# Validation of the chlorophyll fluorescence imaging method (CFI) for early detection of herbicide resistance in weeds

*Validierung der Chlorophyll Fluoreszenz Imaging-Methode (CFI) zur Früherkennung von Herbizidresistenz in Unkräutern*

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## Abstract

The increasing number of herbicide tolerant weed populations is illustrating the increasing demand for reliable methods for an accelerated detection of herbicide tolerance compared to greenhouse studies. Several methods for resistance quick detection have been published in previous years. One of the recent methods is the Chlorophyll Fluorescence Imaging Method (CFI). For this method changes in photosynthetic activity of the target organisms, caused by herbicides, are determined. General assumption of this method in terms of herbicide resistance detection is that each herbicidal compound, independent of the mode of action, will cause changes within the photosynthetic apparatus of the target organisms. This effect already could be confirmed for several modes of action (PSII, ALS, ACCase, EPSPS, synth. Auxins).

Aim of this study is to validate this novel method on the basis of greenhouse experiments and single nucleotide polymorphisms (SNP) analysis. The resistance profiles of 10 black-grass populations (*Alopecurus myosuroides* Huds.) have been determined in greenhouse herbicide efficacy trials and constitutive SNP analyses of the survivors.

With the CFI-method it was possible to detect the resistance profile as well as the resistance frequency within the populations. The results from the greenhouse experiments could be reproduced with conformity of 94%. This result is valid for the tested herbicides mesosulfuron, pyroxsulam as well as clodinafop and pinoxaden.

**Keywords**: ACCase, *Alopecurus myosuroides* Huds., ALS, blackgrass, herbicide resistance, resistance quick detection

## Zusammenfassung

Die ständig steigende Zahl herbizidresistenter Unkrautpopulationen verdeutlicht den wachsenden Bedarf an verlässlichen Methoden welche eine, im Vergleich zu Gewächshausversuchen, beschleunigte Detektion von Herbizidresistenz erlauben. Mehrere solcher Methoden wurden in den vergangenen Jahren publiziert. Eine der neuesten Methoden ist das Chlorophyll Fluoreszenz Imaging (CFI). Hierbei handelt es sich um die bildgebende Erfassung von Veränderungen der Fotosyntheseleistung der Zielorganismen, verursacht durch biotische oder abiotische Stressursachen. Die generelle Annahme dieser Methode in Bezug auf Herbizidresistenz ist, dass jeder herbizide Wirkstoff entweder primär oder sekundär eine Beeinträchtigung der Fotosyntheseleitung der behandelten Pflanzen bewirkt. Dieser Effekt konnte in der Vergangenheit bereits für eine große Anzahl herbizider Wirkmechanismen nachgewiesen werden (PSII, ALS, ACCase, EPSPS, synth. Auxine).

Die hier vorgestellten Versuche haben zum Ziel, diese neue Methode anhand von Gewächshausversuchen sowie genetischen Untersuchungen zu validieren.

Das Resistenzmuster von insgesamt 10 Acker-Fuchsschwanz-Populationen (*Alopecurus myosuroides* L.) wurden in Gewächshausversuchen im Voraus erfasst und darauf aufbauend molekularbiologisch auf bekannte genetische Mutationen an den Wirkorten untersucht.

Mit der CFI-Methode konnte bei allen getesteten Populationen der Resistenzstatus sowie die Frequenz innerhalb der getesteten Population detektiert werden. Im Vergleich zu Gewächshausversuchen sowie den molekularbiologischen Untersuchungen konnten die Ergebnisse zu 94 % reproduziert werden. Dieses Ergebnis ist gültig für die Wirkstoffe Mesosulfuron, Pyroxsulam, Pinoxaden und Clodinafop.

**Stichwörter**: ACCase, Acker-Fuchsschwanz, ALS, Herbizidresistenz, Resistenzschnelltest

## Introduction

The number of herbicide tolerant weed populations is increasing worldwide (HEAP, 2013). Farmers, federal and private consulting services as well as the chemical industry are aware of this situation and started extensive, active and proactive, herbicide resistance monitoring programs in past years (e.g. SIEVERNICH *et al.*, 2013). Due to the increasing number of suspicious herbicide tolerant weed populations to be tested, the demand for screening methods which allow an accelerated detection of resistance patterns, is rising. Several new screening methods have been published, e.g. the Rothamsted Rapid Resistance Test (MOSS, 2000), the Syngenta RISQ-test (KAUNDUN *et al.* 2011) and the Chlorophyll Fluorescence Imaging Method (CFI-Test) (MENEGAT *et al.*, 2011; KAISER *et al.*, 2013). A comprehensive summary and discussion of actual test methods can be found at BURGOS *et al.* (2013). The CFI-Test could demonstrate its feasibility for a wide range of herbicidal compounds as well as for various weed species. The main advantage of the CFI-Test, compared with other quick tests, is the quantitative assessment of herbicide efficacy as well as the large number of repetitions which can be realised per population. The large number of repetitions allows a determination of the resistance frequency within the population.

So far the results obtained from the CFI-Test have not been validated. Therefore the aim of the presented study is to compare the resistance classification for different *A. myosuroides* populations with the classification results obtained from standard greenhouse herbicide efficacy studies.

## Material and Methods

Greenhouse whole-plant herbicide efficacy studies

Ten *A. myosuroides* populations with different resistance profiles were selected for the experiments. The resistance profiles were obtained from standard herbicide efficacy studies under greenhouse conditions (subsequent named whole-plant tests). Therefore *A. myosuroides* populations were pre-germinated in Vermiculite and transplanted into 8 cm Jiffy pots (Jiffy Products International B.V., NL) at growth stage BBCH 09/10. A plant density of 4 plants per pot was realised. The pots were placed in a glasshouse at 15 °C day and 10 °C at night (+- 3 °C), 60% humidity and with 12h additional illumination. Herbicide treatment took place at growth stage BBCH 12-13 with a standard laboratory track sprayer (8002 EVS TeeJet® nozzle, pressure 320 kPa, water amount 200 l/ha). The following three herbicides and dosages were used:

1. Atlantis WG®; 1.0 kg/ha, 0.4 kg/ha, 0.16 kg/ha
2. Broadway®; 687 g/ha, 275 g/ha, 110 g/ha
3. Traxos®; 1.2 l/ha

Each treatment was repeated three times. Atlantis WG® and Broadway® were sprayed with their respective adjuvants. Herbicide efficacy assessment took place 21 days after treatment by visual estimation of plant damage compared to the untreated control of the respective population. Resistance classification was done according to MOSS (1999). For single nucleotide polymorphism analysis (SNP), 10 leaf samples were taken from 10 individual plants which survived the respective herbicide treatment.

Chlorophyll Fluorescence Imaging Resistance Test; herbicide treatment, instrumentation and measurement routine

For preparation of the Chlorophyll Fluorescence Imaging Resistance Test (subsequent named CFI-Test) seeds of the selected *A. myosuroides* populations were pre-germinated on 0.8% sterilized agar (Micro Agar, Duchefa, Germany), containing a nutrient composition according to PEDAS *et al.* (2005). Petri dishes were placed in growth cabinets at a 12h-photoperiod and a temperature regime of 15/5 °C. After germination seedlings were transferred into 24-well multiwell plates (Greiner GmbH, Germany). The multiwell plates were prepared with a mixture of 500 µl herbicide solution and 500 µl sterilized 0.8% agar containing the same nutrient composition used for the

pre-germination. Tested herbicides and dosages can be obtained from Table 1. Each treatment, including the untreated control was repeated 24 times.

Chlorophyll fluorescence parameters were analysed with an IMAGIN-PAM M-Series Chlorophyll Fluorometer (Heinz Walz GmbH, Germany). For evaluation of herbicide efficacy, the maximum quantum efficiency of PSII (subsequently named Fv/Fm) was used. Fv/Fm is calculated by the equation:

$$Fv/fm = (Fm - F0)/Fm$$

*Fm* stands for the maximum fluorescence yield and *F0* for the fluorescence yield of dark adapted plants. For Fv/Fm determination plants were dark adapted for 30 minutes prior to the measurement.

Resistance classification took place at the time at which the Fv/Fm value of the sensitive reference population (2903-STD) tended towards zero and hence the maximum herbicide efficacy was reached. Resistance classification according to Moss (1999) took place 72 hours after treatment for Atlantis WG®, 168 hours after treatment for Broadway® and 48 hours after treatment for Traxos®.

**Tab. 1** Herbicides and their dosages used for the chlorophyll fluorescence imaging resistance test.

***Tab. 1*** *Für den Chlorophyll Fluoreszenz Imaging Resistenz Test verwendete Herbizide und deren Dosierungen.*

| Trade name | Active ingredient | Mode of action (HRAC goup) | Herbicide dose (mM) | Efficacy assessment (h after treatment) |
|---|---|---|---|---|
| Atlantis WG® | Mesosulfuron + iodosufuron | Inhibition of ALS (B) | 1.97* | 72 |
| Broadway® | pyroxsulam + florasulam | Inhibition of ALS (B) | 2.16* | 168 |
| Traxos® | pinoxaden + clodinafop | Inhibition of ACCase (A) | 0.187* | 48 |

*Dosages refer to the active ingredient highlighted in column two

## Results

Herbicide resistance patterns and SNP profiles obtained from whole-plant herbicide efficacy studies

Table 2 shows the herbicide resistance classification of the selected *A. myosuroides* populations as well as the respective SNP profiles. The selected populations are very diverse regarding their SNP-profiles ranging from combined ALS/ACCase target site resistance (population 4411) to non-target-site resistant populations with a multiple resistance against ACCase and ALS (population 4446). Likewise divers are the respective resistance patterns found during the whole-plant herbicide efficacy studies. Aim of the subsequent performed CFI resistance test is to validate the resistance patterns found in the whole-plant efficacy tests.

**Tab. 2** Herbicide resistance patterns and SNP profiles of the selected *A. myosuroides* populations obtained from whole-plant greenhouse herbicide efficacy studies and subsequent SNP analyses of survivors.

***Tab. 2*** *Resistenzmuster und Mutationsprofile der ausgewählten* A. myosuroides *Populationen, basierend auf Resistenztests unter Gewächshausbedingungen und SNP-Analysen an Pflanzen welche die jeweilige Herbizidbehandlungen überlebt haben.*

| | **Tested herbicides** | | | | |
|---|---|---|---|---|---|
| **Population** | **Atlantis WG®** | **Broadway®** | **Traxos®** | **SNP** | **SNP frequency within population** |
| 2903-STD | S | S | S | - | - |
| 4411 | RR | RR | RR | Ser197/Leu574/Leu1781 | 63%/13%/75% |
| 4414 | RR | R? | RR | - | - |
| 4432 | S | S | RR | - | - |
| 4445 | R? | R? | R? | - | - |
| 4446 | R? | R? | R? | - | - |
| 4447 | RR | RR | S | Leu574 | 100% |
| 4449 | RR | RR | S | - | - |
| 4547 | S | S | S | - | - |
| 4549 | RR | RR | RR | Leu574 | 100% |

CFI-resistance test results and comparison with whole-plant efficacy studies

For Atlantis WG® the general resistance classification (sensitive or resistant) obtained by the CFI-Test corresponded in nine of ten cases with the results obtained from the whole-plant greenhouse study. In two cases the resistance classification found with the CFI-Test were higher (RR) than those obtained by the whole-plant essay (R?). Population 4432 was classified as sensitive against Atlantis WG® but was classified as resistant (RR) during the CFI-essay. Additional ALS-SNP analyses with surviving plants out of the CFI-Test were negative. Regardless the possibility of an existing NTSR we classify this case as false positive detection.

For Broadway® the general resistance classification (sensitive or resistant) obtained by the CFI-Test corresponded in nine of ten cases with the results obtained from the whole-plant greenhouse study. In four cases the resistance classification of the CFI-Test was higher than the classification found in the whole-plant test (populations 4411, 4414, 4447 and 4549) and in one case the classification of the CFI-Test was lower (population 4449). Population 4547 was classified as sensitive against Broadway® but was classified as resistant (RR) during the CFI-Test. Additional ALS-SNP analyses with surviving plants out of the CFI-Test were negative. Regardless the possibility of an existing NTSR we classify this case as false positive detection.

For Traxos® the general resistance classification (sensitive or resistant) obtained by the CFI-Test corresponded in nine of ten cases with the results obtained from the whole-plant greenhouse study. In three cases the classification found by the CFI-Test was higher than those found in the whole-plant tests and in one case lower. Population 4547 was classified as RR by the CFI-Test and S by the whole-plant test. Subsequent SNP analyses with surviving plants out of the CFI-Test confirmed an ACCase SNP (Leu1781) with a frequency of 29% within the population. Thus we could confirm the false negative classification of the whole-plant test.

**Tab. 3** Comparison of classification results obtained by the CFI resistance test and the whole-plant test.

***Tab. 3*** *Vergleich der Klassifikationsergebnisse des CFI Resistenztests und des Resistenztests unter Gewächshausbedingungen.*

| Population | | Tested herbicides | | | | |
|---|---|---|---|---|---|---|
| | | Atlantis WG® | Broadway® | Traxos® | SNP | SNP frequency within population |
| 2903-STD | Whole-plant test | S | S | S | - | - |
| | CFI resistance test | S | S | S | - | - |
| 4411 | Whole-plant test | RR | RR | RR | Ser197/Leu574/Leu1781 | 63%/13%/75% |
| | CFI resistance test | RR | RRR | RR | - | - |
| 4414 | Whole-plant test | RR | R? | RR | - | - |
| | CFI resistance test | RR | RR | RR | - | - |
| 4432 | Whole-plant test | S | S | RR | - | - |
| | CFI resistance test | RR | S | RRR | - | - |
| 4445 | Whole-plant test | R? | R? | R? | - | - |
| | CFI resistance test | RR | R? | RR | - | - |
| 4446 | Whole-plant test | R? | R? | R? | - | - |
| | CFI resistance test | RR | R? | RR | Leu1781 | 38% |
| 4447 | Whole-plant test | RR | RR | S | Leu574 | 100% |
| | CFI resistance test | RR | RRR | S | - | - |
| 4449 | Whole-plant test | RR | RR | S | - | - |
| | CFI resistance test | RR | R? | S | - | - |
| 4547 | Whole-plant test | S | S | S | - | - |
| | CFI resistance test | S | RR | RR | Leu1781 | 29% |
| 4549 | Whole-plant test | RR | RR | RR | Leu574 | 100% |
| | CFI resistance test | RR | RRR | R? | - | - |

## Discussion

The presented initial validation was the first step towards a comprehensive validation of the CFI-test. We could show that the classification results obtained by the CFI-test correspond well with the classification results obtained by the whole-plant test, thus conformity of 94% could be achieved for the resistance detection. However, in some cases the resistance classification found in the CFI-test was higher or lower than those found in the whole-plant tests. Within the CFI-test system compounds are not affected by biotic and abiotic degradation accordingly the only existing sink for the compounds are the plants itself. Previous studies could show that ED50 and ED90 values decrease with time since the availability of the compound within the test system is decreasing only marginally. Hence the proper timing for resistance classification is essential to avoid an under- or overestimation of the herbicide efficacy.

The experiments could demonstrate that the CFI-test is a reliable and fast method for herbicide resistance detection. The method is already applicable for a wide range of compounds out of the HRAC groups A, B, C, G and O and for more than 15 monocotyledonous and dicotyledonous weed species. Beyond the laboratory based CFI-test a novel Chlorophyll Fluorescence Imaging System is under development which will allow the herbicide resistance detection directly in the field. This will allow farmers to adapt their herbicide decision within the season and to control the herbicide efficacy within a few days after application, far before herbicide symptoms will be visible.

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