.8	0.8	0.8139
Loc*Tmt*Cult	60	2.4E+03	4.0E+01	1.2	0.1528	51.0	0.9	0.9	0.7691	74.7	1.2	1.2	0.1349
Error (b)	198	6.5E+03	3.2E+01			198.0	1.0			198.0	1.0		
Corrected Total	395	4.5E+05				683.4				723.4			
2014/15	df	S.s.[c]	M.s.[d]	F Value	Pr > F	S.s.	M.s.	F Value	Pr > F	S.s.	M.s.	F Value	Pr > F
Locality (Loc)	2	6.0E+05	3.0E+05	2262.34	<.0001	611.5	305.7	78.6	<.0001	110.2	55.1	25.2	<.0001
Rep (Loc)	6	2.3E+03	3.8E+02	2.91	0.0148	266.6	44.4	11.4	<.0001	9.6	1.6	0.7	0.6244
Treatment (Tmt)	10	2.5E+03	2.5E+02	1.91	0.0617	52.7	5.3	1.4	0.2235	35.5	3.6	1.6	0.1220
Loc*TMT	20	2.8E+03	1.4E+02	1.07	0.4014	58.4	2.9	0.8	0.7574	19.1	1.0	0.4	0.9789
Error (a)	60	8.0E+03	1.3E+02			233.4	3.9			131.1	2.2		
Cultivar (Cult)	3	9.8E+03	3.2E+03	19.42	<.0001	149.2	49.7	49.7	<.0001		45.3	45.3	<.0001
Loc*Cult	6	1.1E+04	1.9E+03	11.54	<.0001	147.0	24.5	24.5	<.0001	108.4	18.1	18.1	<.0001
Tmt*Cult	30	5.4E+03	1.8E+02	1.08	0.3587	33.2	1.1	1.1	0.3314	40.3	1.3	1.3	0.1221
Loc*Tmt*Cult	60	7.9E+03	1.3E+02	0.79	0.8601	65.2	1.1	1.1	0.3305	51.8	0.9	0.7	0.7436
Error (b)	198	3.3E+04	1.6E+02			198.0	1.0			198.0	1.0		
Corrected Total	395	6.8E+05				1815.1				839.9			

[a] – Senescence measured at harvest

[b] – Total plant mass of ten randomly selected sorghum plants (stalk and leaves)

[c] – sum of squares

[d] – mean sum of squares

Table 2. Correlation analysis results between plant mass, senescence and yield at three localities over two seasons.

	2013/14		2014/15	
Greytown	Yield	Plant mass	Yield	Plant mass
Plant mass	0.586 (<0.0001)		0.407 (<0.0001)	
Senescence	-0.311 (0.0003)	-0.283 (0.001)	-0.122 (0.163)	-0.483 (<0.0001)
Potchefstroom				
Plant mass	0.204 (0.019)		0.083 (0.343)	
Senescence	- -	- -	0.098 (0.263)	-0.105 (0.231)
Standerton				
Plant mass	0.131 (0.133)		-0.280 (0.001)	
Senescence	- -	- -	- -	- -

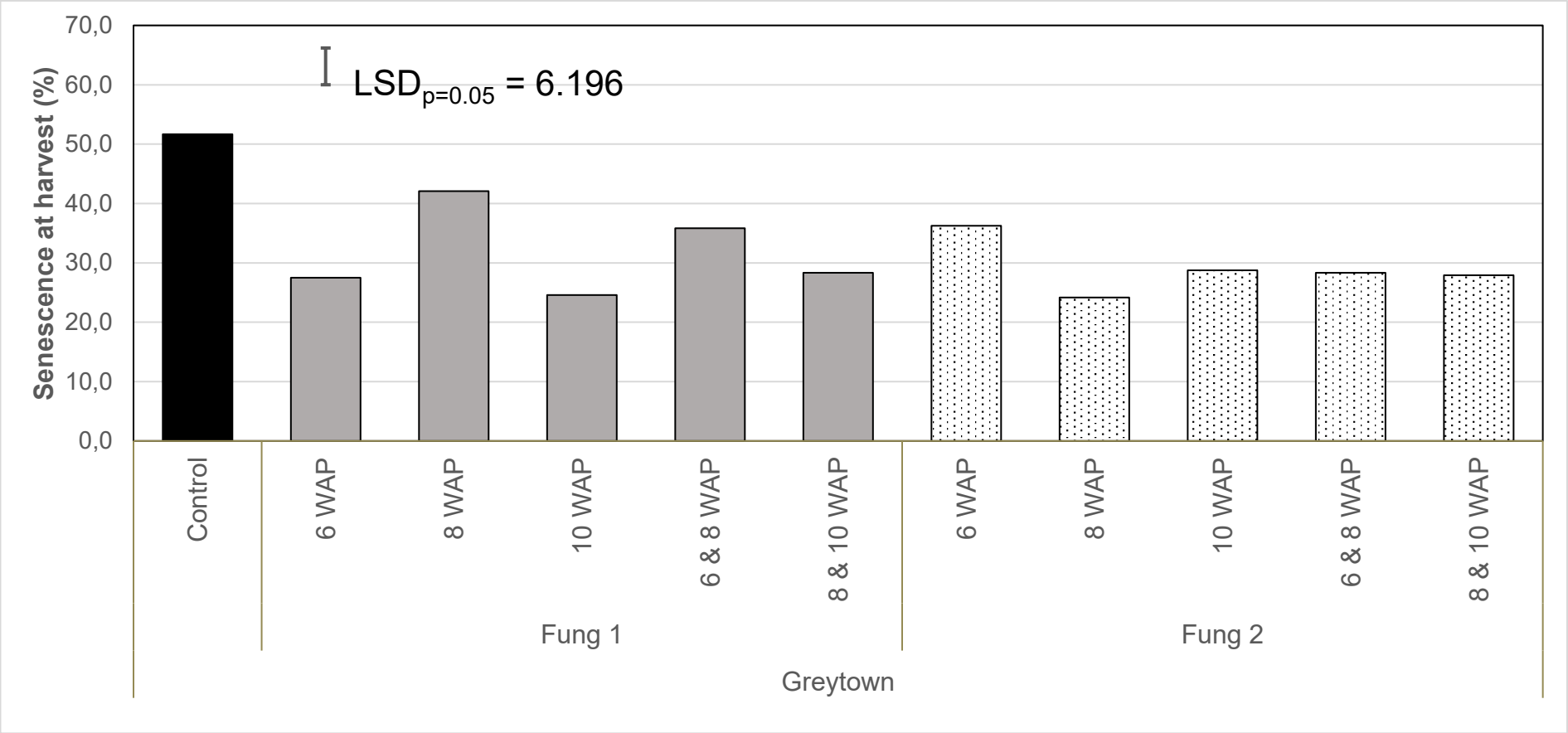

Figure 1. Percentage senescence observed in sorghum at harvest subsequent to the application of two fungicides at five application dates at Greytown during 2013/14. Standerton and Potchefstroom leaf material was fully senesced. (Fung 1 - azoxystrobin/difenoconazole; Fung 2 - epoxiconazole/ pyraclostrobin; WAP - Weeks after planting)

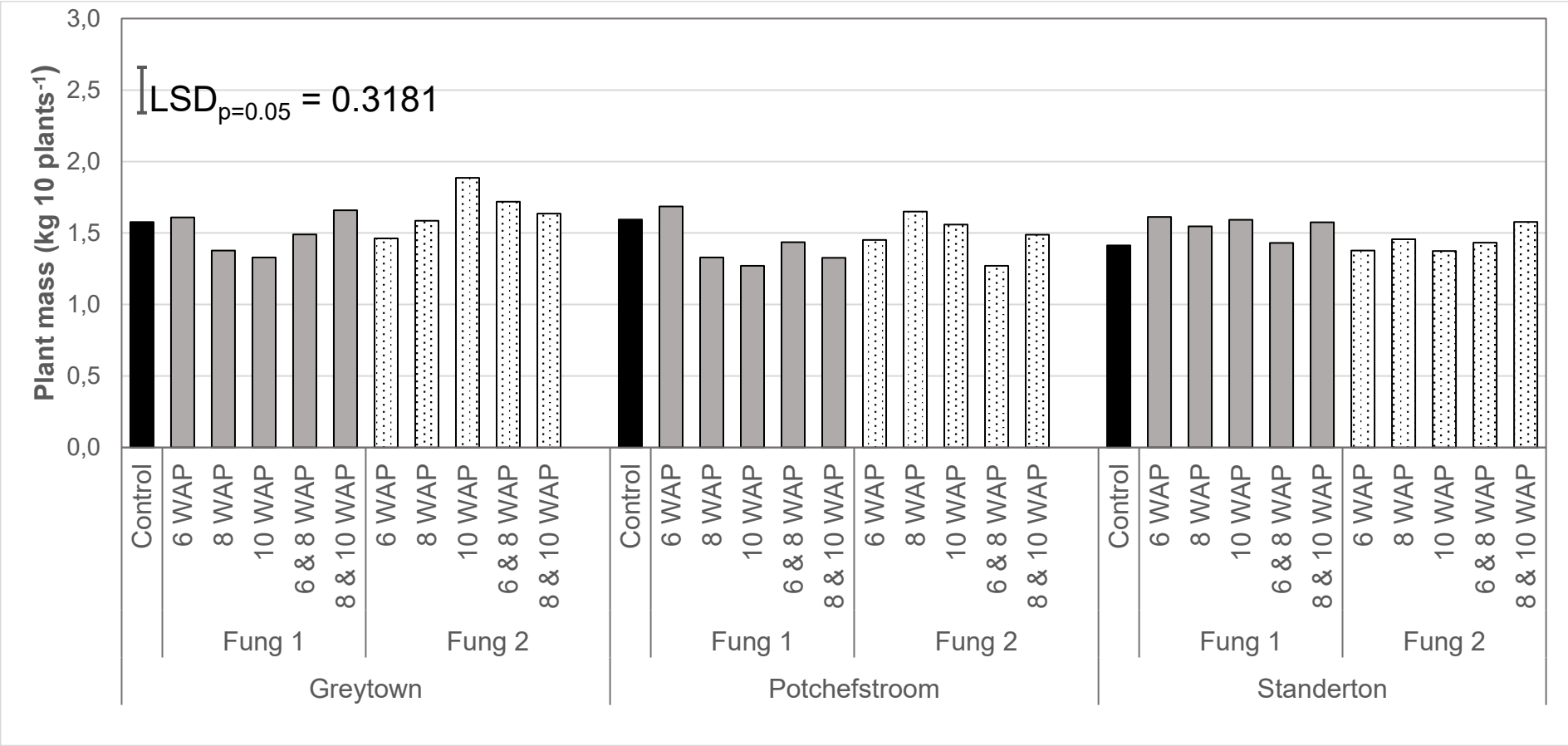

Figure 2. Plant mass of ten sorghum plants at harvest in response to the application of two fungicides at five application dates as evaluated over three localities **(**Greytown, Potchefstroom and Standerton) during 2013/14 (Fung 1 - azoxystrobin/difenoconazole; Fung 2 - epoxiconazole/ pyraclostrobin; WAP - Weeks after planting)

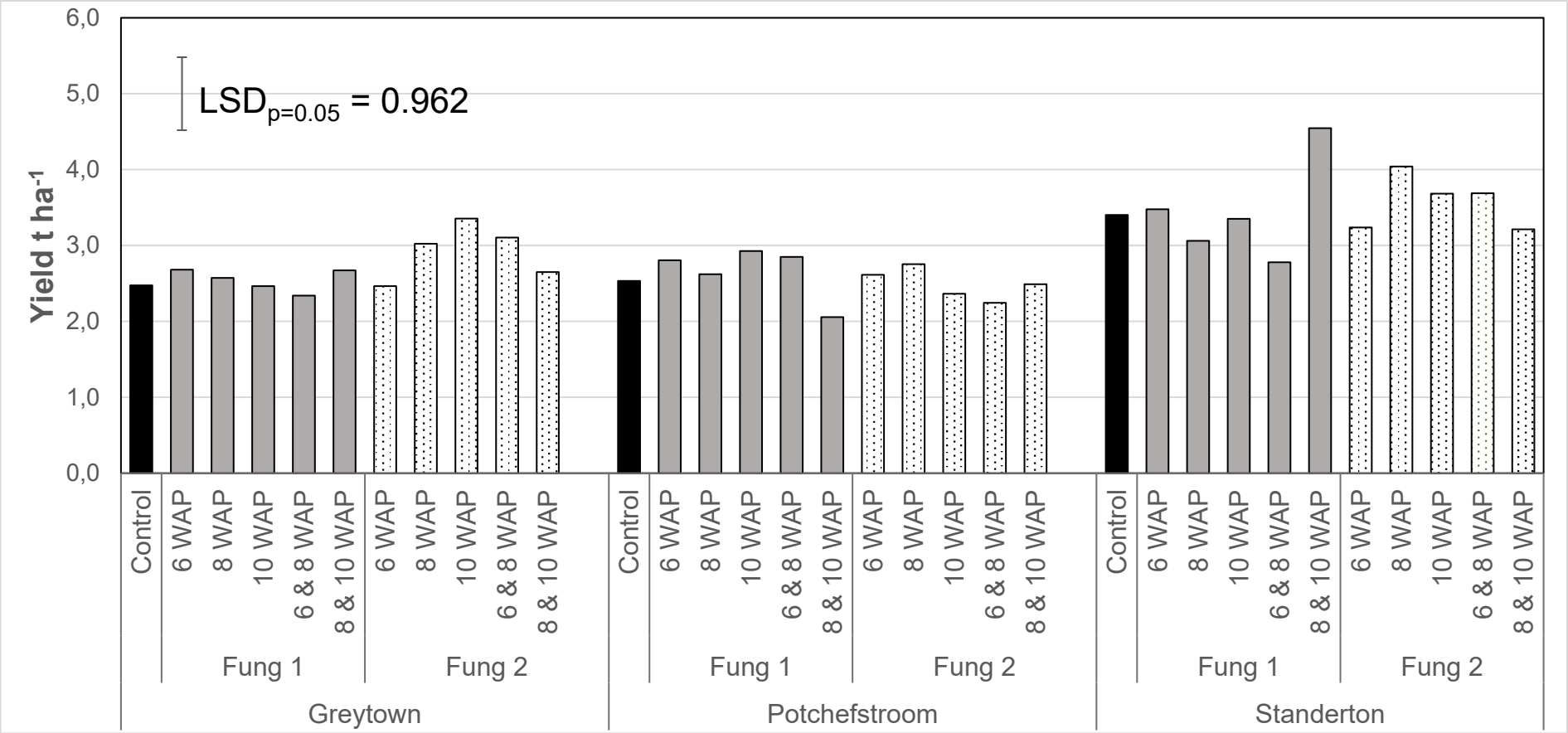

Figure 3. Yield response of sorghum to the application of two fungicides at five application dates as evaluated over three localities **(**Greytown, Potchefstroom and Standerton) during 2013/14 (Fung 1 - azoxystrobin/difenoconazole; Fung 2 - epoxiconazole/ pyraclostrobin; WAP - Weeks after planting)

Section 2: Fungicide effects on grain moulds and translocation of mycotoxins from roots

The University of the Free State was the lead investigator with the study into the effect of fungicide application on grain moulds and the possible translocation of mycotoxins from the roots to the grains. The findings of the study is in the process of being prepared for submission for PhD purposes (Mrs D Van Rooyen) as well as publication in a scientific journal. Results presented in this final report is accordingly preliminary and might differ from the eventual scientific article that will result from this study.

2.1. Introduction

Unlike *E. turcicum*, which is caused by a single fungus, the term “grain mould” refers to diseased appearance of sorghum grains as a result of infection by one or more pathogenic/saprophytic fungi. The disease tends to be important on short and medium sorghum cultivars that mature during the rainy season in humid, tropical and subtropical climates. Grain mould, however, also becomes a problem in the semi-arid production areas of South Africa, where the majority of sorghum is produced (i.e. northern Free State, Standerton, Lichtenburg etc.) when wet conditions are prominent during flowering and grain fill. Species of the genera *Fusarium, Curvularia, Alternaria, Phoma, Bipolaris and Colletotrichum* have been associated with grain mould (Thakur et al., 2003) of which the *Fusarium* spp. are considered to be the most prominent within the grain mould complex (Sharma et al., 2010). Da Silva et al. (2004) indicated that sorghum grain can be contaminated by toxins produced by fungi such as *Aspergillus* and *Fusarium* spp. The various fungi associated with grain mould, however, differ as to when they are problematic and produce toxins. Some grain mould fungi are prevalent pre-harvest whilst others become problematic post-harvest. *F. graminearum* is a pathogen that is shared between maize and sorghum. Not only does it cause root and crown rot of maize, but it can also causes head mould on sorghum.

The question has been raised by the industry as to the effect of fungicide application on grain mould fungi. Janse van Rensburg et al. (2011) found that based on sample collections from the national cultivar evaluation trials, neither *Aspergillus* nor *Fusarium* spp. poses any threat to sorghum production in South Africa. The study did, however, focus on toxins such as aflatoxins (produced by *Aspergillus* spp.) and fumonisin (such as produced by *F. verticillioides* and *F. proliferatum*), of which fumonisins are primarily produced after harvest and aflatoxins during storage. None of the toxins produced by *F. graminearum* did accordingly form part of the study, nor was the effect of fungicides on such toxins evaluated.

Mycotoxins (as a result of grain mould infection) in grain foods are of growing health importance due to their association with a number of human and animal diseases. Mycotoxins that pose human health risks include aflatoxins, deoxynivalenol (DON), fumonisins, ochratoxins and ergot alkaloids. DON, together with zearalenone and nivalenol are produced by *F. graminearum.* As these toxins are produced primarily pre-harvest (Creppy, 2002), fungicide application during the season could possibly affect or limit *F. graminearum* infection of grain and accordingly assist in reducing the amount of toxins produced.

The aim of the study is to determine whether a spray regime developed for the control of leaf blight of sorghum can reduce root rots and control grain moulds and concomitant mycotoxin production. The interest in root rot lies in the potential translocation of mycotoxin from roots to grains.

2.2. Materials and methods

2.2.1. Field trials

The three sorghum field trials plated at Greytown, Potchefstroom and Standerton during 2013/14 and 2014/15 for the evaluation of the efficacy of fungicides for leaf blight (*Exserohilum turcicum*) control (See Section 1), was utilised for this study. Trials consisted of 11 fungicide treatments as main plot effects with four cultivars per fungicide treatment as sub-plots, planted in single row plots. Trials were replicated three times in a randomized split plot experimental design. At various stages after planting trials were sprayed with the respective fungicides using a knapsack spray.

2.2.2. *Root rot severity*

Root systems of ten randomly selected plants were sampled after harvest from each of the cultivars and treatments and disease severity recorded. Root weight were also recorded.

2.2.3. *Grain mould ratings*

Prior to harvest field ratings of grain mould were conducted on a 0-5 scale.

2.2.4. Ergosterol content and HLPC-MS-MS analysis

Harvested grains were analysed for ergosterol content as an indicator of grain colonisation by grain mould fungi. DNA has been extracted from grains for the determination of FgSC levels in grain and mycotoxins were extracted and sent for HLPC-MS-MS analysis.

2.3. Results and discussion

Grain mould ratings for 2013/14 and 2014/15 are presented in Table 2.1. Standerton had the lowest grain mould ratings during 2013/14 while no significant differences were recorded at Potchefstroom and Greytown. During 2014/15, grain mould ratings in plantings at Potchefstroom were the highest. No significant fungicidal effects on visual grain mould rating was observed although significant locality x treatment interactions were recorded. Based on 2013/14 data it was concluded that fungicide spray regimes deployed prior to grain development i.e. to manage vegetative growth leaf pathogens, do not significantly reduce grain moulds.

Similarly, the effect of fungicides on root rot severity during both the 2013/14 and 2014/15 seasons was limited with no significant effects being recorded with the different fungicide regimes although locality effects were significant (Tables 2.2 and 2.3). A slight fungicide effect was recorded with root mass during 2013/14 where Epoxiconazole/Pyraclostrobin applied at 8+10 weeks resulted in a significantly higher root mass than the control (Table 2.2) although this was not recorded during 2014/15. Similarly, the integration of root mass and root rot severity to provide an index of effective root mass did not indicate any significant effects of fungicides on root efficiency during both seasons (Tables 2.2 and 2.3). Results from the ergosterol analyses on roots are currently being studied.

F*g*SC (*Fusarium graminiarum* species complex) DNA levels in the grain were relatively high during 2013/14 with significant locality effects. Colonization was high at Greytown where higher humidity conditions prevailed. During 2014/15 colonization at all localities was low. During 2013/14 significant fungicide effects were recorded, particularly with Epoxiconazole/Pyraclostrobin were applications closer to flowering, in particular 8 weeks after planting, reduced colonization compared with the control. Variation between treatments, however, was high.

FgSC colonization tendencies were reflected in mycotoxin levels with very low levels recorded during 2014/15 with no significant fungicide effects (Table 2.6). During 2013/14 mycotoxins were significantly higher at Greytown although levels were below EU legal limits (Table 2.5).

DON was significantly lower with Azoxystrobin/Difenoconazole applied at 10 weeks although, as above, variation was high and tendencies were not distinct. Similar tendencies were recoded for NIV and ZEA.

Although significant levels of infection were only recorded during 2013/14 and hence only one season's data could be used, it would appear that the fungicide regimes used for leaf blight have a limited effect on root rots, grain moulds and mycotoxin accumulation. Final analyses and conclusions will be provided in the PhD thesis of MS Danelle van Rooyen to be submitted during 2017.

Table 2.1. Grain mould ratings at Greytown, Potchefstroom and Standerton in 11 fungicide regimes (mean of ratings in four cultivars): 2013/14 and 2014/15

2013/14

Locality	Control	Azoxystrobin/difenoconazole					Epoxiconazole/pyraclostrobin					Locality mean
		200/125g/l	200/125g/l	200/125g/l	200/125g/l	200/125g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	
		6 weeks	8 weeks	10 weeks	6 + 8 weeks	8 + 10	6 weeks	8 weeks	10 weeks	6 + 8 weeks	8 + 10	
		500ml/ha	500ml/ha	500ml/ha	500ml/ha	500ml/ha	1l/ha	1l/ha	1l/ha	1l/ha	1l/ha	
Potchefstroom	3.38	3.13	2.96	3.08	3.04	3.25	3.38	3.29	3.38	3.22	3.00	3.19[b]
Greytown	3.17	3.00	3.17	3.13	3.08	3.17	3.04	2.79	3.04	3.33	2.67	3.05[b]
Standerton	1.92	1.83	1.75	1.42	1.75	1.71	1.63	1.63	1.92	1.88	2.04	1.77[a]
Treatment mean	**2.82**	**2.65**	**2.63**	**2.54**	**2.63**	**2.71**	**2.68**	**2.57**	**2.78**	**2.81**	**2.57**	

Interaction LSD (P<0.05) = 0.67

2014/15

Locality	Control	Azoxystrobin/difenoconazole					Epoxiconazole/pyraclostrobin					Locality mean
		200/125g/l	200/125g/l	200/125g/l	200/125g/l	200/125g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	
		6 weeks	8 weeks	10 weeks	6 + 8	8 + 10	6 weeks	8 weeks	10 weeks	6 + 8 weeks	8 + 10	
		500ml/ha	500ml/ha	500ml/ha	500ml/ha	500ml/ha	1l/ha	1l/ha	1l/ha	1l/ha	1l/ha	
Potchefstroom G	3.56	3.80	3.66	3.84	3.89	3.70	3.77	3.87	3.72	3.76	3.78	**3.76**[b]
Potchefstroom B	3.88	3.64	3.88	3.73	3.95	3.82	3.95	4.03	3.82	4.05	3.78	**3.87**[b]
Greytown	2.83	2.24	2.42	3.08	2.79	2.38	2.82	2.67	2.46	2.61	2.58	**2.62**[a]
Standerton	1.94	1.99	1.84	2.13	2.18	2.29	2.47	1.75	1.78	2.33	1.46	**2.01**[a]
Treatment mean	**3.05**	**2.92**	**2.95**	**3.19**	**3.20**	**3.05**	**3.25**	**3.08**	**2.94**	**3.19**	**2.90**	

Interaction LSD (P<0.05) = 0.84

Table 2.2. Root rot severity, root mass and effective root mass in sorghum from Greytown and Potchefstroom in 11 fungicide regimes (mean of ratings in four cultivars): 2013/14

A: Root rot severity (%)

Locality	Control	Azoxystrobin/difenoconazole					Epoxiconazole/pyraclostrobin					Locality mean
		200/125g/l	200/125g/l	200/125g/l	200/125g/l	200/125g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	
		6 weeks	8 weeks	10 weeks	6 + 8 weeks	8 + 10	6 weeks	8 weeks	10 weeks	6 + 8 weeks	8 + 10	
		500ml/ha	500ml/ha	500ml/ha	500ml/ha	500ml/ha	1l/ha	1l/ha	1l/ha	1l/ha	1l/ha	
Potchefstroom	32.92	29.25	27.00	25.85	31.67	30.33	27.58	31.00	28.96	28.54	34.42	**29.46**
Greytown	25.25	28.42	28.42	28.08	27.92	27.25	26.33	25.33	26.42	26.67	25.08	**26.99**
Treatment	**29.08**	**28.83**	**27.71**	**26.97**	**29.79**	**28.79**	**26.96**	**28.17**	**27.69**	**27.60**	**29.75**	

B: Root mass per plant (g)

Locality	Control	Azoxystrobin/difenoconazole					Epoxiconazole/pyraclostrobin					Locality mean
		200/125g/l	200/125g/l	200/125g/l	200/125g/l	200/125g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	
		6 weeks	8 weeks	10 weeks	6 + 8 weeks	8 + 10	6 weeks	8 weeks	10 weeks	6 + 8 weeks	8 + 10	
		500ml/ha	500ml/ha	500ml/ha	500ml/ha	500ml/ha	1l/ha	1l/ha	1l/ha	1l/ha	1l/ha	
Potchefstroom	32.10	31.80	45.84	35.65	32.03	32.55	42.43	35.35	38.15	39.30	50.76	**38.39** [a]
Greytown	37.34	44.47	47.30	50.55	42.44	36.23	47.04	44.84	51.36	48.11	49.27	**46.16** [b]
Treatment	**34.72** [a]	**38.14** [a]	**46.57** [ab]	**43.10** [ab]	**37.23** [a]	**34.39** [a]	**44.74** [ab]	**40.09** [ab]	**44.76** [ab]	**43.70** [ab]	**50.01** [b]	

Interaction LSD (P<0.05) = 16.84

C: Effective root mass per plant (g)

Locality	Control	Azoxystrobin/difenoconazole					Epoxiconazole/pyraclostrobin					Locality mean
		200/125g/l	200/125g/l	200/125g/l	200/125g/l	200/125g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	
		6 weeks	8 weeks	10 weeks	6 + 8 weeks	8 + 10	6 weeks	8 weeks	10 weeks	6 + 8 weeks	8 + 10	
		500ml/ha	500ml/ha	500ml/ha	500ml/ha	500ml/ha	1l/ha	1l/ha	1l/ha	1l/ha	1l/ha	
Potchefstroom	21.57	22.91	33.99	24.35	21.19	22.45	30.44	24.23	22.90	25.54	33.80	**26.18**
Greytown	27.92	32.09	33.74	36.31	30.57	26.50	34.64	33.70	37.95	35.24	37.26	**33.80**
Treatment	**24.74**	**24.74**	**27.50**	**33.87**	**30.33**	**25.88**	**24.48**	**32.54**	**28.96**	**30.43**	**30.39**	

Table 2.3. Root rot severity, root mass and effective root mass in sorghum from Greytown and Potchefstroom in 11 fungicide regimes (mean of ratings in four cultivars): 2014/15

A: Root rot severity (%)

Locality	Control	Azoxystrobin/difenoconazole					Epoxiconazole/pyraclostrobin					Locality mean
		200/125g/l	200/125g/l	200/125g/l	200/125g/l	200/125g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	
		6 weeks	8 weeks	10 weeks	6 + 8 weeks	8 + 10 weeks	6 weeks	8 weeks	10 weeks	6 + 8 weeks	8 + 10 weeks	
		500ml/ha	500ml/ha	500ml/ha	500ml/ha	500ml/ha	1l/ha	1l/ha	1l/ha	1l/ha	1l/ha	
Potchefstroom G	32.50	28.17	27.00	27.17	31.42	27.75	34.75	31.25	34.92	32.17	33.17	**30.78** [ab]
Potchefstroom B	35.00	37.92	35.08	33.25	32.67	33.92	32.50	36.42	31.92	36.92	36.50	**34.71** [b]
Greytown	30.50	26.92	27.50	28.00	27.00	24.08	26.33	28.67	26.83	27.75	27.83	**27.09** [a]
Treatment mean	**32.67**	**31.00**	**29.86**	**29.47**	**30.36**	**28.58**	**31.19**	**32.11**	**31.22**	**32.28**	**32.50**	

B: Root mass per plant (g)

Locality	Control	Azoxystrobin/difenoconazole					Epoxiconazole/pyraclostrobin					Locality mean
		200/125g/l	200/125g/l	200/125g/l	200/125g/l	200/125g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	
		6 weeks	8 weeks	10 weeks	6 + 8 weeks	8 + 10 weeks	6 weeks	8 weeks	10 weeks	6 + 8 weeks	8 + 10 weeks	
		500ml/ha	500ml/ha	500ml/ha	500ml/ha	500ml/ha	1l/ha	1l/ha	1l/ha	1l/ha	1l/ha	
Potchefstroom G	23.56	18.46	23.15	23.28	18.60	17.03	17.69	21.30	24.49	24.74	18.74	**20.75** [a]
Potchefstroom B	28.02	23.00	27.67	21.02	22.12	22.98	26.91	22.81	27.75	25.64	28.78	**24.87** [a]
Greytown	36.43	36.08	39.24	42.05	41.77	38.95	40.88	41.13	39.86	33.07	35.65	**38.87** [b]
Treatment mean	**29.33**	**25.85**	**30.02**	**28.78**	**27.50**	**26.32**	**28.49**	**28.41**	**30.70**	**27.82**	**27.72**	

C: Effective root mass per plant (g)

Locality	Control	Azoxystrobin/difenoconazole					Epoxiconazole/pyraclostrobin					Locality mean
		200/125g/l	200/125g/l	200/125g/l	200/125g/l	200/125g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	
		6 weeks	8 weeks	10 weeks	6 + 8 weeks	8 + 10 weeks	6 weeks	8 weeks	10 weeks	6 + 8 weeks	8 + 10 weeks	
		500ml/ha	500ml/ha	500ml/ha	500ml/ha	500ml/ha	1l/ha	1l/ha	1l/ha	1l/ha	1l/ha	
Potchefstroom G	15.99	13.24	16.60	17.20	12.84	12.58	11.43	14.44	15.57	16.67	12.47	**14.30** [a]
Potchefstroom B	18.21	14.49	17.83	14.18	14.94	15.24	18.32	14.71	19.09	16.62	18.35	**16.37** [a]
Greytown	25.35	26.31	28.41	30.38	30.56	29.51	29.96	29.47	29.08	24.10	25.70	**28.35** [b]
Treatment mean	**19.85**	**18.01**	**20.94**	**20.58**	**19.45**	**19.11**	**19.90**	**19.54**	**21.24**	**19.13**	**18.84**	

Table 2.4. ***F. graminearum*** **DNA as an indicator of colonization by the pathogen in sorghum from Greytown and Potchefstroom in 11 fungicide regimes (mean of ratings in four cultivars): 2013/14 and 2014/15**

2013/14

Locality	Control	Azoxystrobin/difenoconazole					Epoxiconazole/pyraclostrobin					Locality mean
		200/125g/l	200/125g/l	200/125g/l	200/125g/l	200/125g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	
		6 weeks	8 weeks	10 weeks	6 + 8	8 + 10	6 weeks	8 weeks	10 weeks	6 + 8 weeks	8 + 10	
		500ml/ha	500ml/ha	500ml/ha	500ml/ha	500ml/ha	1l/ha	1l/ha	1l/ha	1l/ha	1l/ha	
Potchefstroom	10.81	9.05	10.63	6.74	4.73	4.90	8.23	6.03	9.87	5.89	1.12	**7.09** [a]
Greytown	325.00	326.72	266.65	215.11	255.63	261.67	267.56	168.55	577.05	305.31	413.69	**307.54** [b]
Standerton	11.15	7.04	12.28	9.74	19.68	17.19	7.53	10.34	11.26	13.63	11.97	**11.98** [a]
Treatment mean	**115.66** [b]	**114.27** [b]	**96.52** [ab]	**77.19** [ab]	**93.35** [ab]	**94.59** [ab]	**94.44** [ab]	**61.64** [a]	**199.39** [c]	**108.28** [ab]	**142.26** [b]	

Interaction LSD (P<0.05) = 296.97

2014/15

Locality	Control	Azoxystrobin/difenoconazole					Epoxiconazole/pyraclostrobin					Locality mean
		200/125g/l	200/125g/l	200/125g/l	200/125g/l	200/125g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	
		6 weeks	8 weeks	10 weeks	6 + 8	8 + 10	6 weeks	8 weeks	10 weeks	6 + 8 weeks	8 + 10	
		500ml/ha	500ml/ha	500ml/ha	500ml/ha	500ml/ha	1l/ha	1l/ha	1l/ha	1l/ha	1l/ha	
Potchefstroom G	30.41	17.29	16.50	0.44	0.77	1.41	0.45	0.57	0.63	0.29	1.21	**6.36** [a]
Potchefstroom B	4.31	0.43	0.57	0.99	4.51	4.82	5.22	5.97	6.50	6.35	5.22	**4.08** [a]
Greytown	31.41	22.91	5.49	8.46	11.06	4.81	5.27	10.89	109.84	230.15	178.67	**56.27** [b]
Standerton	0.39	0.33	0.35	0.50	0.19	1.08	2.09	1.74	3.43	2.25	2.12	**1.32** [a]
Treatment mean	**16.63**	**10.24**	**5.73**	**2.60**	**4.13**	**3.03**	**3.26**	**4.80**	**30.10**	**59.76**	**46.81**	

Table 2.5. ***F. graminearum*** **- related mycotoxins in sorghum grain from Greytown and Potchefstroom in 11 fungicide regimes (mean of ratings in four cultivars): 2013/14**

DON 2013/14

Locality	Control	Azoxystrobin/difenoconazole					Epoxiconazole/pyraclostrobin					Locality mean
		200/125g/l	200/125g/l	200/125g/l	200/125g/l	200/125g/l	62.5/62.5g/	62.5/62.5g/	62.5/62.5g/	62.5/62.5g/	62.5/62.5g/l	
		6 weeks	8 weeks	10 weeks	6 + 8	8 + 10	6 weeks	8 weeks	10 weeks	6 + 8	8 + 10 weeks	
		500ml/ha	500ml/ha	500ml/ha	500ml/ha	500ml/ha	1l/ha	1l/ha	1l/ha	1l/ha	1l/ha	
Potchefstroom	1.50	1.63	3.14	3.27	7.58	4.30	1.33	1.81	2.22	2.35	1.29	**2.77** [a]
Greytown	215.58	210.62	210.65	139.17	139.50	254.43	304.27	211.36	387.08	277.07	275.60	**238.66** [b]
Standerton	109.40	2.74	2.48	2.46	88.52	104.47	4.48	7.80	4.51	7.86	5.32	**30.91** [a]
Treatment mean	**108.83** [bc]	**71.66** [ab]	**72.09** [ab]	**48.30** [a]	**78.53** [ab]	**121.06** [c]	**103.36** [bc]	**73.65** [ab]	**131.27** [c]	**95.76** [bc]	**94.07** [bc]	

Interaction LSD (P<0.05) = 218.34

NIV 2013/14

Locality	Control	Azoxystrobin/difenoconazole					Epoxiconazole/pyraclostrobin					Locality mean
		200/125g/l	200/125g/l	200/125g/l	200/125g/l	200/125g/l	62.5/62.5g/	62.5/62.5g/	62.5/62.5g/	62.5/62.5g/	62.5/62.5g/l	
		6 weeks	8 weeks	10 weeks	6 + 8	8 + 10	6 weeks	8 weeks	10 weeks	6 + 8	8 + 10 weeks	
		500ml/ha	500ml/ha	500ml/ha	500ml/ha	500ml/ha	1l/ha	1l/ha	1l/ha	1l/ha	1l/ha	
Potchefstroom	14.14	5.30	12.45	9.28	3.03	12.42	9.39	8.55	24.86	8.85	13.91	**11.11** [a]
Greytown	270.75	261.47	264.95	251.64	309.25	221.50	384.61	279.25	395.91	254.18	316.40	**291.81** [b]
Standerton	12.80	6.75	20.99	17.45	25.63	13.28	20.34	0.66	1.47	0.36	4.55	**11.30** [a]
Treatment mean	**99.23** [ab]	**91.17** [ab]	**99.46** [ab]	**92.79** [ab]	**112.63** [bc]	**82.40** [a]	**138.11** [cd]	**96.15** [ab]	**140.75** [d]	**87.80** [a]	**111.62** [bc]	

Interaction LSD (P<0.05) = 137.84

ZEA 2013/14

Locality	Control	Azoxystrobin/difenoconazole					Epoxiconazole/pyraclostrobin					Locality mean
		200/125g/l	200/125g/l	200/125g/l	200/125g/l	200/125g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	
		6 weeks	8 weeks	10 weeks	6 + 8	8 + 10	6 weeks	8 weeks	10 weeks	6 + 8	8 + 10 weeks	
		500ml/ha	500ml/ha	500ml/ha	500ml/ha	500ml/ha	1l/ha	1l/ha	1l/ha	1l/ha	1l/ha	
Potchefstroom	26.58	7.40	5.05	2.19	0.44	0.51	0.41	0.51	46.64	0.49	0.51	8.25
Greytown	244.28	572.50	818.75	742.75	628.58	257.00	516.70	967.91	1577.63	761.25	1111.00	745.30
Standerton	5.67	132.25	30.67	13.18	276.10	86.81	14.88	9.51	7.15	50.07	0.49	56.98
Treatment mean	**92.18** [a]	**237.39** [bc]	**284.82** [cd]	**252.71** [bc]	**301.71** [cd]	**114.78** [a]	**177.33** [ab]	**325.98** [cd]	**543.81** [e]	**270.60** [bc]	**370.67** [d]	

Interaction LSD (P<0.05) = 584.93

Table 2.6. ***F. graminearum*** **– related mycotoxins in sorghum grain from Greytown and Potchefstroom in 11 fungicide regimes (mean of ratings in four cultivars): 2014/15**

DON 2014/15

Locality	Control	Azoxystrobin/difenoconazole					Epoxiconazole/pyraclostrobin					Locality mean
		200/125g/l	200/125g/l	200/125g/l	200/125g/l	200/125g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	
		6 weeks	8 weeks	10 weeks	6 + 8	8 + 10	6 weeks	8 weeks	10 weeks	6 + 8 weeks	8 + 10 weeks	
		500ml/ha	500ml/ha	500ml/ha	500ml/ha	500ml/ha	1l/ha	1l/ha	1l/ha	1l/ha	1l/ha	
Potchefstroom G	2.64	4.80	3.25	2.44	4.13	5.04	4.42	2.29	6.06	6.23	9.13	**4.58** [a]
Potchefstroom B	9.68	2.42	8.28	10.00	3.05	0.00	1.06	1.62	3.39	1.84	1.66	**3.91** [a]
Greytown	77.54	186.00	74.05	214.65	2.13	194.38	119.39	55.45	84.24	54.94	37.33	**100.01** [b]
Standerton	4.84	3.01	2.49	8.85	1.78	6.53	3.99	6.73	4.38	5.88	2.90	**4.67** [a]
Treatment mean	**23.68**	**49.06**	**22.02**	**58.98**	**2.77**	**51.49**	**32.21**	**16.52**	**24.52**	**17.22**	**12.75**	

NIV 2014/2015

Locality	Control	Azoxystrobin/difenoconazole					Epoxiconazole/pyraclostrobin					Locality mean
		200/125g/l	200/125g/l	200/125g/l	200/125g/l	200/125g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	
		6 weeks	8 weeks	10 weeks	6 + 8	8 + 10	6 weeks	8 weeks	10 weeks	6 + 8 weeks	8 + 10 weeks	
		500ml/ha	500ml/ha	500ml/ha	500ml/ha	500ml/ha	1l/ha	1l/ha	1l/ha	1l/ha	1l/ha	
Potchefstroom G	0.39	0.60	0.39	0.32	0.55	0.23	0.00	0.39	3.06	6.36	4.94	**1.57** [a]
Potchefstroom B	5.96	4.98	3.89	6.28	14.85	10.09	10.69	12.32	13.74	18.26	10.11	**10.10** [a]
Greytown	189.25	161.98	125.43	118.13	135.33	140.27	138.39	117.86	91.33	61.08	82.39	**123.77** [b]
Standerton	9.22	14.32	4.82	1.09	0.58	0.39	2.23	2.16	0.15	0.17	0.11	**3.20** [a]
Treatment mean	**65.20**	**55.85**	**43.23**	**41.57**	**50.24**	**50.19**	**49.69**	**43.52**	**36.04**	**28.56**	**32.48**	

ZEA 2014/2015

Locality	Control	Azoxystrobin/difenoconazole					Epoxiconazole/pyraclostrobin					Locality mean
		200/125g/l	200/125g/l	200/125g/l	200/125g/l	200/125g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	62.5/62.5g/l	
		6 weeks	8 weeks	10 weeks	6 + 8	8 + 10	6 weeks	8 weeks	10 weeks	6 + 8 weeks	8 + 10 weeks	
		500ml/ha	500ml/ha	500ml/ha	500ml/ha	500ml/ha	1l/ha	1l/ha	1l/ha	1l/ha	1l/ha	
Potchefstroom G	1.13	0.62	7.83	0.68	0.31	0.37	0.48	0.37	0.25	0.28	0.33	**1.15** [a]
Potchefstroom B	0.32	0.21	0.33	0.36	0.42	1.39	0.52	0.88	0.75	0.43	0.45	**0.55** [a]
Greytown	459.55	703.60	436.98	250.33	225.00	425.59	477.08	297.94	217.05	188.75	242.08	**356.72** [b]
Standerton	0.30	0.40	0.35	0.52	0.53	0.75	0.44	0.31	0.35	0.46	0.42	**0.44** [a]
Treatment mean	**115.32**	**176.21**	**111.37**	**62.97**	**56.56**	**107.02**	**119.63**	**74.88**	**54.60**	**47.48**	**60.82**	

2.4. References

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Section 3: The evaluation of four sorghum cultivars for their resistance to Leaf blight and the potential yield loss impact associated with *Exserohilum turcicum* infection

3.1. Introduction

Leaf blight is a polycyclic disease, which implies that the causal organism associated with the disease, can cause more than one subsequent infection in a single season. Infection 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.

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).

The aim of the current study was to evaluate four sorghum cultivars for their susceptibility to Leaf blight and to establish potential yield loss associated with *Exserohilum turcicum* infection.

3.2. Materials and methods

3.2.1. Inoculum generation

Two leaf blight isolates obtained from sorghum (reference: ARC-Ht72 and ARC-HT73) were plated out on potato dextrose agar (PDA). The isolates were grown on PDA in petri dishes for two weeks before mycelial plugs were transferred to autoclaved maize kernels in fruit flasks (prepared according to Flett and McLaren, 1994) and allowed to incubate under bench temperature. Flasks were shaken daily. After two weeks, the contents of the flasks were dried (isolates separately) for three days after which the maize kernels were ground in a standard maize mill. The mill was thoroughly cleaned after each isolate batch. After milling equal amounts of each of the two isolates were added and thoroughly mixed to obtain an inoculation mixture.

3.2.2. Field trial

A field trial to evaluate the yield impact of leaf blight on four sorghum cultivars were planted during 2014/15 at Potchefstroom (Photo 3.1.). The trial was planted as a randomised block design with three block replicates. The treatment design was a split-plot with fungicide as main plot and cultivar as sub-plot factor. Two fungicides, azoxystrobin/difenconazole (formulation - 200/125 g l-1; Amistar Top®, Syngenta) and epoxiconazole/pyraclostrobin (formulation - 62.5/62.5 g l-1; Abacus®, BASF) were included in the study. Cultivars were planted as single rows within the main plots and included PAN8816, PAN8906, PAN8625 and NS5511. Each main plot was flanked by a single border row (PAN8816). Plots consisted of six rows, 5 m in length spaced 0.9 m apart. Plant density at all localities was 88 000 plants ha^{-1}.The trial received supplementary water via an overhead sprinkler system. To ensure optimum growth, soil analyses were conducted at each locality and fertilizer was applied accordingly. Frontier® Optima (dimethenamid - 75 g l-1; BASF) was applied pre-emergence and Basagran® (bendioxide 480 g l-1; BASF) post-emergence to prevent weed encroachment. Mid-season stem-borer control was done with Karate® (ambda-cyhalothrin 5.5%, 100 ml ha-1; Syngenta). Weather data was obtained from automatic weathers stations within 50 km of the trials sites.

3.2.3. Inoculation

All the sorghum plots were inoculated with 6g of the inoculum mixture at 5 and 7 leaf stage.

3.2.4. Fungicide treatments

Two foliar fungicide formulations i.e. azoxystrobin/difenconazole (application rate - 500 ml ha-1) and epoxiconazole/pyraclostrobin (application rate - 1 l ha^{-1}), were evaluated at five application times (treatments) i.e. 6 weeks, 6 & 8 weeks, 8 weeks, 8 & 10 weeks and 10 weeks after planting (WAP) and compared to an untreated control (Photo 2). The fungicides were Amistar Top®, the only registered fungicide for the control of LB in South Africa when the trials commenced during 2013, while Abacus® is a product commonly used by producers even though not registered for the control of LB. Fungicides were applied using a CO^2 gas operated knapsack sprayer and a four nozzle (flat fan; 0.9 m spaced) boom. The knap sack sprayer was calibrated to a spray volume of 78 l ha^{-1}.

3.2.5. Crop measurements and analysis

Leaf blight severity, plant mass as well as yield were measured in the current study. Ten randomly selected plants within each row were screened at flower, soft dough stage as well

as hard 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% (Elliot and Jenkins, 1946; Adipala et al., 1993).

Panicles were harvested when the kernel moisture was <15%, even though the plant material was, in some cases, not fully senesced. After the panicles were removed, the remaining plant tissues (stalk and leaves) were used for plant mass determination. Ten plants per cultivar per plot were randomly selected and weighed using a tripod scale. The combined weight (kg) of the ten plants was documented.

Harvested panicles were threshed and grain weight obtained. Grain moisture was determined with a TwistGrain moisture meter (Draminski Elektronics). Yield (t ha^{-1}) was calculated at 12.5% moisture.

Analysis of variance (ANOVA) for a split-plot experimental design was performed with treatment (fungicide application) as the main plot and cultivar as the subplot factor. Student's t-Least Significant Differences were calculated at the 5% level to compare treatment means of significant effects. All the analyses were done using SAS v9.2 statistical software (SAS, 1999).

Linearised forms of the exponential, logistic and Gompertz models were fitted to the disease progress data. The best fitted model was selected based on the coefficient of determination (R^2) and the mean square error (MSE).

3.3. Results and discussion

Disease severity measured at the hard dough stage of the Potchefstroom inoculated trial varied between 4.4% and 69.33% over the various cultivars and treatments (Photo 3.2 and 3.3). Evaluation on the control plot results only, indicated that PAN8906 as well as PAN8625 were the most susceptible cultivars measuring LB disease severity of 65.33 and 59.78% respectively (Figure 3.1). PAN8816 had 52% diseased leaf area, whilst NS5511 demonstrated a high level of disease resistance with only 8.9% disease leaf area measured at hard dough stage.

Both fungicides tested, gave similar control to each other at the various application dates, with the best control being obtained when they were applied twice during the season (8 and 10 weeks after planting)(Figure 3.2). None of the applications significantly reduced the LB

severity with the resistant NS 5511 compared to its control treatment, indicating that even under such severe infections levels, it was not economically viable to spray this specific cultivar in order to control the disease.

Despite the significant differences obtained regarding the application of fungicides on disease severity, yield obtained did not differ significantly between cultivars, treatments or the cultivar x treatment interaction.

The majority of the models fitted to the disease severity data of the various cultivars as measured at flowering, soft dough and hard dough all had very low R^2 values, suggesting that no clear pattern as to how the cultivars reacted regarding yield loss in response to leaf blight severity could be obtained. An interesting observation was that the response obtained by the two fungicides were different, with the best fit in generally being where Amistar Top ® was applied. This observation warrants an in-depth study.

An R^2 value of 0.7877 and R^2=0.8417, were, however, respectively obtained with PAN8625 when the disease severity observed at flowering and soft dough stages were plotted against the eventual yield obtained. (linear fit being achieved). Based on the regression slope, a yield decline of 4.39 kg ha^{-1} was experienced per 1% increase in disease severity at flowering. Expressed differently, a yield loss of 7.9% was experienced for every 10 % increase in disease severity at flowering. A 5.4% yield loss was similarly experienced by this cultivar for every 10% increase observed in leaf blight severity at soft dough stage. These findings are similar to what has been observed with regard to Northern corn leaf blight on maize, where yield losses of between 2 and 8% were observed for every 10% increase in disease severity (Fisher et al., 1976, Bowen and Pedersen, 1988).

Photo 3.1. Field trial planted during 2014/15 at Potchefstroom to evaluate four sorghum cultivars for their susceptibility to leaf blight.

Photo 3.2. Leaf blight severity obtained during 2014/15.

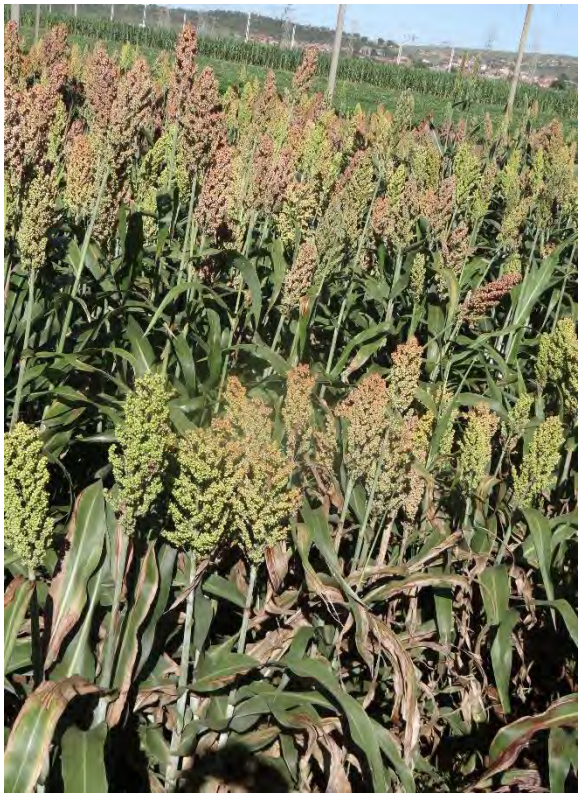

Photo 3.3. Differences in susceptibility to leaf blight observed between two sorghum cultivars (front row as opposed to the back row)

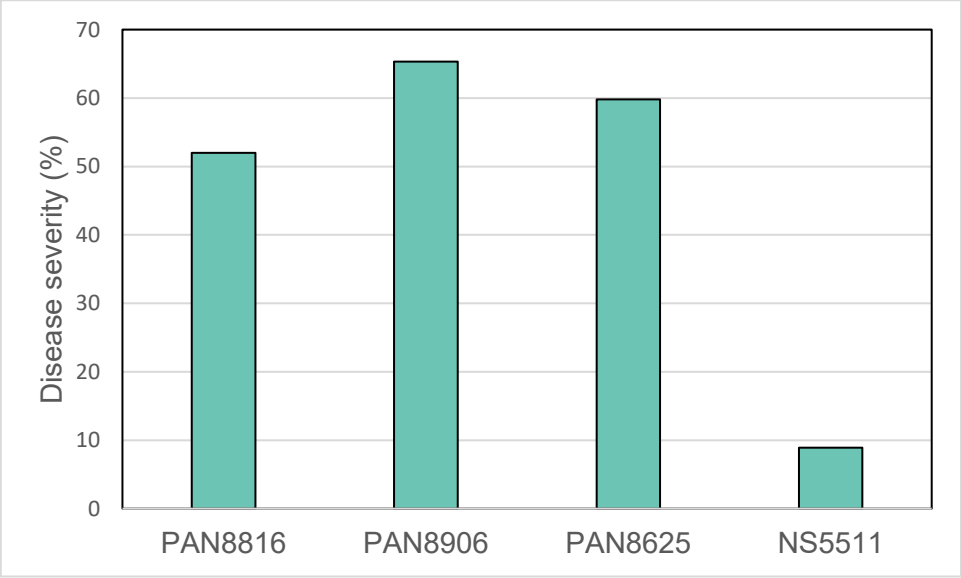

Figure 3.1. Average leaf blight disease severity as measured in the control plots of four sorghum cultivars.

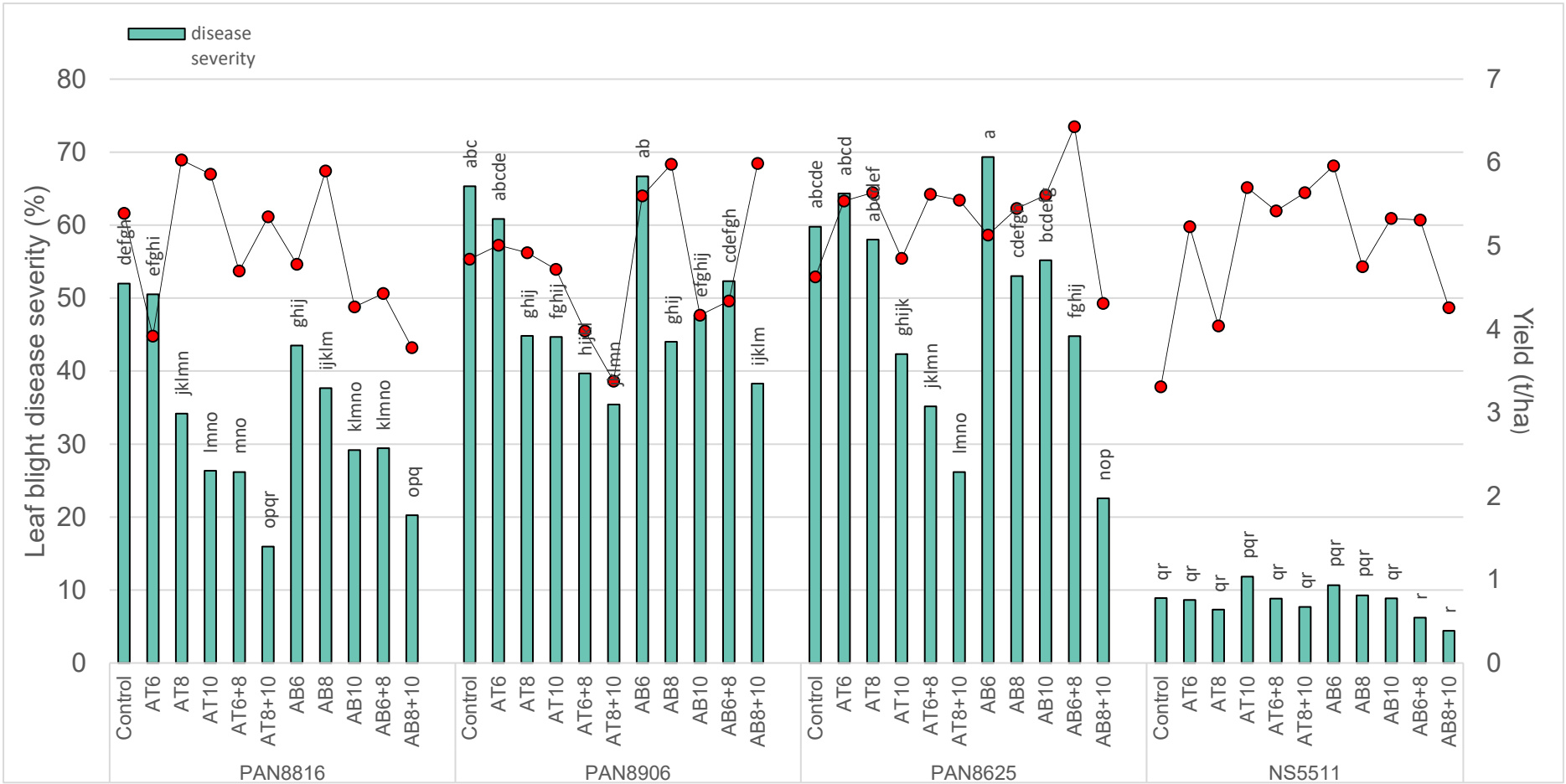

Figure 3.2. Disease severity (%) and subsequent yield (t/ha) obtained on four sorghum cultivars at 11 treatment applications (AT- Amistar TOP®; AB - Abacus; applications at 6, 8, 10, 6+8 and 8+10 weeks after planting).

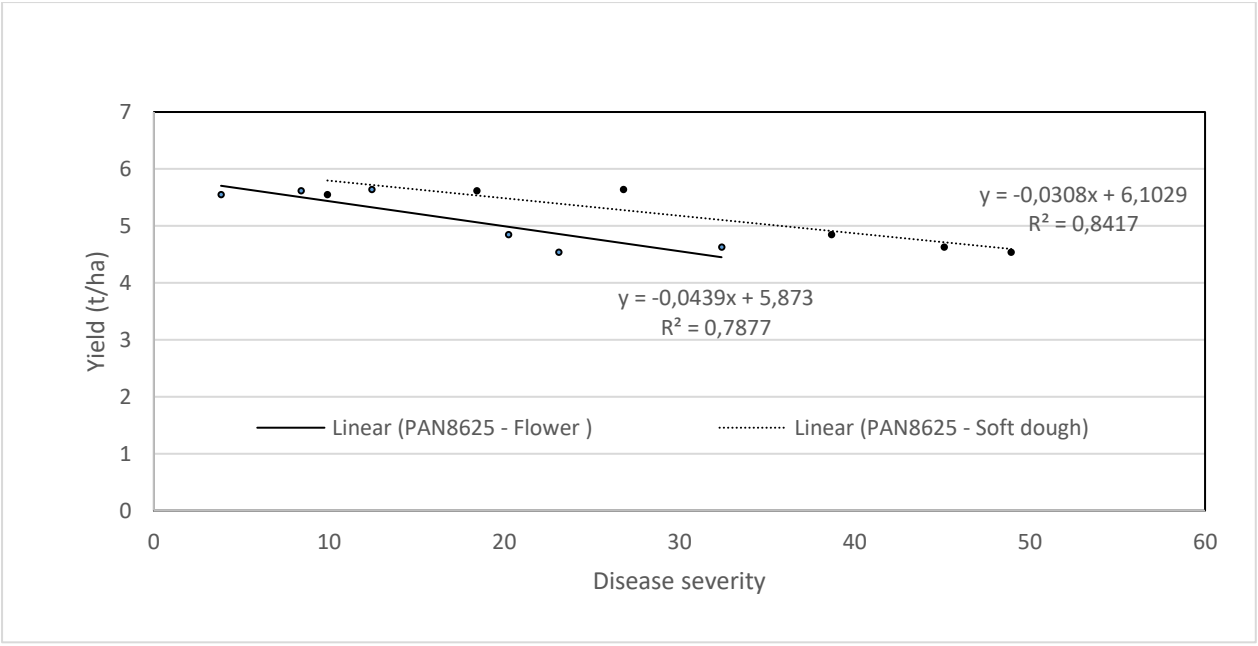

Figure 3.3. Yield loss associated with leaf blight severity as observed at flowering and soft dough stage respectively for PAN8625.

3.4. References

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Section 4: Biochemical and physiological responses of sorghum to fungicide application under glasshouse conditions.

Research presented in this section formed part of an MSc study conducted by Ms K Smith (NWU) - awarded during May 2017.

4.1. Introduction

Results obtained from field trials conducted during 2013/14 and 2014/15 (Section 1) indicated that the prophylactic use of fungicide was not able to increase yield in the absence, or with low levels of leaf blight (LB) development. A delay in plant senescence was, however, observed at some localities, which warranted an in-depth study into the plant's response as a result of fungicide application. Senescence is known to be an oxidative process, characterized by denaturing of proteins, DNA and lipids, 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 results in decreases in active photosynthetic pigments, impacting negatively on the total vitality of the plant.

Chlorophyll, that absorbs light energy, is situated in the chloroplast and is referred to as the photosystem. Different types of chlorophylls are present within plants and differ in their capability to absorb different wavelengths of light. Two photosystems exists i.e. photosystem I (PSL) and photosystem II (PSII). 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 (Lichtenthaler, 1995), and the ability of the plant to photosynthesize is reduced (van Kooten and Snel, 1990). According to Dias (2012), the application of triazoles reportedly lead to a decrease in the quantum yield of PSII (photosystem II) due to decreases in photochemical quenching of the quinone pools. Strobilurins, on the other hand, block the electron transport between PSII and PSI (photosystem I) 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 senescence progress, the generation of reactive oxygen species such as superoxide, hydroxyl radical, hydroperoxyl, hydrogen peroxide, singlet oxygen, and excited carbonyl 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. Reactive oxygen species (ROS) scavenging enzymes such as peroxidase (POD) and superoxide dismutase (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.

As black layer formation is linked 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, this process may be delayed, leading to increased sugar accumulation and higher grain yield.

A glasshouse trial was conducted to examine the effects of fungicide application on sorghum plants' photosynthetic efficiency, anti-oxidative enzyme mechanisms, sugar accumulation and black layer formation in the seeds in order to investigate the biochemical and physiological responses that occur due to the prophylactic fungicide application.

4.2. Materials and methods

4.2.1. Glasshouse trial

Limited differences between cultivars regarding their response to fungicide application could, in general, be observed in the field trials conducted during 2013/14 (See Section 1). 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 the most significant results during the 2013/14 field trial with respect to delays in senescence and yield (See Section 1). 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 (AT8)	500 mL.ha^{-1}
3	Azoxystrobin/difenoconazole		10 weeks (AT10)	
4	Azoxystrobin/difenoconazole		8&10 weeks (AT8&10)	
5	Epoxiconazole/pyraclostrobin	62.5 / 62.5 g.ha^{-1}	8 weeks (AB8)	1L.ha^{-1}
6	Epoxiconazole/pyraclostrobin		10 weeks (AB10)	
7	Epoxiconazole/pyraclostrobin		8 & 10 weeks (AB8&10)	

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). 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.

4.2.2. 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 that 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.

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

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. 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).

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

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

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

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

4.2.3. Analysis of enzyme activity

4.2.3.1. 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, samples were ground to a powder in liquid N_2 and placed in 6 mL scintillation tubes. Samples were stored at -80°C.

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.

4.2.3.2. Peroxidase activity (POD)

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 mL of 40 mM KH_2PO_4 pH 5.5,
- 680 µL d H_2O,
- 200µL 5 mM guaiacol,
- 20 µL enzyme extract, and
- 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. Specific enzyme activity is expressed as nmol guaiacol.mg^{-1}protein.min^{-1}.

4.2.3.3. Superoxide dismutase activity (SOD)

Superoxide dismutase (SOD) activity was determined according to Keppler and Novacky (1987). Leaf material was extracted as described above. Enzyme activity was determined immediately 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 mL of 130 mM methionine,
    - 10mL of 200 µM riboflavin,
    - 1 mL of 10 mM EDTA, and
    - 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 is expressed as nmol NBT.mg^{-1}protein.min^{-1}.

4.2.3.4. Xanthine oxidase activity (XOX)

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:

- 150 mM xanthine was dissolved in a PBS pH 7.5 (substrate),
- 1.45 mL PBS, and
- 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}.

4.2.3.5. 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 dH2O, 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.

4.2.3.6 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 remove 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.

4.2.3. Statistical analysis

The treatment design was a 7x2 factorial consisting of seven treatments (Table 4-1) and two 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. 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.

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.

4.3. Results

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

4.3.1.1. 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. are indicated in Figure 4.1.

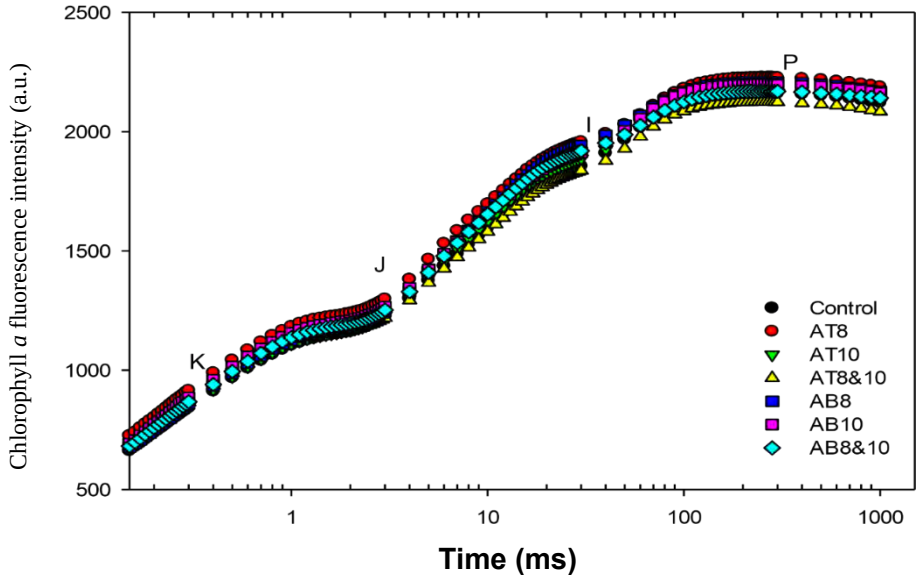

Figure 4.1. OJIP curve for PAN8816 70 days after planting. Treatments are as indicated AT and AB implying treatment with Amistar TOP® and Abacus® respectively and 8, 10 or 8 & 10 the application date in weeks after planting.

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).

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).

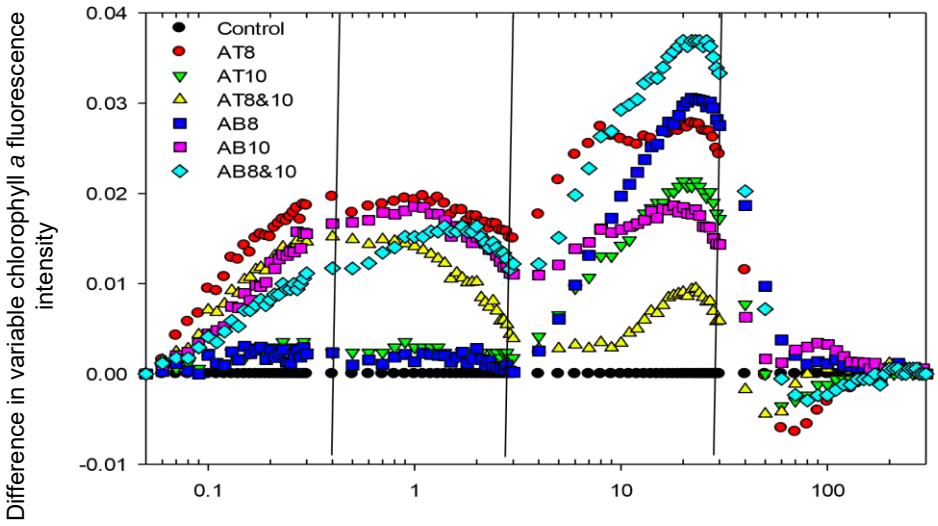

Figure 4.2. Difference in relative variable fluorescence for PAN8816 70 days after planting normalized between 0.05 ms and 300 ms. Treatments are as indicated AT and AB implying treatment with Amistar Top® and Abacus® respectively and 8, 10 or 8&10 the application date in weeks after planting.

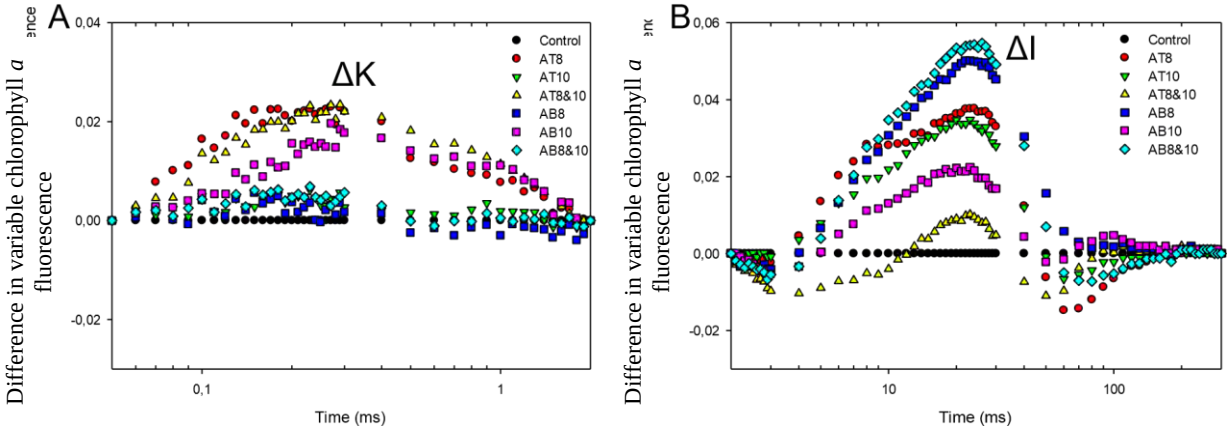

Figure 4.3. A-B: Difference is relative variable fluorescence for PAN8816 70 days after planting normalized between 0.05 ms and 2 ms, and 2 ms and 300 ms respectively. Treatments are as indicated AT and AB implying treatment with Amistar Top® and Abacus® respectively and 8, 10 or 8&10 the application date in weeks after planting.

4.3.1.2. 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. are indicated in Figure 4.4.

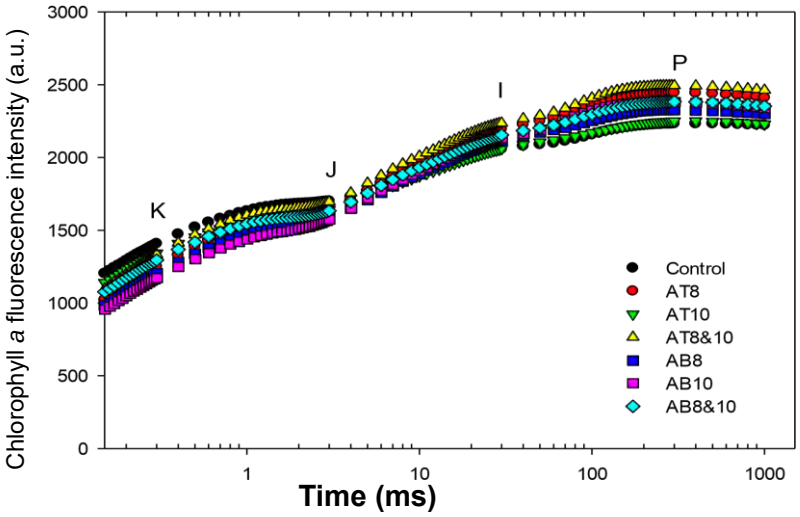

Figure 4.3. OJIP curve obtained 110 days after planting for PAN8816. Treatments are as indicated AT and AB implying treatment with Amistar Top® and Abacus® respectively at 8, 10 or 8 &10 the application date in weeks after planting.

Shapiro-Wilk's paired t-test indicated that no treatment had a significant effect on the difference in relative fluorescence (Figure 4.4).

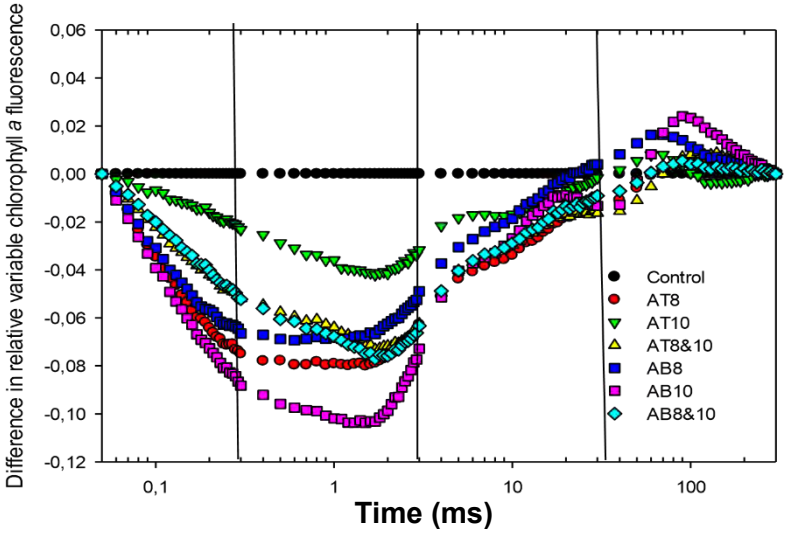

Figure 4.4: Difference in relative variable chlorophyll a fluorescence between 0.05 ms .nd 300 ms. Treatments are as indicated AT and AB implying treatment with Amistar Top® and Abacus® respectively and 8, 10 or 8&10 the application date in weeks after planting.

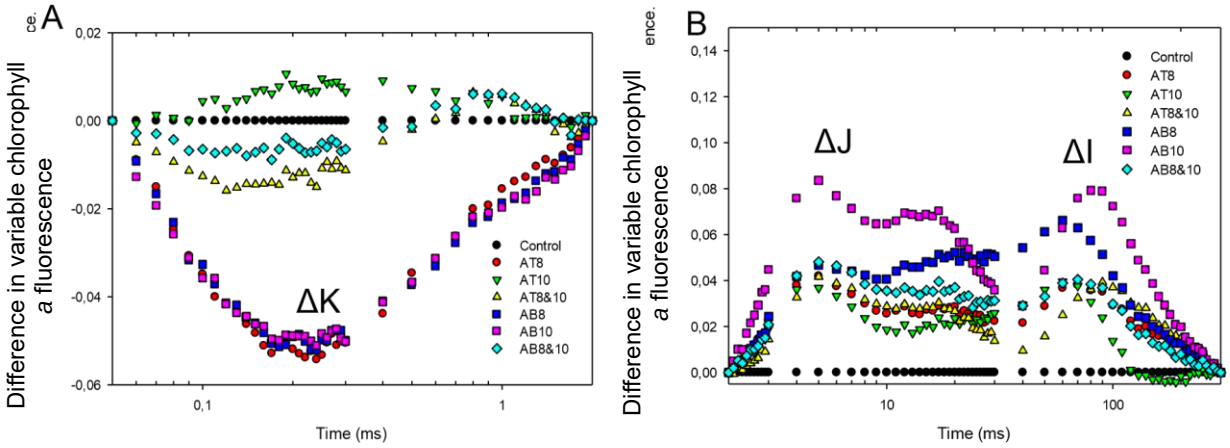

Figure 4.5. A-B: Difference in relative variable chlorophyll a fluorescence between 0.05 ms and 2 ms, and 2 ms and 300 ms respectively 110 day after planting. Treatments are as indicated AT and AB implying treatment with Amistar Top® and Abacus® respectively and 8, 10 or 8&10 the application date in 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 (ΔK-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 ΔJ-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 ΔJ-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 ΔI-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).

4.3.1.3. 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.

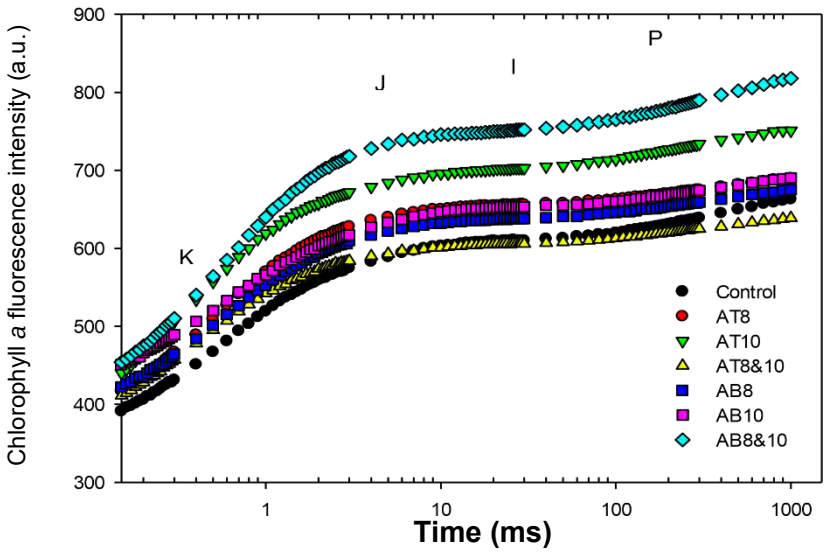

Figure 4.7. OJIP PAN8816 at 150 days after planting. Treatments are as indicated AT and AB implying treatment with Amistar Top® and Abacus® respectively and 8, 10 or 8 & 10 the application date in weeks after planting.

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.

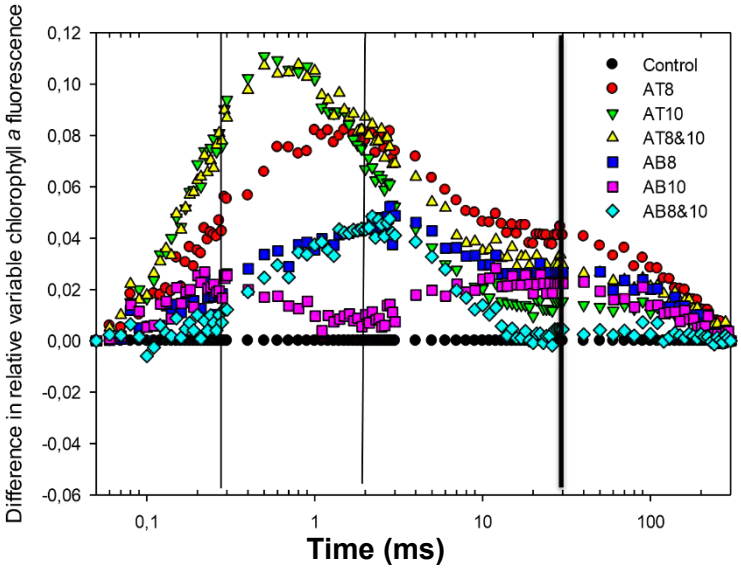

Figure 4.8. Difference in relative variable chlorophyll a fluorescence between 0.05 ms and 300 ms 150 days after planting. Treatments are as indicated AT and AB implying treatment with Amistar Top® and Abacus® respectively and 8, 10 or 8 & 10 the application d date in weeks after planting.

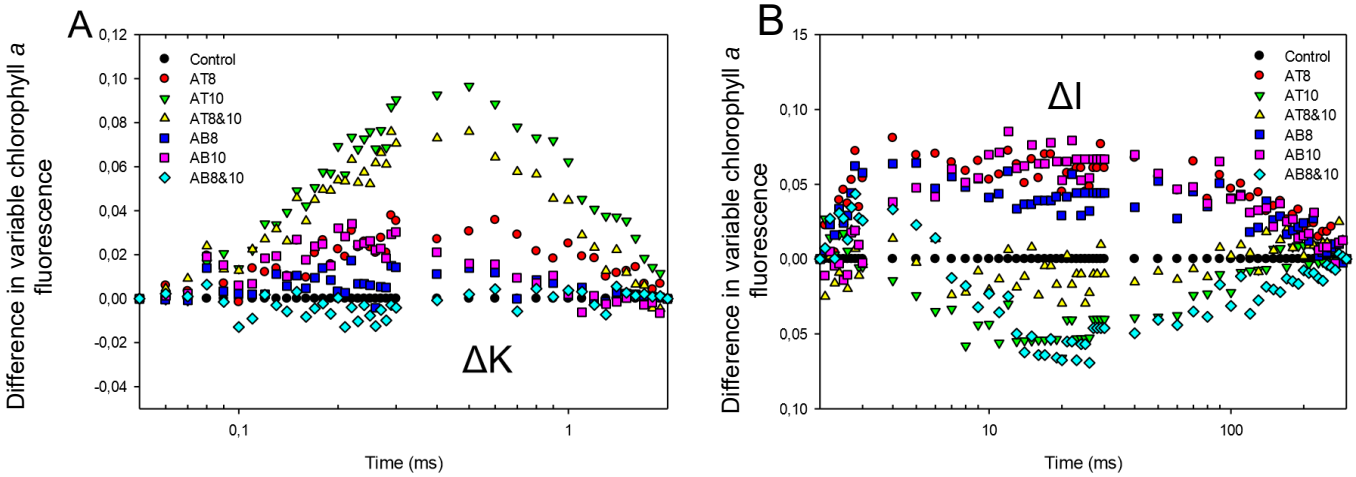

Figure 4.9. A-B: Difference in relative variable chlorophyll a fluorescence between 0.05 ms and 2 ms, and 2 ms and 300 ms 150 days after planting. Treatments are as indicated AT and AB implying treatment with Amistar Top® and Abacus® respectively and 8, 10 or 8 & 10 the application d date in weeks after planting.

4.3.1.4. The performance index and its partial parameters for PAN8816

By arranging the above transients into more quantifiable groups, the following results are obtained. Figure 4.10 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).

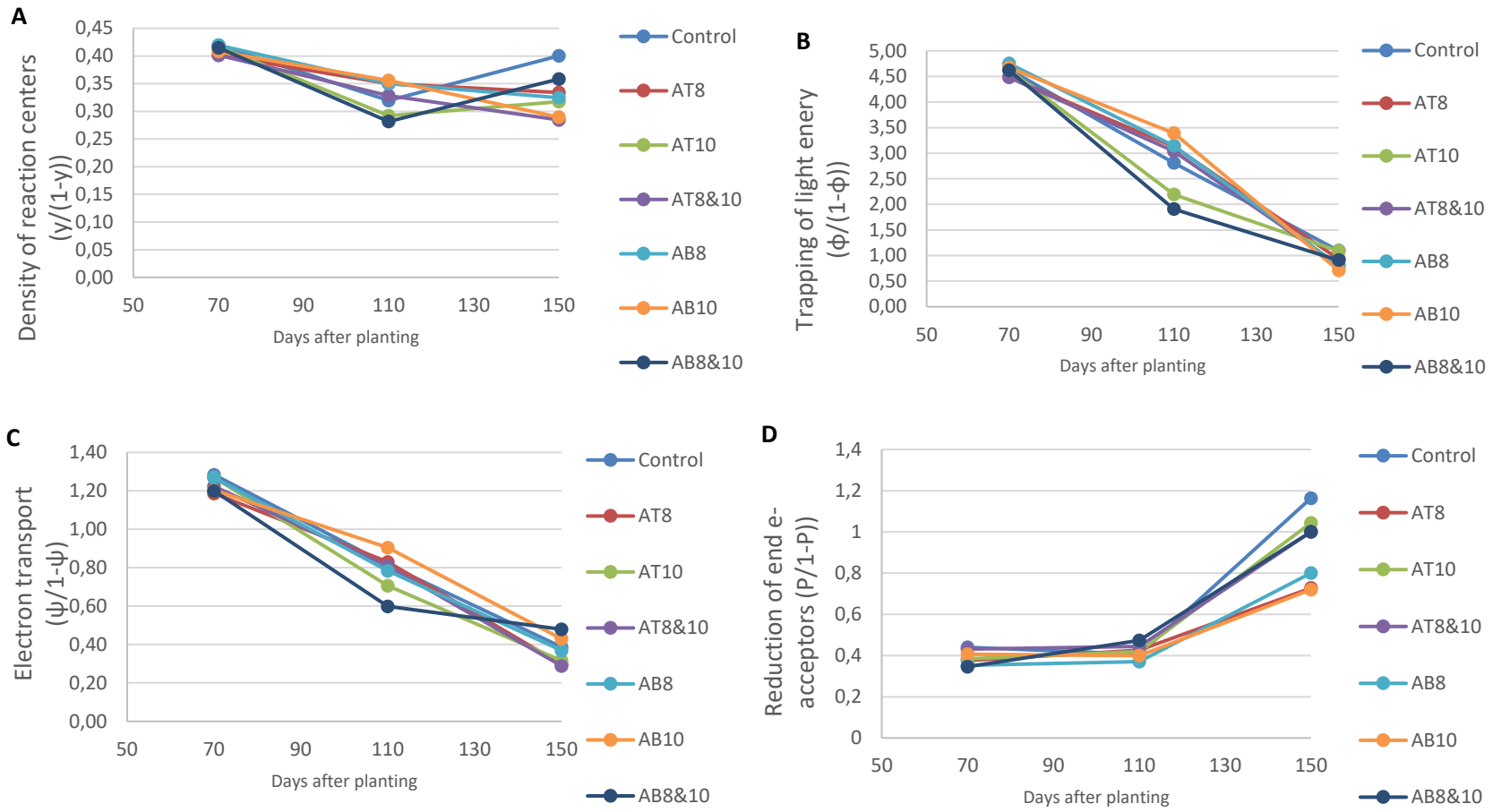

Figure 4.6. A) Absorbance, B) Trapping, C) Dissipation, and D) Probability of reduction of end electron acceptors as function of the total photosynthetic capacity of plants treated and untreated with fungicide as obtained for PAN8816.

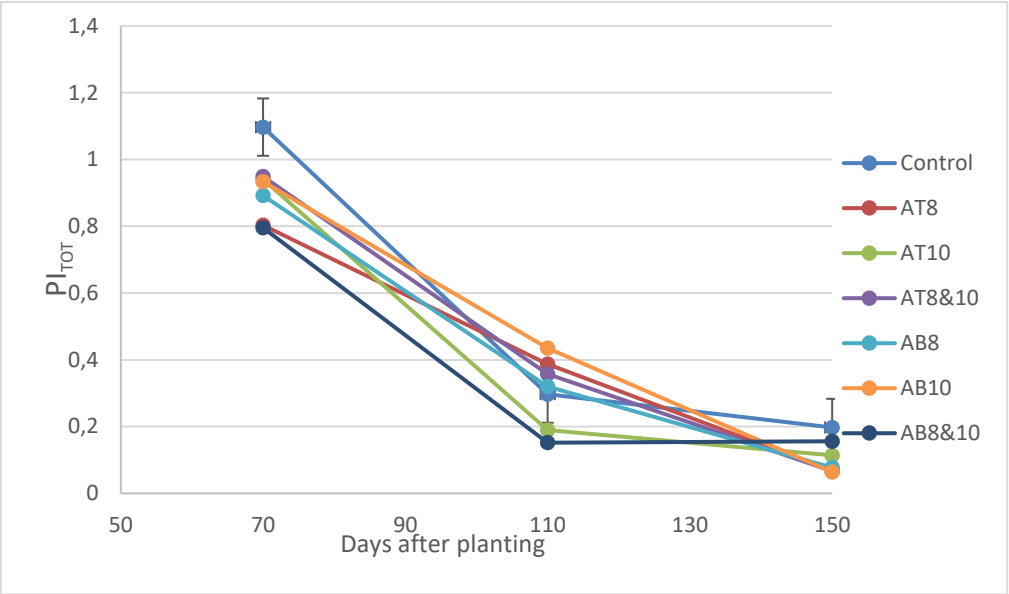

Figure 4.7. Performance index for PAN8816, obtained in the glasshouse trial from 70 to 150 days after planting.

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.

4.3.1.5. 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. are presented in Figure 4.12.

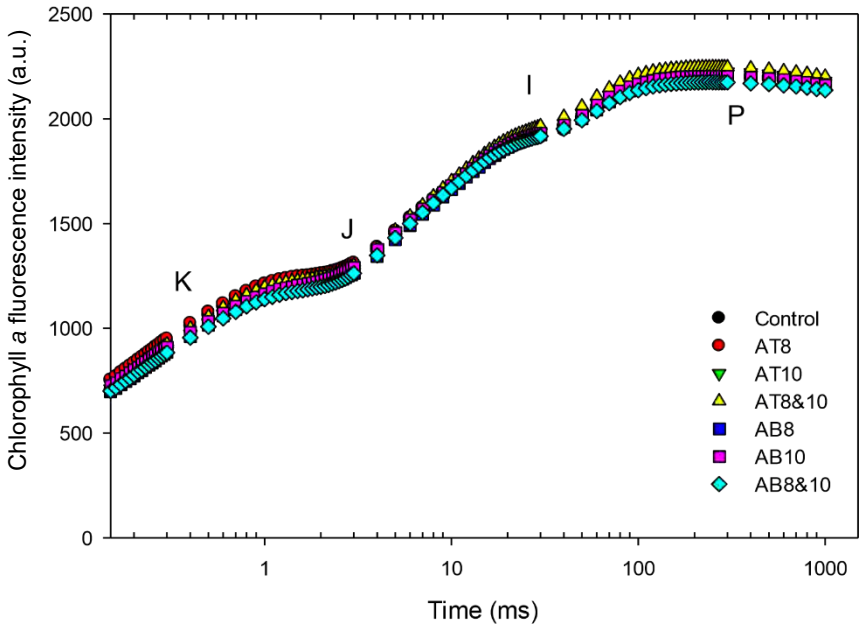

Figure 4.8. OJIP curve obtained for NS5511 70 days after planting. Treatments are as indicated AT and AB implying treatment with Amistar Top® and Abacus® respectively and 8, 10 or 8&10 the application date in weeks after planting

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.

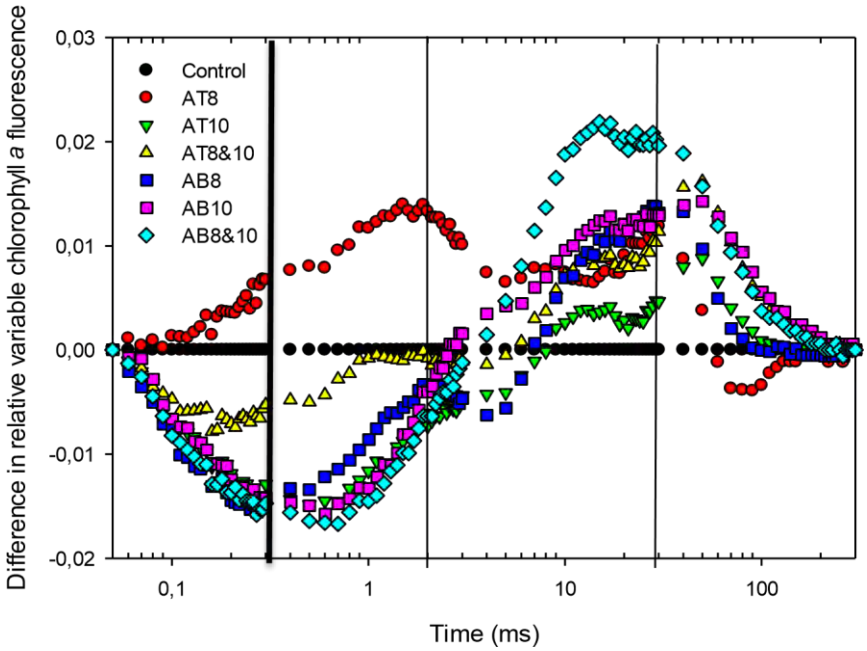

Figure 4.9. **OJIP and variable fluorescence curves for NS5511 at 70 days after planting. Treatments are as indicated AT and AB implying treatment with Amistar Top® and Abacus® respectively and 8, 10 or 8&10 the application date in weeks after planting.**

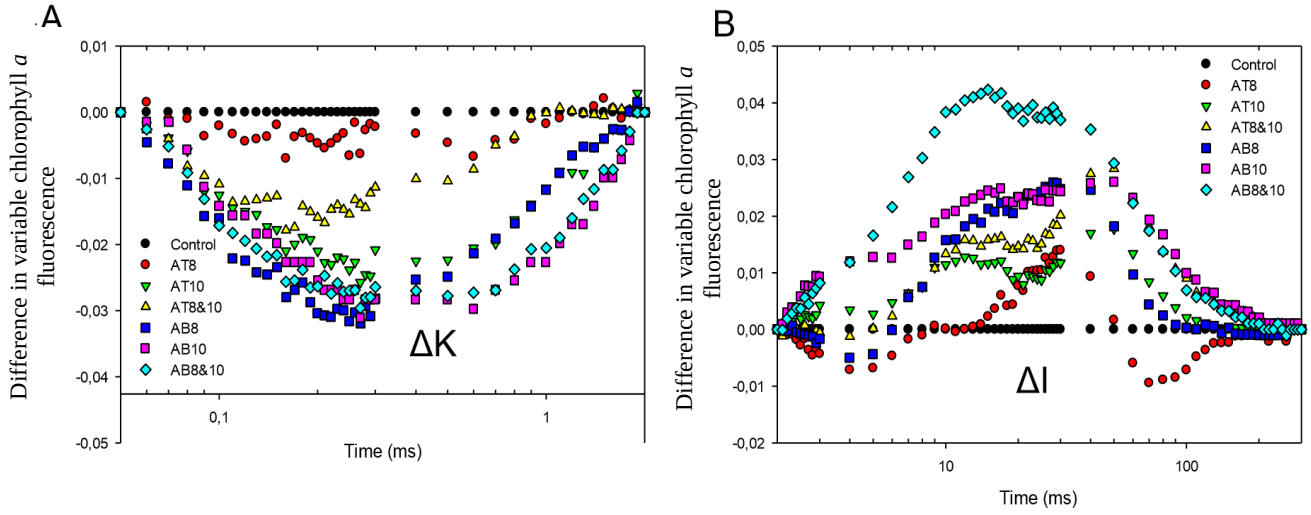

Figure 4.10: **A-B: Difference is relative variable fluorescence for NS5511 70 days after planting normalized between 0.05 ms and 2 ms, and 2 ms and 300 ms respectively. Treatments are as indicated AT and AB implying treatment with Amistar Top® and Abacus® respectively and 8, 10 or 8&10 the application date in weeks after planting**

4.3.1.6. 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.are presented in Figure 4.15.

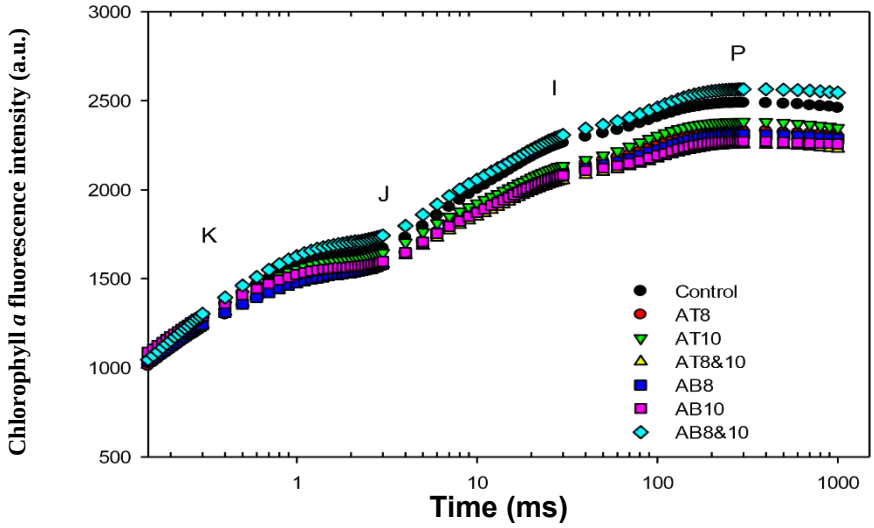

Figure 4.15. OJIP and variable fluorescence curves for NS5511 at 110 days after planting. Treatments are as indicated AT and AB implying treatment with Amistar Top® and Abacus® respectively and 8, 10 or 8&10 the application date in weeks after planting.

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.

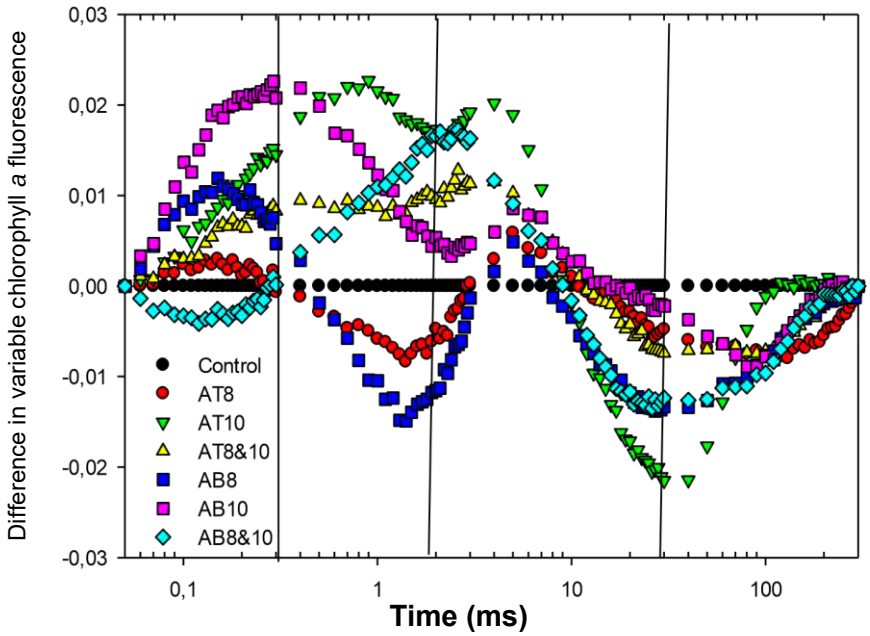

Figure 4.11. **Relative variable fluorescence curves for NS5511 at 110 days after planting. Treatments are as indicated AT and AB implying treatment with Amistar Top® and Abacus® respectively and 8, 10 or 8&10 the application date in weeks after planting.**

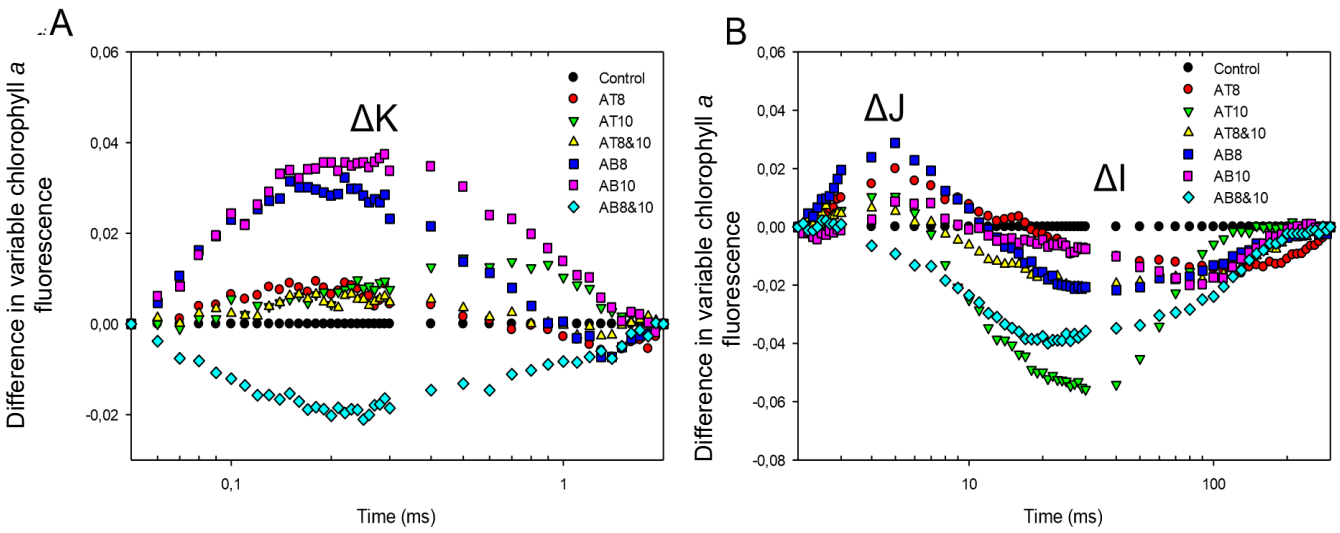

Figure 4.17. **A-B: Difference is relative variable fluorescence for NS5511 110 days after planting normalized between 0.05 ms and 2 ms, and 2 ms and 300 ms respectively over the course of plant development.**

4.3.1.7. 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. are indicated in Figure 4 - 18.

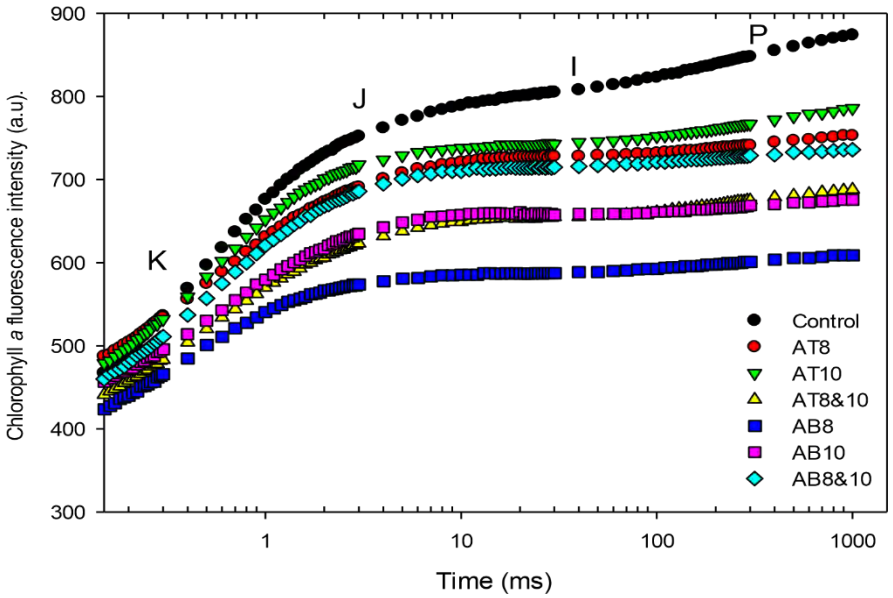

Figure4.18. OJIP and variable fluorescence curves for NS5511 at 150 days after planting.

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 ∆K 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. 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.

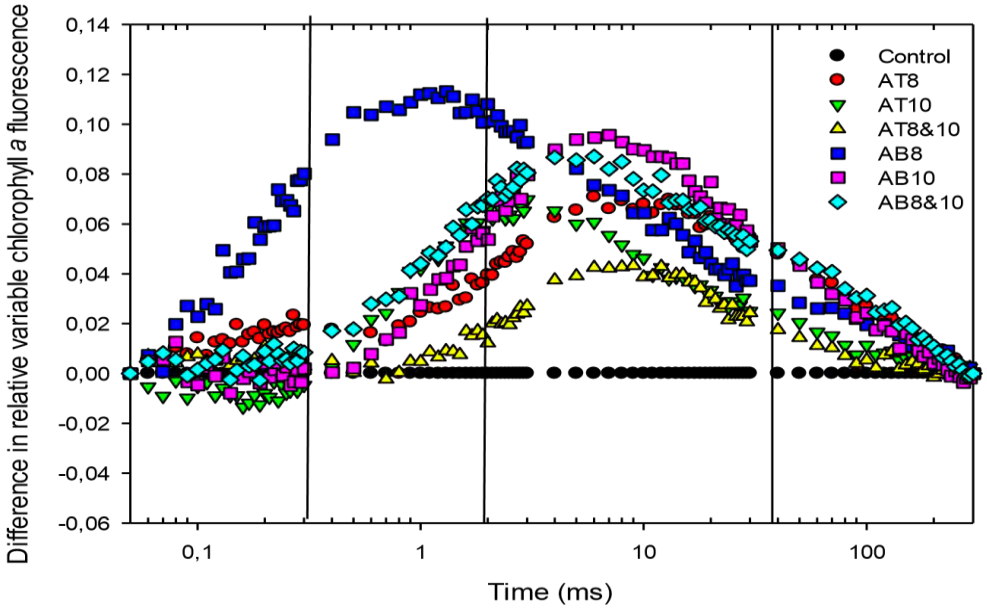

Figure 4.19. Relative variable fluorescence curves for NS5511 at 150 days after planting.

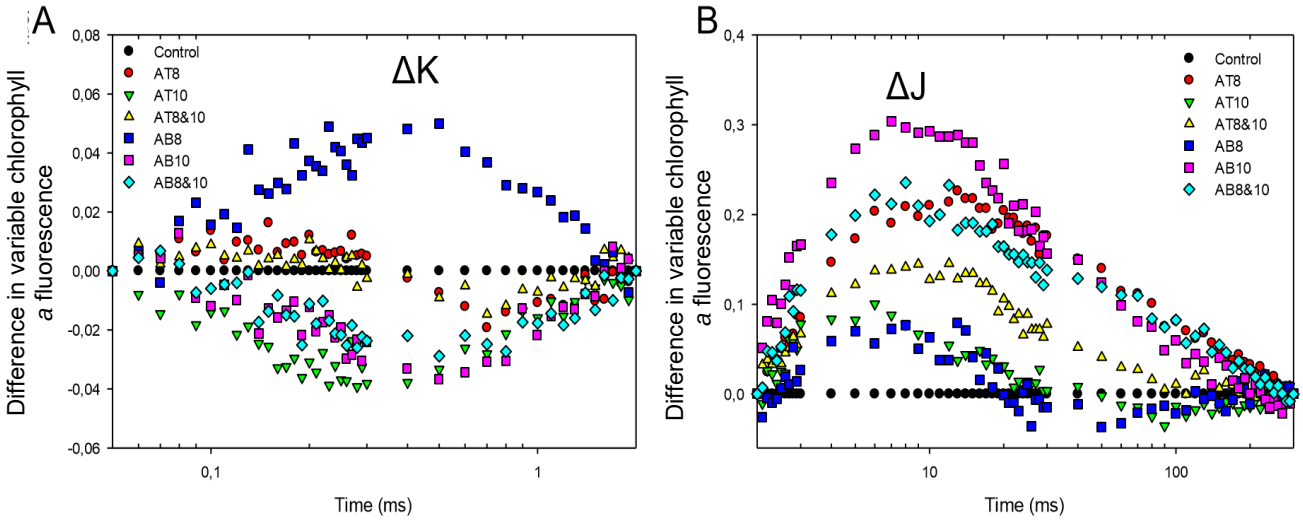

Figure 4.20. A-B: Difference is relative variable fluorescence for NS5511 150 days after planting normalized between 0.05 ms and 2 ms, and 2 ms and 300 ms respectively. Treatments are as indicated AT and AB implying treatment with Amistar Top® and Abacus® respectively and 8, 10 or 8 & 10 the application date in weeks after planting.

4.3.1.8. 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 4.22A-D).

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.

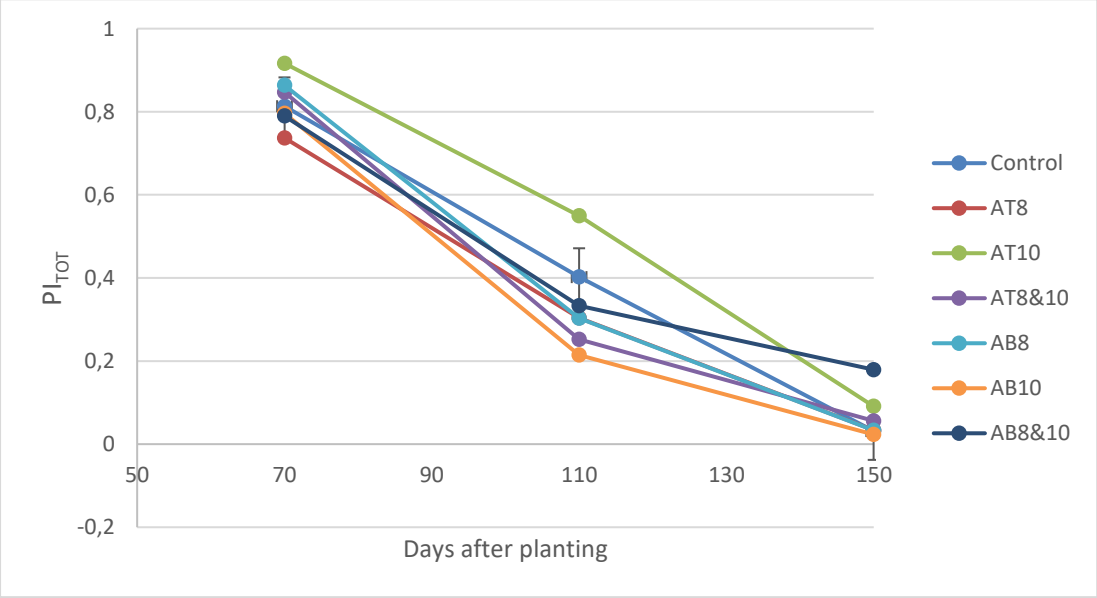

Figure 4.12. PI_{tot} summary for NS5511 as obtained by chlorophyll *a* fluorescence quantification.

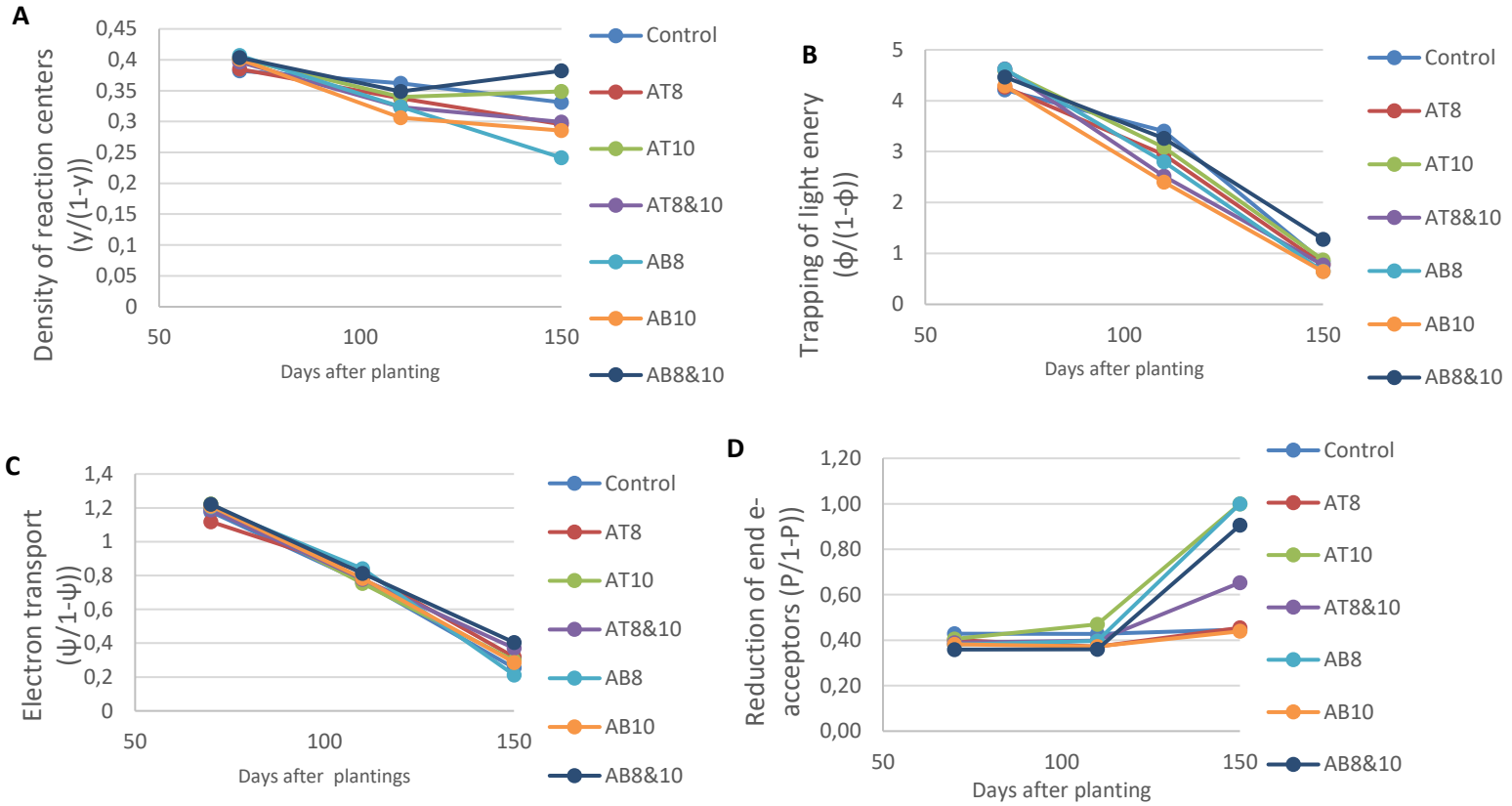

Figure 4.13. A) Absorbance, B) Trapping, C) Dissipation, and D) Probability of reduction of end electron acceptors as function of the total photosynthetic capacity of plants treated and untreated with fungicide as obtained for NS5511.

4.3.1.9. 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 4.23). The performance index has long shown to be very well correlated with the photosynthetic capacity of a plant (Zivcak et al., 2014)

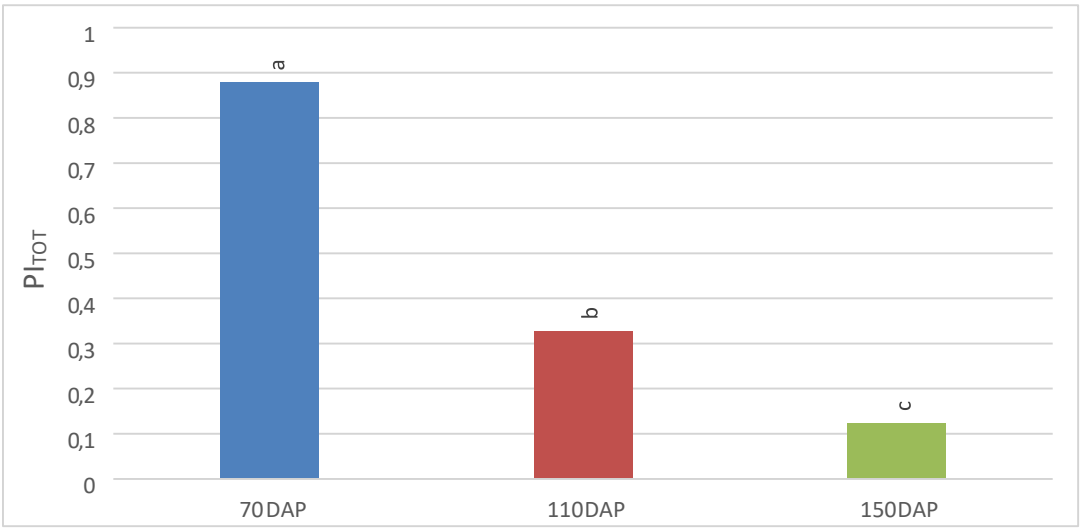

Figure 4.23. The PI_{TOT} values obtained at various sampling dates under glasshouse conditions at 70, 110 and 150 days after planting (DAP)

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.

4.3.2. 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 enzyme 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[a]				SOD[b]				XOX[c]			
		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			

[a] – Peroxidase
[b] - Superoxide dismutase
[c] - Xanthine oxidase

4.3.2.1. 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.

4.3.2.2. 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 & 2010) and Jaleel et al. (2006). These studies, however, only focused on single sampling dates and not coupled to developmental stages of the plants.

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.

4.3.2.3. 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.

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.

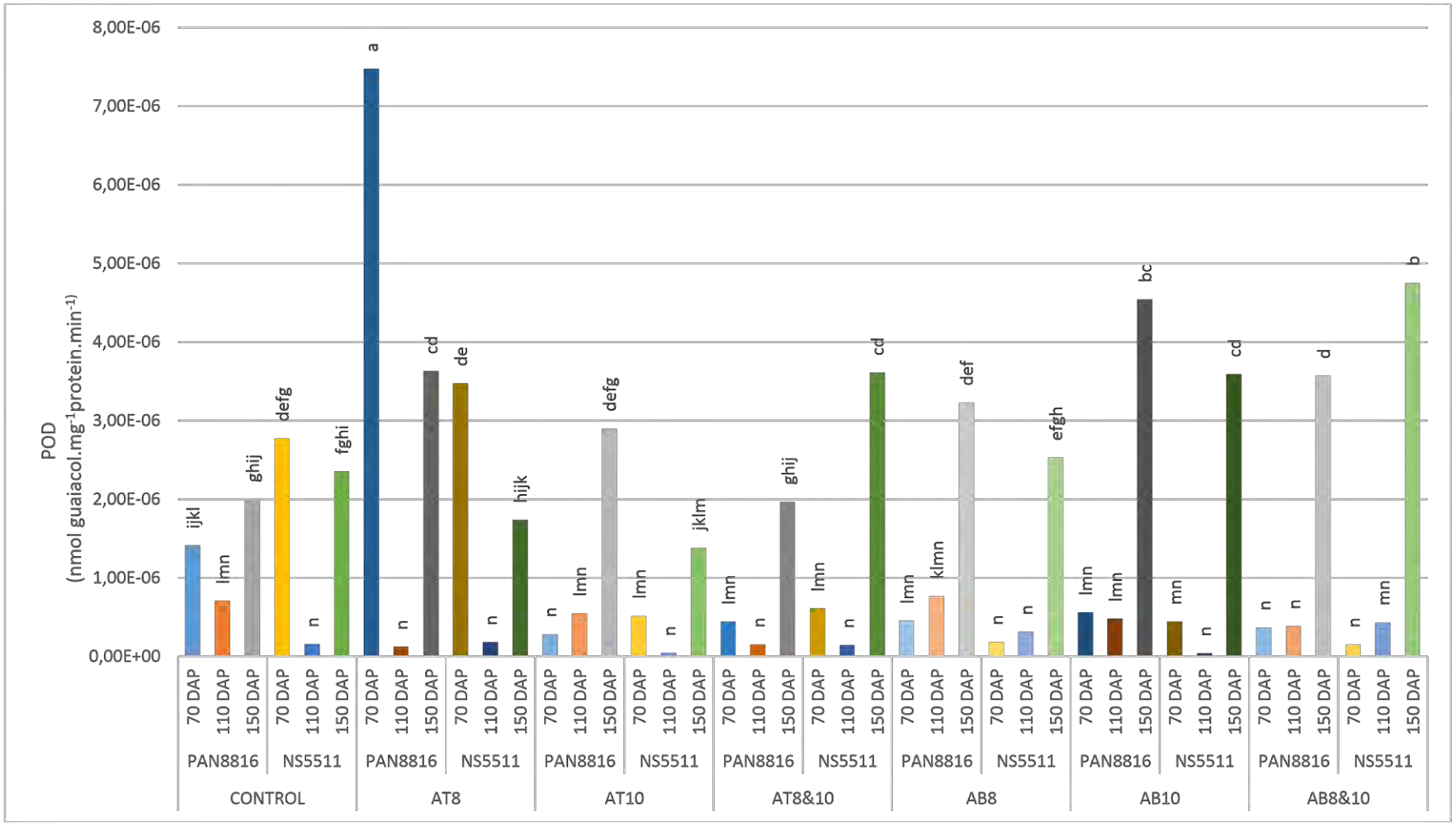

Figure 4.14. The effect of two fungicides applied at three different application dates on the POD activity of two cultivars as measured at three different sampling dates.

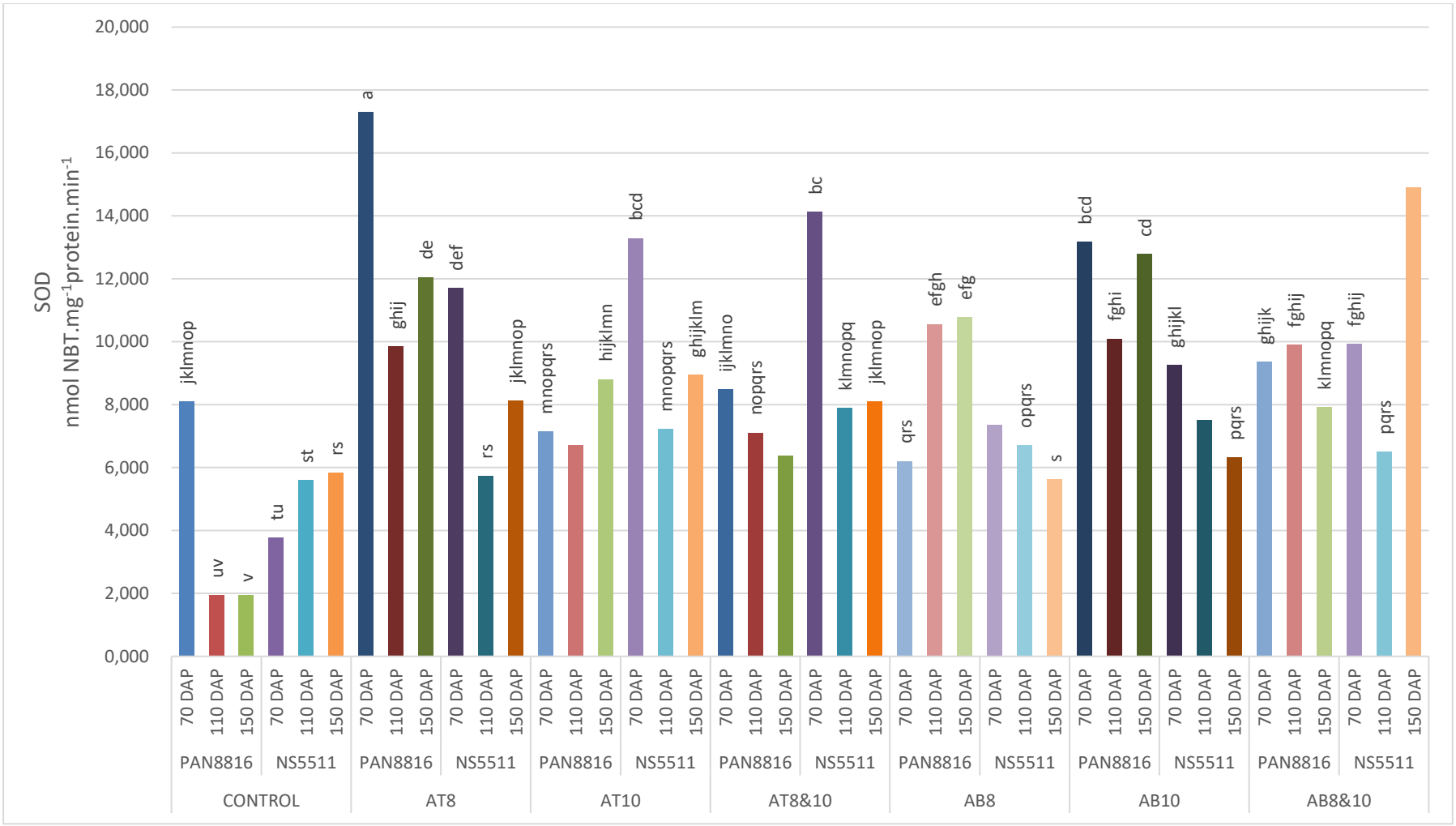

Figure 4.15. The effect of two fungicides applied at three different application dates on the SOD activity of two cultivars as measured at three different sampling dates.

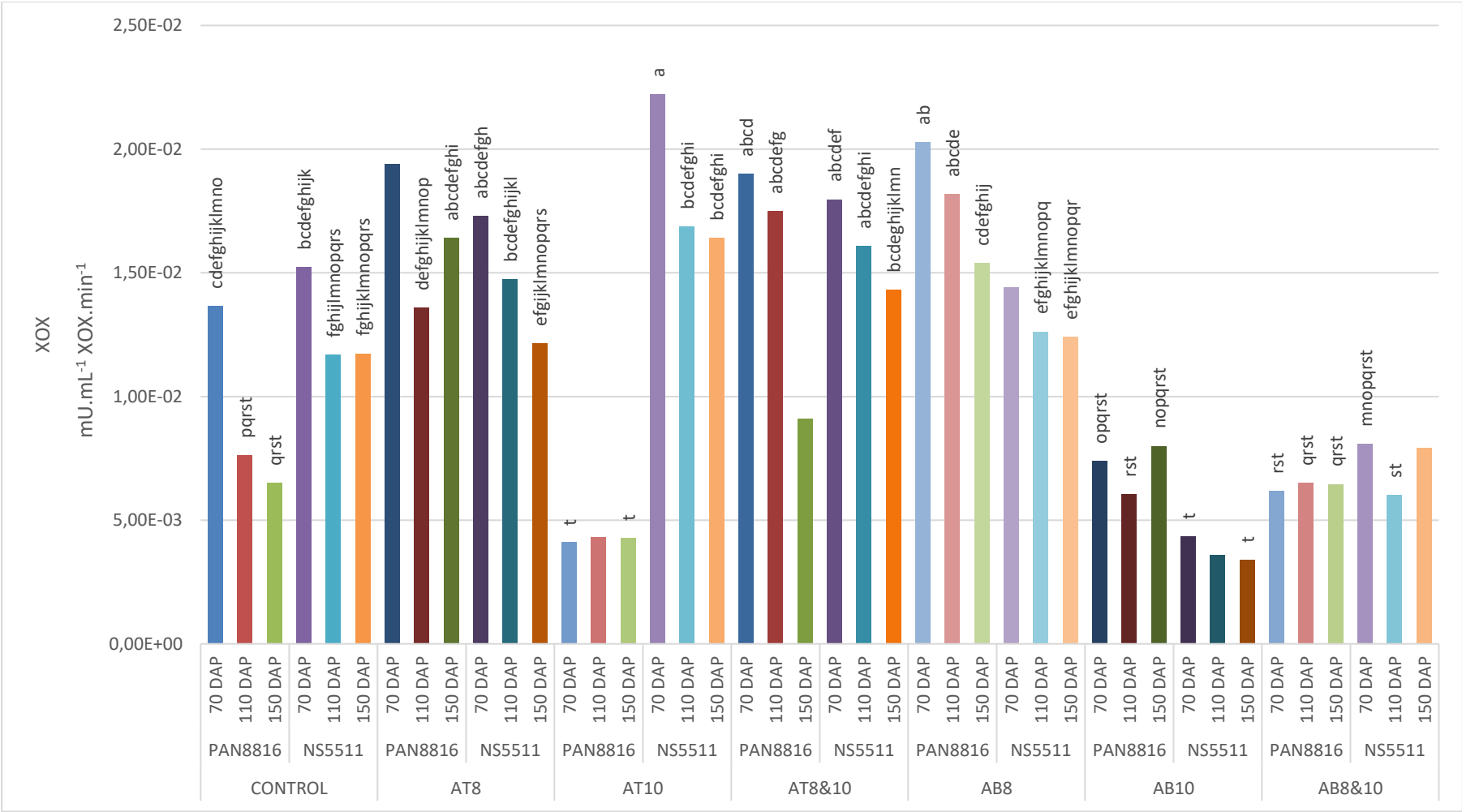

Figure 4.26. The effect of two fungicides applied at three different application dates on the XOX activity of two cultivars as measured at three different sampling dates.

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

4.3.3.1. 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.

Table 4.2. 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

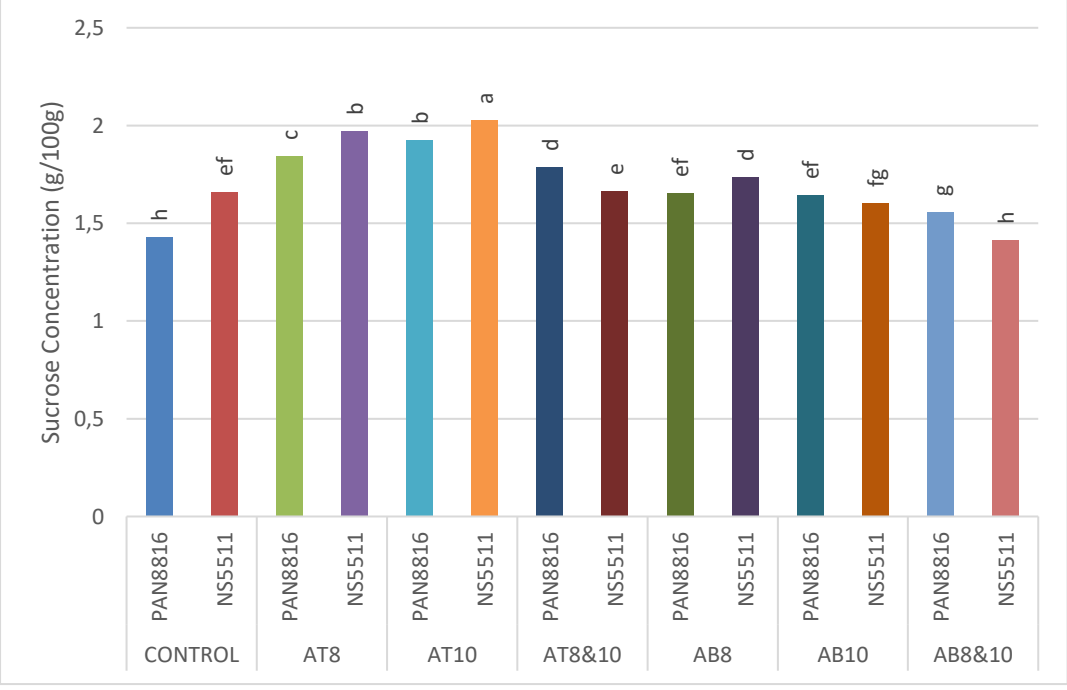

Figure 4.16. Sucrose concentration of sorghum seeds 150 days after planting.

4.3.3.2. 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 4.17. 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).

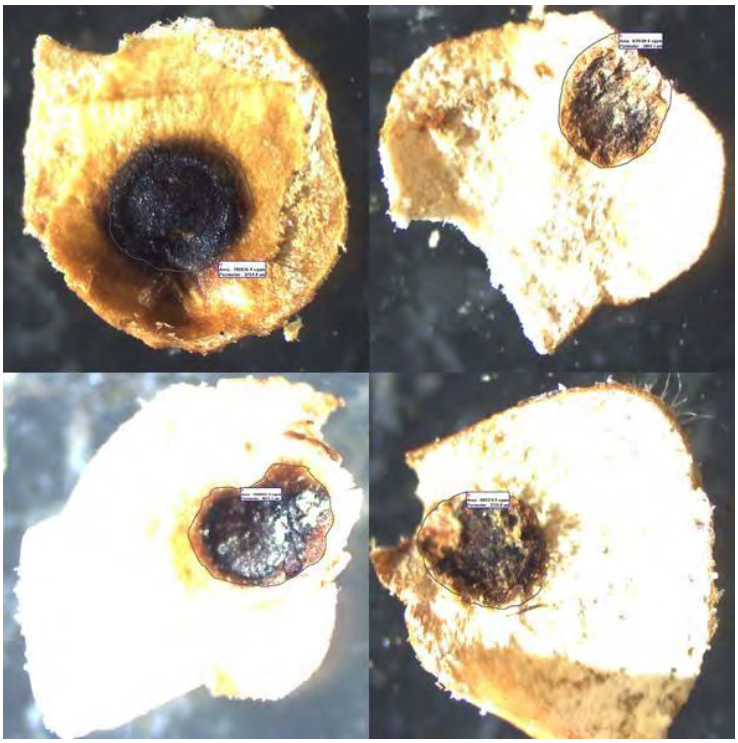

Figure 4.18. Examples of black layer in sorghum seed as seen under a stereomicroscope.

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

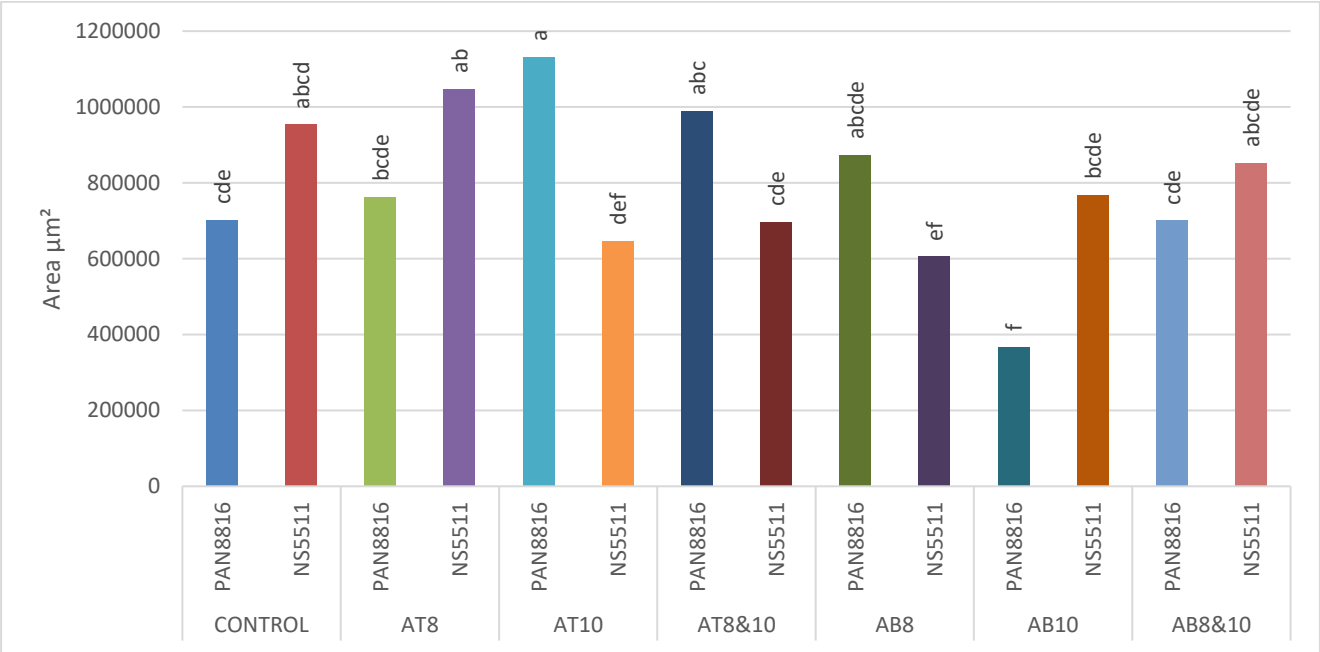

Figure 4.19. Significant differences obtained regarding the area of black layers in the seeds associated with different treatments.

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.

4.3.3.3. 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.

4.4. 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 the 2013/14 season, 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 retard the growth of clevers and clearly reduces the evolution of oxygen (Petit et al. 2012).

Literature 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 determined the effect of fungicide on chlorophyll *a* fluorescence on plants as discussed. The only clear conclusion to be drown from this study is that the photosynthetic efficiency of plants deteriorates as plants senesce.

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.

According to studies by Venancio et al. (2003), Zhang et al. (2000, 2007 & 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 & 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.

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 (1994) 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. 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.

4.4. References

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