USE OF NON-INVASIVE SENSORS TO DETECT BENEFICIAL EFFECTS OF FUNGICIDES ON WHEAT PHYSIOLOGY

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ABSTRACT

The delay of leaf senescence is a beneficial side effect of fungicides described several times in cereal crops. Strobilurins have been shown to extend the green leaf area duration (GLAD) for more than one week compared to untreated plants. An excellent method to assess the effect of fungicides on plant senescence is the use of non-invasive sensors, which allows to detect early changes in leaf pigmentation. The objective of this study was to evaluate the effect of fungicides on wheat physiology by using various sensors. Plants sprayed with fungicides of three chemical classes and untreated control plants were evaluated under disease-free conditions. At four growth stages (GS 70, 75, 80 and 85) photosynthesis, temperature and reflectance of leaves were measured using chlorophyll fluorescence, thermal imaging and non-imaging spectrometry. Spectral vegetation indices were calculated.
Differences in chlorophyll fluorescence between untreated and bixafen treated plants were detected earlier than for the other two fungicide treatments. Significant differences in leaf and ear temperature were detected between fungicide treated and untreated plants. Fungicide application resulted in a decrease of leaf and ear temperature. Significant differences in normalized differenced vegetation index (NDVI) values were verified for untreated and bixafen treated plants; fluoxastrobin and prothioconazole treated plants gave no significant differences. Optical detection of physiological changes in plants with sensor proved to be an accurate technique in order to detect effects of fungicides on plant senescence.

Keywords: Chlorophyll fluorescence, leaf reflectance, plant senescence, thermal imaging, transpiration.

INTRODUCTION

Changes in plant senescence caused by fungicide application may be assessed by destructive methods such as quantification of the chlorophyll content or by visual ratings.

The use of sensors and imaging techniques is an excellent alternative to destructive methods in order to measure the effects of fungicides on plant physiology. They provide the possibility to detect early changes in plant physiology (Mahlein et al., 2012), and as well changes caused by several factors such as biotic and abiotic stresses. Near-range infrared (IR)

thermography is a non-contact measuring technique, which enables the recording of the temperature of plant surfaces depending on differences in transpiration rate. Digital IR thermography was successfully applied to determine significant differences between untreated and fungicide treated plants at different growth stages (Berdugo et al., 2011).

Measurement of the spectral reflectance of plants may evaluate effects of fungicides on plant senescence. Different studies have linked spectral reflectance of radiation to the physiological status of plants (Carter and Knapp, 2001; Asner, 1998; Stylinski et al., 2002). Events occurring in senescing and aging leaves are mainly the progressive loss of photosynthetic pigments (Gitelson and Merzlyak, 1996), unmasking of colored pigments like anthocyanins, carotenoids and xanthophylls, changes in internal cellular structure and decrease in water content (Boyer et al., 1988). Based on the understanding of these principles spectral vegetation indices (SVIs), adapted from specific wavelength of the spectral signature have been developed (Blackburn, 1998; Gitelson et al., 2001). Spectral vegetation indices like the normalized difference vegetation index (NDVI) (Rouse et al., 1974), the pigments specific simple ratio (PSSRa) (Blackburn, 1998) or the modified chlorophyll absorption integral (mCAI) (Laudien et al., 2003) are highly correlated to biochemical and biophysical plant parameters indicating plant health and vitality. In consequence the spectral reflectance measurements and SVIs are applicable for non-destructive assessment of the physiological status and vitality of vegetation (Blackburn, 1998; Richardson, 2001), and may be used to measure physiological side-effects of fungicides in crop plants like delay of senescence.

The main objectives of the present study were 1) to use different non-invasive sensors to assess direct effects of fungicides on wheat senescence; 2) to determine the effects of three fungicidal compounds: bixafen (carboxamide), fluoxastrobin (strobilurin) and prothioconazole (triazole) on wheat physiology over time. A series of experiments was conducted under disease-free conditions in the greenhouse.

MATERIALS AND METHODS

Plant material: Spring wheat (*Triticum aestivum* L.) cultivar Passat, was grown under greenhouse conditions. Twenty wheat kernels were sown 2 cm deep per pot (20 x 20 x 30 cm). Six pots were used per treatment containing a mixture of organic soil (Klasmann-Deilmann GmbH, Germany), sand and C horizon (12:6:2 v/v). Plants were raised at 24/20 °C (day/night), and a photoperiod of 18 h d^{-1} (> 300 µmol m^{-2} s^{-1}, Phillips SGR 140, Hamburg, Germany). Plants were irrigated once per day in order to maintain soil water content (approx. 55 - 85%) to provide a favorable environment for plant growth. A solution of a commercial N-P-K fertilizer (14-10-14, 2 g/l; AGLUKON, Düsseldorf, Germany) was applied once every two weeks to ensure adequate nutrient supply. The plants were carefully inspected to control possible fungal infections. The fungicides Talius™ (active ingredient [a.i.] proquinazid, 200 g L^{-1}, DuPont de Nemours, Neu-Isenburg, Germany) and Vegas™ (a.i. cyflufenamid, 51.3 g L^{-1}, Nisso Chemical Europe GmbH, Düsseldorf, Germany), were applied to keep plants free from powdery mildew (*Blumeria graminis* f.sp. *tritici*). The insecticides Sumicidin™ (a.i. fenvalerate, 25 g L^{-1}, BASF, Limburgerhof, Germany) and Bulldock™ (a.i.

beta-cyfluthrin 125 g L^{-1}, Bayer Cropscience, Monheim, Germany) were applied when necessary to control insect pests.

Fungicide treatments: Three spray treatments and a non-treated control were evaluated. Three active ingredients belonging to different fungicidal groups (carboxamide, triazole and strobilurin group) were used: bixafen [125 g a.i. L^{-1}], prothioconazole [250 g a.i. L^{-1}] and fluoxastrobin [100 g a.i. L^{-1}]. In order to avoid contamination between treatments, plants were moved and placed far away from another for the fungicide application. Fungicidal products were applied at recommended field rates (water 300 L ha^{-1}; 0.35 ml fungicide solution per plant). Fungicides were applied at two different growth stages (GS) according to the BBCH scale (Hack et al., 1992). For the first treatment, fungicides were applied when the flag leaf ligule was visible (GS 39), for the second treatment, the application was done when the emergence of inflorescences was completed (GS 59) and for the third treatment the application was conducted twice at GS 39 and at GS 59. Foliar applications were made with a CO_2 pressurized hand-sprayer (2 L capacity, Meisterwerkzeuge, Wuppertal - Germany) with an adjustable spray. Pots of all treatments were randomized in the greenhouse

IR-thermography: Digital thermal images were used to investigate the potential of IR-thermography for non-contact detection and quantification of effects of fungicides on wheat physiology. Thermographic images were taken at GS 75, GS 80 and GS 85. The images were obtained by a Stirling-cooled infrared scanning camera (VARIOSCAN 3201 ST, Jenopic Laser, Jena, Germany). The camera operates with a spectral sensitivity from 8 to 12 µm, a geometric resolution of 1.5 m radians (240 x 360 pixels focal plane array and a 30° x 20° field of view lens with a minimum focus distance of 0.2 m). The thermal resolution is 0.03 K and the accuracy of absolute temperature measurement is less than ± 2K. Six replicates per treatment were used. The measurements were conducted between 5:00 pm and 7:00 pm in order to avoid physiological and environmental changes among measurements. The software package IRBIS plus V 2.2 (Infratec, Dresden, Germany) was used to analyze the digital thermal images. The temperature of leaves and ears was analyzed independent of each other. In each case fifty pixels were taken from the digital images per replicate per treatment.

Chlorophyll fluorescence: The chlorophyll fluorescence of flag leaves treated with different fungicides was measured at GS 70, GS 75, GS 80 and GS 85. The portable pulse-modulated chlorophyll fluorometer PAM-2000 (Walz, Effeltrich, Germany) was used to perform chlorophyll fluorescence measurements. Plants were kept in total darkness for 30 min at room temperature just before the fluorescence measurements. The experimental protocol of Genty et al. (1989) was followed. The minimal fluorescence (Fo) was measured with a modulated light of (<0.1 mmol $m^{-2}s^{-1}$). Subsequently a 500 ms pulse of high-intensity (10000 umol m^{-2} s^{-1}) was applied. White light was used to produce a transient closure of PS II photochemical reaction centers. The leaves were continuously illuminated with white actinic light (336 mmol $m^{-2}s^{-1}$). All measurements were made between 9:00 a.m. and 4:00 p.m.

Leaf reflectance: Leaf reflectance was measured with a non-imaging spectroradiometer (ASD FieldSpecPro FR spectrometer, Analytic Spectral Devices, Boulder, USA) at GS 70, GS 75, GS 80, and GS 85. A plant probe foreoptic and a leaf clip holder with an integrated 100 W halogen reflector

lamp and a field of view of 10 mm were used for the measurements. The spectral range of the instrument was from 350 to 1100 nm. All measurements were obtained with an integration time of 17 ms per scan. Reflectance data of the wheat leaves was assessed as the average of 25 reflectance spectra per sample. In each treatment, spectra from 5 pots and 3 leaves per pot were taken. The mean reflectance per pot was used for data analysis. Spectral signatures were evaluated and compared for each treatment, respectively. Spectral vegetation indices (SVIs) correlated to biophysical and biochemical parameters were calculated (Tab. 1). In this study the normalized difference vegetation index (NDVI), the pigments specific simple ratio (PSSRa) and the modified chlorophyll absorption integral (mCAI) were used.

Table 1: Spectral vegetation indices and algorithms used in this study.

Index	Equation[a]	Related to	Reference
Normalized difference vegetation index (NDVI)	NDVI = (R800 - R670)/ (R800 + R670)	Plant vitality	Rouse et al. (1974)
Pigment-specific simple ratio (PSSRa)	PSSRa = R800/R680	Chlorophyll (a)	Blackburn (1998)
Modified chlorophyll absorption integral (mCAI)	mCAI = (R545 + R752)/2 * (752 -545) - ((∑R752:R545) * 1.423))	Chlorophyll content	Laudien et al. (2003)

[a] Reflectance at wavelengths indicated

Statistical analysis: All statistical analyses were performed using the Superior Performing System SPSS 17.0 (SPSS Inc. Wacker Drive, Chicago, USA) for Windows. Data were tested for a normal distribution and equality of variances. The data were examined using analysis of variance (ANOVA) with the standard errors (SE) of the means being calculated. The means were compared using Tukey test with a significance level of $p = 0.05$ confidence in order to separate subgroups.

RESULTS AND DISCUSSION

Use of thermography to evaluate the effect of fungicide application on wheat senescence: Fungicide application resulted in significant differences of ear temperature at the first measurement (GS 75); in contrast, at this growth stage no differences in leaf temperature were calculated (Tab. 2). At growth stage 80 the first significant differences in leaf temperature between treatments were detected. Bixafen treated plants had always lower temperatures compared to the other treatments. At GS 80 ear temperature was as well significantly different, and as the leaf temperature, ears of bixafen treated plants were cooler compared to the other treatments (Tab. 2).

Lower absolute temperatures were recorded for bixafen treated plants at GS 85 for all application times; however, when bixafen was applied twice (GS 39+59) the leaf and ear temperature was lower compared to the single applications at growth stage 39 and at growth stage 59.

Table 2: Effect of fungicide treatments on the temperature [°C] of ears and leaves of wheat cv. Passat at growth stages 75, 80 and 85.

Treatment		Temperature [°C]					
		GS 75		GS 80		GS 85	
		ears	leaves	ears	leaves	ears	Leaves
Untreated		21.8 b	21.5 a	24.7 b	23.7 b	23.1 b	22.7 b
Bixafen	GS 39	21.3 ab	21.5 a	23.4 a	22.5 a	22.6 a	21.8 a
	GS 59	20.8 a	21.4 a	23.3 a	22.8 a	22.4 a	21.9 a
	GS 39+59	21.2 a	21.4 a	23.4 a	23.5 b	22.1 a	21.7 a
Fluox.	GS 39	22.2 b	21.7 a	24.9 b	23.7 b	23.1 b	22.3 b
	GS 59	20.7 a	21.4 a	24.3 b	23.2 b	23.1 b	22.8 b
	GS 39+59	21.1 a	21.4 a	24.7 b	24.1 b	23.1 b	22.2 b
Proth.	GS 39	21.1 a	21.5 a	24.5 b	23.3 b	23.1 b	22.8 b
	GS 59	21.6 b	21.4 a	24.2 b	23.6 b	23.1 b	22.8 b
	GS 39+59	21.6 b	21.4 a	24.8 b	23.7 b	23.1 b	22.7 b

Values with same letters in the same column do not differ significantly according to Tukey´s test ($n = 5$, $P \leq 0.05$)

The analysis of the plant surface temperature was useful to detect an effect of fungicide application on plant senescence.

Symptoms of plant senescence were evident first for untreated plants. Since tissue temperature is associated to plant vitality, this technique allows to detect early changes in tissue temperature related to plant senescence. It has been shown that thermography can be used to assess the effect of fungicides on plant senescence. This is in accordance with Lenthe et al. (2007) who reported

that thermography can be used as an accurate technique to detect canopy temperature differences related to plant senescence of wheat plants under field conditions.

Use of chlorophyll fluorescence to evaluate the effect of fungicide application on photosynthesis: The effective quantum yield of photosystem II of flag leaves treated with various fungicides applied at different growth stages was measured four times during the plant growth period. At growth stages 70 and 75 the quantum yield of the photosynthetic electron transport was similar for all treatments for all application times (Fig. 1).

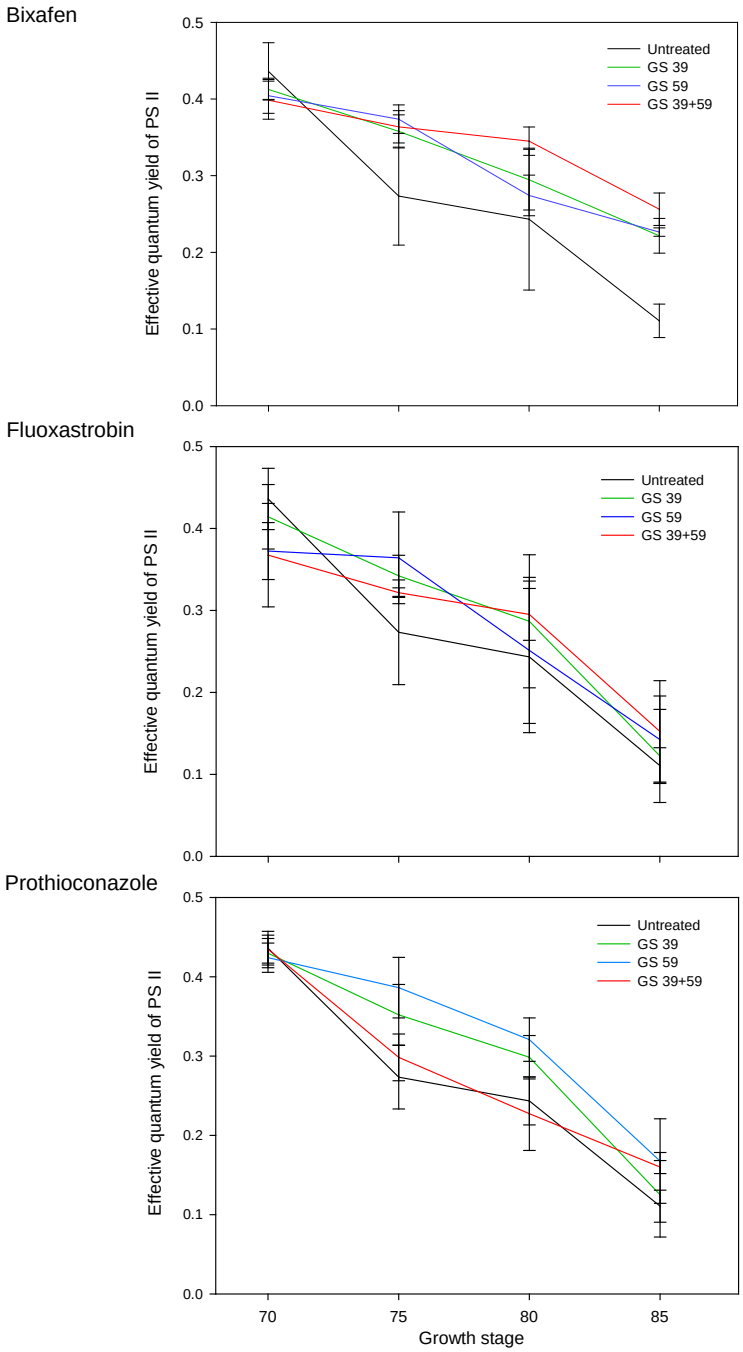

Figure 1: Effect of fungicide application at GS 39, GS 59 and GS 39+59 on the quantum yield of photosystem II. Bars represent the standard error of the mean (n = 5).

The first significant difference to untreated control was detected at GS 80 for plants treated with bixafen at GS 39+59 (Fig. 1).

At GS 85 the effective quantum yield of PS II of all bixafen treatments was significantly higher than for untreated. No significant differences between fluoxastrobin and prothioconazole treatments with respect to the untreated control were calculated for all application times.

The reduction of leaf chlorophyll content associated with a decrease in photosynthetic activity is closely related to plant senescence (Merzlyak et al. 1999). Chlorophyll fluorescence measurements allowed to detect differences in senescence between treatments. It is explained by the fact that one of the most relevant changes during the leaf senescence process is the breakdown of the chlorophyll and chloroplasts, which resulted in a decline of the photosynthetic activity (Lingrui et al. 2007).

Chlorophyll fluorescence was useful to assess differences related to tissue senescence. It was possible to establish differences in the effective quantum yield of PS II between treatments at GS 85. Chlorophyll fluorescence permits to estimate the operating quantum efficiency of electron transport through PSII in leaf tissue (Genty at al., 1990). Since the decrease of photosynthetic activity in senescent leaves is associated to the reduction of the photochemical events of PSI and PSII (Grover and Mohanty 1992), the use of the chlorophyll fluorometer becomes an accurate method to detect changes of the senescence state of plant tissue caused by fungicide application or biotic and abiotic stresses.

Use of spectral reflectance to evaluate the effect of fungicide application on leaf pigmentation: The spectral reflectance curves of all treatments were characteristic for healthy and vital wheat leaves with a strong absorption in the visible (VIS) range and a high reflectance in the near infrared (NIR).

Reflectance measured at GS 70 showed no significant differences between untreated and bixafen, fluoxastrobin, or prothioconazole treated leaves for all application times respectively (Fig. 2). At GS 75 and later changes in reflectance of untreated wheat leaves were strongly correlated to leaf senescence. Higher reflectance in the range 500 to 700 nm was measured for untreated leaves at GS 75.

At GS 80 reflectance of untreated wheat leaves was increased in the VIS compared to all bixafen treatments. At this growth stage similar reflectance curves to untreated control were measured when fluoxastrobin and prothioconazole were applied twice (GS 39+59). Single applications (GS 39 or GS 59) of fluoxastrobin and prothioconazole resulted in lower reflectance at the green peak compared to untreated control.

At GS 85 untreated wheat leaves with advanced senescence exhibited high reflectance in the VIS and an overall increased reflectance in the NIR. Reflectance of all bixafen treatments at this growth stage was similar. Nevertheless, plants treated twice with bixafen (GS 39+59) showed higher absorption in the VIS compared to plants treated only once. For fluoxastrobin treatments, leaf reflectance was lower compared to untreated control. Reflectance of prothioconazole treated leaves differed between all treatments. Plants treated with prothioconazole at GS 59 had leaf reflectance similar to untreated plants.

Reflectance measurements of wheat leaves were highly sensitive to plant vitality. Already at GS 75 differences in leaf reflectance between untreated and fungicide treated plants were detectable. This difference was most evident

for bixafen treatments; untreated plants showed already increasing reflectance in the VIS. Reflectance in the VIS is highly correlated to pigment content and in this way to plant vitality (Carter and Knapp, 2001; Gitelson and Merzlyak, 1996). Therefore lower reflectance of fungicide treated plants in the VIS was an evidence of delayed senescence produced by fungicide application.

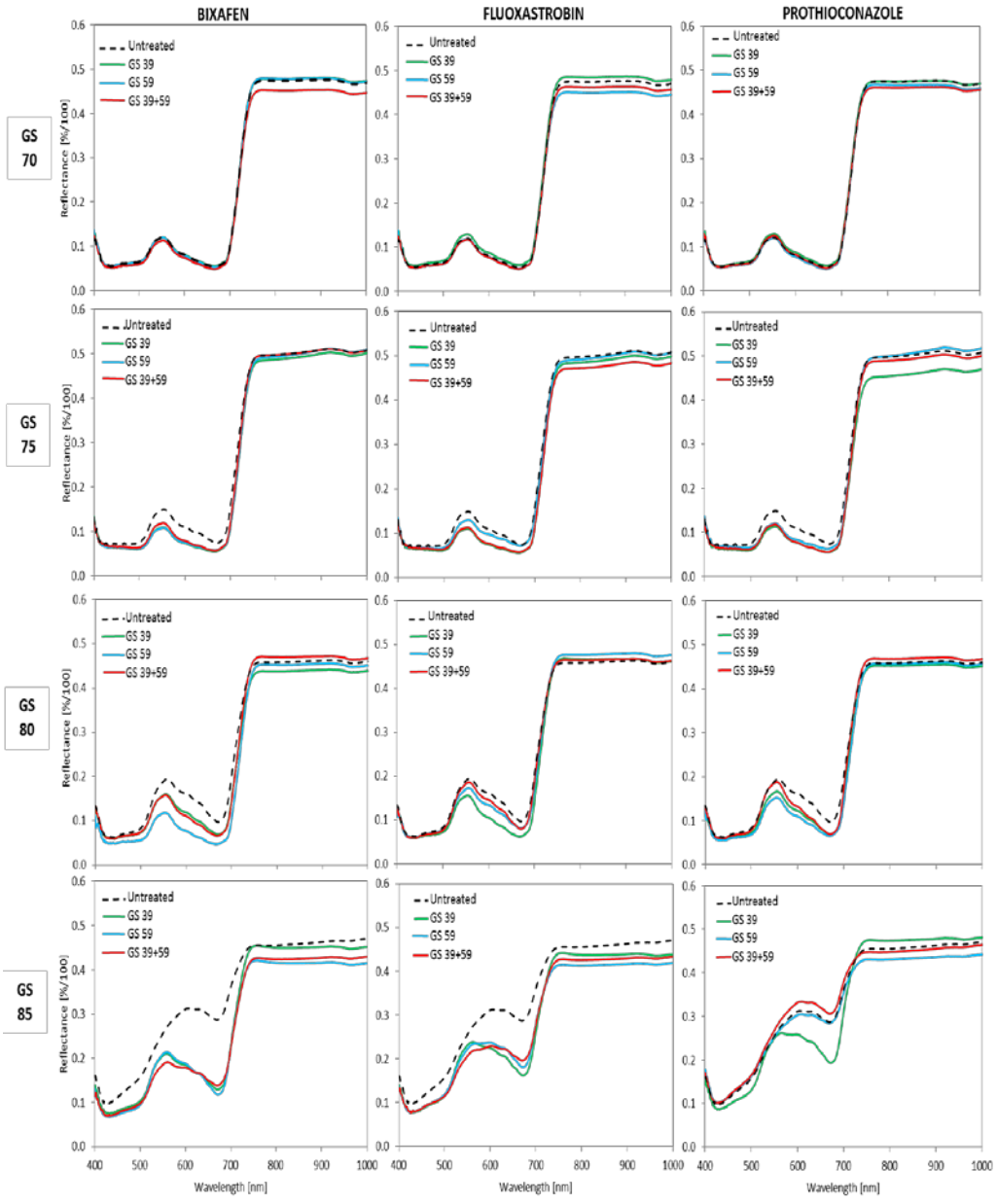

Figure 2: Spectral signatures of wheat leaves treated with fungicide at GS 39, GS 59, and GS 39+59, respectively measured four times during the growth period (GS 70, GS 75, GS 80, and GS 85).

Spectral vegetation indices as indicators of the effect of fungicide application on leaf pigmentation: Three SVIs were calculated for the leaves treated with different fungicides and for each measuring date (Fig. 3). For bixafen treatments the first significant differences to untreated control were detected at GS 80 in the NDVI; at GS 85 all bixafen treatments were

significantly different to the untreated control. Single application with fluoxastrobin and prothioconazole at GS 39 were the only treatments significantly different to untreated plants at GS 85.

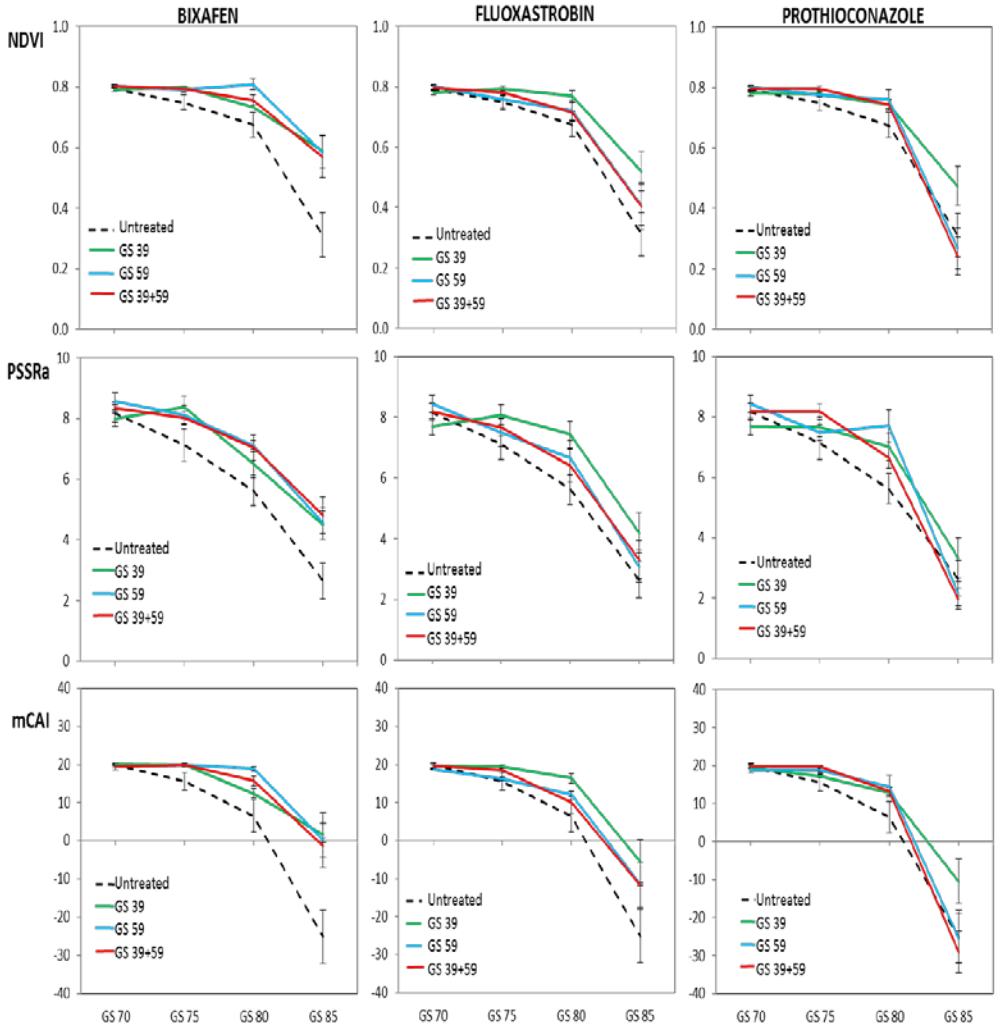

Figure 3: Effect of fungicide application at GS 39, GS 59 and GS 39+59 respectively on spectral vegetation indices of wheat leaves. Bars represent the standard error of the mean (n = 5).

At GS 85 PSSRa values of all bixafen treated plants were significantly higher than those of untreated plants. No significant differences were calculated between prothioconazole and fluoxastrobin treatments with respect to untreated control. Similar results were observed for the mCAI; bixafen treated plants were significantly different to untreated plants at GS 85. Only fluoxastrobin and prothioconazole application at GS 39 were significantly different for mCAI values at GS 85. By calculating spectral vegetation indices it was possible to measure differences in specific leaf traits, deduced from spectral reflectance like the chlorophyll content (Thenkabail et al. 2000).

Two of the SVIs tested in this study (PSSRa and mCAI) are mainly chlorophyll specific. Since reduction of leaf chlorophyll content is closely related to plant senescence (Merzlyak et al. 1999) these SVIs can be used as parameters to estimate differences in plant senescence caused by fungicide

application. These indices have shown potential as a non-destructive measure of fungicidal side effects in this study.

CONCLUSION

In this study, senescence-associated changes in wheat physiology, i.e. degradation of photosynthetic pigments, transpiration of green tissue, photosynthetic electron transport and leaf reflectance, could be quantified by using ground-based optical sensors for chlorophyll fluorescence, plant temperature and spectral reflectance. Furthermore, it was elucidated that non-destructive sensors and imaging methods are useful tools to assess effects of fungicides on wheat physiology.

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