Detecting herbicide-resistant *Apera spica-venti* with a chlorophyll fluorescence agar test

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ABSTRACT

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Reliable tests on herbicide resistance are important for resistance management. Despite well-established greenhouse bioassays, faster and in-season screening methods would aid in more efficient resistance detection. The feasibility of a chlorophyll fluorescence agar-based test on herbicide resistance in *Apera spica-venti* L. was investigated. Herbicide resistant and sensitive *A. spica-venti* seedlings were transplanted into agar containing pinoxaden and pyroxsulam herbicides. Chlorophyll fluorescence was measured and the maximum quantum efficiency of photosystem II (F_v/F_m) was determined 48 h and 72 h after the transplantation to agar, respectively. The F_v/F_m values decreased with increasing herbicide concentration. Dose-response curves and respective ED_{50} values (herbicide concentration leading to 50% decrease of the F_v/F_m value) were calculated. However, each experiment repetition exhibited different sensitivities of the populations for both herbicides. In certain cases, resistant populations demonstrated similar F_v/F_m values as sensitive populations. Contrary to the findings in *Alopecurus myosuroides* Huds., discrimination of sensitive and resistant *A. spica-venti* populations was not feasible. An increased importance of the assessment time due to the herbicide concentrations calibrated for fast responses was assumed in this study.

Keywords: ALS inhibitor; ACCase inhibitor; herbicide-resistant weed; rapid detection; laboratory study; PAM fluorimeter

With the worldwide increase of herbicide-resistant weed populations, there is a need for fast and reliable screening methods for early detection of herbicide-resistant weeds. Several tests on herbicide resistance in weeds exist (Beckie et al. 2000). Until today, the most common method to detect herbicide-resistant weed plants are time-consuming and labour-intensive greenhouse bioassays. The major disadvantage of this test is that the results become available only for the next growing season. One test that was developed to overcome this limitation was the Syngenta rapid in-season

quick test (RISQ) (Kaundun et al. 2011). Here, seedlings are transplanted to herbicide-treated agar and the survivorship is recorded after 10 days. It is a reliable method for testing herbicide resistance in weeds for post-emergent herbicides (Brosnan et al. 2017). While the RISQ provides information if a plant is resistant or not, it does not give estimation about the level of resistance.

To receive information about the resistance level of a weed population, the experimental setup of the RISQ was combined with an assessment of the maximum quantum efficiency of photosys-

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tem II (F_v/F_m) (Kaiser et al. 2013). Especially the F_v/F_m value is a commonly used parameter in plant physiology, reliably quantifying stress of diverse origin in plants (Valladares and Pearcy 1997, Baker 2008). Kaiser et al. (2013) assessed the F_v/F_m values of *Alopecurus myosuroides* Huds. seedlings after transplanting to fenoxaprop-P-ethyl and iodosulfuron + mesosulfuron-treated agar. Then, dose-response curves were fitted based on the assessed F_v/F_m values and resistance factors were calculated. Herewith, the resistant populations could be identified within 48 h to 72 h after transplanting. Due to these promising results a further test of this system in other weed species could diversify the range of screening methods on herbicide resistance.

Apera spica venti (L.) Beauv. can reduce yields up to 30% at infestation levels of 200 plants/m² (Babineau et al. 2017). It has been reported to be resistant to amino lactate synthase (ALS), acetyl-CoA carboxylase (ACCase) and photosystem II inhibitors (Heap 2018). Moreover, it has developed resistance to the active substances frequently used in Europe, i.e. pinoxaden (ACCase inhibitor) and pyroxsulam (ALS inhibitor). Therefore, there is an increasing interest in identifying and classifying herbicide-resistant *A. spica-venti* populations at early vegetation stages.

The objective of this study was to test the suitability of an agar-based herbicide resistance test system for *A. spica-venti*. By chlorophyll fluorescence imaging, it was tried to ascertain a herbicide concentration that discriminates sensitive and resistant *A. spica-venti* populations.

The aims of this study were: (i) to validate the method by Kaiser et al. (2013) with the weed *A. spica-venti* and the active substances pinoxaden

and pyroxsulam; (ii) to find differences in the F_v/F_m values between sensitive and resistant plants depending on herbicide concentrations; (iii) to show the suitability and reliability of a chlorophyll fluorescence-based test on herbicide resistance in *A. spica-venti*.

MATERIAL AND METHODS

Seed origin. In this study, four populations of *Apera spica-venti* (L.) Beauv. A-S1, A-S2, A-R1, A-R2 were examined. The *A. spica-venti* seeds A-S1 were obtained from Herbiseed Ltd., Berkshire, UK and A-S2 was a wild type collected from fields in Germany (Eastern Baden-Württemberg) (Table 1). The seeds of A-R1 and A-R2 were collected from fields in the Czech Republic (Eastern Bohemia) where farmers had reported a repeated lack of control with ALS and ACCase inhibitors.

Herbicide resistance classification. All populations were tested for resistance against the herbicides pyroxsulam (Corello, 74 g a.i. (active ingredient)/kg, WG, Dow AgroScience s.r.o., Prague, Czech Republic) and pinoxaden (Axial Plus, 50 g a.i./L, OD, Syngenta Czech s.r.o., Prague, Czech Republic) in a greenhouse trial. Plants were grown in 8×8 cm² plastic pots, filled with loamy soil, at a 12-h photoperiod and at 15/8°C day/night. Three repetitions were executed, with one pot per repetition. In each pot, 10 plants were grown; A-S1 served as a sensitive reference. The herbicide application was performed at 3-4 leaf stage (BBCH 13-14) of A. spica-venti plants with the recommended doses of 9.25 g a.i./ha pyroxsulam and 45 g a.i./ha pinoxaden, respectively. For the pyroxsulam treatment, the recommended adju-

Table 1. Apera spica-venti (L.) Beauv. populations, seed origin, herbicide efficacy (HE, %), standard error (SE) in a greenhouse bioassay and resistance classification (RC) for pinoxaden and pyroxsulam according to Moss et al. (1999)

Population	C 1:	Pinoxaden			Pyroxsulam		
	Seed origin	HE	SE	RC	HE	SE	RC
A-S1	Herbiseed Ldt., UK	100	0	S	100	0	S
A-S2	Germany	99	1	S	98	0	S
A-R1	Czech Republic	57	3	RR	65	0	RR
A-R2	Czech Republic	55	0	RR	57	3	RR

S - sensitive; RR - moderate resistant

vant alkylfenolalkoxylat (Šaman 990 g a.i./L, Dow AgroScience s.r.o.) was added at a dose of 990 g a.i./ha. Plants, treated with water, served as an untreated control. For the application, a laboratory sprayer was used, equipped with a single flat fan nozzle (8002 EVS, TeeJet Spraying Systems Co., Wheaton, USA) calibrated to deliver 200 L/ha at a speed of 800 mm/s. The plant herbicide damage was visually assessed 28 days after application. It was performed by comparing the fresh biomass reduction of treated plants compared to the untreated control. The respective herbicide resistance classes in the R rating system were determined according to Moss et al. (1999).

Laboratory study. The four *A. spica-venti* populations were tested for herbicide sensitivity in a herbicide treated system modified according to Kaiser et al. (2013). The experiments (EXP) were performed between October and December 2017. The EXP was repeated three times with six repetitions per treatment in a complete randomized design. Six-well plates (Faust Lab Science, Klettgau, Germany) were used in this EXP, with one single well as a repetition. To produce the agar pads, a stock solution was prepared for utilization in a solution series. The respective herbicide was diluted in deionised water, starting at the highest concentration and reduced stepwise (Table 2). The concentrations per active substance were chosen according to the preliminary studies. The target of these studies was to cover the full dose-response range and simultaneously achieve a fast plant

Table 2. Concentrations of pinoxaden and pyroxsulam (nmol a.i.(active ingredient)/mL) in the laboratory study

Treatment	Pinoxaden	Pyroxsulam		
Control	0	0		
C1	849	17 270		
C2	424	8635		
C3	212	4317		
C4	106	2158		
C5	53	1079		
C6	27	540		
C7	14	270		
C8	7*	_		

Recommended field dose (nmol a.i./mL): pinoxaden, 560; pyroxsulam, 110. *only used in Experiment 1

response (less than 3 days). For pyroxsulam, the concentrations were higher than the recommended field dose, while the concentrations of pinoxaden coincided with the recommended field dose. Each well was filled with 1 mL herbicide solution. Then 1 mL 0.8% sterile agar was added. Agar pads for the control treatment were prepared with 1 mL deionised water instead of the herbicide solution. No nutrient solution was added to the pads.

The seeds were germinated on the moist filter paper in a climate chamber with a 12 h photoperiod at day/night temperatures of 20/15°C. The plants were grown until the first leaf was unfolded BBCH 11) and plants had a height of about 10 mm. Five plants were transplanted per a herbicide-treated agar pad. All leaf and root parts of the seedlings were in full contact with the agar. Afterwards, multi-well plates were placed back into the climate chamber at the same temperature and light conditions.

The herbicide-induced stress on the plants was quantified by assessing the $F_{\rm v}/F_{\rm m}$ values for the pinoxaden-treated agar 48 h after transplanting and for the pyroxsulam-treated agar 72 h after transplanting. Plants were dark-adapted around 30 min prior to the fluorescence measurement. Then, the ground fluorescence ($F_{\rm 0}$) and the maximum fluorescence ($F_{\rm m}$) was determined with an Imaging-Pam M-Series chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany). The assessment was done once as the mean of five plants in one well. The $F_{\rm v}/F_{\rm m}$ values were calculated as shown in Eq. 1:

$$\frac{F_{\rm v}}{F_{\rm m}} = \frac{F_{\rm m} - F_{\rm o}}{F_{\rm m}} \tag{1}$$

It describes the percentage proportion of variable fluorescence (F_v) to the F_m . The F_v/F_m value is remarkably constant for non-stressed leafs with the values around 0.83 (Björkman and Demmig 1987). For the Imaging-Pam M-Series, F_v/F_m values of 0.70 \pm 0.05 are common for non-stressed leafs. Lower values indicated stressed plants.

Data analysis. Due to the variability between different populations, the data were normalised by dividing the individual values by the mean value of the control, per population and EXP. The analysis of the data was performed with the statistic program R, version 3.3.1 (Vienna, Austria).

To illustrate differences of herbicide sensitivity between populations, the R package drc was used to calculate dose response curves (DRC) and ${\rm ED}_{50}$ values for each population and herbicide treatment based on relative ${\rm F_v/F_m}$ values (Ritz et

al. 2015). Here, ED_{50} represents 50% decrease of the $\mathrm{F_v/F_m}$ value relative to the control. DRCs were calculated with a three parametric log-logistic model according to Streibig (1988) and the fit of each DRC was checked with a lack of fit test. To detect significant differences in the ED_{50} values per EXP, the function EDcomp was used.

RESULTS AND DISCUSSION

In the greenhouse trial, the herbicides pinoxaden and pyroxsulam had an efficacy of more than 98% on A-S1 and A-S2 (Table 1). Pinoxaden had a decreased efficacy on A-R1 and A-R2 of 57% and 55%, respectively. Likewise, the efficacy of pyroxsulam was lower on A-R1 and A-R2 with 65% and 57%. There were no dead plants in the populations A-R1 and A-R2 28 days after treatment (Figure 1). All those plants had a herbicide damage, but non-lethal. On the other hand, all plants of A-S1 and A-S2 were dead. Based on these results, it can be assumed that the frequency of resistant plants within the populations A-R1 and A-R2 is close to one. To sum up, the greenhouse trial proved A-S1 and A-S2 to be sensitive (S) to pyroxsulam and pinoxaden, while the populations A-R1 and A-R2 were classified as resistant (RR) to both herbicides. Hence, the requirements for finding a threshold herbicide dose to discriminate between sensitive and resistant populations in a laboratory agar test were given.

Kaiser et al. (2013) were able to identify resistant and sensitive *A. myosuroides* populations within 48 h and 72 h after transplanting to fenoxaprop-P-

ethyl and iodosulfuron + mesosulfuron treated agar, respectively. Our preliminary studies showed that sensitive and resistant A. spica-venti populations had a difference of up to 20% in their F_v/F_m values 48 h and 72 h after transplanting to pinoxaden and pyroxsulam-treated agar, respectively. In this study, an observation of the transplanted seedlings was done the consecutive 3 days after the assessment of the F_v/F_m values. The F_v/F_m values of control plants had a range from 0.63 to 0.73 depending on the population and EXP. All standard errors of the control treatments were below 0.02 except in two cases where it was 0.03 and 0.05. To achieve a better comparability, the data were normalised by dividing the individual value by the mean value of the control, per population and EXP. Therefore, the F_v/F_m fluctuations that had been caused by undesired stressors were excluded. Hence, a decrease of the normalized F_v/F_m value represents plant stress induced by the herbicide (Maxwell and Johnson 2000). Even though there were no visual symptoms during the assessment, one day after the assessment all herbicide-treated plants showed a visible herbicide damage.

The F_v/F_m values of treated plants decreased with increasing concentrations of pyroxsulam and pinoxaden. A-S1 in EXP 2 with pyroxsulam had relative F_v/F_m values of 95.6, 76.9 and 34.2% 72 h after transplanting to concentrations of 270, 2158 and 17 270 nmol a.i./mL pyroxsulam, respectively. These findings are in line with the results of Barbagallo et al. (2003), who also examined the chlorophyll fluorescence changes in herbicide-treated plants. Neither of the herbicides damaged the photosystem



Figure 1. Two resistant *Apera spica-venti* (L.) Beauv. populations (A-R1; A-R2) 28 days after treatment with pinoxaden and pyroxsulam compared to their respective untreated control. a.i. – active ingredient

Table 3. Calculated ED_{50} values (nmol a.i.(active ingredient)/mL) and standard errors (SE) of herbicides tested against *Apera spica-venti* (L.) Beauv. populations

Herbicide	Population -	EXP 1		EXI	? 2	EXP 3	
		ED ₅₀	SE	ED ₅₀	SE	ED ₅₀	SE
Pinoxaden	A-S1	198ª	18	162ª	15	72ª	5
	A-S2	251 ^b	22	174 ^a	11	149 ^c	7
	A-R1	364 ^c	34	240^{b}	21	176 ^d	10
	A-R2	357 ^c	28	146 ^a	11	116 ^b	6
Pyroxsulam	A-S1	8528 ^a	1547	5675 ^{ab}	984	2223ª	459
	A-S2	5639 ^a	860	4591 ^a	665	2984 ^a	394
	A-R1	7822ª	1291	9138 ^{bc}	1869	17 327 ^b	4636
	A-R2	7357 ^a	776	12 381 ^{cd}	1846	9150 ^b	1600

Dose-response curves were calculated with normalized F_v/F_m values of three experiments (EXP). F_v/F_m was measured 48 h and 72 h after transplanting to pinoxaden and pyroxsulam-treated agar, respectively. Values with the same letters indicate no statistical difference within one experiment and herbicide ($\alpha = 5\%$)

II directly. Lichtenthaler and Rinderle (1988) reported that differences in chlorophyll fluorescence are detectable in the course of time, independent of the stress factor acting directly or indirectly on the photosynthesis. Likewise prior studies reported an effect on chlorophyll *a* fluorescence in plants after the treatment with ACCase or ALS inhibitors (Norsworthy et al. 1998, Abbaspoor and Streibig 2005, Dayan and Zaccaro 2012).

For all EXPs, the dosage-dependent F_v/F_m values fitted in a three parametric log-logistic DRC model. Yet, the calculated normalized F_v/F_m value-based DRCs of each trial repetition exhibited different sensitivities of the populations to the herbicides. This inconsistency is best expressed for pinoxaden EXPs with A-S1 with an ED₅₀ value of 198 nmol a.i./mL (EXP 1) which declines to 162 nmol a.i./mL (EXP 2) and 72 nmol a.i./mL (EXP 3) (Table 3). Likewise, the ED₅₀ value of A-S1 in EXP 1 with pyroxsulam is 8528 nmol a.i./mL. In EXP 2 and EXP 3, less than 33% and 74% pyroxsulam are needed to decrease the relative F_v/F_m value by 50%, respectively. Only in EXP 1 (pinoxaden) and EXP 3 (pyroxsulam) the ED₅₀ values discriminated A-R1 and A-R2 from A-S1 and A-S2. Merely the DRCs derived by EXP 3 with pyroxsulam clearly showed lower sensitivity against pyroxsulam of A-R1 and A-R2, but not in EXP 1 and 2 (Figure 2). Moreover, the relative sensitivity between the populations changed over the EXPs. In EXP 2 with pinoxaden, A-R2 had the lowest ED₅₀ value and did not differ statistically from A-S1 and

A-S2. A-R2 was statistically even less sensitive than A-S1 in EXP 3, but more sensitive than A-S2. Similar disagreements were found for the sensitivities of the populations against pyroxsulam between the EXPs. A-R1 seemed to have the same sensitivity to pyroxsulam as A-S1, A-S2 and A-R2 at ED $_{50}$ with 7822 nmol a.i./mL (EXP 1). In EXP 2 A-R1 had an ED $_{50}$ value of 9138 nmol a.i./mL and did not statistically differ from A-S1 and A-R2.

The described approach was based on the RISQ test (Kaundun et al. 2011). As an agar-based test on herbicide resistance, the RISQ test was first developed with Lolium multiflorum L. and Lolium rigidum Gaudin. In addition, pinoxaden-resistant and susceptible populations of Avena fatua L., A. myosuroides, A. spica-venti and Phalaris padoxa L. could be discriminated successfully using the RISQ test. Further, it was proved to discriminate resistant and susceptible populations of several broadleaf weeds (Kaundun et al. 2014). Plant seedlings or tillers are transplanted to herbicide-treated agar pads with discriminating dosages and after 7 to 10 days, the survivorship is recorded. The advantage of this test is that weed seedlings from the field can be transplanted into a herbicide solution for testing sensitivity. In contrast to this study, their laboratory results correlated very well with results from the glasshouse. Kaundun et al. (2011) used the RISQ test to test one sensitive and one target site resistant A. spica-venti population with the herbicide pinoxaden and found a discriminating

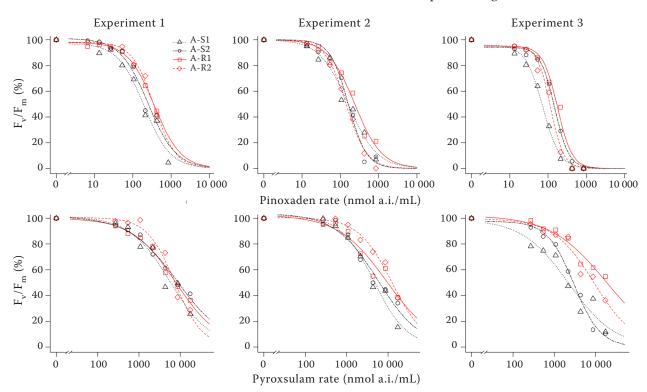


Figure 2. Variation of the maximum quantum efficiency of the photosystem II (F_v/F_m) of different *Apera spica-venti* (L.) Beauv. populations 48 h and 72 h after transplanting to pinoxaden and pyroxsulam-treated agar, respectively. Dose-response curves were calculated with a three-parametric log-logistic model

herbicide dosage of 0.16 µmol. Kaiser et al. (2013) examined resistant and sensitive A. myosuroides populations in a similar agar-based test and calculated DRCs based on the F_v/F_m values. They observed that the herbicide concentration causing a 90% reduction of the F_v/F_m values in resistant populations was more than sevenfold higher compared to a sensitive population. In this study, ED₅₀ values were chosen to compare the herbicide sensitivity, as the DRCs around ED50 are not extrapolated. In addition, due to a lack of robustness in the results, a calculation of resistance factors was foregone. Even if the resistance mechanism in both resistant populations was not examined, the herbicide-induced stress, which is quantified by the F_v/F_m value, is independent of the resistance mechanism. Nevertheless, it was expected that A-R1 and A-R2 have in general higher ED₅₀ values than the populations A-S1 and A-S2 when treated with pinoxaden and pyroxsulam, respectively. This hypothesis could not be verified by the results.

The presented experimental setup tried to reduce as much influencing factors as possible, as they can modify the F_v/F_m value of the plants. It is commonly known that variations in light and

temperature between experiments can cause variability in the results. To overcome the variation produced by light and temperature, the EXPs were conducted in a climate chamber. Additionally, it is well known in the greenhouse screening tests that a timespan of 28 days is necessary for achieving an indisputable result, and time is of essence in these type of experiments (Beckie et al. 2000). Due to the fast response pursued in this EXP, it can be hypothesised that one of the major reasons for the high variation of the results should be the time between the treatment and the measurement.

The transplanting of the seedlings into the agar and the assessment of the F_v/F_m values was done completely randomly. Unfortunately, the exact timespan was not considered as a factor in the EXPs. The transplanting of the seedlings into the agar took about 2.5 h per EXP. The assessment of the F_v/F_m values lasted for about 1.5 h. Therefore, it can be assumed that the timespan from transplanting until measurement was 48 ± 1 h and 72 ± 1 h for pinoxaden and pyroxsulam, respectively. The dosage used created a stress response, higher than anticipated, in this specific time span. Therefore, even this variability in time,

combined with the herbicide concentrations that led to a fast plant response might be the reason for additional variability inside each treatment. Since such a strong variability was not expected, it might be attributed to time. In further studies, this information will be taken into account on a minute level.

A screening method for herbicide resistance has to fulfil, among other things, the criteria of reproducibility (HRAC 1999). This is especially necessary to eliminate wrong resistance classifications. The presented procedure does not give reproducible estimations for the herbicide sensitivity. Deducing a distinct discriminating dosage threshold was not permitted. That was due to the different ED₅₀ values of each population between the EXPs. And, here, drawn conclusions from single results can be strongly misleading. A reliable and robust identification of herbicide-sensitive populations would exclude these sensitive populations from investigation. This could save both resources and labour for the screening facilities. Yet, the current system needs further development in order to achieve a robust outcome, usable as a fast pre-exclusion of the sensitive populations. Achieving a steady time span per sample in the minute scale might give a more robust result. Alternatively, if a better time manipulation does not provide the desired result then, even in the cost of elongating the test period, the used dosages need to be decreased.

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