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Using Leaf Chlorophyll Fluorescence for In-Season Diagnosing Herbicide Resistance in *Echinochloa* Species at Reproductive Growth Stage

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Abstract

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The chlorophyll fluorescence measurement to diagnose herbicide resistant *Echinochloa* species at the reproductive (late) growth stage was applied. The significant correlation between F_v/F_m (chlorophyll fluorescence measurements) and fresh weight (whole plant test) and the statistical similarity of R/S ratios between the two tests demonstrated that the chlorophyll fluorescence test could be reliably used to diagnose herbicide resistant *Echinochloa* spp. at the reproductive growth stage in a shorter period of time (within 10 days) compared with the conventional whole plant test.

Keywords: ACCase inhibitor; ALS inhibitor; chlorophyll fluorescence induction; resistance diagnosis; decision-making

Echinochloa species is one of the most troublesome weeds in rice cultivations worldwide and has caused significant rice yield losses (Azmizmi & Mortimer-ortimer 2000; Moon *et al.* 2010). An increasing number of agriculturally important *Echinochloa* populations such as *E. crus-galli* (barnyardgrass), *E. oryzicola* (late watergrass), and *E. colona* (jungle rice) (Fischer *et al.* 2000; Talbert & Burgos 2007) with multiple resistance to several groups of herbicides have been identified due to heavy reliance upon and repeated use of herbicides for *Echinochloa* control (Heap 2017). In-season herbicide resistance diagnosis is an important tool for timely decision-making regarding herbicide choice. Conventional whole plant herbicide resistance tests usually require seed harvest from plants which survived the herbicide treatment followed by herbicide bioassays under controlled conditions. Such a test system requires several weeks until a result is available for the farmer. A timely adaption of the herbicide choice within the following crop is therefore often impossible.

Currently, most of the existing herbicide diagnostic methods are restricted to diagnosing herbicide resistant weeds at the vegetative growth stage including seed germination and vegetative plant development stage. Seed-based germination tests such as Petri dish (Beckie *et al.* 1990; Tal *et al.* 2000; Kaundun *et al.* 2011, 2014) and growth pouch assays (Zhang *et al.* 2015) are suitable for diagnosing herbicide resistant weeds at the seed germination stage. The conventional whole plant test (HRAC 2016), plant cutting-based methods (Boutsalis 2001; Kim *et al.* 2002; Zhang & Kim 2016), and chlorophyll fluorescence-based tests (Van Oorschot & Van Leeuween 1992; Norsworthy *et al.* 1998; Zhang *et al.* 2016) are commonly used for diagnosing herbicide resistant weeds at the early growth stage. However, concerning the weeds at the reproductive (late) growth stage,

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the development of resistance diagnostic methods on this regard is still imperative.

Utilisation of chlorophyll fluorescence as a biosensor to detect PS II inhibitor resistance in several weed species has been reported, owing to its directly interrupting the electron transport in PS II and causing rapid changes in chlorophyll fluorescence induction (Ahrens *et al.* 1981; Van Oorschot & Van Leeuween 1992; Norsworthy *et al.* 1998; Elahifard *et al.* 2013). Apart from PS II inhibitor, some other inhibitors such as acetyl-CoA carboxylase (ACCase), acetolactate synthase (ALS), and glutamine synthesis (GS) inhibitors, can also affect the induction of chlorophyll fluorescence (Hess *et al.* 2000; Sobye *et al.* 2010). ACCase inhibitors inhibit the enzyme acetyl-CoA carboxylase, which catalyses the first step in *de novo* fatty acid synthesis and is important for membrane synthesis (Focke & Lichtenthaler 1987; Burton *et al.* 1989), while the ALS inhibitors inhibit acetolactate synthase, a key enzyme in the pathway of biosynthesis of the branched-chain amino acids isoleucine, leucine, and valine (LaRossa & Schloss 1984). Those inhibitors by indirectly affecting photosynthesis as a consequence of causing peroxidation of the membrane lipid bilayer or the stability of the photosynthetic apparatus thus result in the slow changes of chlorophyll fluorescence induction (Abbaspoor & Streibig 2005; Sobye *et al.* 2010; Dayan & Zacaro 2012).

Recently, Kaiser *et al.* (2013) using chlorophyll fluorescence imaging successfully detected fenoxaprop-P-ethyl resistant black-grass (*Alopecurus myosuroides*) at the seedling growth stage. This method was further improved by Wang *et al.* (2016) using a mobile fluorescence imaging sensor (weedPAM) for resistance diagnosis in field. All these tests demonstrated that chlorophyll fluorescence is a sensitive bio-signal from weeds in response to herbicide treatment and can be steadily used for diagnosing herbicide resistance. Due to these tests conducted on weeds only at the vegetative (seedling) growth stage, the possibility for detection of herbicide resistance in weeds at the reproductive (late) growth stage is still unknown. Generally, the older growth stage of plants is more tolerant to herbicide damage than the younger growth stage of plants. Our previous study showed that the required time for changes in the leaf chlorophyll fluorescence induction of *Echinochloa* spp. at the younger (4–5 leaf stage) growth stage tested with ACCase and ALS inhibitors was 4 days after herbicide treatment (DAT) (Zhang *et al.* 2016), thus a longer period of time would be predicted if the older growth stage of plant was tested. Therefore, to validate the possibility and applicability of leaf chlorophyll fluorescence test in diagnosing herbicide resistant *Echinochloa* spp. at the reproductive growth stage, we conducted this test using given known herbicide resistant *Echinochloa* spp. at the panicle initiation growth stage and continuously measured leaf chlorophyll fluorescence at an interval of 2 days from 2 DAT to 10 DAT. The chlorophyll fluorescence test was finally compared with the whole plant test.

MATERIAL AND METHODS

Plant material. Two barnyardgrass biotypes, Seosan-5 and Suwon, and two late watergrass biotypes, Gimje and Suwon, were used in this study for the development of a chlorophyll fluorescence test. The Seosan-5 and Gimje biotypes were originally collected from a paddy field in Chungnam and North Jeolla Province, Korea, respectively, and were previously confirmed to be cross-resistant to cyhalofop-butyl and penoxsulam (Zhang *et al.* 2015). The other two Suwon biotypes were both collected in Suwon, Gyounggi Province, Korea, and both were susceptible to cyhalofop-butyl and penoxsulam (Im *et al.* 2009; Zhang *et al.* 2015). Seeds of all four biotypes were pregerminated in Petri dish in an incubation chamber maintained at 33/25°C (day/night) with a 12/12 h photoperiod for 96 hours. The seedlings at the 1-leaf stage were transplanted in the plastic pot (11-cm diameter) containing sandy loam soil at a density of three plants/pot and grown until the plants began the panicle initiation stage in a tropical greenhouse at an experimental farm station of Seoul Nation University, Suwon, Korea maintained at 30/20°C (day/night) with a 14/10 h photoperiod equipped with overhead sodium lamps. The *Echinochloa* spp. used in this study only involved non-target-site-based resistance (Song *et al.* 2011; Kim 2016).

Whole plant test. Barnyardgrass and late watergrass grown to the panicle initiation stage were respectively sprayed with a range of cyhalofop-butyl (ACCase inhibitor, Clincher® EC, 250.0 g active ingredient (ai)/ha; Dongbu Hannong Co. Ltd., Seoul, Korea) (0, 31.3, 62.5, 125.0, and 250.0 g ai/ha) and penoxsulam (ALS inhibitor, Granite® SC; 30.0 g ai/ha; Hankook Samgong Co. Ltd., Seoul, Korea) doses (0, 7.5, 15.0, 30.0 and 60.0 g ai/ha) using a compressor pressurised belt-driven sprayer (R & D Sprayer, Opelousas, USA) equipped

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with an 8002E flat-fan nozzle (Spraying System Co., Glendale Heights, USA) adjusted to deliver 600 l/ha. All the treatments were replicated 3 times and arranged in a completely randomized design. After herbicide application, the plants were maintained in the same tropical greenhouse as described above and the leaf chlorophyll fluorescence was measured accordingly (see the section below). During the leaf chlorophyll fluorescence measurement, the pots were sub-irrigated regularly to avoid any moisture stress affecting chlorophyll fluorescence induction. Aboveground fresh weights were harvested at 30 DAT and weighed.

Chlorophyll fluorescence test. Leaf chlorophyll fluorescence of herbicide sprayed plants in the whole plant test was measured accordingly (Zhang *et al.* 2016) to validate the applicability of this test. Our preliminary tests showed negligible changes in the leaf chlorophyll fluorescence induction of Suwon biotype (susceptible reference) at the panicle initiation stage tested with cyhalofop-butyl or penoxsulam at recommended dose at the first 2 DAT (data not shown). Therefore, the leaf chlorophyll fluorescence measurement in the present study was conducted at an interval of 2 days from 2 to 10 DAT using a portable chlorophyll fluorimeter (Plant Efficiency Analyzer; Hansatech Instruments Ltd., Norfolk, UK) which emits light of 650 nm wavelength with an intensity of 3500 μmol photons/m^2/s for 2 seconds. The measured parameter was F_v/F_m, the quantum yield of PS II [$(F_m - F_o)/F_m$, where: F_o – minimal fluorescence of dark-adapted leaves; F_m – maximal fluorescence], which was rather a probe of overall plant fitness than a selective marker of PS II photochemistry. The measurements were performed on 30-min dark-adapted leaves under a safe light (green light) in darkroom at room temperature (25°C). For each dose of herbicide treatment, 2 newly grown leaves from each plant were randomly chosen, and three different measuring locations of each leaf were measured in triplicate. Therefore, a total of eighteen measurements for each dose of herbicide treatment was determined. The same chosen leaves were used for chlorophyll fluorescence measurement throughout this study. The results were finally compared with fresh weight data obtained from the whole plant dose-response test.

Statistical analysis. Statistical analyses were conducted using R (R Development Core Team 2011). All collected data were tested for normality (Shapiro-Wilk) and homogeneity of variances prior to analysis of variance (ANOVA). As the chlorophyll fluorescence data obtained at 10 DAT and fresh weight data at 30 DAT showed the most significant herbicide dose treatment effects ($P < 0.001$), these data were firstly converted to the percentage of the untreated control, and then subjected to nonlinear regression analysis using the statistical software R with its various dose-response curves package (Knezevic *et al.* 2007). Among the various tested dose-response models, the three-parameter log-logistic dose-response curve (Equation 1) showed the best fit with the data by comparing P values calculated from the lack-of-fit tests and r^2 for each model (Streibig 1980):

$$y = \frac{d}{1 + (x/Z_{50})^b} \quad (1)$$

using the calculated parameters b and Z_{50}, each response level could be determined. Because the decrease of F_v/F_m was small and did not reach 50% inhibition in a chlorophyll fluorescence test, to avoid the inaccuracy for estimate of Z_{50} (GR_{50} or I_{50}), Z_{10} values were calculated using Equation 2 (Streibig *et al.* 1995):

$$Z_x = \frac{Z_{50}}{(x/(100 - x))^b} \quad (2)$$

where: y – plant fresh weight or F_v/F_m as percentage of the untreated control; x – herbicide dose; d – upper limit; b – slope of the line at Z_{50} (GR_{50} or I_{50}); Z_{50} – herbicide dose that results in 50% growth reduction in fresh weight in the whole plant test (GR_{50}) or 50% inhibition in F_v/F_m in the chlorophyll fluorescence test (I_{50}) with regard to the untreated control

A resistance index (R/S ratio) was calculated using GR_{10} or I_{10} values of each resistant biotype compared with the GR_{10} or I_{10} values for the susceptible biotype. GR_{10} or I_{10} values were considered to be statistically different when their respective R/S ratio differed from 1.0 at $\alpha = 0.05$. All treatment means were separated using Fisher's protected *LSD* test at $\alpha = 0.05$ to reflect the differences between biotypes and the effect of the herbicide dose.

RESULTS

In the leaf chlorophyll fluorescence test, the F_v/F_m values of the four *Echinochloa* biotypes at the panicle initiation stage tested with cyhalofop-butyl or penoxsulam were not notably ($P < 0.05$) affected by increasing doses of herbicides at the first 6 DAT (Table 1), thus not allowing the estimation of reliable I_{50} values for each herbicide. However, at 8 DAT for Seosan-5 resistant (R) and Suwon susceptible

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Table 1. Dose responses of barnyardgrass tested with cyhalofop-butyl and late watergrass tested with penoxsulam in the fluorescence rate (F_v/F_m, chlorophyll fluorescence test) at 0, 2, 4, 6, 8, and 10 DAT and fresh weight (whole plant test) at 30 DAT

Species	Dose (g ai/ha)	F_v/F_m						Fresh weight (g) 30 DAT
		0 DAT	2 DAT	4 DAT	6 DAT	8 DAT	10 DAT	
Barnyardgrass (Seosan-5-R)	0	0.779^{A}	0.767^{A}	0.785^{A}	0.783^{A}	0.778^{A}	0.761^{A}	62.9^{A}
	31.4	0.768^{B}	0.757^{BC}	0.766^{C}	0.761^{B}	0.752^{B}	0.741^{B}	57.4^{B}
	62.8	0.785^{A}	0.765^{AB}	0.784^{AB}	0.766^{B}	0.747^{BC}	0.734^{B}	48.9^{C}
	125.0	0.783^{A}	0.754^{BC}	0.764^{C}	0.754^{B}	0.735^{CD}	0.713^{C}	43.1^{D}
	250.0	0.788^{A}	0.752^{C}	0.776^{B}	0.743^{B}	0.726^{D}	0.690^{D}	36.5^{E}
	$LSD_{0.05}$	0.009	0.01	0.008	0.012	0.013	0.009	5.21
	P	0.004	0.015	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	df	89	89	88	89	89	89	44
Barnyardgrass (Suwon-S)	0	0.778^{B}	0.782^{A}	0.788^{A}	0.769^{A}	0.768^{A}	0.765^{A}	66.9^{A}
	31.4	0.770^{A}	0.775^{A}	0.772^{B}	0.749^{B}	0.740^{B}	0.729^{B}	52.4^{B}
	62.8	0.770^{B}	0.769^{Ab}	0.768^{B}	0.748^{B}	0.741^{B}	0.719^{B}	44.7^{C}
	125.0	0.788^{B}	0.749^{BC}	0.778^{Ab}	0.743^{BC}	0.722^{BC}	0.670^{C}	35.0^{D}
	250.0	0.782^{A}	0.745^{C}	0.778^{AB}	0.739^{C}	0.688^{C}	0.585^{D}	31.7^{E}
	$LSD_{0.05}$	0.001	0.022	0.013	0.017	0.030	0.015	3.75
	P	0.001	0.003	0.002	0.001	< 0.001	< 0.001	< 0.001
	df	89	88	89	89	88	89	44
Late watergrass (Gimje-R)	0	0.803^{A}	0.802^{A}	0.807^{A}	0.791^{AB}	0.788^{A}	0.767^{A}	42.1^{A}
	7.5	0.801^{A}	0.800^{AB}	0.805^{A}	0.797^{A}	0.789^{A}	0.762^{AB}	39.9^{A}
	15.0	0.789^{C}	0.793^{BC}	0.797^{B}	0.780^{B}	0.775^{B}	0.754^{BC}	39.0^{A}
	30.0	0.806^{A}	0.793^{BC}	0.801^{AB}	0.783^{B}	0.775^{C}	0.748^{CD}	38.4^{AB}
	60.0	0.791^{B}	0.785^{C}	0.800^{AB}	0.747^{C}	0.761^{C}	0.738^{D}	34.9^{B}
	$LSD_{0.05}$	0.003	0.008	0.008	0.012	0.007	0.010	4.11
	P	< 0.001	< 0.001	0.008	< 0.001	< 0.001	< 0.001	< 0.001
	df	89	89	89	89	89	89	44
Late watergrass (Suwon-S)	0	0.782^{BC}	0.789^{B}	0.796^{AB}	0.775^{A}	0.772^{A}	0.767^{A}	54.4^{A}
	7.5	0.778^{C}	0.789^{B}	0.794^{AB}	0.778^{A}	0.773^{A}	0.753^{AB}	50.7^{A}
	15.0	0.796^{A}	0.795^{AB}	0.793^{AB}	0.774^{A}	0.753^{B}	0.731^{B}	45.8^{B}
	30.0	0.776^{C}	0.801^{A}	0.802^{A}	0.773^{A}	0.749^{B}	0.691^{BC}	40.8^{C}
	60.0	0.789^{AB}	0.787^{B}	0.790^{B}	0.748^{B}	0.741^{B}	0.674^{C}	38.3^{C}
	$LSD_{0.05}$	0.009	0.011	0.010	0.013	0.013	0.024	4.42
	P	< 0.001	0.007	0.016	< 0.001	< 0.001	< 0.001	< 0.001
	df	88	89	89	89	88	89	44

Different letters within a column for each biotype indicated statistical differences according to Fisher's protected *LSD* test at $\alpha = 0.05$

(S) biotypes tested with cyhalofop-butyl, a clear difference in F_v/F_m reduction in both biotypes was observed, and the F_v/F_m value in Suwon-S biotype at the recommended dose decreased to 0.668 compared with 0.726 in Seosan-5-R. At 10 DAT, the continuous reduction of F_v/F_m values in those four biotypes was found by increasing the doses of herbicides (Table 1). However, a reduction of the percentage of untreated control at the highest doses in S biotypes was much greater than in R biotypes (Figures 1A and B). In the case of barnyardgrass tested with cyhalofop-butyl, the percentage of F_v/F_m reduction was 23.5% in Suwon-S biotype compared with 9.4% in Seosan-5-R biotype (Figure 1A). With regard to

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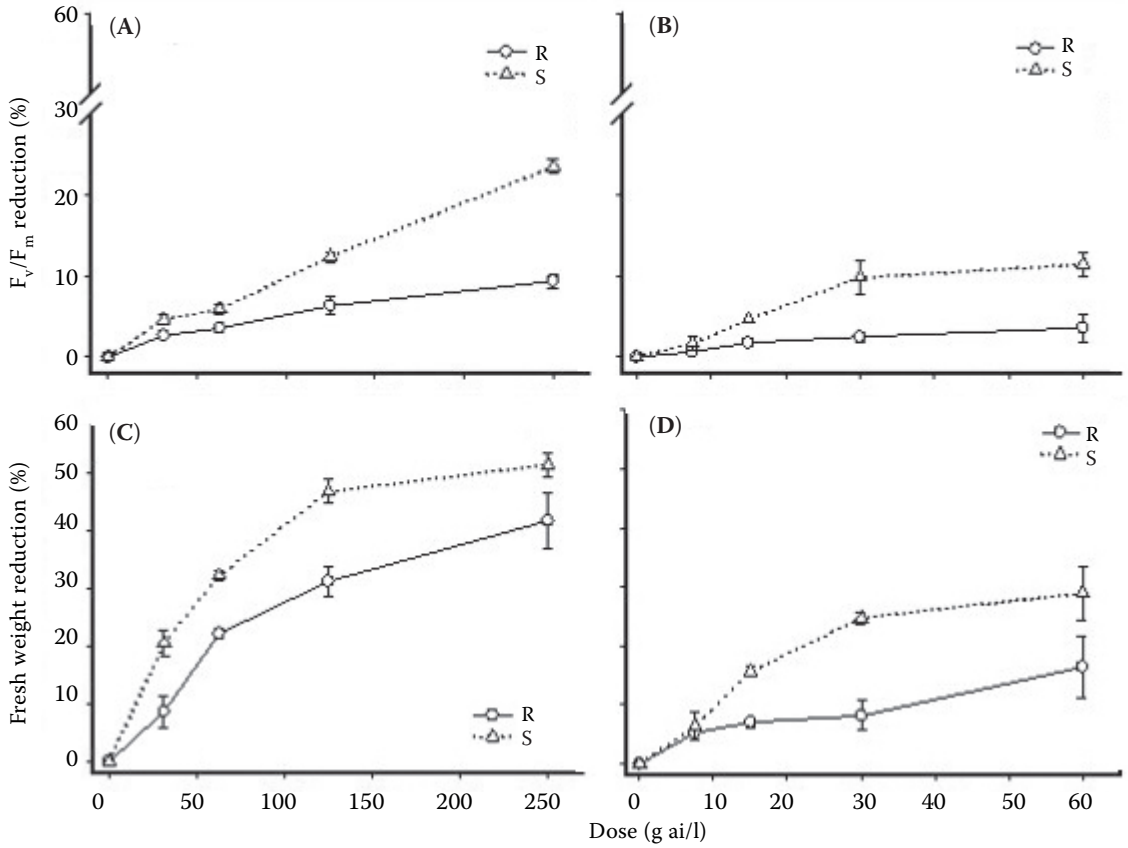

Figure 1. A reduction of the percentage of untreated control in F_v/F_m values (**A**, **B**) and fresh weights (**C**, **D**) of barnyardgrass (Seosan-5-R, Suwon-S biotypes) tested with cyhalofop-butyl (**A**, **C**) and late watergrass (Gimje-R, Suwon-S biotypes) tested with penoxsulam (**B**, **D**) at the panicle initiation stage in chlorophyll fluorescence test at 10 DAT and whole plant test at 30 DAT, respectively

F_v/F_m – variable fluorescence/maximum fluorescence

late watergrass, the percentage of F_v/F_m reduction was 11.5% in Suwon-S biotype in comparison with 3.5% in Gimje-R biotype (Figure 1B). Those F_v/F_m values of the four *Echinochloa* biotypes obtained at 10 DAT were appropriately described by the three-parameter log-logistic dose-response model ($r^2 \geq 0.93$, $P = 0.492$ for a model fit for barnyardgrass tested with cyhalofop-butyl; $r^2 \geq 0.92$, $P = 0.842$ for a model fit for late watergrass tested with penoxsulam), enabling to estimate I_{50} values for the two herbicides (Table 2). As the estimated I_{50} values of barnyardgrass for cyhalofop-butyl (3135.9 and 758.9 g ai/ha for Seosan-5-R and Suwon-S, respectively) and late watergrass for penoxsulam (6956.5 and 933.6 g ai/ha for Gimje-R and Suwon-S, respectively) were much higher than the highest doses applied (cyhalofop-butyl 250.0 g ai/ha; penoxsulam, 60.0 g ai/ha) (Table 2), to give the more accurate estimate of the response level of *Echinochloa* to herbicides, I_{10} values were calculated. The estimate of I_{10} values of barnyardgrass for cyhalofop-butyl at 10 DAT was 267.9 and 101.5 g ai/ha for Seosan-5-R and Suwon-S, respectively, resulting in an R/S ratio of 2.6 (Table 2). For penoxsulam, the I_{10} values of late watergrass at 10 DAT were 274.9 and 40.4 g ai/ha for Gimje-R and Suwon-S, respectively, giving an R/S ratio of 6.8 (Table 2).

In the whole plant test, the aboveground fresh weights of the four biotypes were assessed at 30 DAT. It is worth noting that no plants were killed by herbicides even at the highest doses and no significant ($P > 0.05$) difference was observed in plant height of the four biotypes between the tested herbicide doses (data not shown). However, the fresh weights of the four biotypes were significantly ($P < 0.05$) reduced by increasing the doses of herbicides (Table 1). The reduction of the percentage of untreated control at the highest doses in S biotypes was greater than in R biotypes with the respective values of 52.0 and 40.1% for Suwon-S and Seosan-5-R biotypes tested

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Table 2. Summary of parameter estimates for the log-logistic model of F_v/F_m (I_{50}, g ai/ha) in chlorophyll fluorescence test at 10 DAT and fresh weight (GR_{50}, g ai/ha) in whole plant test at 30 DAT

Herbicide	Species	Chlorophyll fluorescence test							Whole plant test						
		b	*d*	I_{50} (95% CI)[b]	*P*-value for model fit	r^2	I_{10} (95% CI)	R/S (I_{10})	*b*	*d*	GR_{50} (95% CI)	*P*-value for model fit	r^2	GR_{10}	R/S (GR_{10})
Cyhalofopbutyl	barnyardgrass (Seosan-5-R)	0.89 (0.12)	99.5 (0.57)	3135.9 (1051.3–5220.6)	0.492	0.934	267.9 (212.4–323.3)	2.6	0.84 (0.09)	100.4 (1.31)	334.4 (215.3–453.5)	0.486	0.905	24.4 (18.6–30.5)	3.0
	barnyardgrass (Suwon-S)	1.09 (0.08)	99.6 (0.57)	758.9 (616.2–901.1)		0.932	101.5 (84.8–118.2)		0.71 (0.26)	100.1 (0.96)	182.5 (119.8–245.8)		0.918	8.2 (6.8–11.4)	
Penoxsulam	late watergrass (Gimje-R)	0.68 (0.23)	100.0 (1.21)	6956.5 (2649.2-10408.5)	0.842	0.926	274.9 (223.4–312.5)	6.8	0.67 (0.17)	99.7 (0.74)	884.3 (483.0–1877.5)	0.626	0.903	33.2 (26.4–39.6)	4.1
	late watergrass (Suwon-S)	0.70 (0.22)	100.3 (1.02)	933.6 (457.1–1878.1)		0.945	40.4 (24.5–48.8)		0.70 (0.21)	100.5 (1.12)	188.7 (107.7–411.1)		0.894	8.2 (5.7–12.4)	

d – upper limit; *b* –slope of the line at Z_{50} (GR_{50} or I_{50}); I_{50}, I_{10} values – dose required to inhibit the chlorophyll fluorescence of *Echinochloa* by 50 and 10% in chlorophyll fluorescence test, respectively; CI – confidence interval (n = 3); R/S – ratio of the I_{50} or GR_{50} value of the resistant biotype to that of the susceptible reference (Suwon) biotype; GR_{50}, GR_{10} – dose required to inhibit the growth of *Echinochloa* by 50 and 10% in whole plant test, respectively

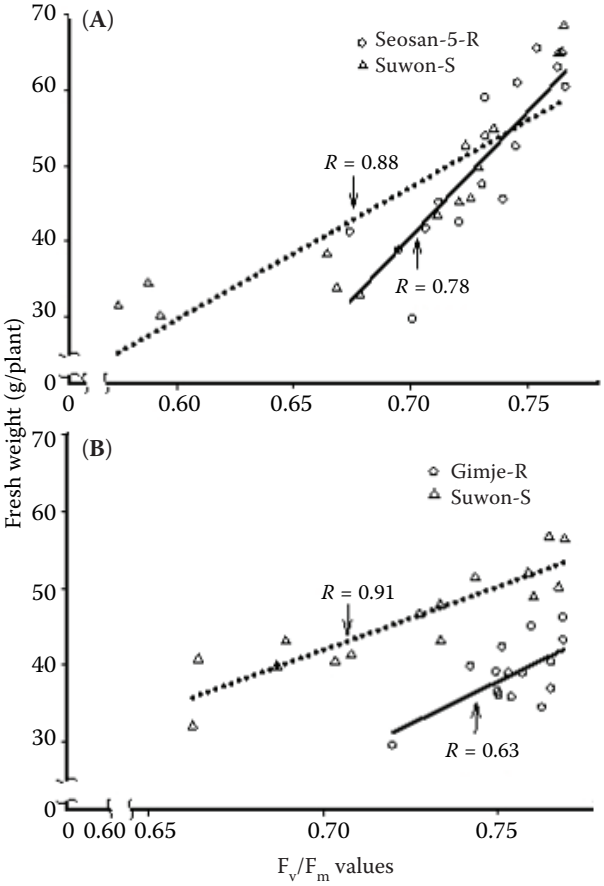

Figure 2. Correlation between F_v/F_m values at 10 DAT (chlorophyll fluorescence test) and fresh weights at 30 DAT (whole plant test) for barnyardgrass (Seosan-5-R, Suwon-S biotypes) tested with cyhalofop-butyl (**A**) and late watergrass (Gimje-R, Suwon-S biotypes) tested with penoxsulam (**B**)

Correlation coefficients (*R*) are shown for the four *Echinochloa* biotypes; F_v/F_m – variable fluorescence/maximum fluorescence

with cyhalofop-butyl (Figure 1C and Table 1), and 29.9 and 16.3% for Suwon-S and Gimje-R biotypes tested with penoxsulam (Figure 1D and Table 1). An acceptable goodness of fit between fresh weight data and the log-logistic model was obtained with $r^2 \geq 0.90$, $P = 0.486$ for a model fit for barnyardgrass tested with cyhalofop-butyl and $r^2 \geq 0.89$, $P = 0.626$ for a model fit for late watergrass tested with penoxsulam (Table 2). The respective GR_{10} values estimated from the log-logistic models were 24.4 and 8.2 g ai/ha for Seosan-5-R and Suwon-S biotypes tested with cyhalofop-butyl at 30 DAT, resulting in an R/S ratio of 3.0 (Table 2). The GR_{10} values were 33.2 and 8.2 g ai/ha for Gimje-R and Suwon-S at 30 DAT, respectively, resulting in an R/S ratio of 4.1 (Table 2).

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Additionally, the linear relationships between F_v/F_m and fresh weight suggested the significant ($P < 0.05$) correlation ranging from 0.73 to 0.91 (Figure 2).

All these data suggested that the chlorophyll fluorescence test could be potentially applied to diagnose *Echinochloa* species resistance to ACCase and ALS inhibitors at the reproductive growth stage.

DISCUSSION

Our previous study (Zhang *et al.* 2016) showed the effects of PS II inhibitor applied at a recommended dose on the fluorescence parameter (F_v/F_m) of jungle rice at the 4–5 leaf stage first commenced at 4 h after treatment (HAT). Other researchers also reported the rapid effects on F_v/F_m at several HAT by other PS II inhibitors, such as triazine, terbuthylazine, and metamitron (Ahrens *et al.* 1981; Abbaspoor *et al.* 2006). Apart from PS II inhibitor, some other inhibitors such as ACCase and ALS inhibitors were also found changing the induction of chlorophyll fluorescence (Abbaspoor & Streibig 2005; Sobye *et al.* 2010; Dayan & Zacaro 2012; Zhang *et al.* 2016). Abbaspoor and Streibig (2005) reported that clodinafop, an ACCase inhibitor, changed the shape of the chlorophyll fluorescence induction in wild oat (*Avena fatua*) and barley at the seedling growth stage at 72 HAT. The chlorophyll fluorescence-based imaging method also showed the changed F_v/F_m of black-grass at the 2–3 leaf stage treated with fenoxaprop-P-ethyl (Kaiser *et al.* 2013) at 96 HAT. These imply the chlorophyll florescence is an important signal from weeds in response to herbicide resistance and has the potential to be applied for herbicide assays and detection of herbicide resistance. In the present study, we found that the required time (> 8 DAT) for the change in F_v/F_m of *Echinochloa* spp. at the reproductive growth stage was much longer than that of plants at the 4–5 leaf growth stage (120 HAT) in our previous study. A similar result was also reported when metamitron affected the fluorescence induction more at the 4- than at the 6-true-leaf stage of sugar beet (Abbaspoor *et al.* 2006). It is common knowledge that the older growth stage of plants is more tolerant to herbicide damage than the younger growth stage of plants, therefore, it explains a slower inhibition of chlorophyll fluorescence induction at the older growth stage of plants.

A significant correlation between F_v/F_m and weight parameters was also found for sugar beet (*Beta vulgaris*) at 4–6-true leaf stages tested with metamitron (PS II inhibitor) at 2 DAT (Abbaspoor *et al.* 2006) and barley (*Hordeum vulgare*) tested with clodinafop (ACCase inhibitor) at 3 DAT (Abbaspoor & Streibig 2005). Additionally, the correlation coefficient calculated for S biotypes tested with each herbicide was more correlated than the value for the corresponding R biotypes (Figure 2), which is in agreement with a pervious study of jungle rice tested with ametryn, a PS II inhibitor herbicide (Elahifard *et al.* 2013). We believed the difference in correlation coefficient between R and S biotypes resulted from different uniformity in the R and S population. The whole S population was tested to be sensitive to cyhalofop-butyl and penoxsulam in the previous whole plant tests (Im *et al.* 2009; Zhang *et al.* 2015), but the confirmed R population may be composed of seeds with different resistance levels to those two herbicides, thus resulting in a less correlated pattern compared with S population. Although the difference in correlation coefficient between R and S biotypes tested with each herbicide was shown, the significant correlation between F_v/F_m and fresh weight demonstrated the agreement and consistence of these two tests in diagnosing *Echinochloa* spp. resistant to ACCase and ALS inhibitors at the reproductive growth stage.

In summary, the present test is quick with results being obtained within 10 days (the diagnostic time is shortened) and allowing the diagnosis of resistance to ACCase and ALS inhibitors late in the season in the case of a failure of *Echinochloa* management at the reproductive growth stage. In addition, as this test does not need mature seeds, this is another advantage compared with tests needing seeds including the whole plant test. In a practical field situation, at least 8 days after herbicide spraying, the randomly selected leaves of plants were treated using clips for a 30-min dark adaption prior to measuring. The diagnostic results will return to the growers in a couple of days, advising them to shift the application of an alternative herbicide or to reapply again if the plants are confirmed to be susceptible or to plan the following season's weed management program.

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