Institut für Nutzpflanzenwissenschaften und Ressourcenschutz (INRES)
der
Rheinischen Friedrich-Wilhelms-Universität Bonn

Effects of fungicides on physiological parameters and yield formation of wheat assessed by non-invasive sensors

Inaugural - Dissertation
zur
Erlangung des Grades

Doktor der Agrarwissenschaften
(Dr. agr.)

der
Landwirtschaftlichen Fakultät
der
Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt am 15.05.2013
von

Carlos Andres Berdugo Agudelo

aus
Tunja, Kolumbien

Referent: Prof. Dr. H.-W. Dehne
Koreferent: Prof. Dr. H. Goldbach
Tag der mündlichen Prüfung: 19.02.2014
Erscheinungsjahr: 2014

ABSTRACT

Apart from fungicidal effects, some fungicide classes have been reported to induce physiological changes in crops such as increased tolerance to abiotic stress, delayed senescence of the photosynthetic leaf area and modifications in the balance of plant growth regulators. The aim of this study was to investigate the effects of different fungicidal groups on physiological parameters of wheat through the use of non-invasive sensors and imaging techniques. Experiments were conducted under field and also under disease-free conditions in the greenhouse.

Under field conditions, application of the azole, carboxamide and strobilurin compounds resulted in low disease incidence. All fungicide treatments delayed the senescence of the uppermost leaf layers; treatments with longer leaf life and lower disease incidence resulted in higher chlorophyll content. The effect of the fungicides on wheat senescence was positively correlated to grain yield and the thousand-kernel weight. However, under field conditions, the presence of the main foliar pathogens of wheat influenced the green leaf area duration as well as the yield, generating a disadvantage for the fungicide treatments with low disease control efficacy.

Under disease-free conditions, an effect produced by the pyrazole carboxamide fungicide bixafen was observed. Bixafen delayed the senescence of leaves and ears resulting in a significantly extended green leaf area duration compared to untreated plants. In addition, an effect produced by this compound on morphogenesis was observed. The combination of the positive effects on physiology and morphogenesis of wheat resulted in a yield advantage of bixafen-treated plants. Furthermore, bixafen had a positive effect on plant tolerance to water stress conditions.

Different non-invasive sensors and imaging techniques were used and compared to measure the effects of fungicidal compounds on wheat physiology. By using ground-based optical sensors it was possible to detect the influence of fungicidal compounds in crop physiology, i.e. degradation of photosynthetic pigments, photosynthetic activity, leaf reflectance, and transpiration of plant tissue earlier than with destructive and visual methods. Chlorophyll fluorescence of leaves was useful to measure differences in the effective quantum yield of photosystem II. Reflectance measurements of wheat leaves were highly sensitive to changes in plant vitality. The spectral vegetation indices were useful to determine differences between treatments in terms of leaf senescence, pigments and water content. Digital infrared images revealed significant differences between untreated and fungicide-treated plants at different growth stages. Moreover, thermography proved to be a suitable technique for distinguishing the beneficial effects of fungicides on plant senescence under different water supply conditions. Through the use of an image analysis software program, leaf senescence differences were successfully detected, thus allowing an early detection of the effect produced by the fungicide on the senescence status of flag leaves. Using hyperspectral imaging, it was possible to study differences in the senescence status of flag leaves. Furthermore, through the analysis of hyperspectral images it was achievable to study the pattern of the senescence process in flag leaves and to determine a delay of senescence of wheat produced by fungicides.

The results of this study demonstrated that non-invasive sensors and imaging techniques are excellent alternatives to conventional screening methods for detecting the beneficial effects of fungicides on plant physiology. Furthermore, among this innovative group of sensors and techniques it was spectrometry, which proved to be the most sensitive and specific method with a high potential for large-scale fungicide screening. Sensors can be incorporated in automatic and reproducible screening of new active ingredients with high efficiency and accuracy. The recent development of hyperspectral imaging techniques will improve future studies to additionally explore plant physiology with high spatial and temporal resolution.

KURZFASSUNG

Für einige fungizide Wirkstoffgruppen ist neben einer fungiziden Wirksamkeit auch eine Wirkung auf die Pflanzenphysiologie, wie zum Beispiel eine erhöhte Widerstandsfähigkeit gegenüber abiotischen Stress, eine Verzögerung der Seneszenz, Veränderungen in der photosynthetisch aktiven Blattfläche und Modifikationen im Verhältnis von Wachstumsregulatoren beschrieben. Das Ziel dieser Arbeit war die Erfassung von Effekten unterschiedlicher fungizider Gruppen auf physiologische Parameter von Weizen mittels nicht-invasiver Sensoren und bildgebender Verfahren. Die Versuche wurden sowohl unter Feldbedingungen als auch unter Krankheits-freien Gewächshausbedingungen durchgeführt. Unter Feldbedingungen herrschte ein geringer Krankheitsdruck bedingt durch die Applikation von Wirkstoffen aus den Gruppen der Azole, Strobilurine und Carboxamide. Alle Funizidapplikationen führten zu einer verzögerten Seneszenz der obersten Blattetage, dies wiederum führte zu einer längeren Vitalität der Blätter bei erhöhtem Chlorophyllgehalt. Der Einfluss der Fungizide auf die Seneszenz von Weizen war positiv mit dem Körnerertrag und dem Tausend-Körner-Gewicht korreliert. Unter Feldbedingungen beeinflusste jedoch das Vorkommen der wichtigsten pilzlichen Pathogene im Weizen die Dauer der grünen Blattfläche sowie den Ertrag. Ein Nachteil konnte bei Varianten mit geringer fungizider Wirksamkeit beobachtet werden. Unter Krankheits-freien Bedingungen wurde ein Effekt von Wirkstoffen aus der Gruppe der Carboxamide beobachtet. Der fungizide Wirkstoff Bixafen verursachte eine Verzögerung der Seneszenz der Blätter und Ähren und eine signifikante Verlängerung der Dauer der grünen Blattfläche im Vergleich zu unbehandelten Pflanzen. Zusätzlich wurde ein Effekt dieses Wirkstoffes auf die Pflanzenmorphogenese beobachtet. Die Kombination positiver Effekte sowohl auf die Physiologie als auch auf die Morphologie von Weizenpflanzen führte zu einem Ertragsvorteil von Pflanzen, behandelt mit Bixafen. Ebenso zeigte Bixafen einen positiven Einfluss gegenüber Trockenstress. Unterschiedliche nicht-invasive Sensoren und bildgebende Verfahren wurden angewendet und verglichen, um den Effekt von Fungiziden auf die Physiologie von Weizen zu messen. Durch den Einsatz von optischen Sensoren war es möglich, den Einfluss von fungiziden Wirkstoffen auf die Pflanzenphysiologie, wie z.B. Abbau von Blattpigmenten, Photosyntheseaktivität, Pflanzenreflektion und Transpiration von Pflanzen, früher als mit herkömmlichen visuellen oder oft destruktiven Methoden zu erfassen. Durch Chlorophyllfluoreszenz-Messungen von Weizenblättern konnten Unterschiede im Effektivem Quantum Yield des Photosystem II gezeigt werden. Reflektionsmessungen waren besonders sensitiv gegenüber Veränderungen in der Pflanzenvitalität. Spektrale Vegetations Indizes wurden berechnet um Unterschiede zwischen den Versuchsvarianten bezüglich der Blattseneszenz, Pigmentgehalt und Wassergehalt zu detektieren. Durch digitale Infrarot-Thermographie konnten signifikante Unterschiede in der Transpiration von Weizen zwischen unbehandelten und Fungizid-behandelten Pflanzen zu unterschiedlichen Entwicklungsstadien gemessen werden. Des Weiteren ist dieses Verfahren geeignet um unterschiedliche Wasserverfügbarkeiten aufzuweisen. Unterschiede in der Blattseneszenz wurden an RGB-Bildern mittels Bildanalyse quantifiziert; hierdurch konnte eine frühzeitige Detektion des Einflusses von Fungiziden auf die Vitalität des Fahnenblattes gemessen werden. Mittels hyperspektraler bildgebender Verfahren war es ebenfalls möglich diese Unterschiede im Seneszenzstatus und im Seneszenzverlauf des Fahnenblatt beobachtet werden. Die Ergebnisse dieser Studie belegen, dass nicht-invasive Sensoren und bildgebende Verfahren hervorragende Alternativen zu konventionellen Screening-Verfahren zur Bewertung positiver Nebeneffekte von Fungiziden auf die Pflanzenphysiologie sind. Es hat sich gezeigt dass Reflektionsmessungen in diesem Zusammenhang besonders sensitiv und spezifisch sind und ein großes Potential für großflächige Fungizid-Screening Anwendungen haben. Optische Sensoren können in automatische Screeningvorgänge implementiert werden und zu neuen Wirkstoffgruppen zuverlässige und reproduzierbare Erkenntnissen liefern.

LIST OF ABBREVIATIONS

ACC	:	1-aminocyclopropane-1-carboxylic acid
ai	:	Active ingredient
ANOVA	:	Analysis of variance
APX	:	Ascorbate peroxidase
ARI	:	Anthocyanin Reflectance Index
AUETC	:	Area under the ear temperature curve
AUGLAC	:	Area under the green leaf area curve
AULTC	:	Area under the leaf temperature curve
AUPC	:	Area under the photosynthesis curve
AUQYC	:	Area under quantum yield of the photosystem II curve
CAT	:	Catalase
Chla	:	Chlorophyll a
Chlb	:	Chlorophyll b
cm	:	Centimeters
CO_2	:	Carbon dioxide
cv	:	Cultivar
das	:	Days after sowing
dasfa	:	Days after the second fungicide application
DMSO	:	Dimethylsulfoxide
EC	:	Emulsifiable concentrate
ETR	:	Electron transport rate
F	:	Flag leaf
F’	:	Fluorescence emission from light-adapted leaf
Fig	:	Figure
Fm	:	Maximum fluorescence from dark-adapted leaf
Fm’	:	Maximum fluorescence from light-adapted leaf
Fo	:	Ground/minimal fluorescence from dark-adapted leaf
Fo’	:	Ground/minimal fluorescence from light-adapted leaf
Fq’	:	Difference in fluorescence between Fm’ and F’
Fq’/Fm’	:	Photosystem II operating efficiency
Fv	:	Variable fluorescence
Fv/Fm	:	Maximum quantum yield of QA reduction
g	:	Grams
GLAD	:	Green leaf area duration
GS	:	Growth Stage
ha	:	Hectare
H_2O	:	Water
IR	:	Infrared radiation
IRGA	:	Infrared gas-analyzer
L	:	Liter
LS	:	Long-term stress
m	:	Meter
min	:	Minutes
ml	:	Milliliters
n	:	Number of replications

N/A	:	Not applicable
NDVI	:	Normalized Difference Vegetation Index
NIR	:	Near Infrared Reflectance
nm	:	Nanometer
ns	:	No significant differences
NS	:	No stress
p	:	Probability of error
PAM	:	Pulse-amplitude-modulated
PAR	:	Photosynthetic active radiation
PRI	:	Photochemical Reflectance Index
PSI	:	Photosystem I
PSII	:	Photosystem II
PSRI	:	Plant Senescence Reflectance Index
PSSR	:	Pigment Specific Simple Ratio
QA	:	Quinone acceptor
qF	:	Fluorescence quenching
qN	:	Nonphotochemical quenching
QoI	:	Quinone outside inhibitors
qP	:	Photochemical quenching
R	:	Reflectance at specific wavelengths
R^2	:	Coefficient of determination
RGB	:	Red Green Blue
RH	:	Relative humidity
s	:	Second
SD	:	Standard deviation
SDHI	:	Succinate dehydrogenase inhibitors
SE	:	Standard error of the mean
SIPI	:	Structural insensitive pigment index
SOD	:	Superoxide dismutase
SS	:	Short-term stress
SVI	:	Spectral Vegetation Indices
SWIR	:	Shortwave Infrared Reflectance
Tab	:	Table
TKW	:	Thousand kernel weight
VIS	:	Visible reflection
WG	:	Water dispersible granules
WI	:	Water Index

CONTENTS

CHAPTER 1: GENERAL INTRODUCTION

1. IMPORTANCE OF WHEAT

Wheat (*Triticum aestivum*) is a monocotyledonous plant, which belongs to the Poaceae family. The origin of wheat is thought to have occurred in the Middle East as a result of the hybridization between the diploid einkorn *Triticum* species with a wild grass-like *Aegilops* species (Jones and Clifford, 1983; Nesbitt, 1998). Wheat is one of the most important crops for human nutrition. Due to the high starch content in the grain (60-70%) wheat has been one of the main sources of calories for the human diet (Shewry, 2009). Wheat has been grown successfully in different environments from Scandinavia to Argentina, covering latitudes from the equator to 60° N and 44° S (Singh *et al*., 2011). It has become in one of the most important elements of the agricultural economy of many countries around the world. In fact, as shown in figure 1.1, total wheat production has seen an increase worldwide during the last 10 years.

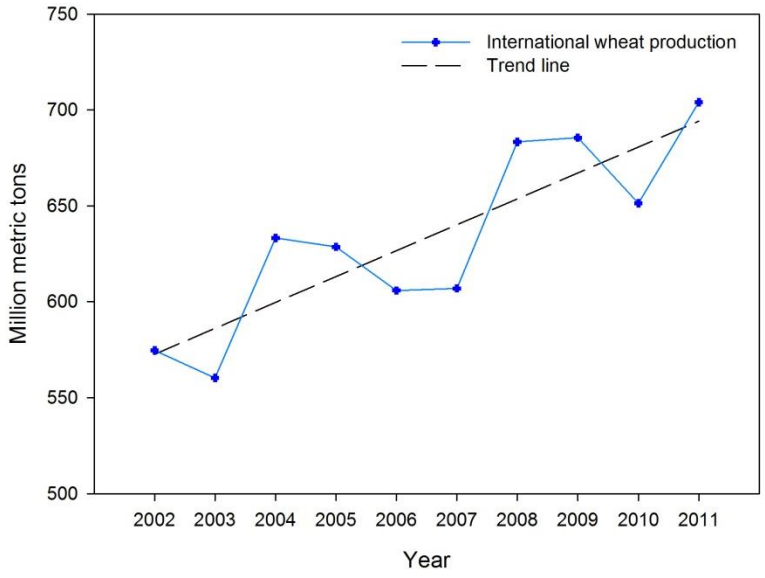

Figure 1.1: Wheat production during the last decade (FAOSTAT, 2011).

The main reasons for the increment in wheat production during the last decade have been a rise of the cultivated area (around 3%) as well as higher yields per hectare (around 10%). According to FAO (2013), the countries that reported the largest productions worldwide in 2011 were China, India, Russia, United States and France.

2. CHEMICAL CONTROL OF FOLIAR WHEAT DISEASES

One of the most limiting factors of wheat production is related to phytosanitary problems, which increase the costs of production, generate yield losses and reduce grain quality. Different organisms such as fungi, bacteria, nematodes, viruses and insects can cause several issues on the plant. In wheat producing countries, the presence of fungi, causes most of the phytosanitary problems. Among the fungal diseases, the most common limiting foliar diseases affecting wheat production are Septoria leaf blotch (*Septoria tritici*), leaf rust (*Puccinia triticina*) and powdery mildew (*Blumeria graminis*) (Shipton *et al*., 1971; Kolmer, 2005; Bennett, 1984). Several strategies, such as crop rotation, use of resistant varieties and application of fungicides have been used to control foliar pathogens. Among these, the use of fungicides has been one of the most successful and widespread methods implemented to sustain wheat production that has prevented yield losses and produced an increase in economic returns.

In addition, a variety of studies have demonstrated that application of fungicides can result in yield increases, which are attributed to the control of foliar pathogens (Caldwell and Starratt, 1987; Loughman and Thomas, 1992). As shown by Wegulo *et al*. (2009) from 27% to 42% yield loss was avoided by applying fungicides in wheat. The advantage of the use of fungicides to control foliar pathogens with respect to the other alternatives is due to the fact that they can be applied when needed depending on disease incidence. However, the widespread use of fungicides has generated an increment of the appearance of pathogen strains resistant to some commercial products (García *et al*., 2003).

The use of chemical compounds to control plant diseases dates back to the 19th century, when in 1807, Prévost demonstrated the control of wheat bunt (*Tilletia caries*) by applying copper sulphate (Russell, 2005). From then until the 1940s, the chemical control of plant diseases was performed mainly through the application of inorganic preparations such as the Bordeaux mixture (copper sulphate, fresh burned lime and water) and Woburn Bordeaux emulsion (copper sulphate, lime water, water and paraffin) (Russell, 2005). Then in 1934, as a result of the screening of chemicals for the vulcanization process of rubber, dithiocarbamates were discovered and patented as fungicides (Russell, 2005). This group was in many cases more efficient than inorganic products such as copper or sulphur (Jones and Clifford, 1983).

An important expansion of the chemical sector occurred during the 60s; compounds like mancozeb, chlorothalonil, among others were introduced. Another important and characteristic development of this time was the introduction of systemic compounds such as benomyl. In the 1970s the first sterol biosynthesis inhibitors (triazoles) were introduced and exhibited great success in the market (Kuck and Vors, 2012). Then in the 1990s,

another important group introduced into the market was the quinone outside inhibitors (QoIs, strobilurins); these fungicidal compounds were discovered and isolated from the mycelium of *Strobilurus tenacellus* a wood-decaying Basidiomycete species (Anke *et al*., 1977).

Finally, the last decade has seen the reintroduction of another fungicidal group: succinate dehydrogenase inhibitors (SDHIs). In the 1960s, the firsts SDHIs were developed mainly to control pathogens such as *Ustilago maydis* and other basidiomycetes (Ulrich and Matre, 1972). The SDHIs belonging to the chemical class of pyrazole carboxamides developed recently such as penthiopyrad or bixafen are characterized to have a broader spectrum of fungicidal activity including also ascomycetes of agronomic importance (Avenot and Michailides, 2010). Bayer CropScience discovered bixafen in 2001, and it shows high efficacy against many cereal pathogens in numerous field trials (Suty-Heinze *et al*., 2011).

Fungicides have been used in wheat production since the introduction of the first systemic fungicides in the 1960s (Hewitt, 1998). To control the main foliar diseases in wheat, the principal fungicidal groups used are triazoles, strobilurins, morpholines, and recently, the SDHIs. In North America the main fungicidal groups used to control foliar diseases in wheat are strobilurins and triazole (Wegulo *et al*., 2012).

The effectiveness of the fungicide application in controlling foliar pathogens is mainly attributed to the accuracy and period of the application as well as the efficacy of the active ingredient. In wheat, to guarantee an optimum grain yield the fungicide application should concentrate on providing protection to the flag leaf, since this leaf layer contributes a majority of the photoassimilates required for grain filling. Application of fungicides in wheat production varies from 0 to 4 applications per season depending on the region and the disease incidence (Jörgensen *et al*., 2008). Farmers usually apply fungicides early in the season to control diseases such as tan spot. Then a second application is performed mainly to protect the flag leaf and is sometimes followed by a third application which is performed at early flowering to reduce the incidence of Fusarium head blight (Wegulo *et al*., 2012).

3. SIDE EFFECTS OF FUNGICIDES ON PLANT PHYSIOLOGY

In addition to the fungicidal effect, several studies have reported a range of benefits resulting from the use of some fungicidal groups on the physiology of wheat. The majority of these experiments demonstrated that the application of some fungicides, especially strobilurins, resulted in increased grain yields and grain protein content, which were associated with the delay of senescence of the flag leaves.

Specifically, the application of pyraclostrobin (Jabs *et al*., 2002), azoxystrobin (Bertelsen *et al*., 2001) and kresoxim-methyl (Grossmann and Retzlaff, 1997) resulted in an extension of the leaf lifetime by delaying senescence processes, and was often associated with increased yields (Grossmann and Retzlaff, 1997).

The extension of the green leaf area duration (GLAD) triggered by strobilurins has been explained by an effect of strobilurins on the metabolism of phytohormones. Kresoxim-methyl increased the endogenous cytokinin level of wheat tissue and reduced the 1-aminocyclopropane-1-carboxylic acid (ACC) content, which catalyzes the first step in the biosynthesis of ethylene (Grossmann and Retzlaff, 1997).

Other observations have reported that the use of fungicides may cause an improvement of plant tolerance to water deficit stress. Application of fungicidal compounds belonging to the strobilurin and azole groups resulted in an increment of plant tolerance to different environmental stresses (Wu and Von Tiedemann, 2002; Jaleel *et al*., 2006). For example, foliar application of pyraclostrobin resulted in a reduction of the ethylene biosynthesis in treated wheat shoots after short-term drought stress (Jabs *et al*., 2002). Similarly, horse chestnuts trees (*Aesculus hippocastanum*) treated with either epoxiconazole, propiconazole, penconazole or paclobutrazol were more tolerant to drought stress than untreated plants (Percival and Noviss, 2008). It was also observed that triazole treated trees had higher photosynthetic rates, total foliar chlorophyll and proline concentration than untreated tress at the end of a three-week drought (Percival and Noviss, 2008).

Another reported effect of fungicides on plant physiology is an increase of the activity of antioxidant enzymes produced by strobilurins as well as by triazole application in plants exposed to water deficit. Wu and Von Tiedeman (2002) reported that azoxystrobin application significantly increased the activity of the antioxidative enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT), protecting barley plants against injurious ozone doses. In contrast to untreated plants, cowpea plants treated with propiconazole were more resistant to the adverse effects of drought stress, and additionally the activity of SOD, APX and CAT was reported to be higher in treated plants (Manivannan *et al*., 2007).

Nevertheless, fungicides may also generate some phytotoxicity in crop plants. Foliage growers in the 1980s reported phytotoxic effects after benzimidazole applications (Van Iersel and Bugbee, 1996). A slight phytotoxicity was observed in cocoa due to azole application (Prior, 1987). Holderness (1990) reported side effects of triazoles such as an inhibition of the stem extension in cocoa plants. Furthermore, fungicide application may generate a fluctuation in the photosynthetic activity of plants. For example, fludioxonil application induced a reduction of the net photosynthetic rate of grapevine (Petit *et al*., 2008).

4. NON-INVASIVE METHODS USED FOR THE ASSESSMENT OF FUNGICIDE EFFECTS ON WHEAT PHYSIOLOGY

4.1. Photosynthetic activity

4.1.1. Gas exchange

The measurement of the photosynthetic activity of plants may provide crucial information regarding plant vitality. Carbon dioxide (CO_2) is one of the main substrates for the transformation of sunlight to chemical energy by the process of photosynthesis. Therefore, assessment of CO_2 uptake becomes an accurate technique to determine the photosynthetic activity of plants. The gas exchange is one of the most commonly utilized tools to determine the photosynthetic activity of plants (Long *et al*., 1996). Gas exchange measurements are based on the placement of a sample in a closed chamber in order to assess fluctuations in the proportion of gases inside the chamber (Millan-Almaraz *et al*., 2009). Gas exchange methods may also be open systems, which measure the CO_2 concentration in the airflow passing over the sample or may be closed systems, where the air passes through a chamber and the CO_2 taken from a fixed volume of air is measured (Millan-Almaraz *et al*., 2009). However, nowadays the open systems are the most commonly used (Gallé and Flexas, 2010). According to Hunt (2003), the open flow gas exchange systems have several advantages, which are: i) photosynthesis and respiration may be assessed continuously, ii) different types of samples (leaves, roots, entire plants) may be monitored due to the diversity of the chamber designs, iii) it is possible to control environmental conditions in a flushed chamber and iv) it is a non-destructive method. Nearly all open flow gas systems consist of the following parts: an air supply unit, a precision flow meter, a sample chamber and the Infrared Gas Analyzers (IRGAs) (Gallé and Flexas, 2010).

In this particular study, the open flow differential infrared gas analyzer system CMS-400 (Walz, Germany) was used. The CMS-400 is based on a central unit, in which two IRGAs measure the CO_2 and H_2O vapor concentration in the air. In brief, in the CMS-400 the air supply passes through a buffer vessel. Before entering into the main unit, the air is adjusted by flowing through a condenser (Fig. 1.2). Upon entering the main unit, the air is divided in two streams, a measuring gas stream and a reference gas stream. The measuring stream passes to the cuvette and the reference stream enters an external compensation vessel. Afterwards, to assess the H_2O concentration differences both gases pass through a H_2O IRGA. Subsequently, both streams pass through a condenser in order to dry the air. Then both gas streams flow through a CO_2 IRGA to determine differences in the CO_2 concentration (ppm) (modified from Mandemaker, 2007).

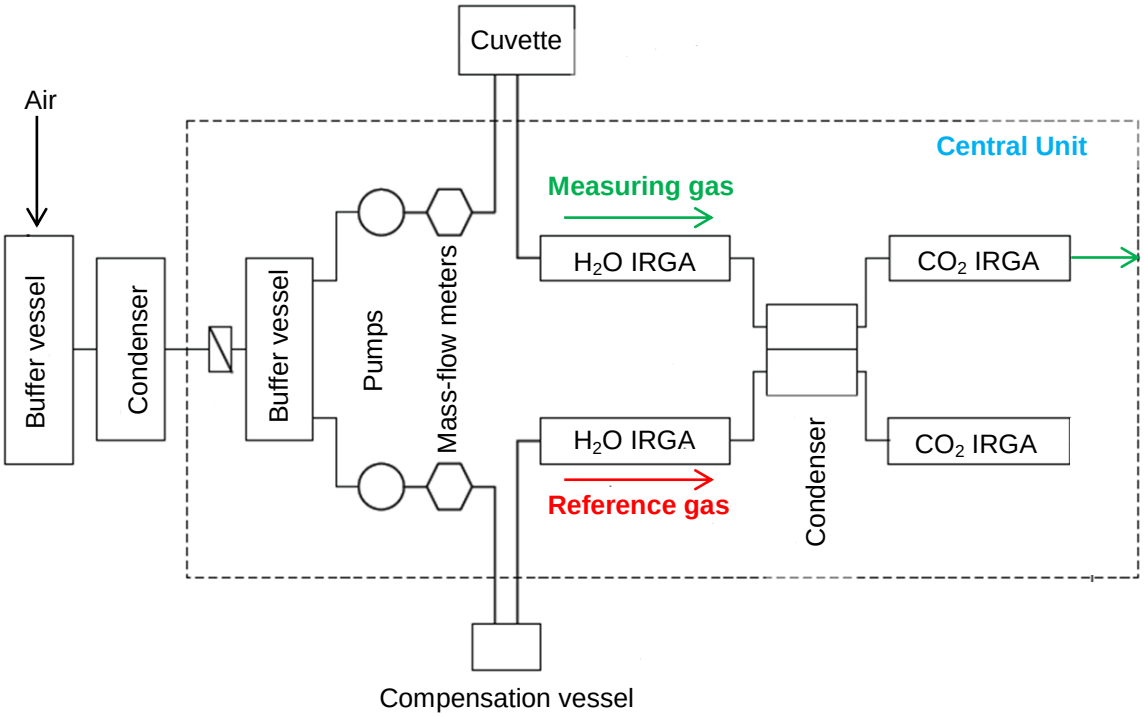

Figure 1.2: Diagram of the air movement in the Walz CMS-400 gas exchange system (modified from Mandemaker, 2007).

Gas exchange measurements have been used with great success to study the effect of fungicides on plant physiology. Beck *et al*. (2001) confirmed by gas exchange measurements a higher photosynthetic activity of strobilurin treated plants in wheat field trials, which resulted in a yield benefit up to 9%.

4.1.2. Chlorophyll fluorescence

When light from the visible spectrum strikes a leaf it can go in one of the three following outcomes: i) it can be absorbed and used as a substrate for photochemical reactions (photochemical quenching - qP), ii) it can be dissipated as heat (nonphotochemical quenching - qN), or iii) it can be re-emitted as light – chlorophyll fluorescence (fluorescence quenching - qF) (Maxwell and Johnson, 2000; Ritchie, 2006; Baker, 2008). More than 80% of the light capture is utilized by photosynthesis (Bürling, 2010) and just 1% - 2% is dissipated as heat and fluorescence (Maxwell and Johnson, 2000).

Nevertheless, the fact that these three possible light pathways occur in competition indicates that an increase in the proficiency of one of them will result in a decrease of light used for the remaining processes (Maxwell and Johnson, 2000). Therefore, the assessment of the chlorophyll fluorescence allows determining changes in the efficiency of the photosynthetic activity of plants. Furthermore, it has been demonstrated that changes in the chlorophyll fluorescence emission are associated to changes in the photosynthetic activity (Baker and Rosenqvist, 2004).

In green plants, chlorophyll and carotenoids capture the absorbed light necessary to drive photosynthesis. Afterwards, the absorbed light (photons) might enter into one of the two reaction centers referred to as: photosystems I and II (PSI and PSII) located on membranes in the chloroplasts (Ritchie, 2006). During photosynthesis, when a photon is absorbed by the PSII an electron becomes exited to a higher energy level and then transferred to the primary quinone acceptor (Q_A). If the former does not occur, the excited electron decays back to the ground state, generating a loss of energy resulting in fluorescent light (Ritchie, 2006). At room temperature, most of the chlorophyll fluorescence (about 90%) is emitted from PSII (Govindjee, 1995). Therefore, chlorophyll fluorescence provides a means to assess the operating quantum efficiency of electron transport through PSII in leaf tissue (Genty *et al*., 1990).

Hans Kautsky and co-workers described the first observations of chlorophyll fluorescence in 1931 (Govindjee, 1995). They reported that dark-adapted photosynthetic material shows an increase in the yield of the chlorophyll fluorescence when illuminated continuously with light, which is now referred to as the Kautsky effect (Govindjee, 1995). The increment of fluorescence emission results from a reduction of the electron acceptors in the photosynthetic pathway (Maxwell and Johnson, 2000).

When a dark-adapted leaf is exposed to a weak modulated measuring beam (ca. 0.1 µmol photons m^{-2} s^{-1}), it results in a minimal level of fluorescence – *Fo*; in this state the PSII reaction centers are maximally open (Fig. 1.3; Baker, 2008). Then, a saturating flash of light is applied (≤ 1 s duration, > 6000 µmol photons m^{-2} s^{-1}) reaching the maximal fluorescence level - *Fm* as a consequence of the maximal reduced Q_A. At this point, the PSII reaction centers are maximally closed incapable of performing photochemistry. The difference between *Fm* and *Fo* is referred to as variable fluorescence - *Fv*. The maximum quantum yield of Q_A reduction is estimated by the ratio of *Fv*/*Fm*. Subsequently, the chlorophyll fluorescence starts to fall due to a much lower steady state. At this point, if the leaf is exposed to a continuous actinic light that will drive photosynthesis (685 µmol photons m^{-2} s^{-1}) it is possible to determine the *F'*. When a brief saturating light pulse is applied the maximal fluorescence level *Fm'* is attained, and the difference between these two parameters can be expressed as *Fq'*. Therefore, the ratio of *Fq'*/*Fm'* represents the quantum yield of PS II before application of a saturating light pulse. This ratio can be used to estimate the non-cyclic electron transport rate (ETR) through PSII (Bürling, 2010).

In this study, the chlorophyll fluorescence technique used was the quenching analysis of modulated fluorescence by the saturation pulse method (PAM). This method is based on an initial light pulse followed by a succession of rapid pulses that overwhelm the electron acceptor pools (Ritchie, 2006). As described previously, figure 1.3 shows a typical PAM fluorescence measurement.

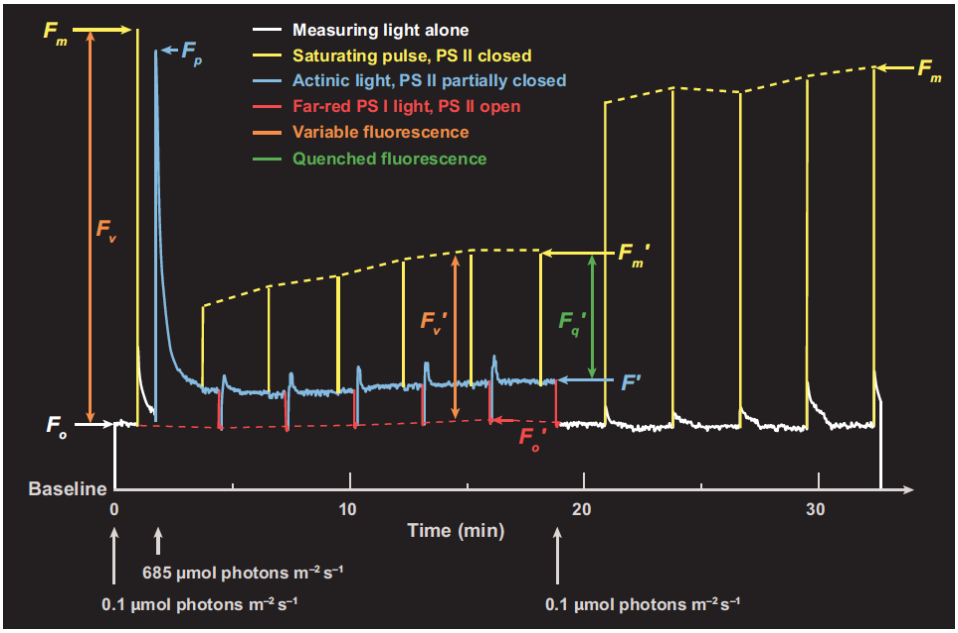

Figure 1.3: Typical chlorophyll emission from a dark-adapted leaf using modulated fluorescence where different quenching parameters are measured (see text, Baker, 2008).

Qingtao *et al*. (2002) reported that in senescent flag leaves of wheat the PSII apparatus remains functional while photosynthetic activity decreases. Since this decrease in senescent leaves is associated to the reduction of the photochemical events of PSI and PSII (Grover and Mohanty, 1992), the use of a chlorophyll fluorometer is an accurate method to detect changes in the senescent states of plant tissue due to fungicide application.

4.2. Transpiration

4.2.1. Infrared thermography

Plant temperature depends mainly on environmental factors such as the temperature of the environment, relative humidity, incoming radiation as well as the water status and the transpiration rate of plants (Oerke and Steiner, 2010). Plant transpiration is the process plants control tissue temperature and it is regulated by stomatal aperture (Jones, 1998). A negative correlation has been observed between plant surface temperature and transpiration rate (Chaerle and van der Straeten, 2000; Jones and Schofield, 2008).

In the past, plant temperature was measured primarily using non-contact thermometers with the disadvantage that most of these instruments were only allowed to measure the temperature at specific points (Oerke and Steiner, 2010; Vadivambal and Jayas, 2011). However, at present most studies are performed using infrared thermography, a real-time measuring technique that allows measuring the plant surface temperature in a non-invasive approach.

The assessment of the surface temperature by infrared thermography is based on the fact that all objects at a temperature above absolute zero (0 K; −273.16 °C; -459.69 °F) emit infrared rays (infrared radiation – IR) in the range of 0.75 - 1000 μm of the electromagnetic spectrum. The radiation intensity emitted by an object is principally related to its surface temperature (Vadivambal and Jayas, 2011). IR thermography provides a visualization of an object's surface temperature by detecting emitted infrared radiation (long-wave infrared [8-14 μm]), which is illustrated in false color images (Chaerle and van der Straeten, 2000). IR thermography translates the emitted radiation into temperature values. In thermographs, each image pixel gives a temperature value of the measured object (Mahlein *et al*., 2012).

Digital infrared thermal imaging techniques have the advantages that they are non-invasive and non-destructive methods, which allows determining the temperature distribution of an object and also a direct comparison of the temperature of plants treated differently under the same climate conditions (Berdugo *et al*., 2012; Mahlein *et al*., 2012).

Thermography has been used successfully in several fields such as medicine, veterinary, environment and agriculture (Meola and Carlomagno, 2004). In the case of the agricultural sector, IR thermography has been useful to monitor changes in the transpiration rate of plants due to factors such as abiotic and biotic stresses (Ayeneh *et al*., 2002; Jones *et al*., 2009; Oerke e*t al*., 2006; Stoll *et al*., 2008). One of the first stages of plant senescence is stomatal closure, which corresponds to a diminution of transpiration rate (Munne-Bosch and Alegre, 2004). This, in turn, can result in an increase of tissue temperature and consequently, a decrease of leaf photosynthetic activity (Kitaya *et al*., 2003). Thermography can then be used as an accurate technique to detect differences in canopy temperature related to plant senescence as reported by Lenthe *et al.* (2007).

Thermography has been used successfully in previous studies to estimate the effect of fungicides on physiological parameters such as the plant tolerance to water deficit stress (Inagaki *et al*., 2009). They reported that thermography allowed to establish differences in leaf temperature between pyraclostrobin treated and untreated plants growing under increasing water deficit conditions. Furthermore, thermograms of wheat plants revealed significant differences between untreated and fungicide treated plants at different growth stages (Berdugo *et al*., 2012). Moreover, this technique was useful to assess the effect of fungicides on vitality and yield of wheat in field trials (Lenthe *et al*., 2007).

4.3. Senescence status

4.3.1. Spectrometry

When the incoming radiation from the sun strikes the surface of an object, it can be reflected (diffuse, specular), transmitted (with refraction) or absorbed. This interaction

depends mainly on the properties of the radiation and also on the properties of the object (Kumar *et al*., 2001). Due to the fact that light absorption is rather important for plant physiological processes, there has been considerable interest in studying the implications of the canopy vitality on plant reflectance.

Plant reflectance results from many interactions of specific plant variables, i) biochemical variables such as pigments, water, lignin and cellulose concentration, and ii) structural variables such as plant and canopy architecture (intercellular space, cell arrangement, anatomy) (Asner, 1998).

The majority of the radiation emitted by the sun occurs in the range of 200 to 2500 nm (Ollinger, 2011). The reflectance of the sunlight is partitioned into three wavelength ranges: the visible (VIS, 400 to 700 nm), near infrared (NIR, 700 to 1100 nm) and short wave infrared (SWIR, 1100 to 2500 nm) range (Fig. 1.4). The reflectance in the VIS region is low as a consequence of the high absorption of light to drive photosynthesis by plant pigments such as: chlorophylls (chlorophyll a and b) that absorb violet-blue (400 to 500 nm) and red light (650 to 700 nm); carotenoids (carotenes and xanthophylls) which absorb blue light (400 to 495 nm); and anthocyanins with a maximum light absorption at 550 nm (Fig. 1.4; Sims and Gamon, 2002; Gitelson *et al*., 2001; Ollinger, 2011). In VIS region, there is a reflectance peak at approximately 540 nm due to a relative lack of absorption in the wavelengths between the two chlorophyll absorption bands (Jensen, 2002). In a typical healthy green leaf the reflectance increases dramatically between 680 and 750 nm; this transition region of the spectra is referred to as the “red-edge”, and it is the transition between the VIS and the NIR region (Filella and Peñuelas, 1994).

In plants, the light reflectance and transmittance in the near-infrared region is high as a consequence of low light absorption by pigments above 700 nm (Fig. 1.4; Merzlyak *et al*., 2002). Comparison of the reflectance and transmission between green and white (albino) corn leaves has shown that pigment concentration did not affect the reflectance in this spectral region (Maas and Dunlap, 1989). Rather, the reflectance in this region is mainly influenced by structural variables such as internal structure and surface characteristics (Jensen, 2002). In this region, between 40 to 60 percent of the incident energy is reflected by the spongy mesophyll and the remaining 45 to 50 percent is transmitted through the leaf resulting in an absorbance between 5 to 10 percent (Jensen, 2002). The reflectance in the short wave infrared region (SWRI) is strongly influenced by the water absorption and it is much lower than in the NIR (Fig. 1.4; Kumar *et al*., 2001). This region is more sensitive to estimate the vegetation water content than the VIS and the NIR (Tucker, 1980). There is a strong correlation between the reflectance in this region and the water content on the leaves (Jensen, 2002).

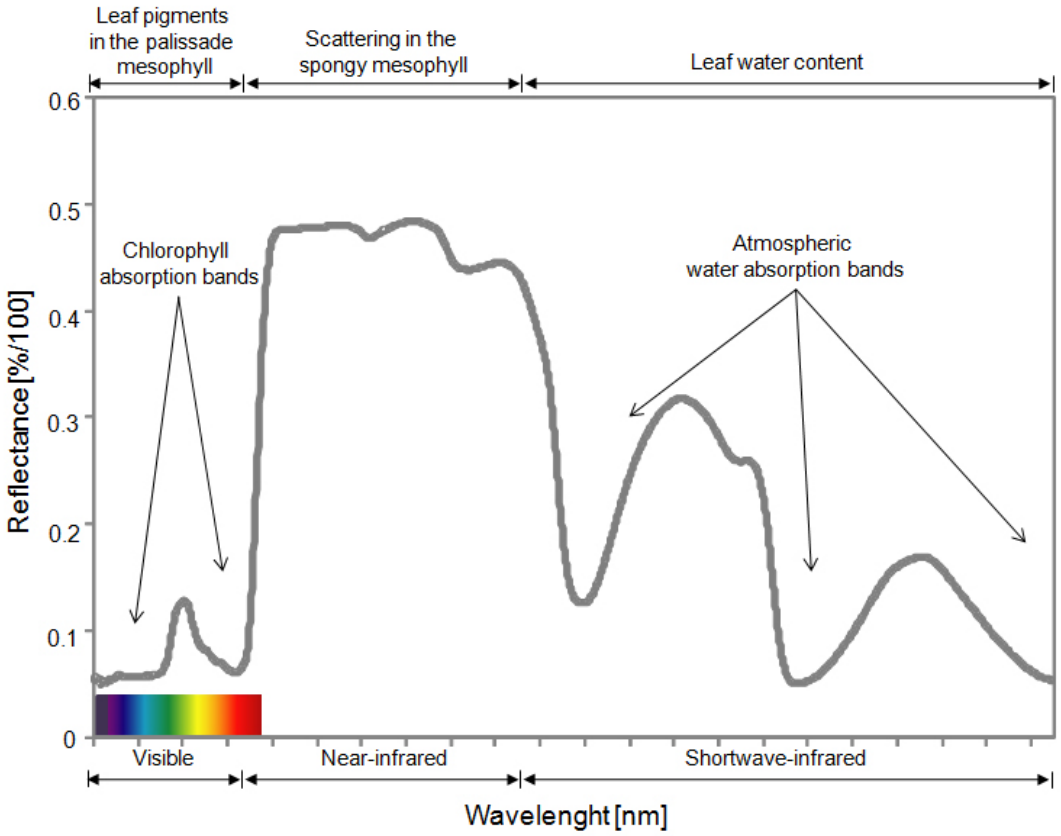

Figure 1.4: Typical reflectance spectra of vegetation in the VIS, NIR, and SWIR and absorption characteristics of biochemical plant components (modified from Curran, 1989; Jensen, 2002; Mahlein, 2011).

Plant reflectance can be measured by imaging and non-imaging sensors (Melesse *et al*., 2007). With non-imaging sensors, it is possible to measure an average of the reflectance of a defined area (Mahlein *et al*., 2010), whereas imaging sensors such as hyperspectral cameras allows the detection of spectral information of a plant with high spatial resolution (Mahlein *et al*., 2012).

In a hyperspectral image, each pixel has a spectrum; therefore, it is possible to spatially map the information of the signal from the studied object (Kumar *et al*., 2001).

In order to analyze the spectral signatures of vegetation, different spectral vegetation indices (SVIs) have been developed during the last years. They are spectral algorithms based on specific wavelengths of the spectral signature (Mahlein *et al*., 2012). SVIs are quantitative measurements, which have more sensitivity than simple spectral bands to estimate different parameters related to plant vitality (Bannari *et al*., 1995).

SVIs like the photochemical reflectance index (PRI, Gamon *et al*., 1992), plant senescence reflectance index (PSRI, Merzlyak *et al*., 1999), anthocyanin reflectance index (ARI, Gitelson *et al*., 2001) and water index (WI, Peñuelas *et al*., 1997) are highly correlated to biochemical and biophysical plant parameters, which are related to plant health and vitality. Therefore, spectral reflectance measurements and SVIs can provide a

non-destructive assessment of changes of the physiological status and vitality of vegetation caused by stress conditions (Blackburn, 1998; Römer *et al*., 2012) and may be used to monitor physiological changes caused by fungicides to crop plants like the delay of senescence.

Spectrometry has been a suitable technique to measure the effect of biotic and abiotic stresses on plant vitality (Mahlein *et al*., 2012). Different studies have linked the spectral reflectance of radiation to the physiological status of plants (Asner, 1998; Carter and Knapp, 2001; Stylinski *et al*., 2002). A healthy leaf represents highly performing photosynthetic machinery, efficiently absorbing radiation in the visible part of the spectrum (Gitelson and Merzlyak, 1994). Therefore, reflectance in the VIS is highly correlated to the pigment content and subsequently to plant vitality (Gitelson and Merzlyak, 1996; Carter and Knapp, 2001).

Another useful application provided by the use of spectrometry is the measurement of the senescence status of plants. During senescence, chlorophyll concentration decreases, whereas carotenoids and anthocyanins become apparent (Gitelson *et al*., 2001; Merzlyak *et al*., 1999); this accounts for the yellowing of senescent leaves. This yellowing causes an increase of reflectance in the VIS, and is measurable with some vegetation indices such as the anthocyanin reflectance index (ARI) in an early stage of senescence. Besides changes in pigmentation, leaf senescence produces differences in leaf structure and leaf water content. This was assessable in the NIR of the reflectance spectrum and by the WI as an indicator of leaf water content (Peñuelas *et al*., 1997; Carter and Knapp, 2001).

Hyperspectral imaging techniques have been used to assess changes produced by pathogen infections in several crops (Rumpf *et al*., 2010; Mahlein *et al*., 2012). Moreover, it has been used effectively to measure the effect of biotic and abiotic stresses on plant vitality (Mahlein *et al*., 2012) and to detect changes in plant senescence (Santos *et al*., 2010).

4.3.2. Analysis of RGB images

The color images captured by digital cameras are composed of three colors (red-R, green-G and blue-B) based on the three-dimensional model color space RGB model (Bock *et al*., 2010). The main reason for using digital cameras is that they are inexpensive and simple to operate (Bock *et al*., 2010). Furthermore, image collection equipment can acquire several images per hour, which can be further analyzed with an imaging analysis software program (Diaz-Lago *et al*., 2003; Mirik *et al*., 2006).

There are many image analysis software programs available, which have been used mainly in the quantification of the severity of plant diseases (Bock *et al*., 2010). The image analysis software programs are based on the process called segmentation, which is the

method to separate areas of interest based on selecting pixels that match a specific criteria (Russ, 1998; Bock *et al*., 2010). The software used in this study was ASSESS© 2.0 (ASSESS©: Image Analysis Software for Plant Disease Quantification, APS Press, St. Paul, MN, USA, Lamari, 2002), which is based on segmentation and it is a popular software used for disease quantification showing it to be more precise than visual assessment (Steddom *et al*., 2005; De Coninck *et al*., 2012). The program analyzes the images based on setting the thresholds between healthy areas and lesion areas (Bock *et al*., 2010) and can likewise to be used to set thresholds between the green area and senescent leaf area. After thresholding, the program calculates the area of interest in pixels (Bock *et al*., 2010).

In order to study the effect of fungicides on the senescence of plants, methods such as chlorophyll extraction and visual assessment have been used. Unfortunately, these approaches have some disadvantages. Chlorophyll extraction is a destructive method; therefore, it is not possible to follow the same replicate in time series experiments. Furthermore, this technique is restrictive to a small part of the leaf. In the case of visual assessment, the disadvantages are that the perception of colors and light may vary among raters (Bock *et al*., 2010) and due to the nature of the senescence, which is not always homogeneous, the rating could be incorrect and imprecise (Hafsi *et al*., 2000). Therefore, the use of image analysis software programs serves as a more suitable method to quantify the differences in the senescence status of plants due to fungicide application. Thus far, imaging analysis software programs have been used successfully to study differences in the senescence status of plants. For example, using digital image analysis software it was possible to quantify the senescence status of cereal leaves (Hafsi *et al*., 2000).

5. SCOPE OF THE STUDY

Most of the research to date has focused on studying the efficacy of fungicides against plant pathogens. However, some fungicides such as strobilurins and triazoles are described to have additional effects on wheat physiology. Strobilurins and triazoles have been reported to induce physiological changes in crops, like increased tolerance against abiotic stress, darker green appearance of leaves, delayed senescence of photosynthetic leaf area and changes in the balance of phytohormones.

Recently, a variety of broad-spectrum fungicide classes have been developed to control cereal pathogens, e.g. succinate dehydrogenase inhibitors (SDHIs) such as bixafen which is a pyrazole carboxamide. There is great interest to further study the effect of the SDHIs on the physiology and yield formation of crop plants due to the fact that positive effects

have been observed previously under field conditions.

The study of the possible beneficial effects produced by fungicides on plant physiology has received more attention nowadays due to several reports describing the positive effect of different fungicidal groups as observed from the evaluation of plant physiological parameters. There are different methods available to measure the effects of fungicides on plant physiology, such as quantification of green leaf area duration, determination of the chlorophyll content, measurement of enzymatic activities associated to senescence, as well as the assessment of changes in the balance of plant growth regulators. However, most of these methods require destructive sampling of the plant tissue, which is laborious and time consuming, and do not allow following the same leaves throughout the crop development. Therefore, an alternative to destructive methods is the use of non-invasive sensors and imaging techniques, which may enable the detection of early changes in plant physiology. Nevertheless, there are still several challenges to determine the potential of these non-invasive methods to study the effects of fungicides on plant physiology.

The overall objective of the present study was to determine the effect of different fungicidal groups (carboxamides, spiroketalamins, strobilurins and triazoles) on physiological parameters such as photosynthesis, transpiration and senescence status of wheat by the employment of non-invasive sensors and imaging techniques.

The specific objectives were to:

I. Compare the effects produced by different fungicidal active ingredients on senescence and yield formation of wheat under field conditions
II. Determine the reliability of field trials to study the potential beneficial effects of fungicides on crop plants
III. Investigate the effects of the pyrazole carboxamide bixafen on the senescence, morphology, photosynthetic rate and yield formation of wheat in comparison to those caused by azoles, strobilurins and spiroketalamins under disease-free conditions
IV. Determine the suitability of different optical sensors to assess direct effects of the fungicides on crop plants
V. Compare different non-invasive and destructive methods and their ability to measure the effect of fungicides on plant senescence
VI. Determine the effects of fungicides on the tolerance of wheat to water deficit by the use of non-invasive techniques

VII. Establish the sensitivity of new non-invasive techniques such as hyperspectral imaging to detect the effects produced by fungicide application on the senescence status of plants.

6. OUTLINE OF THE THESIS

In order to study the effects produced by fungicides on plant physiology, the research design of this study is summarized in figure 1.5:

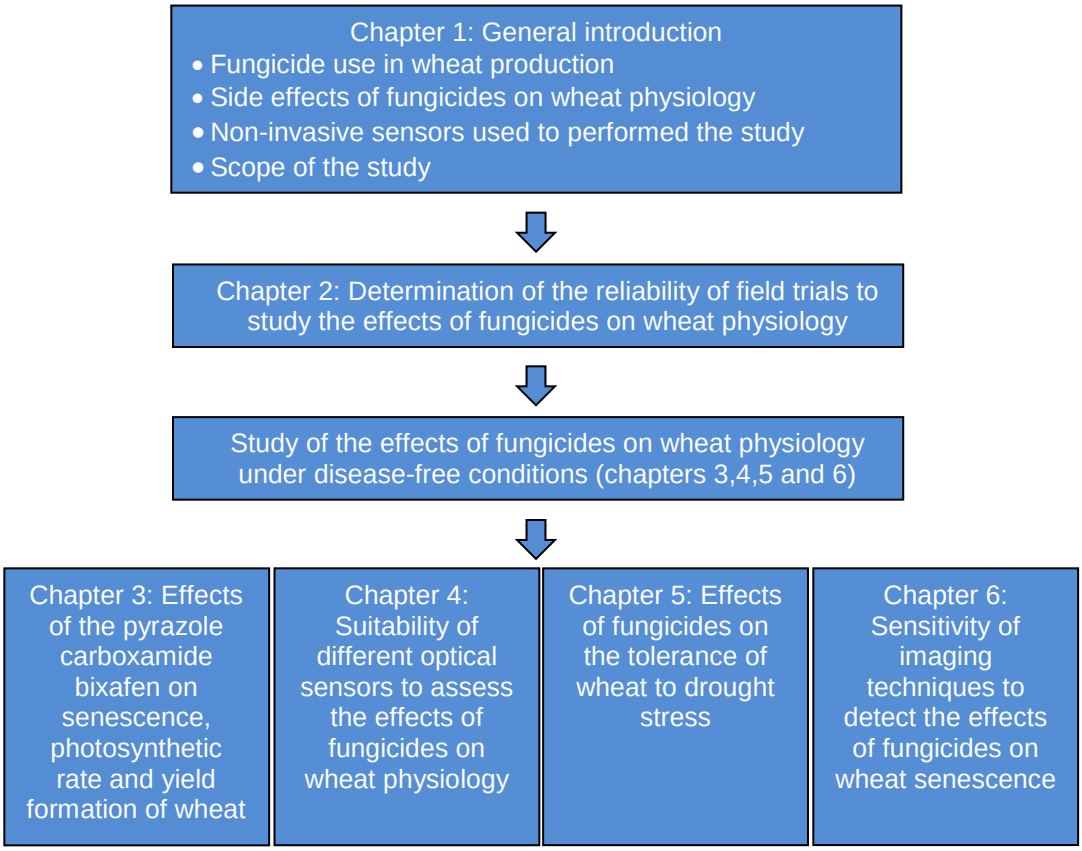

Figure 1.5: Structure of the present study.

In chapter 2, the reliability of field trials to study the effects of fungicides on wheat was assessed. During the field trials, it was possible to observe and register the effect of fungicide application on the senescence and yield formation of wheat. However, the presence of foliar pathogens typically found in wheat influenced the green leaf area duration as well as the yield, generating a disadvantage for fungicide treatments with low efficacy in disease control. Based on the results of chapter 2, the remaining experiments were performed under disease-free conditions in order to separate the fungicidal activity from potential effects that fungicides may cause on crop physiology.

In chapter 3, the effects of the pyrazole carboxamide bixafen on plant physiology are presented. It was observed that the carboxamide group can induce physiological changes in wheat plants such as synchronized ear emergence, increased green leaf area duration, and higher physiological activity of ears during grain filling due to delay of plant senescence. Additionally, tests evaluating the reliability of digital thermography to establish and quantify side effects of fungicides on senescence of leaves and ears are also presented.

Chapter 4 presents the assessment of different types of optical sensors used to record the direct effects of fungicides on crop plants. Different non-invasive and destructive methods were compared to establish their ability to measure the effect of fungicides on plant senescence. The results demonstrate that the use of sensors can reveal changes in the senescence status of wheat plants due to fungicide application. Additionally, these techniques were found to be more sensitive than conventional methods such as destructive and visual assessment.

Studies investigating the influence of fungicide application on the tolerance of wheat to drought stress are presented and discussed in chapter 5. The analysis performed was able to corroborate previous reports that claimed the application of some fungicidal groups had a positive effect on the wheat tolerance to water deficit stress. Specifically, plants treated with the pyrazole carboxamide bixafen were more tolerant to water shortage compared to the untreated control. Moreover, spectral reflectance and thermography revealed significant differences in terms of plant vitality, and both proved to be accurate methods to establish and quantify effects of fungicides on the tolerance of wheat to water deficit. Likewise, these two methods provided sensitive detections of differences between water stress conditions and treatments.

In chapter 6, tests involving two imaging techniques, hyperspectral reflectance and analysis of RGB images are presented. Both methods were evaluated to determine whether they could provide early detection of the effects of fungicide application on wheat senescence. The results of the investigation indicated that the implementation of these techniques can provide early detection of the effects of fungicide application on wheat senescence. Moreover, the results suggest that a combination of non-invasive sensors should be used, which can lead to even earlier detection of changes in the senescence status of plants induced by fungicides. Finally, non-destructive hyperspectral sensing may be implemented in time series experiments to study the effect of new compounds on plant senescence.

7. REFERENCES

Anke, T., Oberwinkler, F., Steglich, W., Schramm, G. (1977). The strobilurins-new antifungal antibiotics from the basidiomycete *Strobiluris tenacellus*. *The Journal of Antibiotics*, 30, 806-810.

Asner, G. (1998). Biophysical and biochemical sources of variability in canopy reflectance. *Remote Sensing of Environment*, 64, 234-253.

Avenot, F.H. and Michailides, T.J. (2010). Progress in understanding molecular mechanisms and evolution of resistance to succinate dehydrogenase inhibiting (SDHI) fungicides in phytopathogenic fungi. *Crop Protection*, 29, 643-651.

Ayeneh, A., van Ginkel, A., Reynolds, M.P. and Ammar, K. (2002). Comparison of leaf, spike, peduncle and canopy temperature depression in wheat under heat stress. *Field Crops Research*, 79, 173-184.

Baker, N.R. and Rosenqvist, E. (2004). Applications of chlorophyll fluorescence can improve crop production strategies: an examination of future possibilities. *Journal of Experimental Botany*, 55, 1607-1621.

Baker, N.R. (2008). Chlorophyll fluorescence: a probe of photosynthesis in vivo. *Annual Review of Plant Biology*, 59, 89-113.

Blackburn, G.A. (1998). Spectral indices for estimating photosynthetic pigment concentrations: a test using senescent tree leaves. *International Journal of Remote Sensing*, 19, 657-675.

Bannari, A., Morin, D., Bonn, F. and Huete, A.R. (1995). A review of vegetation indices. *Remote Sensing Reviews*, 13, 95-120.

Beck, C., Oerke, E.-C. and Dehne, H.-W. (2001). Impact of strobilurins on physiology and yield formation of wheat, Med. Fac. Landbouww, Univ. Gent 67/2, 181-187.

Bennett, F.G.A. (1984). Resistance to powdery mildew in wheat: A review of its use in agriculture and plant breeding programs. *Plant Pathology*, 33, 279-300.

Berdugo, C.A., Steiner, U., Dehne, H.-W. and Oerke, E.-C. (2012). Effect of bixafen on senescence and yield formation of wheat. *Pesticide Biochemistry and Physiology*, 104, 171-177.

Bertelsen, J.R., Neergaard, E.DE. and Smedegaard-Petersen, V. (2001). Fungicidal effects of azoxystrobin and epoxiconazole on phyllosphere fungi, senescence and yield of winter wheat. *Plant Pathology*, 50, 190-205.

Bock, C.H., Poole, G.H., Parker, P.E. and Gottwald, T.R. (2010). Plant disease severity estimated visually, by digital photography and image analysis, and by hyperspectral imaging. *Critical Reviews in Plant Science*, 29, 59-10.

Bürling, K. (2010). Potential of fluorescence techniques with special reference to fluorescence lifetime determination for sensing and differentiating biotic and abiotic stresses in *Triticum aestivum* L. Dissertation, University of Bonn.

Caldwell, C.D. and Starratt, C.E. (1987). Response of Max spring wheat to management inputs. *Canadian Journal of Plant Science,* 67, 645-652.

Carter, G.A. and Knapp, A.K. (2001). Leaf optical properties in higher plants: linking spectral characteristics to stress and chlorophyll concentration. *American Journal of Botany*, 88, 677-684.

Chaerle, L. and Van der Straeten, D. (2000). Imaging techniques and the early detection of plant stress. *Trends in Plant Science*, 5, 495**-**501.

Curran, P.J. (1989). Remote sensing of foliar chemistry. *Remote Sensing of Environment*, 30, 271-278.

De Coninck, B.M.A., Amand, O., Delauré, S.L., Lucas, S., Hias, N., Weyens, G., Mathys, J., De Bruyne, E. and Cammue, B.P.A. (2012). The use of digital image analysis and real-time PCR fine-tunes bioassays for quantification of Cercospora leaf spot disease in sugar beet breeding. *Plant pathology*, 61, 76-84.

Díaz-Lago, J.E., Stuthman, D.D. and Leonard, K.J. (2003). Evaluation of components of partial resistance to oat crown rust using digital image analysis. *Plant Disease*, 87, 667-674.

FAO. (2013). FAOSTAT. Internet source accessed on 13/02/2013. [http://faostat.fao.org/]

Filella, I. and Peñuelas, J. (1994). The red edge position and shape as indicators of plant chlorophyll content, biomass and hydric status. *International Journal of Remote Sensing*, 15, 1459-1470.

Gallé, A. and Flexas, J. (2010). Gas-exchange and chlorophyll fluorescence measurements in grapevine leaves in the field. In: Delrot, S., Medrano, H., Or, E., Bavaresco, L. and Grando, S. eds., Methodologies and results in grapevine research. Springer Science+Business Media B.V. Heidelberg, Germany, pp. 107-121.

Gamon, J.A., Peñeulas, J. and Field, C.B. (1992). A narrow-waveband spectral index that tracks diurnal changes in photosynthetic efficiency. *Remote Sensing of Environment*, 41, 35-44.

García, P.C., Rivero, R.M., Ruiz, J.M. and Romero, L. (2003). The role of fungicides in the physiology of higher plants: implications for defense responses. *Botanical Review*, 69, 162-172.

Genty, B., Harbinson, J., Briantais, J.-M. and Baker, N.R. (1990). The relationship between non-photochemical quenching of chlorophyll fluorescence and the rate of photosystem II photochemistry in leaves. *Photosynthesis Research*, 25**,** 249-257.

Gitelson, A. and Merzlyak, M.N. (1994). Quantitative estimation of chlorophyll-a using reflectance spectra: experiments with autumn chestnut and maple leaves. *Journal of Photochemistry and Photobiology B: Biology*, 22, 247-252.

Gitelson, A.A. and Merzlyak, M.N. (1996). Signature analysis of leaf reflectance spectra: Algorithm development for remote sensing of chlorophyll. *Journal of Plant Physiology*, 148, 494-500.

Gitelson, A.A., Merzlyak, N.M. and Chivkunova, B.O. (2001). Optical properties and nondestructive estimation of anthocyanin content in plant leaves. *Photochemistry and Photobiology*, 74, 38-45.

Govindjee. (1995). Sixty-three years since Kautsky: Chlorophyll a fluorescence. *Australian Journal of Plant Physiology*, 22, 131-160.

Grossmann, K. and Retzlaff, G. (1997). Bioregulatory effects of the fungicidal strobilurin kresoxim-methyl in wheat (*Triticum aestivum*). *Pesticide Science*, 50, 11-20.

Grover, A. and Mohanty, P. (1992). Leaf senescence-induced alterations in structure and function of higher plant chloroplasts. In: Abrol, Y.P., Mohanty, P. and Govindjee, eds., Photosynthesis: Photoreactions to Plant Productivity. Kluwer Academic Publishers, The Netherlands, pp. 225-255.

Hafsi, M., Mechmeche, W., Bouamama, L., Djekoune, A., Zaharieva, M. and Monneveux, P. (2000). Flag leaf senescence, as evaluated by numerical image analysis, and its relationship with yield under drought in durum wheat. *Journal of Agronomy and Crop Science,* 185, 275-280.

Hewitt, H.G. (1998). Fungicides in Crop Protection, CAB International, ISBN 0-85199-164-5, Wallingford, UK.

Holderness, M. (1990). Control of vascular-streak dieback of cocoa with triazole fungicides and the problem of phytotoxicity. *Plant Pathology*, 39, 286-293.

Hunt, S. (2003). Measurements of photosynthesis and respiration in plants. *Physiologia Plantarum*, 117, 314-325.

Inagaki, M., Mori, M. and Nachit, M.M. (2009). Effect of a strobilurin-class fungicide on water use in synthetic bread wheat genotypes grown under increasing water deficit conditions. *Cereal Research Communications*, 37, 513-519.

Jabs, T., Pfirrmann, J., Schäfer, S., Wu, Y. and Tiedemann, AV. (2002). Anti-oxidative and anti-senescence effects of the strobilurin pyraclostrobin in plants: A new strategy to cope with environmental stress in cereals. In: Proceedings of the BCPC Conference - Pest and Diseases, 18-21 November 2002, Brighton, UK, pp. 941-946.

Jaleel, C.A., Gopi, R., Alagu Lakshmanan, G.M. and R. Panneerselvam. (2006). Triadimefon induced changes in the antioxidant metabolism and ajmalicine production in *Catharanthus roseus* (L.) G. Don. *Plant Science*, 171, 271-276.

Jensen, J.R. (2002). Remote sensing of the environment – An earth resource perspective. [reprint.] edition. The MIT Press and MIT Press, Upper Saddle River, NJ, USA.

Jones, D.G. and Clifford, B.C. (1983). Cereal diseases: their pathology and control. John Wiley, Chichester.

Jones, H.G. (1998). Stomatal control of photosynthesis and transpiration. *Journal of Experimental Botany*, 49**,** 387-398.

Jones, H.G. and Schofield, P. (2008). Thermal and other remote sensing of plant stress. *General and Applied Plant Physiology*, 34, 19-32.

Jones, H.G., Serraj, R., Loveys, B.R., Xiong, L., Wheaton, A. and Price, A.H. (2009). Thermal infrared imaging of crop canopies for the remote diagnosis and quantification of plant responses to water stress in the field. *Functional Plant Biology*, 36, 978-989.

Jörgensen, L.N., Jahn, M., Clark, B., Antichi, D., Góral, T., Schepers, H., Lucas, P., Rolland, B., Gouache, D. and Hornok, L. (2008). Control of wheat diseases – optimizing control strategies. In: Proceeding of the Endure conference, 12-15 October 2008, La Grande Motte, France.

Kitaya, Y., Kavai, M., Tsruyama, J., Takahashi, H., Tani, A., Goto, E., Saito, T. and Kiyota, M. (2003). The effect of gravity on surface temperatures of plant leaves. *Plant, Cell and Environment*, 26**,** 497-503.

Kolmer, J.A. (2005). Tracking wheat rust on a continental scale. *Current Opinion in Plant Biology*, 8, 441-449.

Kuck, K.-H. and Vors, J.-P. (2012). Sterol biosynthesis inhibitors. In: Krämer, W. and Schirmer, U. eds., Modern crop protection compounds. Wiley-VCH Verlag GmbH & Co, Weinheim, Germany, pp. 605-650.

Kumar, L., Schmidt, K.S., Dury, S. and Skidmore, A.K. (2001). Imaging spectrometry and vegetation science. In: van der Meer, F. and de Jong, S.M. eds., Imaging Spectrometry. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 111-155.

Lamari, L. (2002). ASSESS: Image Analysis Software for Plant Disease Quantification. APS Press, St. Paul, MN.

Lenthe, J.H., Oerke, E.-C. and Dehne, H.-W. (2007). Digital infrared thermography for monitoring canopy health of wheat. *Precision Agriculture*, 8, 15-26.

Long, S.P., Farage, P.K. and Garcia, R.L. (1996). Measurement of leaf and canopy photosynthetic CO2 exchange in the field. *Journal of Experimental Botany*, 47, 1629-1642.

Loughman, R. and Thomas, G.J. (1992). Fungicide and cultivar control of Septoria diseases of wheat. *Crop Protection*, 11, 349-354.

Mahlein, A.-K., Steiner, U., Dehne, H.-W. and Oerke, E.-C. (2010). Spectral signatures of sugar beet leaves for the detection and differentiation of diseases. *Precision Agriculture*, 11, 413-431.

Mahlein, A.-K. (2011). Detection, identification, and quantification of fungal diseases of sugar beet leaves using imaging and non-imaging hyperspectral techniques. Dissertation, University of Bonn.

Mahlein, A.-K., Oerke, E.-C., Steiner, U. and Dehne, H.-W. (2012). Recent advances in sensing plant diseases for precision crop protection. *European Journal of Plant Pathology*, 133, 197-209.

Mandemaker, A.J. (2007). Winter leaf yellowing in ‘hass’ avocado. Master thesis, The University of Waikato.

Manivannan, P., Abdul Jaleel, C., Kishorekumar, A., Sankar, B., Somasundaram, R., Sridharan, R. and Panneerselvam, R. (2007). Changes in antioxidant metabolism of Vigna unguiculata (L.) Walp. by propiconazole under water deficit stress. *Colloids and Surfaces B: Biointerfaces*, 57, 69-74.

Maas, S. and J. Dunlap. (1989). Reflectance, transmittance, and absorbance of light by normal, etiolated, and albino corn leaves. *Agronomy Journal*, 81, 105-110.

Maxwell, K. and Johnson, G.N. (2000). Chlorophyll fluorescence-a practical guide. *Journal of Experimental Botany*, 51, 659-668.

Melesse, A.M., Weng, Q., Thenkabail, P.S. and Senay, G.B. (2007). Remote sensing sensors and applications in environmental resources mapping and modeling. *Sensors*, 7, 3209-3241.

Meola, C., and Carlomagno, G.M. (2004). Recent advances in the use of infrared thermography. *Measurement Science and Technology*, 15, 27-58.

Merzlyak, M.N., Gitelson, A.A., Chivkunova, O.B. and Rakitin, V.Y. (1999). Non-destructive optical detection of pigment changes during leaf senescence and fruit ripening. *Physiologica Plantarum*, 106, 135-141.

Merzlyak, M.N., Chivkunova, O.B., Melø, T.B. and Naqvi, K.R. (2002). Does a leaf absorb radiation in the near infrared (780–900 nm) region? A new approach to quantifying optical reflection, absorption and transmission of leaves. *Photosynthesis Research*, 72, 263-270.

Millan-Almaraz, J., Guevara-Gonzalez, R., de Jesus Romero-Troncoso, R., Osornio-Rios, R., and Torres-Pacheco, I. (2009). Advantages and disadvantages on photosynthesis measurement techniques: A review. *African Journal of Biotechnology*, 8, 7340-7349.

Mirik, M., Michels Jr, G.J., Kassymzhanova-Mirik, S., Elliott, N.C., Catana, V., Jones, D.V. and Bowling, R. (2006). Using digital image analysis and spectral reflectance data to quantify damage by greenbug (Hemitera: Aphididae) in winter wheat. *Computers and Electronics in Agriculture*, 51, 86-98.

Munne-Bosch, S. and Alegre, L. (2004). Die and let live: leaf senescence contributes to plant survival under drought stress. Functional Plant Biology*,* 31**,** 203-216.

Nesbitt, M. (1998). Where was einkorn wheat domesticated?. *Trends in Plant Science,* 3, 1360-1385.

Oerke, E.-C., Steiner, U., Dehne, H.-W., and Lindenthal, M. (2006). Thermal imaging of cucumber leaves affected by downy mildew and environmental conditions. *Journal of Experimental Botany*, 57, 2121-2132.

Oerke, E.-C. and Steiner, U. (2010). Potential of digital thermography for disease control. In: Oerke, E.-C., Gerhards, R., Menz, G. and Sikora, R.A. eds., Precision crop protection **-** the challenge and use of heterogeneity. Springer, Dordrecht, The Netherlands, pp. 67**-**182.

Ollinger, S.V. (2011). Sources of variability in canopy reflectance and the convergent properties of plants. *New Phytologist*, 189, 375-394.

Peñuelas, J., Pinol, R.O., Ogaya, R. and Filella, I. (1997). Estimation of plant water concentration by the reflectance Water Index WI (R900/R970). *International Journal of Remote Sensing*, 18, 2869-2875.

Percival, G.C. and Noviss, K. (2008). Triazole induced drought tolerance in horse chestnut (Aesculus hippocastanum). *Tree Physiology*, 28, 1685-1692.

Petit, A.N., Fontaine, F., Clément, C. and Vaillant-Gaveau, N. (2008). Photosynthesis limitations of grapevine after treatment with the fungicide fludioxonil. *Journal of Agricultural and Food Chemistry*, 56, 6761-6767.

Prior, C. (1987). Chemical control of vascular-streak dieback disease of cocoa in Papua New Guinea. *Plant Pathology*, 36, 355-360.

Qingtao,L., Congming, L., Jianhua, Z. and Tingyun, K. (2002). Photosynthesis and chlorophyll a fluorescence during flag leaf senescence of field-grown wheat plants. *Journal of Plant Physiology*, 159, 1173-1178.

Trethowan, R.M., Morgunov, A., He, Z., Pauw, D.R., Crossa, J., Warburton, M., Baytasov, A., Zhang, C., Mergoum, M. and Alvarado, G. (2006). The global adaptation of bread wheat at high latitudes. *Euphytica*, 152, 303-316.

Ritchie, G.A. (2006). Chlorophyll fluorescence: what is it and what do the numbers mean?. In: Riley, L.E., Dumroese, R.K., Landis, T.D., technical coordinators. National Proceedings: Forest and Conservation Nursery Associations-2005. Fort Collins (CO): USDA Forest Service, Rocky Mountain Research Station. Proceedings RMRS-P-43, pp. 34-44.

Rumpf, T., Mahlein, A-K., Steiner, U., Oerke, E-C., Dehne, H-W. and Plümer, L. (2010). Early detection and classification of plant diseases with Support Vector Machines based on hyperspectral reflectance. *Computers and Electronics in Agriculture*, 74, 91-99.

Russ, J.C. (1998). The Image Processing Handbook, 3rd edn. Boca Raton, FL, USA: CRC Press.

Russell P.E. (2005). A century of fungicide evolution. *Journal of Agricultural Sciences*, 143, 11-25.

Römer, C., Wahabzada, M., Ballvora, A., Pinto, F., Rossini, M., Panigada, C., Behmann, J., Léon, J., Thurau, C., Bauckhage, C., Kersting, K., Rascher, U. and Plümer, L. (2012). Early drought stress detection in cereals: simplex volume maximisation for hyperspectral image analysis. *Functional Plant Biology*, 39, 878-890.

Santos, M.J., Greenberg, J.A. and Ustin, S.L. (2010). Using hyperspectral remote sensing to detect and quantify southeastern pine senescence effects in red-cockaded woodpecker (*Picoides borealis*) habitat. *Remote Sensing of Environment*, 114, 1242-1250.

Sims, D.A. and Gamon, J.A. (2002). Relationship between leaf pigment content and spectral reflectance across a wide range of species, leaf structures and developmental stages. *Remote Sensing of Environment*, 81, 337-354.

Singh, R.P., Hodson, D.P., Huerta-Espino, J., Jin, Y., Bhavani, S., Njau, P., Herrera-Foessel, S., Singh, P.K., Singh, S. and Govindan, V. (2011). The emergence of Ug99 races of the stem rust fungus is a threat to world wheat production. *Annual Review Phytopathology*, 49, 465-481.

Shewry, P.R. (2009). Wheat. *Journal of Experimental Botany*, 60, 1537-1553.

Shipton, W.A., Boyd, W.R.J., Rosielle, A.A. and Shearer, B.l. (1971). The common Septoria diseases of wheat. *The Botanical Review*, 37, 231-262.

Steddom, K., Bredehoeft, M.W., Khan, M. and Rush, C.M. (2005). Comparison of visual and multispectral radiometric disease evaluations of Cercospora leaf spot of sugar beet. *Plant Disease*, 89, 153-8.

Stoll, M., Schultz, H.R., Baecker, G. and Berkelmann-Loehnertz, B. (2008). Early pathogen detection under different water status and the assessment of spray application in vineyards through the use of thermal imagery. *Precision Agriculture*, 9, 407**-**417.

Stylinski, C.D., Gamon, J.A. and Oechel, W.C. (2002). Seasonal patterns of reflectance indices, carotenoid pigments and photosynthesis of evergreen chaparral species. *Oecologia*, 131**,** 366-374.

Suty-Heinze, A., Dunkel, R., Krieg, U. and Rieck, H. (2011). Bixafen - the new cereal fungicide with yield boosting effects. In: Dehne, H.-W., Deising, H.B., Gisi, U., Kuck, K.H., Russell, P.E. and Lyr, H., eds., Modern Fungicides and Antifungal Compounds VI, Proceedings of the 16th International Reinhardsbrunn Symposium, 25-29 April 2010, Friedrichroda, Germany, Lebenshilfe gGmbH, Braunschweig, pp. 69-74.

Tucker, C.J. (1980). Remote sensing of leaf water content in the near-infrared. *Remote Sensing of Environment*, 10, 23-32.

Ulrich, L.L. and Matre, D.E. (1972). Mode of action of oxathiin systemic fungicides V. Effect on electron transport system of *Ustilago maydis* and *Saccharomyces cerevisiae*. *Journal of Bacteriology*, 110, 628-632.

Vadivambal, R. and Jayas, D.S. (2011). Applications of thermal imaging in agriculture and food industry - A review. *Food and Bioprocess Technology*, 4, 186-199.

Van Iersel, M.W. and Bugbee, B. (1996). Phytotoxic effects of benzimidazole fungicides on bedding plants. *Journal of the American Society for Horticultural Science*, 121, 1095-1102.

Wegulo, S.N., Breathnach J.A. and Baenziger P.S. (2009). Effect of growth stage on the relationship between tan spot and spot blotch severity and yields in winter wheat. *Crop Protection*, 28, 696-702.

Wegulo, S., Stevens, J., Zwingman, M. and Baenziger, P.S. (2012). Yield response to foliar fungicide application in winter wheat, Fungicides for plant and animal diseases, Dhanasekaran, D., ed., ISBN: 978-953-307-804-5, InTech, Available from: http://www.intechopen.com/books/fungicides-for-plant-and-animal-diseases/yield-response-to-foliar-fungicideapplication-in-winter-wheat.

Wu, Y.X. and Von Tiedemann, A. (2002). Impact of fungicides on active oxygen species and antioxidant enzymes in spring barley (*Hordeum vulgare* L.) exposed to ozone. *Environmental Pollution*, 116, 37-47.

CHAPTER 2: EFFECT OF FUNGICIDES ON SENESCENCE AND YIELD FORMATION OF WHEAT UNDER FIELD CONDITIONS

ABSTRACT

The effect of different fungicides on diseases, senescence and yield formation of wheat was assessed in a set of field experiments. Compounds belonging to different fungicidal groups were applied at flag leaf emergence (GS 39) and at ear emergence (GS 59). Application of the azole, carboxamide and strobilurin compounds resulted in lower disease incidence compared to the remaining treatments. However, lower disease incidence was assessed when the pyrazole carboxamide bixafen was applied. The fungicidal efficacy was enhanced by mixing the carboxamide and the azole compounds resulting in lower disease incidence at F, F-1 and F-2.

All fungicide treatments delayed the senescence of the uppermost leaf layers. This effect was also reflected in the leaf chlorophyll content; treatments with lower disease incidence and longer leaf area duration resulted in higher chlorophyll content. All fungicide treatments produced a higher grain yield compared to the untreated control. The extra yield produced by fungicide application varied between 5 and 19%. The effect of fungicides on wheat senescence was positively correlated to grain yield and thousand kernel weight. However, a better correlation was calculated between the extension of the flag leaf life and the grain yield compared to the other leaf layers. It was demonstrated that the carboxamide fungicide bixafen produced similar effects on the green leaf area extension as reported for strobilurins and azoles under field conditions, and in some cases was found to have a higher effect than the other two treatments.

Keywords: Azole, carboxamide, strobilurins, disease incidence, leaf senescence, chlorophyll content.

1. INTRODUCTION

Cultivation and production of wheat (*Triticum aestivum* L.) is one of the most important components of the agricultural economy in many countries (FAOSTAT, 2013). Biotic and abiotic stresses are the primary constraints in wheat production worldwide. Within the biotic stresses, foliar diseases are the main restrictive factors affecting grain yields especially in intensive crops. Septoria leaf blotch (*Septoria tritici*), leaf rust (*Puccinia triticina*) and powdery mildew (*Blumeria graminis*) are the most common limiting diseases

affecting wheat production (Shipton *et al*., 1971; Kolmer, 2005; Bennett, 1984). These diseases on the uppermost three leaf layers reduce the photosynthetic leaf area resulted in lower grain yields (Shaw and Royle, 1989; Wiik, 2009). Oerke and Dehne (1997) reported that yield losses produced by pathogens in susceptible wheat cultivars may be up to 20%. Therefore, different strategies and methods have been implemented to control foliar pathogens of wheat. Application of fungicides and use of resistant and/or tolerant cultivars are necessary to reduce the yield losses caused by foliar pathogens (Bancal *et al*., 2007). However, chemical strategies are the methods most used to control foliar pathogens in intensive wheat production around the world. Since the introduction of the fungicides in the late 1960s, they have been used broadly in crop production resulting in yield benefits (Hewitt, 1998; Wegulo, 2011).

Grain weight is defined during the period between anthesis and physiological maturity (Calderini *et al*., 1999). There are reports regarding negative effects of foliar disease on grain weight as a consequence of the shortened leaf life (Gooding *et al*., 2000; Dimmock and Gooding, 2002). Therefore, a correct and precise disease control after anthesis will result in lower yield losses. Significant positive yield responses due to an effective control of foliar pathogens by fungicide application have been reported for wheat (Caldwell and Starratt, 1987; Loughman and Thomas, 1992).

In the past, most studies were focused on the investigation of the efficacy of fungicidal compounds to control the main foliar diseases (Bateman and Fitt, 1991; Milus, 1994). It was possible to demonstrate the high efficacy of azoles and strobilurins controlling the major foliar pathogens of wheat (Ruske *et al*., 2003). Nevertheless, during the last decades the study of possible beneficial effects of fungicides on wheat physiology has been of great interest. The most reported effect is the extension of the leaf life as a consequence of a delay of the senescence produced by azoles and strobilurins (Cromey *et al*., 2004; Pepler *et al*., 2005).

Additional positive changes reported for strobilurin application have been the greener appearance of leaves with longer green leaf duration compared to leaves treated with other fungicidal compounds (Dimmock and Gooding, 2002). The senescence delay produced by strobilurins application also resulted in higher grain yields. It is cited as being a consequence of the prolongation of the photosynthetic period, which leads to a higher transport of photoassimilates required for grain filling (Bertelsen *et al*., 2001).

Under field conditions, applications of either strobilurins such as kresosim-methyl, azoxystrobin or different azole compounds resulted in longer green leaf area duration compared to the untreated control. However, this extension has been linked in most of the cases to the effective and accurate control of foliar pathogens produced by these two fungicidal groups.

During the last years, the main fungicide producing companies has developed new active ingredients, e.g. the succinate dehydrogenase inhibitors (SDHIs). The fungicidal compound bixafen a pyrazole carboxamide belongs to this group. Bixafen controls fungal pathogens by inhibiting the enzyme succinate dehydrogenase in the respiration chain of fungi (Hoersefield *et al*., 2006). This active ingredient was discovered by Bayer CropScience in 2001 (Suty-Heinze *et al*., 2011), and since then the application of this compound resulted in high control of the major foliar diseases of cereal crops in numerous field trials. Suty-Heinze *et al*. (2011) reported that bixafen has a beneficial effect on the green leaf duration, resulting in a significant yield increase in barley and wheat. Therefore, the goal of this study was to measure and compare the effects of different fungicidal groups including the pyrazole carboxamide bixafen on the leaf area duration and the yield formation of wheat under field conditions.

The overall objectives of this chapter were to:

i. Compare the effects of different fungicidal compounds on senescence and yield formation of wheat under field conditions
ii. Determine the reliability of field trials to study the potential beneficial effects of fungicides on crop plants.

2. MATERIALS AND METHODS

2.1. Experimental design

In order to determine the possible side effects produced by fungicides on the senescence of wheat plants, field trials were conducted during the growing seasons of 2008 and 2009 at the Poppelsdorf research station of the University of Bonn (N50° 43`37 E7° 05`05 - altitude of 59 m above sea level). The annual mean temperature is 10.9 °C, the average precipitation is 634 mm per year (Görtz, 2009). A completely randomized block design was used (Fig. 2.1).

The winter wheat (*Triticum aestivum* L.) cultivar Ritmo was grown. The sowing was performed on November 4th of 2008. In the last week of January wheat plants were fertilized with 60 kg/ha NPK (12:12:17).

For the control of grass weeds, cleavers and other broad-leaved weeds, herbicide application was done (Axial®, active ingredient [a.i.] pinoxaden 45 g a.i. L^{-1}, application rate 1l/ha, Syngenta Agro GmbH, Maintal, Germany; Hoestar® Super, a.i. amidosulfuron 125 g a.i. Kg^{-1}, iodosulfuron-methyl-natrium 12.5 g a.i. Kg^{-1}, mefenpyr-diethyl 125 g a.i. Kg^{-1}, application rate 200 g/ha, Bayer CropScience, Monheim, Germany).

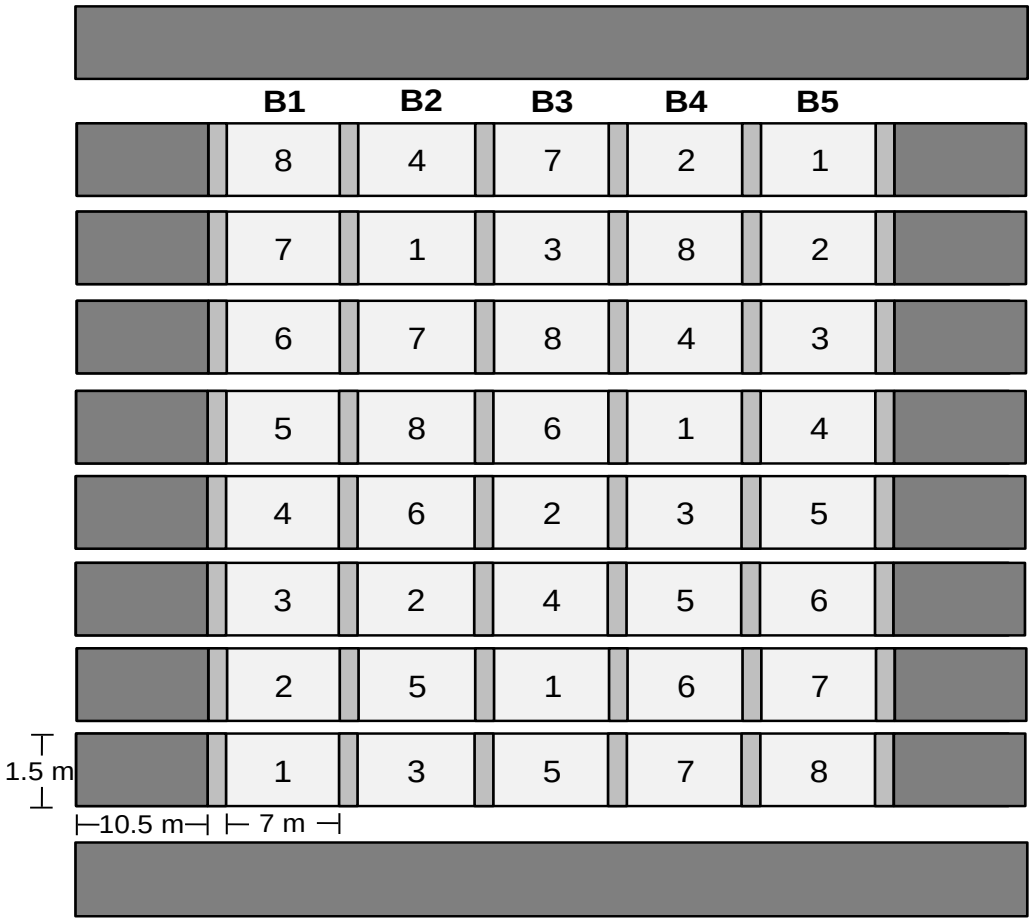

Figure 2.1: Experimental design of field trails at the Poppelsdorf research station, University of Bonn 2008/09; five blocks (B), 8 treatments (1-untreated, 2-bixafen, 3-fluoxastrobin, 4-prothioconazole, 5-spiroxamine, 6-boscalid, 7-bixafen+prothioconazole and 8-spiroxamine+prothioconazole).

Insecticides were applied to control pests (Sumicidin®, a.i. fenvalerate 25 g a.i. L^{-1}, application rate 0.2 l/ha, BASF, Limburgerhof, Germany; Bulldock®, a.i. beta-cyfluthrin 125 g a.i. L^{-1}, application rate 0.1 l/ha, Bayer CropScience, Monheim, Germany).

To avoid fungal inoculum pressure from the plants located in the border area, the fungicide Stratego® was applied (a.i. propiconazole 125 g a.i. L^{-1} and trifloxystrobin g a.i. L^{-1}, application rate 0.5 l/ha, Bayer CropScience, Monheim, Germany).

Each treatment consisted of five plots with a dimension of 7 m x 1.5 m. All plots were harvested individually on the 5th of August 2009 with a cereal plot combine-harvester (Haldrup, Ilshofen, Germany). The activities related to the field experiment are summarized in table 2.1.

Table 2.1: Workflow of the field experiment conducted at the Poppelsdorf research station of the University of Bonn.

Activity	Product	Dose	Date
Sowing of wheat cultivar Ritmo	N/A[1]	N/A	November 4th 2008
Application of granular fertilizer	NPK 12:12:17	60 kg/ha	January 29th 2009
Herbicide application	Axial+Hoestar	1l/ha	April 01st 2009
Plot design	N/A	N/A	May 07th 2009
First fungicide application	Treatments	(Tab. 2.2)	May 11th 2009
Borders fungicide application	Stratego®	0.5 l/ha	May 20th 2009
Second fungicide application	Treatments	(Tab. 2.2)	May 28th 2009
Insecticide application	Sumicidin®	0.2 l/ha	May 29th 2009
1st disease and GLAD rating	N/A	N/A	June 4th 2009
2nd disease and GLAD rating	N/A	N/A	June 11th 2009
Borders application	Stratego®	0.5 l/ha	June 17th 2009
3rd disease and GLAD rating	N/A	N/A	June 18th 2009
1st leaf sampling (chlorophyll content)	N/A	N/A	June 18th 2009
Insecticide application	Bulldock®	0.1 l/ha	June 19th 2009
4th disease and GLAD rating	N/A	N/A	June 25th 2009
5th disease and GLAD rating	N/A	N/A	July 02nd 2009
6th disease and GLAD rating	N/A	N/A	July 09th 2009
2nd leaf sampling (chlorophyll content)	N/A	N/A	July 11th 2009
7th disease and GLAD rating	N/A	N/A	July 16th 2009
Harvest of wheat plots	N/A	N/A	August 05th 2009

[1]N/A: not applicable.

2.2. Fungicide treatments

Seven fungicide spray treatments and a non-treated control were evaluated (Tab. 2.2). Active ingredients belonging to the following fungicidal groups were applied: carboxamides (bixafen, Bayer CropScience, Monheim, Germany and boscalid, BASF, Limburgerhof, Germany), strobilurins (fluoxastrobin, Bayer CropScience, Monheim, Germany), azoles (prothioconazole, Bayer CropScience, Monheim, Germany), and spiroketalamines (spiroxamine, Bayer CropScience, Monheim, Germany).

Fungicidal products were applied at recommended field rates (water 400 L ha^{-1}) at two growth stages (GS) according to the BBCH scale (Hack *et al*., 1992). The first application was performed on the 11th of May when the flag leaf ligule was visible (GS 39), and the second application was carried out on the 28th of May when the emergence of the inflorescences was completed (GS 59). The fungicides were applied with a CO_2 pressurized backpack sprayer (Solo, Hampton, USA) with an adjustable spray.

Table 2.2: Fungicide treatments evaluated in the field experiment conducted at the Poppelsdorf research station of the University of Bonn, 2008/09.

Active ingredient (s)	Formu-lation	Active ingredient content [g a.i./l]	Application rate [l/ha]	Amount applied [g/ha]
Bixafen (Bix.)	EC[1]	125	1	125
Fluoxastrobin	EC	100	2	200
Prothioconazole (Prot.)	EC	250	0.8	200
Spiroxamine (Spir.)	EC	500	0.75	375
Boscalid	WG[2]	500 g/kg	1 kg/ha	500 g/ha
Bixafen+Prothioconazole (Bix.+Prot.)	EC	75 + 150	1.25	94 + 188
Spiroxamine+Prothioconazole (Spir.+Prot.)	EC	300 + 160	1.25	375 + 200

[1] EC: Emulsifiable concentrate
[2] WG: Water dispersible granules

2.3. Disease incidence

After the second fungicide application (GS 59), plots were inspected periodically until harvest to determine the presence of foliar pathogens. Eight random plants per plot were selected to inspect the presence of fungal pathogens on the top three leaves (F, F-1 and F-2). Symptoms causes by the main fungal pathogens of wheat (*Septoria tritici, Puccinia triticina* and *Blumeria graminis f. sp. Tritici*) were recorded.

The disease incidence was determined in percentage by the relation between the affected plants and the total number of inspected plants:

$$\text{Disease incidence [\%]: } \frac{\text{number of infected plants} \times 100}{\text{number of inspected plants}}$$

2.4. Green leaf area duration

Visual assessment of the green leaf area was performed weekly until harvest after the second fungicide application (GS 59). It was measured as percentage of the green area of the top three leaves (F, F-1 and F-2). Every week eight randomly selected plants per replicate per treatment were selected to assess the green leaf area.

The area under the green leaf area curve (AUGLAC) was calculated using the equation given by Cromey *et al*. (2004),

$$AUGLAC = \sum_{i=1}^{n} 1/2(Y_i + Y_{i-1})(X_i - X_{i-1})$$

Where Y_i= percent green leaf area at the *i*th observation, X_i = time (days after the second fungicide application) at the *i*th observation, *n* = total number of observations.

The AUGLAC difference was calculated by subtracting the mean of the AUGLAC of fungicide treatments from the mean AUGLAC of the untreated control.

2.5. Chlorophyll extraction

Eight random tillers were collected from every trial plot for chlorophyll extraction. At two growth stages (GS 75 and GS 85) the chlorophyll content of F, F-1 and F-2 from all treatments was measured. The chlorophyll content was extracted following the protocol of Hiscox and Israelstam (1979).

The total chlorophyll content of F, F-1 and F-2 from all treatments was measured according to Blanke (1990). Dimethyl sulfoxide 99% (DMSO) was used as the extraction solvent for the chlorophyll content measurements. Eight leaf discs with a diameter of 1 cm from the center of the leaves were weighted and placed in glass tubes containing DMSO. The tubes were kept under dark conditions for 24 hours. A double beam UV/VIS spectrophotometer Uvikon 933A (BioTek Instruments, USA) was used to measure the absorbance at specific wavelengths (470, 645 and 663 nm).

2.6. Yield parameters

At GS 71, the number of ears per plot was determined. In each trial plot a square with an area of 1 m^2 was randomly released twice, the ears inside the square were counted and the number of ears was extrapolated to the total area of the plot.

At harvest the straw from every plot was weighted using an electronic balance (Bizerba GmbH & Co. KG, Balingen, Germany). Plots were harvested individually and the grain yields were determined. Subsamples of 2 kg were taken from every plot to determine the thousand kernels weight. Yield parameters such as grain yield, number of ears and thousand kernel weights were calculated.

2.7. Statistical analysis

Data were analyzed using the statistical program SPSS for Windows (IBM Deutschland GmbH, Ehningen, Germany), version 17.0. Data were tested for 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 at 95% confidence in order to separate subgroups. The correlations were

tested at a probability level of 0.05 using the Pearson's correlation coefficient.

3. RESULTS

3.1. Effect of fungicide application on disease incidence

3.1.1. Septoria leaf blotch (Septoria tritici)

For flag leaf (F) the first Septoria symptoms were rated in the untreated plots twenty-one days after the second fungicide application (dasfa; Fig. 2.2).

Fungicide applications significantly reduced the incidence of Septoria leaf blotch. On the last measurement date (49 dasfa), the disease incidence on F was 100% for untreated plants, while in fungicide treated plots it was lower than 20% (Fig. 2.2).

For F-1 the first significant differences between treatments were registered fourteen dasfa; the incidence of Septoria leaf blotch on untreated plots was significantly higher compared to fungicide treated plots. Twenty-one dasfa the disease incidence in spiroxamine and boscalid treated plots started to increase with respect to the remaining fungicide treatments.

From the fourth (28 dasfa) until the last measuring date (49 dasfa) spiroxamine and boscalid treatments had significantly higher disease incidence compared to the other fungicide treatments. Plots treated with bixafen and Bix.+Prot. had significantly lower disease incidence compared to all other treatments from the fifth until the last measuring date (Fig. 2.2).

The disease incidence levels were higher for F-2 compared to the two uppermost leaf layers. At this leaf level the first disease symptoms were rated seven dasfa in untreated plots.

From the first (7 dasfa) to the fourth (28 dasfa) measuring date untreated plots had significantly higher disease incidence than fungicide treatments. The disease incidence was significantly lower for bixafen and Bix.+Prot. treated plots from the fourth measurement onwards.

3.1.2. Leaf rust (Puccinia triticina)

Analysis of rust incidence for the flag leaf showed a general increase for all treatments compared to the Septoria leaf blotch incidence. Furthermore, at the flag leaf level rust symptoms were registered two weeks earlier than Septoria symptoms (Fig. 2.2). From the first until the last measuring date, untreated, spiroxamine and boscalid treated plots were found to have significantly higher rust incidence compared to the other treatments. No significant differences were calculated between the remaining treatments.

For F-1 and F-2, the rust incidence trend was similar than for F. Untreated, spiroxamine

and boscalid treatments had higher rust incidence compared to the other treatments from the first to the last measuring date. However, the disease incidence calculated for all treatments was higher for F-2 compared to the two upper leaf layers (Fig. 2.2).

For all leaf layers, from the third to the last measuring date spiroxamine and boscalid treatments did not differ significantly from the untreated control. On the last assessment date, no significant differences were observed between the remaining treatments for all leaf layers (Fig. 2.2).

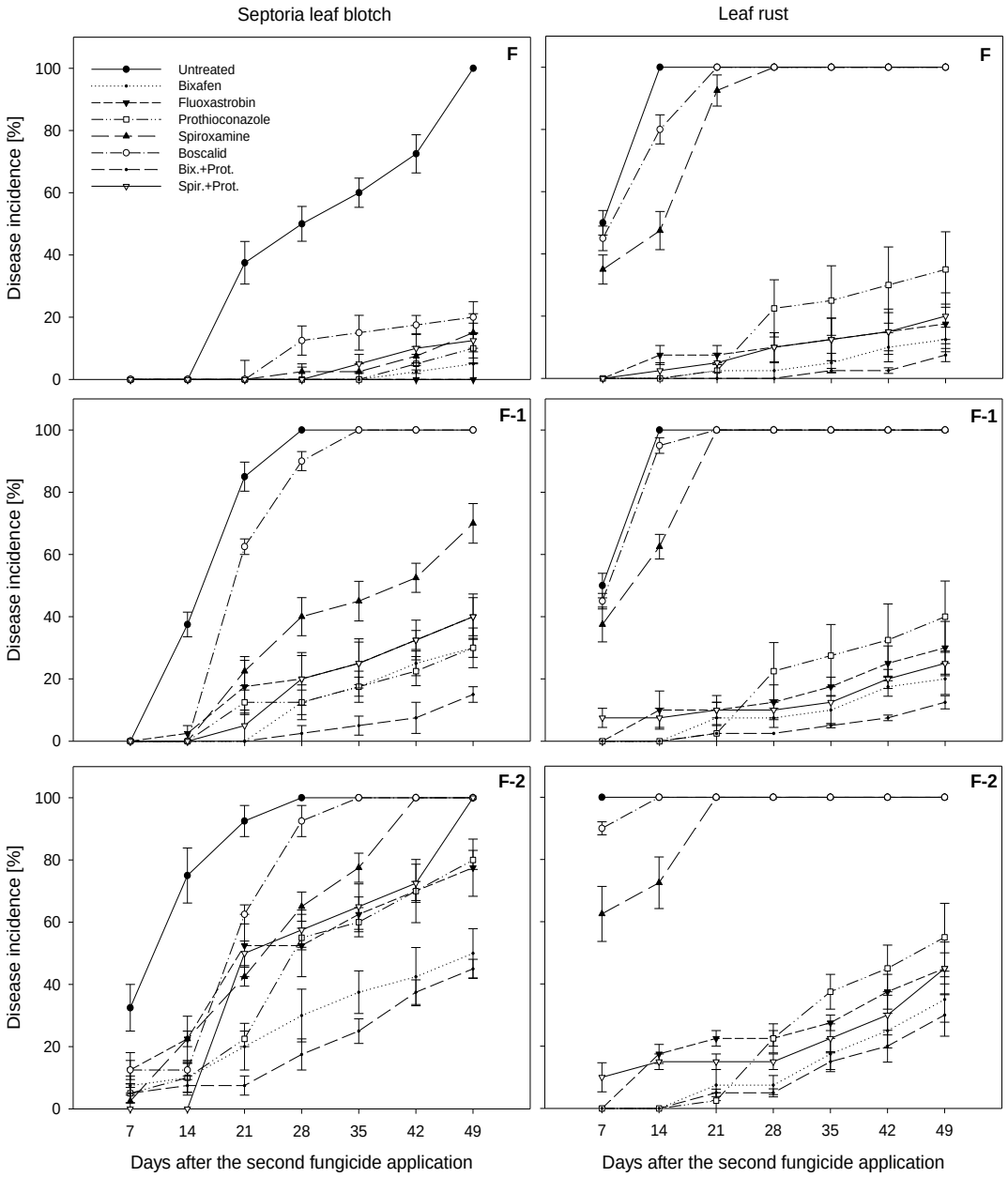

Figure 2.2: Effect of fungicide applications at GS 39 and GS 59 on Septoria leaf blotch and leaf rust incidence on F, F-1 and F-2 of wheat (cv. Ritmo). Field experiment conducted at the Poppelsdorf research station of the University of Bonn, 2008/09. Vertical bars indicate SE ($p \leq 0.05$; $n = 5$).

3.2. Effect of fungicide application on green leaf area duration

The results of the weekly evaluation of GLAD showed that all fungicide treatments significantly extended the GLAD of F compared to the untreated control (Fig. 2.3). However, the delay of senescence produced by spiroxamine and boscalid treatments was lower compared to the one produced by the other fungicides.

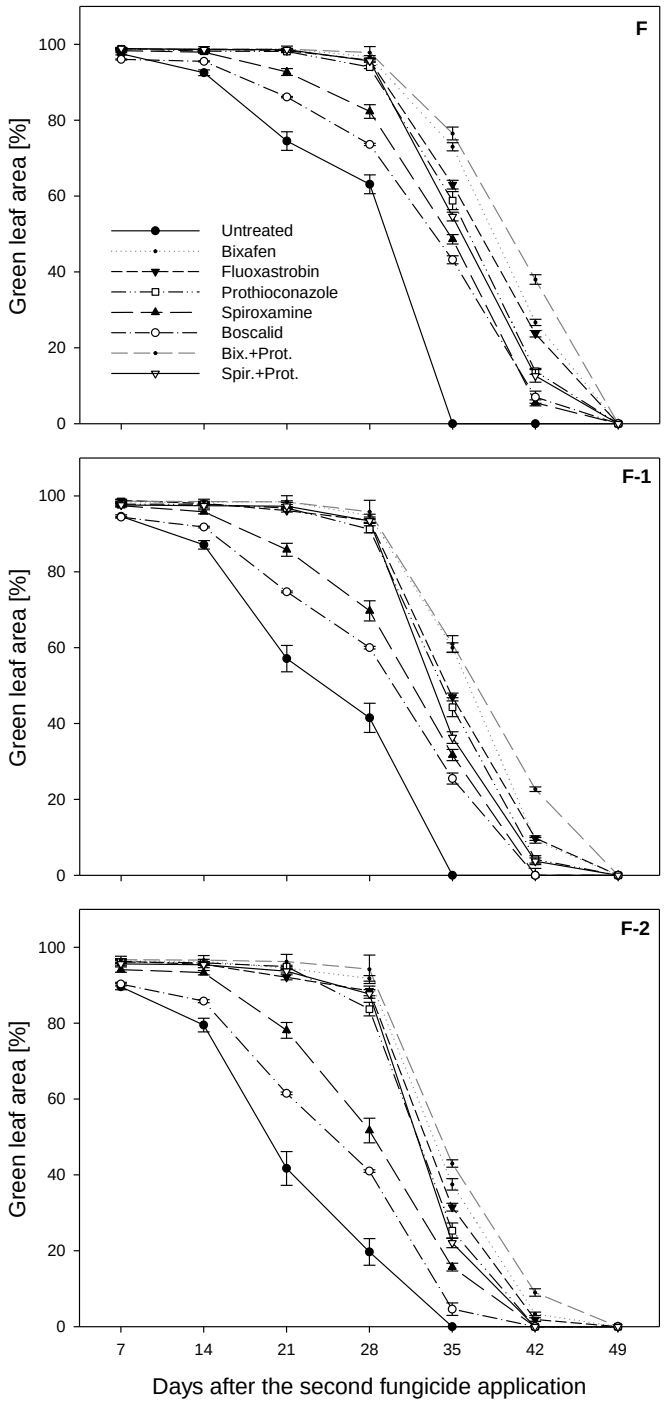

Figure 2.3: Effect of fungicide applications at GS 39 and GS 59 on the green leaf area duration of F, F-1 and F-2 of wheat (cv. Ritmo). Field experiment conducted at the Poppelsdorf research station of the University of Bonn, 2008/09. Vertical bars indicate SE ($p \leq 0.05$; $n = 5$).

The GLAD was extended by fungicide treatments on average by one week. However, bixafen and Bix.+Prot. treated plots had longer GLAD than the other fungicide treatments. Thirty-five dasfa, no green leaf area was rated for the untreated flag leaves.

At the sixth (42 dasfa) evaluating date, Bix.+Prot. treated plots had a higher green leaf area than the other fungicidal treatments (Fig. 2.3).

In the case of F-1, the GLAD started to decay earlier compared to F. Lower green leaf area was rated in untreated plots followed by spiroxamine and boscalid treatments. Significant differences were calculated between treatments from the first to the fifth measurement. As for F, Bix.+Prot. treated plots has longer GLAD than the other treatments.

All fungicide treatments delayed F-2 senescence with respect to the untreated control. Twenty-eight dasfa it was possible to distinguish three different groups of treatments: untreated plots were showing the lowest GLAD, spiroxamine and boscalid treatments both showed an average of 50% of GLA, and the remaining treatments with the highest GLA.

On the next measuring date, it was possible to differentiate bixafen treated plots from the rest of the treatments. Forty-two dasfa, Bix.+Prot. treatment had higher GLAD compared to the other treatments.

The area under the green leaf area curve (AUGLAC) difference to untreated was calculated for all leaf layers (Fig. 2.4).

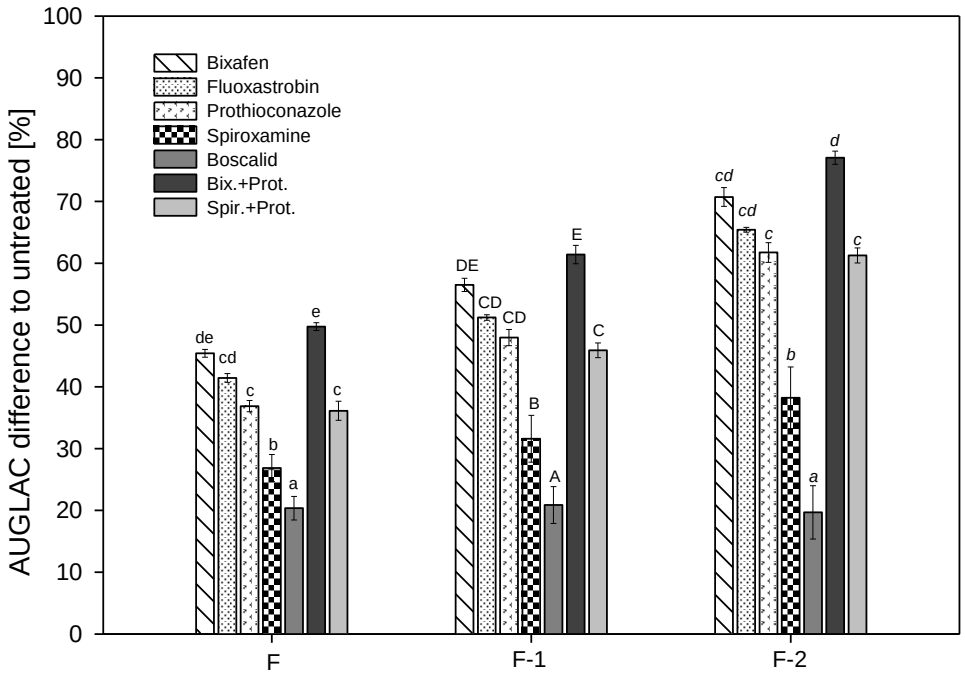

Figure 2.4: Effect of fungicide applications at GS 39 and GS 59 on the area under the green leaf area curve (AUGLAC) differences for F, F-1 and F-2 as compared to untreated control. Field experiment conducted at the Poppelsdorf research station of the University of Bonn, 2008/09. Vertical bars indicate SE. Different letters indicate significant differences according to Tukey's test ($p \leq 0.05$; $n = 5$).

All fungicide treatments resulted in higher AUGLAC than the untreated control at all leaf levels. However, this difference was higher for F-2 and in most cases it was more than 60% compared to the untreated control (Fig. 2.4). For all leaf layers the highest AUGLAC difference to the untreated control was calculated when bixafen, fluoxastrobin, prothioconazole and the two fungicide mixtures were applied. In contrast, the lowest AUGLAC differences to untreated were calculated when spiroxamine and boscalid were applied (Fig. 2.4).

3.3. Effect of fungicide application on chlorophyll content

Samples of F, F-1 and F-2 leaves were collected at GS 75 and GS 85 and the chlorophyll content was calculated. At GS 75, fungicide treated plots had higher chlorophyll content for all leaf layers compared to the untreated control (Fig. 2.5). However, for F, fluoxastrobin treatment was the only one that was significantly different to the untreated control.

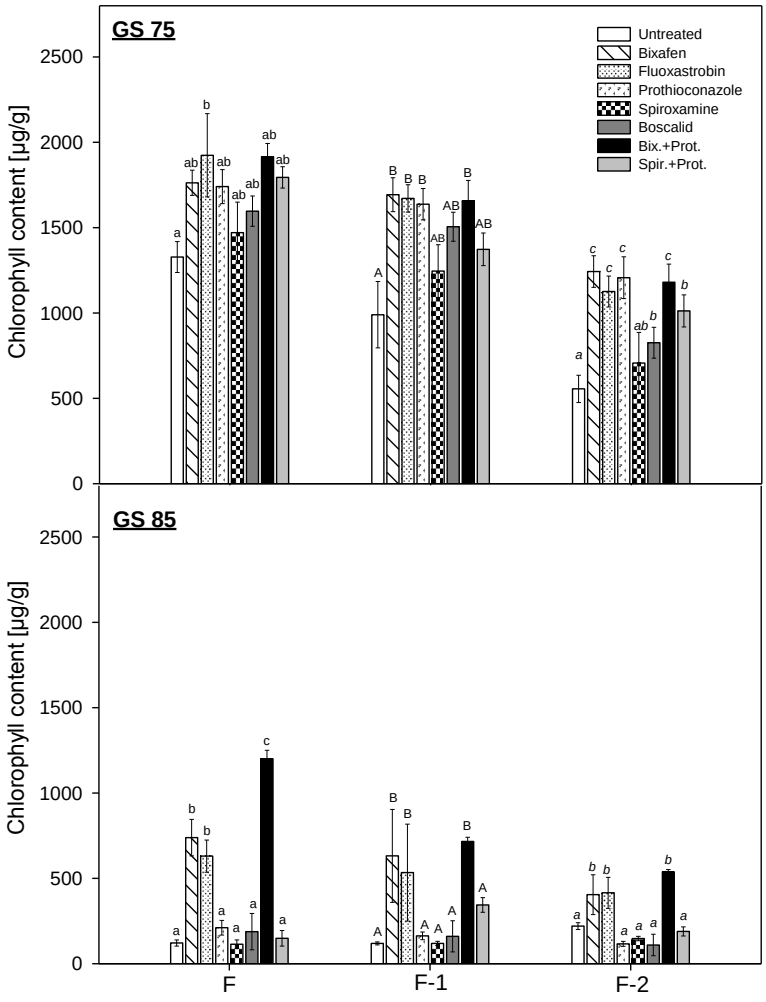

Figure 2.5: Effect of fungicide application at GS 39 and GS 59 on the chlorophyll content of F, F-1 and F-2 leaves of wheat at GS 75 and GS 85. Field experiment conducted at the Poppelsdorf research station of the University of Bonn, 2008/09. Vertical bars indicate SE. Different letters indicate significant differences according to Tukey's test ($p \leq 0.05$; $n = 8$).

In the case of F-1, application of bixafen, fluoxastrobin, prothioconazole and Bix.+Prot. resulted in significant higher chlorophyll content that the untreated control. For F-2, the spiroxamine treatment was the only one that did not result in significant differences to untreated (Fig. 2.5).

Likewise, at GS 85, bixafen, fluoxastrobin and Bix.+Prot. treatments had significantly higher chlorophyll content for all leaf layers compared to the untreated control and the remaining fungicide treatments (Fig. 2.5). Bix.+Prot. application resulted in higher chlorophyll content for F with respect to all other treatments.

3.4. Effect of green leaf area duration on grain yield and thousand kernel weight

The area under the green leaf area curve (AUGLAC) summarized for three uppermost leaves was positively correlated to grain yield and TKW (Fig. 2.6). However, the correlations between these two yield parameters and the AUGLAC tended to vary between treatments.

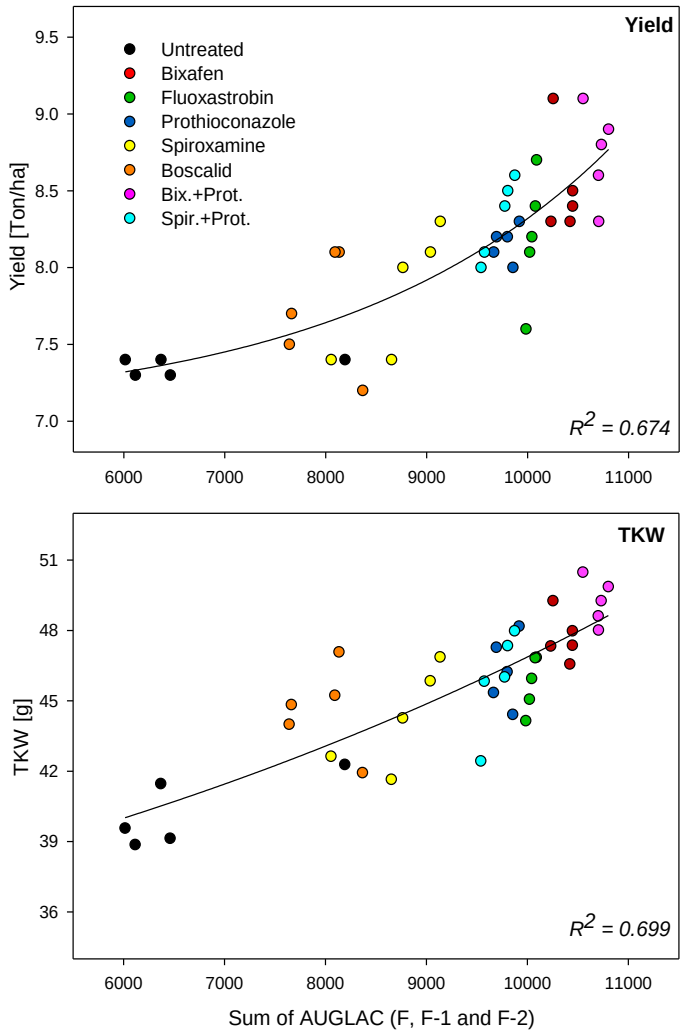

Figure 2.6: Relationship between yield and TKW and the area under the green leaf area duration summarized for the three uppermost leaves (F, F-1 and F-2) of wheat. Field experiment conducted at the Poppelsdorf research station of the University of Bonn, 2008/09.

For all treatments, there was a strong relationship between the yield parameters and AUGLAC. A strong correlation (> 0.6) was calculated on average for all treatments. Treatments with a high AUGLAC resulted in both a high grain yield and TKW, which were observed for bixafen, prothioconazole, fluoxastrobin and the two fungicide combinations. All fungicide treatments produced a higher AUGLAC of the uppermost three leaves compared to the untreated control, which was clearly reflected in the yield for all treatments excluding spiroxamine and boscalid treatments, where grain yield and TKW increment for these two treatments was minute.

It was possible to observe a relationship between the extension of the green leaf area duration of every leaf layer produced by fungicide application and grain yield and TKW (Fig. 2.7).

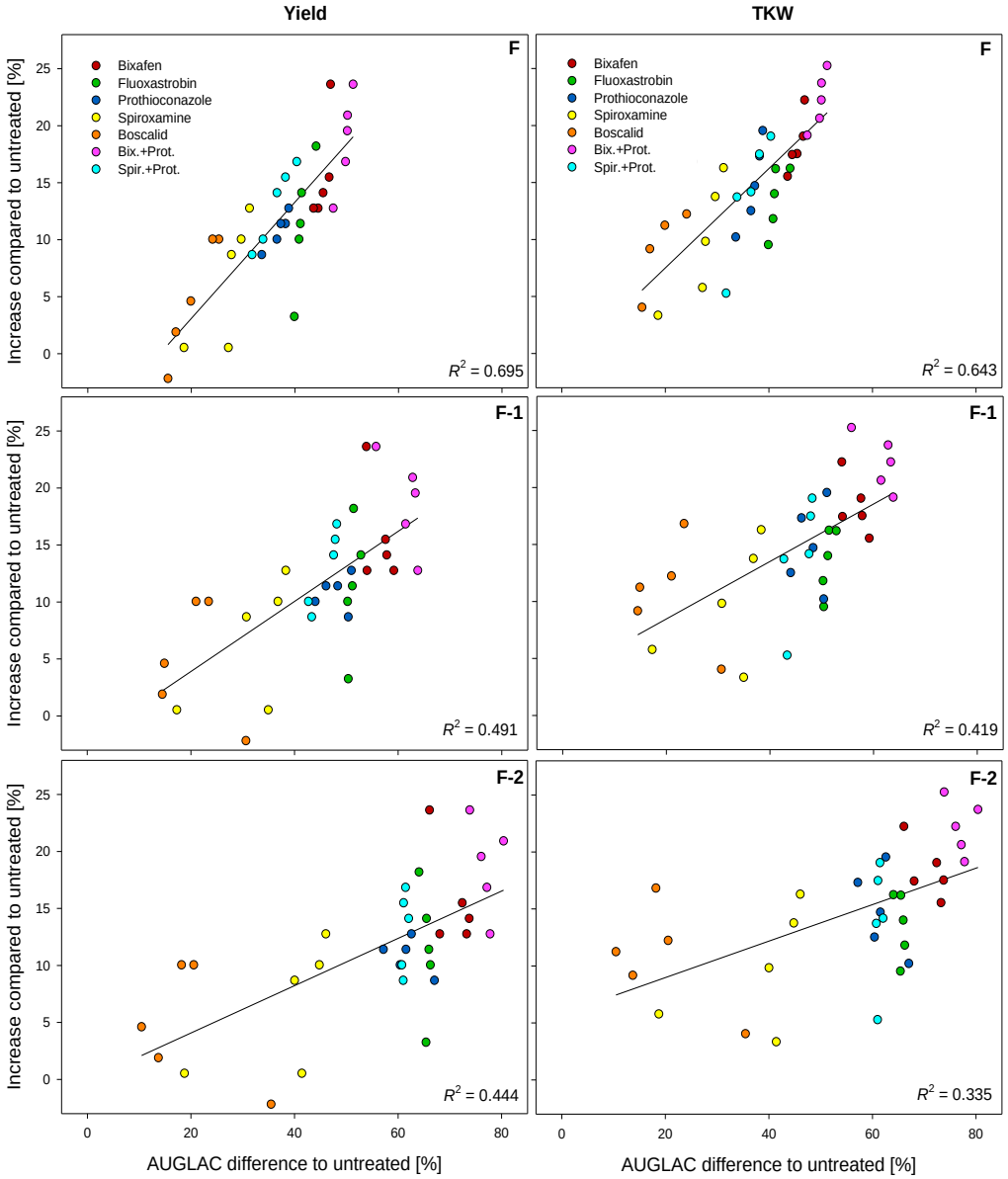

Figure 2.7: Relationship between the increase of yield and TKW compared to the untreated control and the difference to the untreated control of the area under the green leaf area curve. Field experiment conducted at the Poppelsdorf research station of the University of Bonn, 2008/09.

The strongest correlation was calculated between the green leaf area of the flag leaf and the yield parameters. The correlation coefficient was >0.6 when AUGLAC of the flag leaf was correlated to both the grain yield and TKW (Fig. 2.7). The correlation of the AUGLAC of F-1 and F-2 to the yield parameters had R^2 values < 0.5. Furthermore, the slope (m) of the linear regression for the AUGLAC of the flag leaf and grain yield parameters was steeper than the slope for the other two leaf layers.

The AUGLAC difference between fungicide treatments and untreated control for all leaf layers resulted in higher grain yield and TKW. The higher the difference in AUGLAC, the larger the yield increment compared to the untreated control. The same trend was observed for all leaf layers.

3.5. Effect of fungicide application on yield parameters

No significant differences were calculated for the number of ears per plot between treatments (Tab. 2.3). Foliar fungicide application had an effect on straw weight, TKW and grain yield, all fungicide treatments resulted in a higher straw weight, TKW and grain yield compared to the untreated control. Nevertheless, not all fungicide treatments produced significant changes compared to the untreated control. It was only the case for TKW, where all fungicide treatments resulted in a significantly higher values compared to the untreated control.

Table 2.3: Effects of fungicide application at GS 39 and GS 59 on yield components of wheat (cv. Ritmo). Field experiment conducted at the Poppelsdorf research station of the University of Bonn, 2008/09.

Treatment	Number of ears per plot	Straw weight [kg/plot]	TKW [g]	Yield [Ton.ha^{-1}]
Control	4118 a	4.9 a	40.3 a	7.3 a
Bixafen	4384 a	6.2 b	47.7 de	8.5 cd
Fluoxastrobin	4296 a	6.1 b	45.8 bc	8.2 bcd
Prothioconazole	4168 a	5.6 ab	46.3 cd	8.1 bc
Spiroxamine	4296 a	5.4 ab	44.2 b	7.7 ab
Boscalid	4658 a	5.4 ab	44.6 bc	7.8 ab
Bix.+Prot.	4104 a	6.3 b	49.2 c	8.7 d
Spir.+Prot.	4150 a	5.9 b	45.9 bc	8.3 bcd

Values with the same letter in the same column do not differ significantly according to Tukey´s test ($p \leq 0.05$; $n = 5$).

4. DISCUSSION

The main objective of this study was to investigate whether different fungicides influence the grain yield, specifically by prolonging the green leaf area duration. There was a yield benefit linked to the life extension of the uppermost leaves caused by the application of fungicides. All fungicide treatments resulted in a higher grain yield compared to the untreated control. The extra yield induced by fungicide application varied between 5 and 19%.

Experiments under field conditions confirmed that the effect of strobilurins and azoles on the leaf life extension resulted in higher grain yields compared to the untreated control (Bayles, 1999; Gooding *et al*., 2000; Blandino *et al*., 2009). Furthermore, in this study it was possible to observe the same effect generated by the pyrazole carboxamide bixafen on the leaf life extension of wheat leaves. Additionally, bixafen application also resulted in a longer green leaf area duration compared to the strobilurin and the azole compounds for all leaf layers.

Several studies have demonstrated the benefit produced by strobilurins and azoles on grain yield due to longer green leaf area duration (Dimmock and Gooding, 2002; Bryson *et al*., 2000). In this particular study the existence of a correlation of the extension of the green leaf area duration with the yield increment produced by fungicide application was shown. This is in accordance to Pepler *et al*. (2005) who reported an increment of grain yield associated to the extension of the green leaf area duration due to fungicide application.

Previous studies demonstrated a strong association between a longer grain-filling period and a higher grain yield, which is consistent with our results (Evans, 1993; Ojanperä *et al*., 1998). In particular, Gelang *et al*. (2000) reported a positive correlation between the thousand kernel weight and the grain yield with the grain filling period.

Grain yield and TKW were positively correlated to the area under the green leaf area curve of the three uppermost leaves of wheat. A higher correlation was calculated between the extension of flag leaf life and grain yield compared to the other two leaf layers. This is due to the fact that the photosynthetic activity of the flag leaf is the most important source for the grain filling in wheat (Thorne, 1965; Evans *et al*., 1975). Furthermore, the flag leaf life has a strong influence on the grain yield because this leaf layer intercepts more light than F-1 and F-2 and is the closest leaf to the ears. It results in a higher transport of assimilates required for grain filling (Gooding *et al*., 2000; Cromey *et al*., 2004).

The results confirmed that the longer period of photosynthetic activity of the flag leaf positively influences the grain filling resulting in a higher TKW. The AUGLC was a suitable

parameter to compare the effect of different fungicidal compounds on grain yield and could also be used to determine the contribution of every leaf layer for the grain filling.

The positive effect of the fungicides on the leaf life extension is mainly attributed to a delay of senescence. In higher plants, one of the first steps of the senescence process is the degradation of photosynthetic pigments such as chlorophyll (Matile *et al*., 1999; Hörtensteiner, 2006). Therefore, measurement of the chlorophyll content can be an adequate tool to evaluate the senescent status of plants (Lim *et al*., 2007). In our study, it was not just possible to observe extended leaf area duration following fungicide application as reported by Pepler *et al*. (2005), rather an additional effect on chlorophyll concentration was also detected. However, the chlorophyll content was mainly related to the maintenance of the green leaf area and the reduction of the disease incidence caused by fungicides. Treatments with low disease incidence and long leaf life period resulted in high chlorophyll content. Previous studies revealed a reduction of the chlorophyll loss produced by strobilurin and azole application (Grossmann and Retzlaff, 1997; Jaleel *et al*., 2007). Here, we report an effect of the carboxamide fungicide bixafen on the reduction of the chlorophyll loss due to delayed plant senescence.

The fungicidal efficacy controlling the main foliar pathogens of wheat differed between treatments. The present study confirms that leaf rust and Septoria leaf blotch are effectively controlled by azoles and strobilurins (Ruske *et al*., 2003). However, even lower disease incidence was rated when the carboxamide fungicide bixafen was applied. The fungicidal efficacy was enhanced by mixing the carboxamide and the azole compounds, this combination resulted in lower disease incidence at all leaf layers. Furthermore, the broad spectrum activity of bixafen against the most important foliar diseases of cereal crops has been described in previous reports (Suty-Heinze *et al*., 2011).

Grain yield parameters were also strongly influenced by the incidence of Septoria leaf blotch and leaf rust. Fungicide treatments with higher effectiveness against these diseases resulted in higher yield compared to the untreated control. Various studies have shown the relevance of the application of fungicides in diminishing the negative effects of foliar pathogens on wheat (Vamshidhar *et al*., 1998; Kelley, 2001; Wegulo *et al*., 2009). Wegulo *et al*., (2011) showed especially the benefits of fungicide application due to disease control which depends on various factors such as favorable conditions for disease development, disease intensity and fungicidal efficacy among others.

Studies to determine the fungicidal effects on plant physiology have been conducted under field, greenhouse and lab conditions (McCartney *et al*., 2007; Berdugo *et al*., 2012; Grossmann *et al*., 1999). Under field conditions, wheat plants tend to express their higher yield potentials better than under greenhouse conditions (Quinlan and Sagar, 1965). However, under field conditions it is complicated and almost impossible to avoid pathogen

infections even in years with low disease pressure. This creates a disadvantage for the untreated control since untreated plots would be most affected by foliar pathogens. Furthermore, there would be a clear reflection of this on the green leaf area duration and consequently on grain yield.

One of the objectives of this study was to evaluate and determine whether fungicides can induce beneficial effects on wheat physiology. However, from the field trials it was difficult to separate the fungicidal effect of these active ingredients from the effect that they might produce on the physiology and yield formation of wheat. In this study, there was a direct relation between disease incidence and green leaf area duration as well as grain yield. It provides the main reason why it is important to set up experiments in the greenhouse under disease-free conditions where it might be possible to assess the beneficial effects of fungicides on wheat physiology apart from the fungicidal activity of these compounds.

5. CONCLUSION

Use of fungicides in intensive wheat production provides benefits for growers such as control of the main foliar pathogens, which will result in accurate yields, and thus, provide a higher profit for growers. In addition to the fungicidal effect, some active ingredients generate an extension of the life of the most important leaf layers for grain filling. This effect provides an extra advantage to the disease control produced by these active ingredients.

The foliar application of the azole, carboxamide and the strobilurin compounds resulted in higher grain yield compared to the other fungicide treatments as a result of an accurate disease control. Furthermore, it was possible to observe and register the effect of the carboxamide fungicide bixafen on the senescence and yield formation of wheat. The effect on the senescence of wheat produced by bixafen was similar and in some cases, better than those caused by azoles and strobilurins.

Under field conditions, the presence of the main foliar pathogens of wheat influenced the green leaf area duration as well as the yield generating a disadvantage for the fungicide treatments with low disease control efficacy. Thus, in order to study the potential beneficial effects of fungicides on plant physiology it is advised to perform the studies under disease-free conditions. In the greenhouse, it is possible to control the temperature and the relative humidity in order to prevent the development of the major foliar pathogens of wheat. Hence, under greenhouse conditions it would be possible to separate the fungicidal activity from the potential effects that those active ingredients may cause on crop physiology.

6. REFERENCES

Bancal, M.O., Robert, C. and Ney, B. (2007). Modeling wheat growth and yield losses from late epidemics of foliar diseases using loss of green leaf area per layer and pre-anthesis reserves. *Annals of Botany*, 100, 777-789.

Bateman, G.L. and Fitt, B.D.L. (1991). Effects of fungicides on eyespot in five successive crops of winter wheat in plots with different initial or fungicide-selected populations of *Pseudocercosporella herpotrichoides*. *Crop Protection*, 10, 479-484.

Bayles, R. (1999). The interaction of strobilurin fungicides with cereal varieties. *Plant Varieties Seeds*, 12, 129-140.

Berdugo, C.A., Steiner, U., Dehne, H.-W. and Oerke, E.-C. (2012). Effect of bixafen on senescence and yield formation of wheat. *Pesticide Biochemistry and Physiology*, 104, 171-177.

Bertelsen, J.R., Neergaard, E.De. and Smedegaard-Petersen, V. (2001). Fungicidal effects of azoxystrobin and epoxiconazole on phyllosphere fungi, senescence and yield of winter wheat. *Plant Pathology*, 50, 190-205.

Blandino, M., Pilati, A. and Reyneri, A. (2009). Effect of foliar treatments to durum wheat on flag leaf senescence, grain yield, quality and deoxynivalenol contamination in North Italy. *Field Crops Research*, 114, 214-222.

Blanke, M.M. (1990). Chlorophyll determination using DMF. *Wein-Wissenschaft*, 45, 76-78.

Bryson, R.J., Leandro, L. and Jones, D.R. (2000). The physiological effects of kresosim-methyl on wheat leaf greenness and the implication for crop yield. In: Proceedings of the BCPC Conference - Pest and Diseases, 13-16 November 2000, British Crop Protection Council, Farnham, UK, pp. 739-746.

Bennett, F.G.A. (1984). Resistance to powdery mildew in wheat: A review of its use in agriculture and plant breeding programs. *Plant Pathology*, 33, 279-300.

Calderini, D.F., Abeledo, L.G., Savin, R. and Slafer, G.A. (1999). Final grain weight in wheat as affected by short periods of high temperature during pre- and post-anthesis under field conditions. *Functional Plant Biology*, 26, 453-458.

Caldwell, C.D. and Starratt, C.E. (1987). Response of Max spring wheat to management inputs. *Canadian Journal of Plant Science,* 67, 645-652.

Cromey, M.G., Butler, R.C., Mace, M.A. and Cole, A.L.J. (2004). Effects of the fungicides azoxystrobin and tebuconazole on *Didymella exitialis*, leaf senescence and grain yield in wheat. *Crop Protection*, 23, 1019-1030.

Dimmock, J.P.R.E. and Gooding, M.J. (2002). The effects of fungicides on rate and duration of grain filling in winter wheat in relation to maintenance of flag leaf green area. *Journal of Agricultural Science*, 138, 1-16.

Evans, L.T., Wardlaw, I.F. and Fischer, R.A. (1975). Wheat. In: Evans, L.T. ed., Crop Physiology, Cambridge University Press, London, UK, pp. 101-150.

Evans, L.T. (1993). Crop Evolution, Adaptation and Yield. Cambridge University Press, Cambridge, UK. ISBN 0-521-22571.

FAO. (2013). FAOSTAT. Internet source accessed on 13/02/2013. [http://faostat.fao.org/]

Gelang, J., Pleijel, H., Sild, E., Danielsson, H., Younis, S., and Selldén, G. (2000). Rate and duration of grain filling in relation to flag leaf senescence and grain yield in spring wheat (Triticum aestivum) exposed to different concentrations of ozone. *Physiologia Plantarum*, 110, 366-375.

Gooding, M.J., Dimmock, J.P.R.E., France, J. and Jones, S.A. (2000). Green leaf area decline of wheat flag leaves: the influence of fungicides and relationships with mean grain weight and grain yield. *Annals of Applied Biology*, 136, 77-84.

Görtz, A. (2009). Auftreten der Fusarium-Kolbenfäule im Maisanbau in Deutschland und Maßnahmen zur Vermeidung der Mykotoxinbelastung in Maiskörnern. Dissertation, University of Bonn.

Grossmann, K. and Retzlaff, G. (1997). Bioregulatory effects of the fungicidal strobilurin kresoxim-methyl in wheat (*Triticum aestivum*). *Pesticide Science*, 50, 11-20.

Grossmann, K., Kwaltowski, J. and Caspar, C. (1999). Regulation of phytohormone levels, leaf senescence and transpiration by the strobilurin kresoxim-methyl in wheat (*Triticum aestivum*). *Journal of Plant Physiology*, 154, 805-808.

Hack, H., Bleiholder, H., Buhr, L., Meier, U., Schnock-Fricke, U., Weber, E. and Witzenberger, A. (1992). Einheitliche Codierung der phänologischen Entwicklungsstadien mono- und dikotyler pflanzen – Erweiterte BBCH-Skala, Allgemein. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes*, 44, 265-270.

Hewitt, H.G. (1998). Fungicides in Crop Protection. CAB International, Wallingford.

Hiscox, J.D. and Israelstam, G.F. (1979). A method for the extraction of chlorophyll from leaf tissue without maceration. *Canadian Journal of Botany*, 57, 1332-1334.

Hoersefield, R., Yankovskaya, V., Sexton, G., Whittingham, W., Shiomi, K., Omura, S., Byrne, B., Cecchini, G. and Iwata, S. (2006). Structural and computational of the quinone-binding site of the complex II (succinate–ubiquinone oxidoreductase): a mechanism of electron transfer and proton conduction during ubiquinone reduction. *Journal of Biological Chemistry*, 281, 7309-7316.

Hörtensteiner, S. (2006). Chlorophyll degradation during senescence. *Annual Review of Plant Biology*, 57, 55-77.

Jaleel, C.A., Manivannan, P., Sankar, B., Kishorekumar, A., Sankari, S. and Panneerselvam, R. (2007). Paclobutrazol enhances photosynthesis and ajmalicine production in *Catharanthus roseus*. *Process Biochemistry*, 42, 1566-1570.

Kelley, K.W. (2001). Planting date and foliar fungicide effects on yield components and grain traits of winter wheat. *Agronomy Journal*, 93, 380-389.

Kolmer, J.A. (2005). Tracking wheat rust on a continental scale. *Current Opinion in Plan Biology*, 8, 441-449.

Kaul, R. (1974). Potential net photosynthesis in flag leaves of severely drought-stressed wheat cultivars and its relationship to grain yield. *Canadian Journal of Plant Science*, 54, 811-815.

Lim, P.O., Kim, H.J. and Nam, H,G. (2007). Leaf senescence. *Annual Review of Plant Biology*, 58, 115-136.

Loughman, R. and Thomas, G.J. (1992). Fungicide and cultivar control of Septoria diseases of wheat. *Crop Protection*, 11, 349-354.

Matile, P., Hörtensteiner, S. and Thomas, H. (1999). Chlorophyll degradation. *Annual Review of Plant Physiology and Plant Molecular Biology*, 50, 67-95.

McCartney, C., Mercer, P.C., Cooke, L.R. and Fraaije, B.A. (2007). Effects of a strobilurin-based spray programme on disease control, green leaf area, yield and development of fungicide-resistance in *Mycosphaerella graminicola* in Northern Ireland. *Crop Protection*, 26, 1272-1280.

Milus, E.A. (1994). Effect of foliar fungicides on disease control, yield and test weight of soft red winter wheat. *Crop Protection,* 13, 291-295.

Oerke, E.-C. and Dehne, H.-W. (1997). Global crop production and the efficacy of crop protection–current situation and future trends. *European Journal of Plant Pathology*, 103, 203-215.

Ojanperä, K., Pätsikkä, E. and Yläranta, T. (1998). Effects of low ozone exposure of spring wheat on net CO_2 uptake, Rubisco, leaf senescence and grain filling. *New Phytologist*, 138, 451-460.

Pepler, S., Gooding, M.J., Ford, K.E., Ellis, R.H. and Jones, S.A. (2005). A temporal limit to the association between flag leaf life extension by fungicides and wheat yields. *European Journal of Agronomy*, 22, 405-416.

Quinlan, J.D. and Sagar, G.R. (1965). Grain yield in two contrasting varieties of spring wheat. *Annals of Botany*, 29, 683-697.

Ruske, R.E., Gooding, M.J. and Jones, S.A. (2003). The effects of adding picoxystrobin, azoxystrobin and nitrogen to a triazole programme on disease control, flag leaf senescence, yield and grain quality of winter wheat. *Crop Protection*, 22, 975-987.

Shaw, M.W. and Royle, D.J. (1989). Estimation and validation of a function describing the rate at which *Mycosphaerella graminicola* causes yield loss in winter wheat. *Annals of Applied Biology*, 115, 425-442.

Shipton, W.A., Boyd, W.R.J., Rosielle, A.A. and Shearer, B.I. (1971). The common Septoria diseases of wheat. *The Botanical Review*, 37, 231-262.

Suty-Heinze, A., Dunkel, R., Krieg, U. and Rieck, H. (2011). Bixafen - the new cereal fungicide with yield boosting effects. In: Dehne, H.-W., Deising, H.B., Gisi, U., Kuck, K.H., Russell, P.E. and Lyr, H., eds., Modern Fungicides and Antifungal Compounds VI, Proceedings of the 16th International Reinhardsbrunn Symposium, 25-29 April 2010, Friedrichroda, Germany, Lebenshilfe gGmbH, Braunschweig, pp. 69-74.

Thorne, G.N. (1965). Photosynthesis of ears and leaves of wheat and barley. *Annals of Botany*, 29, 317-329.

Vamshidhar, P., Herrman, T.J., Bockus, W.W. and Loughin, T.M. (1998). Quality response of twelve hard red winter wheat cultivars to foliar disease across four locations in central Kansas. *Cereal Chemistry*, 75, 94-99.

Wegulo, S.N., Breathnach, J.A. and Baenziger, P.S. (2009). Effect of growth stage on the relationship between tan spot and spot blotch severity and yield in winter wheat. *Crop Protection*, 28, 696-702.

Wegulo, S.N., Zwingman, M.V., Breathnach, J.A. and Baenziger, P.S. (2011). Economic returns from fungicide application to control foliar fungal diseases in winter wheat. *Crop Protection*, 30, 685-692.

Wiik, L. (2009). Yield and disease control in winter wheat in southern Sweden during 1977–2005. *Crop Protection*, 28, 82-89.

CHAPTER 3: EFFECT OF BIXAFEN ON SENESCENCE AND YIELD FORMATION OF WHEAT

(Published in: Pesticide Biochemistry and Physiology, 2012, 104, 171-177)

ABSTRACT

Bixafen, a pyrazole carboxamide inhibiting succinate dehydrogenase of the fungal respiratory chain, is a new broad-spectrum fungicide developed for the control of pathogens in cereals. The effects of bixafen on senescence and yield formation of wheat plants were studied and compared to those caused by azoles, strobilurins and spiroketalamins under disease-free conditions in the greenhouse. Fungicide applications delayed the appearance and senescence of wheat ears. Application of bixafen, fluoxastrobin and prothioconazole delayed also the senescence of leaves and significantly extended the green leaf area duration compared to untreated and spiroxamine treated plants. Differences in the senescence of leaves and ears between treatments were confirmed by measurements of the temperature of wheat tissue as an indicator of transpiration activity. Digital infrared images revealed significant differences between untreated and fungicide-treated plants at different growth stages (GS). At GS 75, photosynthetic activity of untreated plants was lower compared to fungicide-treated plants; however, only bixafen gave a significant effect. All fungicide treatments increased grain yield. Application of bixafen significantly increased the yield compared to all other treatments. The temperature of ears and leaves was negatively correlated to grain yield. Lower tissue temperature of fungicide-treated plants was a suitable indicator of tissue vitality and higher photosynthetic activity due to the retardation of ear and leaf senescence. The combination of positive effects on physiology of wheat resulted in a yield advantage of bixafen-treated plants.

Keywords: Flag leaf, grain yield, green leaf area, plant senescence, thermography.

1. INTRODUCTION

Several tools are used to control fungal diseases in wheat (*Triticum aestivun* L.) production in order to prevent yield losses and reductions in grain quality. In intensive production the use of fungicides is one of the most widespread methods among these strategies. An effective control of foliar diseases during the period between flag leaf emergence and milk stage of kernels is important to achieve optimum crop yields,

because the flag leaf is the most important organ associated with grain yield (Reynolds *et al*., 2000). Almost 45 % of the carbohydrates used for grain filling are produced in the flag leaf (Lupton, 1972). Successful control of fungal pathogens on the upper leaves results in a longer period of light interception associated with higher assimilate production and their transport into the ear for grain filling (Gooding *et al*., 2000; Blandino and Reyneri, 2009). Effects of fungicides on yield and grain weight were superior when applied just after flag leaf emergence (Cook and Thomas, 1990).

The efficacy of fungicides against pathogens has been the parameter usually studied. However, some fungicides are described to have additional effects on plant physiology. Application of strobilurins (Qo inhibitors) has been repeatedly shown to result in increased grain yields, kernel weights and protein contents associated with a delay in the senescence of flag leaves (Bayles, 1999; Dimmock and Gooding, 2002a; Dimmock and Gooding, 2002b; Bryson *et al*., 2000; Zhang *et al*., 2010). In addition to the fungicidal activity, strobilurins – which inhibit fungal growth by blocking the electron transport at the cytochromr-bc_1 complex in mitochondrial respiration (Sauter *et al*., 1995) – have been reported to induce physiological changes in crops, like increased tolerance against abiotic stress (Köhle *et al*., 2002), darker green appearance of leaves (Konradt *et al*., 1996), delayed senescence of photosynthetic leaf area (Wu and von Tiedemann, 2001), changes in the balance of phytohormones (Grossmann and Retzlaff, 1997), and increased CO_2-uptake (Beck *et al*., 2001). Extension of the green leaf area duration (GLAD) triggered by strobilurins is one of the most positive side effects reported in literature (Habermeyer *et al*., 1998). It has been explained by an effect of strobilurins on the metabolism of phytohormones. Kresoxim-methyl increased the endogenous cytokinin level of wheat tissue and reduced the 1-aminocyclopropane-1-carboxylic acid (ACC) content, which catalyzes the first step in the biosynthesis of ethylene (Grossmann and Retzlaff, 1997).

Several studies have investigated also the effect of azole fungicides on plant physiology (Buchenauer and Rohner, 1981; Lorens and Cothren, 1989; Grossmann *et al*., 1999). Triadimefon increased the chlorophyll content of dark-adapted winter wheat by 4 to 40% under disease-free conditions; however, the fungicide had no effect on GLAD (Lorens and Cothren, 1989). The application of benomyl, dichlofuanid and zineb on wheat resulted in a delay of senescence compared to untreated plants (King *et al*., 1983). Assessment of the percentage green leaf area is the main method to evaluate the effect of fungicides on plant senescence. This method has the disadvantage that the estimation of real green leaf area is subjective and may vary among individuals. Reliable differences often have to be obvious. The use of non-destructive methods such as infrared (IR) thermography may be an alternative to establish differences in terms of plant senescence. The method gives the possibility to have an early detection of changes in canopy vitality. Near-range IR

thermography records the temperature of plant surfaces depending on the transpiration rate. Infrared sensing of the canopy temperature has been used since the early 1980s (Grant *et al*., 2006) and variations in the surface temperature caused by biotic and abiotic stresses may be recorded by imaging techniques (Chaerle and Van Der Straeten, 2000).

Recently, various fungicide classes to control cereal pathogens have been developed, e.g. succinate dehydrogenase inhibitors (SDHIs), which control fungal pathogens by inhibiting the enzyme succinate dehydrogenase in the mitochondrial respiration chain of fungi (Hoersefield *et al*., 2006). The first SDHI fungicides were developed in the 1960s to control pathogens such as *Ustilago maydis* and other basidiomycetes (Ulrich and Matre, 1972) and were used especially for seed dressing. The SDHIs belonging to the chemical class of pyrazole carboxamides developed recently such as penthiopyrad or bixafen are characterized to have a broader spectrum of fungicidal activity including also ascomycetes of agronomic importance (Avenot and Michailides, 2010). Bayer CropScience discovered bixafen in 2001, and it shows high efficacy against many cereal pathogens in numerous field trials (Suty-Heinze *et al*., 2011).

The aim of this chapter was to investigate the effects of bixafen, a pyrazole carboxamide, on the senescence and yield formation of wheat in comparison to those caused by azoles (triazolinthione), strobilurins and spiroketalamins. A series of experiments was conducted under disease-free conditions in the greenhouse. Non-invasive techniques were used in order to assess direct effects of the fungicides on wheat plants.

2. MATERIALS AND METHODS

2.1. Plant material

Spring wheat (*Triticum aestivum* L., cv. Passat) was grown under greenhouse conditions. Twenty wheat kernels were sown two cm deep per pot (20 x 20 x 30 cm). Ten 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 grown at 24/20 °C (day/night), 70 ± 10% relative humidity (RH), photoperiod of 16 h d^{-1} with supplemental illumination (> 300 µmol m^{-2} s^{-1}, lamps Philips SGR 140, Philips, Hamburg, Germany). Pots were watered once per day in order to maintain soil water content (approx. 55 - 85%) favorable for plant growth. A solution of a commercial N-P-K fertilizer (14-10-14, 2 g/l; Aglukon GmbH, Düsseldorf, Germany) was applied once every two weeks to ensure adequate nutrient supply. Plants were carefully inspected in order to control possible fungal infections. According to the BBCH scale (Hack *et al*., 1992) at growth stage (GS) 33 the fungicide Talius® (active ingredient [a.i.] proquinazid, 200 g L^{-1}, DuPont de Nemours, Neu-Isenburg, Germany) and at GS 70 the fungicide Vegas® (a.i. cyflufenamid, 51.3 g L^{-1},

Spiess-Urania, Hamburg, 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.

2.2. Fungicide treatments

Four fungicide spray treatments and a non-treated control were evaluated. Active ingredients belonging to the –azoles (prothioconazole, 250 g a.i. L^{-1}), carboxamides (bixafen, 125 g a.i. L^{-1}), spiroketalamines (spiroxamine, 500 g a.i L^{-1}), and strobilurins (fluoxastrobin, 100 g a.i. L^{-1}) were supplied by Bayer CropScience, (Monheim, Germany). Fungicidal products were applied at recommended field rates (water 300 L ha^{-1}; 0.35 ml fungicide solution per plant) at two growth stages; first application when the flag leaf ligule was visible (GS 39), second application when emergence of inflorescences was completed (GS 59). Foliar applications were made with a CO_2 pressurized hand-sprayer (Meisterwerkzeuge, Wuppertal, Germany) with an adjustable spray. Plants of different treatments were separated before fungicide application in order to avoid contamination between treatments. After spraying replicates of each treatment were randomized. The experiments were conducted twice.

2.3. Assessment of green leaf area

The green leaf area was assessed weekly after the second fungicide application (GS 59) until harvest and was measured as percentage of green area of the top three leaves (F, F-1 and F-2). Every week three randomly selected plants per replicate per treatment were selected to assess the green leaf area. The area under the green leaf area curve (AUGLAC) was calculated using the equation given by Cromey *et al*. (2004),

$$\text{AUGLAC} = \sum_{i=1}^{n} 1/2(Y_i + Y_{i-1})(X_i - X_{i-1})$$

Where Y_i= percent green leaf area at the *i*th observation, X_i = time (days after sowing) at the *i*th observation, *n* = total number of observations.

2.4. Gas exchange measurements

Measurements of gas exchange were performed at GS 75. Two ears per replicate were used and five replicates per treatment were measured. The minicuvette gas exchange system (CMS-400 Heinz Walz GmbH, Effeltrich, Germany) was used to measure net assimilation and respiration of wheat ears. Measurements on ears were conducted under light conditions simulating the sun spectrum by the use of the lighting unit FL-440 (Walz,

Effeltrich, Germany, ± 950 µmol m^{-2} s^{-1}) after 20 min of equilibration to measure CO_2 uptake (photosynthetic activity), and under dark conditions measuring CO_2 release (respiration) after 10 min of equilibration.

2.5. Measurement of surface temperature of plant tissue

Thermographic images were taken at GS 75, 80, 85 and 90. The images were recorded using 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 mrad (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); thermal resolution is 0.03 K. Ten replicates per treatment were recorded. 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. For each replicate fifty pixels representing the tissue of interest in the digital images were used. Temperature data from the pixels were exported from IRBIS™ plus to MS Office Excel (Microsoft Deutschland, Unterschleißheim, Germany). The area under the leaf temperature curve (AULTC) and the area under the ear temperature curve (AUETC) were calculated using the equation:

$$AUL(E)TC = \sum_{i=1}^{n} 1/2(Y_i + Y_{i-1})(X_i - X_{i-1})$$

Where Y_i= absolute temperature value at the *i*th observation, X_i = time (days after sowing) at the *i*th observation, *n* = total number of observations.

2.6. Ear development and yield parameters

Parameters related to ear development were measured throughout the experiments. Plants were inspected every day to count the total number of emerged ears (GS 51) per pot. Ear senescence was evaluated daily after the second fungicide application at GS 59, ears with at least 25 % of the surface with typical signs of senescence were counted as senescent ears. The percentage of the emerged and senescent ears was calculated with respect to the total number of ears per pot. At harvest the total shoot biomass was weighed and the total number of ears per pot was determined. Ear weight was measured and the kernels were separated from the ears with a mini combine (Reichhardt Electronic Innovations, Inc, West Fargo, USA). Subsequently, the total weight of kernels was measured and the total number of grains was counted using an electronic grain counter (Pfeuffer GmbH, Kitzingen, Germany). Yield parameters e.g. grain yield, thousand kernels weight, number of ears, and number of kernels per ear was assessed.

2.7. Statistical analysis

Data were analyzed using the statistical program SPSS for Windows (IBM Deutschland GmbH, Ehningen, Germany), version 17.0. Data were tested for 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 at 95% confidence in order to separate subgroups. The grain yield and the area under the ear and leaf temperature curve were tested for correlation at a probability level of 0.05 using Pearson's correlation coefficient.

3. RESULTS

3.1. Ear emergence and green leaf area duration

The emergence of wheat ears was delayed by fungicide applications. First ears emerged in untreated plants followed by plants treated with spiroxamine and fluoxastrobin (Fig. 3.1).

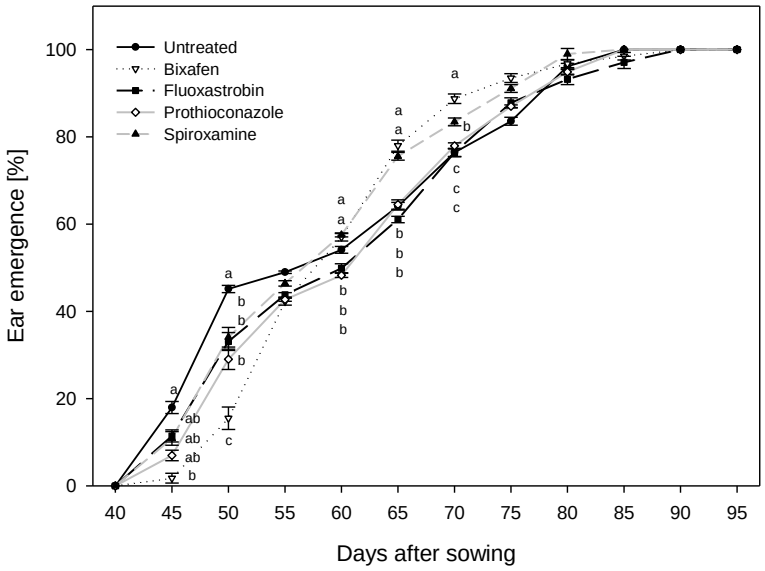

Figure 3.1: Effect of fungicide treatments at GS 39 and GS 59 on ear emergence of wheat (cv. Passat). Vertical bars indicate SE. Same letters for a time indicate non-significant differences ($p \leq 0.05$; $n = 10$).

Bixafen and prothioconazole delayed the start of ear emergence by four and two days, respectively. Differences between untreated and fungicide treatments were significant for the first 15 days after the first ear had appeared. Despite of the delay in ear emergence in the first phase, fungicides did not retard the end of ear emergence; they rather synchronized the period of ear emergence. Leaf senescence was delayed when plants were treated with bixafen, fluoxastrobin and prothioconazole. These fungicides produced a significant effect on GLAD compared to the two other treatments (Fig. 3.2).

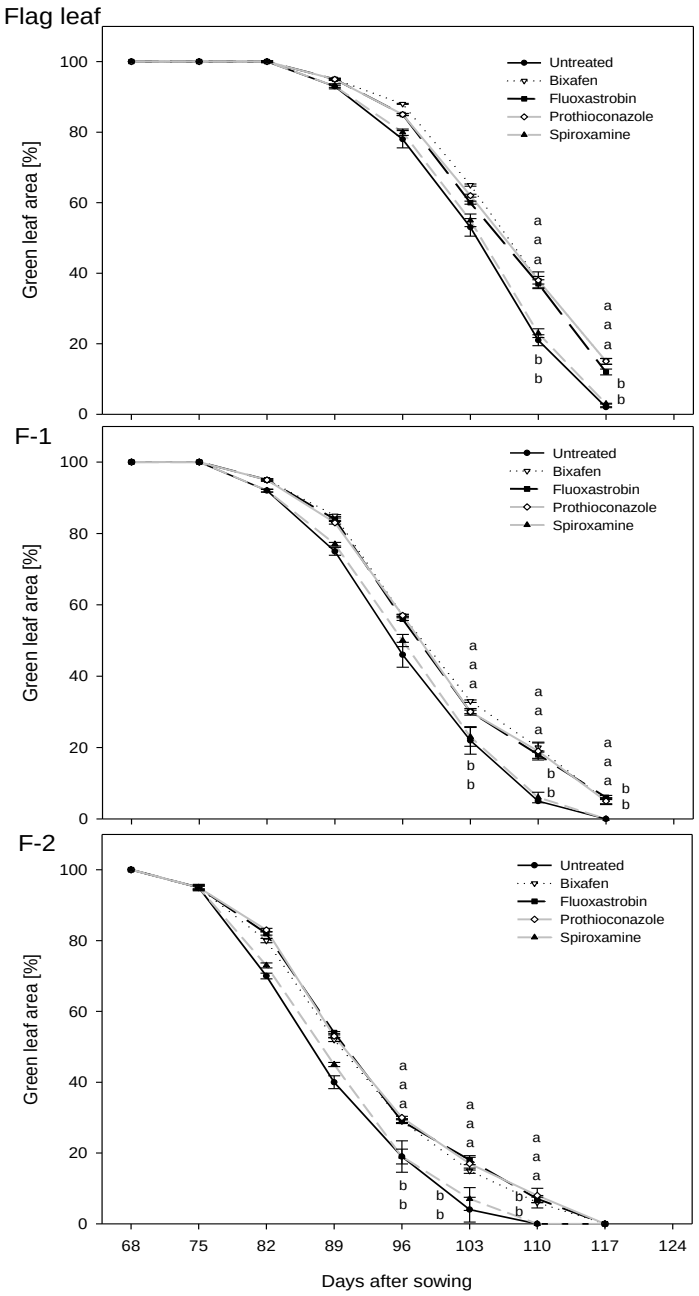

Figure 3.2: Effect of fungicide treatments on the green leaf area duration of flag leaf, F-1 and F-2 of wheat (cv. Passat). Vertical bars indicate SE. Same letters for a time indicate non-significant differences ($p \leq 0.05$; $n = 10$).

The first differences in GLAD between these two groups became evident for leaf F-2 76 days after sowing (das), and 82 and 89 das for leaf F-1 and flag leaf, respectively. For flag leaves, the higher differences between untreated and bixafen, prothioconazole and fluoxastrobin treatments were observed 110 das. For F-2, the biggest differences between treatments were recorded 103 das (Fig. 3.2). Bixafen, fluoxastrobin and prothioconazole treatments resulted in GLAD of all leaf layers five to seven days longer than that of untreated and spiroxamine-treated wheat.

For all fungicide treatments except spiroxamine, the area under green leaf area curve (AUGLAC) of flag leaves, F-1 and F-2 was increased by 7%, 12% and 19%, respectively, as compared to untreated plants (Fig. 3.3).

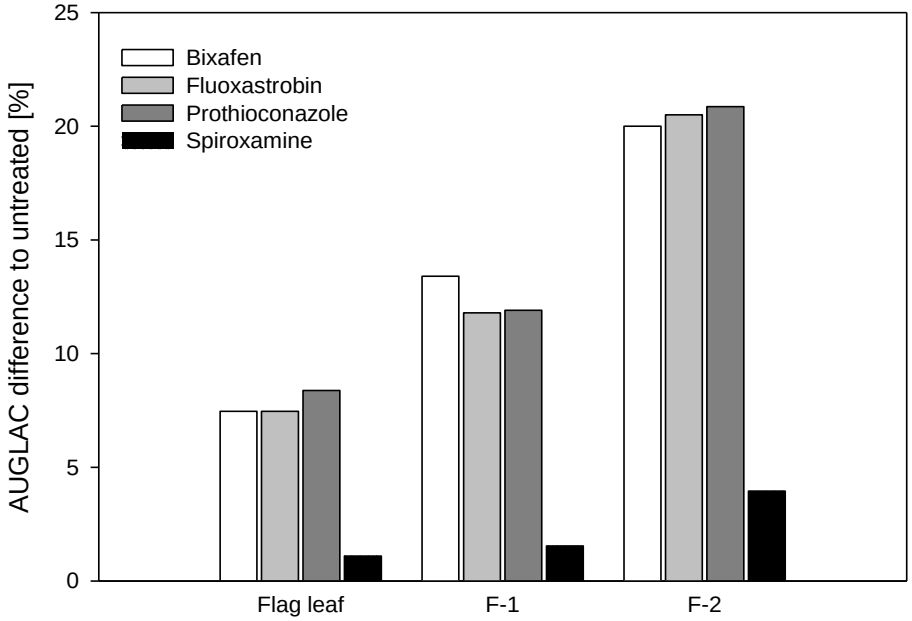

Figure 3.3: Effect of fungicide treatments on the area under green leaf area curve; differences for flag leaf, F-1 and F-2 as compared to untreated control.

3.2. Gas exchange of ears

At growth stage 75 the CO_2 net assimilation of wheat ears was significantly affected by fungicide treatments (Tab. 3.1). Early senescence of untreated ears resulted in a lower photosynthetic rate compared to fungicide treatments. At the same time, dark respiration of untreated ears was also lower than for fungicide-treated ears. Statistical analyses revealed that bixafen was the only fungicide treatment that resulted in a significant difference to untreated in photosynthesis and respiration.

Table 3.1: Effect of fungicide application at GS 39 and GS 59 on the photosynthetic activity and respiration of wheat ears (cv. Passat) at GS 75.

Treatment	Photosynthesis [µmol CO_2 m^{-2} s^{-1}]	Respiration [µmol CO_2 m^{-2} s^{-1}]
Untreated	-0.98 a	0.80 a
Bixafen	-5.94 b	6.12 b
Fluoxastrobin	-2.44 a	2.96 a
Prothioconazole	-1.86 a	2.12 a
Spiroxamine	-2.36 a	2.82 a

Values with the same letter in the same column do not differ significantly according to Tukey´s test ($p \leq 0.05$; $n = 5$).

3.3. Temperature of ears and leaves

The temperature of leaves and ears was significantly different between fungicide-treated and untreated plants (Tab. 3.2). Ear temperature of fungicide-treated plants was significantly lower than of untreated ears at GS 75.

Table 3.2: Effect of fungicide application at GS 39 and GS 59 on the temperature of ears and leaves of wheat (cv. Passat) at growth stages 75, 80, 85 and 90.

Treatment	Temperature [°C]							
	GS 75		GS 80		GS 85		GS 90	
	Ears	Leaves	Ears	Leaves	Ears	Leaves	Ears	Leaves
Untreated	23.7c	22.5b	20.4d	18.1b	25.3b	24.4c	24.3a	23.4b
Bixafen	22.3a	22.1a	18.2a	16.4a	24.5a	23.3a	24.1a	22.3a
Fluoxas.	23.1b	22.3ab	18.9b	16.5a	24.8ab	23.4ab	23.9a	22.7a
Prothioc.	23.1b	22.4ab	19.5c	16.6a	24.8ab	23.7 b	24.2a	23.1b
Spirox.	23.2b	22.4ab	20.2d	16.4a	25.1ab	23.7b	24.2a	23.2b

Values with the same letter in the same column do not differ significantly according to Tukey´s test ($p \leq 0.05$; $n = 10$).

In reflectance images ears of untreated plants showed typical signs of senescence, which were also evident in IR images (Fig. 3.4). At this growth stage bixafen was the only fungicide treatment resulting in average leaf temperature significantly different from that of untreated plants.

Ear temperature significantly differed among treatments in the period from GS 75 to GS 85, while there were no differences at GS 90. At this growth stage, wheat treated with spiroxamine, prothioconazole and untreated plants did not differ in leaf temperature, whereas the temperature of leaves treated with fluoxastrobin and bixafen was significantly lower than that of untreated wheat.

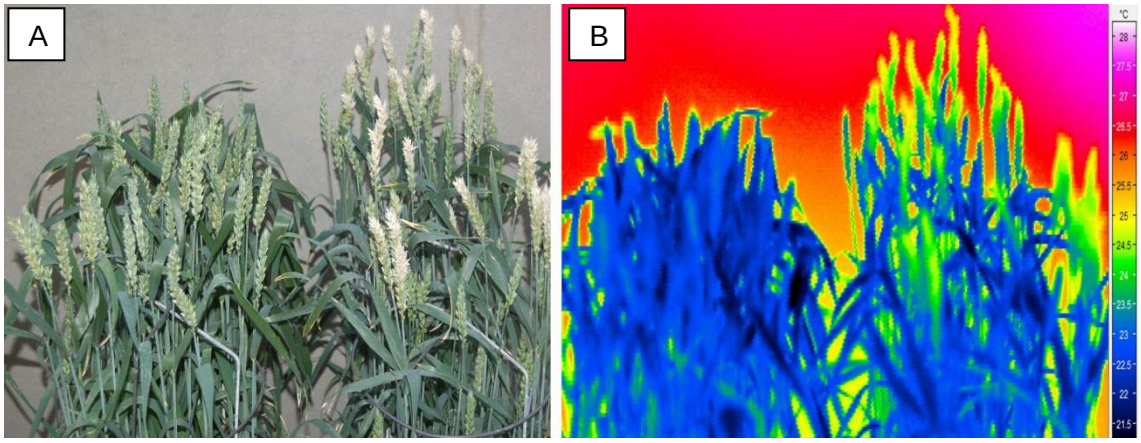

Figure 3.4: Reflectance image (A) and thermogram (B) of bixafen treated (left) and untreated (right) wheat plants (cv. Passat) at GS 75.

3.4. Plant growth and yield parameters

Fungicide applications delayed the senescence of wheat ears. Ears of untreated plants showed first symptoms of senescence 75 das, while senescent ears were recorded three days later for fungicide-treated wheat (Fig. 3.5).

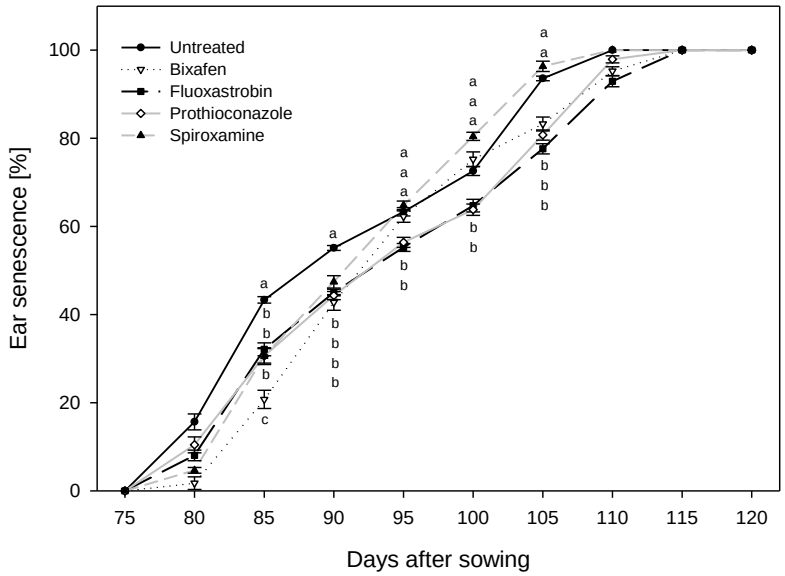

Figure 3.5: Effect of fungicide application on the senescence of wheat ears (cv. Passat). Vertical bars indicate SE. Same letters at a time indicate non-significant differences ($p \leq 0.05$; $n = 10$).

All ears of untreated and spiroxamine-treated plants were senescent 110 das, while for fluoxastrobin, prothioconazole and bixafen treated plants this stage was reached five days later. All fungicide treatments increased the grain yield. However, only the application of bixafen significantly increased the yield compared to the other treatments (Tab. 3.3). The number of ears per pot and the total number of kernels per ear showed no significant differences among treatments. The thousand kernel weight was increased by all fungicide treatments compared to untreated with bixafen resulting in a significant effect.

Table 3.3: Effects of fungicide application at GS 39 and GS 59 on yield components of spring wheat (cv. Passat).

Treatment	No. of ears per pot	Ears weight [g]	No. of kernels per ear	TKW [g]	Yield [g / pot]
Untreated	39.0 a	38.9 a	22.7 a	33.5 a	29.6 a
Bixafen	40.0 a	47.3 b	25.1 a	37.5 b	37.5 b
Fluoxastrobin	41.0 a	41.2 a	21.9 a	34.3 a	30.5 a
Prothioconazole	43.1 a	41.8 a	22.2 a	33.5 a	32.1 a
Spiroxamine	40.9 a	41.8 a	23.2 a	35.5 ab	33.4 a

Values with the same letter in the same column do not differ significantly according to Tukey´s test ($p \leq 0.05$; $n = 10$).

The area under ear temperature curve (AUETC) and the area under leaf temperature curve (AULTC) were negatively correlated to grain yield (Fig. 3.6). Regression coefficients were very similar for all treatments for both correlations, however, the intercepts slightly varied. For all treatments the correlation coefficients R^2 were >0.9 when AUETC was correlated to grain yield.

In contrast, the correlation coefficient for the relation between AULTC and grain yield were always lower that 0.9 for all treatments. For the correlation between AUETC and grain yield is clear how the slope of linear regression curves varied between treatments. Untreated and spiroxamine treatments showed curves with steeper gradient compared to the other treatments (Fig. 3.6). The overall result of the linear regression analysis demonstrated that ear temperature has a higher effect on the grain yield than leaf temperature.

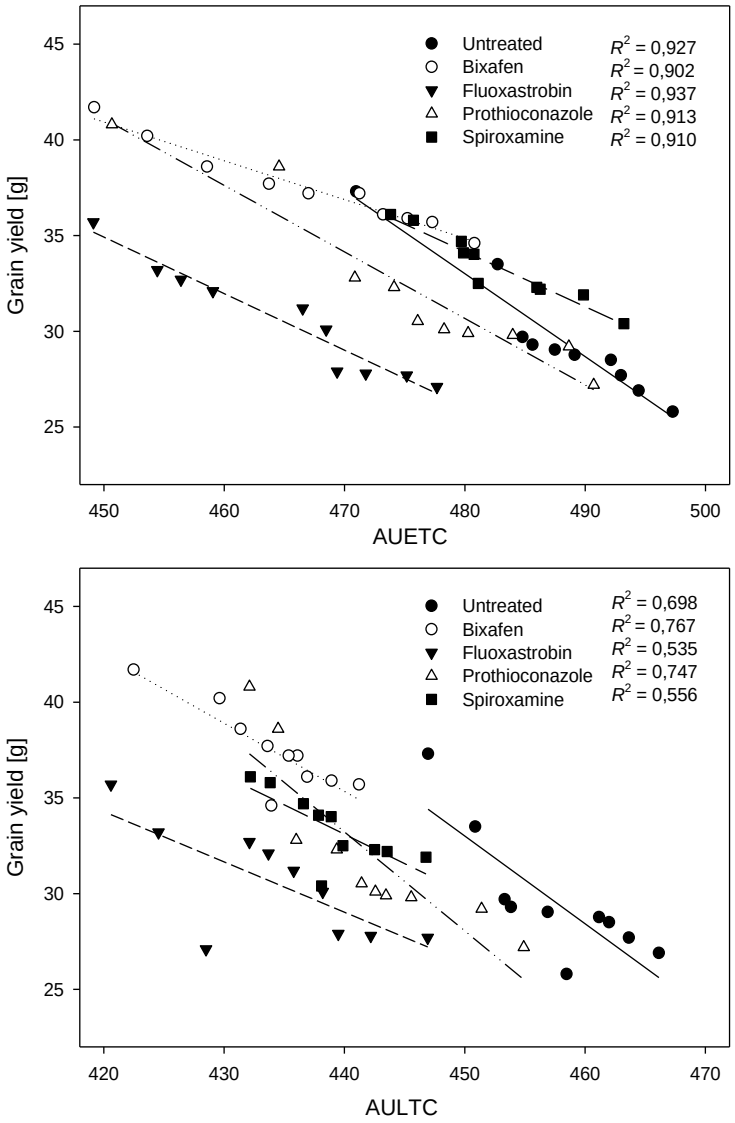

Figure 3.6: Correlations between the area under ear temperature curve (AUETC) and area under leaf temperature curve (AULTC) and grain yield of wheat (cv. Passat), by fungicide.

4. DISCUSSION

The experiments under disease-free conditions indicated that the fungicides belonging to the chemical groups pyrazole carboxamides, strobilurins and azoles had positive effects on plant development and grain yield. All treatments including untreated completed ear emergence at the same time. Therefore bixafen, prothioconazole and fluoxastrobin harmonized the period of heading compared to untreated wheat plants. This effect produced by fungicides has not been reported before and may be positive especially in years with high risk of *Fusarium* infection of ears as the infection risk increases with the period of heading.

Bixafen, fluoxastrobin and prothioconazole extended GLAD of the upper three leaf layers irrespective of fungicide activity. This is in agreement with previous reports that strobilurins and azoles result in longer GLAD of flag leaves (Dimmock and Gooding, 2002c; Ruske *et al*., 2004; Peppler *et al*., 2005). Grossmann *et al*. (1999) demonstrated in an assay with wheat leaf discs that the positive effect on chlorophyll content increased with the dose of the strobilurin pyraclostrobin.

Most of the studies investigating the effect of fungicides on plant physiology have been conducted under field conditions where effects of the fungicides on GLAD are attributed to the control of the major foliar pathogens of wheat (Cook and Thomas, 1990; Gooding *et al*., 2000; Peppler *et al*., 2005). Dimmock and Gooding (2002) reported that the effect on GLAD was more pronounced when famoxadone and flusilazole were applied in plots with the highest disease pressure. Gooding *et al*. (2000) concluded that the effect of fungicides on GLAD is closely related to factors such as crop genotype, active ingredient and incidence of foliar pathogen. In the present study the only factor differing between treatments was the fungicide applied, since the main objective was to investigate the effect(s) of fungicides on wheat physiology.

The delay of senescence may be attributed to the control of saprophytic fungi on the plant surface. They are reported to play a role in leaf senescence and since fungicides inhibit spore germination and mycelial growth of saprophytic fungi and minor pathogens, this control may contribute to retarded senescence of crops (Bertelsen *et al*., 2001). Tolstrup (1984) confirmed that saprophytic fungi may influence leaf senescence in barley.

The extension of the green leaf area duration and the greening effect caused by fungicides has been associated with yield increases (Jörgensen *et al*., 1999; Jörgensen and Olesen, 2002). A direct relation between the extension of GLAD and extra grain yield has been shown in several studies (Gooding *et al*., 2000; Beck *et al*., 2001; Peppler *et al*., 2005). Bixafen, and to a lesser degree the other fungicides maintained vitality of ears and leaves at high levels - even in later growth stages. Due to delay of ear senescence

bixafen positively influenced the photosynthetic activity of wheat ears and promoted physiological activity of ears associated with improved grain filling.

Application of the azole, carboxamide and strobilurin fungicides resulted in higher grain yield, although it was not always significantly different from the yield of untreated wheat. The yield increase from bixafen was clearly related to improved / extended grain filling resulting in increased kernel weight. The number of tillers was not affected, since the fungicides had been applied only at growth stages after the determination of this yield component during wheat ontogenesis.

Infrared sensing of the canopy temperature has been used mainly to study changes caused by biotic and abiotic stress conditions (Guiliani and Flore, 2000; Chaerle *et al*., 2004; Oerke *et al*., 2006). In this study IR thermography was used to establish differences in plant vitality and senescence represented in surface temperature of leaves and ears. A direct relation could be established between leaf temperature and green leaf area (duration) because plants with retarded senescence had higher tissue vitality associated with higher levels of transpiration. Infrared thermography was suitable for the assessment of beneficial effects of fungicides on plant vitality due to the high thermal sensitivity of the camera. Significant differences in leaf temperature between treatments could be measured even before characteristic symptoms of senescence became visible.

Since plant temperature was negatively correlated to vitality and grain yield, IR thermography has the potential for remote sensing of fungicide effects also in screening assays – under controlled conditions as well as in the field. Lenthe *et al*., (2007) demonstrated the use of aerial thermography for the assessment of direct effects of fungicides on wheat yield formation without differentiating between fungicidal effect and phytotonic effects of the fungicides applied.

The use of non-destructive sensors for the recording of plant vitality is especially useful in time series experiments, since the assessment of plant senescence is objective – without subjective errors of the rater(s) – and may be automatized for larger sample numbers.

5. CONCLUSION

This study demonstrates that the SDHI fungicide bixafen induces physiological changes in wheat plants such as synchronized the ear emergence, increased green leaf area duration, higher physiological activity of ears during grain filling due to delay of plant senescence. The combination of these effects resulted in a yield advantage of bixafen treated plants compared to the other fungicide treatments.

Digital thermography for the assessment of plant surface temperature revealed significant differences between untreated and fungicide-treated plants, it proved to be a precise

method to establish and quantify side effects of fungicides on senescence of leaves and ears. This method was more sensitive than visual assessment of green leaf area duration.

6. REFERENCES

Avenot, F.H. and Michailides, T.J. (2010). Progress in understanding molecular mechanisms and evolution of resistance to succinate dehydrogenase inhibiting (SDHI) fungicides in phytopathogenic fungi. *Crop Protection*, 29, 643-651.

Bayles, R. (1999). The interaction of strobilurin fungicides with cereal varieties. *Plant Varieties Seeds*, 12, 129-140.

Beck, C., Oerke, E.-C. and Dehne, H.-W. (2001). Impact of strobilurins on physiology and yield formation of wheat, Med. Fac. Landbouww, Univ. Gent 67/2, 181-187.

Bertelsen, J.R., Neergaard, E.De. and Smedegaard-Petersen, V. (2001). Fungicidal effects of azoxystrobin and epoxiconazole on phyllosphere fungi, senescence and yield of winter wheat. *Plant Pathology*, 50, 190-205.

Blandino, M. and Reyneri, A. (2009). Effect of fungicide and foliar fertilizer application to winter wheat at anthesis on flag leaf senescence, grain yield, flour bread making quality and DON contamination. *European Journal of Agronomy*, 30, 275-282.

Bryson, R.J., Leandro, L. and Jones, D.R. (2000). The physiological effects of kresosim-methyl on wheat leaf greenness and the implication for crop yield. In: Proceedings of the BCPC Conference - Pest and Diseases, 13-16 November 2000, British Crop Protection Council, Farnham, UK, pp. 739-746.

Buchenauer, H. and Rohner, E. (1981). Effect of triadimefon and triadimenol on growth of various plant species as well as on gibberellin content and sterol metabolism in shoots of barley seedlings. *Pesticide Biochemistry of Physiology*, 15, 58-70.

Chaerle, L. and Van Der Straeten, D. (2000). Imaging techniques and the early detection of plant stress. *Trends in Plant Science*, 5, 495-501.

Chaerle, L., Hagenbeek, D., De Bruyne, E., Valcke, R. and Van Der Straeten, D. (2004). Thermal and chlorophyll-fluorescence imaging distinguish plant-pathogen interactions at an early stage. *Plant Cell Physiology*, 45, 887-896.

Cook, R.J. and Thomas, M.R. (1990). Influence of site factors on yield response of winter wheat to fungicide programs in England and Wales, 1979-1987. *Plant Pathology*, 39, 548-557.

Cromey, M.G., Butler, R.C., Mace, M.A. and Cole, A.L.J. (2004). Effects of the fungicides azoxystrobin and tebuconazole on *Didymella exitialis*, leaf senescence and grain yield in wheat. *Crop Protection*, 23, 1019-1030.

Dimmock, J.P.R.E. and Gooding, M.J. (2002a). The effects of fungicides on rate and duration of grain filling in winter wheat in relation to maintenance of flag leaf green area. *Journal of Agricultural Science*, 138, 1-16.

Dimmock, J.P.R.E. and Gooding, M.J. (2002b). The effects of fungicides on Hagberg falling number and blackpoint of winter wheat. *Crop Protection*, 21, 475-487.

Dimmock, J.P.R.E. and Gooding, M.J. (2002c). The influence of foliar diseases, and their control by fungicides, on the protein concentration in wheat grain: a review. *Journal of Agricultural Science*, 138, 349-366.

Gooding, M.J., Dimmock, J.P.R.E., France, J. and Jones, S.A. (2000). Green leaf area decline of wheat flag leaves: the influence of fungicides and relationships with mean grain weight and grain yield. *Annals of Applied Biology*, 136, 77-84.

Grant, O.M., Chaves, M.M. and Jones, H.G. (2006). Optimizing thermal imaging as a technique for detecting stomatal closure induced by drought stress under greenhouse conditions. *Physiologia Plantarum*, 127, 507-518.

Grossmann, K. and Retzlaff, G. (1997). Bioregulatory effects of the fungicidal strobilurin kresoxim-methyl in wheat (*Triticum aestivum*). *Pesticide Science*, 50, 11-20.

Grossmann, K., Kwaltowski, J. and Caspar, C. (1999). Regulation of phytohormone levels, leaf senescence and transpiration by the strobilurin kresoxim-methyl in wheat (*Triticum aestivum*). *Journal of Plant Physiology*, 154, 805-808.

Guiliani, R. and Flore, J.A. (2000). Potential use of infrared thermometry for the detection of water stress in apple trees. *Acta Horticulturae*, 537, 383-392.

Habermeyer, J., Gerhard, M. and Zinkernagel, V. (1998). The impact of strobilurins on the plant physiology of wheat. In: Proceedings of 7th International Congress of Plant Pathology, 9-16 August, 1998, Edinburgh, Scotland, B3 2PP.

Hack, H., Bleiholder, H., Buhr, L., Meier, U., Schnock-Fricke, U., Weber, E. and Witzenberger, A. (1992). Einheitliche Codierung der phänologischen Entwicklungsstadien mono- und dikotyler pflanzen – Erweiterte BBCH-Skala, Allgemein. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes*, 44, 265-270.

Hoersefield, R., Yankovskaya, V., Sexton, G., Whittingham, W., Shiomi, K., Omura, S., Byrne, B., Cecchini, G. and Iwata, S. (2006). Structural and computational of the quinone-binding site of the complex II (succinate–ubiquinone oxidoreductase): a mechanism of electron transfer and proton conduction during ubiquinone reduction. *Journal of Biological Chemistry*, 281, 7309-7316.

Jörgensen, L.N., Henriksen, K.E. and Nielsen, G.C. (1999). Adjusting thresholds for *Septoria* control in winter wheat using strobilurins. In: Van Ginkel, M., Mc Nab, A., and Krupinsky, J., eds., *Septoria* and *Stagonospora* Diseases of Cereals: A Compilation of Global Research. CIMMYT, Mexico, pp. 173-175.

Jörgensen, L.N. and Olensen, J.E. (2002). Fungicide treatments affect yield and moisture content of grain and straw in winter wheat. *Crop Protection*, 21, 1023-1032.

King, K.E., Jenkins, J.E.E. and Morgan, W.A. (1983). The estimation of yield losses in wheat from severity of infection by *Septoria* species. *Plant Pathology*, 32, 239-249.

Konradt, M., Kappes, E.M., Hiemer, M. And Petersen, H.H. (1996). Amistar - ein Strobilurin zur Bekämpfung von Getreidekrankheiten. *Gesunde Pflanzen*, 48, 126-134.

Köhle, H., Grossmann, K., Jabs, T., Gerhard, M., Kaiser, W., Glaab, J., Conrath, U., Seehaus, K. and Herms, S. (2002). Physiological effects of the strobilurin fungicide F 500 on plants. In: Dehne, H.-W., Gisi, U., Kuck, K.-H., Russell, P.E. and Lyr, H., eds., Modern Fungicides and Antifungal Compounds III, Proceedings of the 13th International Reinhardsbrunn Symposium, 14-18 May 2001, Friedrichroda, Germany, AgroConcept GmbH, Bonn, pp. 61-74.

Lenthe, J.H., Oerke, E.-C. and Dehne, H.-W. (2007). Digital infrared thermography for monitoring canopy health of wheat. *Precision Agriculture*, 8, 15-26.

Lorens, E.J. and Cothren, J.T. (1989). Photosynthesis and yield of wheat (*Triticum aestivum*) treated with fungicides in a disease-free environment. *Plant Disease*, 73, 25-27.

Lupton, F.G.H. (1972). Further experiments on photosynthesis and translocation in wheat. *Annals of Applied Biology*, 71, 69-79.

Oerke, E.-C., Steiner, U., Dehne, H.-W. and Lindenthal, M. (2006). Thermal imaging of cucumber leaves affected by downy mildew and environmental conditions. *Journal of Experimental Botany*, 57, 2121-2132.

Pepler, S., Gooding, M.J., Ford, K.E., Ellis, R.H. and Jones, S.A. (2005). A temporal limit to the association between flag leaf life extension by fungicides and wheat yields. *European Journal of Agronomy*, 22, 405-416.

Reynolds, M.P., Delgado, M.I., Gutiérrez-Rodríguez, M. and Larque-Saavedra, A. (2000). Photosynthesis in wheat in a warm, irrigated environment I: genetic diversity and crop productivity. *Field Crop Research*, 66, 37-50.

Ruske, R.E., Gooding, M.J. and Dobraszczyk, B.J. (2004). Effects of triazole and strobilurin fungicide programmes, with and without late-season nitrogen fertilizer, on the baking quality of Malacca winter wheat. *Journal of Cereal Science*, 40, 1-8.

Sauter, H., Ammermann, A. and Röhl, F. (1995). Strobilurins - from natural products to a new class of fungicides. In: Copping, L.G. ed., Crop protection agents from nature, Royal Society of Chemistry, Cambridge, UK, pp. 50-81.

Suty-Heinze, A., Dunkel, R., Krieg, U. and Rieck, H. (2011). Bixafen - the new cereal fungicide with yield boosting effects. In: Dehne, H.-W., Deising, H.B., Gisi, U., Kuck, K.H., Russell, P.E. and Lyr, H., eds., Modern Fungicides and Antifungal Compounds VI, Proceedings of the 16th International Reinhardsbrunn Symposium, 25-29 April 2010, Friedrichroda, Germany, Lebenshilfe gGmbH, Braunschweig, pp. 69-74.

Tolstrup, K. (1984). Saprophytic fungi in the phyllosphere of barley and their effects on the plants growth and grain yield. Dissertation, Royal Veterinary and Agricultural University.

Ulrich, L.L. and Matre, D.E. (1972). Mode of action of oxathiin systemic fungicides V. Effect on electron transport system of *Ustilago maydis* and *Saccharomyces cerevisiae*. *Journal of Bacteriology*, 110, 628-632.

Wu, Y.X. and Von Tiedemann, A. (2001). Physiological effects of azoxystrobin and epoxiconazole on senescence and the oxidative status of wheat. *Pesticide Biochemistry of Physiology*, 71, 1-10.

Zhang, X.J., Zhang, X., Chen, C.J., Zhou, M.J. and Wang, H.C. (2010). Effects of fungicides JS399-19, azoxystrobin, tebuconazole, and carbendazim on the physiological and biochemical indices and grain yield of winter wheat. *Pesticide Biochemistry and Physiology*, 98, 151-157.

CHAPTER 4: SENSORS AND IMAGING TECHNIQUES FOR THE ASSESSMENT OF THE DELAY OF WHEAT SENESCENCE INDUCED BY FUNGICIDES

(Published in: Functional Plant Biology, 2013, DOI: 10.1071/FP12351)

ABSTRACT

Near range and remote sensing techniques are excellent alternatives for destructive methods to measure beneficial effects of fungicides on plant physiology. Different non-invasive sensors and imaging techniques have been used and compared to measure the effects of three fungicidal compounds, bixafen, fluoxastrobin and prothioconazole on wheat physiology under disease-free conditions in the greenhouse. Depending on the fungicidal treatment changes in green leaf area and in yield parameters were observed. Chlorophyll fluorescence of leaves was useful to measure differences in the effective quantum yield of photosystem II. Reflectance measurements of wheat leaves were highly sensitive to changes in plant vitality. The spectral vegetation indices were useful to determine differences between treatments in terms of leaf senescence, pigments and water content. The analysis of ear and leaf surface temperature was reliable to detect effects of fungicides on plant senescence.

Using non-destructive sensors, it was possible to assess a delay in senescence of wheat due to fungicide application. Furthermore, it was deduced that sensors and imaging methods are useful tools to estimate the effects of fungicides on wheat physiology**.** Physiological parameters measured by the sensors were actually more sensitive than yield parameters to assess the effect caused by fungicide application on wheat physiology.

Keywords: Leaf reflectance, IR thermography, chlorophyll fluorescence, green leaf area, grain yield.

1. INTRODUCTION

Fungicide application is the common practice to control fungal pathogens in intensive wheat crops. Various fungicidal compounds with different modes of action are available on the market. The development of active ingredients with novel mode of action is essential for a sustainable disease control. Currently, fungicide manufacturing companies have developed new active ingredients to control the major foliar pathogens of cereals (Walter,

2011). Succinate dehydrogenase inhibitors (SDHIs) are one example; they affect pathogens by inhibiting succinate dehydrogenase in the mitochondrial respiratory chain (Hoersefield *et al*., 2006). This fungicidal group has a broader spectrum activity than older compounds from the same chemical group (Avenot and Michailides, 2010).

Several papers report the beneficial effects of some fungicidal groups on the physiology of crops (Gooding *et al*., 2000; Bayles, 1999). The most reported effect is the delay of leaf senescence caused by strobilurins. Application of pyraclostrobin (Jabs *et al*., 2002), azoxystrobin (Bertelsen *et al*., 2001) and kresoxim-methyl (Grossmann and Retzlaff, 1997) resulted in an extension of leaf lifetime by delaying senescence processes, which was often associated with increased yields (Grossmann and Retzlaff, 1997).

There are a variety of methods for measuring beneficial effects of fungicides on wheat physiology, such as quantification of green leaf area duration (Gooding *et al*., 2000), photosynthetic activity (Beck *et al*., 2002), chlorophyll content (Grossmann *et al*., 1999), activity of enzymes associated to senescence (Köhle *et al*., 2002), and assessment of changes in the balance of plant growth regulators (Grossmann *et al*., 1999). However, most of these methods require destructive sampling of plant tissue, which is laborious and time consuming, and do not allow following the same leaves throughout the crop development. An excellent alternative to destructive methods is the use of non-invasive sensors and imaging techniques, which may enable the detection of differences in growth rate and early changes in plant physiology (Mahlein *et al*., 2012a). These techniques have been implemented successfully in phenotyping programs (Munns *et al*., 2010; Passioura, 2012). Non-invasive methods provide a faster approach in the screening of a large number of genotypes, resulting in the optimization of crop breeding programs (Langridge and Fleury, 2011).

Infrared (IR) thermography is a technique that records the temperature of plant surfaces, which corresponds to differences in transpiration rate (Chaerle and Van der Straeten, 2001). Thermograms of wheat plants revealed significant differences between untreated and fungicide treated plants at different growth stages (Berdugo *et al*., 2012). Moreover, this technique is useful to assess the effect of fungicides on vitality and yield of wheat in field trials (Lenthe *et al*., 2007).

The chlorophyll content is typically used as a parameter to estimate differences in photosynthetic activity of plants. The total chlorophyll content may be measured by destructive preparations, followed by the determination of the absorbance at specific wavelengths by spectrophotometric measurements (Richardson *et al*., 2002). Alternatively, the photosynthetic electron transfer can be assessed by chlorophyll fluorometers (Chaerle *et al*., 2003). This method has been used to study differences of the photosynthetic activity caused by biotic and abiotic stresses over the leaf area (Scholes

and Rolfe, 2009; Bürling *et al*., 2011).

Different studies have linked spectral reflectance of radiation to the physiological status of plants (Asner, 1998; Carter and Knapp, 2001; Stylinski *et al*., 2002). Therefore, spectral reflectance measurements may be used to assess plant vitality. The reflectance of plant leaves is characterized by low reflectance in the visible range (VIS, 400 to 700 nm) due to light absorption by photoactive plant pigments (Merzlyak *et al*., 2008; Gitelson and Merzlyak, 1996), and high reflectance in the near infrared (NIR, 700 to 1100 nm) influenced by the internal structure and epidermal layers of the leaves (Asner, 1998; Carter and Knapp, 2001).

The progressive loss of chlorophyll a and b results in the unmasking of pigments like anthocyanins, carotenoids and xanthophylls, as well as changes in internal cellular structure and a 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 reflectance have been developed (Blackburn, 1998; Gitelson *et al*., 2001; Mahlein *et al*., 2013). SVIs like the photochemical reflectance index (PRI, Gamon *et al*., 1992), plant senescence reflectance index (PSRI, Merzlyak *et al*., 1999), anthocyanin reflectance index (ARI, Gitelson *et al*., 2001) and water index (WI, Peñuelas *et al*., 1997) are highly correlated to biochemical and biophysical plant parameters related to plant health and vitality. Consequently, spectral reflectance measurements and SVIs are applicable for non-destructive assessment of changes of the physiological status and vitality of vegetation caused by stress conditions (Blackburn, 1998; Römer *et al*., 2012) and may be used to monitor physiological changes in crop plants caused by fungicides such as delay of senescence. Furthermore, spectral reflectance might become an accurate alternative to conventional methods in determining the effects caused by fungicides on plant physiology. In this research we studied the suitability of non-invasive sensors to determine the influence of fungicides on physiological plant parameters such as photosynthesis and senescence over time. For this purpose, different non-invasive methods were employed and compared in order to determine advantages and disadvantages of every technique.

The main objectives of this chapter were to:

i. Use different optical sensors to assess direct effects of the fungicides on crop plants
ii. Compare different non-invasive and destructive methods and their ability to measure the effect of fungicides on plant senescence
iii. Determine the effects of different fungicidal compounds on wheat senescence.

2. MATERIALS AND METHODS

2.1. Plant material

Spring wheat (*Triticum aestivum* L. cv. Passat) was grown under greenhouse conditions. Five pots (experimental units, 20 x 20 x 30 cm) with 20 plants were used per treatment containing a mixture of organic soil (Klasmann-Deilmann GmbH, Geeste, Germany), sand and C horizon (12:6:2 v/v). Pots were randomized into the greenhouse. Plants were raised at 24/20 °C (day/night), 70 ± 10% relative humidity (RH), photoperiod of 16 h d^{-1} with supplemental illumination (> 300 µmol m^{-2} s^{-1}, lamps Philips SGR 140, Philips, Hamburg, Germany). Plants were irrigated once per day in order to maintain soil water content (approx. 55 - 85%) to provide favorable growth conditions. 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. Plants were carefully inspected to control possible fungal infections. According to the BBCH scale (Hack *et al*., 1992) all plants were treated at growth stage (GS) 31 with the fungicide Talius® (active ingredient [a.i.] proquinazid, 200 g L^{-1}, DuPont, Neu-Isenburg, Germany) and the fungicide Vegas® (a.i. cyflufenamid, 51.3 g L^{-1}, Spiess-Urania, Hamburg, Germany) was applied at GS 65 to keep plants free from powdery mildew infections. To control insect pests, all plants were treated at GS 41 with the insecticide Sumicidin® (a.i. fenvalerate, 25 g L^{-1}, BASF, Limburgerhof, Germany) and at GS 69 with the insecticide Bulldock® (a.i. beta-cyfluthrin 125 g L^{-1}, Bayer CropScience, Monheim, Germany). The experiments were conducted twice. In order to avoid interactions between treatments, all plants were treated in the same way and at the same time with the compounds used to prevent the presence of powdery mildew and insect pests.

2.2. Experimental fungicides

Plants treated with one of the three fungicidal compounds and a non-treated control were evaluated. Active ingredients belonging to different fungicidal groups - carboxamides, strobilurins and triazoles - were used: bixafen (125 g a.i. L^{-1}, Emulsifiable concentrate (EC)), fluoxastrobin (100 g a.i. L^{-1}, EC) and prothioconazole (250 g a.i. L^{-1}, EC), the compounds were supplied by Bayer CropScience (Monheim, Germany). Fungicides were applied at the recommended field rates at two growth stages (GS). The first time of fungicide application was when the flag leaf ligule was visible (GS 39), the second time when emergence of inflorescences was completed (GS 59); in a third treatment the application was conducted twice at GS 39 and GS 59. Foliar applications were made with a CO_2 pressurized hand-sprayer (2 L capacity, Meisterwerkzeuge, Wuppertal, Germany) with an adjustable spray.

2.3. Green leaf area and yield parameters

The green leaf area was assessed by visual assessment at GS 71, GS 75, GS 81 and GS 85. It was recorded as percentage of green area of the flag leaf. At every measurement three randomly selected plants per replicate (pot) were selected to assess the green leaf area. The average of the green leaf area of three leaves per pot was used for the statistical analysis (n=5).

The number of ears per pot was counted and the total biomass was weighted at harvest. The ear weight was measured and kernels were separated from ears with a mini combine (Reichhardt Electronic Innovations, Inc, West Fargo, USA). Subsequently, the total weight of kernels was measured and the grains were counted by an electronic grain counter (Pfeuffer GmbH, Kitzingen, Germany). Yield parameters such as number of ears, single ear weight, number of kernels per ear and the grain yield per ear were calculated.

2.4. Chlorophyll extraction

The chlorophyll content was extracted following the protocol of Hiscox and Israelstam (1979). The total chlorophyll content of flag leaves from all treatments was measured at GS 75 and at GS 85 according to Blanke (1990). Dimethyl sulfoxide (99%) was used for chlorophyll extraction. Five leaf discs per treatment (n=5) with a diameter of 1 cm were weighted and kept under dark conditions for 24 h. A double beam UV/VIS spectrophotometer Uvikon 933A (BioTek Instruments, USA) was used to measure the absorbance at specific wavelengths (470, 645 and 663 nm).

2.5. Chlorophyll fluorescence

The chlorophyll fluorescence of wheat flag leaves was measured at GS 71, GS 75, GS 81 and GS 85 with a portable pulse-modulated chlorophyll fluorometer PAM-2000 (Walz, Effeltrich, Germany). Plants were kept in total darkness for 30 min at room temperature directly before 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 (1000 µmol m^{-2} s^{-1}) was applied. White light was used to produce a transient closure of PS II photochemical reaction centers. Leaves were continuously illuminated with white actinic light (336 mmol m^{-2} s^{-1}). Four replicates were used per treatment (n=4). All measurements were made between 9:00 a.m. and 4:00 p.m. The effective quantum yield of photosystem II (yield) was assessed with a PAMWIN data acquisition system (Walz, Effeltrich, Germany).

2.6. Leaf reflectance

Leaf reflectance was measured with a non-imaging spectroradiometer (ASD FieldSpecPro FR spectrometer, Analytic Spectral Devices, Boulder, USA) at GS 71, GS 75, GS 81 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 was used for measurements. The spectral range of the instrument was from 350 to 1100 nm. Since the reflectance spectra were noisy at the extremes, only values from 400 to 1000 nm were included in data analysis. The instrument was warmed up 90 min before measurements to ensure high quality and homogeneity of reflectance data. All measurements were obtained with an integration time of 17 ms per scan. For instrument optimization and reflectance calibration the average of 25 dark current measurements was calibrated to the average of 25 barium sulphate white reference (Spectralon, LabSphere, North Sutton, NH, USA) measurements. Reflectance data of the wheat leaves was assessed as the average of 25 spectra per sample. In each treatment, spectra from 5 pots and 3 plants per pot were taken. Mean reflectance per pot was used for data analysis. Spectral signatures were evaluated and compared for all treatments. The reflectance difference was calculated by subtracting the mean reflectance of fungicide treated wheat from mean reflectance of untreated wheat at each wavelength. To evaluate the efficiency of spectral vegetation indices (SVIs) to identify and describe side effects of fungicides on wheat physiology, SVIs related to photosynthetic efficiency, plant senescence and to the pigment and water content were calculated (Tab. 4.1). In this study the photochemical reflectance index (PRI), the plant senescence index (PSRI), anthocyanin reflectance index (ARI), and water index (WI) were used.

Table 4.1: Spectral vegetation indices and algorithms used in this study.

Index	Equation [a]	Related to	Reference
Photochemical reflectance index	$PRI= (R_{531}-R_{570}) / (R_{531}+R_{570})$	Epoxidation state xanthophylls cycle, pigments and photosynthetic radiation use efficiency	Gamon *et al*., (1992)
Plant senescence reflectance index	$PSRI= (R_{680}-R_{500}) / R_{750}$	Plant senescence	Merzlyak *et al*., (1999)
Anthocyanin reflectance index	$ARI= (1 / R_{550}) - (1 / R_{700})$	Anthocyanin	Gitelson *et al.*, (2001)
Water index	$WI= R_{900} / R_{970}$	Water content	Peñuelas *et al*., (1997)

[a] Reflectance at wavelengths indicated.

2.7. IR thermography

Thermographic images were taken using a Stirling-cooled infrared scanning camera (VARIOSCAN 3021 ST, JENOPTIK, Jena, Germany). Characteristics are spectral sensitivity 8 to 12 µm, geometric resolution 1.5 m radians (240 x 360 pixels focal plane array, 30° x 20° field of view lens, minimum focus distance 0.2 m), thermal resolution 0.03 K, and accuracy of absolute temperature measurement less than ± 2K. All pots were assessed by thermography, 5 pots per treatment. The temperature of leaves and ears was analyzed independent of each other. In each case fifteen pixels were taken from the digital images per pot per treatment. The mean ear and leaf temperature per pot was used for statistical analysis (n=5). The plant temperature was measured at three growth stages: GS 75, GS 81 and GS 85, 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.

2.8. 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 normal distribution and equality of variances. The data were examined using analysis of variance (One way-ANOVA) with the standard errors (SE) of the means being calculated. The means were compared using the Tukey test with a significance level of p = 0.05 confidence in order to separate subgroups.

3. RESULTS

3.1. Leaf senescence and yield

The green leaf area duration was extended by all fungicides. Application of bixafen, fluoxastrobin and prothioconazole prolonged the green leaf area compared to untreated control. However, significant differences were calculated just at the last measurement (GS 85; Fig. 4.1).

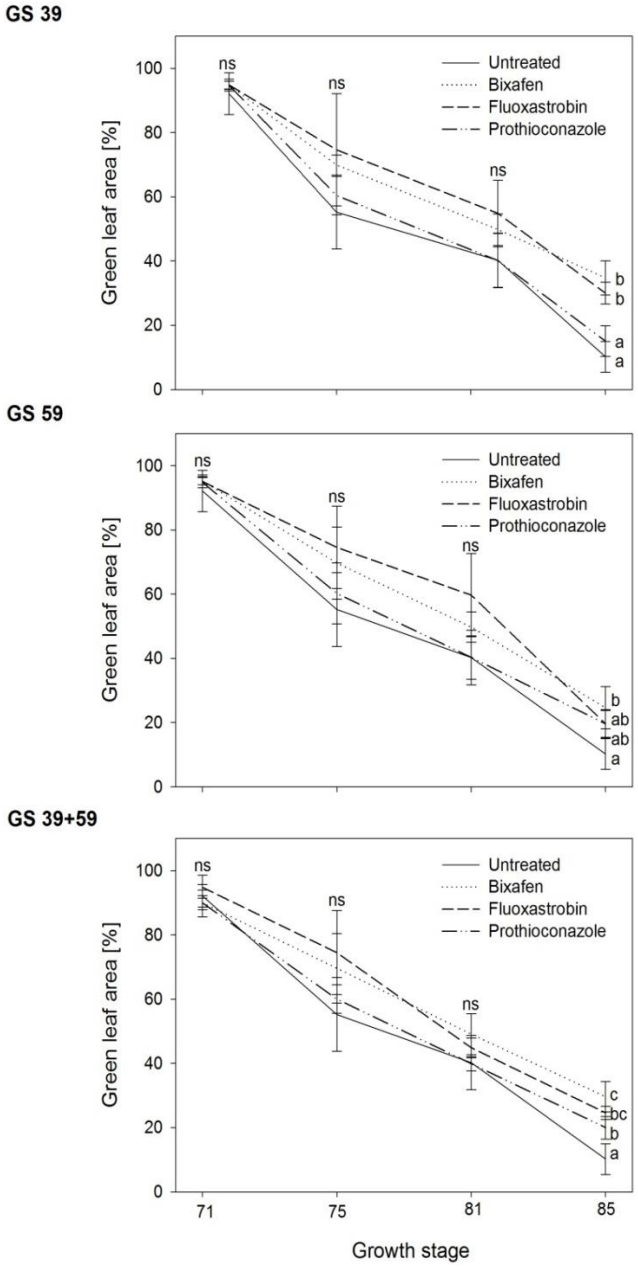

Figure 4.1: Effect of fungicides applied at GS 39, GS 59 and GS 39+59 on the green leaf area of wheat flag leaves measured by visual assessment. Bars represent standard error of the mean ($p \leq 0.05$; $n = 5$); ns, not significant.

At harvest, yield parameters were assessed. Irrespective of the application time, fungicide treatments had no statistically significant effect on the number of ears per pot, ear weight and number of kernels per ear (Tab. 4.2). Double bixafen application at GS 39 and 59 resulted in significant higher yield in comparison to the other treatments.

Table 4.2: Effects of fungicides applied at GS 39, GS 59 and GS 39+59, respectively, on yield components of cv. Passat spring wheat.

Application time	Treatment	No. of ears / pot	Ear weight [g]	No. of kernels per ear	Yield per ear [g]
GS 39	Untreated	39.4a	1.4a	24.5a	1.1a
	Bixafen	39.4a	1.5a	26.0a	1.2a
	Fluoxastrobin	38.6a	1.7a	26.1a	1.1a
	Prothioconazole	40.0a	1.4a	24.6a	1.0a
GS 59	Untreated	39.4a	1.4a	24.5a	1.1a
	Bixafen	42.2a	1.5a	24.6a	1.2a
	Fluoxastrobin	40.2a	1.6a	25.0a	1.1a
	Prothioconazole	37.6a	1.4a	25.6a	1.2a
GS 39+59	Untreated	39.4a	1.4a	24.5a	1.1a
	Bixafen	39.0a	1.6a	25.8a	1.3b
	Fluoxastrobin	38.2a	1.6a	26.1a	1.2ab
	Prothioconazole	39.6a	1.6a	25.7a	1.1a

Values with same letter within one application time do not differ significantly according to tukey´s test ($p \leq 0.05$; $n = 5$).

3.2. Chlorophyll content

At GS 75, no significant differences in chlorophyll content of wheat leaves were detectable between treatments (Tab. 4.3).

Table 4.3: Effect of fungicide treatments on the chlorophyll content of flag leaves of wheat cv. Passat at growth stages 75 and 85.

Application time	Treatment	Chlorophyll content [mg/g]	
		GS 75	GS 85
GS 39	Untreated	5.26a	1.36a
	Bixafen	5.15a	1.85a
	Fluoxastrobin	5.42a	1.42a
	Prothioconazole	5.11a	1.38a
GS 59	Untreated	5.26a	1.36a
	Bixafen	5.56a	2.40a
	Fluoxastrobin	5.06a	1.62a
	Prothioconazole	5.19a	1.29a
GS 39+59	Untreated	5.26a	1.36ab
	Bixafen	5.03a	3.30b
	Fluoxastrobin	5.77a	1.57ab
	Prothioconazole	5.39a	1.24a

Values with same letter within one application time do not differ significantly according to tukey´s test ($p \leq 0.05$; $n = 5$).

At GS 85, single fungicide applications at GS 39 and GS 59 did not result in significant differences of chlorophyll content between treatments. However, bixafen and fluoxastrobin treated leaves had higher chlorophyll content compared to the other treatments. Double fungicide application at GS 39+59 resulted in a significant difference in chlorophyll content. Additionally, in average plants treated twice with bixafen (GS 39+59) had higher chlorophyll content compared to plants treated just once.

3.3. Photosynthetic activity

The effective quantum yield of photosystem II (PS II) of wheat flag leaves was not affected by fungicide treatments at GS 71, 75 and 81 (Fig. 4.2). However, at GS 75 and at GS 81 all plants treated with fungicides at GS 39 had higher yields of PS II compared to untreated plants.

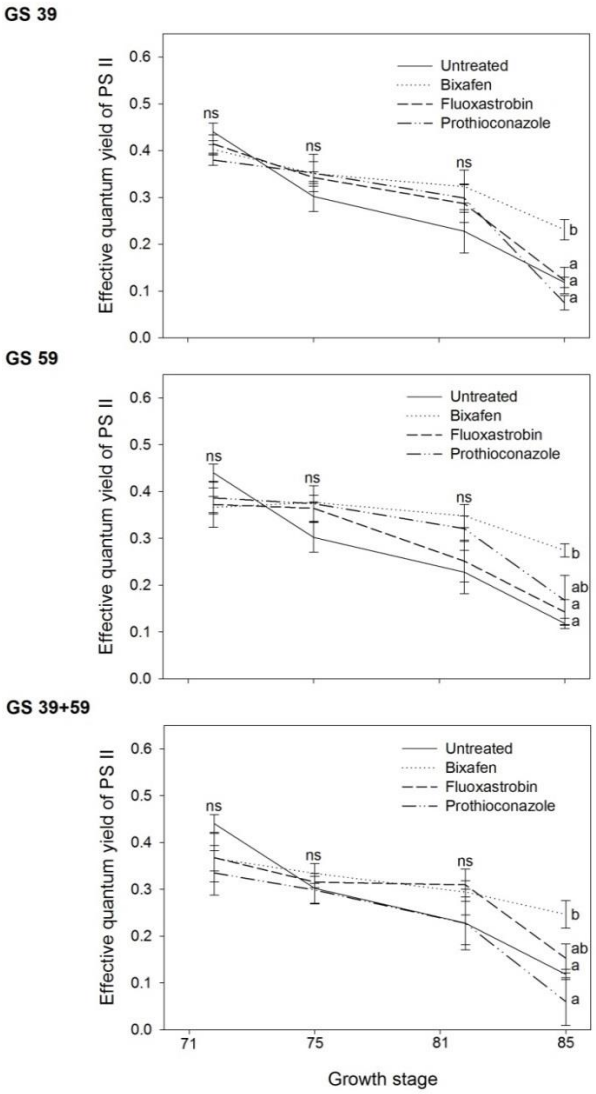

Figure 4.2: Effect of fungicides applied at GS 39, GS 59 and GS 39+59 on the quantum yield of photosystem II of wheat plants measured by chlorophyll fluorescence. Bars represent standard error of the mean ($p \leq 0.05$; $n = 4$); ns, not significant.

Significant differences between treatments were detected at GS 85. The same trend was observed when the fungicides were applied at GS 59; significant differences were observed for the last measurement time. Double fungicide application (GS 39+59) did not result in significant differences between treatments at the first three measurement times. At GS 85 bixafen application resulted in significant differences in the effective quantum yield of photosystem II. Plants treated twice with bixafen (GS 39+59) tendencially had higher PS II yields than the other treatments.

3.4. Spectral reflectance

At GS 71 leaf reflectance showed no differences between untreated plants and plants treated with bixafen, fluoxastrobin, or prothioconazole irrespective of the application time (Fig. 4.3). Reflectance spectra of all treatments were characteristic for healthy and vital wheat leaves. Changes in reflectance of untreated wheat leaves started at GS 75 and were strongly related to leaf senescence. Higher reflectance in the ranges 500 to 700 nm and 700 to 1000 nm were measured for untreated leaves at GS 75. Single prothioconazole application at GS 39 resulted in higher reflectance of leaves at the green peak (550 nm), whereas leaves treated with bixafen and fluoxastrobin showed higher absorbance in the VIS. Double applications of fungicides resulted in similar reflectance curves for all fungicide treatments.

At GS 81, reflectance of untreated flag leaves was higher between the 400 to 670 nm range compared to all fungicide treated leaves. Reflectance of plants with single application at GS 39 was similar for bixafen and fluoxastrobin treated leaves with a marginal increase for prothioconazole. Single application at GS 59 resulted in reflectance differences between treatments. Bixafen caused lower reflectance in the VIS than fluoxastrobin and prothioconazole. Plants treated twice with bixafen (GS 39+59) had lower reflectance in the VIS than plants treated twice with fluoxastrobin and prothioconazole.

At GS 85, untreated wheat leaves were in an advanced stage of senescence and had high reflectance in the range 450 to 650 nm and an overall increased reflectance in the NIR. In contrast, plants treated with bixafen (all applications) still were vital and had significantly lower reflectance than the other treatments. Reflectance of plants treated with fluoxastrobin or prothioconazole at GS 39 showed increased reflectance compared to the previous measuring time in the VIS and in the NIR only for prothioconazole. Application at GS 59 and double applications of these fungicides gave similar results.

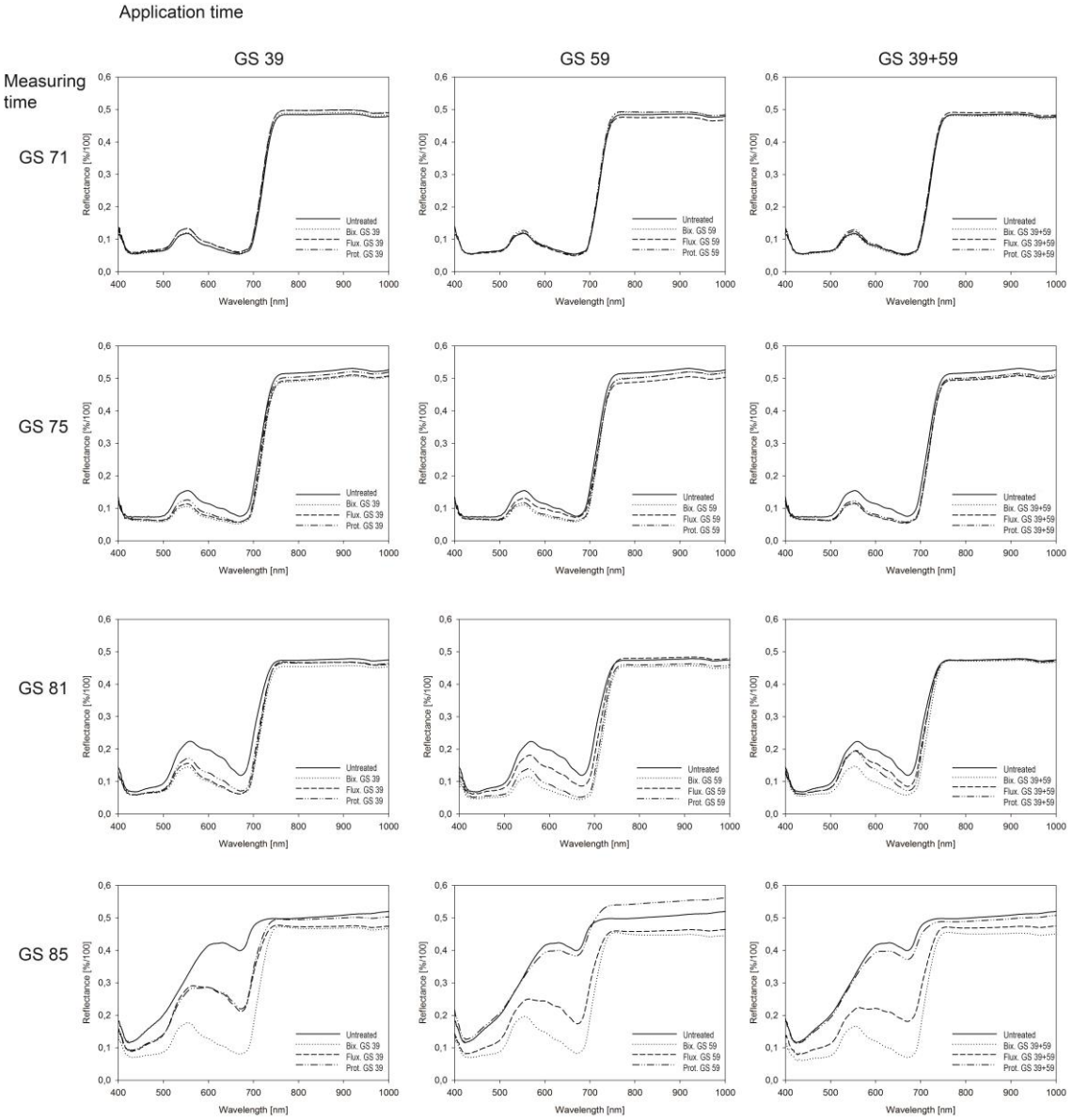

Figure 4.3: Spectral signatures of wheat leaves treated with fungicides at GS 39, GS 59, and GS 39+59 respectively, measured four times during the growth period (GS 71, GS 75, GS 81 and GS 85).

Plotting the differences in reflectance to untreated control demonstrated that bixafen and fluoxastrobin had an effect on leaf reflectance (Fig. 4.4). At GS 71, all fungicide treatments exhibited only marginal differences to untreated plants. The differences increased at later growth stages especially in the VIS from 550 to 650 nm for all treatments and at the red edge inflection point. At GS 85, reflectance of bixafen treated plants differed highly in the VIS with respect to the untreated plants, the effect of fluoxastrobin on leaf reflectance was intermediate and prothioconazole affected leaf reflectance only when applied at GS 39.

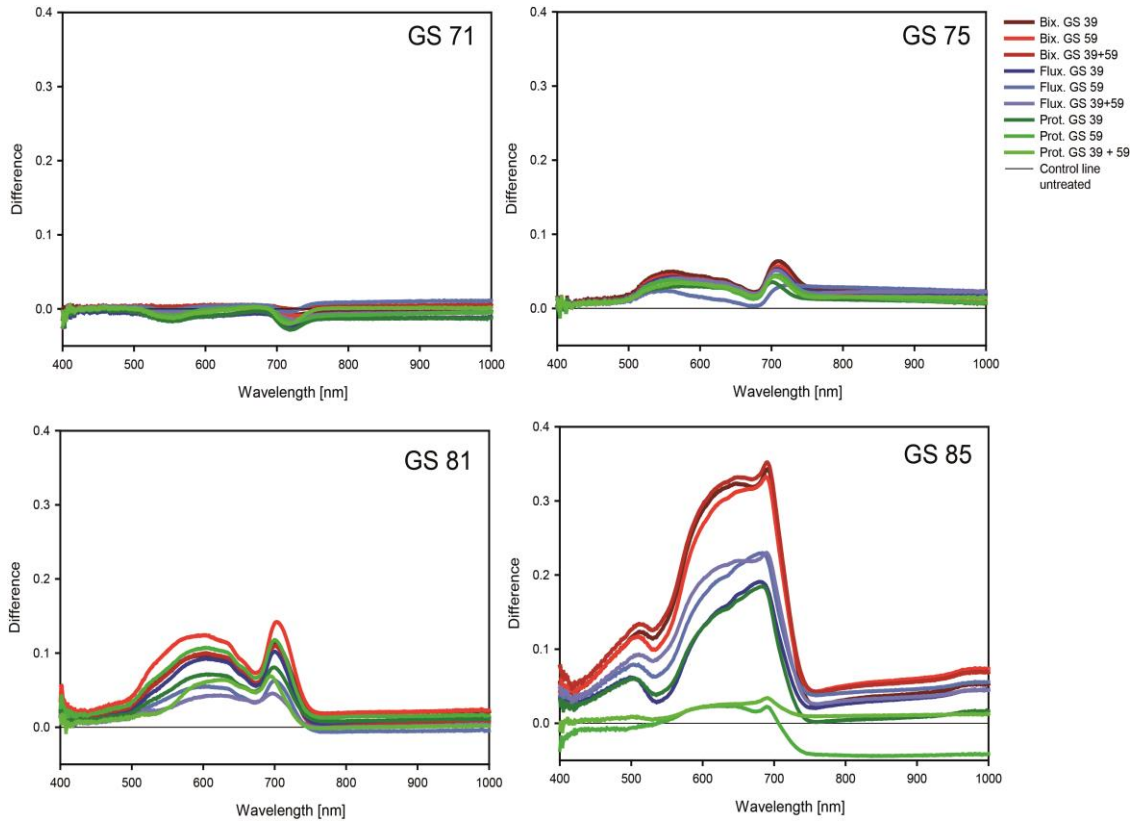

Figure 4.4: Difference spectra of wheat leaves treated with fungicides at GS 39, GS 59, and GS 39+59, respectively. Non-dimensional differences were computed by subtracting reflectance of untreated leaves and fungicide treated leaves.

3.5. Spectral vegetation indices

Four SVIs related to physiological parameters were calculated for the fungicide treated wheat leaves (Tab. 4.4). For all indices, no significant differences were present at the first two measurement times. At GS 81, single fungicide application at GS 39 resulted in different WI values. Fluoxastrobin treated plants were significantly different to untreated, bixafen and prothioconazole treated plants.

Single application of bixafen at GS 59 resulted in significant differences to the untreated control in ARI and WI values at GS 81. At this time, no relevant changes were calculated for all indices between fluoxastrobin, prothioconazole and untreated plants. At GS 81, when fungicides were applied twice (GS 39+59) all fungicide treatments exhibited significant differences in WI values compared to the untreated control.

At GS 85, all bixafen treatments (single and double application) were significant different to the untreated control for all SVIs. Leaves of bixafen treated plants were less senescent than those of the untreated plants at GS 85. For all indices, single fluoxastrobin application at GS 39 resulted in significant differences to untreated. For prothioconazole, only the WI was significantly different to untreated.

Table 4.4: Effect of fungicide application at GS 39, GS 59 and GS 39+59 on spectral vegetation indices of wheat leaves.

Index	Application time	Treatment	GS 71	GS 75	GS 81	GS 85
PRI	GS 39	Untr.	0.015a	0.008a	-0.046a	-0.138a
		Bix.	0.016a	0.022a	-0.009a	-0.015c
		Fluox.	0.012a	0.025a	0.001a	-0.073bc
		Proth.	0.011a	0.013a	-0.017a	-0.100ab
	GS 59	Untr.	0.015a	0.008a	-0.046a	-0.138a
		Bix.	0.019a	0.015a	0.006a	-0.016b
		Fluox.	0.022a	0.003a	-0.026a	-0.081a
		Proth.	0.022a	0.012a	-0.002a	-0.130a
	GS 39+59	Untr.	0.015a	0.008a	-0.046ab	-0.138a
		Bix.	0.022a	0.027a	-0.002b	-0.024b
		Fluox.	0.020a	0.028a	-0.049a	-0.079b
		Proth.	0.019a	0.026a	-0.023ab	-0.138a
PSRI	GS 39	Untr.	-0.014a	0.007a	0.042a	0.358b
		Bix.	-0.011a	0.000a	-0.007a	-0.002a
		Fluox.	-0.009a	0.000a	-0.010a	0.133a
		Proth.	-0.011a	-0.002a	-0.004a	0.163ab
	GS 59	Untr.	-0.014a	0.007a	0.042a	0.358b
		Bix.	-0.013a	0.002a	-0.008a	-0.006a
		Fluox.	-0.016a	0.018a	0.017a	0.113a
		Proth.	-0.015a	0.003a	-0.010a	0.332b
	GS 39+59	Untr.	-0.014a	0.007a	0.042ab	0.358bc
		Bix.	-0.013a	-0.002a	-0.007ab	-0.003a
		Fluox.	-0.013a	-0.006a	0.027b	0.153ab
		Proth.	-0.014a	-0.004a	-0.009a	0.383c
ARI	GS 39	Untr.	-1.538a	0.003a	-0.207a	0.960c
		Bix.	-1.431a	0.045a	-0.848a	-0.301a
		Fluox.	-1.226a	0.040a	-0.864a	0.204ab
		Proth.	-1.169a	0.064a	-0.802a	0.563bc
	GS 59	Untr.	-1.538a	0.003a	-0.207b	0.960bc
		Bix.	-1.526a	0.144a	-1.325a	-0.500a
		Fluox.	-1.724a	0.255a	-0.571b	0.369b
		Proth.	-1.529a	0.278a	-1.002ab	1.161c
	GS 39+59	Untr.	-1.538a	0.003a	-0.207a	0.960bc
		Bix.	-1.586a	0.053a	-0.885a	-0.118a
		Fluox.	-1.458a	-0.102a	-0.302a	0.443ab
		Proth.	-1.370a	-0.114a	-0.540a	1.013c
WI	GS 39	Untr.	0.979a	0.987a	0.988b	1.006b
		Bix.	0.980a	0.991a	0.987b	0.989a
		Fluox.	0.979a	0.989a	0.982a	0.990a
		Proth.	0.980a	0.991a	0.986b	0.998a
	GS 59	Untr.	0.979a	0.987a	0.988b	1.006bc
		Bix.	0.977a	0.989a	0.983a	0.988a
		Fluox.	0.979a	0.991a	0.986ab	0.994ab
		Proth.	0.977a	0.992a	0.985ab	1.013c
	GS 39+59	Untr.	0.979a	0.987a	0.988c	1.006b
		Bix.	0.978a	0.986a	0.983a	0.988a
		Fluox.	0.978a	0.985a	0.984ab	0.994ab
		Proth.	0.980a	0.987a	0.986ab	1.011b

Values with same letters within one application time do not differ significantly according to Tukey´s test ($p \leq 0.05$; $n = 5$). *PRI* photochemical reflectance index, *PSRI* plant senescence reflectance index, *ARI* anthocyanin reflectance index and *WI* water index.

Application of fluoxastrobin at GS 59 just resulted in significant different PSRI values to untreated control at the last measuring time. In contrast, single prothioconazole

application at GS 59 did not result in significant differences to the untreated control. Double fluoxastrobin application resulted in significant differences to the untreated control only by the PRI. There were no significant differences to the untreated control for all indices when prothioconazole was applied twice.

3.6. Temperature of leaves and ears

Fungicide application resulted in significant differences in ear temperature at GS 75 (Tab. 4.5). In contrast, the mean temperature of wheat leaves at this growth stage was similar for all active ingredients and application times. The first relevant differences in leaf temperature between treatments were detected at growth stage 81. Leaves of bixafen treated plants had lower temperature compared to the other treatments (Tab. 4.5).At GS 85, lower ear temperature was observed for bixafen treated plants for all application times; however, double application (GS 39+59) resulted in lower ear temperature compared to the single applications. Single and double fungicide applications showed similar effects on leaf and ear temperature. The first differences in ear temperature became evident at GS 75, at this growth stage, no significant differences in leaf temperature were measured. First differences in leaf temperature occurred at GS 81 for all application times. Differences in ear and leaf temperature between untreated and plants treated twice with bixafen were recorded at GS 75 (Fig. 4.5).

Table 4.5: Effect of fungicide treatments on the temperature [°C] of ears and leaves of wheat cv. Passat at growth stages 75, 81 and 85.

Application time	Treatment	Temperature [°C]					
		GS 75		GS 81		GS 85	
		Ears	Leaves	Ears	Leaves	Ears	Leaves
GS 39	Untreated	21.8 b	21.5 a	24.7 bc	23.7bc	23.1 b	22.7 b
	Bixafen	21.3 a	21.5 a	23.4 a	22.5 a	22.6 a	21.8 a
	Fluoxastrobin	22.2 c	21.7 a	24.9 c	23.7 c	23.1 b	22.3 ab
	Prothioconazole	21.1 a	21.5 a	24.5 b	23.3 b	23.1 b	22.8 b
GS 59	Untreated	21.8 b	21.5 a	24.7 c	23.7 c	23.1 b	22.7 b
	Bixafen	20.8 a	21.4 a	23.3 a	22.8 a	22.4 a	21.7 a
	Fluoxastrobin	20.7 a	21.4 a	24.3 b	23.2 b	23.1 b	22.8 b
	Prothioconazole	21.6 b	21.4 a	24.2 b	23.6 bc	23.1 b	22.8 b
GS 39+59	Untreated	21.8 b	21.5 a	24.7 b	23.7 ab	23.1 b	22.7 b
	Bixafen	21.2 a	21.4 a	23.4 a	23.5 a	22.1 a	21.7 a
	Fluoxastrobin	21.1 a	21.4 a	24.7 b	24.1 b	23.1 b	22.2 ab
	Prothioconazole	21.6 b	21.4 a	24.8 b	23.7 ab	23.1 b	22.7 b

Values with same letter within one application time do not differ significantly according to Tukey´s test ($p \leq 0.05$; $n = 5$).

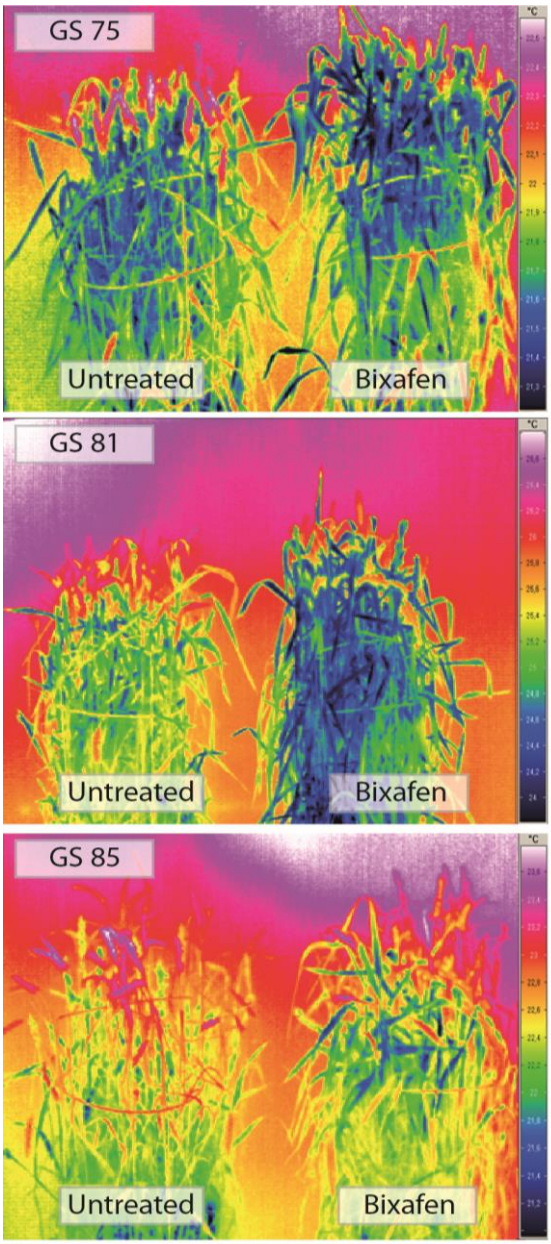

Figure 4.5: Thermograms of untreated wheat plants and plants treated twice (GS 39+59) with bixafen measured at GS 75, GS 81 and GS 85.

4. DISCUSSION

This study demonstrates the potential of different non-invasive sensors to assess the delay of wheat senescence induced by fungicides. The effect of strobilurins and triazoles on the leaf senescence processes have been studied during the last decade, with the conclusion that these fungicidal groups may influence the level of phytohormons and as well as a reduction of oxidative stress associated to plant senescence (Cromey *et al*., 2004; Jaleel *et al*., 2006). There is more information available about the effect of these two fungicidal groups on plant physiology, whereas there are few reports related to the effect of carboxamide fungicides on plant senescence. Recently, studies on different

crops demonstrated that carboxamide application may delay senescence and positively influence the yield (Horsfield *et al*., 2010; Berdugo *et al*., 2011). In the present study, the higher grain yield of carboxamide-treated plants was mainly attributed to the delay of senescence of leaves and ears, which resulted in a longer grain filling period compared to the untreated control. Application of bixafen, fluoxastrobin and prothioconazole delayed the senescence of leaves and extended the green leaf area duration compared to the untreated plants. However, visual assessments of the green leaf area by human raters are influenced by different constraints (Bock *et al*., 2010). The reliability of visual assessed senescence may differ between different rater or on different days and experiments (Nutter *et al*., 1993). Thus, it is subjective and differences in green leaf area have to be obvious. Differences between treatments in terms of green area at early stages were not noticeable by the naked eye.

Since the green leaf area duration is correlated to chlorophyll content, pigment extraction is a common tool to determine leaf vitality. The reduction of leaf chlorophyll content associated with a decrease in the photosynthetic activity is closely related to plant senescence (Merzlyak *et al*., 1999). The breakdown of the chlorophyll and chloroplasts, which result in a decline of the photosynthetic activity, is one of the most relevant processes during senescence (Lingrui *et al*., 2007). However, it was not possible to detect significant differences between treatments at an early growth stage (GS 75). Capabilities of pigment extraction for fungicide screening or phenotyping are limited due to the destructive matter. It is not possible to follow the same individual over time and the sample size is restricted, and as a result may be not representative.

Chlorophyll fluorescence permits the assessment of the operating quantum efficiency of electron transport through PSII in leaf tissue (Genty *et al*., 1990). Qingtao *et al.* (2002) reported that in senescent flag leaves of wheat the PSII apparatus remains functional while photosynthetic activity decreases. Since the decrease in 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 a chlorophyll fluorometer is an accurate method to detect changes in the senescence states of plant tissue due to fungicide application or biotic and abiotic stresses. Furthermore, compared to destructive chlorophyll analysis, the use of a chlorophyll fluorometer allows the observation of the same leaves throughout an investigation, and it is less laborious.

Reflectance measurements of wheat leaves were highly sensitive to plant vitality. Already at GS 75 leaf reflectance differences between untreated and fungicide treated plants were detected. This difference was most evident for double fungicide application; untreated plants showed already increasing reflectance compared to the previous measuring time in the VIS (low leaf absorption of light by leaf pigments), whereas fungicide treated plants

revealed no difference in reflectance. A healthy leaf represents highly performing photosynthetic machinery, efficiently absorbing radiation in the visible part of the spectrum (Gitelson and Merzlyak, 1994). Therefore, reflectance in the VIS is highly correlated to the pigment content and subsequently to plant vitality (Carter and Knapp, 2001; Gitelson and Merzlyak, 1996). During senescence, a progressive loss of photosynthetic pigments is described. As mentioned by Gitelson and Merzlyak (1994), events occurring in senescing and aging leaves are very close to processes in plants under abiotic and biotic stress conditions. By calculating spectral vegetation indices, it was possible to measure differences in specific leaf traits deduced from spectral reflectance like the anthocyanin content or the water content. In wheat leaves treated twice with bixafen, the concentration of photosynthetic pigments like chlorophyll a and b was higher than in leaves of the other treatments. This difference was measurable with the PRI, which is associated to photosynthetic radiation use efficiency and photosynthetic pigments, and with the PSRI, which is linked to plant senescence (Gamon *et al*., 1992). Comparison of spectral vegetation indices with destructive measures suggests fungicide application-specific relationships with leaf senescence. Thenkabail *et al.* (2000) established a strong relationship between spectral vegetation indices and agricultural crop biophysical characteristics. Besides the observation of vegetation, these indices have shown potential as a non-destructive measure of fungicidal side effects on the leaf level in this study. During senescence, chlorophyll concentration decreases whereas carotenoids and anthocyanins become apparent (Gitelson *et al*., 2001; Merzlyak *et al*., 1999); this accounts for the yellowing of senescing leaves. This yellowing caused an increase of reflectance in the green peak, and it and it was measurable in this study with the ARI in an early stage of senescence. Besides changes in pigmentation, leaf senescence produces differences in leaf structure and leaf water content. This was assessable in the NIR of the reflectance spectrum and by the WI as an indicator of leaf water content (Carter and Knapp, 2001; Peñuelas *et al*., 1997). Wheat plants treated with bixafen revealed higher turgor resulting in differences in WI index values compared to the other treatments. It has been shown that the indices used in our study are reliable indicators of fungicidal side effects and can differentiate senescent stages of wheat leaves. Our results are in accordance with Sims and Gamon (2002) who stated that optical methods are more suitable to assess pigment levels than destructive chemical methods, which estimate leaf pigment content by an area-based average. A further advantage of these vegetation indices to assess beneficial side effects of fungicides resides in their simplicity. The complex hyper-dimensional spectrum is reduced to a set of two or three relevant wavelengths. Thus, the method can be easily incorporated in online application or in fungicide screening processes.

Use of thermal imaging allowed the comparison of the temperature of plants treated differently under the same climate conditions directly in one picture (Berdugo *et al*., 2012; Mahlein *et al*., 2012b). The analysis of plant surface temperature was useful to detect and verify an effect of fungicide application on plant senescence. Plants treated with fungicides showed higher transpiration rates during grain filling explained by longer plant vitality compared to untreated plants. Stomatal aperture enables plants to exchange gases needed to run photosynthesis and to regulate plant temperature by water evaporation (Jones, 1998). One of the first stages of plant senescence is stomatal closure and is thus a diminution of transpiration (Munne-Bosch and Alegre, 2004). The reduction of the transpiration rate causes an increase of tissue temperature and consequently, a decrease of leaf photosynthetic activity (Kitaya *et al*., 2003). Since tissue temperature is associated to the plant senescence status, it was possible to assess the effect of fungicides on plant senescence by thermography. This is in accordance with Lenthe *et al.* (2007) who reported that thermography can be used as an accurate technique to detect differences in canopy temperature related to plant senescence of wheat plants under field conditions.

Studies under field conditions demonstrated that the effect of fungicides on senescence is closely related to the active ingredient, the crop genotype and the incidence of foliar diseases (Gooding *et al*., 2000). Additionally, the effects of fungicides on yield have been mainly attributed to disease control (Cook and Thomas, 1990; Peppler *et al*., 2005). Application of prothioconazole and fluoxastrobin often resulted in higher grain yield than untreated control in experiments under field conditions (Haidukowski *et al*., 2012; Sadowski *et al*., 2009). The yield increment was mainly associated to the effective control of foliar pathogens by these active ingredients. Under greenhouse conditions, it is possible to control various environmental factors which can significantly affect disease development (Larkin and Fravel, 2002). By controlling temperature and moisture in the greenhouse, it is possible to avoid the major foliar pathogens of wheat.

Yield assessment under greenhouse conditions bears several limitations. Environmental factors in the greenhouse such as temperature, UV-B radiation and CO_2 concentration can diminish the yield potential of some plant species (Rajan and Blackman, 1975; Teramura and Murali, 1986; Lawlor and Mitchell, 1991). It may generate difficulties to detect and distinguish the effects produced by fungicide application through yield parameters. In the present study it was not always achievable to recognize beneficial effects of fungicidal compounds by yield parameters. However, by the use of sensors and imaging techniques it was possible to detect the effects of fungicides on plant senescence and physiology. Furthermore, by non-invasive techniques it was feasible to measure beneficial effects of fungicides on crop plants already at an early stage of the vegetation

period. In this comparative study, different methods have been applied and compared to monitor the effect of fungicides on wheat physiology (Tab. 4.6). These methods are based on different measuring principles and are linked to specific plant parameters. The physiological parameters recorded by technical sensors were more sensitive than visual assessments and yield parameters. The assessment of photosynthetic activity, surface temperature and leaf reflectance were suitable to establish differences between treatments. Based on these results it can be deduced that leaf reflectance measurement is the most sensitive and specific method with a high potential for large-scale fungicide screening processes. Recent hyperspectral imaging techniques should be incorporated in future studies to additionally explore plant physiology in high spatial resolution.

Table 4.6: Comparison of different techniques to assess the delay of wheat senescence induced by fungicides.

Method	Parameter	Physics	Expertise required	Input		Accuracy		Conclusions and remarks
				Time (length)	Cost	Sensitivity	Specificity	
Reflectance spectrometry	Light absorption and reflectance due to leaf structure and pigment content	Visible range (400 - 700 nm) Near infrared (700 - 1000 nm)	High	Short	High	High	High	- Non-invasive - Imaging and non-imaging - Reproducible and objective - Repeated measurement of individuals - Analysis to be standardized
Thermography	Surface temperature influenced by transpiration rate	Infrared radiation (8 - 14 µm)	Medium	Medium	High	High	Medium	- Non-invasive - Imaging technique - Comparison of different treatments in one image - Strong effect of environmental conditions
Chlorophyll fluorescence	Photosynthetic activity	Fluorescence emission of PSII (PSI)	High	Long	High	High	Medium	- Non-invasive - Imaging and non-imaging - Dark adaptation is time consuming
Green leaf area duration	Green leaf area	Visual assessment	Low	Short	Low	Low	Low	- Non-invasive - Simplest and fastest method - Subjective estimation - May vary among raters
Pigment extraction	Pigment content (chlorophyll a/b, carotenoids, anthocyanin)	Light absorption measurements with spectrophotometer at specific wavelengths	Medium	Long	Medium	Medium	High	- Destructive - High specificity - Small sample size, not representative for the whole plant
Yield	Grain yield	n/a	Low	Medium	High	Medium	Medium	- Destructive - Influenced by various environmental factors - Long period between treatment and assessment - Highly variable

5. CONCLUSION

It was possible by the use of sensors to reveal changes in the senescence status of wheat plants due to fungicide application. In this study, senescence-associated changes in wheat physiology, i.e. degradation of photosynthetic pigments, photosynthetic activity, leaf reflectance, and transpiration of plant tissue was quantified by using ground-based optical sensors earlier than with destructive and visual methods.

Non-invasive sensors and imaging techniques are an excellent alternative to traditional screening methods. New insights from non-invasive sensors can contribute to a better understanding of the effects of fungicides on plant senescence. These sensors can be incorporated in automatic and reproducible screening and phenotyping systems with high efficiency and accuracy.

6. REFERENCES

Asner, G.P. (1998). Biophysical and biochemical sources of variability in canopy reflectance. *Remote Sensing of Environment*, 64, 234-253.

Avenot, F.H. and Michailides, T.J. (2010). Progress in understanding molecular mechanisms and evolution of resistance to succinate dehydrogenase inhibiting (SDHI) fungicides in phytopathogenic fungi. *Crop Protection*, 29, 643-651.

Bayles, R. (1999). The interaction of strobilurin fungicides with cereal varieties. *Plant Varieties and Seeds,* 12, 129-140.

Beck, C., Oerke, E.-C. and Dehne, H.-W. (2002). Effect of strobilurins on the yield formation of wheat – Influence of variety and environmental conditions. In: Dehne, H.-W., Gisi, U., Kuck, K.H., Russell, P.E. and Lyr, H., eds., Modern Fungicides and Antifungal Compounds III, Proceedings of the 13th International Reinhardsbrunn Symposium, 14-18 May 2001, Friedrichroda, Germany, AgroConcept GmbH, Bonn, pp.41-47.

Berdugo, C.A., Steiner, U., Oerke, E.-C. and Dehne, H.-W. (2011). Effects of the new SDHI-fungicide bixafen on the physiology and yield of wheat plants. In: Dehne, H.-W., Deising, H.B., Gisi, U., Kuck, K.H., Russell, P.E. and Lyr, H., eds., Modern Fungicides and Antifungal Compounds VI, Proceedings of the 16th International Reinhardsbrunn Symposium, 25-29 April 2010, Friedrichroda, Germany, Lebenshilfe gGmbH, Braunschweig, pp. 81-84.

Berdugo, C.A., Steiner, U. and Dehne, H.-W. and Oerke, E.-C. (2012). Effect of bixafen on senescence and yield formation of wheat. *Pesticide Biochemistry and Physiology*, 104, 171-177.

Bertelsen, J.R., Neergaard, E.DE. and Smedegaard-Petersen, V. (2001). Fungicidal effects of azoxystrobin and epoxiconazole on phyllosphere fungi, senescence and yield of winter wheat. *Plant Pathology*, 50, 190-205.

Blackburn, G.A. (1998). Spectral indices for estimating photosynthetic pigment concentrations: a test using senescent tree leaves. *International Journal of Remote Sensing*, 19, 657-675.

Blanke, M.M. (1990). Chlorophyll determination using DMF. *Wein-Wissenschaft*, 45, 76-78.

Bock, C.H., Poole, G.H., Parker, P.E. and Gottwald, T.R. (2010). Plant disease severity estimated visually, by digital photography and image analysis, and by hyperspectral imaging. *Critical Reviews in Plant Science*, 29, 59-107.

Boyer, M., Miller, J., Nelanger, M. and Hare, E. (1988). Senescence and spectral reflectance in leaves of northern pin oak (*Quercus palustis* Munich.). *Remote Sensing of Environment*, 25, 71-87.

Bürling, K., Hunsche, M. and Noga, G. (2011). Use of blue-green and chlorophyll fluorescence measurements for differentiation between nitrogen deficiency and pathogen infection in winter wheat. *Journal of Plant Physiology*, 168, 1641-1648.

Carter, G.A. and Knapp, A.K. (2001). Leaf optical properties in higher plants: linking spectral characteristics to stress and chlorophyll concentration. *American Journal of Botany*, 88, 677-684.

Chaerle, L. and Van der Straeten, D. (2001). Seeing is believing: imaging techniques to monitor plant health. *Biochimica et Biophysica Acta*, 1519, 153-166.

Chaerle, L., Valcke, R. and Van der Straeten. (2003). Imaging techniques in plant physiology and agronomy: from simple to multispectral approaches. In: Hemantaranjan, A., ed., Advances in Plant Physiology, Volume 5, Jodhpur: Scientific Publishers, pp.135-155.

Cook, R.J. and Thomas, M.R. (1990). Influence of site factors on yield response of winter wheat to fungicide programs in England and Wales, 1979-1987. *Plant Pathology*, 39, 548-557.

Cromey, M.G., Butler, R.C., Mace, M.A. and Cole, A.L.J. (2004). Effects of the fungicides azoxystrobin and tebuconazole on *Didymella exitialis*, leaf senescence and grain yield in wheat. *Crop Protection*, 23, 1019-1030.

Gamon, J.A., Peñuelas, J. and Field, C.B. (1992). A narrow-waveband spectral index that tracks diurnal changes in photosynthetic efficiency. *Remote Sensing of Environment*, 41, 35-44.

Genty, B., Briantais, J.M. and Baker, N.R. (1989). The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta (BBA) – General Subjects*, 990, 87-92.

Genty, B., Harbinson, J., Briantais, J.-M. and Baker, N.R. (1990). The relationship between non-photochemical quenching of chlorophyll fluorescence and the rate of photosystem II photochemistry in leaves. *Photosynthesis Research*, 25, 249-257.

Gitelson, A. and Merzlyak, M.N. (1994). Quantitative estimation of chlorophyll-a using reflectance spectra: experiments with autumn chestnut and maple leaves. *Journal of Photochemistry and Photobiology B: Biology*, 22, 247-252.

Gitelson, A.A. and Merzlyak, M.N. (1996). Signature analysis of leaf reflectance spectra: Algorithm development for remote sensing of chlorophyll. *Journal of Plant Physiology*, 148, 494-500.

Gitelson, A.A., Merzlyak, N.M. and Chivkunova, O. (2001). Optical properties and nondestructive estimation of anthocyanin content in plant leaves. *Photochemistry and Photobiology*, 74, 38-45.

Gooding, M.J., Dimmock, J.P.R.E., France, J. and Jones, S.A. (2000). Green leaf area decline of wheat flag leaves: the influence of fungicides and relationships with mean grain weight and grain yield. *Annals of Applied Biology*, 136, 77-84.

Grossmann, K. and Retzlaff, G. (1997). Bioregulatory effects of the fungicidal strobilurin kresoxim-methyl in wheat (*Triticum aestivum*). *Pesticide Science*, 50, 11-20.

Grossmann, K., Kwiatkowski, J. and Retzlaff, G. (1999). Regulation of phytohormone levels, leaf senescence and transpiration by the strobilurin kresoxim-methyl in wheat (*Triticum aestivum*). *Journal of Plant Physiology*, 154, 805-808.

Grover, A. and Mohanty, P. (1992). Leaf senescence-induced alterations in structure and function of higher plant chloroplasts. In: Abrol, Y.P., Mohanty, P. and Govindjee, eds., Photosynthesis: Photoreactions to Plant Productivity. Kluwer Academic Publishers, The Netherlands, pp. 225-255.

Hack, H., Bleiholder, H., Buhr, L., Meier, U., Schnock-Fricke, U., Weber, E. and Witzenberger, A. (1992) Einheitliche Codierung der phänologischen Entwicklungsstadien mono- und dikotyler pflanzen – Erweiterte BBCH-Skala, Allgemein. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes*, 44, 265-270.

Haidukowski, M., Visconti, A., Perrone, G., Vanadia, S., Pancaldi, D., Covarelli, L., Balestrazzi, R. and Pascale, M. (2012). Effect of prothioconazole-based fungicides on *Fusarium* head blight, grain yield and deoxynivalenol accumulation in wheat under field conditions. *Phytopathologia Mediterranea*, 51, 236-246.

Hiscox, J.D. and Israelstam, G.F. (1979). A method for the extraction of chlorophyll from leaf tissue without maceration. *Canadian Journal of Botany*, 57, 1332-1334.

Horsefield, R., Yankovskaya, V., Sexton, G., Whittingham, W., Shiomi, K., Omura, S., Byrne, B., Cecchini, G. and Iwata, S. (2006). Structural and computational of the quinone-binding site of the complex II (succinate-ubiquinone oxidoreductase): a mechanism of electron transfer and proton conduction during ubiquinone reduction. *Journal of Biological Chemistry*, 281, 7309-7316.

Horsfield, A., Wicks, T., Davies, K., Wilson, D. and Paton, S. (2010). Effect of fungicide use strategies on the control of early blight (*Alternaria solani*) and potato yield. *Australasian Plant Pathology*, 39, 368-375.

Jabs, T., Pfirrmann, J., Schäfer, S., Wu, Y. and Tiedemann, AV. (2002). Anti-oxidative and anti-senescence effects of the strobilurin pyraclostrobin in plants: A new strategy to cope with environmental stress in cereals. In: Proceedings of the BCPC Conference - Pest and Diseases, 18-21 November 2002, Brighton, UK, pp. 941-946.

Jaleel, C.A., Gopi, R., Alagu Lakshmanan, G.M. and Panneerselvam, R. (2006). Triadimefon induced changes in the antioxidant metabolism and ajmalicine production in *Catharanthus roseus* (L.) G. Don. *Plant Science*, 171, 271-276.

Jones, H.G. (1998). Stomatal control of photosynthesis and transpiration. *Journal of Experimental Botany*, 49, 387-398.

Kitaya, Y., Kavai, M., Tsruyama, J., Takahashi, H., Tani, A., Goto, E., Saito, T. and Kiyota, M. (2003). The effect of gravity on surface temperatures of plant leaves. *Plant, Cell and Environment*, 26, 497-503.

Köhle, H., Grossmann, K., Jabs, T., Gerhard, M., Kaiser, W., Glaab, J., Conrath, U., Seehaus, K. and Herms, S. (2002). Physiological effects of the strobilurin fungicide F 500 on plants. In: Dehne, H.-W., Gisi, U., Kuck, K.-H., Russell, P.E. and Lyr, H., eds., Modern Fungicides and Antifungal Compounds III, Proceedings of the 13th International Reinhardsbrunn Symposium, 14-18 May 2001, Friedrichroda, Germany, AgroConcept GmbH, Bonn, pp. 61-74.

Langridge, P. and Fleury, D. (2011). Making the most of 'omics' for crop breeding. *Trends in Biotechnology*, 29, 33-40.

Larkin, R. and Fravel, D. (2002). Effects of varying environmental conditions on biological control of *Fusarium* wilt of tomato by nonpathogenic *Fusarium* spp. *Phytopathology*, 92, 1160-1166.

Lawlor, D.W. and Mitchell, R.A.C. (1991). The effects of increasing CO2 on crop photosynthesis and productivity: a review of field studies. *Plant, Cell and Environment*, 14, 807-818.

Lenthe, J.H., Oerke, E.-C. and Dehne, H.-W. (2007). Digital infrared thermography for monitoring canopy health of wheat. *Precision Agriculture*, 8, 15-26.

Lingrui, Z., Da, X., Junsheng, W., and Lingling, L. (2007). Rapid and non-invasive detection of plants senescence using a delayed fluorescence technique. *Photochemical and Photobiological Science*, 6, 635-641.

Mahlein, A.-K., Steiner, U., Hillnhütter, C., Dehne, H.-W. and Oerke, E.-C. (2012a). Hyperspectral imaging for small-scale analysis of symptoms caused by different sugar beet diseases. *Plant Methods,* 8, 3; doi:10.1186/1746-4811-8-3.

Mahlein, A.-K., Oerke, E.-C., Steiner, U. and Dehne, H.-W. (2012b). Recent advances in sensing plant diseases for precision crop protection. *European Journal of Plant Pathology*, 133, 197-209.

Mahlein, A.-K., Rumpf, T., Welke, P., Dehne, H.-W., Plumer, L., Steiner, U. and Oerke, E.-C. (2013). Development of spectral indices for detecting and identifying plant diseases. *Remote Sensing of Environment*, 128, 21-30.

Merzlyak, M.N., Gitelson, A.A., Chivkunova, O.B. and Rakitin, V.Y. (1999). Non-destructive optical detection of pigment changes during leaf senescence and fruit ripening. *Physiologia Plantarum*, 106, 135-141.

Merzlyak, M.N., Melo, T.B. and Naqvi, K.R. (2008). Effect of anthocyanins, carotenoids and flavonols on chlorophyll fluorescence excitation spectra in apple fruit: signature analysis, assessment, modeling, and relevance to photoprotection. *Journal of Experimental Botany*, 59, 349-359.

Munne-Bosch, S. and Alegre, L. (2004). Die and let live: leaf senescence contributes to plant survival under drought stress. Functional Plant Biology, 31, 203-216.

Munns, R., James, R.A., Sirault, X.R.R., Furbank, R.T. and Jones, H.G. (2010). New phenotyping methods for screening wheat and barley for beneficial responses to water deficit. *Journal of Experimental Botany*, 61, 3499-3507.

Nutter, F.W. Jr, Gleason, M.L., Jenco, J.H. and Christians, N.L. (1993). Accuracy, Intra-rater repeatability, and inter-rater reliability of disease assessment systems. *Phytopathology*, 83, 806-812.

Passioura, J.B. (2012). Phenotyping for drought tolerance in grain crops: when is it useful to breeders?. Functional Plant Biology, 39, 851-859.

Peñuelas, J., Pinol, R.O., Ogaya, R. and Filella, I. (1997). Estimation of plant water concentration by the reflectance Water Index WI= (R900/R970). *International Journal of Remote Sensing*, 18, 2869-2875.

Peppler, S., Gooding, M.J., Ford, K.E., Ellis, R.H. and Jones, S.A. (2005). A temporal limit to the association between flag leaf life extension by fungicides and wheat yields. *European Journal of Agronomy*, 22, 405-416.

Qingtao, L., Congming, L., Jianhua, Z. and Tingyun, K. (2002). Photosynthesis and chlorophyll a fluorescence during flag leaf senescence of field-grown wheat plants. *Journal of Plant Physiology*, 159, 1173-1178.

Rajan, A.K. and Blackman, G.E. (1975). Interacting effects of light and day and night temperatures on growth of 4 species in vegetative phase. *Annals of Botany*, 39, 733-743.

Richardson, A.D., Duigan, S.P. and Berlyn, G.P. (2002). An evaluation of noninvasive methods to estimate foliar chlorophyll content. *New Phytologist*, 153, 185-194.

Römer, C., Wahabzada, M., Ballvora, A., Pinto, F., Rossini, M., Panigada, C., Behmann, J., Léon, J., Thurau, C., Bauckhage, C., Kersting, K., Rascher, U. and Plümer, L. (2012). Early drought stress detection in cereals: simplex volume maximisation for hyperspectral image analysis. *Functional Plant Biology*, 39, 878-890.

Sadowski, C., Lenc, L., Lemanczyk, G. and Panka, D. (2009). Occurrence of *Fusarium* head blight of winter wheat (*Fusarium culmorum* - DON chemotype) in dependence on fungicidal protection programs. *Progress in Plant Protection*, 49, 1344-1348.

Scholes, J.D. and Rolfe, S.A. (2009). Chlorophyll fluorescence imaging as tool for understanding the impact of fungal diseases on plant performance: a phenomics perspective. *Functional Plant Biology*, 36, 880-892.

Sims, D.A. and Gamon, J.A. (2002). Relationship between pigment content and spectral reflectance across a wide range of species, leaf structures and developmental stages. *Remote Sensing of Environment*, 81, 337-354.

Stylinski, C.D., Gamon, J.A. and Oechel, W.C. (2002). Seasonal patterns of reflectance indices, carotenoid pigments and photosynthesis of evergreen chaparral species. *Oecologia*, 131, 366-374.

Teramura, A.H. and Murali, N.S. (1986). Intraspecific differences in growth and yield of soybean exposed to ultraviolet-B radiation under greenhouse and field conditions. *Environmental and Experimental Botany*, 26, 89-95.

Thenkabail, P., Smith, R. and Pauw, E. (2000). Hyperspectral vegetation indices and their relationships with agricultural crop characteristics. *Remote Sensing of Environment*, 71, 158-182.

Walter, H. (2011). New fungicides and new modes of actions. In: Dehne, H.-W., Deising, H.B., Gisi, U., Kuck, K.H., Russell, P.E. and Lyr, H., eds., Modern Fungicides and Antifungal Compounds VI, Proceedings of the 16th International Reinhardsbrunn Symposium, 25-29 April 2010, Friedrichroda, Germany, Lebenshilfe gGmbH, Braunschweig, pp. 47-54.

CHAPTER 5: EFFECTS OF FUNGICIDES ON WHEAT TOLERANCE TO WATER DEFICIT

ABSTRACT

A series of experiments was conducted under greenhouse conditions, in order to determine the effects of fungicides on the tolerance of wheat to two water deficit conditions. Additionally, the potential of non-invasive methods, such as thermography and spectral reflectance, to determine the effects of fungicide application on plant tolerance to water deficit were assessed. Drought stress severely affected plant development; however, the application of bixafen and prothioconazole had a positive effect on plant tolerance to this stress condition. Specifically, plants treated with bixafen were more tolerant to drought compared to the untreated control. Moreover, plants treated with this active ingredient were found to be more tolerant to both water deficit conditions compared to the other fungicide treatments.

Plant surface temperature and plant reflectance were suitable parameters for detecting the effect of fungicide application on wheat tolerance to water deficit. The use of infrared thermography and plant reflectance allowed the quantification of differences in plant vitality between fungicide treatments. IR thermography proved to be a suitable technique for assessing the effect of drought on transpiration of wheat as affected by various fungicides. Furthermore, changes in the reflectance of plants treated with different fungicidal groups were detected. Thus, it was possible to demonstrate the potential of non-invasive techniques to evaluate the effects of fungicides on the tolerance of wheat plants to adverse conditions such as drought.

Keywords: Leaf reflectance, IR thermography, Spectral vegetation indices, stress tolerance.

1. INTRODUCTION

Abiotic and biotic stresses are the main limiting factors affecting crop production around the world (Boyer, 1982). Among the various types of abiotic stresses, water deficit generates main yield losses in intensive wheat production (Dash and Mohanty, 2001; Rampino *et al*., 2012). A rise in the global average temperature and a diminution of the precipitation due to climate change have resulted in a reduction in the supply of natural water sources, which have caused a decline in the amount of water available for crop

irrigation over the last years (Habash *et al*., 2009). Several alternatives to counteract the low water availability for crops have been implemented. The use of drought tolerant varieties and irrigation systems are the most common strategies applied worldwide to maximize the water use efficiency (Passioura, 2004). However, it is necessary to advance research to explore new and diverse alternatives to counter the negative effects caused by drought conditions.

Apart from the fungicidal effect, the application of various active ingredients induced physiological changes in wheat plants (Nason *et al*., 2007). One reported effect is the substantial increase of plant tolerance to drought. Application of fungicidal compounds belonging to the strobilurin and azole groups resulted in an increment of plant tolerance to different environmental stresses (Wu and Von Tiedemann, 2002; Jaleel *et al*., 2006). For example, foliar application of pyraclostrobin resulted in a reduction of the ethylene biosynthesis in treated wheat shoots after short-term drought stress (Jabs *et al*., 2002). Similarly, horse chestnut trees (*Aesculus hippocastanum*) treated with either epoxiconazole, propiconazole, penconazole or paclobutrazol were more tolerant to drought stress than untreated plants (Percival and Noviss, 2008). Triazole treated trees had higher photosynthetic rates, total foliar chlorophyll and proline concentration than untreated trees at the end of a three-week drought (Percival and Noviss, 2008). Another reported effect is an increase in the antioxidant enzymes activity produced by strobilurins or triazole application in plants exposed to stress conditions. Wu and Von Tiedeman (2002) reported that azoxystrobin application significantly increased the activity of the antioxidative enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT), protecting barley plants against injurious ozone doses. In contrast to untreated plants, cowpea plants treated with propiconazole were more resistant to the adverse effects of drought stress, and the activity of SOD, APX and CAT were reported to be higher in treated plants (Manivannan *et al*., 2007). Nevertheless, the measurement of enzymatic activity to reduce the oxidative stress produced by the reactive oxygen species (ROS) required the destruction of the tissue, which is laborious and time consuming. Therefore, this has generated interest to explore alternatives and methods to measure the influence of fungicide application on plant tolerance to abiotic stresses.

Thermography has been widely used to measure the damage caused by abiotic stresses in crop plants (Ayeneh *et al*., 2002; Jones *et al*., 2009) and to determine the effects caused by fungicide application on plant physiology (Berdugo *et al*., 2012). Canopy temperature is directly correlated to the evapotranspiration rate, which is affected by water deficit, making infrared sensing a suitable tool to measure the effects of drought conditions on the transpiration rate (Leinonen and Jones, 2004).

Another non-invasive method to monitor detrimental effects caused by abiotic stress

conditions on plant development is the assessment of spectral reflectance. Canopy reflectance is influenced by biophysical characteristics such as tissue structure and biochemical characteristics like chlorophyll content (Mahlein *et al*., 2012). One of the first indicators of plant water deficit stress is the reduction of the content of photosynthetic pigments (Pirzad *et al*., 2011). Therefore, by the use of spectrometry it will be possible to measure the effect of water stress conditions on plant vitality. Several studies have described the use of spectral reflectance to detect early damage caused by abiotic stresses (Sonmez *et al*., 2008).

One of the most popular methods to use information from spectral reflectance measurements is the calculation of spectral vegetation indices (SVIs), which are algorithms related to specific plant parameters such as plant vitality, senescence, pigment concentration, among others characteristics. They provide the possibility to determine specific changes in crop biomass or pigments content due to stress conditions (Thenkabail *et al*., 2000; Römer *et al*., 2012).

In order to determine the effect of fungicide application on the wheat tolerance to water deficit, three fungicide treatments (bixafen, fluoxastrobin and prothioconazole) were compared to untreated control plants. The potential of thermography and spectral reflectance to determine the effect of fungicides on wheat tolerance to water deficit was assessed in a series of experiments conducted under greenhouse conditions.

The main objectives of this chapter were to:

i. Determine the effects of fungicides on the tolerance of wheat to water deficit
ii. Investigate the response of wheat plants under two water deficit conditions
iii. Use of non-invasive techniques to detect the effects of water deficit on plant vitality.

2. MATERIALS AND METHODS

2.1. Plant material

Twenty seeds of wheat, cultivar Passat were sown in plastic pots (20 x 20 x 30 cm) 2 cm deep. Eight pots were used per treatment. The substrate consisted of a mixture of organic soil (Klasmann-Deilmann GmbH, Germany), sand and C horizon (12:6:2 v/v). Plants were grown at 24/20 °C (day/night), 70 ± 10% relative humidity (RH), photoperiod of 16 h d^{-1} with supplemental illumination (> 300 µmol m-2 s^{-1}, lamps Philips SGR 140, Philips, Hamburg, Germany). Plants were fertilized every two weeks with 400 ml of a 0.2% solution of a commercial N-P-K fertilizer (14-10-14, 2 g/l; Aglukon GmbH, Düsseldorf, Germany). Plants were carefully inspected in order to control possible fungal infections. According to the BBCH scale (Hack *et al*., 1992) all plants were treated at growth stage

(GS) 33 with the fungicide Talius® (active ingredient [a.i.] proquinazid, 200 g L^{-1}, DuPont, Neu-Isenburg, Germany) and the fungicide Vegas® (a.i. cyflufenamid, 51.3 g L^{-1}, Spiess-Urania, Hamburg, Germany) was applied at GS 65 to keep plants free from powdery mildew infections. To control insect pests, all plants were treated at GS 41 with the insecticide Sumicidin® (a.i. fenvalerate, 25 g L^{-1}, BASF, Limburgerhof, Germany) and at GS 71 with the insecticide Bulldock® (a.i. beta-cyfluthrin 125 g L^{-1}, Bayer CropScience, Monheim, Germany).

2.2. Fungicide treatments and water stress regimes

Three fungicide treatments were compared to an untreated control: bixafen (125 g a.i. L^{-1}), fluoxastrobin (100 g a.i. L^{-1}) and the mixture of bixafen and prothioconazole (Bix.+Prot. / 75 + 150 g a.i. L^{-1}). The active ingredients were supplied by Bayer CropScience, (Monheim, Germany). Fungicides were applied at recommended field rates (water 300 L ha^{-1}). Plants were treated twice, first at GS 39 and the second fungicide application was conducted at GS 59. A CO_2 pressurized hand-sprayer (Meisterwerkzeuge, Wuppertal, Germany) with an adjustable spray was used for fungicide application. To avoid contamination between treatments plants were separated and treated far away from each other. After fungicide application plants were randomized into the greenhouse.

Wheat plants were subjected to two different water stress regimes. Control plants without drought stress were watered once per day (400 ml/pot) in order to maintain soil water content favorable for plant growth (approx. 55 - 85%). Plants under drought stress conditions received half the amount of water compared to plants under no stress. The long-term drought stress condition started just after the first fungicide application (GS 39), and the short-term stress condition started after the second fungicide application (GS 59).

2.3. Plant height and yield parameters

Plant development parameters were assessed throughout the experiments. Plant height was measured at GS 62 from the base of the stem to the tip of the ear. The average of three measurements per pot was the value of the replicate. Eight replicates were used per treatment. At the end of the cycle, yield parameters were measured. The total number of ears per pot was counted and the single ear weight was calculated. At harvest the straw was weighed per pot. Eight replicates were measured per treatment.

2.4. Measurement of plant reflectance

At growth stage 65, 73, 81 and 85 plant reflectance was measured with a non-imaging spectroradiometer (ASD FieldSpecPro FR spectrometer, Analytic Spectral Devices, Boulder, USA). A pistol grip foreoptic was mounted on a tripod, 1 m above the plant

canopy. The measurements of the wheat canopy were conducted between 9 pm and 12 am. During the measurements plants were constantly illuminated by six ASD-Pro-Lamps (Analytic Spectral Devices (ASD), Boulder, USA). Reflectance data of the wheat plants were assessed at an average of 15 spectra per sample. In each treatment, spectra from five pots and three measurements per pot were taken. Mean reflectance per pot was used for data analysis.

The spectral range of the instrument is from 350 to 1100 nm. Since the reflectance spectra were noisy at the extremes, only values from 400 to 1000 nm were included in data analysis. The instrument was warmed up 90 min before measurements were taken to ensure high quality and homogeneity of the reflectance data. A barium sulphate white reference was used for reflectance normalization. The white reference was placed under the pistol grip at the same level as the wheat canopy.

Five spectral vegetation indices (SVIs) related to plant vitality and pigments content were calculated to categorize the effects of fungicide application on wheat tolerance to water deficit (Tab. 5.1).

Table 5.1: Spectral vegetation indices and algorithms used in this study.

Index	Equation[a]	Related to	Reference
Normalized difference vegetation index	NDVI= $(R_{800}-R_{670})/(R_{800}+R_{670})$	Biomass, plant vitality	Rouse *et al*., (1974)
Water index	WI= R_{900} / R_{970}	Water content	Peñuelas *et al*., (1997)
Pigment specific simple ratio	PSSRa= R_{800} / R_{680}	Chlorophyll a content	Blackburn, (1998)
	PSSRb= R_{800} / R_{635}	Chlorophyll b content	
	PSSRc= R_{800} / R_{470}	Carotenoid content	

[a] Reflectance at wavelengths indicated

2.5. IR thermography

Thermographic images were taken using an infrared scanning camera (VarioCAM® hr head 500T, InfraTec GmbH, Dresden, Germany) with the following characteristics: spectral sensitivity 7.5 to 14 µm, geometric resolution of 768 x 576 pixels, temperature measuring range -40°C to 1200 °C. For thermographic measurements plants were arranged in two different ways. In order to measure absolute temperature, pictures from every replicate from each treatment were taken; eight replicates were used per treatment. To compare the treatments in the same image, four replicates from each treatment were

arranged together and the pictures were taken one meter and a half above from the plants canopy. Plant temperature was measured at four growth stages: GS 65, GS 73, GS 81 and GS 85. Measurements were conducted between 5:00 pm and 7:00 pm in order to avoid physiological and environmental changes among measurements. Images were processed using the software package IRBIS 3 (InfraTec, Dresden, Germany). The temperature of leaves and ears were analyzed independently of each other.

2.6. Statistical analysis

Data were analyzed using the statistical program SPSS statistics for Windows (IBM Deutschland GmbH, Ehningen, Germany), version 20.0. Data were tested for normal distribution and equality of variances. The data were examined using analysis of variance (ANOVA) and the standard errors (SE) of the means were calculated. The means were compared using Tukey test at 95% confidence in order to separate subgroups.

3. RESULTS

3.1. Plant development and yield parameters

Plant height was severely affected by the long-term water stress condition. The long-term stress condition reduced plant height by 11% on average for all treatments in comparison to plants cultivated under the no stress condition (Tab. 5.2). However, no significant differences in plant height were calculated between treatments. Furthermore, fungicide application did not influence this parameter even under the no stress condition. The number of ears per pot was reduced by both drought conditions. This reduction was more severe when drought stress started at GS 39 (long-term stress). However, no significant differences were calculated between treatments regarding the number of ears per pot.

The ear weight was affected by stress conditions; it was reduced on average for all treatments by 21% by the short-term drought stress and 29% by the long-term drought stress condition.

At harvest, the straw weight was measured; under no stress condition, no significant differences were calculated between treatments. However, all fungicide treatments had higher straw weight than the untreated control. In contrast, under both drought stress conditions significant differences were calculated between treatments. Application of bixafen and Bix.+Prot. resulted in a higher plant biomass compared to the untreated and fluoxastrobin treatments (Tab. 5.2). On average, the short-term stress reduced the straw weight by 23% compared to normal water conditions. Under the long-term stress condition, the reduction was 42%.

Table 5.2: Effect of fungicide treatments on wheat development parameters under different water supply conditions.

Water regime	Fungicide Treatment	Plant height [cm]	No. of ears per pot	Ears weight [g/pot]	Straw weight [g/pot]
No stress	Untreated	55.3 a	42.0 a	30.7 a	60.7 a
	Bixafen	56.3 a	40.7 a	31.0 a	66.4 a
	Fluoxastrobin	56.2 a	42.4 a	29.0 a	66.6 a
	Bix.+Prot.	54.1 a	38.6 a	28.6 a	72.3 a
Short-term stress	Untreated	55.6 a	34.1 a	23.4 a	49.1 a
	Bixafen	55.6 a	34.5 a	25.4 a	55.2 b
	Fluoxastrobin	55.8 a	32.0 a	22.6 a	47.6 a
	Bix.+Prot.	55.7 a	34.1 a	22.6 a	52.3 ab
Long-term stress	Untreated	49.7 a	28.7 a	20.4 a	37.5 ab
	Bixafen	49.3 a	31.5 a	23.8 a	40.9 b
	Fluoxastrobin	49.7 a	27.2 a	20.2 a	35.3 a
	Bix.+Prot.	49.4 a	27.4 a	20.9 a	40.5 b

Values with same letters within the same water supply condition do not differ significantly according to Tukey´s test ($p \leq 0.05$; $n = 8$).

3.2. Plant reflectance

At the first measurement (GS 65) the reflectance spectra of wheat for all water supply conditions were similar for all fungicide treatments (Fig. 5.1). However, plants under long-term stress showed higher reflectance in the visible (VIS) range compared to plants under the other two water conditions at this growth stage.

At GS 73, first differences in plant reflectance between plants under no stress and short-term stress conditions were measured. The reflectance of plants under short-term stress conditions was higher in the VIS range and in the near infrared (NIR) compared to the reflectance of plants under normal water conditions. Comparable to the first measurement date, plants under long-term stress had higher reflectance compared to plants under no stress and short-term stress conditions. Bixafen and Bix.+Prot. treated plants had lower reflectance in the green peak compared to the other treatments for both water deficit regimes.

At the third measuring date (GS 81), higher reflectance in the range from 500 to 700 nm was measured for untreated and fluoxastrobin treatments compared to bixafen and Bix.+Prot. treatments. In the case of short-term stress, bixafen treated plants had lower reflectance at the VIS and at the NIR compared to the other treatments. Under long-term stress conditions application of Bix.+Prot. resulted in a lower reflectance at the green peak (550 nm) compared to the remaining treatments.

At the last measurement date (GS 85), the reflectance in the VIS was lower for plants treated with bixafen and Bix.+Prot. under no stress conditions compared to the untreated

control. Application of fluoxastrobin resulted in a spectrum similar to the untreated control. Under the short-term stress condition, the bixafen treatment was the only treatment with lower reflectance in the VIS than the untreated control. Plants under long-term stress conditions treated with bixafen and Bix.+Prot. had a lower reflectance in the VIS and higher reflectance in the NIR compared to the untreated and fluoxastrobin treatments. In general the plant reflectance for all treatments was lower under the no stress condition than under both stress conditions (Fig. 5.1).

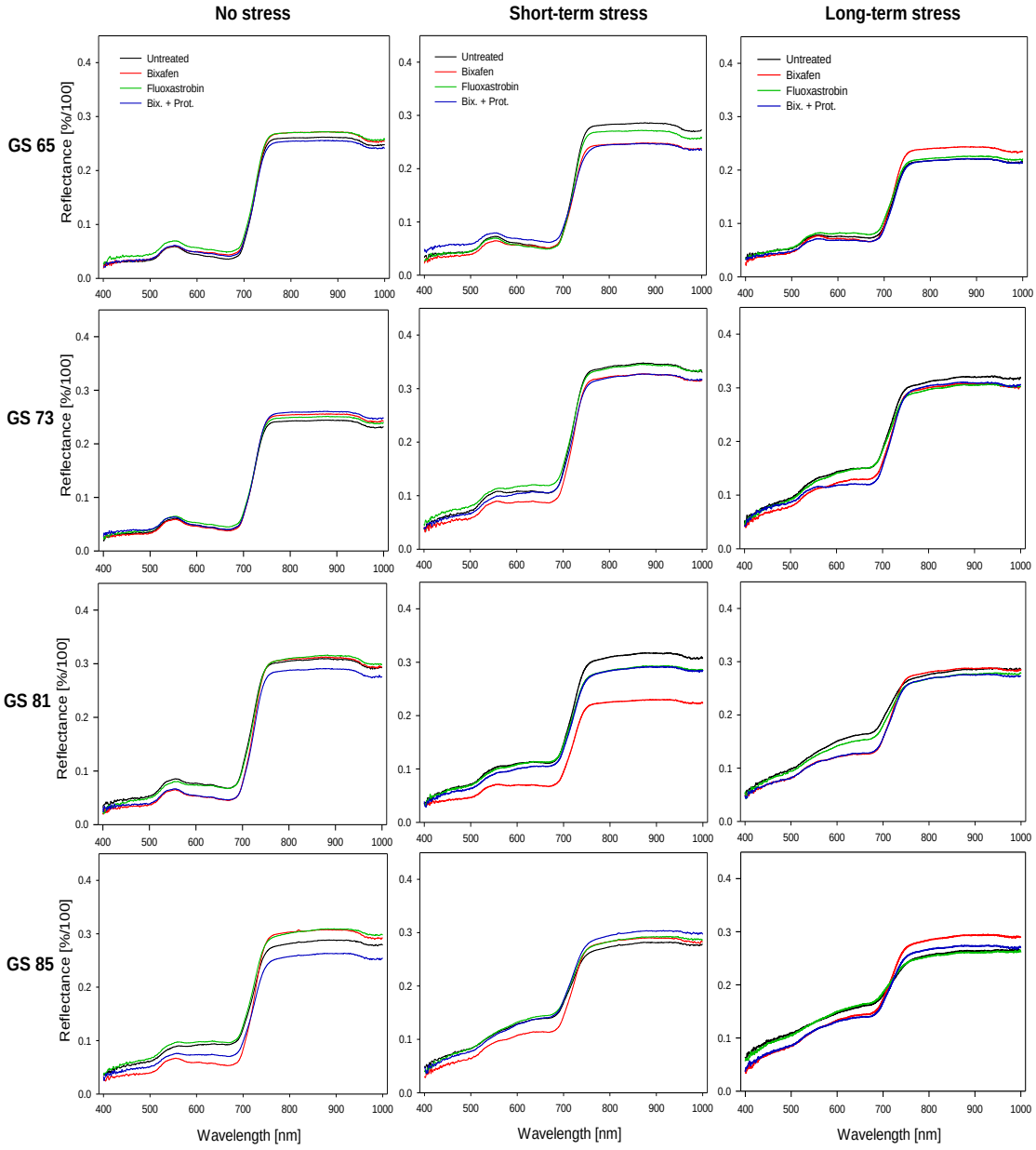

Figure 5.1: Spectral reflectance of fungicide treated and untreated wheat plants under different drought stress conditions, measured four times during the growth period (GS 65, GS 73, GS 81 and GS 85).

The first significant differences between the fungicide treatments and the untreated control were detected at GS 81 (Fig. 5.2). Under all water conditions, bixafen treated plants had

significantly lower reflectance from 500 to 700 nm than the untreated control. In the case of the fluoxastrobin treatments, no significant differences were calculated at this growth stage with respect to the untreated control. Plants treated with Bix.+Prot. had lower reflectance than the untreated control. However, significant differences in the spectrum were calculated under no stress conditions; plants treated with Bix.+Prot. had significantly lower reflectance from 500 to 700 nm than the untreated control.

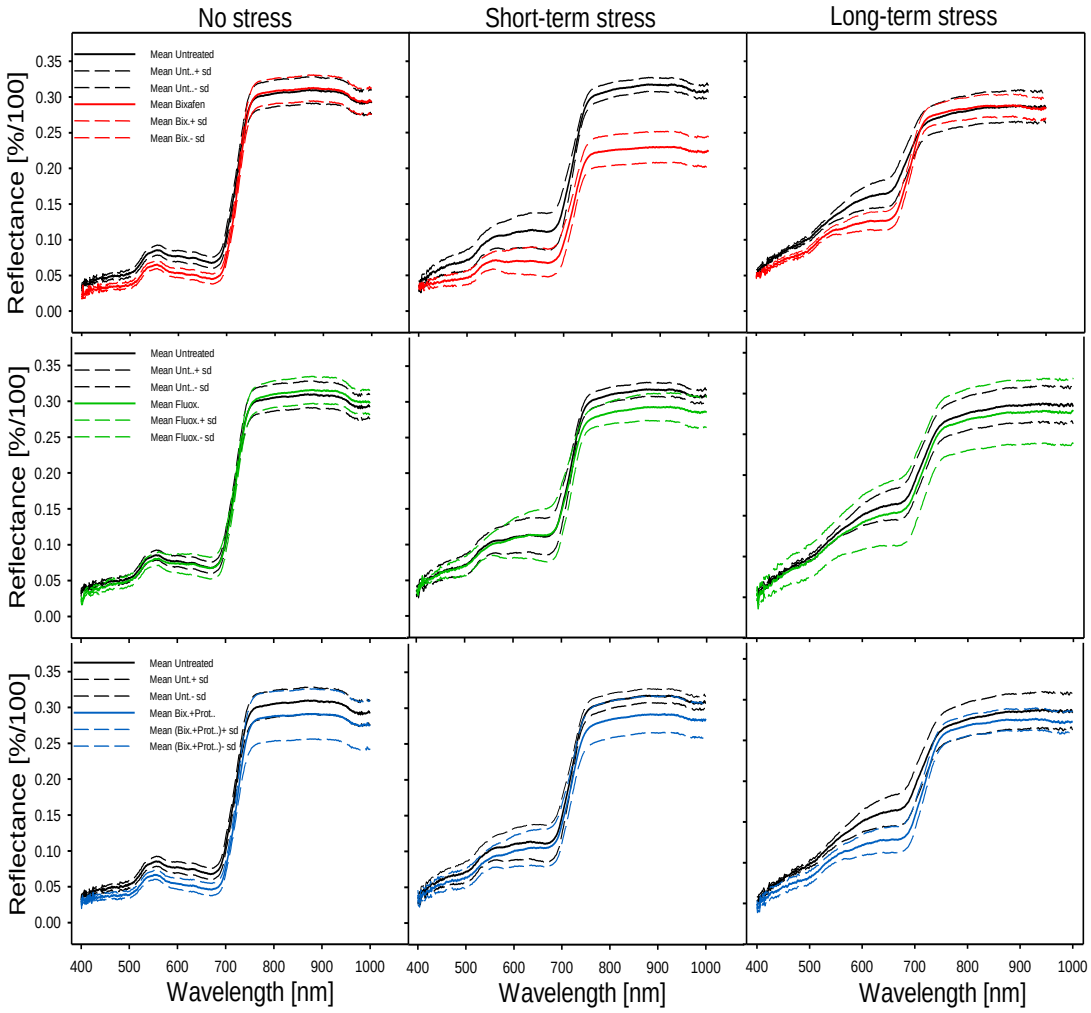

Figure 5.2: Spectral signatures together with the standard deviation (sd) of fungicide treated and untreated plants under different water supply conditions, measured at GS 81.

By plotting the standard deviation, it was possible to observe different trends in the variability of the spectra within treatments (Fig. 5.3). Under no stress conditions the higher variability occurring within the treatments was calculated in the near infrared range. In contrast, the standard deviation under stress conditions was high from 500 to 700 nm and the variability of reflectance within treatments increased with time.

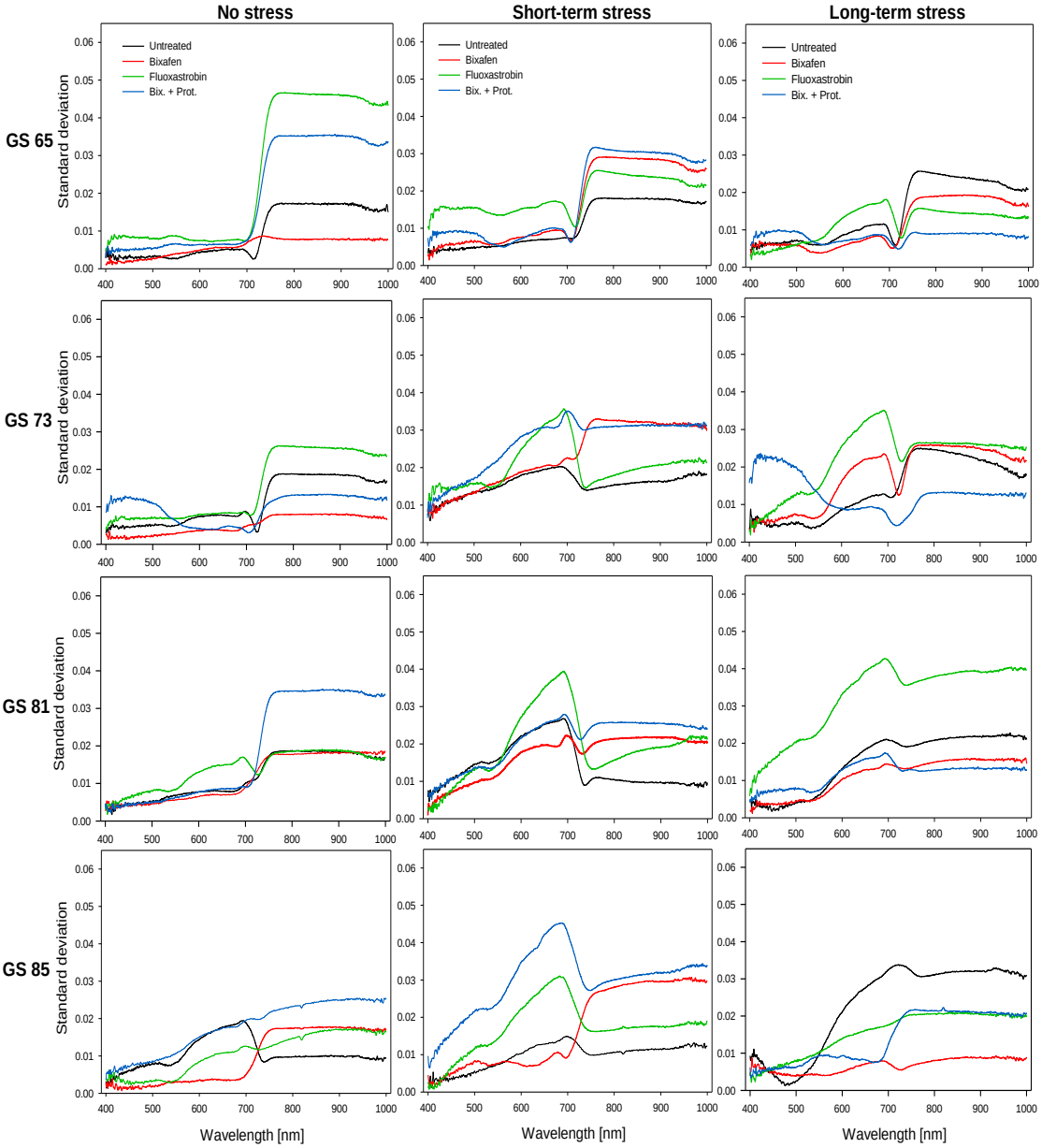

Figure 5.3: Standard deviation of the spectral reflectance of fungicide treated and untreated wheat plants under different water supply conditions, measured four times during the growth period (GS 65, GS 73, GS 81 and GS 85).

3.3. Spectral vegetation indices

The normalized difference vegetation index (NDVI) was calculated as an indicator of plant vitality. At the first two measurement times, no significant differences were observed between treatments under all water supply conditions (Fig. 5.4). At GS 81 and GS 85 bixafen application resulted in significant differences in the NDVI values compared to the untreated control. Furthermore, plants subjected to the stress conditions had lower NDVI values than plants grown under no stress conditions (Fig. 5.4).

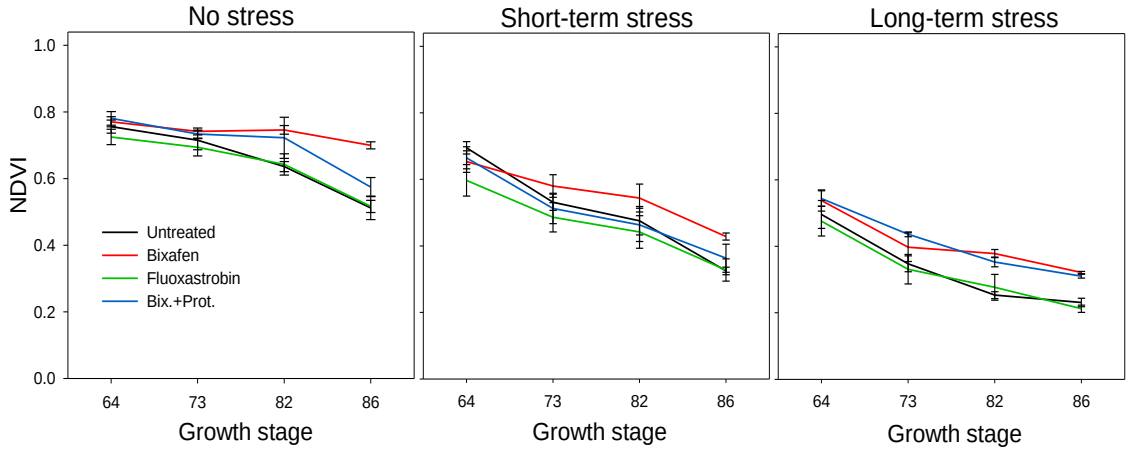

Figure 5.4: Effect of fungicide treatments on the normalized difference vegetation index (NDVI) of wheat plants under normal water supply conditions, short-term and long-term water stress.

Four spectral vegetation indices related to water and pigments content were calculated from the reflectance curves at every measuring date.

For all water supply conditions, no significant differences were calculated at the first two measurement times (Tab. 5.3). Under no stress conditions, significant differences were detected in PSSRa, PSSRb and PSSRc at GS 81. Bixafen and Bix.+Prot. treatments were significantly different to the untreated and fluoxastrobin treatments. Under long-term stress conditions significant differences were calculated for all vegetation indices (Tab. 5.3). At the last measurement time, significant differences were calculated for the majority of the indices under all water supply conditions. However, for WI, no significant differences were calculated under the short stress condition.

Table 5.3: Effect of fungicide application at GS 39 and at GS 59 on spectral vegetation indices of wheat plants under three water supply conditions; no stress (NS), short-term stress (SS) and long-term stress (LS).

Index	Treatment	Growth stage											
		65			73			81			85		
		NS	SS	LS	NS	SS	LS	NS	SS	LS	NS	SS	LS
WI	Untreated	0.95 a	0.95 a	0.98 a	0.95 a	0.97 a	0.99 a	0.95 a	0.98 a	1.00 b	0.97 b	0.99 a	1.01 b
	Bixafen	0.94 a	0.96 a	0.97 a	0.95 a	0.97 a	0.99 a	0.95 a	0.97 a	0.99 a	0.95 a	0.98 a	0.99 a
	Fluoxastrobin	0.95 a	0.96 a	0.97 a	0.95 a	0.97 a	1.00 a	0.96 a	0.97 a	1.00 b	0.97 b	0.99 a	1.00 b
	Bix. + Prot.	0.94 a	0.96 a	0.97 a	0.95 a	0.97 a	0.98 a	0.95 a	0.97 a	0.99 a	0.96 ab	0.99 a	0.99 a
PSSRa	Untreated	6.99 a	5.35 a	2.98 a	5.88 a	3.20 a	2.03 a	4.35 a	2.81 a	1.65 a	3.20 a	1.92 a	1.57 a
	Bixafen	7.35 a	4.66 a	3.27 a	6.39 a	3.70 a	2.33 a	6.59 c	3.40 a	2.17 c	5.47 b	2.44 b	1.90 b
	Fluoxastrobin	6.14 a	4.05 a	2.84 a	5.38 a	2.92 a	1.99 a	4.62 ab	2.61 a	1.76 ab	3.06 a	1.95 ab	1.51 a
	Bix. + Prot.	7.81 a	4.96 a	3.32 a	6.16 a	3.14 a	2.48 a	6.15 bc	2.79 a	2.06 bc	3.65 a	2.12 ab	1.86 b
PSSRb	Untreated	6.63 a	5.10 a	2.94 a	5.66 a	3.20 a	2.09 a	4.13 a	2.82 a	1.72 a	3.21 a	2.01 a	1.65 a
	Bixafen	6.92 a	4.54 a	3.20 a	6.01 a	3.72 a	2.39 a	6.21 c	3.40 a	2.11 bc	5.29 b	2.48 b	1.93 b
	Fluoxastrobin	5.92 a	3.86 a	2.79 a	5.23 a	2.93 a	2.08 a	4.40 ab	2.63 a	1.84 ab	3.06 a	2.04 ab	1.59 a
	Bix. + Prot.	7.39 a	4.72 a	3.25 a	5.97 a	3.18 a	2.53 a	5.70 bc	2.85 a	2.22 c	3.58 a	2.23 ab	1.98 b
PSSRc	Untreated	7.84 a	6.50 a	4.09 a	7.20 a	4.90 a	3.31 a	5.82 a	4.46 a	2.80 a	4.68 a	3.29 a	2.35 a
	Bixafen	8.63 a	6.47 a	4.67 a	7.77 a	5.88 a	3.85 a	8.30 c	4.97 a	3.43 b	7.62 b	4.43 b	3.28 b
	Fluoxastrobin	6.92 a	5.14 a	4.11 a	6.66 a	4.27 a	3.27 a	6.33 ab	4.13 a	2.89 a	4.53 a	3.44 a	2.39 a
	Bix. + Prot.	8.58 a	6.01 a	4.40 a	6.79 a	5.18 a	3.60 a	7.57 bc	4.68 a	3.29 ab	5.20 a	3.93 ab	3.11 b

Values with same letters within the same index do not differ significantly according to Tukey´s test ($p \leq 0.05$; $n = 5$). WI water index, PSSR pigment specific simple ratio (PSSRa, chlorophyll a; PSSRb, chlorophyll b and PSSRc, carotenoids).

3.4. Plant surface temperature

At the first measuring date (GS 65), no significant differences in terms of ear and leaf temperature were observed between treatments under all water supply conditions (Fig. 5.5).

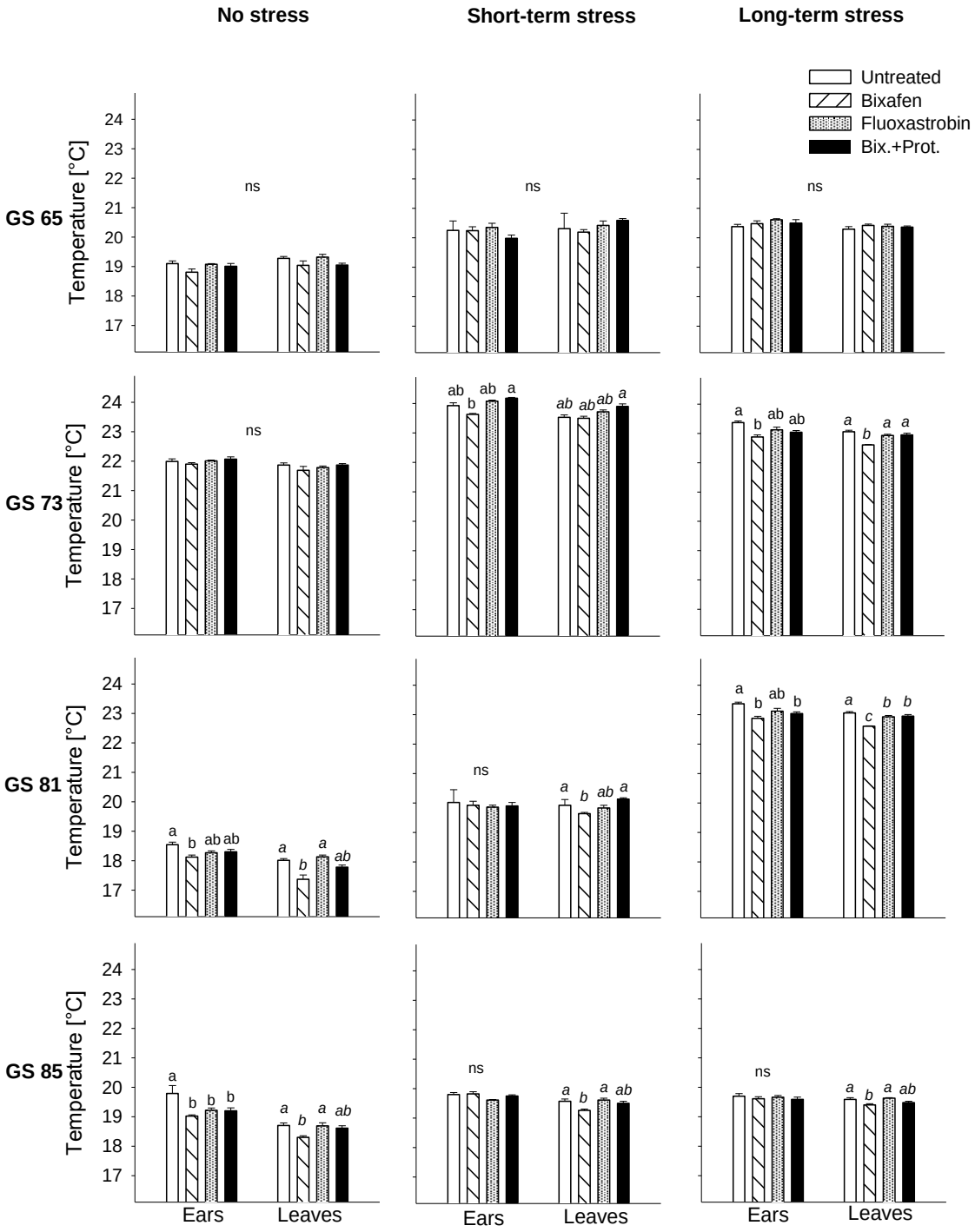

Figure 5.5: Ear and leaf temperature of wheat plants under no drought stress, short-term and long-term drought stress. Vertical bars indicate SE. Different letters indicate significant differences according to Tukey's test ($p \leq 0.05$; $n = 8$); ns, no significant difference.

At GS 73, thermographs showed that plants treated under normal water supply conditions had lower surface temperatures than those plants experiencing stress conditions (Fig. 5.6). Ear and leaf temperatures were no significant different among plants under the no stress condition; in contrast, for both stress conditions, the first significant differences between treatments were observed.

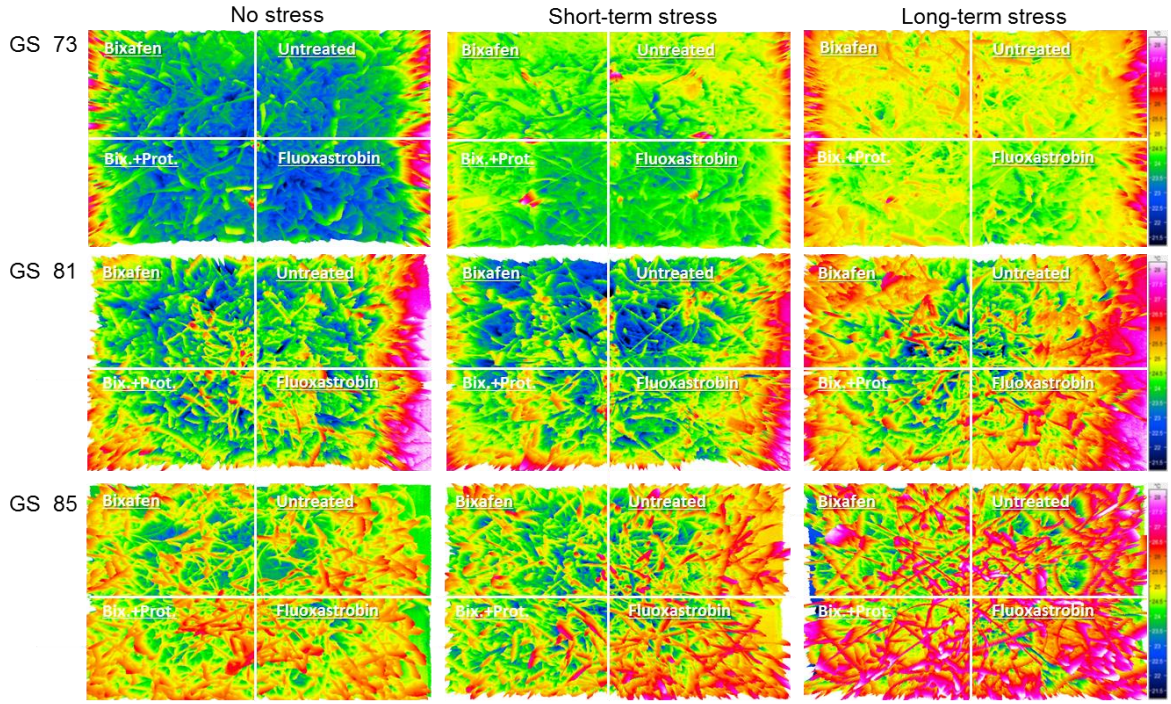

Figure 5.6: Effect of fungicide application on wheat canopy temperature, thermograms at GS 73, GS 81 and GS 85 of untreated plants and fungicide treated plants under three different drought stress conditions.

At GS 81, fungicide application resulted in lower ear temperatures compared to the untreated plants. Bixafen treated plants had a significantly lower leaf temperature than the untreated control (Fig. 5.5).

At the last measurement time (GS 85), plants subjected to drought were more senescent compared to plants under normal water supply, which was reflected in the canopy temperature (Fig. 5.6). No significant differences in ear temperature were calculated between treatments under both stress conditions. Under the no stress condition, all fungicide treatments had significantly lower ear temperatures compared to the untreated control. Bixafen treated plants had a significantly lower leaf temperature relative to the untreated control for all water supply conditions.

The surface temperature under no stress conditions was always lower than the temperature of wheat under drought. For plants under long-term stress conditions, the temperature differences with respect to the untreated control were higher compared to the other two water regimes.

4. DISCUSSION

Plants grown under long-term water deficit stress completed their life cycle faster than plants under normal water supply and short-term water stress conditions. It resulted in a shortening of the duration of every growth stage, and as a consequence it generated a decrement in the total plant biomass. It was possible to observe how wheat susceptibility to drought conditions was influenced by the phenological stage during which the stress began.

Plants growing under water deficit had lower plant height and total biomass than plants growing under the normal water supply condition. The negative impact on the total biomass weight and plant height was more severe when plants were subjected to a longer water deficit period. This is supported by previous studies conducted either under greenhouse or field conditions, where the negative effect of drought on the total plant biomass was observed and quantified (Pan *et al*., 2003; McMaster and Wilhelm, 2003; Sibel and Birol, 2007).

The reduction of plant biomass caused by water deficit might be explained by the fact that in these plants the abscisic acid level increases generating a reduction in shoot elongation; this is a plant strategy to counteract the negative effects caused by the stress condition (Spollen *et al*., 2000). Wheat plants treated with bixafen and Bix.+Prot. were less affected by drought in terms of plant biomass than the untreated control. These results confirm previous reports concerning how triazole application ameliorated the negative effects of water shortage on plant growth (Percival *et al*., 2008; Hojati *et al*., 2011). Hassanpour *et al*. (2012) reported that penconazole application on *M. pulegium* alleviates the negative effects caused by drought stress conditions. There are also reports about the increment of plant tolerance to stress conditions produced by bixafen application. Barley plants treated with bixafen had higher stress-tolerance reflected in a higher survival rate compared to untreated plants exposed to drought stress (Suty-Heinze *et al*., 2011).

The water deficit severely reduced the ear weight compared to plants growing with an appropriate water supply; however, the reduction produced by the long-term stress varied between treatments. For untreated plants this reduction was 34%, whereas, for plants treated with bixafen and Bix.+Prot. it was 23% and 27% respectively. These two active ingredients increased the tolerance of wheat to drought stress.

The negative effect of water shortage on plant vitality was detected at an early stage by reflectance measurements of wheat plants. Already at GS 65 it was possible to observe a higher reflectance in the VIS region (low leaf absorption of light by leaf pigments) of plants exposed to water stress. Under drought stress the reflectance increased in the range linked to light absorption by leaf pigments. This difference was more noticeable in plants

subjected to long-term stress. Water deficit generates a degradation of the photosynthetic pigments in plants that resulted in a transient decrease of the photochemical efficiency (Shao *et al*., 2008). Behera *et al*. (2002) reported that the chlorophyll (a+b) loss was faster in wheat leaves exposed to moderate water stress conditions compared to the control. As plant reflectance in the VIS is mainly related to the pigment concentration (Carter and Knapp, 2001), the decline of the pigment content caused by water deficit was clearly detected in this study by the measurement of the canopy reflectance, as well as by the chlorophyll related spectral vegetation indices.

NDVI is one of the most frequently used vegetation index in remote sensing to assess plant vitality (Peñuelas *et al*., 1993; Krumov *et al*., 2008). Plants growing under water stress conditions had lower NDVI values compared to plants growing under normal water supply. Moreover, at later stages plants treated either with bixafen or Bix.+Prot. had higher NDVI values compared to the other treatments. It provided an indication of higher vitality of plants treated with these two fungicidal compounds under both water deficit conditions.

The water index (WI) is a useful parameter to estimate water stress in plants (Peñuelas *et al*., 1997). WI has been used successfully to measure plant growth under water deficit conditions (Serrano *et al*., 2000; Claudio *et al*., 2006). In this study the calculation of the WI was an accurate measurement to establish differences between treatments in terms of water content. Already at GS 81, significant differences were calculated between treatments; bixafen and Bix.+Prot. treated plants were significantly different to the other treatments under long-term stress. It was an indication of the higher tolerance to water deficit for plants treated with these two compounds compared to the untreated plants.

The PSSRc demonstrated significant differences between treatments at GS 81. This index is correlated to the carotenoid concentration of plant tissue. Fungicide treatments had higher PSSRc values compared to the untreated control. Carotenoids are regarded as very important pigments for photosynthesis; however, this class of pigments also plays a valuable role in generating plant tolerance towards stress conditions (Strzalka *et al*., 2003). Several studies demonstrated the efficiency of carotenoids to reduce the activity of reactive oxygen species thus protecting pigments from oxidative damage (Conn *et al*., 1991; Edge *et al*., 1997).

Thermography has been used successfully in previous studies to assess the effect of fungicides on plant tolerance to water deficit (Inagaki *et al*., 2009). They reported that by the employment of thermography it was feasible to establish differences in leaf temperature between pyraclostrobin treated and untreated plants growing under increasing water deficit conditions. The average canopy temperature of plants treated with pyraclostrobin was lower than the untreated plants. Previous studies also demonstrated

the effectiveness of IR thermography to detect water stress in plants (Gonzalez-Dugo *et al*., 2006; Wang *et al*., 2010).

Digital infrared thermography was an effective technique to measure significant differences in surface temperature between treatments for all water supply conditions. Specifically, this method enabled the detection of differences in the response of plants treated with different fungicidal compounds to water deficit. Already at GS 73, it was possible to detect significant differences in the canopy temperature between treatments. These findings corroborate that thermography can provide an early detection of changes in the plant surface temperature influenced by the canopy water status (Jones *et al*., 2002). Use of thermography also made it possible to detect the higher tolerance of plants treated with bixafen compared to the untreated control under both water deficit conditions.

Remote sensing techniques have been used before to optimize the application of fungicides to control pathogens (Nicholas, 2004; Dammer *et al*., 2008). This research also served to demonstrate the potential of remote sensing methods to evaluate the effects of fungicides on the tolerance of crop plants to adverse conditions such as water deficit.

5. CONCLUSION

This study demonstrated that the application of some fungicidal groups had a positive effect on the wheat tolerance to water deficit stress. Plants treated with the pyrazole carboxamide bixafen were more tolerant to water shortage compared to the other treatments. These conclusions are based on the assessment of some plant developmental parameters and as well by the measurement of the canopy reflectance and temperature. Furthermore, the use of sensors revealed differences in the tolerance to water deficit between the untreated control and the fungicide treatments, demonstrating its utility. The employment of spectral reflectance and thermography revealed significant differences in terms of plant vitality, and both proved to be accurate methods that can establish and quantify the potential effects of fungicides on the tolerance of wheat to water deficit. Likewise, these two methods provided sensitive detections of differences between water stress conditions and treatments.

6. REFERENCES

Ayeneh, A., van Ginkel, A., Reynolds, M.P. and Ammar, K. (2002). Comparison of leaf, spike, peduncle and canopy temperature depression in wheat under heat stress. *Field Crops Research*, 79, 173-184.

Behera, R.K., Mishra, P.C. and Choudhury, N.K. (2002). High irradiance and water stress induce alterations in pigment composition and chloroplast activities of primary wheat leaves. *Journal of Plant Physiology*, 159, 967-973.

Berdugo, C.A., Steiner, U., Dehne, H.-W. and Oerke, E.-C. (2012). Effect of bixafen on senescence and yield formation of wheat. *Pesticide Biochemistry and Physiology*, 104, 171-177.

Blackburn, G.A. (1998). Quantifying chlorophylls and carotenoids at leaf and canopy scale: an evaluation of some hyperspectral approaches. *Remote Sensing of Environment*, 66, 273-285.

Boyer, J.S. (1982). Plant productivity and environment. *Science*, 218, 443-448.

Carter, G.A. and Knapp, A.K. (2001). Leaf optical properties in higher plants: linking spectral characteristics to stress and chlorophyll concentration. *American Journal of Botany*, 88, 677-684.

Claudio, H., Cheng, Y., Fuentes, D., Gamon, J., Luo, H. and Oechel, W. (2006). Monitoring drought effects on vegetation water content and fluxes in chaparral with the 970nm water band index. *Remote Sensing of Environment*, 103, 304-311.

Conn, P., Schalch, W. and Truscott, T.G. (1991). The single oxygen and carotenoid interaction. *Journal of Photochemistry and Photobiology B: Biology*, 11, 41-47.

Dammer, K.H., Wollny, J. and Giebel, A. (2008). Estimation of the leaf area index in cereal crops for variable rate fungicide. *European Journal of Agronomy*, 28, 351-360.

Dash, S. and Mohanty, N. (2001). Evaluation of assays for the analysis of thermo-tolerance and recovery potentials of seedlings of wheat (*Triticum aestivum* L.) cultivars. *Journal of Plant Physiology*, 158, 1153-1165.

Edge, R., McGarvey, D.J. and Truscott, T.G. (1997). The carotenoids as antioxidants - A review. *Journal of Photochemistry and Photobiology B: Biology*, 41, 189-200.

Gonzalez-Dugo, M.P., Moran, M.S., Mateos, L. and Bryant, R. (2006). Canopy temperature variability as an indicator of crop water stress severity. *Irrigation Science*, 24, 233-240.

Habash**,** D.Z., Kehel, Z. and Nachit, M. (2009). Genomic approaches for designing durum wheat ready for climate change with a focus on drought. Journal of Experimental Botany, 60, 2805-2815.

Hack, H., Bleiholder, H., Buhr, L., Meier, U., Schnock-Fricke, U., Weber, E. and Witzenberger, A. (1992). Einheitliche Codierung der phänologischen Entwicklungsstadien mono- und dikotyler pflanzen – Erweiterte BBCH-Skala, Allgemein. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes*, 44, 265-270.

Hassanpour, H., Khavari-Nejad, R.A., Niknam, V., Najafi, F. and Razavi, K. (2012). Effects of penconazole and water deficit stress on physiological and antioxidative responses in pennyroyal (*Mentha pulegium* L.). *Acta Physiologiae Plantarum*, 34, 1537-1549.

Hojati, M., Modarres-Sanavy, S.A.M., Ghanati, F. and Panahi, M. (2011). Hexaconazole induces antioxidant protection and apigenin-7-glucoside accumulation in *Matricaria chamomilla* plants subjected to drought stress. *Journal of Plant Physiology*, 168, 782-791.

Inagaki, M., Mori, M. and Nachit, M.M. (2009). Effect of a strobilurin-class fungicide on water use in synthetic bread wheat genotypes grown under increasing water deficit conditions. *Cereal Research Communications*, 37, 513-519.

Jabs, T., Pfirrmann, J., Schäfer, S., Wu, Y. and Tiedemann, AV. (2002). Anti-oxidative and anti-senescence effects of the strobilurin pyraclostrobin in plants: A new strategy to cope with environmental stress in cereals. In: Proceedings of the BCPC Conference - Pest and Diseases, 18-21 November 2002, Brighton, UK, pp. 941-946.

Jaleel, C.A., Gopi, R., Alagu Lakshmanan, G.M. and R. Panneerselvam. (2006). Triadimefon induced changes in the antioxidant metabolism and ajmalicine production in *Catharanthus roseus* (L.) G. Don. *Plant Science*, 171, 271-276.

Jones, H.G., Stoll, M., Santos, T., de Sousa, C., Chaves, M.M. and Grant, O.M. (2002). Use of infrared thermography for monitoring stomatal closure in the field: application to grapevine. *Journal of Experimental Botany*, 53, 2249-2260.

Jones, H.G. and Schofield, P. (2008). Thermal and other remote sensing of plant stress. *General and Applied Plant Physiology*, 34, 19-32.

Jones, H.G., Serraj, R., Loveys, B.R., Xiong, L., Wheaton, A. and Price, A.H. (2009). Thermal infrared imaging of crop canopies for the remote diagnosis and quantification of plant responses to water stress in the field. *Functional Plant Biology*, 36, 978-989.

Krumov A., Nikolova, A., Vassilev, V. and Vassilev, N. (2008). Assessment of plant vitality detection through fluorescence and reflectance imagery. *Advances in Space Research*, 41, 1870-1875.

Leinonen, I. and Jones, H.G. (2004). Combining thermal and visible imagery for estimating canopy temperature and identifying plant stress. *Journal of Experimental Botany*, 55, 1423-1431.

Mahlein, A.-K., Steiner, U., Hillnhütter, C., Dehne, H.-W. and Oerke, E.-C. (2012). Hyperspectral imaging for small-scale analysis of symptoms caused by different sugar beet diseases. *Plant Methods,* 8, 3; doi:10.1186/1746-4811-8-3.

McMaster, G.S. and Wilhelm, W.W. (2003). Phenological responses of wheat and barley to water and temperature: Improving simulation models. *Journal of Agricultural Science*, 141, 129-147.

Manivannan, P., Abdul Jaleel, C., Kishorekumar, A., Sankar, B., Somasundaram, R., Sridharan, R. and Panneerselvam, R. (2007). Changes in antioxidant metabolism of *Vigna unguiculata* (L.) Walp. by propiconazole under water deficit stress. *Colloids and Surfaces B: Biointerfaces*, 57, 69-74.

Nason, M.A., Farrar, J. and Bartlett, D. (2007). Strobilurin fungicides induce changes in photosynthetic gas exchange that do not improve water use efficiency of plants grown under conditions of water stress. *Pest Management Science*, 63, 1191-1200.

Nicholas, H. (2004). Using remote sensing to determine the date of a fungicide application on winter wheat. *Crop Protection*, 23, 853-863.

Pan, X.Y., Wang, G.X., Yang, H.M. and Wei, X.P. (2003). Effect of water deficits on within-plot variability in growth and grain yield of spring wheat in Northwest China. *Field Crops Research*, 80, 195-205.

Passioura, J.B. (2004). Increasing crop productivity when water is scarce - from breeding to field management. In: Proceedings of the Fourth International Crop Science Congress, pp. 1-17.

Peñuelas, J., Gamon, J.A., Griffin, K.L. and Field, C.B. (1993). Assessing community type, plant biomass, pigment composition, and photosynthetic efficiency of aquatic vegetation from spectral reflectance. *Remote Sensing of the Environment*, 46, 110-118.

Peñuelas, J., Pinol, R.O., Ogaya, R. and Filella, I. (1997). Estimation of plant water concentration by the reflectance Water Index WI= (R900/R970). *International Journal of Remote Sensing*, 18, 2869-2875.

Percival, G.C. and Noviss, K. (2008). Triazole induced drought tolerance in horse chestnut (*Aesculus hippocastanum*). *Tree Physiology*, 28, 1685-1692.

Pirzad, A., Shakiba, M.R., Zehtab-Salmasi, S., Mohammadi, S.A., Darvishzadeh, R., and Samadi, A. (2011). Effect of water stress on leaf relative water content, chlorophyll, proline and soluble carbohydrates in *Matricaria chamomilla* L. *Journal of Medicinal Plants Research*, 5, 2483-2488.

Rampino, P., Mita, G., Fasano, P., Borelli, G., Aprile, A., Dalessandro, G., De Bellis, L. and Perrota, C. (2012). Novel durum wheat genes up-regulated in response to a combination of heat and drought stress. *Plant Physiology and Biochemistry*, 56, 72-78.

Rouse, J.W., Haas, R.H., Schell, J.A. and Deering, D.W. (1974). Monitoring vegetation systems in the Great Plains with ERTS. In: Proceedings of the 3th Earth Resources Technology Satellite-1 Symposium. Greenbelt, MD NASA, 301-317.

Römer, C., Wahabzada, M., Ballvora, A., Pinto, F., Rossini, M., Panigada, C., Behmann, J., Léon, J., Thurau, C., Bauckhage, C., Kersting, K., Rascher, U. and Plümer, L. (2012). Early drought stress detection in cereals: simplex volume maximisation for hyperspectral image analysis. *Functional Plant Biology*, 39, 878-890.

Serrano, L., Ustin, S.L., Roberts, D.A., Gamon, J.A. and Peñuelas, J. (2000). Deriving water content of chaparral vegetation from AVIRIS data. *Remote Sensing of Environment*, 74, 570-581.

Sibel, T. and Birol, T. (2007). Some physiological responses of drought stress in wheat genotypes with different ploidity in Turkiye. *World Journal of Agricultural Sciences*, 3, 178-183.

Sonmez, N.K., Emekli, Y., Sari, M. and Bastug, R. (2008). Relationship between spectral reflectance and water stress conditions of Bermuda grass (*Cynodon dactylon* L). *New Zealand Journal of Agricultural Research*, 51, 223-233.

Spollen, W.G., LeNoble, M.E., Samuels, T.D., Bernstein, N. and Sharp, R.E. (2000). Abscisic acid accumulation maintains maize primary root elongation at low water potentials by restricting ethylene production. *Plant Physiology*, 122, 967-976.

Shao, H.B., Chu, L.Y., Jaleel, C.A. and Zhao, C.X. (2008). Water deficit stress induced anatomical changes in higher plants. *Comptes Rendus Biologies*, 331, 215-225.

Suty-Heinze, A., Dunkel, R., Krieg, U. and Rieck, H. (2011). Bixafen - the new cereal fungicide with yield boosting effects. In: Dehne, H.-W., Deising, H.B., Gisi, U., Kuck, K.H., Russell, P.E. and Lyr, H., eds., Modern Fungicides and Antifungal Compounds VI, Proceedings of the 16th International Reinhardsbrunn Symposium, 25-29 April 2010, Friedrichroda, Germany, Lebenshilfe gGmbH, Braunschweig, pp. 69-74.

Strzalka, K., Kostecka-Gugala, A. and Latowski, D. (2003). Carotenoids and environmental stress in plants: Significance of carotenoid-mediated modulation of membrane physical properties. *Russian Journal of Plant Physiology*, 50, 168-172.

Thenkabail, P.S., Smith, R.B. and De Pauw, E. (2000). Hyperspectral vegetation indices and their relationships with agricultural crop characteristics. *Remote Sensing of Environment*, 71, 158-182.

Wang, X., Yang, W., Wheaton, A., Cooley, N. and Moran, W. (2010). Automated canopy temperature estimation via infrared thermography: A first step towards automated plant water stress monitoring. *Computers and Electronics in Agriculture*, 73, 74-83.

Wu Y.X. and Von Tiedemann, A. (2002). Impact of fungicides on active oxygen species and antioxidant enzymes in spring barley (*Hordeum vulgare* L.) exposed to ozone. *Environmental Pollution*, 116, 37-47.

CHAPTER 6: USE OF NON-INVASIVE SENSORS TO STUDY THE EFFECTS OF BIXAFEN ON PHOTOSYNTHESIS, MORPHOGENESIS AND SENESCENCE OF WHEAT

ABSTRACT

A series of experiments was conducted under greenhouse conditions, in order to determine the effects of bixafen on the photosynthetic rate and senescence of wheat plants. In conjunction with these experiments, the sensitivity of different non-invasive techniques to detect the effect of fungicides on the senescence status was tested.

Application of bixafen significantly increased the leaf area of wheat. Chlorophyll fluorescence and gas exchange measurements were found to be adequate diagnostics for detecting differences between bixafen treated and untreated plants in the photosynthetic rate of flag leaves. Differences in leaf senescence were detected using image analysis software, thus allowing an early detection of the fungicide effect on the senescence status of flag leaves. With hyperspectral imaging, it was possible to study differences in the senescence status of flag leaf segments. Spectral vegetation indices indicated differences between treatments earlier for the leaf tip than for the base and the middle of the flag leaf. Both non-invasive techniques allowed the detection and quantification of differences in the senescence stage between fungicide-treated and non-treated plants. The potential of chlorophyll fluorescence, digital imaging analysis and hyperspectral reflectance for early non-invasive detection of fungicide effects on plant senescence was demonstrated in this study. Moreover, the results of this study suggest that a combination of non-invasive sensors may lead to the detection of earlier changes produced by fungicides on the senescence status of plants. Non-destructive hyperspectral sensing may be implemented in time series experiments to study the effect of new compounds on plant senescence.

Keywords: leaf area, photosynthetic rate, RGB image, leaf reflectance, spectral vegetation indices.

1. INTRODUCTION

Apart from the fungicidal effect, the application of various active ingredients induced physiological changes in wheat and barley (Nason *et al*., 2007). The effect of fungicides on plant senescence has been studied extensively during the last decades. Many researchers reported that application of some fungicidal groups can induce a delay of

senescence (Cromey *et al*., 2004; Pepler *et al*., 2005).

Leaf senescence is a complex process that involves several physiological and biochemical events (Gan and Amasino, 1997). During senescence, one of the primary effects is the reduction of the photosynthetic rate as a consequence of the disassembly of the photosynthetic apparatus (Grover and Mohanty, 1992). Chlorophyll fluorescence has been shown to be a viable diagnostic tool to evaluate the influence of biotic and abiotic stresses on the photosynthetic activity of plants (Berger *et al*., 2007; Bürling *et al*., 2011). Using chlorophyll fluorescence, Lu *et al*. (2002) observed a substantial decrease in the photosynthetic activity during flag leaf senescence. This suggests that the assessment of the photosynthetic activity of flag leaves by chlorophyll fluorescence might be a useful probe for investigating the effects of fungicides on plant senescence.

The photosynthetic activity of the flag leaf has a strong influence on the final grain yield, rendering it an important topic of research that can potentially be used to improve the grain yield of wheat. There are many reports regarding the importance of the flag leaf for grain filling (Yoshida, 1972; Evans *et al*., 1972; Makunga *et al*., 1978), this organ contributes with most of the assimilates required for the grain filling (Rawson *et al*., 1983). The senescence period of the flag leaf of wheat is highly correlated to the period of grain filling. Gong *et al*. (2005) reported that a 'stay green' hybrid had higher grain yield mainly due to the delay of senescence.

Application of different fungicidal groups resulted in greener flag leaves of wheat (Dimmock and Gooding, 2002). Color differences due to senescence are mainly attributed to the degradation of chlorophyll (Hörtensteiner, 2006). Methods such as chlorophyll extraction and visual assessment have been used to study the effect of fungicides on the senescence of plants. These approaches have some disadvantages; chlorophyll extraction is a destructive method; therefore, it is not possible to follow the same replicate in time series experiments. Furthermore, this technique is restricted to a small part of the leaf. The disadvantages of visual assessment of senescence are that the perception of colors and light may vary among raters (Bock *et al*., 2010) and due to the nature of the senescence, which is not always homogeneous; the rating could be incorrect and imprecise (Hafsi *et al*., 2000). This makes the employment of non-invasive sensors such as reflectance spectrometry and the use of digital image analysis software an alternative to assess the effect of fungicide application on the senescence status of crops.

As shown by Hafsi *et al*. (2000) for cereal leaves the use of digital image analysis software is an adequate alternative to quantify the senescence status of leaves. It has also been used successfully for the quantification of disease severity showing it to be more precise than visual assessment (Steddom *et al*., 2005; De Coninck *et al*., 2012).

Another non-invasive alternative for the assessment of the senescence status of crop

plants is the use of hyperspectral imaging techniques. This non-invasive method has been used to assess changes produced by pathogen infections in different crops (Rumpf *et al*., 2010; Mahlein *et al*., 2012a). Moreover, it has been used effectively to measure the effect of biotic and abiotic stresses on plant vitality (Mahlein *et al*., 2012b).

Santos *et al*. (2010) demonstrated the suitability of this technique to detect changes in plant senescence. Therefore, hyperspectral imaging systems are an alternative to destructive and visual assessment of the senescence status of plants. Additionally, using hyperspectral imaging systems renders the possibility to study the senescence status of different sections of the leaf in order to determine the senescence pattern and when and where the differences between treatments might be observed.

Thus, sensor based methods for the detection of the possible effects of fungicidal compounds on plant physiology will improve the screening for new active ingredients. New techniques are available such as non-invasive sensors and imaging techniques previously discussed which will provide the possibility to detect differences in plant vitality between fungicide and non-treated plants earlier than with conventional techniques.

The overall objectives of this chapter were to:

i. Determine the effect of the bixafen dose on the morphology and photosynthetic rate of wheat plants
ii. Investigate the effect of bixafen application on wheat senescence by the use of non-invasive sensors
iii. Compare the sensitivity of different techniques to detect the effects produced by fungicide application on some physiological parameters.

2. MATERIALS AND METHODS

2.1. Plant material

Four seeds of wheat, cultivar Passat (KWS GmbH, Einbeck, Germany) were sown in plastic pots (13 cm Ø) 1 cm deep. Ten pots were used per treatment. The substrate consisted of a mixture of organic soil (Klasmann-Deilmann GmbH, Germany), sand and C horizon (12:6:2 v/v). Plants were grown at 24/20 °C (day/night), 70 ± 10% relative humidity (RH), photoperiod of 16 h d^{-1} with supplemental illumination (> 300 µmol m^{-2} s^{-1}, lamps Philips SGR 140, Philips, Hamburg, Germany). Plants were fertilized every two weeks with 100 ml of a 0.2% solution of a commercial N-P-K fertilizer (14-10-14, 2 g/l; Aglukon GmbH, Düsseldorf, Germany). Plants were carefully inspected in order to control possible fungal infections. In order to keep plants free from powdery mildew the fungicide Talius® (active ingredient [a.i.] proquinazid, 200 g L^{-1}, DuPont de Nemours, Neu-Isenburg, Germany) and the fungicide Vegas® (a.i. cyflufenamid, 51.3 g L^{-1}, Spiess-Urania,

Germany) were applied. 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.

2.2. Fungicide treatments

Two doses of the fungicide bixafen were compared to an untreated control: recommended dose (125 g a.i. L^{-1}) and half of the recommended dose. The active ingredient was supplied by Bayer CropScience, (Monheim, Germany). The fungicide was applied at recommended field rates (water 300 L ha^{-1}). Plants were treated once at growth stage (GS) 13 according to the BBCH scale (Hack *et al*., 1992). A CO_2 pressurized hand-sprayer (Meisterwerkzeuge, Wuppertal, Germany) with an adjustable spray was used for fungicide application. To avoid contamination between treatments plants were separated and treated far away from each other. After fungicide application plants were randomized into the greenhouse.

2.3. Plant development and yield parameters

Plant height was measured and the number of tillers was counted per replicate on a weekly basis after fungicide application. There were a total of 10 replicates, and for each replicate, plant height and the number of tillers from two plants were assessed. The mean plant height and the mean number of tillers per pot was used for data analysis. The length, width and the total leaf area of the top three leaves (F, F-1 and F-2) were measured at GS 75. The portable laser leaf area meter CI-203 (CID Bio-Science, Camas, USA) was used in a non-destructive way. The device was gently slid from the base to the leaf tip to measure the total leaf area. Two plants per replicate per treatment were used to determine leaf size. At harvest, the total weight of kernels per ear was measured.

2.4. Assessment of photosynthetic activity

2.4.1. Chlorophyll fluorescence

Chlorophyll fluorescence of flag leaves (F) and F-1 of wheat was measured at GS 71, GS 75, GS 81 and GS 85 with a portable pulse-modulated chlorophyll fluorometer PAM-2000 (Walz, Effeltrich, Germany). Plants were kept in total darkness for 30 min at room temperature directly before 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 (1000 µmol m^{-2} s^{-1}) was applied. White light was used to produce a transient closure of the PS II photochemical reaction centers. Leaves were continuously illuminated with white actinic light (336 mmol m^{-2} s^{-1}). Seven replicates per treatment were used. All measurements

were made between 9:00 a.m. and 4:00 p.m. The effective quantum yield of photosystem II was assessed with a PAMWIN data acquisition system (Walz, Effeltrich, Germany).
The area under quantum yield of the PS II curve (AUQYC) was calculated using the equation:

$$AUQYC = \sum_{i=1}^{n} 1/2(Y_i + Y_{i-1})(X_i - X_{i-1})$$

Where Y_i= effective quantum yield of the PS II value at the *i*th observation, X_i = measurement number at the *i*th observation, *n* = total number of observations.

2.4.2. Leaf gas exchange

Gas exchange was measured at GS 71, GS 75, GS 81 and GS 85. One flag leaf per replicate was used and seven replicates per treatment were measured. The minicuvette gas exchange system (CMS-400 Heinz Walz GmbH, Effeltrich; Germany) was used to measure the net assimilation and respiration of wheat leaves. Measurements on the flag leaves were conducted under light conditions simulating the sun spectrum by the use of the lighting unit FL-440 (Walz, Effeltrich; Germany, ± 950 µmol m^{-2} s^{-1}) after 20 minutes of equilibration to measure net CO_2 uptake (photosynthetic activity), and under dark conditions measuring CO_2 release (respiration) after 10 minutes of equilibration.

The area under the photosynthesis curve (AUPC) was calculated using the equation:

$$AUPC = \sum_{i=1}^{n} 1/2(Y_i + Y_{i-1})(X_i - X_{i-1})$$

Where Y_i= CO_2 uptake value at the *i*th observation, X_i = measurement number at the *i*th observation, *n* = total number of observations.

2.5. Assessment of senescence

Two image analysis techniques were used to study the effect of early application of bixafen on wheat senescence. The recommended dose (125 g a.i. L^{-1}) was applied at GS 13 and GS 39 (Hack *et al*., 1992) and was compared to an untreated control.

2.5.1. Image analysis software

After the end of heading (GS 59), RGB images of leaves were taken every three days until the fully ripe stage (GS 89). The recommended dose of bixafen was compared to the untreated control. Eight leaves were assessed per treatment. The images were taken using a camera (Canon PowerShot Pro 1) with the following features: 8.0 Megapixel, aspect ratio 4.3, focal length 7.2 - 50.8 mm (35 mm film equivalent: 28-200 mm), zoom

optical 7.0x, digital approx. 3.2x, combined approx. 22x. The images were stored in high resolution - JEPG format. The same leaves were measured and subsequently observed until harvest. Leaves were mounted into a special structure constructed in order to have the same distance from the camera lens to the leaf surface (45 cm). The image analysis software program ASSESS 2.0 (ASSESS 2.0; L. Lamari, American Phytopathological Society, St. Paul, MN, USA) was used to analyze the images. The total leaf area, the total green leaf area and the total senescent area were calculated in terms of the number of pixels (Fig. 6.1). The total senescent area was calculated as percentage of the total leaf area ((senescence pixels/total leaf pixels) x 100).

The area under the green leaf area curve (AUGLAC) was calculated using the equation given by Cromey *et al*. (2004),

$$\text{AUGLAC}= \sum_{i=1}^{n} 1/2(Y_i + Y_{i-1})(X_i - X_{i-1})$$

Where Y_i= percent green leaf area at the *i*th observation, X_i = time (days after the end of heading) at the *i*th observation, *n* = total number of observations.

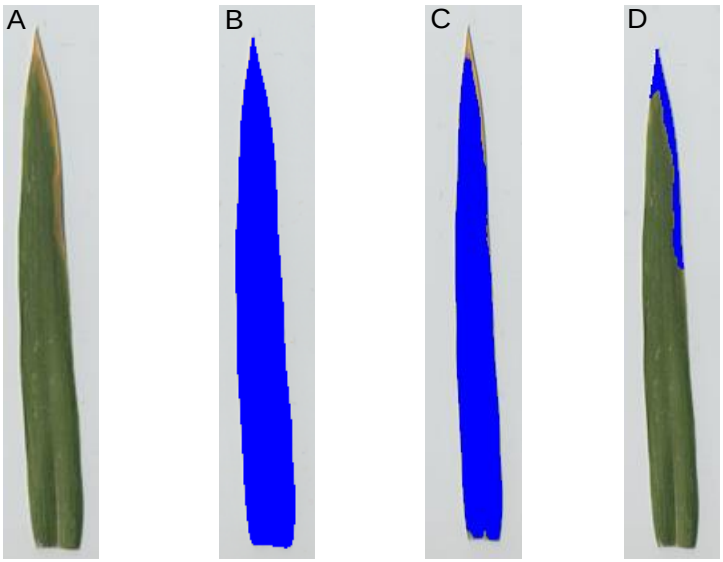

Figure 6.1: Illustration of the methodology to estimate the senescence of flag leaves using the software ASSESS 2.0; blue color represented the area calculated by the program; RGB image (A), measurement of total leaf area (B), measurement of total green leaf area (C) and measurement of total senescent area (D).

2.5.2. Hyperspectral imaging

Hyperspectral images of untreated and bixafen treated flag leaves were taken at four growth stages (GS 65, GS 71, GS 75 and GS 81). Six flag leaves were measured per treatment. The leaves were fixed with a rubber band horizontally on a black background in

order to keep them flat for the measurement. The images were taken using a line scanner with a spectral resolution up to 2.8 nm and a spectral range from 400-1000 nm (ImSpector V10E, Spectral Imaging Ltd., Oulu, Finland). The images were taken under dark conditions. Four ASD Pro-Lamps (Analytical Spectral Devices Inc., Boulder, CO, USA) were used to provide an accurate illumination for the assessment. A white and black test target (Spectral Imaging Ltd., Oulu, Finland) was used to focus the camera. The software SpectralCube (Spectral Imaging Ltd., Oulu, Finland) was used to record the images. Four images were taken: A white reference image, a dark current image, a raw white reference and a raw image. The four images were normalized using the software program ENVI 4.6 + IDL 7.0 (ITT Visual Information Solutions, Boulder, CO, USA). After normalization, the Savitzky-Golay filter (Savitzky and Golay, 1964) was applied to smooth the reflectance curves. For the reflectance spectra, the sampling method was to sub-divide the leaf in three parts for further analysis: leaf base, leaf middle and leaf tip. Specific spectra were extracted from an area consisting of 12.000 pixels on average for every region of interest from each replicate. The pre-processed images were used to calculate three spectral vegetation indices (SVIs; Tab. 6.1) related to senescence and biochemical parameters using the ENVI 4.6 + IDL 7.0 software. The normalized difference vegetation index (NDVI, Rouse *et al*., 1974), the plant senescence reflectance index (PSRI, Merzlyak *et al*., 1999) and the structure insensitive pigment index (SIPI, Peñuelas *et al*., 1995) were calculated to detect special and temporal senescence changes in wheat flag leaves at the leaf subdivisions.

Table 6.1: Spectral vegetation indices and algorithms used in this study.

Index	Equation [a]	Related to	Reference
Normalized difference vegetation index	$NDVI= (R_{800}-R_{670})/(R_{800}+R_{670})$	Biomass, plant vitality	Rouse *et al*., (1974)
Plant senescence reflectance index	$PSRI= (R_{680}-R_{500})/R_{750}$	Plant senescence	Merzlyak *et al*., (1999)
Structure insensitive pigment index	$SIPI= (R_{800}-R_{445})/(R_{800}+R_{680})$	Carotenoid: chlorophyll a ratio	Peñuelas *et al.,* (1995)

[a] Reflectance at wavelengths indicated.

2.6. Statistical analysis

Data were analyzed using the statistical program SPSS statistics for Windows (IBM Deutschland GmbH, Ehningen, Germany), version 20.0. Data were tested for normal

distribution and equality of variances. The data were examined using analysis of variance (ANOVA) and the standard errors (SE) on the means were calculated. The means were compared using the Tukey test at 95% confidence in order to separate subgroups. The correlations between grain yield and different parameters were tested for correlation significance using Pearson's correlation coefficient at a probability level of 0.05. The t-test was used ($p < 0.05$) for analysis of the senescence status and the spectral vegetation indices between untreated control and bixafen treatment. The Levene´s test was used to assess the equality of the variances between treatments.

3. RESULTS

3.1. Plant development and yield parameters

Plant height and the number of tillers were assessed weekly from GS 23 until GS 59. No significant differences in plant height were calculated between the untreated control and the bixafen treatments (Fig. 6.2). At the last measurement bixafen treatments had significantly more tillers than the untreated control (Fig. 6.2).

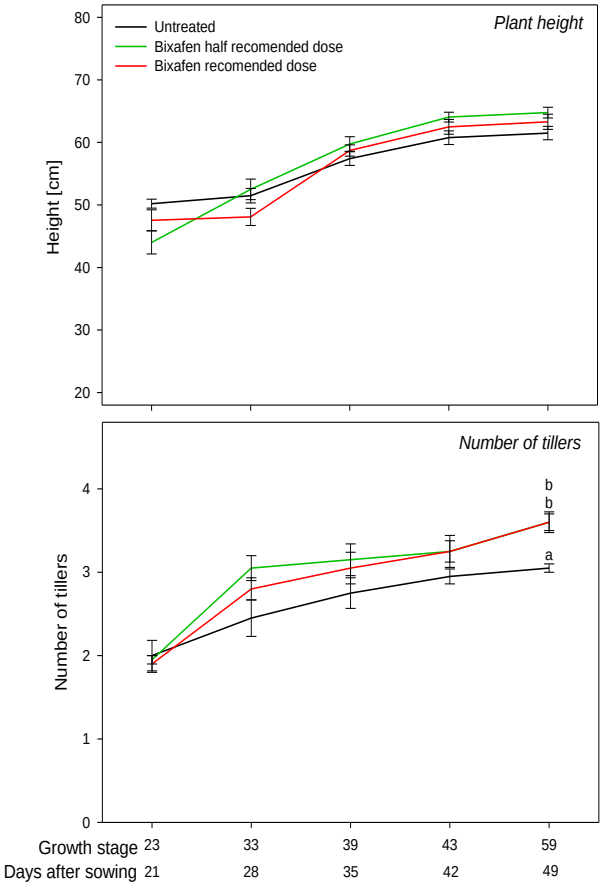

Figure 6.2: Effect of two bixafen doses on plant height and number of tillers of wheat plants (cv. Passat). Vertical bars indicate SE, different letters indicate significant differences according to Tukey's test ($p \leq 0.05$; $n = 10$).

The recommended bixafen dose was significantly different to the untreated control in terms of the area of F and F-1 (Fig. 6.3). Plants treated with the recommended bixafen dose had significantly longer F and F-1 leaves compared to untreated and half bixafen dose treated plants. No significant differences were calculated for leaf width for these two leaf layers (Fig. 6.3). For F-2, bixafen application did not result in significant differences for the leaf size parameters compared to the untreated control.

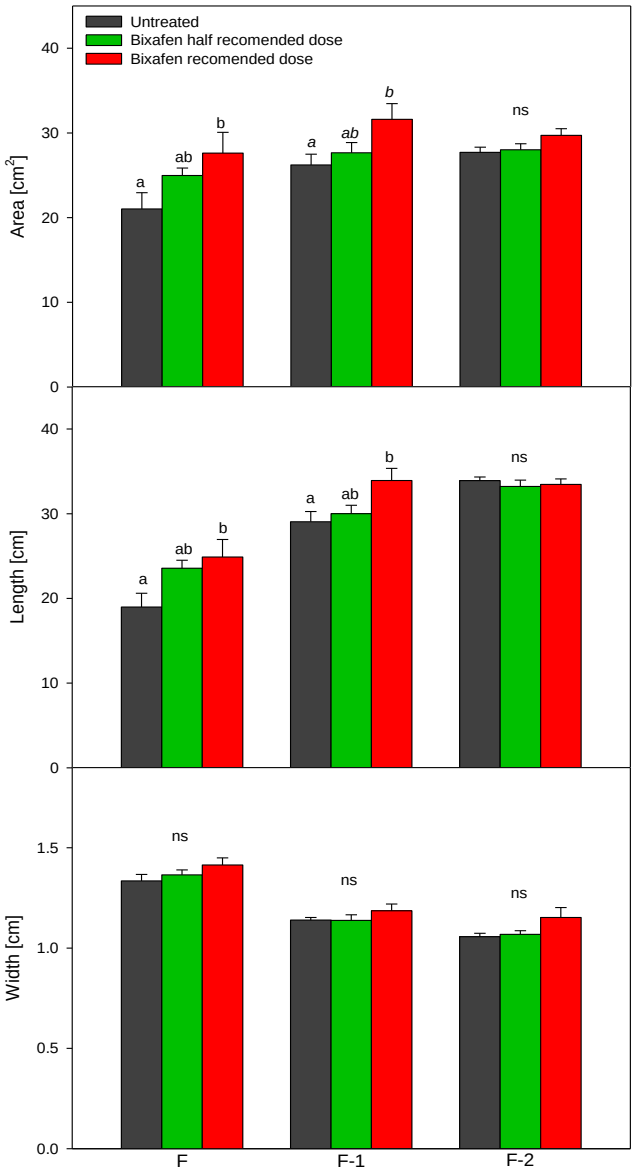

Figure 6.3: Effect of two bixafen doses on area, length and width of F, F-1 and F-2 leaves of wheat plants (cv. Passat). Vertical bars indicate SE, different letters indicate significant differences according to Tukey's test ($p \leq 0.05$; $n = 10$); ns, no significant difference.

Plants were harvested and the grain weight per ear was determined. Significant differences were calculated between treatments, with the bixafen recommended dose application resulting in a higher grain yield compared to the other treatments (Fig. 6.4).

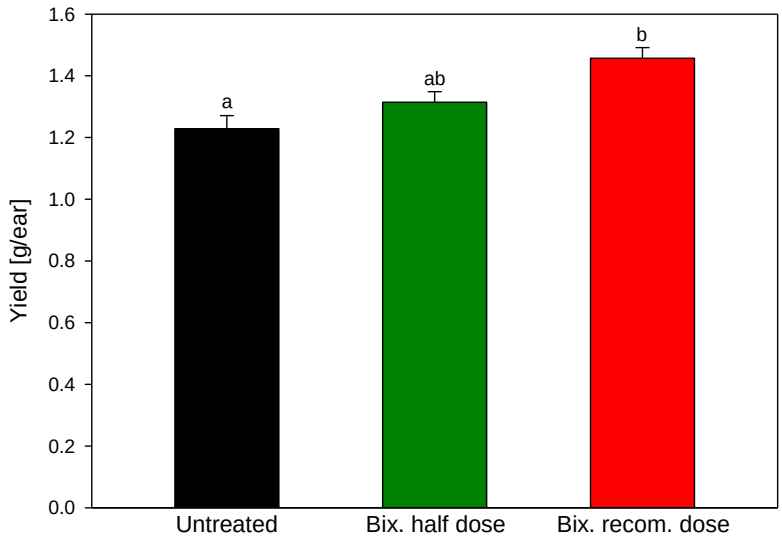

Figure 6.4: Effect of two bixafen doses on the grain yield per ear of wheat plants (cv. Passat). Vertical bars indicate SE, different letters indicate significant differences according to Tukey's test ($p \leq 0.05$; $n = 10$).

The total flag leaf area was positively correlated to the grain yield per ear (Fig. 6.5). A coefficient of determination R^2 of 0.670 was calculated between these two parameters. In contrast, no significant correlation was calculated for the size of F-1 and F-2 leaves and total grain yield per ear. The slope (m) of the linear regression for flag leaf area and grain yield was steeper than the other two linear regressions. The overall result of the linear regression analysis demonstrated that flag leaf size has a higher effect on grain yield compared to the other two leaf layers.

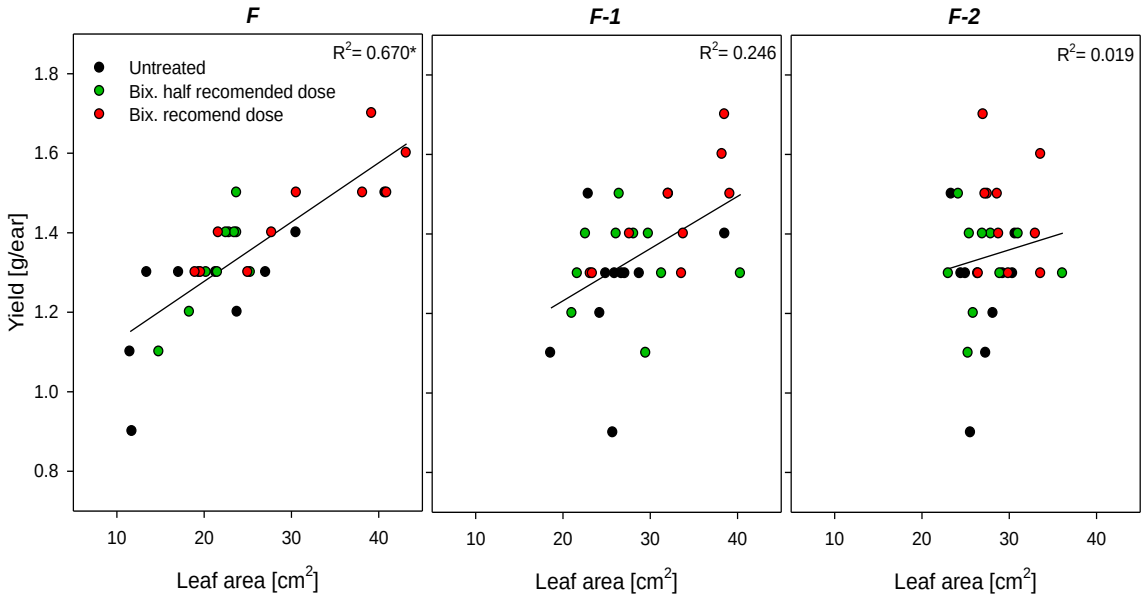

Figure 6.5: Correlations between F, F-1 and F-2 area and the grain yield per ear of wheat (cv. Passat). Asterisk indicates significant correlation according to the Pearson's test.

3.2. Photosynthetic activity

3.2.1. Chlorophyll fluorescence

Significant differences were calculated between treatments concerning the effective quantum yield of photosystem II (PS II) of the flag leaves (Fig. 6.6). At the first two measuring dates, no significant differences were detected between treatments. At GS 81 and GS 85 application of bixafen recommended dose resulted in significantly higher effective quantum yield of PSII compared to the other treatments.

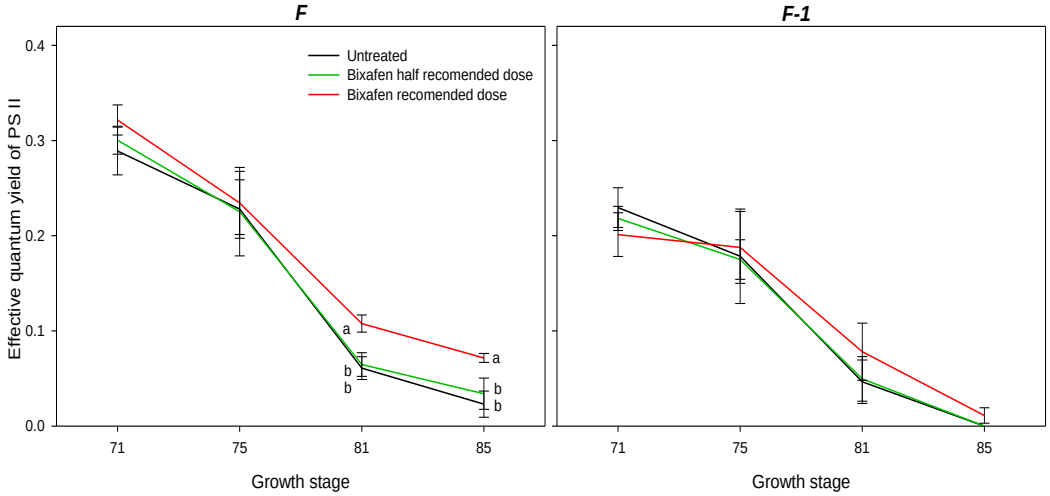

Figure 6.6: Effect of two bixafen doses on the quantum yield of photosystem II of leaves F and F-1 of wheat plants (cv. Passat). Vertical bars indicate SE, different letters indicate significant differences according to Tukey ($p \leq 0.05$; $n = 7$).

The effective quantum yield of photosystem II of F-1 did not significantly differ between treatments. The application of the half dose had not effect on the quantum yield of F and F-1 compared to the untreated control. The area under quantum yield of PS II curve and grain yield per ear showed a positive linear correlation (Fig. 6.7A).

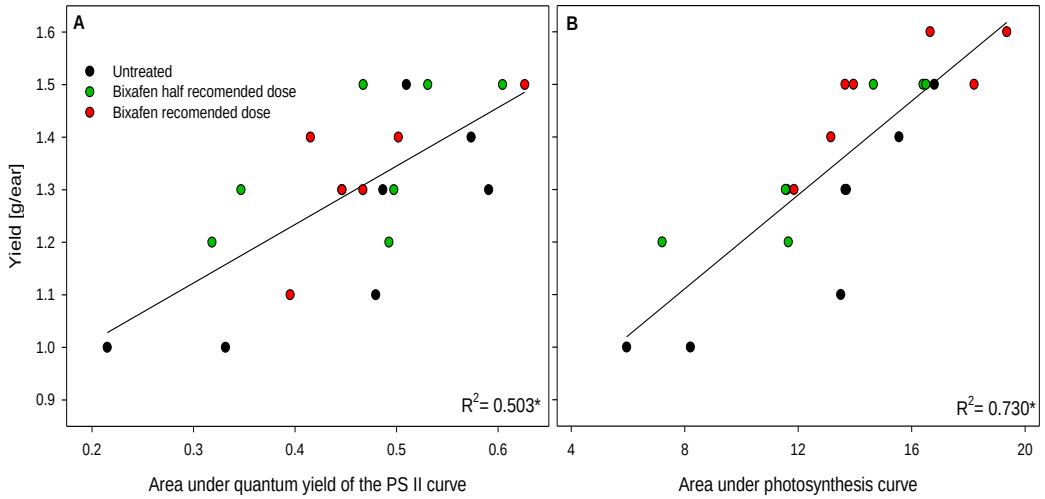

Figure 6.7: Correlations between the area under quantum yield of the PS II curve (A) and the area under photosynthesis curve (B) and grain yield per ear of wheat (cv. Passat). Asterisk indicates significant correlation according to Pearson's test.

3.2.2. Gas exchange

Net photosynthesis and respiration of treatments were not significant different at the first measurements (GS 71 and GS 75, Fig. 6.8). At GS 81, the net CO_2 assimilation of wheat flag leaves was significantly influenced by the bixafen recommended dose. The early senescence of untreated flag leaves resulted in a lower photosynthetic rate compared to bixafen treated leaves. Moreover, at this growth stage, no significant differences were calculated between treatments in terms of dark respiration. The last measurements at GS 85 showed that the recommended dose was significantly higher than the other treatments for both photosynthesis and respiration. The statistical analysis revealed that the half dose of bixafen did not produce any significant differences in photosynthesis and respiration compared to the untreated control.

The Pearson's correlation coefficient revealed a significant positive linear correlation between the area under photosynthesis curve and the grain yield per ear (Fig. 6.7B).

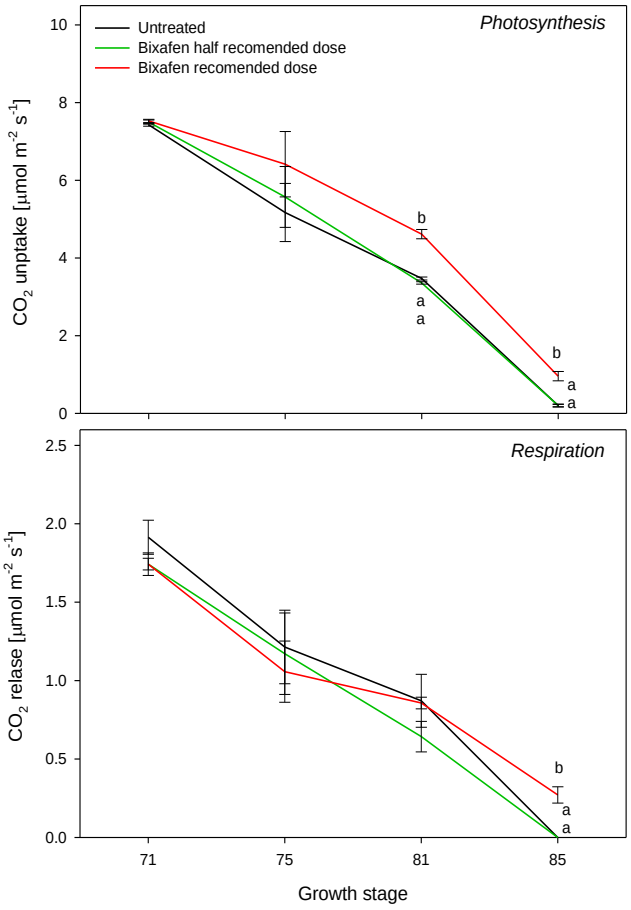

Figure 6.8: Effect of two bixafen doses on the photosynthetic activity and respiration of wheat flag leaves (cv. Passat). Vertical bars indicate SE, different letters indicate significant differences according to Tukey's test ($p \leq 0.05$; $n = 7$).

3.3. Plant senescence

3.3.1. Assessment of leaf senescence in digital RGB images

It was possible by using the digital analysis software (ASSESS 2.0) to detect differences

in flag leaf senescence between treatments eighteen days after the end of heading (Fig. 6.9). Flag leaf senescence was delayed in plants treated with the recommended bixafen dose compared to the untreated control. Bixafen application extended the leaf life compared to the untreated control on average 6 days. The biggest differences between these two treatments were calculated forty-four days after the end of heading (Fig. 6.9).

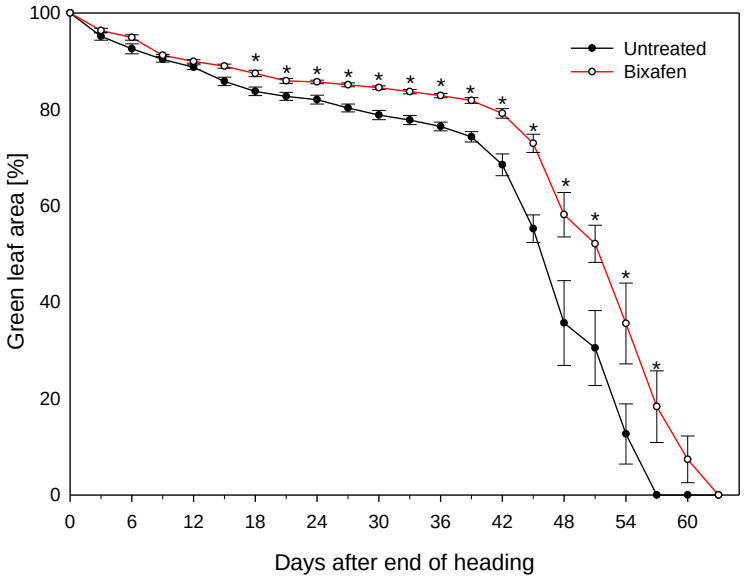

Figure 6.9: Effect of bixafen application on the green leaf area duration of flag leaves of wheat (cv. Passat) assessed by ASSESS 2.0. Vertical bars indicate SE, asterisk indicate significant differences according to t-test ($p \leq 0.05$; $n = 8$).

The area under the green leaf area duration (AUGLAD) was correlated with the grain yield (Fig. 6.10). A positive correlation was calculated between these two parameters; a significant correlation coefficient of 0.614 was calculated.

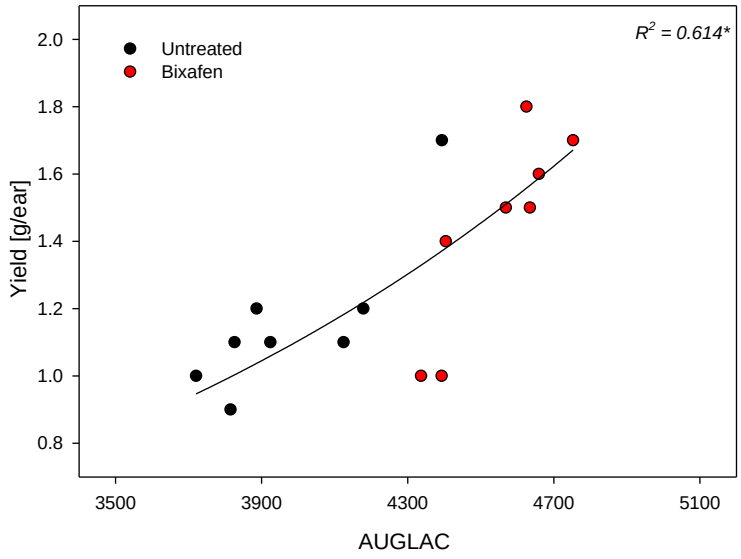

Figure 6.10: Correlation between the area under the green leaf area curve (AUGLAC) and grain yield per ear of wheat (cv. Passat). Asterisk indicates significant correlation according to the Pearson's test.

3.3.2. Plant reflectance and spectral vegetation indices

At GS 65, leaf reflectance at the base and at the middle of the leaf showed no differences between untreated plants and plants treated with bixafen in the visible range (VIS) (Fig. 6.11). In contrast, in the near infrared (NIR) bixafen treated leaves had higher reflectance than untreated plants. The tip of untreated leaves had higher reflectance in VIS and lower in the NIR than bixafen treated leaves. Moreover, the reflectance was always higher for both treatments at the leaf tip compared to the other two leaf sections analyzed.

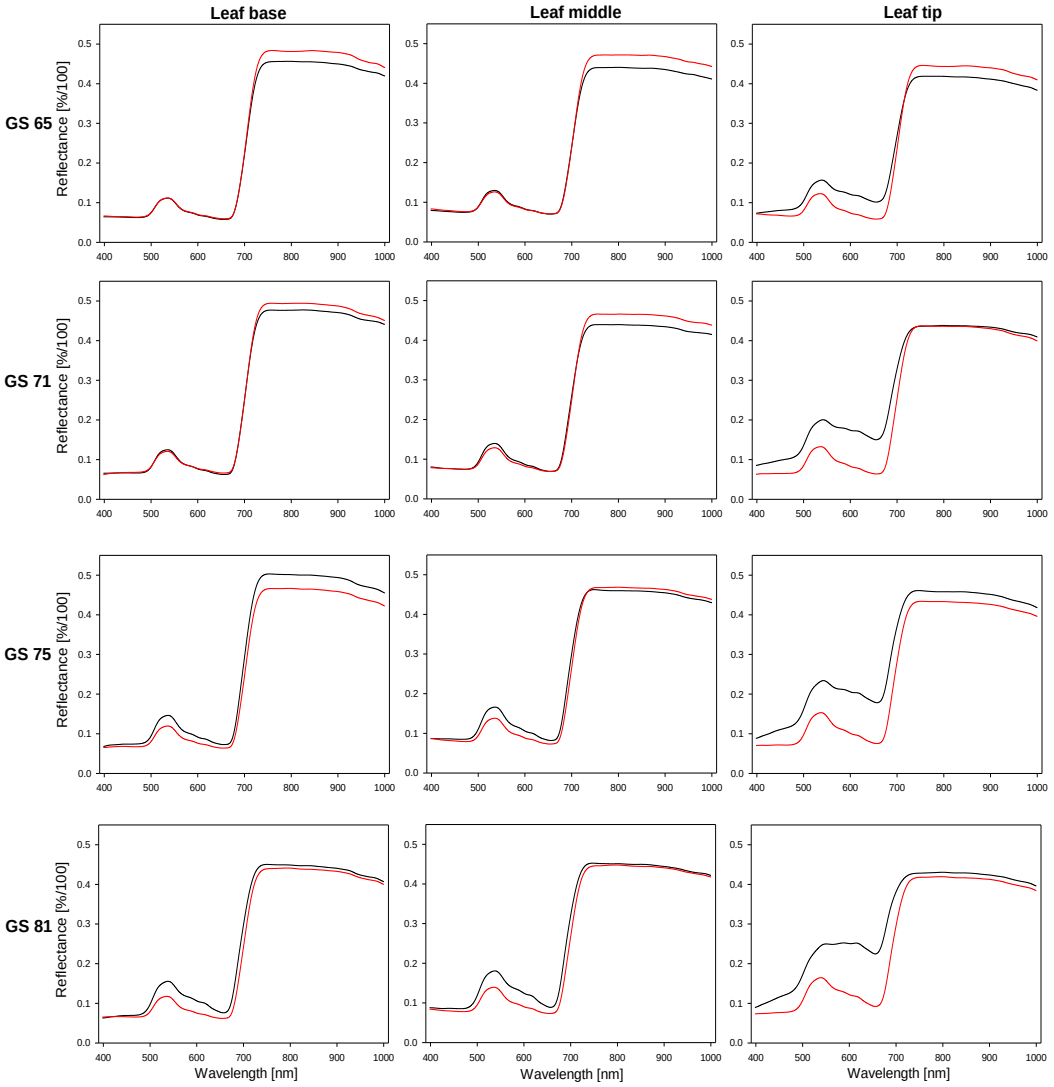

Figure 6.11: Spectral signatures of bixafen treated and untreated flag leaves of wheat (cv. Passat), measured four times during the growth period (GS 65, GS 71, GS 75 and GS 81). Spectral curves of the base, middle and tip of flag leaves.

At GS 71, no differences in reflectance were observed for the leaf base compared to the previous measuring date. In contrast, the first differences for the middle of the leaves were observed in the VIS between treatments. In the case of the leaf tip, the tendency in the VIS was the same compared to the previous measurement time; however, the reflectance

difference at the NIR was lower compared to the earlier measurement.

At GS 75, the first differences were observed in the VIS between treatments at the leaf bases. Reflectance in the range of 500 to 700 was higher for untreated than for bixafen treated leaves. At the middle of leaves reflectance differences in the green peak were much higher compared to the earlier measurement. The same effect was observed for the leaf tip. Untreated leaves had higher reflectance in the VIS for all leaf sections evaluated compared to bixafen treated leaves.

At GS 81, untreated wheat leaves were in an advanced stage of senescence compared to bixafen treated leaves and had higher reflectance in the range 450 to 650 nm and an overall increased reflectance in the NIR at the three different leaf sections. Bixafen treated leaves were more vital and had lower reflectance at the green peak in all leaf segments.

Three SVIs related to physiological parameters were calculated from reflectance spectra (Tab. 6.2). For the leaf base, no significant differences between treatments were observed during any of the measuring dates.

Table 6.2: Effect of bixafen on spectral vegetation indices of wheat (cv. Passat) flag leaves.

Index	Growth stage	Treatment	Leaf base	Leaf middle	Leaf tip
NDVI	65	Untreated	0.762	0.708	0.576
		Bixafen	0.769	0.725	0.748
	71	Untreated	0.752	0.705	0.446
		Bixafen	0.749	0.725	0.719
	75	Untreated	0.725	0.665	0.399
		Bixafen	0.744	0.711	0.665
	81	Untreated	0.662	0.615	0.259
		Bixafen	0.734	0.694	0.587
PSRI	65	Untreated	0.015	0.019	0.084
		Bixafen	0.012	0.014	0.015
	71	Untreated	0.022	0.026	0.155
		Bixafen	0.023	0.020	0.035
	75	Untreated	0.036	0.044	0.171
		Bixafen	0.023	0.026	0.062
	81	Untreated	0.081	0.082	0.286
		Bixafen	0.029	0.035	0.103
SIPI	65	Untreated	0.729	0.675	0.604
		Bixafen	0.738	0.692	0.705
	71	Untreated	0.719	0.666	0.529
		Bixafen	0.724	0.690	0.689
	75	Untreated	0.693	0.631	0.498
		Bixafen	0.712	0.671	0.646
	81	Untreated	0.649	0.599	0.436
		Bixafen	0.701	0.657	0.599

Measuring dates highlighted in gray indicate significant differences between treatments according to t-test ($p \leq 0.05$; $n = 6$). NDVI, Normalized difference vegetation index, PSRI, plant senescence reflectance index and SIPI, Structure insensitive pigment index.

For the middle of leaves, the first significant differences between treatments were calculated at GS 75. The NDVI of bixafen treated plants was significantly higher than for untreated leaves. No significant differences between treatments were detected for the other vegetation indices. At GS 81, significant differences between treatments were calculated for all vegetation indices.

For the leaf tip, from the first to the last measuring date, significant differences were calculated between treatments for all vegetation indices.

Figure 6.12 illustrates the spatial distribution of three spectral vegetation indices at GS 65 and at GS 81 calculated from the hyperspectral imaging data of flag leaves treated with bixafen and the untreated control. The NDVI, PSRI and SIPI images demonstrated the development of senescence increasing from the tip to the base of flag leaves. Moreover, it is possible to observe how the main differences between treatments in the senescence status of flag leaves are located at the leaf tip. In contrast, no clearly distinguishable differences between treatments were found at the leaf bases.

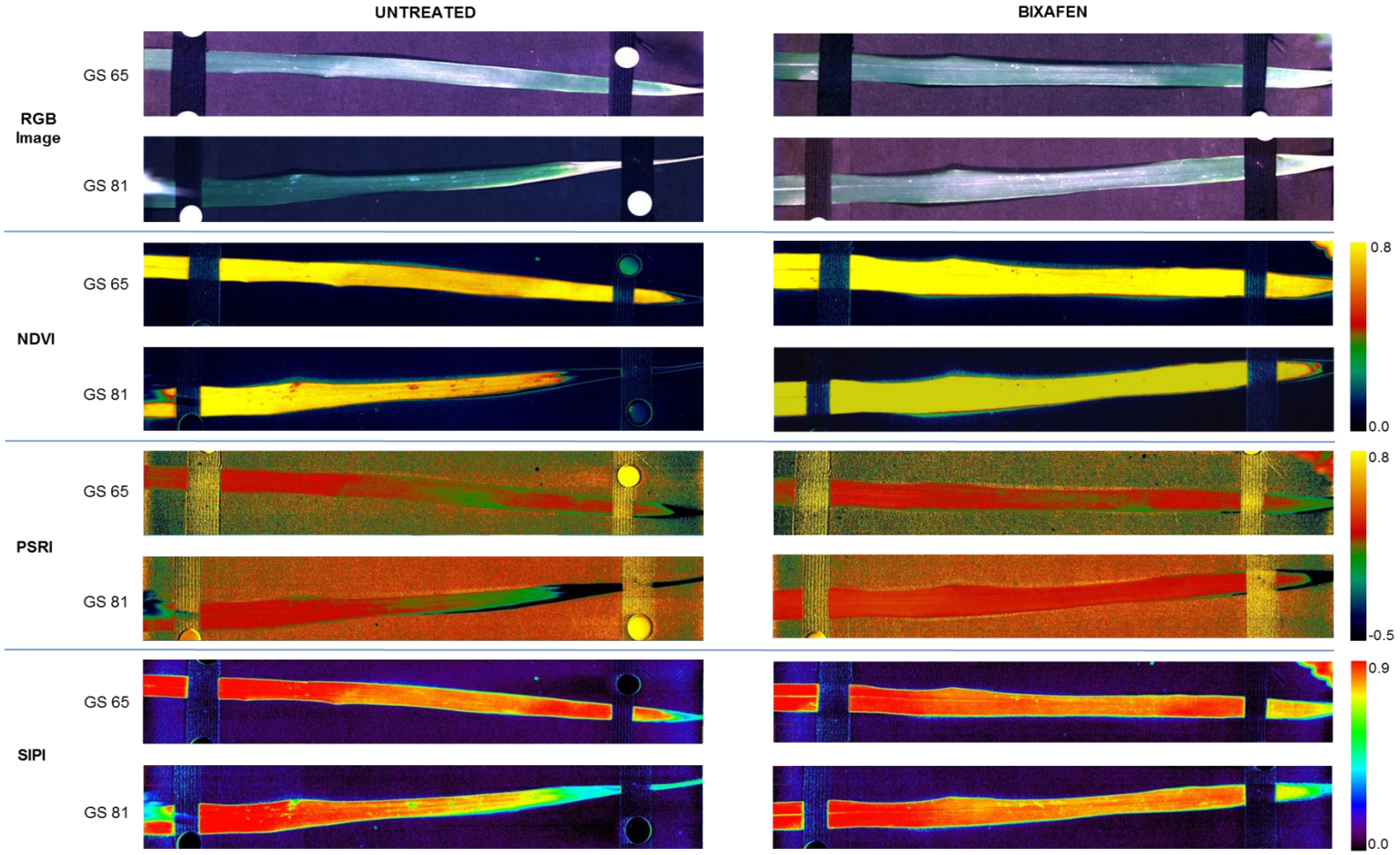

Figure 6.12: Use of spectral vegetation indices calculated from hyperspectral imaging data of bixafen treated and untreated flag leaves of wheat (cv. Passat), for the illustration of senescence process from GS 65 to GS 81. NDVI, normalized difference vegetation index, PSRI, plant senescence reflectance index and SIPI, structure insensitive pigment index.

4. DISCUSSION

Early application of bixafen recommended dose resulted in a higher number of tillers and also in differences in the leaf area of F and F-1 compared to untreated plants. Application of various fungicidal groups such as triazoles resulted in modifications of the morphology of different crops. Triadimefon and diclobutrazol induced morphological changes in maize plants like reduced plant height, mainly attributable to the reduction of shoot elongation; and shorter leaves compared to the untreated control (Khalil *et al*., 1990). A reduction in plant height and leaflet area was found in Galium plants treated with epoxiconazole seven days after treatment (Benton and Cobb, 1995). Epoxiconazole treated plants had thicker leaves and higher chlorophyll content compared to control plants. The effect of azoles on leaf anatomy has been reported by many authors. One example is the application of triazole compounds, which resulted in variations in leaf anatomy such as elongated palisade and spongy mesophyll (Benton and Cobb, 1995). Furthermore, treated leaves had a higher number of cells per unit of area in the palisade than untreated control (Gopi *et al*., 2008). The effects of triazoles on plant morphology have been mainly attributed to the influence of this fungicidal group on the balance of plant growth regulators (Sankhla, *et al*., 1991; Grossman *et al*., 1994).

There are many reports on the effects of fungicides on phytohormonal balance; nevertheless, research should target the possible influence of carboxamides on the balance of plant growth regulators. This fungicidal group may alter the balance of auxins and cytokinins since these phytohormones are highly associated to shoot branching (Tanaka *et al*., 2006; Müller and Leyser, 2011). Both hormones also play an important role in leaf growth; cytokinins are involved in the regulation of cell division (Hirose *et al*., 2008) and auxines in cell expansion (Perrot-Rechenmann, 2010). Juvany *et al*. (2013) reported an increase in the endogenous levels of cytokinins during phases of leaf growth. The leaf cell production was highly reduced in cytokinin-deficient tobacco compared to the wild type (Werner *et al*., 2001).

The effect of bixafen on leaf area was reflected in grain yield illustrated by a significant correlation between flag leaf area and grain yield. This results from the fact that the light interception by the flag leaf is strongly influenced by the leaf size. Bigger leaves will intercept more photosynthetic active radiation (PAR) resulting in higher photosynthetic rate. In contrast, a low correlation was calculated between the leaf area of F-1 and F-2 and the grain yield.

Chlorophyll fluorescence and gas exchange measurements were methods suitable to detect differences between bixafen and untreated plants. Both photosynthetic parameters were highly correlated to grain yield. Flag leaves treated with bixafen were less senescent

than untreated leaves and had higher photosynthetic activity. This is in agreement with many reports indicating that grain filling is mainly associated to the photosynthetic activity of the flag leaves (Lupton, 1968; Hsu and Walton, 1971; Kaul, 1974).

Chlorophyll fluorescence was used as a non-invasive technique to investigate the influence of fungicide application may have on crop senescence. This method made it possible to follow the same samples over time and to identify early differences in the senescence status of fungicide treated and untreated wheat. Therefore, chlorophyll fluorescence is an accurate alternative to destructive methods such as chlorophyll extraction, which is both time consuming and laborious.

Analysis of RGB images was used to measure the effect of bixafen application on senescence of flag leaves. Image analysis allowed the differentiation between fungicide treated and non-treated plants already eighteen days after heading. Several studies have demonstrated differences in the green leaf area between fungicide treated and untreated plants; however, in most studies senescence was determined by visual assessment. The results are in agreement with previous studies that have shown the usefulness of image analysis to measure the senescence status of cereal leaves (Adamsen *et al*., 1999; Hafsi *et al*., 2000; Guendouz and Maamari, 2011).

Previous studies reported the extension of green leaf area duration caused by fungicide application (Bayles, 1999; Gooding *et al*., 2000; Blandino *et al*., 2009). Green leaf area duration was positively correlated to grain yield. This result confirms previous studies, which reported a yield increment due to fungicide application mainly attributable to the extension of the grain filling period (Dimmock and Gooding, 2002; Pepler *et al*., 2005).

Hyperspectral reflectance images were taken to detect early differences in the senescence status of fungicide treated and untreated plants. The analysis of hyperspectral images allowed to study the pattern of the senescence process in flag leaves and to determine the effect of bixafen on the senescence of wheat.

Differences in reflectance spectra between fungicide treated and untreated plants were detected for the leaf tip already at GS 65. This was in contrast to the leaf base; where the first differences in the VIS were observed at GS 75. Senescence normally starts from the tip to the base of the leaf (Lim *et al*., 2007; Gregersen and Holm, 2007). In contrast to leaves of dicotyledonous plants, wheat leaves have basal meristems; therefore, the oldest cells are located in the leaf tip and the leaf base is mainly compound by the youngest cells (Boffey *et al*., 1980; Mullet, 1988). Tewari *et al*. (2012) demonstrated that activity of the antioxidant enzymes increased from the base to the leaf tip, which served as an early indicator of the spatial pattern of leaf senescence.

The spectral reflectance of fungicide treated and untreated plants were used to calculate vegetation indices for additional analysis. The spectral vegetation indices used in this

study are highly associated to the senescence status and plant vitality (Blackburn, 1998; Di Bella *et al*., 2004; Castro and Sanchez-Azofeifa, 2008).

The NDVI of the leaf middle resulted in significant differences between treatments earlier than the other two indices. This provides evidence of the suitability of this index as an indicator for plant senescence (Di Bella *et al.,* 2004).

5. CONCLUSION

Application of bixafen caused a delay of wheat senescence and increased the photosynthetic activity of flag leaves. Non-invasive techniques used in this study were suitable to detect early differences between fungicide and non-treated plants confirming the utility of these techniques to study the effect of fungicide application on plant senescence.

Early differences in the senescence status of flag leaves were detectable by analysis of RGB images and hyperspectral imaging. These imaging techniques allowed the screening of the possible effects of new fungicidal compounds on the senescence of crop plants.

The combination of non-invasive sensors might enable the detection of early modifications of plant metabolism related to senescence caused by various chemicals. The potential of non-invasive methods such as chlorophyll fluorescence, image analysis and hyperspectral imaging for an early detection of the effect of fungicide application on plant senescence was demonstrated in this study. Non-destructive hyperspectral sensing may be implemented in time series experiments to study the effect of new compounds on plant senescence.

6. REFERENCES

Adamsen, F.J., Pinter Jr., P.J., Barnes, E.M., LaMorte, R.L., Wall, G.W., Leavitt, S.W. and Kimball, B.A. (1999). Measuring wheat senescence with a digital camera. *Crop Science*, 39, 719-724.

Bayles, R. (1999). The interaction of strobilurin fungicides with cereal varieties. *Plant Varieties Seeds*, 12, 129-140.

Benton, J.M. and Cobb, A.H. (1995). The plant growth regulator activity of the fungicide, epoxiconazole, on *Galium aparine* L. (cleavers). *Plant Growth Regulation*, 17, 149-155.

Berger, S., Benediktyová, Z., Matouš, K., Bonfig, K., Mueller, M.J., Nedbal, L. and Roitsch, T. (2007). Visualization of dynamics of plant-pathogen interaction by novel combination of chlorophyll fluorescence imaging and statistical analysis: differential effects of virulent and avirulent strains of *P. syringae* and of oxylipins on *A. thaliana*. *Journal of Experimental Botany*, 58, 797-806.

Blackburn, G.A. (1998). Spectral indices for estimating photosynthetic pigment concentrations: a test using senescent tree leaves. *International Journal of Remote Sensing*, 19, 657-675.

Blandino, M., Pilati, A. and Reyneri, A. (2009). Effect of foliar treatments to durum wheat on flag leaf senescence, grain yield, quality and deoxynivalenol contamination in North Italy. *Field Crops Research*, 114, 214-222.

Bock, C.H., Poole, G.H., Parker, P.E. and Gottwald, T.R. (2010). Plant disease severity estimated visually, by digital photography and image analysis, and by hyperspectral imaging. *Critical Reviews in Plant Science*, 29, 59-10.

Boffey, S., Selldén, G. and Leech, R. (1980). Influence of cell age on chlorophyll formation in light-grown and etiolated wheat seedlings. *Plant Physiology*, 65, 680-684.

Bürling, K., Hunsche, M. and Noga, G. (2011). Use of blue-green and chlorophyll fluorescence measurements for differentiation between nitrogen deficiency and pathogen infection in wheat. *Journal of Plant Physiology*, 168, 1641-1648.

Castro, K.L. and Sanchez-Azofeifa, A. (2008). Changes in spectral properties, chlorophyll content and internal mesophyll structure of senescing *Populus balsamifera* and *Populus tremuloides* leaves. *Sensors*, 8, 51-69.

Cromey, M.G., Butler, R.C., Mace, M.A. and Cole, A.L.J. (2004). Effects of the fungicides azoxystrobin and tebuconazole on *Didymella exitialis*, leaf senescence and grain yield in wheat. *Crop Protection*, 23, 1019-1030.

De Coninck, B.M.A., Amand, O., Delauré, S.L., Lucas, S., Hias, N., Weyens, G., Mathys, J., De Bruyne, E. and Cammue, B.P.A. (2012). The use of digital image analysis and real-time PCR fine-tunes bioassays for quantification of Cercospora leaf spot disease in sugar beet breeding. *Plant pathology*, 61, 76-84.

Di Bella, C.M., Paruelo, J.M., Becerra, J.E., Bacour, C. and Baret, F. (2004). Effect of senescent leaves on NDVI-based estimates of *f*APAR: experimental and modelling evidences. *International Journal of Remote Sensing*, 25, 5415-5427.

Dimmock, J.P.R.E. and Gooding, M.J. (2002). The effects of fungicides on rate and duration of grain filling in winter wheat in relation to maintenance of flag leaf green area. *Journal of Agricultural Science*, 138, 1-16.

Evans, L.T., Bingham, J., Jackson, P. and Sutherland, J. (1972). Effect of awns and drought on the supply of photosynthate and its distribution within wheat ears. *Annals of Applied Biology*, 70, 67-76.

Gan, S. and Amasino, R.M. (1997). Making sense of senescence: molecular genetic regulation and manipulation of leaf senescence. *Plant Physiology*, 113, 313-319.

Genty, B., Briantais, J.M. and Baker, N.R. (1989). The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta (BBA) – General Subjects*, 990**,** 87-92.

Gong, Y., Zhang, J., Gao, J., Lu, L. and Wang, J. (2005). Slow export of photoassimilate from stay-green leaves during late grain-filling stage in hybrid winter wheat (*Triticum aestivum* L.). *Journal of Agronomy and Crop Science*, 191, 292-299.

Gooding, M.J., Dimmock, J.P.R.E., France, J. and Jones, S.A. (2000). Green leaf area decline of wheat flag leaves: the influence of fungicides and relationships with mean grain weight and grain yield. *Annals of Applied Biology*, 136, 77-84.

Gopi, R., Jaleel, C.A. and Panneerselvam, R. (2008). Leaf anatomical responses of Amorphophallus campanulatus to triazoles fungicides. *EurAsian Journal of Biosciences*, 2, 46-52.

Guendouz, A. and Maamari, K. (2011). Evaluating durum wheat performance and efficiency of senescence parameter usage in screening under Mediterranean conditions. *Electronic Journal of Plant Breeding*, 2, 400-404.

Gregersen, P.L. and Holm, P.B. (2007). Transcriptome analysis of senescence in the flag leaf of wheat (*Triticum aestivum* L.). *Plant Biotechnology Journal*, 5, 192-206.

Grover, A. and Mohanty, P. (1992). Leaf senescence-induced alterations in structure and function of higher plant chloroplasts. In: Abrol, Y.P., Mohanty, P. and Govindjee, eds., Photosynthesis: Photoreactions to Plant Productivity. Kluwer Academic Publishers, The Netherlands, pp. 225-255.

Grossman, K., Kwiathowski, J., Häuser, C. and Siefert, F. (1994). Influence of the triazole growth retardant BAS. III. W on phytohormone levels in senescing intact pods of oilseed rape. *Journal of Plant Growth Regulation*, 14, 115-118.

Hack, H., Bleiholder, H., Buhr, L., Meier, U., Schnock-Fricke, U., Weber, E. and Witzenberger, A. (1992). Einheitliche Codierung der phänologischen Entwicklungsstadien mono- und dikotyler pflanzen – Erweiterte BBCH-Skala, Allgemein. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes*, 44, 265-270.

Hafsi, M., Mechmeche, W., Bouamama, L., Djekoune, A., Zaharieva, M. and Monneveux, P. (2000). Flag leaf senescence, as evaluated by numerical image analysis, and its relationship with yield under drought in durum wheat. *Journal of Agronomy and Crop Science,* 185, 275-280.

Hirose, N., Takei, K., Kuroha, T., Kamada-Nobusada, T., Hayashi, H. and Sakakibara, H. (2008). Regulation of cytokinin biosynthesis, compartmentalization and translocation. *Journal of Experimental Botany*, 59, 75-83.

Hörtensteiner, S. (2006). Chlorophyll degradation during senescence. *Annual Review of Plant Biology*, 57, 55-77.

Hsu, P. and Walton, P.D. (1971). Relationships between yield and its components and structures above the flag leaf node in spring wheat. *Crop Science*, 11, 190-193.

Juvany, M., Müller, M. and Munné-Bosch, S. (2013). Plant age-related changes in cytokinins, leaf growth and pigment accumulation in juvenile mastic trees. *Environmental and experimental Botany*, 87, 10-18.

Kaul, R. (1974). Potential net photosynthesis in flag leaves of severely drought-stressed wheat cultivars and its relationship to grain yield. *Canadian Journal of Plant Science*, 54, 811-815.

Khalil, I.A., Mercer, E.I. and Wang, Z.X. (1990). Effect of triazole fungicides on the growth, chloroplast pigments and sterol biosynthesis of maize. *Plant Science*, 66, 21-28.

Lim, P.O., Kim, H.J. and Nam, H.G. (2007). Leaf Senescence. *Annual Review of Plant Biology*, 58, 115-136.

Lu, Q., Lu, C., Zhang, J. and Kuang, T. (2002). Photosynthesis and chlorophyll a fluorescence during flag leaf senescence of field-grown wheat plants. *Journal of Plant Physiology*, 159, 1173-1178.

Lupton, F.G.H. (1968). The analysis of grain yield of wheat in terms of photosynthetic ability and efficiency of translocation. *Annals of Applied Biology*, 61, 109-119.

Mahlein, A.-K., Steiner, U., Hillnhütter, C., Dehne, H.-W. and Oerke, E-C. (2012a). Hyperspectral imaging for small-scale analysis of symptoms caused by different sugar beet diseases. *Plant Methods*, 8, 3; doi:10.1186/1746-4811-8-3.

Mahlein, A.-K., Oerke, E.-C., Steiner, U. and Dehne, H.-W. (2012b). Recent advances in sensing plant diseases for precision crop protection. *European Journal of Plant Pathology*, 133, 197-209.

Makunga, O.H.D., Pearman, I., Thomas, S.M. and Thorne, G.N. (1978). Distribution of photosynthate produced before and after anthesis in tall and semi-dwarf winter wheat as affected by nitrogen fertilizer. *Annals Applied Biology*, 88, 429-437.

Merzlyak, M.N., Gitelson, A.A., Chivkunova, O.B. and Rakitin, V.Y. (1999). Non-destructive optical detection of pigment changes during leaf senescence and fruit ripening. *Physiologica Plantarum*, 106, 135-141.

Montfort, F., Klepper, B.L. and Smiley, R.W. (1996). Effects of two triazole seed-treatments, triticonazole and triadimenol, on growth and development of wheat. *Pesticide Science*, 46, 315-322.

Mullet, J.E. (1988). Chloroplast development and gene expression. *Annual Review of Plant Physiology and Plant Molecular Biology*, 39, 503-532.

Müller, D. and Leyser, O. (2011). Auxin, cytokinin and the control of shoot branching. *Annals of Botany*, 107, 1203-1212.

Nason, M.A., Farrar, J. and Bartlett, D. (2007). Strobilurin fungicides induce changes in photosynthetic gas exchange that do not improve water use efficiency of plants grown under conditions of water stress. *Pest Management Science*, 63, 1191-1200.

Pepler, S., Gooding, M.J., Ford, K.E., Ellis, R.H. and Jones, S.A. (2005). A temporal limit to the association between flag leaf life extension by fungicides and wheat yields. *European Journal of Agronomy*, 22, 405-416.

Peñuelas, J., Baret, F. and Filella, I. (1995). Semiempirical indices to assess carotenoids/chlorophyll a ratio from leaf spectral reflectance. *Photosynthetica*, 31, 221-230.

Perrot-Rechenmann, C. (2010). Cellular responses to auxin: division versus expansion. *Cold Spring Harbor Perspectives in Biology*, 2: a001446.

Rawson, H., Hindmarsh, J., Fischer, R. and Stockman, Y. (1983). Changes in leaf photosynthesis with plant ontogeny and relationship with yield per ear in wheat cultivars and 120 progeny. *Australian Journal of Plant Physiology*, 10, 503-514.

Rouse, J.W., Haas, R.H., Schell, J.A. and Deering, D.W. (1974). Monitoring vegetation systems in the Great Plains with ERTS. In: Proceedings of the 3th Earth Resources Technology Satellite-1 Symposium. Greenbelt, MD NASA, 301-317.

Rumpf, T., Mahlein, A-K., Steiner, U., Oerke, E-C., Dehne, H-W. and Plümer, L. (2010). Early detection and classification of plant diseases with Support Vector Machines based on hyperspectral reflectance. *Computers and Electronics in Agriculture*, 74, 91-99.

Sankhla, N., Davis, T.D., Gehlot, H.S., Upadhyaya, A., Sankhla, A. and Sankhla, D. (1991). Growth and organogenesis in moth bean callus cultures as influenced by triazole growth regulators and gibberellic acid. *Journal of Plant Growth Regulation*, 10, 41-45.

Santos, M.J., Greenberg, J.A. and Ustin, S.L. (2010). Using hyperspectral remote sensing to detect and quantify southeastern pine senescence effects in red-cockaded woodpecker (*Picoides borealis*) habitat. *Remote Sensing of Environment*, 114, 1242-1250.

Savitzky, A. and Golay, J.M.E. (1964). Smoothing and differentiation of data by simplified least squares procedures. *Analytical Chemistry*, 36, 1627-1639.

Steddom, K., Bredehoeft, M.W., Khan, M. and Rush, C.M. (2005). Comparison of visual and multispectral radiometric disease evaluations of Cercospora leaf spot of sugar beet. *Plant Disease*, 89, 153-8.

Tanaka, M., Takei, K., Kojima, M., Sakakibara, H. and Mori, H. (2006). Auxin controls local cytokinin biosynthesis in the nodal stem in apical dominance. *The Plant Journal*, 45, 1028-1036.

Tewari, R.K., Singh, P.K. and Watanabe, M. (2012). The spatial patterns of oxidative stress indicators co-locate with early signs of natural senescence in maize leaves. *Acta Physiologiae Plantarum*; doi: 10.1007/s11738-012-1138-0.

Yoshida, S. (1972). Physiological aspects of grain yield. *Annual Review of Plant Physiology*, 23, 437-464.

Werner, T., Motyka, V., Strnad, M. and Schmülling, T. (2001). Regulation of plant growth by cytokinin. *PNAS*, 98, 10487-10492.

SUMMARY

The main objective of the present thesis was to investigate the effect of different fungicidal groups on different physiological parameters of wheat by the employment of non-invasive sensors and imaging techniques. Therefore, different studies were conducted under field as well as greenhouse conditions to determine the physiological response of wheat plants to the application of various fungicidal groups. The present study focused on the reliability of non-invasive sensors to detect and differentiate the effects of fungicides on plant physiology. The effect of four fungicidal groups on wheat physiology was assessed by the employment of five different non-invasive techniques. Furthermore, the first studies were performed to determine the reliability of field trials to study the potential beneficial effects of fungicides on crop plants and to determine the best environment for the development of the further studies.

The results obtained in this study can be summarized as follows:

- Foliar application of the carboxamide, the strobilurin and the azole compounds ministered an accurate disease control, which resulted in higher grain yield compared to the other fungicide treatments under field conditions. Furthermore, it was possible to observe and register the effect of the carboxamide fungicide bixafen on the senescence and yield formation of wheat. However, under field conditions, the presence of the main foliar pathogens of wheat influenced the green leaf area duration as well as the yield, generating a disadvantage for the fungicide treatments with low efficacy in disease control. Thus, in order to study the potential beneficial effects of fungicides on plant physiology it is necessary to perform the studies under disease-free conditions such as in the greenhouse, where it would be possible to separate the fungicidal activity from the potential effects that those active ingredients may cause on crop physiology.
- This study demonstrates that the pyrazole carboxamide bixafen induces physiological changes in wheat plants such as delayed start of ear emergence, increased green leaf area duration and higher physiological activity during grain filling due to the delay of plant senescence. In addition, an effect produced by this compound on morphogenesis was observed.
- It was possible in this study to determine the suitability of different sensors and imaging techniques to reveal changes in the senescence status of wheat plants

due to fungicide application. The physiological parameters recorded by the sensors were more sensitive than destructive methods, visual assessments or yield parameters. In this study, senescence-associated changes in wheat physiology were quantified by using ground-based optical sensors earlier than with the conventional methods. The potential of non-invasive methods such as gas exchange, chlorophyll fluorescence, spectrometry, imaging analysis and hyperspectral reflectance for an early detection of the effect of fungicide application on plant senescence was demonstrated in this study.

- In this study, the effects of some fungicidal groups on the tolerance of wheat to water deficit stress were also observed. Specifically, plants treated with the carboxamide compound bixafen were more tolerant to drought compared to the untreated control. Moreover, plants treated with this active ingredient were found to be more tolerant to different water deficit conditions compared to the other fungicide treatments. These findings are based on the assessment of some plant developmental parameters as well by the measurement of the canopy reflectance and temperature.

In summary, the results obtained in this study demonstrated an effect generated by the bixafen on wheat physiological parameters such as leaf life extension. Also, morphological changes such as increment of the leaf area and shoot branching were observed when this fungicidal group was applied. Therefore, a future goal should be to establish the possible effect that carboxamides may have on the balance of auxins and cytokinins since these two phytohormones are highly associated to shoot branching and leaf growth.

Additionally, the potential of non-invasive sensors to detect early changes in the senescence status of flag leaves due to fungicide application was demonstrated. Therefore, non-invasive sensors and imaging techniques serve as an excellent alternative to conventional screening methods. In addition, use of these types of techniques will allow the screening of the possible effects of new compounds on the senescence of crop plants. Finally, non-destructive hyperspectral sensing may be implemented in time series experiments for the screening of new compounds to study the possible effect that they might produce on the senescence status of plants.

ACKNOWLEDGEMENTS

First of all, I would like to thank **Prof. Dr. H.-W. Dehne** for agreeing to supervise me in this research effort and for his guidance and encouragement. Thank you for your mentorship while completing this work and for your constructive suggestions.

I would like to thank my second supervisor, **Prof. Dr. H. Goldbach** for agreeing to evaluate this thesis.

I would like to thank **Dr. Ulrike Steiner** and **Dr. Erich-Christian Oerke** for their outstanding support during my research work. They were always available to answer my questions and I have learned a lot from you both. Thank you for your patience and help.

I would like to acknowledge the financial support from **Bayer CropScience** and for the very interesting scientific discussions.

I would like to express my gratitude to my friend **Anne-Katrin Mahlein**, for her support, guidance and time, but most important for our friendship. Thank you Anita!

I am very grateful to my family (**Martin, Beatriz and Juan**) for their support and advice. They were always there supporting me and helping me through those tough moments. With their encouragement I feel that I was able to reach this goal in my professional career. Muchas gracias!

I would like to thank all members of **my family in Bonn** (my Latino-Spanish-French Italian-German-Iranian community) without their support and friendship I would never have accomplished this work.

I would like to thank **Jennifer Pollack** for reading and correcting my thesis.

Last but not the least; I would like to thank the **PK team**, for the good environment and good moments that we had together.