Institute of Phytomedicine

University of Hohenheim

Department of Weed Science (360b)

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From greenhouse to field practice:

Herbicide resistance detection using chlorophyll-fluorescence-imaging technology

Dissertation

submitted in fulfillment of the regulations to acquire the degree “Doktor der Agrarwissenschaften”

(Dr. sc. agr. in Agricultural Sciences)

to the

Faculty of Agricultural Sciences

presented by

Pei Wang

born in Nanjing, China

October 2016

This thesis was accepted as a doctoral thesis (Dissertation) in fulfillment of the regulations to acquire the doctoral degree "Doktor der Agrarwissenschaften by the Faculty of Agricultural Sciences at University of Hohenheim on 13.03.2017 (date: see the letter from the chairperson of the doctoral committee to the doctoral candidate)

Date of the oral examination: 13.03.2017

Examination Committee

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List of abbreviations

ACCase	Acetyl coenzyme A carboxylase
ALS	acetolactate synthase
ANOVA	analysis of variance
Asp	aspartic acid
ATP	adenosine triphosphate
BBCH	Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie
CCD	charge-coupled device
CoA	Coenzyme A
CF	chlorophyll fluorescence
CS	capsule suspensions
DAT	days after treatment
DOXP	1-Deoxy-D-xylulose 5-phosphate
EC	emulsifiable concentrate
EPSP	5-enolpyruvylshikimate-3-phosphate
et al.	et alia, and others
etc.	et cetera
EU	European Union
F_0	Dark fluorescence yield
Fm	maximal fluorescence yield

Fv/Fm	maximal PSII quantum yield
Gly	glycine
GR	Glyphosate Resistant
HRAC	Herbicide Resistance Action Committee
HSD	honest significant difference
IL	Illinois
Ile	isoleucine
LED	light-emitting diode
LD_{50}	median lethal dose
MoA	mode of action
N	nitrogen
PAM	pulse-amplitude-modulation
Pro	proline
PS I	photosynthesis system I
PS II	photosynthesis system II
Q_B	a protein-bound plastoquinone
RR	roundup ready
SC	suspension concentrates
SG	water-soluble granules
SL	soluble (liquid) concentrates
Trp	tryptophan

UK	United Kingdom
USA	United States of America
US$	United States Dollars
VLCFA	very long chain fatty acid
WG	water dispersible granules
WHC	water holding capacity
WSSA	Weed Science Society of America

From greenhouse to field practice: Herbicide resistance detection using chlorophyll fluorescence imaging technology

This thesis is structured in four chapters. First, the introduction described the objectives of the thesis. In the second part, the publications of the research project were listed. In the main chapter, the scientific manuscripts are included. In the fourth chapter, a general discussion was fixed.

Chapter 1 Introduction

Weed control is one of the most important aspects in cultivation agriculture. It enhances the crops' possibility for the competition of the light, water, nutrition and space, etc. Instead of manual hoeing or removal of weeds before 19th century, today, machinery and chemicals are the most common tools of farmers for the weed control. This thesis focuses on an important issue in weed science, the herbicide resistance, which was first reported after the use of herbicide since 1950s (Shaner and Beckie, 2014). The objectives of this thesis are,

(i) to clarify that if the sensitive and herbicide resistant weeds can be identified by a chlorophyll fluorescence sensor shortly after herbicide application; and if abiotic factors can influence the identification;

(ii) to verify that if the chlorophyll fluorescence imaging technology on herbicide resistance detection is capable for field application;

(iii) to evaluate its robustness concerning herbicide resistant weed detection;

(iv) to investigate that if the chlorophyll fluorescence imaging technology is capable of evaluating herbicide stress on crops shortly after emergence.

1. Herbicide and herbicide resistance

Herbicides are the chemicals used to control weeds. The application of herbicides is now one of the most efficient tools by the farmers for weed control. Since 1945, when the selective herbicide 2,4-D (2,4-Dichlorophenoxyacetic acid) was discovered, the market of herbicide has expanded to US$ 22.3 billion until 2014 (Transparency Market Research, 2015). Why there is such a high demand? It is estimated that each dollar invested in the USA on agrochemicals can bring the farmer five dollars income. And similarly, each pound in the UK can generate six pounds (Cobb and Reade, 2010).

Furthermore, the use of pesticide provides not only financial rewards to the farmers, but also improved the crop yield and food quality. Lots of safer, lower dosage and more environmentally protected products are replacing the old compounds (Rüegg *et al.*, 2007). Farmers and consumers have all benefit from the increasing competition of the agrochemical industry.

According to the site of actions, the Herbicide Resistance Action Committee made a classification for all herbicides. There are more than 20 groups of herbicides on market right now. Among them, the photosynthesis system II inhibitors, branched chain amino acid synthesis inhibitors (acetolactate synthase inhibitor, ALS inhibitor) and lipid synthesis inhibitors (Acetyl coenzyme A carboxylase inhibitor, ACCase inhibitor) are the mainly concerned herbicides in this thesis.

However, with decades' use of herbicides, some weeds generate the inherited ability to survive the herbicides toxicity. As the result, less or even none weeds are controlled after the herbicide application. This character is named resistance by weed scientists (WSSA, 1998). To be specified, the definition of herbicide resistant and herbicide tolerance should be introduced.

Herbicide resistance: "Herbicide resistance is the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type. In a plant, resistance may be naturally occurring or induced by such techniques as genetic engineering or selection of variants produced by tissue culture or mutagenesis."

Herbicide tolerance: "Herbicide tolerance is the inherent ability of a species to survive and reproduce after herbicide treatment. This implies that there was no selection or genetic manipulation to make the plant tolerant; it is naturally tolerant." (WSSA, 1998)

The first herbicide resistance case was reported in the United States in 1950's. Resistance by field bindweed to 2,4-D was found in 1964 and resistance by common groundsel to triazine herbicides was appeared in 1970 (Cobb and Reade, 2010). There

are already 471 reported herbicide resistant cases with 250 species, which covers more than 90 percentages of all the herbicide groups (Heap, 2016). Furthermore, the recent survey shows that it takes shorter and shorter period for the weeds to generate the resistant ability after a new herbicide being introduced to the market. First resistant cases were reported to ALS and ACCase herbicides within 5 years after the introduction of these herbicide groups, while it took more than 20 years to find the first reported resistant species to 2,4-D (Shaner, 1992). Glyphosate Resistant (GR) crops were commercially introduced in 1996 for the first time. The farmers rapidly adopted GR crops because of the simple and effective application. However, the glyphosate resistant weeds were discovered in the same year within ryegrass and goose grass (Heap, 2016). Now, the glyphosate resistant weeds have been discovered in many countries and become a worldwide challenge (Powles, 2008).

Meanwhile, many reported cases showed the fact that resistant species are surviving more than one single herbicide (Heap, 2013). The cross resistance and multiple resistance have evolved in many weed species. The cross resistant weeds can survive two or more herbicides with the same mode of action. Usually, the cross resistant weed has only one resistance mechanism. The multiple resistant plants are resistant to two or more herbicides with different modes of action. In this case, the multiple resistant weeds may have more resistance mechanisms (HRAC, 2009; Vencill *et al.*, 2012).

Many herbicide resistance problems were attributed to the inappropriate use of herbicides. Due to the cultivation practice by farmers and weed scientists, both reduced and high rates of herbicide applications may induce resistance problems (Norsworthy *et al.*, 2012). Long-term high rate application of herbicides in same mode of action would cause target site resistance. With high rate applications, only the genetically unique populations will survive. In this case, cross-resistance against the herbicides in the same mode of action, especially in the same chemical class, may be generated (de Carvalho, *et al.* 2009). On the other hand, repeated use of reduced rates of herbicides by years would lead to metabolic resistance. The reduced rate

application cannot kill the majority weeds or even be failed to kill the weeds. It enables the weeds to survive after the metabolism and then reproduce the next generations that can fit the herbicide application. Multiple resistance happens frequently but not only in the metabolic resistant populations (Powles and Preston, 1995; Foes *et al.* 1999).

2. Mechanism and detection of herbicide resistance

2.1 Target site and non-target site resistance

Herbicide resistance can be described as the plants' capacity to survive, grow and reproduce after certain herbicide treatment(s). A plant may express "target site" and "non-target site" resistance (Prather *et al.*, 2000). The target sites are usually some enzymes of in the plants. In these enzymes, the active ingredients of the herbicides can bind and interfere the plants' physiological processes (Nandula, 2010). If a mutation takes place on a gene that expresses a certain herbicide target enzyme, the molecular structure of this enzyme will be altered. As a result, the herbicide can never bind on the enzyme and inhibit its activities again (Neve, 2007). Beside gene sequence mutations, gene overexpression and gene duplication are also known as mechanisms of target site resistance (Beffa *et al.*, 2012). The target site resistance is the main mechanism of weeds against ALS-, ACCase- and PS II-inhibitors (Powles and Preston, 1995, 2006; Öttmeier, 1999; Powles and Yu, 2010).

The non-target site resistance includes different mechanisms apart from the mechanism of target-site resistance. Some non-target site resistant weeds, for instance, the barnyard grass and velvetleaf, may have enhanced metabolism capacity to detoxify the active ingredients of the herbicides (Anderson and Gronwald, 1991; Carey *et al.*, 1997). Reduced absorption and translocations can prevent herbicide ingredients reaching the plants' target site (Powles and Preston, 2006). Sequestration was reported by Ge *et al.* (2010) to be another way of weed to inactive glyphosate's physiological effects in vivo the weeds. It means that the herbicide may bind to other molecular such

as a sugar moiety. The herbicides can also be removed from active metabolic region of the plant cell (Yuan *et al.*, 2007).

2.2 Detection of herbicide resistance

To make efficient strategy for controlling herbicide resistant weeds, reliable methods should be established to diagnose the herbicide resistance (Beffa *et al.*, 2012). Greenhouse and laboratory tests are most commonly adopted to identify the herbicide resistant populations. Moreover, in order to understand the mechanism of the recognized resistant cases, biochemical, molecular and analytical assays are also invested.

Greenhouse bioassay, or whole plant assessment, usually includes several groups of herbicides. The results are based on the visual evaluation, mortality rates, as well as measuring the dry and fresh weight of the plants under different treatments (Moss, *et al.*, 1998). It can identify resistant weed populations without concerning the plant is target site resistant or non-target site resistant. The results can be influenced by environmental conditions of each greenhouse, such as temperature, humidity, light intensity and period. Thus, standardized monitoring protocol was claimed (Beckie *et al.*, 2000). In most whole plants assessment cases, herbicides are applied with series of doses, so that more quantitative results like LD_{50} (median lethal dose), resistance factor (R/S ratio) or dose response curves can be calculated (Streibig, 1988; Northworthy *et al.*, 1998; Beckie *et al.*, 2000). Even laboratory-based bioassays have already been designed to reduce some time and space consumption (using filter paper or agar), both methods require high expense and seasoned evaluators (Beffa *et al.*, 2012).

Biochemical assays, also known as enzyme assays, provide knowledge about the target site resistant biotypes. It focuses on the known mutations in the gene sequence. That means the target enzyme (protein) is re-expressed and recognized by the evaluators before assays. The test is usually performed with enough purified protein from high mount of harvested living plants. Nevertheless, the performance needs

much time and can only be operated by well-trained technicians. This method cannot detect non-target site resistant and multiple resistant biotypes (Powles and Yu, 2010).

More detailed knowledge of the target site resistance mechanism can be gained by molecular assays (Beffa *et al.*, 2012; Kaundun *et al.*, 2011a). It introduces the gene sequence analyses technology to learn about the known mutations as well as search for the novel mutations (Kaundun and Windass, 2006; Kaundun *et al.*, 2006; Petersen *et al.*, 2010). The technology of pyrosequencing has been widely used in analysis for ALS and ACCase target site mutations (Powles and Yu, 2010). Some detailed protocols were described (Petersen *et al.*, 2010; Hess *et al.*, 2012). Furthermore, this technology can also be applied in the investigation of non-target site resistance (Beffa *et al.*, 2012). Several approaches have been developed to evaluate gene copy number (Gaines *et al.*, 2010) and gene expression capacity (Peng *et al.*, 2010; Yuan *et al.*, 2010). However, the herbicides metabolism chains more mostly displayed by analytical assays using ^{14}C-radiolabelled compounds. With this method, it is possible to quantitatively and qualitatively determine the enhanced metabolic resistance in most weeds (Beffa *et al.*, 2012).

Even though so many approaches for herbicide resistance detection exist, the mentioned assays can only be done after realizing the problem. Besides, they are time consuming, expensive and profession-dependent.

3. Photosynthesis system II, chlorophyll fluorescence and sensor-based stress detection

Photosynthesis system II (PS II) is the first light reaction center of oxygenic photosynthesis. Within the photosystem, electron transfer is active by the enzymes, known as chlorophyll dimers (P680 for PSII, P700 for PS I), from the energy of light photons (Rutherford and Faller, 2003). Besides photochemistry, the rest energy of light photons quenches as heat and fluorescence radiation. This fluorescence radiation is called chlorophyll fluorescence (CF) (Maxwell and Johnson, 2000).

In 1960, Kautsky *et al.* (1960) found a rapid rise in fluorescence from PS II, followed by a slow decline, upon illumination of a dark-adapted leaf. It is called the *Kautsky Effect*. At lower PS II reaction level, the CF will increase, as more energy from absorbed light needs to be emitted. This happens when the PS II reaction center is closed. In which state, the PS II has not passed electrons to a subsequent electron carrier. And as the result, the chlorophyll dimers P680 cannot accept more electrons (Maxwell and Johnson, 2000).

Measurement of PS II chlorophyll fluorescence has been widely used in the researches for plants and algae physiologies under biotic and abiotic stresses (Quick and Horton, 1984; Maxwell and Johnson, 2000). Janka *et al.* (2015) reported a significant reduction of quantum yields of PS II under high irradiance and high temperature in chrysanthemum. The low temperature and frost also inhibit the efficiency of PS II activities according to many publications (Ottander and Öquist, 1991; Örlander, 1993; Lundmark *et al.*, 1998b). Besides, other abiotic impacts, such as salt and drought, can enhance the photo-inhibition as well (Neale and Melis, 1989; Burke, 2007; Burke *et al.*, 2010). In weed science research, the parameter Maximum PS II Quantum Efficiency (*Fv/Fm*) is frequently used for the description of herbicide efficacy as PS II inhibitors (Flores *et al.*, 2013). Recently, a chlorophyll fluorescence imaging method was applied to assist dose response screening and herbicide resistance detection in laboratory and greenhouse (Kaiser *et al.*, 2013). The herbicide efficacy was determined two to four days after treatment. The results of this test correlated well with the conventional whole-plant assessment.

With the technology of chlorophyll fluorescence imaging measurements, researchers can rapidly assess the herbicide treatments quantitatively and qualitatively. But the system is based in laboratory. Seeds collection is required by most greenhouse and laboratory bioassays. The in-season field detection of herbicide efficacy is not available. Thus, the farmers can only benefit from the existing detection methods for next growing season. But the yield loss risk cannot be avoided due to the herbicide resistance problems in their fields.

4. Identification herbicide stress on crops

Herbicides could injure the crops when inappropriate application is taken in the fields, such as overrate treatment, improper application time or incorrect mixture application with several ingredients (Salzman and Renner, 1992; Johnson *et al.*, 2002). Visual assessment is the most common method to evaluate the herbicide stress on crops. Many researches have demonstrated the correlation for yield loss and injury symptoms of the stressed crops (Weidenhamer, *et al.*, 1989; Bailey and Kapusta, 1993). New technologies like machine vision and spectrum measurement enable agronomists to evaluate the relative yield loss according to parameters such as percentage of ground cover, light reflectance and etc. (Adcock *et al.*, 1990; Donald, 1998). Nevertheless, these approaches can only be applied when the stress symptoms have developed. It requires relatively a long period of time so that the features can be distinct. As chlorophyll fluorescence imaging system is a sensitive method to identify physiological rather than physical features of the stress on plants (Maxwell and Johnson, 2000; Schreiber, 2004; Janka *et al.*, 2015), it can be a potential faster approach to detect the herbicide stress on crops. This would support farmers to make proper cultivation strategies to overcome herbicide stress effects on soybeans in the same growing season.

Chapter 2 Publications

The present thesis consists of three scientific articles as reflected in Chapter 3 by Section I - III. These articles have been published or under peer review.

Section I

Wang, P., Peteinatos, G., Li, H., Braendle, F., Pfuendel, E., Drobny, H.G., Gerhards, R. Rapid stress monitoring on *Alopecurus myosuroides* using chlorophyll fluorescence imaging technology. Journal of Plant Diseases and Protection. (under review)

Section II

Wang, P., Peteinatos, G., Li, H., Gerhards, R. (2016) Rapid in-season detection of herbicide resistant *Alopecurus myosuroides* using a mobile fluorescence sensor. Crop Protection 89, 170-177.

Section III

Wang, P., Weber, J., Gerhards, R. Early identification of herbicide stress on soybean (*Glycine max* (L.) Merr.) using chlorophyll fluorescence imaging technology. Biosystems Engineering (under review)

Beyond the three articles included in the main Chapter of this thesis, five contributions were published in proceedings of international conferences as well as one more paper as co-author published in a peer reviewed journal.

Wang, P., Peteinatos, G., Gerhards, R. (2017) In field identification of herbicide resistant *Apera spica-venti* using chlorophyll fluorescence. Proceedings of the 11th European Conference on Precision Agriculture, Edinburgh, UK. (Accepted)

Wang, P., Peteinatos, G., Li, H., Gerhards, R. (2016) Temperature effects on chlorophyll fluorescence of *Alopecurus myosuroides*. 7th International Weed Science Congress, Prague, Czech Republic.

Koecher, C., Braendle, F., **Wang, P.**, Pfuendel, E., Drobny, H.G., Gerhards, R. (2016) QWERT® – a innovative system to reduce weed resistance in-season. 7th International Weed Science Congress, Prague, Czech Republic.

Wang, P., Li, H., Gerhards, R. (2016) Chlorophyll fluorescence response to herbicide stress in *Alopecurus myosuroides*. Proceedings 27th German Conference on Weed Biology and Weed Control, February 23-25, 2016, Braunschweig, Germany. pp. 57-67.

Wang, P., Kaiser, Y.I., Menegat, A., Gerhards, R. (2015) Weed PAM: A rapid in-season herbicide resistance detector. 17th European Weed Research Society Symposium, Montpellier, France.

Li, H., Qi, L., **Wang, P.** (2014) 3-D simulation for airflow field and droplets deposition of hanging cold sprayer. Transactions of the Chinese Society of Agricultural Machinery, 45(4):103-109, 122.

Chapter 3 Scientific Contributions

Section I

This article focuses on testing whether PS II activities of herbicide sensitive and resistant *A. myosuroides* can be stressed after application of ingredients in different action modes. Meanwhile, abiotic factors influence on PS II activities of *A. myosuroides*, including drought and nitrogen shortage stress, were clarified to determine its impact on herbicide resistance level classification.

Rapid stress monitoring on *Alopecurus myosuroides* using chlorophyll fluorescence imaging technology

Submitted to: Journal of Plant Diseases and Protection (Under review)

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Abstract

Sensor based stress recognition is an effective tool for improving herbicide efficacy and selectivity. In this study, a chlorophyll fluorescence imaging sensor was used for measuring Maximal Photosystem II Quantum Yield (*Fv/Fm*). Six herbicides were tested on herbicide sensitive and resistant *Alopecurus myosuroides* populations. Furthermore, it was investigated, how abiotic stress due to water shortage and nitrogen deficiency influenced the *Fv/Fm* of the sensitive populations. Results showed that ALS- and ACCase-inhibiting herbicides significantly reduced *Fv/Fm* of sensitive populations compared to the untreated plants three days after treatment. ALS- and ACCase-inhibiting herbicides did not affect resistant populations. *Fv/Fm* values were equal to the untreated control plants. The PSII-inhibiting herbicides *isoproturon* reduced *Fv/Fm* of sensitive and resistant populations. A differentiation of sensitive and resistant weeds based on *Fv/Fm* values was possible only after 4 DAT. Water shortage stress was detected seven days after treatment, when the visual symptoms from water shortage were quite severe on the plants. Nitrogen deficiency could not be identified using chlorophyll fluorescence imaging. We conclude that the chlorophyll-

fluorescence-imaging sensor has a great potential for identifying herbicide resistant weed populations in the field shortly after herbicide application.

Keywords: chlorophyll fluorescence, Maximal PS II Quantum Yield, herbicide resistance, abiotic stress, *Alopecurus myosuroides*

1. Introduction

Alopecurus myosuroides (black-grass) is one of the most problematic weeds in Western Europe. It can severely infest winter cereal fields and cause yield losses of 20% at infestations of 100 plants/m^2 (Blair *et al.*, 1999). With higher proportions of winter cereals in the rotation, earlier sowing dates and the application of non-inversion tillage systems, the infestation levels of *A. myosuroides* have increased during the last 30 years (Melander, 1995). The high infestations of plants enabled some populations to survive the herbicide applications with standard doses. Many *A. myosuroides* populations in Europe were recorded being resistant to herbicides inhibiting acetolactate synthase (ALS), acetyl CoA carboxylase (ACCase) and photosynthesis system II (PS II) (Drobny *et al.*, 2006; Neve, 2007).

In order to manage herbicide resistant weed populations, a reliable detection procedure is needed. A common approach for herbicide resistance screening is the whole-plant bioassay in the greenhouse (HRAC, 1999). The tests require collecting seeds of suspicious weed populations in the field. Emerged plants are sprayed with different herbicides and rate of herbicides. Efficacy is measured and estimated approximately 3-4 weeks after application in relation to an untreated control. In general, the test is simple and gives reliable results of herbicide sensitivity. However, it is relatively expensive and time consuming. Moreover, results are available only for the next growing season. Detailed knowledge of the target site resistance mechanism can be gained by molecular assays (Beffa *et al.*, 2012). Target genes are sequenced and analyzed for mutations causing herbicide resistance. Kaundun *et al.* (2011b) introduced an in-season method to create herbicide resistance profiles of weed species. Seedlings of *Lolium rigidum* L. and *Lolium multiflorum* Lam. were transplanted into agar containing herbicide solutions. A visual assessment of root and shoot

development was made 10 days after transplanting. The results of this agar-based method correlated well with the conventional whole-plant assessment. Kaiser *et al.* (2013) presented a similar method for *A. myosuroides* in the greenhouse. However, herbicide efficacy in weed seedlings was quantified using chlorophyll fluorescence imaging technology. The herbicide efficacy was determined two to four days after treatment. The results of this test correlated well with the conventional whole-plant assessment.

Chlorophyll fluorescence is a sensitive indicator of the physiological status of plants. It can be used to detect abiotic and biotic stress in crops and weeds (Roháček and Barták, 1999; Maxwell and Johnson, 2000; Roháček, 2002; Baker, 2008). The parameter Maximal PS II Quantum Yield (*Fv/Fm*) correlated well with herbicide stress in plants shortly after treatment (Ahrens *et al.*, 1981; Ali and Machado, 1981; Hensley, 1981; Vencill and Foy, 1988; van Oorschot and van Leeuwen, 1992). However only in the latest studies, chlorophyll fluorescence imaging was applied to differentiate between sensitive and resistant weed populations (Kaiser *et al.*, 2013; Wang *et al.*, 2016).

The first objective of this study was to investigate the temporal response of sensitive and resistant populations of *A. myosuroides* to herbicide treatments with different modes of action using a new chlorophyll fluorescence imaging sensor. It was tested when herbicide sensitive and resistant *A. myosuroides* populations could be differentiated. The second objective was to test how a sensitive population of *A. myosuroides* responded to abiotic stress induced by water shortage and nitrogen deficiency.

2. Materials and Methods

2.1 Chlorophyll fluorescence imaging sensor (Weed PAM®)

The sensor Weed PAM® is a mobile version of Imaging-PAM® fluorescence meter by Heinz Walz GmbH, Effeltrich, Germany. The sensor uses LEDs that excite blue light with 460 nm wavelength. These LEDs alternate actinic illumination light

with strong pulses to saturate photosystem II. Chlorophyll fluorescence is detected with an imaging camera containing a cut-off filter at 620 nm wavelength in front of the lens. The camera was centrally mounted on the head containing the LEDs. The measured signal corresponds to the fluorescence excited by the saturated pulses and, thus, variation in the measuring signal can be attributed to changes in chlorophyll levels. The background noise was removed by software as described in Kaiser *et al.* (2013). The Weed PAM® fluorescence meter was operated using the software "ImagingWin for Weed PAM®" (Heinz Walz GmbH, Effeltrich, Germany). The software controls LED function and generates pictures of various fluorescence levels. This work uses images of F_0 (minimal fluorescence in the dark-acclimated state) and *Fm* (maximal fluorescence in the dark-acclimated state in the presence of a saturation pulse). From these two fluorescence levels, the parameter *Fv/Fm* was calculated for each pixel according to the equation.

$$F_V/F_m = \frac{F_m - F_0}{F_m}$$

2.2 Experimental setup

Two independent experiments were conducted in the greenhouse to investigate the herbicide effects on chlorophyll fluorescence of sensitive and resistant *A. myosuroides* at the University of Hohenheim, Germany in 2015. Another two independent experiments were conducted in the greenhouse to study the impact of water shortage and nitrogen deficiency on *A. myosuroides* in 2015. Experiment 2 was a repetition of experiment 1 and experiment 4 was carried out in the same way as experiment 3. Data were pooled over both repeated experiments. For all experiments, the plants were cultivated in plastic pots with a diameter of 10 cm filled with a mixture of 50% clay, 25% silt, and 25% sand. One *A. myosuroides* was transplanted in each pot, when it was in the 1-leaf-stage. Plants were grown in a light cycle of 16 h day and 8 h night. The temperature was kept at 25 °C during the day and 15 °C at night. All pots were placed in a complete randomized block design with three blocks. Each treatment contained three pots.

The first experiment was done to identify the effect of herbicides on *Fv/Fm* of *A. myosuroides*. Two populations of *A. myosuroides* were used, one herbicide sensitive population (Herbiseed, UK) and one multi-resistant population collected from a field near Heilbronn, Germany. The latter population was proved to be multi-resistant to ALS-, ACCase- and PS II-inhibitors in a standard greenhouse bioassay at the University of Hohenheim (Gerhards, 2013). The population did not have any mutation on alleles Pro-197, Trp-574, Ile-1781, Trp-2027, Ile-2041, Asp-2078 and Gly-2096. DNA sequencing was conducted at Identxx GmbH, Stuttgart, Germany.

Treatments of the first and second experiment are listed in Table 2. A laboratory track sprayer chamber mounted with a single flat fan nozzle was used for the herbicide application (8002 EVS, TeeJet Spraying Systems Co., Wheaton, IL, USA). The sprayer was calibrated for an applying volume of 200 L/ha, at a speed of 800 mm/s and a spraying pressure of 300 kPa. Foliar applications were performed 50 cm above the soil surface.

In the third and fourth experiment, the effect of water shortage and nitrogen deficiency on a herbicide sensitive *A. myosuroides* population was investigated using chlorophyll fluorescence imaging. For each factor, 2 levels were used: stressed and non-stressed. Drought was induced by keeping the plants constantly at 40% of the soil Water Holding Capacity (WHC). Non-stressed plants were kept at 70% of the WHC. Irrigation of all plants was performed twice per day. The amount of water was determined according to the weight of the pots. Calibration was done before the setup of the study. Plants with nitrogen deficiency did not receive any nitrogen fertilizer. Non-stressed plants were fertilized with 70 mg N per pot, when the plants were in the 2-leaf stage. Experiments started when the plants of the control group were in the 2-leaf stage. Plants were cut for measuring biomass 2-3 weeks later, when they had produced 3 tillers. A summary of the four treatment combinations is presented in Table 3.

Prior to the *Fv/Fm* measurements, plants were dark-adapted underneath measuring boxes (Figure 1) for 20 minutes. In first and second experiment, plants

were measured daily from 1 DAT until 7 DAT and then again 10 DAT and 14 DAT. In the third and fourth experiment, plants were measured every two days for two weeks after stress induction.

Visual assessments in the first and second experiment were performed 21 DAT. The plants were classified as “dead” (sensitive) and “alive” (resistant). The herbicide resistance level was classified according to Moss et al. (2000). Mortality of 100-81% was classified as "S", sensitive, 80-73% as "R?", slightly resistant, 72-37% as "RR", partly resistant, 36-0% as "RRR", resistant. Plants of the third and fourth experiment were cut 0.5 cm above ground at DAT 21 and then dried at 80° C. After 48 h, dry biomass was determined. Plants were classified as “stressed”, when dry biomass was significantly lower than the unstressed control plants.

2.3 Statistical analysis.

Data analysis was done with RStudio 0.98.490 (R Development Core Team, 2013). Analysis of variance (ANOVA) was carried out for *Fv/Fm*, data and biomass measurements. Means were compared by *Tukey's HSD* test. Data were tested for normal distribution using the *Shapiro-Wilk* test ($p > 0.05$). Equality for heterogeneity of variances for resistant and sensitive and stressed/unstressed groups was tested using *Levene's* test for each date and treatment ($p > 0.05$). Data of equal DAT per experiment were pooled together.

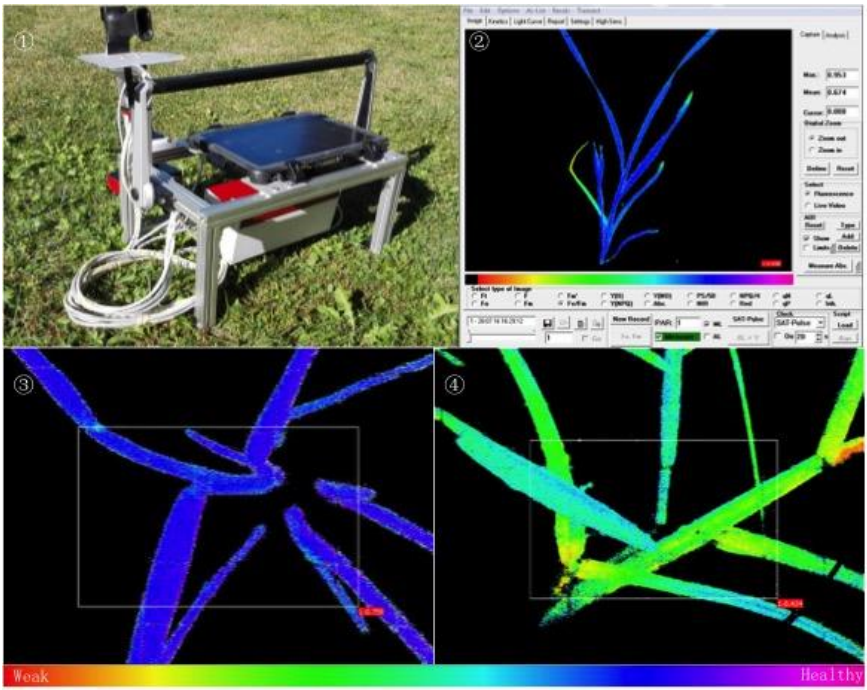


Figure 1. The Weed PAM® chlorophyll fluorescence imaging sensor, ① A picture of the sensor. It consists of the camera control unit and the computer including software. ② The software graphical interface. ③ A resistant *A. myosuroides* to *pinoxaden* (ACCase inhibitor) measured 3 DAT. The blue shift responds to higher *Fv/Fm* and higher vitality. ④ A sensitive plant to *pinoxaden* (ACCase inhibitor) measured 3 DAT. The red shift responds to lower *Fv/Fm* and stress.

Table 1. Profile of the standard greenhouse bioassay for the *A. myosuroides* population near Heilbronn, Germany. The herbicide efficacy was evaluated according to Moss *et al.(2000).* a.i.g/ha = active ingredients gram per hectare; M = *mesosulfuron*; I = *iodosulfuron*.

Herbicide	**Dose (a.i.g/ha)**	**Effect**
meso-/iodosulfuron	15 (M) + 3 (I)	RR
pinoxaden	60	RR
isoproturon	600	RRR

Table 2. Details of the herbicide treatments. The rates used for the treatment were prepared according to the manufacturer recommendation, which was calculated for a spray volume of 200 l ha^{-1} with the active ingredient rate showed in the table. MoA = Mode of Action by HRAC groups; A = acetolactate synthase (ALS) inhibitors; B = acetyl coenzyme-A carboxylase (ACCase) inhibitors; C = PSII inhibitors; M = *mesosulfuron*; I = *iodosulfuron*; a.i.g/ha = active ingredients gram per hectare

No.	**Trade Name**	**Active Ingredient**	**Formulation**	**MoA**	**Rate (a.i.g ha^{-1})**	**Provider**
1	Control	-		-	-	-
2	Atlantis® WG	*29.2 g/kg mesosulfuron, 5.6 g/kg iodosulfuron+ adjuvant*	WG	B	15 (M) + 3 (I)	Bayer CropScience
3	Attribut®	*700 g/kg propoxycarbazone*	SG	B	70	Bayer CropScience
4	Lexus® SX®	*500 g/kg* flupyrsulfuron	WG	B	20	DuPont de Nemours
5	Topik® 100	*89.1 g/L clodinafop*	EC	A	107	Syngenta Agro
6	Axial® 50	*50 g/L pinoxaden*	EC	A	60	Syngenta Agro
7	Arelon® Top	*500 g/L isoproturon*	SC	C	600	Cheminova Deutschland

Table 3. Treatments of nitrogen deficiency and water shortage stress; plants of *A. myosuroides* were exposed to different irrigation and fertilizing levels. Water shortage stress was achieved by reducing the water content of soil close to the wilting point, and the nitrogen deficiency stress was obtained by omitting fertilization. Abbreviations: W= drought stress; N = nitrogen deficiency stress.

Treatment	**Water Content**	***KNO_3*(mg)**
Control	70%	505.55
N	70%	0
W	40%	505.55
W + N	40%	0

3. Results and Discussions

3.1 Herbicide effect on chlorophyll fluorescence experiments

The visual assessment 21 DAT and the standard greenhouse biotest showed that the sensitive *A. myosuroides* population obtained from HerbiSeed was susceptible to all tested herbicides. The population from Heilbronn (Germany) was resistant against all tested herbicides. This agrees with the findings of Gerhards (2013), where this resistant population was firstly reported. In the unsprayed control treatments, no significant differences of *Fv/Fm* were found between the sensitive and resistant populations (Figure 2). Yet, the values for both treatments varied over the period of measurements. In herbicide treatments, *Fv/Fm* of the resistant populations were higher than the sensitive plants. This corresponds to Kaiser *et al.* (2013), who found that a *meso-/iodosulfuron* treatment reduced *Fv/Fm* of a susceptible *A. myosuroides* population but not in the three resistant populations. In a field survey of 50 *A. myosuroides* populations, Wang *et al.* (2016) could also differentiate between resistant and sensitive populations 5 DAT with different ACCase- and ALS-inhibitors using chlorophyll fluorescence imaging.

In all three treatments with ALS inhibitors, the *Fv/Fm* of the sensitive population rapidly decreased within 3 DAT. Greatest *Fv/Fm* difference between the sensitive and resistant populations were recorded 3 DAT. Then, at 4 DAT a reduction of *Fv/Fm* was also observed for the resistant population. Despite this decrease, the *Fv/Fm* of the resistant population was still significantly higher than of the sensitive population. Afterwards, the resistant population recovered gradually to the level around the beginning of measurement. ALS-inhibitors interfere with the production of the amino acids valine, leucine and isoleucine (Whitcomb, 1999; Oettmeier, 1999). These are essential amino acids for the turnover of the D protein in the PSII-electron transport chain. That results in higher degradation rate of the protein than the synthesis rate. (Xiong *et al.*, 1996; Rutherford and Faller, 2003). Hence, electrons are transferred much slower in the electron transport chain. This results in a suboptimal function of

PS II, which can be observed as a lower *Fv/Fm* (Allen and Williams, 1998; Ventrella *et al.*, 2010).

Table 4. Mortality rate (%) of *A. myosuroides* 21 days after herbicide treatment and resistance classification according to Moss e*t al.* (2000): 100-81% = "S", sensitive, 80-73% = "R?", slightly resistant, 72-37% = "RR", partly resistant, 36-0% = "RRR", resistant

Population	**Treatment**						
	Control	*Meso-/iodosulfuron*	*Propoxycarbazone*	*Flupyrsulfuron*	*Clodinafop*	*Pinoxaden*	*Isoproturon*
Sensitive	0	100	94	83	83	100	94
	-	S	S	S	S	S	S
Resistant	0	38	11	0	6	0	28
	-	RR	RRR	RRR	RRR	RRR	RRR

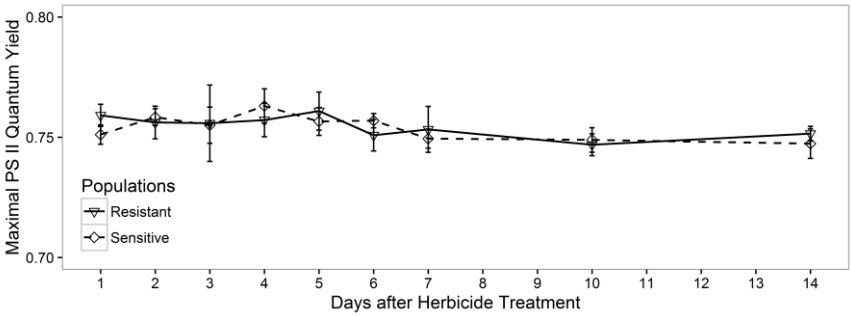
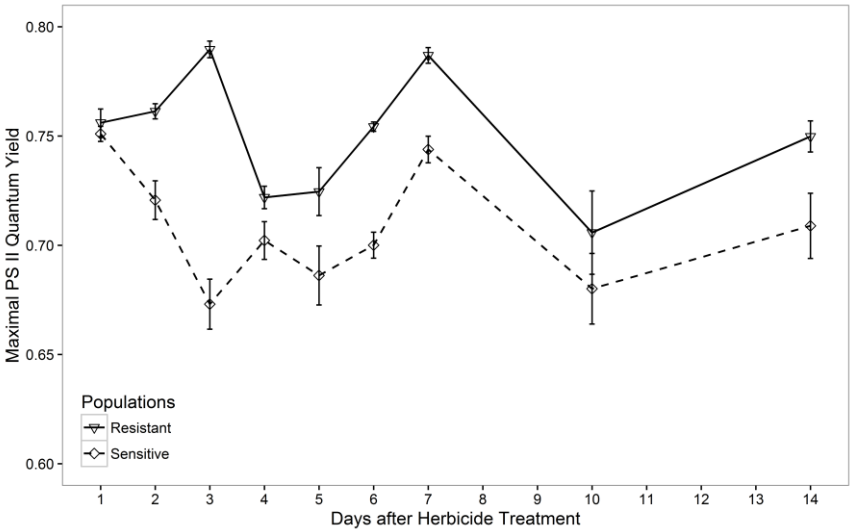


Figure 2. Daily presentation of the *Fv/Fm* of the control treatment. Mean *Fv/Fm* and the standard error are shown for the sensitive and the resistant populations of *A. myosuroides*.


Maximal PS II Quantum Yield
0.80
0.75
0.70
0.65
0.60
Populations
Resistant
Sensitive
1
2
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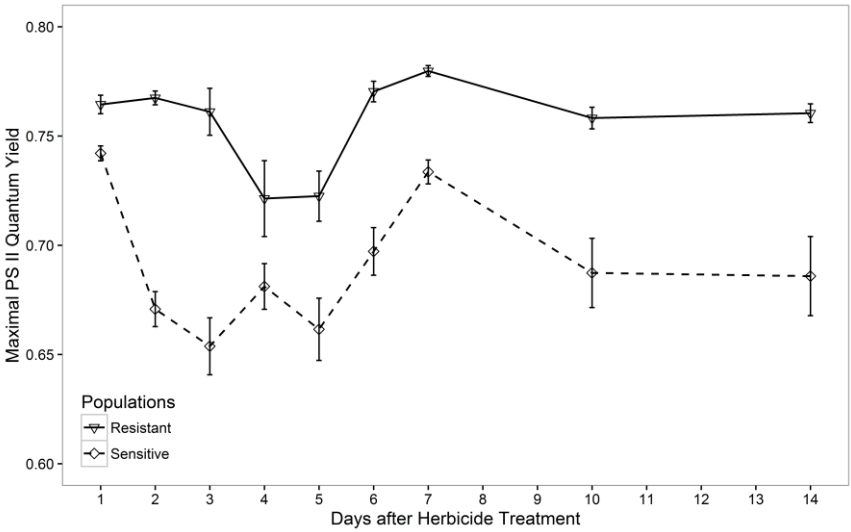
13
14
Days after Herbicide Treatment

(a)


Maximal PS II Quantum Yield
0.80
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Populations
Resistant
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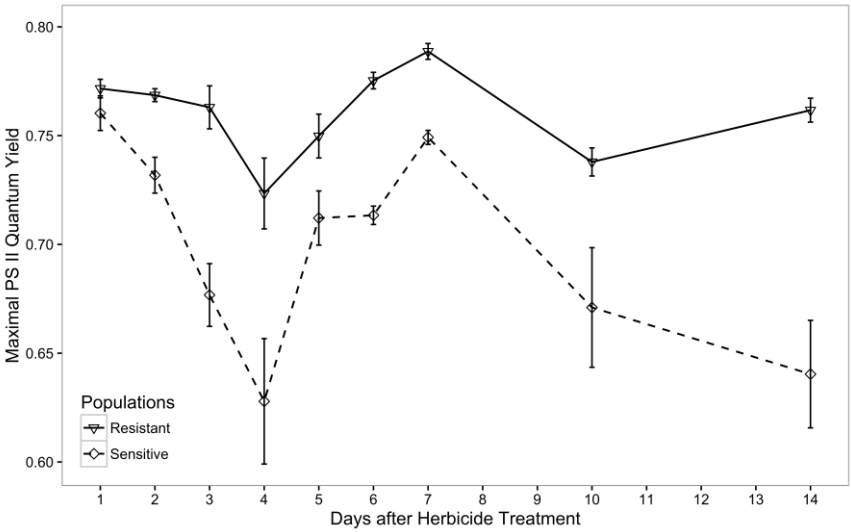
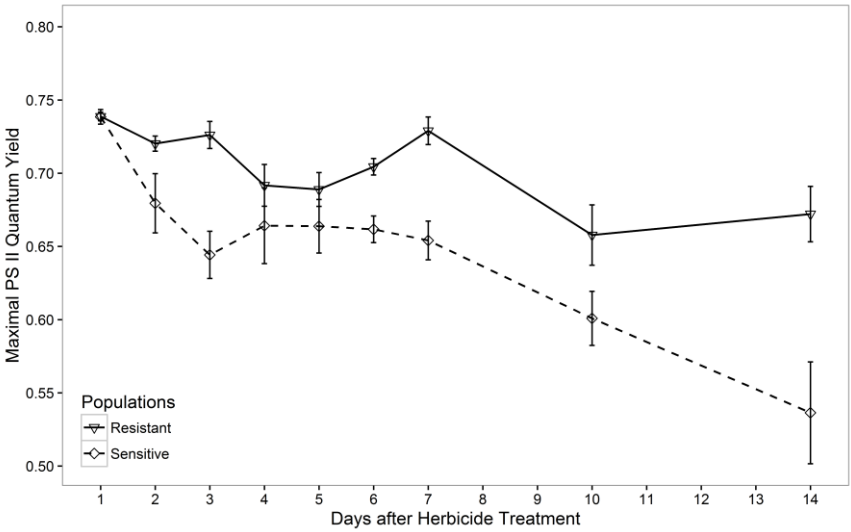
14
Days after Herbicide Treatment

(b)

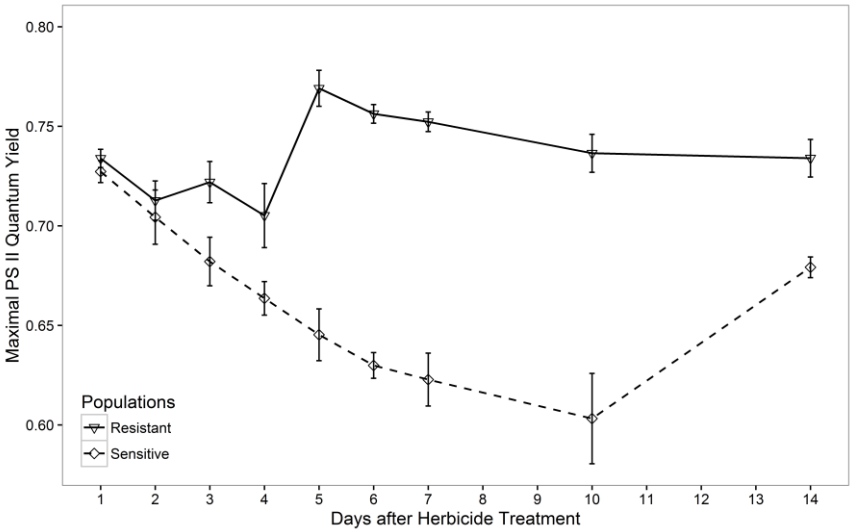


(c)

Figure 3. Daily presentation of the *Fv/Fm* of the ALS inhibitor treatments. (a), Treatment No. 2 (*meso-/iodosulfuron*); (b), Treatment No 3 (*propoxycarbazone*); (c), Treatment No 4 (*flupyrsulfuron*). Mean *Fv/Fm* and the standard error are shown for the sensitive and the resistant populations of *A. myosuroides.*



(a)



(b)

Figure 4. Daily presentation of the *Fv/Fm* of the ACCase inhibitor treatments. (a), Treatment No. 5 (*clodinafop*); (b), Treatment No 6 (*pinoxaden*). Mean *Fv/Fm* and the standard error are shown for the sensitive and the resistant population of *A. myosuroides*.

The *Fv/Fm* of sensitive plants continuously reduced over the period of measurement after the application of ACCase inhibitors (Figure 4). *Fv/Fm* was significantly lower in the sensitive population compared to the resistant plants 3 DAT Resistant plants recovered from a slight decrease 5 DAT while *Fv/Fm* of the sensitive population continuously decreased. Acetyl-CoA carboxylase is an important enzyme in the chloroplasts of many plants (Harwood, 1988). It is essential for the de-novo lipid acid synthesis (Kukorelli *et al.*, 2013). Sensitive grasses treated with ACCase inhibitors stop producing new leaves due to the fewer lipids for membrane production (Sasaki and Nagano, 2004). Therefore, the photosynthetic activity will slow down (Harwood, 1988), and the *Fv/Fm* decreases rapidly after the ACCase inhibitor treatment.

The PS II inhibitor *isoproturon* reduced *Fv/Fm* of both populations within a few hours. Compared to the untreated plants, *Fv/Fm* of the treated resistant plants were significantly lower. *Fv/Fm* of the treated sensitive population were significantly lower than the resistant and untreated population. Figure 5 shows that, from DAT 1 to 4, *Fv/Fm* differed between the sensitive and resistant population by 0.1. After 5 DAT, the resistant population slightly recovered, whereas the sensitive population died and *Fv/Fm* values remained low at about 0.2. *Isoproturon* inhibits PS II rapidly by interrupting the electron transfer chain of photosynthesis reaction (Ventrella *et al.*, 2010). As Pietsch *et al.* (2006) presented, photosynthetic oxygen release of *Ceratophyllum demersum* L. had reduced to half of the original level 6 hours after treatment with 20 µg/L *isoproturon*. After absorption, *isoproturon* inhibits Q_B in PS II activity center due to higher competitive binding kinetics to the binding niche in the D1 Protein (Hess, 2000). Thus, the electron transfer chain of PS II is stopped. However, *isoproturon* cannot bind in the target-site resistant population (Pfister *et al.*, 1981). The PS II activity of target site resistant plants will not be affected after treatment (Pfister and Arntzen, 1979). Therefore, we assume that the population in our study had a non-target-site resistance to *isoproturon*. The applied rate of *isoproturon* in this experiment might have been higher than the *A. myosuroides* population could metabolize. Several researches have presented that the urea (a sub-group of PS II

inhibitors) can be detoxified by enhanced and modified metabolism. Burnet *et al.* (1991) and Moss (1990a) have reported high rates of enhanced N-demethylation and ring alkyl-oxidation in *L. rigidum* and *A. myosuroides*. Some ingredients can still bind to D1 protein of the resistant plants even in lower rate than in the sensitive plants. Therefore, the PS II activity of the non-target site resistant plants was then stressed.

Over all treatments, the *Fv/Fm* of both populations did not remain stable over time. There is a daily differentiation after the treatment. Despite these differentiations, *Fv/Fm* of sensitive and resistant populations were significantly different. It is apparent from Figure 3 that treatments with ALS inhibitors induced much greater fluctuations than those observed in the control treatment. The ACCase inhibitors led to continuous reduction for *Fv/Fm* of the sensitive population. Due to the high fluctuation of the *Fv/Fm* that both populations have for almost all herbicides, there was a need to identify the origins of this fluctuation. Therefore, the second experiment was designed, where plants were exposed to water shortage and nitrogen deficiency.

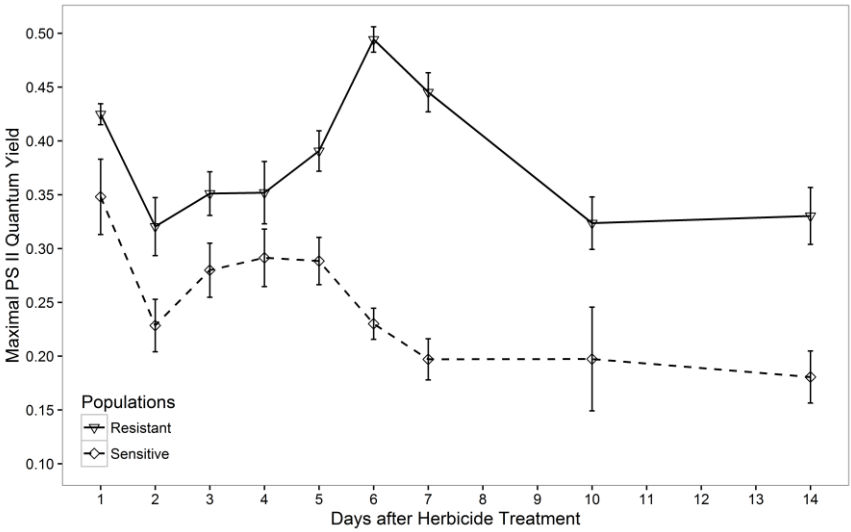
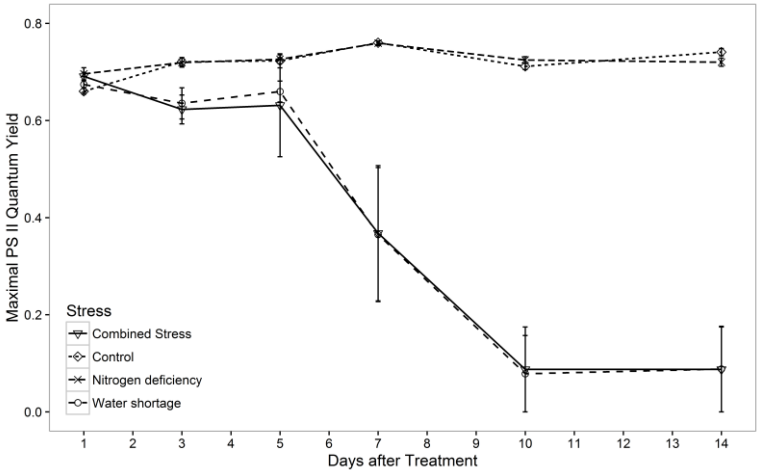


Figure 5. Daily presentation of the *Fv/Fm* in the *isoproturon* treatment. Mean *Fv/Fm* and the standard error are shown for the sensitive and the resistant population of *A. myosuroides*.

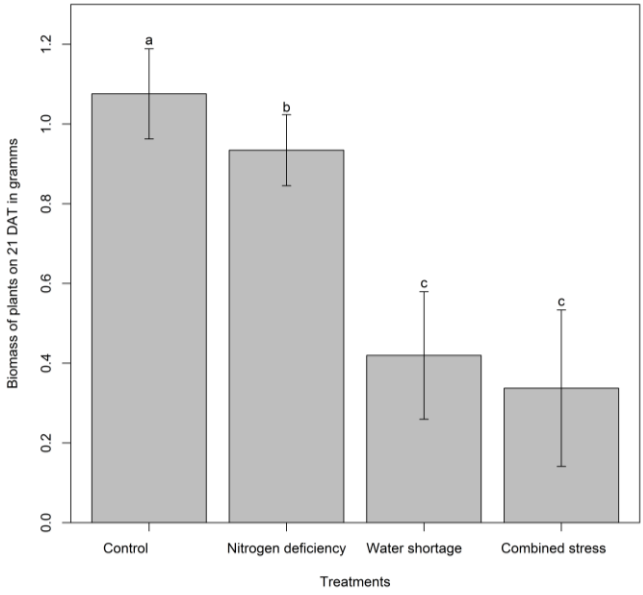
3.2 Water shortage and Nitrogen deficiency effect on chlorophyll fluorescence

Visual and biomass assessments of *A. myosuroides* plants exposed to water shortage and nitrogen deficiency were taken at DAT 21. All the plants under drought stress were wilting and had significantly lower biomass than the unstressed plants. Nitrogen shortage significantly reduced leaf length, number of tillers and biomass compared to the control group. No stress symptoms were visualized in the plants of the control group. These plants had the highest biomass and *Fv/Fm* values.

Water shortage significantly reduced *Fv/Fm*, while nitrogen deficiency did not affect *Fv/Fm* compared to the unstressed control plants. This corresponds to Baker and Rosenqvist (2004), who also could not find any reduction of *Fv/Fm* in plants deficient to nitrogen. Drougt significantly reduced *Fv/Fm* in *A. myosuroides* 7 days after exposure to water shortage. Then *Fv/Fm* values in the plants under water shortage had extremely *Fv/Fm* values with 0.1 compared to plants without stress (0.7). This agrees to Carmo-Silva *et al.* (2008), who reported that the *Fv/Fm* could be used for severe water stress after about seven days without water. The drought stress can decrease the CO_2 availability and alternate the photochemistry and carbon metabolism (Ashraf and Harris, 2013). The stomata usually close during the initial stages of drought stress resulting in increased water using efficiency (Chaves *et al.*, 2009). However, under severe drought stress, dehydration of mesophyll cells takes place causing a marked inhibition of basic metabolic processes of photosynthesis as well as a reduction of plant water using efficiency (Damayanthi *et al.*, 2010).



(a)



(b)

Figure 6. Results for drought and nitrogen stress experiment. (a) represents the *Fv/Fm* of *A. myosuroides* plants under different stresses . (b) shows the biomass of *A. myosuroides* plants at DAT 21. The letters "a,b, and c" represent significant difference according to Tukey's HSD-Test ($P < 0.05$).

4 Conclusions

The new chlorophyll fluorescence imaging sensor identified herbicide sensitive and resistant populations of *A. myosuroides* three days after treatment. At that time, no visual symptoms appeared on the leaves of *A. myosuroides*. Therefore, we conclude that the sensor can be used for in-season herbicide resistance screening. However, *Fv/Fm* values of herbicide damage was similar to water deficiency. Therefore, abiotic stress needs to be excluded for herbicide resistance detection. This can be achieved by measuring untreated control plots or by recording soil water content when the measurements take place. More input is needed in order to create a classfier to automaticially classify sensitive and resistant *A. myosuroides*. This can be achieved by training *Fv/Fm* values of large amounts of sensitive and resistant plants under different environments (Rumpff *et al.*, 2012) Further researches is needed to apply this herbicide resistance detection sensor for other weed species.

Acknowledgements

The authors would like to thank Dr. Jörg Kolbowski, Dr. Cornelia Köcher Dr. Alexander Menegat, Dr. Yasmin Kaiser and Dr. Hans G. Drobny for their valuable advice and technical support. This project was funded by the Federal Ministry of Food and Agriculture in Germany ("BLE-HerbiResistenz" - FKZ 2814705011) and the Chinese Scholarship Council (CSC, China, No. 201306350053).

Section II

The second article focuses on the field application capability of Weed PAM® sensor and the optimized measurement date on resistant population detection. 50 populations were tested for their resistance to ALS/ACCase herbicides in several field locations and application time.

Rapid in-season detection of herbicide resistant *Alopecurus myosuroides* using a mobile fluorescence imaging sensor

Published in: Crop Protection, 2016, 89, 170-177.

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DOI: 10.1016/j.cropro.2016.07.022

Abstract

WeedPAM has been introduced as a new chlorophyll fluorescence imaging sensor to detect herbicide stress in weeds a few days after treatment (DAT). In this study, it was investigated if the sensor could differentiate between 50 sensitive and herbicide resistant populations of *Alopecurus myosuroides* 5 DAT. Resistance profile of all populations had been analyzed in standard greenhouse bioassays. Populations were sown in winter wheat at several locations in Germany over two years. At 3-7 leaves growth stage, they were treated with four ALS- and three ACCase-inhibitors at recommended dosages. Five DAT, maximum quantum efficiency of PS II was measured with the WeedPAM sensor on 40 *A. myosuroides* plants per treatment. Based on the sensor data, populations were classified into sensitive and resistant populations. Classification was verified by a visual assessment of all treatments and populations 21 DAT. In total, 95% of the WeedPAM classifications 5 DAT were correct. We could demonstrate that WeedPAM is capable to detect herbicide resistant *A. myosuroides* populations shortly after treatment. This allows selecting alternative weed control methods against resistant weed populations in the same growing season.

Keywords: herbicide resistance; sensor detection; chlorophyll fluorescence; greenhouse biotest

1. Introduction

Alopecurus myosuroides Huds. often occurs in Western European winter cereals. It is highly competitive in winter wheat production causing relative yield losses of 20% at 100 plants m^{-2} (Blair *et al*., 1999). Seeds mostly germinate in autumn (Moss, 1990b). They persist more than 5 years in the soil (Moss, 1985). Densities increased due to higher proportions of winter cereals in the rotation, earlier sowing dates and conservation tillage systems (Melander, 1995; Lutman *et al*., 2013). With the capability of many populations to survive standard herbicide applications, *A. myosuroides* developed to the most problematic weed species in winter wheat in Western Europe (Neve, 2007). Populations with evolved resistance to herbicides inhibiting acetolactate synthase (ALS), acetyl CoA carboxylase (ACCase) and photosynthesis system II (PS II) have been documented mainly in England, France, Germany, parts of Belgium and the Netherlands (Drobny *et al*., 2006; Neve, 2007; Délye *et al.*, 2007; Heap, 2014). Herbicide resistance tests play an important role for resistance management. Conventional whole-plant bioassay in the greenhouse is most often used to screen for herbicide resistance (HRAC, 1999). It provides a resistance profile usually for several herbicides with different modes of action. However, this test requires mature weed seeds and therefore, provides results only for the next season. Furthermore, it is relatively expensive and requires a lot of space in the greenhouse. In many whole-plant bioassays, dose-response data are also included (Kaiser *et al*., 2013). Molecular assays are most commonly used to verify target-site single nucleotide mutations (Beffa *et al*., 2012). Several in-season tests have been developed to rapidly provide results of herbicide resistance in weed species (Burgos *et al*., 2013). Kaundun *et al*. (2011b) transplanted *Lolium rigidum* Gaudin and *Lolium multiflorum* L. and other grass-weeds into petri-dishes filled with agar and herbicide solutions. After 10 days, a visual assessment of the grasses was made to classify into sensitive and resistant plants. The results of this qualitative agar-based method correlated well with classical whole-plant bioassays. Kaiser *et al*. (2013) presented a similar approach for *A. myosuroides*. However, classification was based on quantitative measurements of chlorophyll fluorescence imaging approximately two

days after treatment. The results of this quantitative quick-test again correlated well with classical whole-plant greenhouse tests. Chlorophyll fluorescence imaging is a non-destructive sensor system that can be used as a very sensitive indicator of abiotic and biotic plant stress (Quick and Horton, 1984; Maxwell and Johnson, 2000; Schreiber, 2004; Janka *et al*., 2015). Riethmüller-Haage *et al*. (2006a,b), Kempenaar *et al*. (2011) and Kaiser *et al*. (2013) showed that herbicides with different modes of action including PS II-inhibitors, inhibition of amino acid and fatty acid synthesis and auxin-like herbicides caused a rapid decrease of relative quantum efficiency of photosystem II and photosystem I electron transport in different sensitive weed species shortly after herbicide application. A few studies showed that chlorophyll fluorescence after herbicide application was significantly higher in sensitive weed populations than in resistant biotypes (Ahrens et al., 1981; Ali & Machado, 1981; Hensley, 1981; Vencill and Foy, 1988; Van Oorschot and Van Leeuwen, 1992; Kaiser *et al*. 2013). However, studies and measurements were carried out under laboratory and greenhouse conditions. The objective of this study was to test if herbicide resistant and sensitive *A. myosuroides* populations can be identified few days after herbicide application in the field using a new mobile chlorophyll fluorescence imaging sensor.

2. Materials and methods

2.1 Experimental design

Three field experiments were conducted with *A. myosuroides* in winter wheat in 2014 and 2015. The first field trial was located at the University of Hohenheim research station. Seeds of sensitive *A. myosuroides* population (HerbiSeed, Twyford, UK) were sown at the same date as winter wheat on October 5th 2014 at a density of 1000 seeds m^{-2}. Germination rate was determined to be 15% resulting in approximately 150 emerged seedlings m^{-2}. The field had been free of *A. myosuroides* in the previous two years. Inversion tillage was done 10 days before sowing followed by seedbed preparation. The experiment was set up as randomized complete block design with four blocks and six treatments. Treatments are listed in Table 5. Herbicides were sprayed with an electric motorized plot sprayer equipped with

Lechler IDK 120-02 nozzles at a volume of 200 L ha^{-1} when *A. myosuroides* had approximately 3-7 leaves. The size of each plot was 2 × 5 m.

The second set of experiments was conducted in spring 2015 at 8 winter wheat sites in Germany in the states of Baden-Württemberg, Rheinland-Pfalz, Nordrhein-Westfalen and Niedersachsen. All fields were heavily infested with *A. myosuroides* at densities of 100-500 plants m^{-2}. Farmers had reported that either ALS- or ACCase-inhibiting herbicides did not provide sufficient efficacy in the previous years. All experiments were set up as completely randomized design with four replicates. The size of each plot was 2 × 5 m. *A. myosuroides* was treated with herbicides listed in Table 6 when plants had 3-7 leaves. An untreated control was included at each site.

The third experiment was conducted in autumn 2015. Winter wheat and 42 *A. myosuroides* populations originating from Baden-Württemberg were sown at the research station Ihinger Hof of the University of Hohenheim on October 5th 2015 (Table 7). Resistance to either ALS-, ACCase- or PS II- inhibiting herbicides had been proofed in 39 populations in a standard greenhouse biotest. One sensitive biotype from the company HerbiSeed in the UK was included in the experiment. *A. myosuroides* was sown at a density of 1000 seeds m^{-2} by hand and then seeds were incorporated into the soil with a harrow. Approximately 150 seedlings m^{-2} emerged in all plots. The field had been planted to maize for two years before this experiment was set up. It was free of *A. myosuroides*. One week before sowing, inversion tillage was conducted, followed by seedbed preparation. The experiment was set up as a randomized split-plot design with four replicates, *A. myosuroides* population was the main factor and herbicide treatments was the subplot factor. Each subplot had a size of 2 × 10 m. At 3-7 leave stage, *A. myosuroides* was treated with 1.2 L ha^{-1} Axial® 50 (50 g a.i. L^{-1} *pinoxaden*, EC, Syngenta Agro) and 500 g ha^{-1} Atlantis® WG (29.2 g a.i. kg^{-1} *mesosulfuron*, 5.6 g a.i. kg^{-1} *iodosulfuron*, WG, Bayer CropScience) with its recommended adjuvant (27% *fatty alcohol ether sulphate*). An untreated control was included for all populations.

Table 5. Herbicides applied in experiment 1 in autumn 2014 and spring 2015; WG = Water Dispersible Granules, SG = Water Soluble Granules, EC = Emulsifiable Concentrate, A = Lipid synthesis inhibition (inhibition of ACCase), B = Inhibition of ALS (branched chain amino acid synthesis).

No.	Trade name	Active ingredients rates	Formulation	HRAC MoA	Application rates	Provider
1	control	-	-	-	-	-
2	Atlantis® WG +	*29.2 g kg^{-1} mesosulfuron, 5.6 g kg^{-1} iodosulfuron*	WG	B	500 g ha^{-1}	Bayer CropScience
	adjuvant	*27% fatty alcohol ether sulphate*			1 L ha^{-1}	Bayer CropScience
3	Attribut®	*700 g kg^{-1} propoxycarbazone-Na*	SG	B	100 g ha^{-1}	Bayer CropScience
4	Broadway® +	*68.3 g kg^{-1} pyroxsulam, 22.8 g kg^{-1} florasulam*	WG	B	220 g ha^{-1}	Dow AgroSciences
	adjuvant	*99.9% methyl esters in rapeseed oil*			1 L ha^{-1}	Dow AgroSciences
5	Axial® 50	*50 g L^{-1} pinoxaden*	EC	A	1.2 L ha^{-1}	Syngenta Agro
6	Topik® 100	*89.1 g L^{-1} clodinafop*	EC	A	0.6 L ha^{-1}	Syngenta Agro

Table 6. Locations and treatment details of experiment 2 in spring 2015; EC = Emulsifiable Concentrate, SC = Suspension Concentrate, WG = Water Dispersible Granules, SG = Water Soluble Granules.

State	Location	Trade name	Active ingredients rates	Formulation	Application rates	Provider
		control				
		Axial® 50	*50 g L^{-1} pinoxaden*	EC	0.9 L ha^{-1}	Syngenta Agro
		Bacara® Forte	*120 g L^{-1} diflufenican, 120 g L^{-1} flurtamone, 120 g L^{-1} flufenacet*	SC	0.9 L ha^{-1}	Bayer CropScience
	Ihinger Hof, Renningen	Axial® 50	*50 g L^{-1} pinoxaden*	EC	0.9 L ha^{-1}	Syngenta Agro
		Herold® SC	*200 g L^{-1} diflufenican, 400 g L^{-1} flufenacet*	SC	0.5 L ha^{-1}	Bayer CropScience
Baden-Wuerttemberg		Axial® 50	*50 g L^{-1} pinoxaden*	EC	0.9 L ha^{-1}	Syngenta Agro
		Malibu®	*300 g L^{-1} pendimethalin, 60 g L^{-1} flufenacet*	EC	4.0 L ha^{-1}	BASF
		Axial® Komplett	*5 g L^{-1} florasulam, 45 g L^{-1} pinoxaden*	EC	1.0 L ha^{-1}	Syngenta Agro
	Wurmberg1	control				
		Broadway®	*68.3 g kg^{-1} pyroxsulam, 22.8 g kg^{-1} florasulam* + adjuvant	WG	220 g ha^{-1}	Dow AgroSciences
	Wurmberg2	control				
		Atlantis® WG	*29.2 g kg^{-1} mesosulfuron, 5.6 g kg^{-1} iodosulfuron* + adjuvant	WG	500 g ha^{-1}	Bayer CropScience
	Anderten	control				
		Atlantis® WG	*29.2 g kg^{-1} mesosulfuron, 5.6 g kg^{-1} iodosulfuron* + adjuvant	WG	500 g ha^{-1}	Bayer CropScience

		Atlas®	*264 g L^{-1} clethodim*	EC	0.438 L ha^{-1}	Altitude Crop Innovations
		control				
Niedersachsen		Caliban® Duo	*9.3 g kg^{-1} iodosulfuron, 159.2 g kg^{-1} propoxycarbazone* + adjuvant	WG	250 g ha^{-1}	Cheminova Deutschland GmbH
	Jeinsen	Broadway®	*68.3 g kg^{-1} pyroxsulam, 22.8 g kg^{-1} florasulam* + adjuvant	WG	220 g ha^{-1}	Dow AgroSciences
		Atlantis® WG	*29.2 g kg^{-1} mesosulfuron, 5.6 g kg^{-1} iodosulfuron* + adjuvant	WG	500 g ha^{-1}	Bayer CropScience
		Traxos®	*22.3 g L^{-1} clodinafop, 25 g L^{-1} pinoxaden*	EC	1.2 L ha^{-1}	Syngenta Agro
		control				
		Axial® 50	*50 g L^{-1} pinoxaden*	EC	1.2 L ha^{-1}	Syngenta Agro
	Appel	Attribut®	*700 g kg^{-1} propoxycarbazone-Na + adjuvant*	SG	100 g ha^{-1}	Bayer CropScience
		Atlantis® WG	*29.2 g kg^{-1} mesosulfuron, 5.6 g kg^{-1} iodosulfuron* + adjuvant	WG	500 g ha^{-1}	Bayer CropScience
		control				
Rheinland-Pfalz	Schleich	Axial® 50	*50 g L^{-1} pinoxaden*	EC	1.2 L ha^{-1}	Syngenta Agro
		Attribut®	*700 g kg^{-1} propoxycarbazone-Na* + adjuvant	SG	100 g ha^{-1}	Bayer CropScience
		Atlantis® WG	*29.2 g kg^{-1} mesosulfuron, 5.6 g kg^{-1} iodosulfuron* + adjuvant	WG	500 g ha^{-1}	Bayer CropScience
		control				
Nordrhein-Westfalen	Muenster	Broadway®	*68.3 g kg^{-1} pyroxsulam, 22.8 g kg^{-1} florasulam* + adjuvant	WG	220 g ha^{-1}	Dow AgroSciences

Table 7. Origins of resistant *A. myosuroides* seeds for the field experiment in winter wheat at Ihinger Hof in 2015; resistance status of the populations was determined in a standard greenhouse biotest; A1: resistance to *clodinafop*, A2: resistance to *fenoxaprop*, A3: resistance to *pinoxaden*, A4: resistance to other FOPs, A5: resistance to DIMs, B1: resitance to *flupyrsulfuron-methly*, B2: resistance to *meso-/iodosulfuron*, B3: resistance to *propxycarbazone*, B4: resistance to *pyroxsulam*, C: resistance to *isoproturon*.

No.	Origin	Resistance history	Collection year
1	Hohenheim(HerbiSeed)	Sensitive	2015
2	Niedersachsen	A5, B2	2015
3	Niedersachsen	A5, B2	2015
4	Blaufelden-Herrentierbach	A1, A2, A3, A4, A5, B1, B2, B4	2012
5	Lehrden	A1, A2, A3, B1, B2	2012
6	Nenenstetten	A1, A2, A3, A4, B1, B2, B4	2012
7	Tübingen	A1, A2, A3, A4, B1, B2, B4	2013
8	Tübingen	A1, A2, A3, A4, B1, B2, B4	2013
9	Tübingen	A1, A2, A3, A4, B1, B2, B4	2013
10	Öhringen	A1, A2, A3, A4, B1	2014
11	Douaueschingen	A4, B1	2014
12	Douaueschingen	A2, A3, B1	2014
13	Wurmberg	B2, B4, C	2014
14	Wurmberg	B2, B4, C	2014
15	Heilbronn	A2, A3, A4, A5, B2, B4	2014
16	Heilbronn	A1, A2, A3, A5	2014
17	Heilbronn	B2, B4	2014
18	Heilbronn	A2, A3, A4, A5, B2, B4	2014
19	Tübingen	A1, A2, A3	2014
20	Waldshut	A3	2014
21	Ahorn-Bach	B2, B3	2014

22	Rhein-Neckar-Kreis	B1, B2, B3, B4	2014
23	Rhein-Neckar-Kreis	Sensitive	2014
24	Schwäbisch Hall	A3, A4, B4	2014
25	Schwäbisch Hall	A1, A2, A3, B1, B2, B3, B4	2014
26	Schwäbisch Hall	A4, A5, B2, B4	2014
27	Alb-Donau-Kreis	A1, A2, A3, B1, B2, B3, B4	2014
28	Main-Taber-Kreis	A3, A4, B4	2014
29	Main-Taber-Kreis	A3, A4, B4	2014
30	Rottweil	A1, A2, A3, B1, B2, B3, B4	2014
31	Rottweil	A1, A2, A3, B1, B2, B3, B4	2014
32	Karlsruhe	Sensitive	2014
33	Reutlingen	A3, A4	2014
34	Reutlingen	A2, A3, A4	2014
35	Reutlingen	A1, A2, A3, A4, B1	2014
36	Calw	A2, A3, A4	2014
37	Calw	A2, A3, A4	2014
38	Calw	A1, A2, A3, A4, B4	2014
39	Nekar-Odenwald	B1, B2, B3, B4	2014
40	Nekar-Odenwald	B1, B2, B3, B4	2014
41	Nekar-Odenwald	A2, B1, B2, B3, B4	2014
42	Nekar-Odenwald	A2, B1, B2, B3, B4	2014

In all experiments, herbicides were sprayed at temperatures above 10 °C. No rainfall was recorded within 24 hours after treatment.

2.2 WeedPAM-sensor

WeedPAM is a mobile version of IMAGING-PAM® fluorescence sensor (MINI version, Heinz Walz GmbH, Germany). It contains 40 dark adaption and measuring boxes for plants, a measuring head with a CCD camera, a water proof tablet computer and a control unit (Figure 7). The efficacy of photosynthesis system II (PS II) of the plants was determined by measuring the maximum quantum efficiency of PS II (*Fv/Fm*) using the following equation:

$$F_v/F_m = \frac{F_m - F_0}{F_m}$$

where F_0 is the basic fluorescence emission when only 2634 μM m^{-2} s^{-1} photosynthetic photon flux density is given to the plants in dark acclimated state. F_m is the maximum fluorescence yield of dark acclimated plants after a saturation pulse. Chlorophyll fluorescence was induced by blue LED lights of 460 nm wavelength. An optical red long pass filter of > 680nm wavelength was mounted in front of the camera lens. The WeedPAM system (Figure 7) was controlled by the software "ImagingWin for WeedPAM" (Heinz Walz GmbH, Germany). The software removed all pixels from the image that did not contain green plants (Kaiser *et al.,* 2013). *Alopecurus myosuroides* plants were automatically classified into sensitive and resistant based on the *Fv/Fm* values measured.

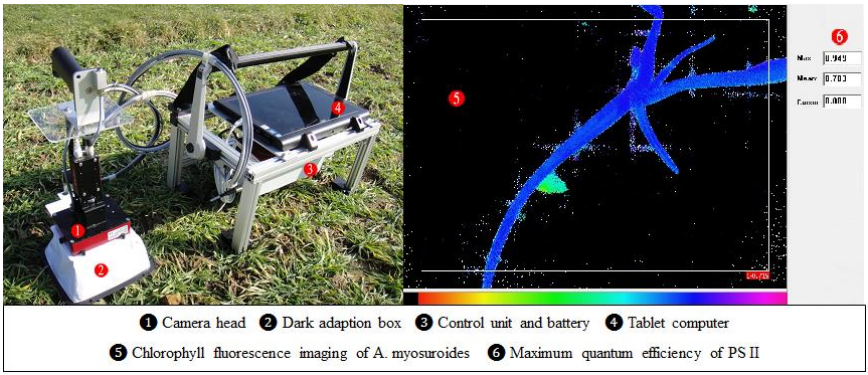


Figure 7. The WeedPAM sensor; left: operation in the field, right: a screenshot of one measurement of *A. myosuroides*, blue und violet represent high maximum quantum efficiency of PS II, red color indicates stress.

2.3 Measurements and data analysis

In all three experiments, maximum quantum efficiency of PS II of 40 *A. myosuroides* plants per plot was measured. In the first experiment, measurements were done every day during seven days after herbicide treatment (DAT) and again 10 DAT and 14 DAT. In the second and third experiments, images were taken 5 DAT. Plants were dark adapted for 25-30 minutes underneath the measuring boxes. Values of all 40 plants were averaged. During sensor measurements each plant was marked with a stick and label. A visual classification of "dead" (sensitive) and "alive" (resistant) plants was made 21 DAT. The ratio of dead plants was computed. Then, herbicide resistance level was determined similar to Moss e*t al.* (2000): "S" = sensitive, 100-81% dead; "R?" = slightly resistant, 80-73% dead; "RR" = resistant, 72-37% dead; "RRR" strongly resistant, 36-0% dead.

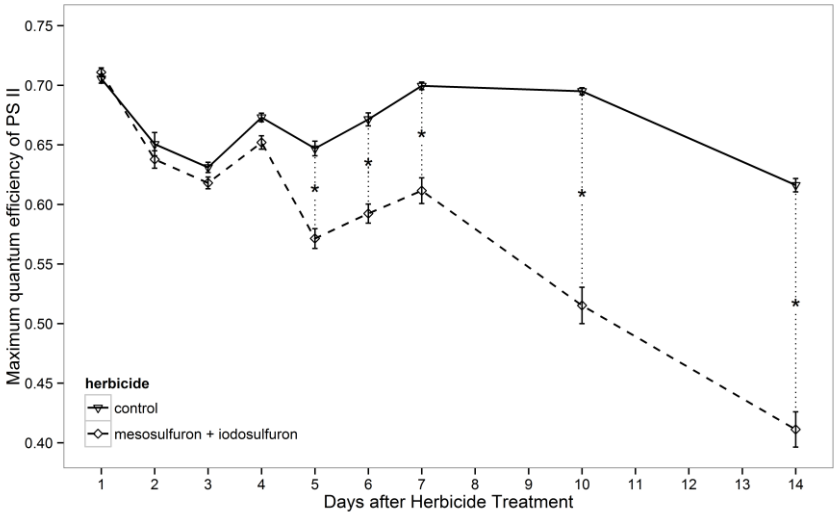
Data were analyzed with R (Version 3.0.2) and the packages *agricolae* and *lawstat* (R Development Core Team, 2013). First, ANOVA was conducted to determine if herbicide treatments significantly affect *Fv/Fm* followed by a Tukey's HSD test. All the datasets were proved to be normally distributed by the Shapiro-Wilk test ($p > 0.05$). Homogeneity of variances was analyzed using Levene's test ($p > 0.05$).

3. Results and Discussion

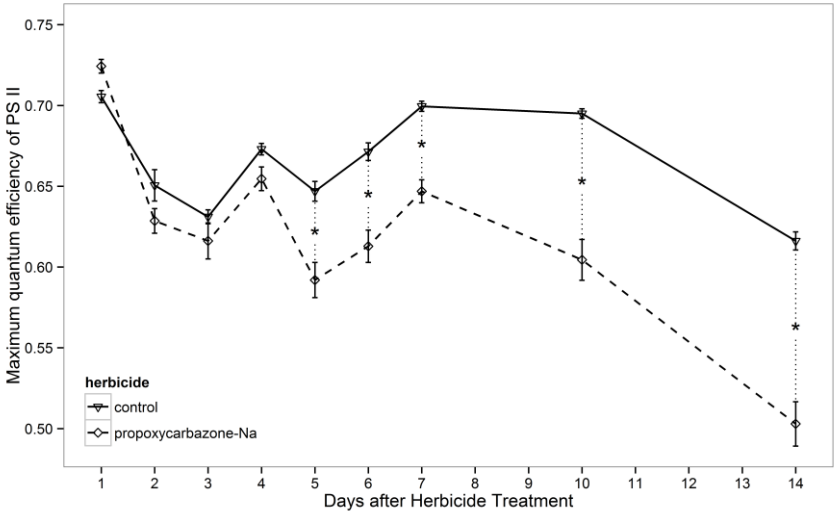
3.1 Field tests with WeedPAM

At the time of herbicide application on November 2nd 2014, in average 150 *A. myosuroides* plants m^{-2} had emerged and produced 3-7 leaves. Less than 1 plant m^{-2} emerged in the band area around the experiment indicating that *A. myosuroides* plants in the experiments mostly arose from the seeds incorporated in the soil at time of winter wheat sowing. In all herbicide treatments, *Fv/Fm* of *A. myosuroides* was significantly lower than in the untreated control plots from 5 DAT until the end of measurements 14 DAT (Figure 8). *Alopecurus myosuroides* plants did not recover from herbicide applications. Differences of *Fv/Fm* values between treated and untreated plants were slightly higher for ALS-inhibitors than for ACCase-inhibitors.

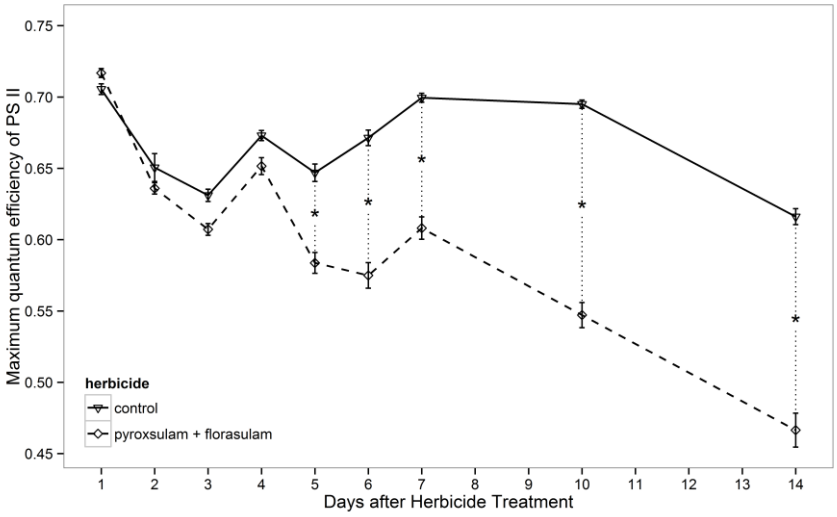
The differences of *Fv/Fm* values in *A. myosuroides* plants treated with ACCase inhibitors compared to untreated plants increased until 5 DAT and then kept stable until the end of measurements. Acetyl-CoA carboxylase is an important enzyme catalyzing lipid acid synthesis using ATP from photosynthesis. Lipids are essential for membrane production in young leaves (Sasaki and Nagano, 2004). Synthesis of ACCase is inhibited in sensitive grasses after application of ACCase inhibitors and thus, less ATP is used. Therefore, photochemistry reaction is lowered (Kukorelli *et al.*, 2013). ALS-inhibiting herbicides showed a stronger and faster response in sensitive *A. myosuroides* plants 4-5 DAT. Inhibition of acetolactate synthase in susceptible plants decreases PS II activity and photosynthesis rate, because less branched-chain amino acids such as valine, leucine and isoleucine are produced for regeneration of proteins (e.g. D-protein) involved in electron transport chains (LaRossa and Schloss, 1984; Öttmeier, 1999; Whitcomb, 1999; Rutherford and Falle, 2003; Matham, 2009).


Maximum quantum efficiency of PS II
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
herbicide
control
mesosulfuron + iodosulfuron
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Days after Herbicide Treatment

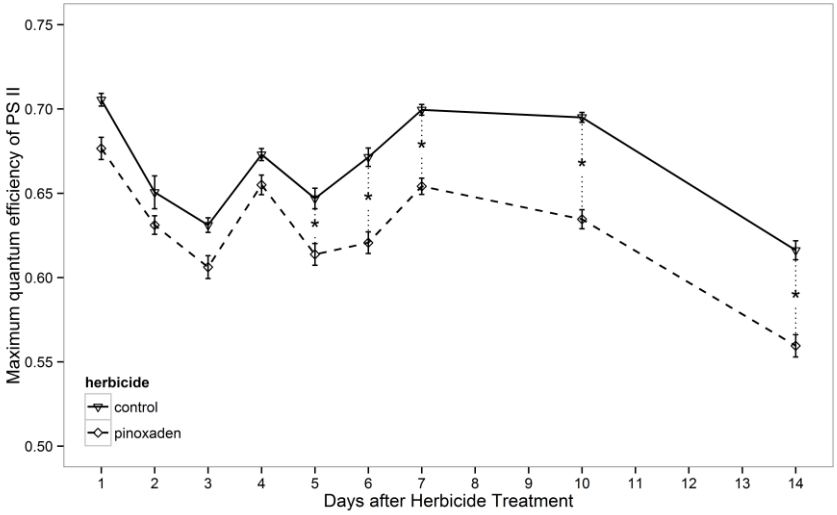
(a)


Maximum quantum efficiency of PS II
0.75
0.70
0.65
0.60
0.55
0.50
herbicide
control
propoxycarbazone-Na
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Days after Herbicide Treatment

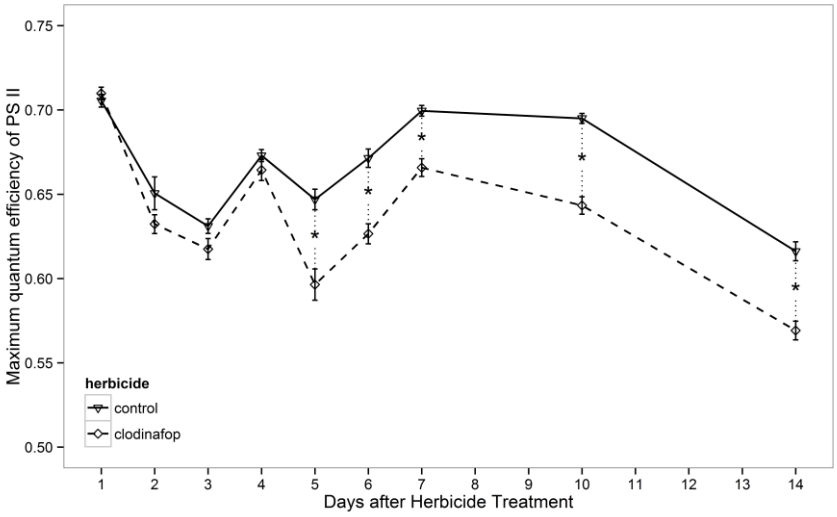
(b)


Maximum quantum efficiency of PS II
0.75
0.70
0.65
0.60
0.55
0.50
0.45
herbicide
control
pyroxsulam + florasulam
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Days after Herbicide Treatment

(c)


Maximum quantum efficiency of PS II
0.75
0.70
0.65
0.60
0.55
0.50
herbicide
control
pinoxaden
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Days after Herbicide Treatment

(d)



(e)

Figure 8. Variation of maximum quantum efficiency of PSII (*Fv/Fm* values) in a sensitive population of *A. myosuroides* plants after application of (a) *meso-/iodosulfuron*; (b) *propoxycarbazone-Na*; (c) *pyrox-/florasulam*; (d) *pinoxaden*; and (e) *clodinafop*. The values are means ± standards error (n = 40). Stars "*" indicate significant differences of *Fv/Fm* values between treated and untreated plots (ANOVA, $P < 0.05$).

Table 8. Maximum quantum efficiency of PSII (*Fv/Fm* values) in 8 field populations of *A. myosuroides* five days after treatment with ALS- and ACCase inhibitors compared to untreated control plots; significant differences between mean values are indicated by different letters (Tukey's Test, $P < 0.05$).

Location	Treatment	*Fv/Fm*	significance	Resistance level Visual Assessment	Classification True/False
Baden-Württemberg 1	control	0.6763	a		
	pinoxaden	0.6611	a	RRR	true
	florasulam, pinoxaden	0.6583	a	RRR	true
Baden-Württemberg 2	control	0.6463	a		
	pyrox-/florasulam	0.6304	b	R?	false
Baden-Württemberg 3	control	0.6298	a		
	meso-/iodosulfuron	0.6346	a	RR	true
Niedersachsen 1	control	0.6505	a		
	meso-/iodosulfuron	0.6461	a	RRR	true
	meso-/iodosulfuron + clethodim	0.6331	a	RRR	true
Niedersachsen 2	control	0.6625	a		
	iodosulfuron, propoxycarbazone	0.5564	b	S	true
	pyrox-/florasulam, cloquintocet-methyl	0.5751	b	S	true
	meso-/iodosulfuron	0.539	b	S	true
	clodinafop, pinoxaden	0.6099	b	S	true
Niedersachsen 3	control	0.6831	a		
	pinoxaden	0.6149	b	S	true
	propoxycarbazone-Na	0.5925	b	S	true
	meso-/iodosulfuron	0.6081	b	S	true

Rheinland-Pfalz	control	0.65875	a		
	pinoxaden	0.6473	a	RRR	true
	propoxycarbazone-Na	0.6382	a	RRR	true
	meso-/iodosulfuron	0.6168	a	R?	true
Nordrhein-Westfalen	control	0.66265	a		
	pyrox-/florasulam	0.61025	a	RR	true

Table 9. Maximum quantum efficiency of PSII (*Fv/Fm* values) in 42 populations of *A. myosuroides* five days after treatment with *meso-/iodosulfuron* and *pinoxaden* compared to untreated control plots; significant differences between mean values are indicated by different letters (Tukey's Test, $P < 0.05$).

Population No.	**Measurement date**	**Temperature at 5cm over ground**	**Treatments**						
			Fv/Fm Control	*meso-/iodosulfuron* (ALS)			*pinoxaden* (ACCase)		
				Fv/Fm	Resistance level - Visual Assessment	Accuracy false/true	*Fv/Fm*	Resistance level - Visual Assessment	Accuracy false/true
1	Nov. 17	7 °C	0.7288a	0.6971b	S	true	0.6708b	S	true
2			0.7199a	0.7329a	RRR	true	0.7067a	RRR	true
3			0.7396a	0.7471a	RRR	true	0.6830b	S	true
4			0.7310a	0.7236a	RRR	true	0.7333a	RRR	true
5			0.7302a	0.7212a	RRR	true	0.7330a	RRR	true
6			0.7282a	0.7299a	R?	true	0.7279a	RRR	true
7	Nov. 18	12 °C	0.7128a	0.6961a	RRR	true	0.6936a	RRR	true
8			0.7245a	0.7121a	RRR	true	0.7174a	RR	true
9			0.7221a	0.7134a	RRR	true	0.7063a	RRR	true
10			0.7244a	0.7179a	RRR	true	0.7138a	RR	true

11			0.7291a	0.7171a	RR	true	0.6946b	S	true
12			0.7283a	0.7261a	RRR	true	0.7177a	RRR	true
13			0.7364a	0.7183a	RR	true	0.6681b	S	true
14			0.7402a	0.7472a	RRR	true	0.7254a	RR	true
15	Nov. 19	10 °C	0.7492a	0.7326a	RRR	true	0.7028b	S	true
16			0.7451a	0.7363a	RR	true	0.7293a	RR	true
17			0.7366a	0.7273a	RRR	true	0.6845b	S	true
18			0.7377a	0.7483a	RRR	true	0.7082a	RRR	true
19			0.7300a	0.7453a	RR	true	0.7073a	RR	true
20	Nov. 20	14 °C	0.7276a	0.7468a	RR	true	0.7015a	RR	true
21			0.7420a	0.7447a	RR	true	0.7110b	S	true
22			0.7363a	0.7407a	RRR	true	0.6625b	S	true
23			0.6528a	0.6323b	S	true	0.5923b	S	true
24			0.6675a	0.6514a	RRR	true	0.6557a	RRR	true
25	Nov. 27	-3 °C	0.6683a	0.6678a	RRR	true	0.6001b	R?	false
26			0.6856a	0.6768a	RRR	true	0.6651a	RRR	true

27			0.6751a	0.6697a	RRR	true	0.6082b	S	true
28			0.6851a	0.6386b	S	true	0.6205b	RR	false
29			0.6558a	0.6289a	RRR	true	0.5766b	S	true
30			0.6675a	0.5891b	RRR	false	0.5440b	S	true
31			0.6683a	0.6274b	RRR	false	0.5422b	RRR	false
	Nov. 28	-3 °C							
32			0.6856a	0.5949b	S	true	0.5168b	S	true
33			0.6751a	0.6070b	RR	false	0.5830b	RRR	false
34			0.6871a	0.5858b	S	true	0.5300b	S	true
35			0.5809a	0.6103a	R?	true	0.6013a	RR	true
36			0.5960a	0.5908a	RR	true	0.5523b	S	true
37			0.6065a	0.5802a	RR	true	0.5786b	S	true
38			0.6438a	0.5842b	S	true	0.5601b	S	true
	Dec. 10	-2 °C							
39			0.6342a	0.6396a	RRR	true	0.5613b	S	true
40			0.6584a	0.6052b	RRR	false	0.6232b	S	true
41			0.6163a	0.6047a	RRR	true	0.5863b	R?	false
42			0.6170a	0.6053a	RRR	true	0.5491b	R?	false

Analysis of maximum quantum efficiency of PS II (*Fv/Fm*) 5 DAT in winter wheat fields at eight locations infested with *A. myosuroides* in spring 2015 resulted in 94% correct classifications of sensitive and resistant populations (Table 8). Resistant populations showed equal *Fv/Fm* values to untreated populations and sensitive populations had significantly lower *Fv/Fm* values than untreated and resistant plants. Only one population from Baden-Württemberg was misclassified as slightly resistance after treatment with *pyroxsulam* and *florasulam.*

In the third experiment, 95% of the field classifications were equal to the results of the standard greenhouse biotests with 42 *A. myosuroides* populations (Table 7). Therefore, we conclude that the methodology to measure and sample 40 plants plot^{-1} resulted in correct classifications into sensitive and resistant populations. WeedPAM-measurements of maximum quantum efficiency of PS II (*Fv/Fm*) 5 DAT in the winter wheat field resulted in 88% correct classifications of sensitive and resistant *A. myosuroides* populations (Table 9). Resistant populations showed equal *Fv/Fm* values to untreated populations and sensitive populations had significantly lower *Fv/Fm* values.

In several earlier studies (Ahrens *et al*., 1991; Ali and Machado, 1981; Hensley, 1981; Van Oorschot and Van Leeuwen, 1992) chlorophyll fluorescence increased after application of PS II-inhibitors. We proved in this study that chlorophyll fluorescence in sensitive *A. myosuroides* was also higher shortly after treatment with ACCase- and ALS-inbibitors. Those modes of action play a highly important role in controlling grass-weeds in arable crops.

3.2 Effects on the WeedPAM measurement

Fv/Fm values of treated and untreated *A. myosuroides* plants varied over different locations and the period of measurements (Figure 8) indicating that environmental conditions such as temperature and sunlight intensity affect chlorophyll fluorescence. This has also been proved for *Musa sp.*, *Allium ursinum* and *Dendranthema grandiflora* (Dongsansuk *et al.*, 2013; Janka *et al.*, 2015). In the second experiment at eight different locations, *Fv/Fm* values of untreated plants were different at each

location. This proves that *Fv/Fm* values of plants are affected by abiotic factors (Adams and Demmig-Adams, 2004). In the third experiment at Ihinger Hof Research Station, we found no significant difference of *Fv/Fm* values among the untreated plants measured at the same date. This indicates that the *A. myosuroides* population did not influence *Fv/Fm* values. So, in this experiment variation of *Fv/Fm* values was only due to herbicide treatments and resistance status of *A. myosuroides* populations. In experiment 3, only four populations treated with *meso-/iodosulfuron* and 6 populations sprayed with *pinoxaden* were misclassified (Table 9). All false classifications occurred, when WeedPAM-measurements were taken at temperatures below freezing point. Low temperatures reduced *Fv/Fm* values. Measurements of populations 1-22 were carried out at temperatures between 7°C - 14.0°C with average *Fv/Fm* values of untreated *A. myosuroides* plants of 0.72. *Fv/Fm* values of untreated *A. myosuroides* plants dropped to 0.65, when temperature decreased to -3°C. Similar results were reported by Krause (1994), Lundmark *et al.* (1998a) and Janka *et al.* (2015). Frost may damage chloroplast and therefore *Fv/Fm* values are lower (Örlander, 1993). Therefore, we conclude that WeedPAM measurements need to be taken at temperatures above 0°C. As herbicides are usually applied at temperatures above 0°C during the vegetation period, the effect of cold temperature does not limit the application of WeedPAM for detecting herbicide resistance in weeds.

4. Conclusion

ACCase- and ALS-inhibitors caused a significant reduction of maximum quantum efficiency of PS II in sensitive field populations of *A. myosuroides* already 5 DAT before typical visual symptoms of herbicide efficacy appear on the plants. In herbicide resistant populations treated with standard herbicide rates, maximum quantum efficiency of PS II was equal to untreated control plants. Therefore, we conclude that the new mobile chlorophyll fluorescence imaging sensor WeedPAM is capable to identify herbicide resistant *A. myosuroides* field populations early enough to apply alternative control methods in the same growing season.

Acknowledgements

The authors highly appreciate helpful comments, ideas and technical help from Dr. Jörg Kolbowski, Dr. Erhard Pfündel, Dr. Cornelia Köcher, Dr. Frank Brändle, Dr. Hans G. Drobny, Dr. Thomas Uhl, Dr. Yasmin I. Kaiser and Dr. Alexander Menegat. We thank the Federal Office for Agriculture and Food (BLE, Germany) and the Chinese Scholarship Council (CSC, China) for financial support.

Section III

In the third article, greenhouse and field experiments were conducted to investigate the application potential of the chlorophyll fluorescence imaging technology in the early identification of herbicide stress on soybeans.

Early identification of herbicide stress in soybean (Glycine max (L.) Merr.) using chlorophyll fluorescence imaging technology

Submitted to: Biosystems Engineering

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Abstract

Herbicides may damage soybean in conventional production systems. Chlorophyll fluorescence imaging technology has been applied to identify herbicide stress in weed species few days after application. In this study, greenhouse experiments followed by field experiments at five sites were conducted to investigate if the chlorophyll fluorescence imaging is capable for identifying herbicide stress in soybean shortly after application. Measurements were carried out from emergence until three-four-leaf stage of soybean. Results showed that maximal photosynthesis system II (PS II) quantum yield and shoot dry biomass was significantly reduced in soybean by herbicides compared to the untreated control plants. The stress of PS II inhibiting herbicides occurred on the cotyledons of soybean and plants recovered after one week. The stress induced by DOXP synthase-, microtubule assembly- or cell division-inhibitors was measured from the two-leaf stage until four-leaf stage. We could demonstrate that the chlorophyll fluorescence imaging technology is capable for detecting herbicide stress in soybean. The system can be applied under both greenhouse and field conditions. This helps farmers to select weed control strategies with less phytotoxicity in soybean and avoid yield losses due to herbicide stress.

Keywords: Herbicide stress, phytotoxicity, soybean, chlorophyll fluorescence imaging

1. Introduction

Soybean (*Glycine max* (L.) Merr.) is a worldwide cultivated crop. More than 80% of the soybeans overall production were supplied by the USA, Brazil, Argentina (FAOSTAT, 2016). Since 1996, the Roundup-Ready (RR) Soybean system has been introduced in the USA, Brazil and Argentina. Farmers can apply *glyphosate* as a simple, selective and effective method for weed control without concerning of crop injury. In the European Union, weed control in soybean is only performed with conventional herbicides and non-chemical methods. Soybean production in Germany has rapidly increased during the last seven years. The production has increased more than ten times in Germany since 2009 (FAOSTAT, 2016). Pre- and post-emergent herbicide application is a conventional and effective approach for weed control in soybean cultivations. Occasionally, the herbicides can also damage the crops, delay crop growth and reduce crop yield when applied under unfavourable soil conditions or at incorrect timing or mixture (Salzman and Renner, 1992; Johnson *et al.*, 2002). Thus, early identification of herbicide stress can help farmers to make proper crop management decisions.

Conventional estimation of herbicide damage on crops was conducted by visual assessment (Donald, 1998). For instance, the soybean yield loss could be correlated to the injury symptoms of the stressed plants (Weidenhamer *et al.*, 1989; Bailey and Kapusta, 1993). Advances in computer and photography technology enabled a quantitative assessment method by measuring crop ground cover (Donald, 1998). Linear relationship was presented between the relative soybean yield and percentage of ground cover. The light reflectance was also used to evaluate the herbicide injury to herbicide (Adcock *et al.*, 1990). However, these methods evaluate the crop healthiness according to the visible features. It usually requires a relatively long period of time so that the phytotoxic symptoms can be identified on the plants or the plants can grow large enough for the ground cover rates distinction. Chlorophyll fluorescence imaging technology is a non-destructive method to investigate the physiological reaction of photosynthesis system II (PS II) of plants. This sensor is very sensitive for abiotic and

biotic stress detection on plants (Maxwell and Johnson, 2000; Schreiber, 2004; Janka *et al.*, 2015). Some laboratory and greenhouse research demonstrated that, after herbicide application, the chlorophyll fluorescence quantum of sensitive weeds was markedly higher than the resistant populations (Ahrens *et al.*, 1981; Ali and Machado, 1981; Hensley, 1981; Vencill and Foy, 1988; van Oorschot and van Leeuwen, 1992; Kaiser *et al.*, 2013; Zhang *et al.*, 2016). Wang *et al.* (2016) successfully practiced this technology in fields for a survey for resistance profiles of 40 *Alopecurus myosuroides* populations. By applying the chlorophyll fluorescence imaging technology, herbicide efficacy on weeds was observed within five days in above researches. However, the studies and measurements were carried out to distinct herbicide injured sensitive weeds from unstressed resistant population. The recovery of herbicide stress in crops has not been investigated.

The objective of this study is to test if herbicide stress in soybean and recovery can be identified shortly after herbicide application under greenhouse and field conditions using the chlorophyll fluorescence imaging technology.

2. Materials and methods

2.1. Experimental design

2.1.1. Greenhouse experiment

A greenhouse experiment was conducted in the Univeristy Hohenheim from November 2013 until April 2014. Soybeans (Sultana, R.A.G.T. Saaten, Germany) were sown in pots filled with 6.5 kg soil mixture of 50% clay, 25% silt, and 25% sand. The depth of soil mixture was about 80 mm. The soybeans were sown in depth of 45 mm with three seeds per pot (equivalent to 96 seeds m^{-2}). Plants were grown in a light cycle of 16 h day and 8 h night. The temperature was kept at 25 °C during the day and 15 °C at night. All pots were placed in a randomized complete block design with four blocks. Three herbicide combinations with recommended dosages were selected for the treatments, respectively, including, 1) 0.3 kg ha^{-1} Sencor® WG (700 g a.i. L^{-1} *metribuzin*, WG, Bayer CropScience) plus 0.25 L ha^{-1} Centium® 36 CS (360 g a.i. L^{-1}

clomazone, CS, Cheminova Deutschland GmbH) plus 0.8 L ha^{-1} Spectrum® (720 g a.i. L^{-1} *dimethenamid-P*, EC, BASF); 2) 2.0 kg ha^{-1} Artist® (175 g a.i. kg^{-1} *metribuzin*, 240 g a.i. kg^{-1} *flufenacet*, WG, Bayer CropScience), plus Harmony® SX® (500 g a.i. kg^{-1} *thifensulfuron*, SG, Du Pont); 3) Harmony® SX® (500 g a.i. kg^{-1} *thifensulfuron*, SG, Du Pont), Basagran® (480 g a.i. L^{-1} *bentazon*, SL, BASF), plus Fusilade® MAX (125 g a.i. L^{-1} *fluazifop-P-butyl*, EC, Syngenta). Additionally, herbicide combinations in half recommended dosages were also applied as separate treatments. Untreated control pots with and without hand weeding were included respectively in each block. Herbicide treatments were performed pre- and post-emergence depending on the registrations of the products. The application time is given in Table 10. A laboratory track sprayer chamber mounted with a single flat fan nozzle was used for the herbicide application (8002 EVS, TeeJet Spraying System Co., Wheaton, IL USA). The sprayer was calibrated for an applying volume of 200 L ha^{-1}. The applications were performed 500 mm above soil surface.

Table 10. The herbicide application time in greenhouse experiment (in days after sowing of soybeans). H1, herbicide combination 1; H2, herbicide combination 2; H3, herbicide combination 3; E, early application; L, late application; D1, recommended dosage; $D_{0.5}$, half recommended dosage.

Treatments	Days after sowing						
	Before emergence		After emergence				
	4	11	24	31	33	38	45
H1ED1	*metribuzin, clomazone, dimethenamid-P*						
H1ED$_{0.5}$							
H1LD1		*metribuzin, clomazone, dimethenamid-P*					
H1LD$_{0.5}$							
H2ED1	*metribuzin, flufenacet*			*thifensulfuron*			
H2ED$_{0.5}$							
H2LD1		*metribuzin, flufenacet*				*thifensulfuron*	
H2LD$_{0.5}$							
H3ED1			*thifensulfuron, bentazon*		*thifensulfuron, fluazifop-P-butyl*		
H3ED$_{0.5}$							
H3LD1					*thifensulfuron, bentazon*		*thifensulfuron, fluazifop-P-butyl*
H3LD$_{0.5}$							

2.1.2. Field experiment

Five field experiments were conducted in 2015. The field trials were located in Southwest Germany at Böblingen, Calw, Nürtingen, Renningen and Tübingen. All the herbicide combinations were selected according to the local practice of the farmers during the last three years. Seeds of soybeans (Sultana, R.A.G.T. Saaten，Germany) were sown at a depth of 45 mm between 14th April and 15th May. Approximately 70 seeds m^{-2} were sown with row distance of 170 mm in the fields. The experiments were set up as randomized complete block design with four blocks and five treatments. The size of each plot was 2×5 m. Herbicide application was carried out three days after sowing with i) 2.0 kg ha^{-1} Artist® (175 g a.i. kg^{-1} *metribuzin*, 240 g a.i. kg^{-1} *flufenacet*, WG, Bayer CropScience), ii) 1.5 kg ha^{-1} Stomp® Aqua (455 g a.i. L^{-1} *pendimethalin*, CS, BASF) plus 2.0 L ha^{-1} Quantum® (600 g a.i. L^{-1} *pethoxamid*, EC, Cheminova Deutschland GmbH), iii) 0.4 L ha^{-1} Sencor® Liquid (600 g a.i. L^{-1} *metribuzin*, SC, Bayer CropScience) plus 0.25 L ha^{-1} Centium® 36 CS (360 g a.i. L^{-1} *clomazone*, CS, Cheminova Deutschland GmbH) and iv) 0.4 L ha^{-1} Sencor® Liquid (600 g a.i. L^{-1} *metribuzin*, SC, Bayer CropScience) plus 0.25 L ha^{-1} Centium® 36 CS (360 g a.i. L^{-1} *clomazone*, CS, Cheminova Deutschland GmbH) plus 0.8 L ha^{-1} Spectrum® (720 g a.i. L^{-1} *dimethenamid-P*, EC, BASF). An untreated control was included in each block at all sites. Herbicides were sprayed with an electric motorized plot sprayer with Lechler IDK 120-02 nozzles (Metzingen, Germany). The spraying volume was calibrated to 200 L ha^{-1}. No rainfall was recorded within 24 hours after treatments.

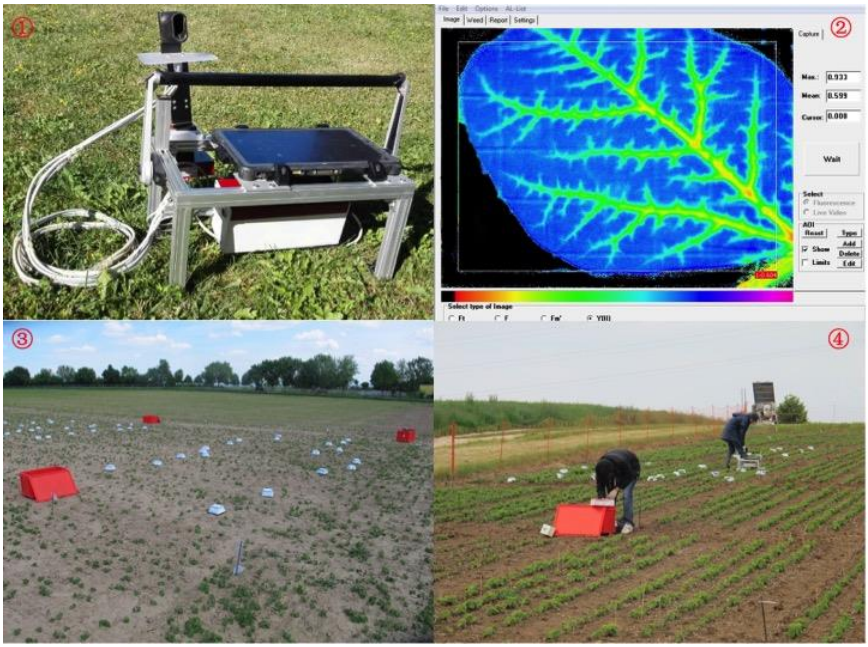

Figure 9. The mobile fluorescence sensor, Weed PAM® system. ① The designed setup of the sensor. It consists of the camera control unit and the computer including software. ② The software interface when measuring a herbicide treated leaf of soybean. The purple pixels represent leaf area with higher *Fv/Fm* values, while the red pixels donate leaf area with lower *Fv/Fm* values. ③ The distribution of dark adaption cover boxes when conducting first measurement at one-leaf stage of soybeans at site Böblingen. ④ The measurement at two-leaf stage of the soybeans at site Nürtingen.

2.2. Chlorophyll fluorescence sensor

The mobile fluorescence sensor, Weed PAM® system (Heinz Walz GmbH, Germany), was used to measure the chlorophyll fluorescence in this research. It contains 40 dark adaption cover boxes, a camera head, a tablet computer and a central control unit. LED lights with 460 nm wavelengths were mount on the camera head to induce chlorophyll fluorescence. The camera detects fluorescence signals above 680 nm after an optical red long pass filter. The efficiency of photosynthesis system II (PS II) of soybeans was determined by measuring the maximal PS II quantum yield (*Fv/Fm*). It is calculated as

$$Fv/Fm = \frac{Fm - Fo}{Fm}$$

where *Fo* is the dark fluorescence yield, *Fm* is the maximal fluorescence yield (Maxwell and Johnson, 2000). The Weed PAM® system was operated by the software "ImagingWin" (Heinz Walz GmbH, Germany). With this software, the background noise can be removed as described by Kaiser *et al.* (2013).

2.3. Measurements and data analysis

For the greenhouse experiment, all the measurements with the Weed PAM® system were conducted 19, 21, 26, 31, 38 and 47 days after sowing (at least one plant had emerged in each pot). One plant per pot was selected for the measurement. All the plants were dark adapted with the dark adaption cover boxes for 30 min before measuring. Whole plants of soybeans were collected and washed 67 days after sowing. The root and above ground biomass were cut and dried separately. After 48 h drying in a drying chamber under 80 °C, the dry biomass was measured.

For the field trials, three measurements were taken at each site, respectively, when the soybeans were at one-leaf stage (BBCH 10), two-leaf stage (BBCH 11) and three-leaf stage (BBCH 12). Ten soybean plants were measured in each plot. All the plants were dark adapted with the dark adaption cover boxes for 25-30 min before measuring. Values of all 40 plants were averaged. During the measurement, each plant

was marked with an orange stick and label. Above ground biomass was cut on 15th July 2015 (ten to twelve weeks after sowing) at all five sites. Plants were cut in each plot from an area of 0.5 m^2. The dry aboveground biomass of soybean was weighted after 48 h drying in a drying chamber under 80 °C.

Data were analysed with R (Version 3.0.2) and the package *agricolae* and *lawstat* (R Development Core Team, 2008). The significance of herbicide effect on soybean plants was determined by ANOVA. Then the Tukey's HSD test of the ANOVA model was taken. All the datasets were proved to be normally distributed using *Shapiro-Wilk test* ($p>0.05$). Homogeneity of variances was analysed by *Levene's test* ($p>0.05$).

3. Results

3.1. Greenhouse experiment

In the greenhouse test, at least one plant emerged in each pot since 19 days after sowing. Due to Table 11, all three herbicide combinations reduced *Fv/Fm* of the soybeans (several results were ignored because of overexposure during the measurement). High emissions of chlorophyll fluorescence in treatments 1 and 2 occurred already after soybean emergence. The *Fv/Fm* of soybeans with pre-emergent herbicide treatments were significantly lower than the control plants during the first three weeks after application. However, the *Fv/Fm* of plants with post-emergent herbicide application dropped to lower level only for one week after treatment. Meanwhile, soybeans in treatments with half of the recommended dosage mostly presented no significantly different PS II reaction level than the untreated control plants. Both early and late application of herbicide could lead to *Fv/Fm* reduction of the soybean plants. Dry biomass measurement demonstrated that soybean plants in untreated group without hand-weeding had lowest weight. The soybean plants in untreated group with hand-weeding had relatively high biomass. However, the difference to the herbicide treated groups was not significant.

Table 11. The results of chlorophyll fluorescence measurements of the greenhouse experiment. H1, herbicide combination 1; H2, herbicide combination 2; H3, herbicide combination 3; E, early application; L, late application; D1, recommended dosage; $D_{0.5}$, half recommended dosage; ConH, control with hand weeding; Con, control without hand weeding; significant differences between mean values are indicated by different letters (Tukey's HSD Test, $p<0.05$).

Treatments	Days after sowing											
	19		21		26		31		38		47	
H1ED1	0.264	b	0.241	cd	0.271	cd	0.484	bc	0.717	a	0.724	a
H1ED$_{0.5}$	0.425	ab	0.520	abc	0.483	abc	0.608	abc	0.739	a	0.731	a
H1LD1	0.330	b	0.386	bcd	0.361	bcd	0.605	abc	0.740	a	0.725	a
H1LD$_{0.5}$	0.463	ab	0.466	abcd	0.405	abcd	0.577	abc	0.708	a	0.716	a
H2ED1	0.296	b	0.285	cd	0.295	cd	0.476	bc	0.723	a	-	
H2ED$_{0.5}$	0.420	ab	0.419	abcd	0.336	cd	0.515	abc	0.720	a	0.697	a
H2LD1	0.235	b	0.201	d	0.152	d	0.432	c	0.720	a	0.705	a
H2LD$_{0.5}$	0.306	b	0.267	cd	0.345	cd	0.567	abc	0.727	a	0.714	a
H3ED1	0.655	a	0.695	a	0.425	abcd	0.644	abc	0.737	a	0.724	a
H3ED$_{0.5}$	0.652	a	0.690	a	0.537	abc	0.679	ab	0.746	a	0.729	a
H3LD1	0.641	a	0.691	a	0.667	a	0.668	ab	0.666	a	0.705	a
H3LD$_{0.5}$	0.616	a	0.671	ab	0.650	ab	0.673	ab	0.707	a	0.722	a
ConH	0.641	a	0.674	a	0.694	a	0.720	a	-		0.751	a
Con	0.636	a	0.636	ab	0.643	ab	0.672	ab	-		0.733	a

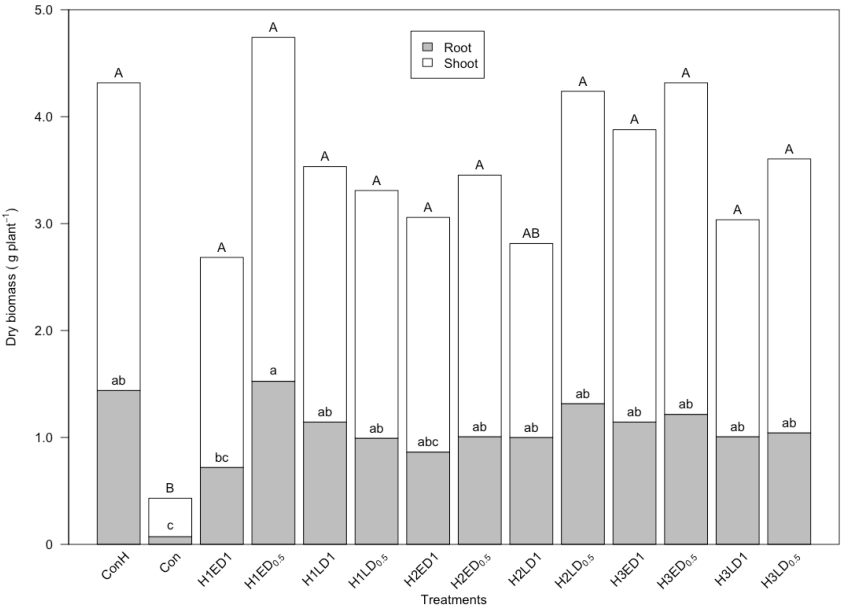

Figure 10. Root and shoot dry biomass per soybean plant on 67 days after sowing. ConH, control with hand weeding; Con, control without hand weeding; H1, herbicide combination 1; H2, herbicide combination 2; H3, herbicide combination 3; E, early application; L, late application; D1, recommended dosage; $D_{0.5}$, half recommended dosage; significant differences between mean values are indicated by different letters (Tukey's HSD Test, $p<0.05$).

Table 12. The results of chlorophyll fluorescence and dry biomass measurements of the field experiment. MoA, Mode of Action; C1, Inhibition of PS II; F4, Inhibition of DOXP synthase; K1, Inhibition of microtubule assembly; K3, Inhibition of cell division (VLCFA); *, significant stress efficacy in both measurement; significant differences between mean values are indicated by different letters (Tukey's HSD Test, $p<0.05$).

Sites	Treatment	MoA	*Fv/Fm*			Biomass ($g\ m^{-2}$)	Significant stress
			Date 1	Date 2	Date 3		
Böblingen	Control	-	0.575a	0.587a	0.666a	310b	
	i	C1 K3	0.423b	0.503a	0.681a	394b	*
	ii	K1 K3	0.543a	0.607a	0.639a	476a	
	iii	C1 F4	0.490ab	0.567a	0.674a	450a	
	iv	C1 F4 K3	0.428b	0.524a	0.639a	356b	*
Calw	Control	-	0.584a	0.558ab	0.672a	40b	
	i	C1 K3	0.575a	0.524bc	0.645ab	296a	
	ii	K1 K3	0.585a	0.571ab	0.647ab	226ab	
	iii	C1 F4	0.596a	0.464c	0.563c	130b	*
	iv	C1 F4 K3	0.585a	0.593a	0.627b	248ab	
Nürtingen	Control	-	0.586a	0.602a	0.722a	580a	
	i	C1 K3	0.629a	0.531ab	0.706a	548a	
	ii	K1 K3	0.586a	0.516b	0.644b	490b	*
	iii	C1 F4	0.583a	0.592a	0.714a	558a	
	iv	C1 F4 K3	0.601a	0.577ab	0.709a	526a	
Renningen	Control	-	0.411a	0.472a	0.645ab	102b	
	i	C1 K3	0.440a	0.513a	0.613b	206a	
	ii	K1 K3	-	0.474a	0.666a	242a	
	iii	C1 F4	0.498a	0.490a	0.426c	136b	*
	iv	C1 F4 K3	-	0.514a	0.632ab	216a	
Tübingen	Control	-	0.545a	0.545a	0.662a	85b	
	i	C1 K3	0.529a	0.478a	0.659a	147a	
	ii	K1 K3	0.555a	0.472a	0.663a	125a	
	iii	C1 F4	0.517a	0.518a	0.658a	150a	
	iv	C1 F4 K3	0.545a	0.520a	0.667a	110a	

3.2. Field experiment

At Böblingen, the *Fv/Fm* of soybean seedlings in the treatment i and iv was significantly lower than in the untreated control plants already at the first measurement. But the plants recovered until the second measurement. The biomass weight of soybean plants with treatment i and iv was significantly lower than the soybean plants of all other treatments. The biomass of soybean in the plots without herbicide treatment was lowest probably due to weed competition.

At Calw, the soybean plants presented lower photosystem efficiency in treatment iii. Unlike at Böblingen, the herbicide stress on PS II appeared, when plants produced the second leaf. Moreover, the stress lasted until the end of measurement. Biomass measurement showed significantly lower weight of soybean in the control and treatment iii than in the other treatments.

A significant response of PS II was observed in treatment ii at Nürtingen. *Fv/Fm* in soybean of treatment ii was reduced from second measuring date until the end of measurement similar to the trial at Calw. Weed infestation at this site was very low. Therefore, biomass of soybeans was not reduced in the untreated plots.

First measurement results of treatment ii and iv at Renningen were lost due to unexpected power failure when exporting the data from sensor. At this site, *Fv/Fm* reduction occurred in treatment iii. But the difference could only be distinct until the third leaf of soybeans was produced. The biomass measurement also showed lower weight of soybeans in control group and under treatment iii.

At Tübingen, except the biomass of soybeans in untreated plots, no markedly different PS II quantum yield and biomass were observed between the treatments.

4. Discussion

The chlorophyll fluorescence measurement showed that herbicide induced stress on PS II of young soybeans plants in all treatments in the greenhouse, as well as at four sites out of the five field trials. Herbicides with six modes of action were included

in the study, which were PS II inhibition, DOXP synthase inhibition, microtubule assembly inhibition, cell division inhibition, ALS- and ACCase inhibitors. Several authors support our findings, that most herbicides reduce light reactions of photosystems shortly after application, especially when the herbicide dose absorbed by the plants exceeded their metabolism capability (Dayan and Watson, 2011; Dayan and Zaccaro, 2012; Wang *et al.*, 2016).

Metribuzin rapidly inhibits the PS II after treatment by binding at the Q_B site of *plastoquinone* and interrupting the electron transfer flow (Ventrella *et al.*, 2010). Most cultivars of soybean are tolerant to *metribuzin*. Therfore, *metribuzin* provides selective weed control in soybean (Hardcastle, 1974; Barrentine *et al.*, 1976). Sultana which was selected for this research is a *metribuzin* tolerant cultivar. According to Falb and Smith (1984), tolerant soybean cultivators can detoxify *metribuzin* within 106 hours after treatment. These finding corresponded to our chlorophyll fluorescence imaging measurements revealing a rapid recovery from *metribuzin* treatments mainly in the field trial at Böblingen. In treatment 3 of the greenhouse test, the stress could also be induced by the PS II inhibitor *bentazon*, as the separated application of *thifensulfuron* and *fluazifop-P-butyl* caused no effect on the *Fv/Fm* of the soybean plants. Post-emergent ALS- and ACCase-inhibiting herbicides did not cause any stress to soybeans. However, their activity against weed species is limited as well. That is why pre-emergent herbicides in soybean production play a major role in weed management.

In the greenhouse study, early occurrence and long duration of stress effect took place after treatment of herbicide combination 1 and 2. Apart from the PS II inhibitor, DOXP synthase- and cell division- inhibitors were also included in the herbicide mixtures. Thus, other stress mechanism might take place as well in these groups.

In field experiments, inhibition on PS II of soybeans at site Calw and Renningen also occurred later and lasted longer than the photosystem regulation at site Böblingen. Besides *metribuzin*, *clomazone* (inhibitor of DOXP synthase) was also involved in the stressed treatments. Non-mevalonate 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway is a main biosynthesis approach for plastidic isoprenoids, such as carotenoids,

phytol (a side-chain of chlorophylls), plastoquinone-9, isoprene, mono-, and diterpenes (Lichtenthaler, 1999). Most of the biosynthesis proceeded inside the chloroplast (Lichtenthaler *et al.*, 1997). Chlorophyll production could be reduced as less phytol was provided due to the DOXP synthase inhibition. Therefore, the photosystem efficiency of DOXP synthase stressed soybeans was lower than the unstressed ones when the plants grew larger. The *Fv/Fm* reduction of soybean plants in treatment iii at site Calw and Renningen could be attribute to the application of *clomazone*.

The combination application of *pendimethalin* (microtubule assembly inhibitor) and *pethoxamid* (cell division inhibitor) induced stress on PS II at site Nürtingen. Dinitroanilines herbicides like *pendimethalin* bind to α-tubulin (Morrissette *et al.*, 2004). Thus, the free tubulin could not group into ploymetrization as microtubule. Early research noted that dinitronanilines could interfere with the photosynthesis system dramatically by oxygen evolution (Morland *et al.*, 1972a, b). Chloroacetamides inhibits very-long-chain fatty acids (VLCFA) synthase. The herbicide markedly reduces VLCFA content in plasma membrane and results in cell death (Böger, 2003). Some chloroacetamides (e.g. *carbetamide*) could inhibit electron transport up to 50% as a secondary effect of membrane destabilization (Weisshaar and Böger, 1987; Dayan and Zaccaro, 2012). Therefore, the chlorophyll fluorescence of plants could be altered. It correlated well to the *Fv/Fm* regulation of soybean under combination treatment 2 of *metribuzin* and *flufenacet* in the greenhouse test. However, *metribuzin* was not to be the only compound causing stress in soybean. As the herbicides inhibiting either cell division or VLCFA synthase might induce the regulation on photosystem, the stress mechanism in the treatment ii at site Nürtingen still could not be clearly explained. Furthermore, considering the long period stress on soybeans under combination treatment 1 in the greenhouse experiment, it could also be induced by the combined effect of DOXP synthase- and cell division-inhibitors after the effect of PS II inhibitor *metribuzin*.

The biomass assessment on herbicide treated soybean significantly distinct the stressed or non-stressed groups in the field. Apparently, the biomass assessment results correlated well with the sensor measurements. This correlation was also observed in the greenhouse study.

Weed PAM® technology allows quantifying soybean response to herbicide treatments. The variation of plants' chlorophyll fluorescence emission could be detected shortly after treatment. Thus, herbicide damage to soybean can be avoided by proper selection of products. Since soybean cultivars respond differently to herbicides, Weed PAM® technology can help to select the most tolerant cultivars.

5. Conclusion

Herbicides interfere directly or indirectly with the photosystem of plants and can reduce quantum use efficiency of PSII in soybean plants and result in lower biomass. With the chlorophyll fluorescence imaging technology, it is capable to identify the stress rapidly in young growth stages. This achievement will support farmers to avoid herbicide combination that reduce crop growth.

Acknowledgements

The authors appreciate Simon Hotz, Moritz Sauter and Dr. Hui Li for collecting the data, and our colleagues from the State Plant Protection Services for preparing the field trails. We thank the Chinese Scholarship Council (grant number 201306350053) and the Ministry of Ländlicher Raum Baden-Württemberg for financial support.

Chapter 4 General discussion

In the previous chapter, the new instruments Weed PAM® was introduced for the detection of herbicide resistance in field trials and farms. With this technology, the researchers and farmers will be available to conduct herbicide resistance test trials in field with less time, financial and labor expense. Due to the results, more proper herbicide applications can be selected. Additionally, fewer chemicals will be sprayed into fields and environment as inefficient input.

1. Herbicide resistance detection with Weed PAM®

1.1 Greenhouse and field tests with Weed PAM® measurement

The experiments, which were presented in Chapter 3 Section I and II, used the sensor Weed PAM® to identifying the herbicide and abiotic stresses on the weed *Alopecurus myosuroides*. The system capability was firstly proved in greenhouse and then practiced in the field trials.

Due to the greenhouse test, significant difference of photosynthesis system II (PS II) activities of resistant and sensitive populations was clarified after herbicide treatment. The *A. myosuroides* displayed different PS II activity level according to the each mode of action, which presented the consistency to many former researches (Ahrens *et al.*, 1981; Ali and Machado, 1981; Kaiser *et al.*, 2013). Therefore, the Weed PAM® system can be applied with various principles for herbicide resistance detection.

The acetolactate synthesis (ALS) inhibitors rapid up the D protein turnover and resulted in slower electron transfer rate in PS II system in sensitive plants (Öttmeier, 1999; Whitcomb, 1999). The greenhouse experiment results showed rapid reduction of *Fv/Fm* values of sensitive *A. myosuroides* during the first three days after the treatment of ALS inhibitors. Little expand was observed afterwards. Meanwhile, even there were still fluctuations of the *Fv/Fm* values of resistant plants during the measuring period. The difference to the value of control plants was not significant.

Thus, ALS resistant populations could be identified as soon as three days after treatment (DAT) in the greenhouse test.

Acetyl coenzyme-A carboxylase (ACCase) inhibitors slow down the synthesis activities of lipid acids which consuming a lot of ATPs (Kukorelli *et al.,* 2013). The PS II activities were then inhibited according to the lower demand of ATP consumption (Harwood, 1988). According to the results in Chapter 3 Section I, long stress duration of ACCase inhibitors on the sensitive *A. myosuroides* plants was observed. The significant differences of *Fv/Fm* values between the sensitive and resistant plants could also be identified from 3 DAT onwards.

The PS II inhibitor *isoproturon* affected the photosystem reaction of both sensitive and resistant *A. myosuroides* populations that were tested in this research. The *Fv/Fm* values of sensitive plants were much lower than the resistant plants after treatment. Meanwhile, the *Fv/Fm* values of resistant plants were significantly lower than the control groups. This phenomenon presented different effect of PS II inhibitors on photosynthesis activities of target site resistant grass. The PS II inhibitors cannot influence the PS II activities of target site resistant plants significantly (Pfister and Arntzen, 1979). However, the non-target site resistant plants can be partly impacted. The herbicide ingredients would be gradually detoxified by the non-target site resistant plants due to N-demethylation and ring alkyl-oxidation (Burnet *et al.,* 1991; Moss 1990b). Thus, there will be lower rates of herbicide ingredients binding to the target site of D1 protein (Hess, 2000). Therefore, it was assumed that the resistant *A. myosuroides* population in this study has non-target site resistance to *isoproturon*. Rather than just identifying resistant populations, this result presented a potential for the Weed PAM® sensor to recognize the resistance mechanism against PS II inhibitors, as either target site or non-target site types. However, further investigation on both resistant biotypes should be conducted to clarify that.

While the greenhouse test has shown high capability of the Weed PAM® to detect herbicide resistant populations, many field trials were then conducted. The first field trial was conducted very similar to the greenhouse herbicide treatments but with

only sensitive *A. myosuroides* sown in a winter wheat field. Due to the results, the sensitive population could also be identified after the herbicide treatment due to their *Fv/Fm* reduction, however on DAT 5. Based on the outcome of this experiment, larger scales of field experiments were conducted to test this detection system's application capability in ten locations including 50 populations. Both sensitive and resistant populations were involved in these biotypes. Measurements were carried out on DAT 5. In addition, visual assessments were conducted on DAT 21 to verify the detection results. The results showed that in total 95% of these populations were correctly classified. It means that the Weed PAM® sensor is capable to detect herbicide resistant *A. myosuroides* populations in the same growing season as winter cereals. This method would contribute to adopting alternative weed control strategies promptly for the farmers.

1.2 Effects on the Weed PAM® measurement

Fluctuations of the *Fv/Fm* values of untreated plants were observed in both greenhouse and field trials. Concerning that, many abiotic stresses were considered to be the origin for this influence (Rohacek, 2002; Baker, 2008; Burke *et al.*, 2010). In the greenhouse test, two common abiotic factors were investigated for their influence on PS II of *A. myosuroides*. Water shortage stress was clearly visible with the sensor data, while the *Fv/Fm* measurement could not differentiate between nitrogen deficiency stressed and control plants. It corresponded to Baker and Rosenqvist (2004)'s publication, where the authors could not find any reduction of *Fv/Fm* values with nitrogen deficiency stress until the plants were severely stressed and this stress was visibly assessable. According the results, the *Fv/Fm* values reduced slightly at the beginning of measurement when the plants were treated with lower water supply. The reduction only got significant since five days after the water shortage treatment when the plants were severely stressed. The water shortage stress can reduce the CO_2 availability resulting in change of the photochemistry and carbon metabolism (Ashraf and Harris, 2013). During the initial period of water shortage stress, the stomata on plant leaves will close. This would lead to the increase of water using efficiency

(Chaves *et al.*, 2009). However, if the drought stress existing in long term, dehydration of mesophyll cells will take place, resulting in a significant inhibition of metabolic processes of photosynthesis and a reduction of plant water using efficiency (Damayanthi *et al.*, 2010).

Moreover, due to the field trials, temperature was recognized to be one of the main factors that could affect the PS II reaction of *A. myosuroides*. Even up to 95% of the weed resistance classification of the field tests were correct, 5% of the populations were false classified. All the false classification occurred, when the measurements were conducted at temperatures below freezing point. The low temperatures, especially the frost, would damage the chloroplast and reduce the photosystem activity levels (Krause, 1994; Lundmark *et al.*, 1998b). Therefore, the *Fv/Fm* values are reduced. Due to the frost impact, it is suggested that the in field detection for herbicide resistance with Weed PAM® system should be applied above 0 °C.

2. The herbicide stress detection in soybeans at early growth stage

The field experiment in Chapter 3 Section III demonstrated the extended application of Weed PAM® system in the detection of herbicide stress on the crop soybean. Several herbicide combinations were involved in this study, including ALS-, ACCase-, PS II-, DOXP synthase-, microtubule assembly- and cell division-inhibitors. The greenhouse experiment demonstrated that post-emergence application of ALS- and ACCase-inhibitors could not induce stress on the soybeans' PS II. Both greenhouse and field experiments showed that, significant reduction of *Fv/Fm* occurred under the stress of pre-emergence herbicides. Lower *Fv/Fm* of soybeans with PS II inhibitors treatment displayed usually since emergence. The PS II inhibition could attribute to the ingredients' binding at Q_B shortly after the absorption of herbicide (Ventrella *et al.*, 2010). While Sultana is a *metribuzin* tolerant soybean cultivator, the stress would be overcome within 106 hours due to the fast metabolism (Falb and Smith, 1984, 1987). Apart from PS II inhibitor, another photosystem-based herbicide, DOXP synthase inhibitor, could also induce stress on the PS II of soybeans. It occurred in both greenhouse experiment and some sites of the field experiments.

Non-mevalonate 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway is a main biosynthesis approach for plastidic isoprenoids, such as carotenoids, phytol (a side-chain of chlorophylls), plastoquinone-9, isoprene, mono-, and diterpenes (Lichtenthaler, 1999). Mechanism of PS II inhibition by DOXP synthase inhibitor was described in Chapter 3 Section III. Less chlorophyll was produced in soybeans when the plants were treated with DOXP synthase inhibitor. Thus, the photosystem efficiency would reduce. That correlated with the later and longer stress period of DOXP synthase inhibition than PS II inhibition. The microtubule assembly inhibitor dinitronanilines could interfere the photosynthesis system dramatically by oxygen evolution (Moreland *et al.*, 1972a, b). In the greenhouse test, it took place as a stress extension on photosystem after PS II inhibition from *metribuzin*. Cell division inhibitor chloroacetamides inhibit very-long-chain fatty acids (VLCFA) synthase. Former studies showed that it could regulate half of the electron transport in plant (Weisshaar and Böger, 1987). The photosystem efficiency could decrease as a secondary effect and demonstrated lower *Fv/Fm* values (Dayan and Zaccaro, 2012). Due to the investigation with greenhouse and field studies, it could be concluded that the herbicide stress on soybeans could be identified shortly after emergence. The chlorophyll fluorescence imaging technology could be used for the detection of herbicide stress on soybeans at early growth stage. Farmers can benefit from this knowledge to take integrated cultivation strategies in the same growth season.

3. Further work to improve the Weed PAM® system

As protocol type of a new method, even the Weed PAM® system had been proved on the detection *A. myosuroides* resistance against ALS-, ACCase- and PS II-inhibitors, some problems were found during the greenhouse and field application. Besides, there were some more factors should be included in the analysis modeling and several suggestions maybe helpful to improve the application.

According to the analysis of the results of the greenhouse experiments and existing literatures, it indicated that the resistance mechanisms of target site resistance

and non-target site resistance against PS II inhibitors might be identified using the method of chlorophyll fluorescence measurement (Chapter 3 Section I). However, further researches including both resistant biotypes should be done to clarify this hypothesis. Furthermore, only the PS II inhibitors in the group of Ureas (C2 in HRAC code) were reported to have different effect on chlorophyll fluorescence emission of target site and non-target site resistant populations (Moss, 1990b; Burnet, 1991). For wider spectrum of application, the photosynthesis activities under stress of PS II inhibitors in other subgroups should be observed in further experiments.

The system was successfully practiced in detecting ALS- and ACCase- inhibitors in the field experiments. But the resistance to PS II inhibitors could not be identified due to the lack of sensitive standard samples in the fields. For the detection of non-target site resistance to PS II inhibitors, a wider field investigation on the herbicide effect on the photosystem of the biotypes is required. So that a reduction range of the *Fv/Fm* values can be standardized. With this improvement, the non-target site PS II inhibitor resistant populations will be recognized just by comparing with the control plants, but without the sensitive standard samples anymore.

Many abiotic factors have been proved to have influence on the photosystem activities of plants (Maxwell and Johnson, 2000). The greenhouse experiment indicated that the nitrogen deficiency could not affect the PS II activity, as the *Fv/Fm* values did not decreased under the lack of nitrogen fertilizer application during the tillering stage. Even the water shortage effect reduced the *Fv/Fm* values of plants five days after application, observation during the experiment showed that the soil water content had reached the wilting point. Not only the chlorophyll fluorescence differentiation could be measured, but also the drought stress symptoms were already visible. As the herbicide application and on field detection of resistant population are conducted in autumn and early spring when the soil moisture is usually suitable for plant germination and growth, the water shortage effect might be exclude from the negative impacts for the Weed PAM® sensor. Due to the results of field experiments, the fluctuation of *Fv/Fm* values were displayed for the average values of control plants

which were measured on different days. In addition, false detections were taken place in some cold days. In these incorrect detections, low *Fv/Fm* values were measured of the weeds. That proved the frost damage to photosystem II as many researches have reported (Örlander, 1993; Lundmark *et al.*, 1998b). Therefore, an experiment should be established on the temperature effects on photosystem II. Beside normal temperatures, the factor should include both frost and heat stress levels (Janka *et al.*, 2015).

Resistance against ALS-, ACCase- and PS II-inhibitors are the most common cases of resistant *A. myosuroides* biotypes in Germany and even in Western Europe. However, many other different herbicide resistance cases are reported overall the world. Detection cases should be conducted with other common resistant species including monocots like ryegrass (*Lolium multiforum*), blue grass (*Poa annua*), barnyard grass (*Echinochloa crus-galli*), etc. and dicots like common lambsquarters (*Chenopodium album*), corn poppy (*Papaver rhoeas*), redroot pigweed (*Amaranthus retroflexus*), etc. Furthermore, herbicides in other groups of mode of action should be tested, especially for many dicot species which are resistant to Synthetic Auxins (O in HRAC code), EPSP synthase inhibitors (G in HRAC code), PPO inhibitors (E in HRAC code), etc. The Weed PAM® system will be more capable for commercial practice when these optimizations are finished.

4. Integrated herbicide resistance management

Herbicide resistance should be managed in integrated strategies including chemical, biological and mechanical contributions. The proper herbicide application can slow the development of herbicide resistance while the non-chemical methods can control the weeds without concerning the resistant problems.

The proper use of herbicide plays a significant aspect. Firstly, the application time is an important factor for herbicide effect on the weeds. Researches on post emergence herbicides have shown that early application of herbicides could enhance the herbicide efficacy on weed control and crop yield than the later applications when

plants grew larger (Carey and Kells, 1995). In addition, herbicides in proper mode of action (MoA) should be selected for the treatment. A rotation of herbicides in different MoAs will help to reduce the risk of resistance development (Norsworthy *et al.*, 2012). Furthermore, it is suggested by many research and modeling results that the herbicides should be applied at recommended dosage (Neve, 2007). Long term high rate applications of herbicide in same mode of action would cause target site resistance, while the repeated use of reduced rates of herbicides would lead to metabolic resistance (Powles and Preston, 1995; de Carvalho *et al.*, 2009).

The output of this thesis contributed to all the points of the chemical strategy mentioned above, application time, herbicide's MoA and dosage selection. With the Weed PAM® system, the resistant populations may be recognized shortly after the herbicide application. Therefore, farmers will get the possibility to make further strategies and ensure the weed control efficacy before the weeds growing up to much larger size. In addition, the sensor will be helpful to evaluate the herbicide efficacy in dose response test shortly prior to the herbicide application on the whole field. The proper ingredient and suitable dose can be selected soon after the efficacy determination using the Weed PAM® system. This will optimize the herbicide application strategy.

Cultivation practices like crop competition are also commonly adopted to control the herbicide resistant weeds (Norsworthy *et al.*, 2012). Crop competitiveness can be employed to reduce the weed emergence and growth (Mhlanga *et al.*, 2016; Peerzada *et al.*, 2016). Effective approaches include cultivator selection, increasing seed rate, using narrow row space, optimizing sowing dates, improving irrigation and fertilizing management, and involving crop rotation (Schreiber, 1992; Jordan, 1993; Webster *et al.* 2009). Each method does not guarantee the commercial benefit of the weed control. Growers should concern the integrated effect for the equipment modification, increasing production costs, availability of labor and farming size, and cropping season length individually.

Other common biological management of weed resistance is practiced using cover crops and mulching. During growing season, the cover crops or mulch can establish barriers among crop rows. Thus, less light and growth space are available for the weeds (Banarwa *et al.*, 2011). Allelopathy, plants' ability to affect other plants growth by releasing allelochemicals, is another aspect of using cover crops as biological weed management. It has been proved in many crop cultivations, including cereal rye, wheat, oat, sunflower, maize and sorghum (Norsworthy *et al.*, 2012). However, growers should carefully choose the cover crop species. Because, some cover crop would also have negative influence on main crop's growth. In addition, the growers should also take care of the factors that may affect the allelopathic ability when using cover crops for weed control, for instance, the environment conditions of field, the management practice and the cover crop's biomass.

Last but not least, mechanical weed control is one of the most traditional and effective weed management practices. Using tillage, the small weeds can be buried, the roots can be damaged and the broadleaf weeds can be cut. The conventional hoeing or harrowing can only control the weeds inter rows. But with the finger weeder, it is now available to control weeds inner rows (Kunz *et al.*, 2015). Tillage contributes also to reduce the seedbank in soil. It stimulates the weed seed germination at beginning of crop season but prevents the seed input with further weed control (Gallandt, 2006). Intensive tillage such as plow, can invert soil layers and bury weed seeds deep enough to prevent them from germination (Bennett, 2011).

According to the discussion above, we can conclude that applying herbicide seems to be the most simple and fast method to control weeds. However, herbicide resistance can develop by improper or repeated application of herbicides in same mode of action. The early detection of herbicide resistant species can help growers to make right decisions on integrated weed control strategies for crop cultivation.

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Summary

All over the world, herbicide resistance has developed to one of the most important barriers in weed control, making the implementation of the weed control strategy more complicated. One of the first steps to deal with this challenge is to identify the herbicide resistant species. Many approaches have been designed for that purpose. However, most of the currently available methods are adopted for laboratory or greenhouse use. Until now there is no feasible measuring method in the field. Furthermore, these methods are cost and labor intensive.

There is an intense need for a rapid, cheap and reliable method to conduct in field detection of herbicide resistant weed populations. In the current thesis with the use of chlorophyll fluorescence imaging technology, such a method is implemented and tested in field conditions. Therefore, the objectives of this study were (i) to clarify if a chlorophyll fluorescence sensor can be used to identify the sensitive and herbicide resistant weeds, (ii) to verify the applicability of the chlorophyll fluorescence imaging technology in the field, (iii) to evaluate its robustness concerning herbicide resistant weed detection, and (iv) to investigate if the chlorophyll fluorescence imaging technology is capable of identifying herbicide stress on the crop plants shortly after a herbicide application. In order to realize these objectives a series of experiments were designed and carried out. The data gathered from these experiments were compiled under three paper articles.

Paper 1. In this study, a greenhouse experiment was conducted to verify if the parameter, Maximal Photosystem II Quantum Yield (*Fv/Fm*), could possibly indicate the herbicide efficacy. The chlorophyll-fluorescence-imaging sensor, Weed PAM®, was selected for the measurements. The experiment was separated into two parts. In the first part it was investigated if the *Fv/Fm* value could differentiate between herbicide sensitive and resistant plants. In the second part two important abiotic stress factors were tested if they affected the *Fv/Fm* value. I) Six herbicides were tested on herbicide sensitive and resistant *Alopecurus myosuroides* populations; II) Water shortage and nitrogen deficiency were applied on a herbicide sensitive population to

observe their influence on the plants. The sensitive plants presented significantly lower *Fv/Fm* values than the resistant plants three days after the treatment for the ALS and ACCase inhibitors. On the same day, and for the same treatments the *Fv/Fm* values of the resistant plants were not affected and similar to the control. Appling a PS II inhibitor, as a herbicide, reduced the *Fv/Fm* values of both sensitive and resistant plants rapidly. Yet, sensitive and resistant plants could clearly be separated, four days after treatment, based on the different *Fv/Fm* values. On the other hand, nitrogen deficiency did not influence the photosystem II measurements. Water shortage reduced rapidly the *Fv/Fm* value of the plants seven days after the application, yet at this point plant symptoms included the death of the plants. According to this experiment, the Weed PAM® sensor has proved its capability to identify the sensitive and resistant *A. myosuroides* populations shortly after the herbicide application.

Paper 2. In this study, a verification of the above results was made under field conditions for different *A. myosuroides* populations and different locations. On the first part 50 populations in total including both sensitive and herbicide resistant populations were tested in this experiment. On the second part field experiments were conducted in ten locations around Germany over two years with the local field population mix. It was investigated if the Weed PAM® sensor could separate between herbicide sensitive and resistant *A. myosuroides* populations five days after treatment (DAT). The 50 different populations were sown in a winter wheat field. Two ACCase- and three ALS- inhibitors were applied. In all herbicide treatments, *Fv/Fm* values of *A. myosuroides* were significantly lower than the untreated plants at the 5th DAT. At the different locations three ACCase- and four ALS- inhibitors were applied when the weeds were at the 3-7 leaves growth stage. For each location, 40 sample plants per treatment were selected, and measured with the sensor at 5 DAT. A visual measurement, to verify the result, was carried out at 21 DAT. In both cases, 95% of the plants were correctly identified as sensitive or resistant. This demonstrated the ability of the Weed PAM® sensor to conduct in field real time detection of herbicide resistant *A. myosuroides* populations shortly after treatment.

Paper 3. In this study, greenhouse and field experiments were carried out to investigate if the chlorophyll fluorescence of soybean plants was altered, under herbicide stress. Herbicide combinations including inhibitors of PS II, DOXP synthase, cell division and microtubule assembly were selected for different pre-emergence treatments. Herbicide combinations including inhibitors of PS II, ALS and ACCase were applied in post-emergence treatments. Chlorophyll fluorescence was measured from the emergence of soybeans until the three/four-leaf stage. Furthermore the stress effect of the different treatments on the soybean plants was determined by measuring their dry biomass. In the greenhouse, post-emergence treatments with ALS and ACCase inhibitors did not seem to induce stress on the soybean plants. As expected, it originally demonstrated low *Fv/Fm* values when stressed by PS II inhibitors. But the PS II system recovered soon, one week after emergence. Stress induced by other pre-emergence herbicides occurred one week after emergence and lasted longer than the stress induced by the PS II inhibitors. Dry biomass collaborated with the sensor result.

Based on the current thesis, the Weed PAM® system can be an important tool in the identification of herbicide resistant weed populations, in a timely manner. It has proven its capabilities both in *A. myosuroides* as a weed and in soybean plants. Yet its applicability needs to be proven in more grass and broad-leaved weeds or crop plants. This technology will help farmers to take more suitable weed control strategies, as well as less economic and environmental risks.

Zusammenfassung

Die weltweite Zunahme an Herbizidresistenzen stellt eine der größten Herausforderungen der heutigen Unkrautbekämpfung dar. Die Früherkennung einer Resistenz könnte einer zunehmenden Ausbreitung entgegenwirken. Bislang gibt es nur wenige Ansätze zur Erkennung von Herbizidresistenzen. Häufig findet die Identifikation einer Resistenz über aufwendige Labor- oder Gewächshausversuche statt , welche sehr zeit-, kosten und arbeitsintensiv sind.

Die heutige Landwirtschaft verlangt eine effiziente, kostengünstige und zuverlässige Methode um Herbizidresistenzen an Unkräutern direkt im Feld zu erkennen. Darauf aufbauend wurden folgende Ziele entlang dieser Arbeit formuliert: Die Untersuchung, (i) ob ein Chlorophyllfluoreszenz-Sensor in der Lage ist zwischen sensitiven und resistenten Unkräutern zu unterscheiden, (ii) ob eine bildgebende Chlorophyllfluoreszenz-Technologie im Stande ist Herbizidresistenzen unter Feldbedingungen zu erfassen. Weiter sollte erforscht werden, (iii) ob das System konstante Ergebnisse in Bezug auf herbizidresistente Unkrautdetektion zeigt, und (iv) ob die bildgebende Chlorophyllfluoreszenz-Technologie Herbizidstress an Kulturpflanzen kurz nach deren Feldaufgang erfassen kann. Im Hinblick auf diese Ziele wurden mehrere Experimente durchgeführt, welche dazu verwendet wurden, um drei wissenschaftliche Artikel zu verfassen.

Experiment 1. In diesem Teil der Arbeit wurde ein Gewächshausexperiment mit dem bildgebenden Chlorophyllfluoreszenz-Sensor Weed PAM® durchgeführt. Um die Effektivität von Herbiziden festzustellen wurde der vielversprechende Parameter „Maximaler Photosystem II Quantenertrag (Fv/Fm)“ mit dem Sensor gemessen. Das Experiment wurde in zwei Versuche unterteilt. Im ersten Versuch wurden sechs verschiedene Herbizide an sensitiven sowie resistenten Alopecurus myosuroides Populationen getestet. Im zweiten Versuch wurden sensitive Populationen Wasserknappheit und Stickstoffmangel ausgesetzt, um deren Stressreaktionen zu beobachten. Dieser Versuch trug dazu bei die Einflüsse von abiotischen Faktoren auf die Fv/Fm-Werte zu erkennen.

Die Ergebnisse zeigten, dass drei Tage nach einer Behandlung mit ALS- und ACCase-Hemmern die sensitiven Pflanzen signifikant geringere Fv/Fm-Werte aufwiesen, als die resistenten Populationen. Es zeigte sich nur ein geringer Einfluss des Herbizidstresses auf das Photosystem II der resistenten Pflanzen nach der Behandlung mit ALS- und ACCase-Hemmern. Die Fv/Fm-Werte sensitiver und resistenter Pflanzen fielen unter dem Einfluss von PS II Hemmern jedoch rapide ab. Vier Tage nach der Behandlung zeigte sich, dass die Fv/Fm-Werte der beiden Populationen sich signifikant unterschieden.

Stickstoffmangel hatte während der Messungen keinen signifikanten Einfluss auf das Photosystem II, wohingegen sieben Tage nach Initiierung der Wasserknappheit eine schnelle Reduktion der Fv/Fm-Werte aufgetreten ist. Nach den Ergebnissen dieses Experimentes ist der Weed PAM® Sensor dazu in der Lage kurz nach einer Herbizidbehandlung sensitive und resistente Populationen von A. myosuroides zu erkennen.

Experiment 2. Dieses Experiment untersuchte die Erkennung von Herbizideffektivität auf sensitive Pflanzen unter Feldbedingungen mithilfe des Weed PAM® Sensors. Zudem wurde getestet, ob der Sensor in der Lage ist unter diesen Bedingungen auch herbizidresistente A.myosuroides Populationen fünf Tage nach Applikation (TNA) festzustellen. Auf einem Winterweizenschlag wurde eine herbizidsensitive Population von A. myosuroides ausgesät und mit zwei ACCase- und drei ALS-Hemmern behandelt. Die Fv/Fm-Werte der A.myosuroides Pflanzen waren fünf TNA in allen Herbizidbehandlungen signifikant geringer im Vergleich zu den unbehandelten Pflanzen. Innerhalb von 2 Jahren wurden in einem weiteren Experiment insgesamt 50 sensitive und resistente Populationen an zehn Standorten getestet. Im 3-7 Blatt Stadium der Ungräser wurden als Behandlungen drei ACCase- und vier ALS-Hemmer appliziert. In jeder Population wurden 5 TNA 40 Pflanzen pro Behandlung für die Sensormessungen ausgewählt. Eine visuelle Bonitur erfolgte 21 TNA. Die Ergebnisse zeigten, dass 95% der Erkennungen korrekt durchgeführt wurden. Dies zeigt die hohe Genauigkeit des Weed PAM® Sensors für eine direkte

Herbizidresistenz-Erkennung von A.myosuroides Populationen kurz nach der Applikation unter Feldbedingungen.

Experiment 3. In diesem Teil der Arbeit wurden in Gewächshaus- und Feldversuchen Sojabohnen einem Herbizidstress ausgesetzt, um zu untersuchen, ob sich die Chlorophyllfluoreszenz-Emissionen nach einer Herbizidapplikation ändern. Herbizid-Kombinationen mit Hemmern des PS II-Systems, der DOXP Synthase und der Zellteilung, sowie des Mikrotubuliaufbaus stellten die Vorauflauf-Varianten dar. Die Nachauflauf-Varianten bestanden aus Herbizidmischungen mit PS II, ALS- und ACCase hemmern. Die Chlorophyllfluoreszenz wurde direkt nach dem Auflaufen der Sojabohnen bis zum 3-4 Blatt Stadium gemessen. Durch die Messung der Trockenmasse der Sojabohnen wurde das Stressniveau bestimmt. Im Gewächshausexperiment wurde beobachtet, dass kein Stress durch die Nachauflauf-Varianten in den Sojabohnen induziert wurde. Pflanzen, die durch PS II-Hemmer gestresst wurden, zeigten nach dem Auflaufen geringe Fv/Fm-Werte. Das Photosystem II der Pflanzen erholte sich jedoch innerhalb einer Woche auf das Niveau der unbehandelten Kontrolle. Der Stress durch andere Nachauflauf-Varianten trat eine Woche nach dem Auflauf auf und dauerte länger an als die Variante mit PS II-Hemmern. Die Aufnahmen der Trockenmasse bestätigten die Erkenntnisse der auf Chlorophyllfluoreszenz basierten Stresserkennung.

Unter Zuhilfenahme des Weed PAM® Systems ist es Landwirten möglich die richtigen Unkrautbekämpfungsmaßnahmen noch in derselben Vegetationsperiode zu ergreifen in der das Resistenzproblem in ihrem Feld erkannt wurde. Zusammenfassend hilft diese neue Technologie den Landwirten geeignetere Unkrautbekämpfungsstrategien zu entwickeln und geringere ökonomische und ökologische Risiken einzugehen.

Acknowledgement

Firstly, I would like to deeply thank Mr. Prof. Dr. Roland Gerhards for offering me the opportunity to work on these interesting projects of sensor based herbicide resistance detection and the Weed PAM® development. I really appreciate him not only for his supervise on the scientific projects and publications, but also for the living and emotional support during my four years in Germany.

It is a great honor for me that Mr. Prof. Dr. Jan Petersen and Ms. PD Dr. Regina Belz agreed to be the reviewers and examiners of my PhD examination committee.

Then I would like to give the special acknowledgement to Dr. Gerassimos Peteinatos and Matthias Schumacher. I cannot count how much support I have got from their side.

In addition, I feel so grateful to get the living expense funding from Chinese Scholarship Council (CSC, China) and the project funding from Federal Ministry of Food and Agriculture (BLE, Germany). Meanwhile, thanks for the support from other project partners, Dr. Jörg Kolbowski, Dr. Erhard Pfündel, Dr. Cornelia Köcher, Dr. Frank Brändle, Dr. Hans G. Drobny, Dr. Thomas Uhl, Dr. Yasmin I. Kaiser and Dr. Alexander Menegat. In the same context many thanks to Sebastian Bökle, Kevin Leitenberger and Hans-Jörg Ruess at the research stations of University Hohenheim for their valuable technical support in the field trials. I would also like to thank Rijk Heinrich, Simon Hotz, Alexander Zeller, Tobias Schmidt, Sarenqimuge and Moritz Sauter for their cooperation of conducting the experiments.

The best thanks and wishes go to my dear colleagues of the Weed Science Department. Time flies! There were so many unforgettable events during the last four years, the nice movie-nights and game-nights that were kindly organized by Gerrassimos, the big party at beer festival in Wasen, and the group hiking, etc. It was a wonderful four years duration and unforgettable experience with you nice guys here.

Last but not least, I would like to thank all my friends and family members, especially to my parents, my uncle Nianhua Yang and my wife Hui Li. Without their support, it could be really hard for me to go over this period. Every minute together with you was so precious in the last four years, that I would save them deeply in my memory. I love you all!

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