CHLOROPHYLL FLUORESCENCE MICROSCREENING AS A RAPID DETECTION METHOD FOR HERBICIDE RESISTANCE IN GRASS WEEDS IN NORTH CHINA PLAIN WINTER WHEAT PRODUCTION SYSTEMS AND BEYOND

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ABSTRACT

The North China Plain (NCP) is one of the most important winter wheat production areas in the world. A double cropping system of winter wheat followed by summer maize in one year is the most common cropping practice in the NCP. However new crops and agricultural practices including chemical weed control measures were recently introduced in this area. Alopecurus spp., Aegilops squarrosa L. and Bromus japonicus Thunb. were found to be the most abundant grass weeds in the NCP winter wheat production system. In 2008 and 2009, A. japonicus seeds were collected from different locations in the NCP to conduct herbicide efficacy studies. Besides conventional glasshouse bioassays a rapid herbicide resistance test has been developed and tested. This new resistance test is based on chlorophyll fluorescence microscreenings for evaluation of the efficacy of herbicides on grass weeds grown in tissue culture plates filled with an agar-herbicide solution. In glasshouse bioassays for chlorotoluron a resistance factor of 3.5 was found for one of the NCP biotypes compared to the sensitive control biotype. The chlorophyll fluorescence microscreening clearly verified this result. Further studies showed that this method is also suitable for other herbicide modes of action like ALS and ACC inhibitors. Furthermore this method is easily transferable to other important grass weeds. Using the chlorophyll fluorescence assay, it becomes possible to evaluate efficacy for a large number of biotypes with a minimum requirement of time and space. Therefore it is well suited for high throughput resistance screenings, especially at locations where glasshouse space is a limiting factor. An accelerated identification of resistant grass weed biotypes and thus a prompt resistance management plan for the field will be of great importance for the North China Plain and other intensive agricultural areas in the world.

Key words: *Alopecurus japonicus* Steud., chlorophyll fluorescence, herbicide bioassay, herbicide resistance.

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INTRODUCTION

The North China Plain (NCP) is the most important agricultural area in China. It comprises parts of the six provinces Beijing, Tianjin, Hebei, Shandong, Henan, Anhui, and Jiangsu and covers an area of 31 million hectares. More than half of this area is used for agriculture which accounts for only 20% of the total agricultural area in China, but 50% of the total Chinese winter wheat production (Wu *et al*. 2006). Winter wheat (*Triticum aestivum* L.) production in the NCP is implemented as a double cropping system of winter wheat followed by summer maize (Z*ea mays* L.) over one year.

In 2009 and 2010 weed surveys were conducted in the NCP to investigate the major weed species in winter wheat (*Triticum aestivum*). As one of the most abundant grass weeds Japanese foxtail (*Alopecurus japonicus*) was widely distributed. In 2009, seeds of *A. japonicus* were collected from three different locations in the NCP for herbicide efficacy studies. For the efficacy studies an herbicide resistance microscreening method was developed, based on chlorophyll fluorescence measurements. Rapid herbicide resistance screening methods are receiving increased attention, because worldwide the number of herbicide resistant weeds is rapidly increasing and therefore more accelerated screening methods become necessary. Beside molecular genetic analysis, there are several test methods described in literature; for example, the "Rothamsted Rapid Resistance Test" (Moss, 2000) or the Syngenta RISQ test (Kaundun *et al*., 2011). Except for molecular genetic analysis, all methods have several commonalities including lack of useful methodology to quantify herbicide resistance or difficult, time-consuming method for measuring plant response to herbicides. Chlorophyll fluorescence measurement has been widely used in weed science, especially in mode of action studies and crop sensitivity studies (Korres *et al.,* 2003; Abbaspoor and Streibig, 2007).

The aim of this study is to introduce and evaluate the chlorophyll fluorescence microscreening test to accelerate the detection of herbicide resistance with a minimum requirement of space and labor. Furthermore we wish to jointly utilize existing chlorophyll fluorescence based herbicide screening and photosynthesis evaluation methods and to develop them further. The method used in this study describes the effect of chlorotoluron upon *A. japonicus*.

MATERIALS AND METHODS
Seed Origin and Seed Germination

A. Japonicus seeds were collected from different locations in the North China Plain. Three biotypes originating from the Hebei province (subsequently named Hebei1, Hebei2 and Hebei3) and one biotype

originating from the Jiangsu province (subsequently named Jiangsu1) were obtained. The collected biotypes were compared to a chlorotoluron sensitive biotype (subsequently referred to as sensitive).

The collected seeds were pre-germinated on three folds of filter paper in glass petri dishes. For germination, 6.0 mL of nutrient solution, formulated according to Pedas *et al.* (2005), was added to each petri dish. The petri dishes were placed in a growth cabinet at a day/night cycle of 18/10°C, 12/12h.

Herbicide Treatment

After cotyledons were fully developed, plants were transplanted into six-well tissue culture plates (TPP, Switzerland). Each well was filled with 3 ml of a 0.4% Agar solution (Micro Agar, Duchefa, Germany). The same nutrient solution as used for seed germination was used to prepare the plant Agar in order to guarantee optimal nutrient availability. Chlorotoluron (Lentipur™, 700 g a.i. L^{-1}) was added to the Agar solution at nine concentrations, ranging from 0.18 µM a.i. to 0.00070313 µM a.i.. Each treatment was repeated six times.

Chlorophyll Fluorescence Instrumentation and Measurement Routine

Chlorophyll fluorescence parameters were analyzed with an IMAGING-PAM *M-Series* Chlorophyll Fluorometer (Heinz Walz GmbH, Germany). For evaluation of herbicide efficacy, maximum quantum efficiency of PS II photochemistry, is defined as *Fv/Fm*. It is calculated by the equation *Fv/Fm = (Fm - Fo)/Fm*, were *Fm* is the maximal fluorescence yield and *Fo* the dark fluorescence yield. For determination of *Fo*, plants were dark adapted for the duration of 30 minutes prior to the measurement. The measurement was carried out in a dark room under green illumination to avoid other phytosynthetic active radiation except that emitted by the IMAGING PAM light source. After dark adaption, leaves were illuminated with a light saturation pulse of 580 µM $m^{-2} s^{-1}$ and a wavelength of 450 nm for *Fv/Fm* determination.

In unstressed leaves *Fv/Fm* is approximately 0.83. This value is independent of the plant species. Lower values indicate that a proportion of the PS II reaction centers are inhibited due to stress conditions, for example due to the presence of a herbicide. This phenomenon is called photoinhibition (Strasser and Stirbet, 2001). Beside other fluorescence parameters *Fv/Fm* is suitable especially for the study of PS II inhibitors (Korres *et al.,* 2003).*Fv/Fm* determination began 3 HAT (HAT, hours after transplanting) and was repeated at 24 HAT, 48 HAT, 72 HAT, 120 HAT and 192 HAT, for analysis of dose response relationships.

Whole- Plant Herbicide Efficacy Studies

For validation of the chlorophyll fluorescence microscreening experiment results, standard glasshouse dose-response studies were

carried out. Therefore, seeds were pre-germinated in vermiculite and transplanted to plastic pots of 9x9 cm^2 size after the cotyledon was fully developed. In each pot 2 plants were transplanted. Chlorotoluron (Lentipur™, 700 g a.i. L^{-1}) was sprayed in nine descending dosages ranging from 2100 g a.i. ha^{-1} to 8.2 g a.i. ha^{-1} 10 days after transplanting in a precision application chamber (Aro, Langenthal, Switzerland). The nozzle (8004 EVS, Teejet Spraying Systems Co., Wheaton, IL, USA) was calibrated to spray a volume of 400 L ha^{-1} resulting in a speed of the nozzle of 800 mm s^{-1}, a distance from the sprayed surface of 500 mm and a spraying pressure of 300 kPa. Each treatment was replicated 4 times. Before and after the herbicide treatment, pots were placed in a glasshouse at a day/night cycle of 18/10°C,12/12h. The plants were cut at ground level 12 days after herbicide treatment and dried at 80°C for 48 h for dry weight determination.

Statistical Analysis

Statistical analysis was carried out with the statistical software *R* (*R* Development Core Team, 2011) and the R extension package *drc* (Ritz and Streibig 2005). For the analysis of dose response relationships the non-linear four parameter model after Streibig (1988) was used. The model follows the equation: $y = C + ((D - C) / (1 + exp(b \ln(x / ED_{50})))$, where y represents the plant response (Fv/Fm or dry weight), D the upper limit of the curve, C the lower limit, and b is proportional to the slope around ED50, the dose at which the plant response is reduced by 50%. Dose–response curves were compared by horizontal assessment (*F* test, α=0.05) after data normalization according to Streibig *et al.* (1995). All experiments were repeated twice.

RESULTS
Chlorophyll Fluorescence Microscreening Herbicide Efficacy Tests

The time response analysis of the sensitive biotype showed a reduction of *ED$_{50}$* and *ED$_{90}$* values with increased time of exposure (Figure 1). In order to determine the optimal timing for dose response comparison among biotypes, data were analysed with regard to the timing, where the maximum herbicide dosage resulted in a 90% *Fv/Fm*- reduction *(ED$_{90}$)*. Therefore, *ED$_{90}$* was reached 48 h after transplanting (Table-1). The standard error decreased over time and therefore horizontal comparison between biotypes was not to be calculated earlier than 48 HAT in order to get meaningful results.

For the tested *A. japonicus* biotypes Hebei2, Hebei3 and Jiangsu1 the horizontal curve assessment resulted in no significant differences (Table-2). The Hebei1 biotype showed an increased

tolerance to chlorotoluron compared to the Sensitive biotype (Figure 2) resulting in a resistance factor ($ED_{50Hebei1}/ED_{50Sensitive}$) of *RF*=3.39 (Table-2).

Table-1. Effective dosages of chlorotoluron causing a 90% (*ED90*) reduction of *Fv/Fm* over time in sensitive biotypes.

	Estimate $ED_{90Fv/Fm}$ [μM a.i.]	Std. Error
3 HAT	0.92961	2.0806
24 HAT	0.551237	0.6135
48 HAT	0.185193	0.2056
120 HAT	0.073922	0.0663
192 HAT	0.049844	0.0462
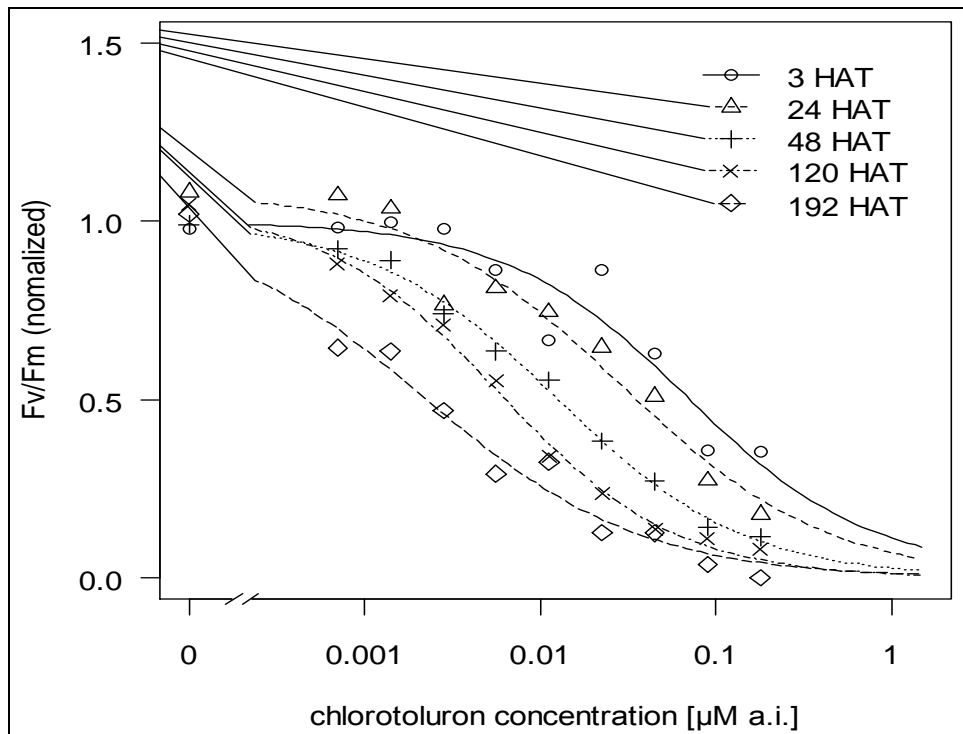
Figure 1. Chlorotoluron dose-response relationship for the sensitive biotype in dependency of the timing of *Fv/Fm* measurement (HAT, hours after transplanting).

Table-2. Resistance factor calculations based on *Fv/Fm*-determination for the evaluated *A. japonicus* biotypes, p<0.05 indicating significant differences in *ED$_{50}$* values.

ED$_{50}$/ED$_{50}$	Estimate	Std. Error	p-value
Hebei1/Sensitive: ED$_{50}$/ED$_{50}$	3.39	0.9211	0.0107
Hebei2/Sensitive: ED$_{50}$/ED$_{50}$	0.79	0.1418	0.1324
Hebei3/Sensitive: ED$_{50}$/ED$_{50}$	0.77	0.1411	0.1070
Jiangsu1/Sensitive: ED$_{50}$/ED$_{50}$	0.98	0.1681	0.1390

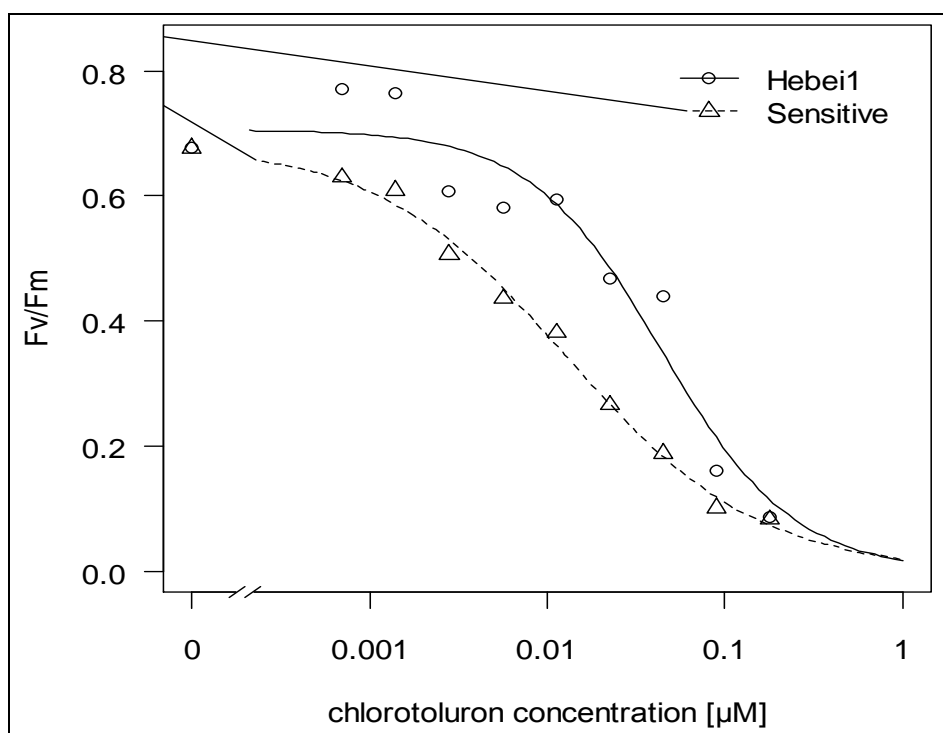**Figure 2. Chlorotoluron dose-response relationship for the sensitive biotype compared to the Hebei1 biotype. *ED50$_{Sensitive}$*=0.0129 µM a.i., *ED50$_{Hebei1}$*=0.0439 µM a.i.**

Whole Plant Herbicide Efficacy Studies

All tested *A. japonicus* biotypes were controlled sufficiently by application of chlorotoluron. For the Hebei2, Hebei3 and Jiangsu1 biotypes the horizontal curve assessment resulted in non significant difference compared to the sensitive biotype (Figure 3, Table-3).

Table-3.   Resistance factor calculation based on whole-plant dose-response studies for the evaluated *A. japonicus* biotypes; p<0.05 indicating significant differences in ED$_{50}$ values.

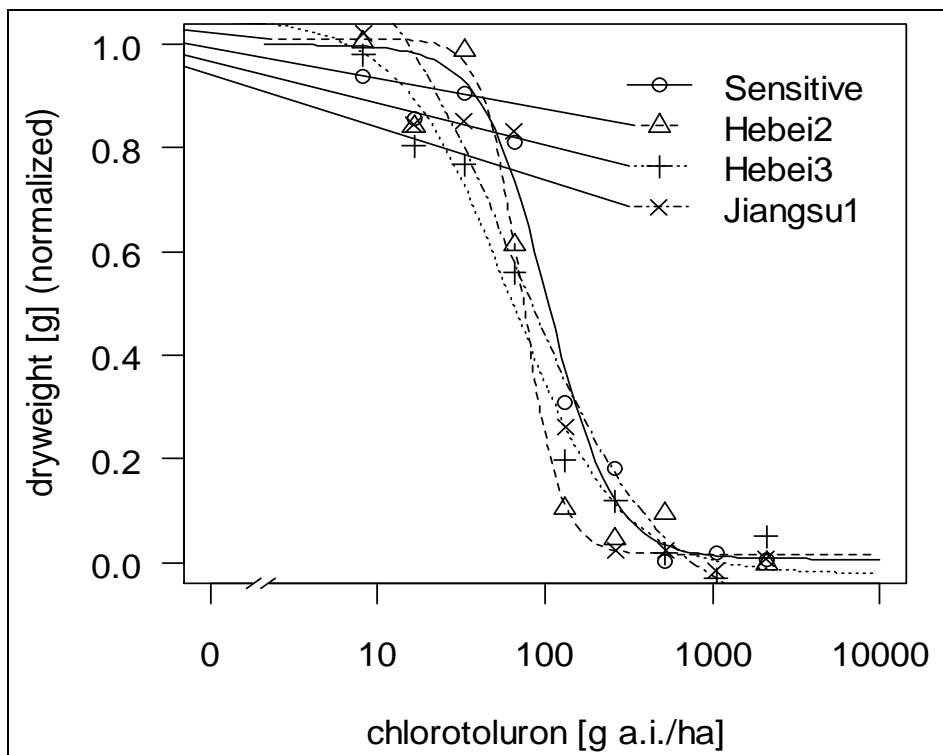| ED$_{50}$/ED$_{50}$	Estimate	Std. Error	p-value
Hebei1/Sensitive: ED$_{50}$/ED$_{50}$	3.52	0.2235	0.0023
Hebei2/Sensitive: ED$_{50}$/ED$_{50}$	0.70	0.1756	0.0952
Hebei3/Sensitive: ED$_{50}$/ED$_{50}$	0.59	0.1722	0.0515
Jiangsu1/Sensitive: ED$_{50}$/ED$_{50}$	0.63	0.2361	0.1259

Figure 3.   Whole-plant chlorotoluron dose- response results for the *A. japonicus* biotypes Hebei2 and Hebei3 and Jiangsu1 compared to the sensitive biotype.

The Hebei1 biotype showed an increased tolerance to chlorotoluron compared to the Sensitive biotype (Figure 4) resulting in a resistance factor (*ED$_{50Hebei1}$/ED$_{50Sensitive}$*) of RF=3.5 (Table-3).

DISCUSSION

All biotypes under evaluation were controlled by chlorotoluron sufficiently even at lower dosages than the recommended maximum

dosage of 2100 g a.i. ha^{-1}. The Hebei1 biotype required the highest dosage to gain 90% control of 1134.51 g a.i.; under field conditions it is assumed the full dosage is needed to control this biotype completely. All other biotypes were controlled under field conditions even with lower dosages of chlorotoluron than recommended.
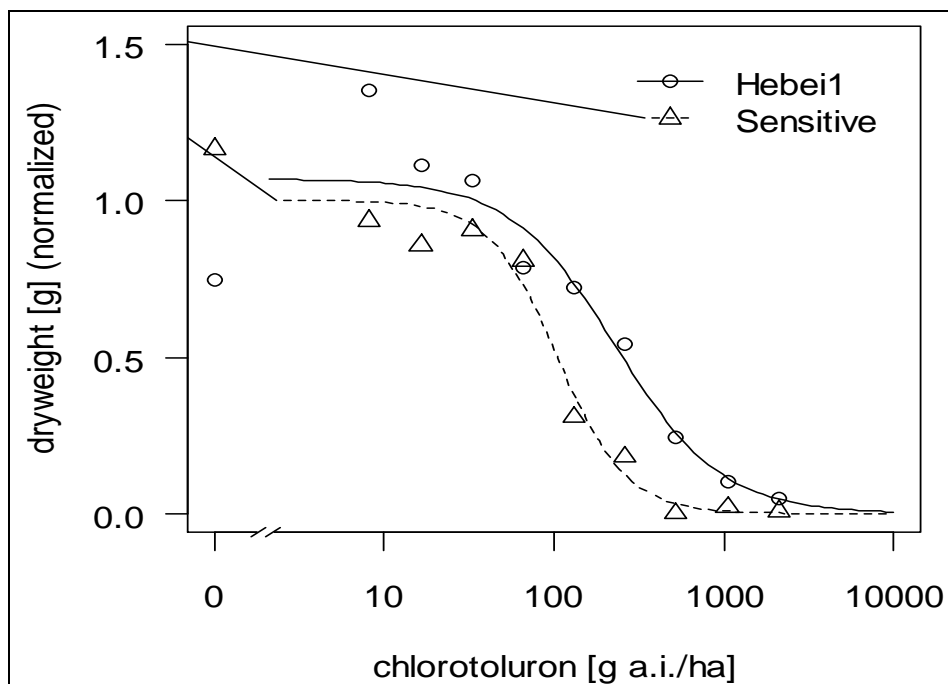

Figure 4. Whole-plant chlorotoluron dose response results for the *A. japonicus* biotypes Hebei1 compared to the sensitive biotype.
$ED_{50Sensitive}$=65.68 g a.i. ha^{-1}, $ED_{50Hebei1}$=230.97 g a.i. ha^{-1}.

The timing of 48 HAT for *Fv/Fm* measurement seems to be a good compromise between reduction of standard error and the evaluation of the maximum differences between the biotypes. The results of the whole-plant herbicide efficacy study verified clearly the results of the chlorophyll fluorescence microscreening. This new method can serve as a prospective rapid resistance test not only for chlorotoluron, but also for other inhibitors. Further experiments already demonstrated that this method is also applicable for ALS- and ACCase herbicides and transferable to other grass weeds like *Bromus japonicus, Lolium rigidum* and *Apera spica-venti.* For this novel rapid resistance test, neither a glasshouse nor a precision track sprayer was necessary. Results available within 72 h after transplanting are crucial

for testing during the field production season. This makes it possible for farmers to adjust weed control decisions within the season if weed seedlings from the field are directly transferred into the chlorophyll fluorescence microscreening test system.

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REFERENCES CITED

Abbaspoor, M. and J.C. Streibig. 2007. Monitoring the efficacy and metabolism of phenylcarbamates in sugar beet and black nightshade by chlorophyll fluorescence. Pest Manag. Sci. 585: 576-585.

Kaundun, S.S., S.J. Hutchings, R.P. Dale, G.C. Bailly and P. Glanfield. 2011. Syngenta RISQ test: a novel method for detecting resistance to post-emergence ACCase and ALS inhibitor herbicides in grass weeds. Weed Res. 51: 284-293.

Korres, N.E., R.J. Froud-Williams and S.R. Moss. 2003. Chlorophyll fluorescence technique as a rapid diagnostic test of the effects of the photosynthetic inhibitor chlorotoluron on two winter wheat cultivars. Annals Appl. Biol. 143: 53-56.

Moss, S.R. 2000. The "Rothamsted Rapid Resistance Test" for detecting herbicide-resistance in annual grass-weeds. Weed Science Society of America, Annual Meeting, Toronto, 40, Abstract 102.

Pedas, P., C.A. Hebbern, J.K. Schjoerring, P.E. Holm and S. Husted. 2005. Differential capacity for high-affinity manganese uptake contributes to differences between barley genotypes in tolerance to low manganese availability. Plant Physiol. 139: 1411–1420.

R Development Core Team. 2011. R: A language and environment for statistical computing. R Foundation for Statistical Computing Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.

Ritz, C. and J.C. Streibig. 2005. Bioassay Analysis using R.J. Statistical Software, 12:5.

Strasser, R.J. and A.D. Stirbet. 2001. Estimation of the energetic connectivity of PS II centres in plants using the fluorescence rise O–J–I–P; fitting of experimental data to three different PS II models. Math. Comput. Simul. 56: 451–461.

Streibig, J.C., A. Walker, A.M. Blair, G. Anderson-Taylor, D.J. Eagle, H. Friedländer, E. Hacker, W. Iwanzik, P. Kudsk, C. Labhart, B.M. Luscombe, G. Madafiglio, P.C. Nel, W. Pestemer, A. Rahman, G. Retzlaff, J. Rola, L. Stefanovic, H.J.M. Straathof and E.P. Thies. 1995. Variability of bioassays with metsulfuron-methyl in soil. Weed Res. 35: 215–224.

Streibig, J. 1988. Herbicide bioassay. Weed Res. 28:479-484.

Wu, D., Q. Yu, C. Lu and H. Hengsdijk. 2006. Quantifying production potentials of winter wheat in the North China Plain. Europ. J. Agron. 24: 226-235.