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Short Title: CYHALOFOP RESISTANCE DIAGNOSIS IN ECHINOCHLOA

Diagnosing ACCase Inhibitor– Cyhalofop-butyl Resistance in *Echinochloa crus-galli* at Various Growth Stages

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Abbreviations: ACCase, acetyl coenzyme-A carboxylase; ALS, acetolactate synthase; DAT, days after treatment; POST, post-emergence; PS II, photosystem II; R/S, resistance to sensitivity; VLCFAs, very long chain fatty acids

Cyhalofop-butyl-resistant *Echinochloa crus-galli* has been wide-spreading, so rapid diagnosis of herbicide resistance in *E. crus-galli* at various growth stages is crucial for timely and effective management of herbicide resistant *E. crus-galli* throughout the season. This study was thus conducted to diagnose cyhalofop-butyl resistance in *E. crus-galli* at various stages of growth using rapid diagnostic test methods. Growth pouch, trimmed seedling and stem node tests were conducted on *E. crus-galli* at seed germination, juvenile and heading stages, respectively and the diagnostic results were then compared with the conventional whole plant test. All rapid diagnostic tests discriminated resistant and susceptible biotypes on the basis of R/S ratios (the ratio of GR₅₀ values of resistant and susceptible biotypes) within 7 days after herbicide treatment (DAT). The statistical agreement in R/S ratios between the rapid diagnostic tests (R/S ratios, 2.0-4.8) and the whole plant test (R/S ratio, 3.6) demonstrated that the rapid diagnostic tests could be reliably applied to diagnose cyhalofop-butyl resistance in *E. crus-galli* at various stages of growth with significant time and cost savings compared to conventional whole plant test. In addition, the presented diagnostic results coupled with discrimination of herbicide resistant weeds in previous studies suggest that the rapid diagnostic tests are capable of detecting herbicide resistance regardless of herbicide in many different weed species.

Key words: ACCase inhibitor, bioassay, *Echinochloa*, herbicide resistance, rapid diagnosis

Echinochloa crus-galli continues to pose a serious threat to lowland rice production both in transplanted and direct-seeded fields in many parts of the world (Holm et al. 1977). It is more competitive than rice, by reducing the number of tillers during the rice early growth stage, resulting in significant reductions in rice yields (Moon et al. 2010 and 2014). Several herbicides with different sites-of-action such as photosystem II (PS II), acetyl CoA carboxylase (ACCase) and acetolactate synthase (ALS)-inhibitors have been applied for the post-emergence (POST) control of *E. crus-galli*. Cyhalofop-butyl is an ACCase inhibitor belonging to aryloxyphenoxy-propionate (AOPP) family and has been used in Asian lowland rice fields for the POST control of *E. crus-galli* (Gronwald 1994; Im et al. 2009; Rahman et al. 2010). The repeated and continuous use of cyhalofop-butyl for POST grass weed control in rice field has resulted in the evolution of resistant *E. crus-galli* populations. Cyhalofop-butyl-resistant *E. crus-galli* was first reported in rice fields of United States in 2000 followed by Thailand in 2001, Korea in 2008, and Malaysia in 2010 (Heap 2016). Moreover, multiple herbicide resistant *E. crus-galli* populations has also been reported, such as PS II and very long chain fatty acids (VLCFAs)-inhibitors resistant *E. crus-galli* in the Philippines in 2005, ACCase and ALS-inhibitors resistant *E. crus-galli* in Italy, Korea, Turkey in 2009, ACCase, ALS, PS II and cellulose biosynthesis-inhibitors resistant *E. crus-galli* in United States, etc. (Heap 2016).

The early and rapid detection of herbicide resistance is an essential component of decision making for weed management and herbicide application (rotation). Several

diagnostic methods for detecting herbicide resistance in weeds have been developed, including the conventional whole plant test (HRAC), the seed-based germination test (Moss 1990; Ghanizadeh et al. 2015), the enzyme activity and molecular test (Reade and Cobb 2002; Delye et al. 2009), chlorophyll fluorescence-based non-destructive tests (Norsworthy et al. 1998; Kaiser et al. 2013; Zhang et al. 2016), etc. Various tests for the detection of herbicide resistance in *Echinochoa* spp. have also been developed. One recently developed rapid test, namely the growth pouch test, involves incubation of pre-germinated seeds in a growth pouch containing a range of herbicide concentrations and discriminating the ACCase (cyhalofop-butyl) and ALS (penoxsulam)-inhibitors resistance in *E. crus-galli* and *E. oryzicola* at 6 DAT (Zhang et al. 2015). Plant cutting-based methods such as the juvenile plant test, tiller test and stem node test were also developed to rapidly detect PS II (propanil) and ACCase (fenoxaprop)-inhibitors resistance in *E. colona* at a wide range of growth stages (Kim et al. 2000). The cutting method was further successfully extended to detect ALS (imidazolinone)-inhibitor resistance in *E. crus-galli* at 7 DAT (Matzenbacher et al. 2010). Compared with the required period of 30 days by the whole plant test, these tests showed a significantly shorter period of resistance detection (within 7 days). In addition, these tests are very versatile and flexible with respect to resistance detection at various stages of growth ranging from seed germination to the heading stage.

Cyhalofop-butyl-resistant *E. crus-galli* has become one of the most problematic weeds since its first report in a reclaimed paddy field in Korea in 2007 (Im et al. 2009).

For farmers, the rapid diagnosis of cyhalofop-butyl-resistant *E. crus-galli* and finding alternative herbicides with different sites of action are crucial for timely and effective control of this weed throughout the season. The aforementioned herbicide resistance confirmation methods have shown good potential for rapid feedback concerning the resistance status of suspected samples to farmers in the same growing season (Kim et al. 2000; Boutsalis 2001). Although the successful detection of resistance to several inhibitors in *Echinochloa* spp. has been documented by those tests; however, its suitability for the detection of ACCase-inhibitor resistance in *E. crus-galli* at various growth stages still requires clearer and more concrete validation. It is necessary to validate the applicability of those tests for detecting cyhalofop-butyl resistance in *E. crus-galli*. Therefore, in this study, we used the growth pouch, trimmed seedling and trimmed stem node tests to detect cyhalofop-butyl resistance in *E. crus-galli* at various growth stages from seed germination to the heading stage.

MATERIALS AND METHODS

Plant Materials

Seed Source

Three *Echinochloa crus-galli* biotypes, namely Seosan-3 (susceptible to cyhalofop-butyl), Seosan-5 (resistant to cyhalofop-butyl) and Suwon (susceptible reference), were used throughout the tests, which included whole plant, growth pouch, trimmed seedling and trimmed stem node tests with cyhalofop-butyl. The former two

Seosan biotypes were originally collected from a paddy field in Seosan Hyundai farm station, Chungnam province, Korea, and Suwon biotype was collected from a paddy field in Suwon, Gyeonggi province, Korea. Seosan-3 and Suwon biotypes were pre-confirmed susceptible to cyhalofop-butyl, while Seosan-5 was resistant to cyhalofop-butyl based on a whole plant dose-response study (Im et al. 2009). In particular, Suwon biotype was also proved to be susceptible to most of herbicides registered in Korea, so has been used as a reference susceptible biotype for evaluating the level of resistance of other suspected biotypes (Kim et al. 2011; Zhang et al. 2015).

Seedling Growth and Sampling

Seeds of the three *E. crus-galli* biotypes were germinated in petri dishes in an incubation room maintained at 33/25°C (day/night) and left until the first leaf stage. Seedlings at the first leaf-stage were then transplanted in plastic pots (11-cm diameter) containing sandy loam soil at a density of three plants per pot and grown in a tropical greenhouse at an Experimental Farm Station of Seoul Nation University, Suwon, Korea maintained at $26 \pm 3^\circ\text{C}$ temperature regime with a 12/12 hour photoperiod supplemented by overhead sodium lamps. The plants were grown up to the four specific growth stages, namely 1) the 4 leaf-1 tiller stage, 2) 1-2 tillers stage, 3) 2-3 tillers stage and 4) 3-4 tillers stage, and sampled for the trimmed seedling test. Plants grown to the heading stage were sampled for trimmed stem node tests and the pre-germinated seeds were used in the growth pouch test.

Diagnosis of Herbicide Resistance

Determination of optimized test conditions

Initially, a series of common tests with the three *E. crus-galli* biotypes at those specific growth stages (as described above) were conducted to determine the optimized test conditions including cyhalofop-butyl concentration range and culturing method for each corresponding test. To determine appropriate range of cyhalofop-butyl concentrations suitable for discriminating between susceptible and resistant biotypes in each test, a wide range of cyhalofop-butyl concentrations were tested for growth pouch, trimmed seedling and trimmed stem node tests. To optimize methods for plant culture and assessment in each test, each test was conducted several times under different incubation conditions and then the most reliable methods for plant culture before and after herbicide treatment and assessment were determined. For assessment, the most responsive growth parameters to herbicide treatment were determined: root length for growth pouch test, new shoot length and fresh weight for trimmed seedling test, and new root length and fresh weight for trimmed stem node test. Under the optimized test condition, each test was repeated several times to validate the reproducibility of discrimination between susceptible and resistant biotypes. In this paper, the data presented here are those of the final repeat of each test. Finally, these results were compared with those from the conventional whole plant test to validate the suitability of those tests for detection of cyhalofop-butyl resistance in *E. crus-galli*.

Whole Plant Test

A conventional whole plant test was conducted in the greenhouse at an Experimental Farm Station of Seoul National University, Suwon, Korea, using the three biotypes (Seosan-3, Seosan-5 and Suwon) of *E. crus-galli* to determine the resistance status among biotypes (Fig. 1). The plants grown up to the 5-leaf-stage were sprayed with a range of cyhalofop-butyl (ACCaseinhibitor, Clincher[®], EC, 250.0 g active ingredient (a.i.) ha⁻¹, FarmHannong Co. Ltd., Korea) doses (0, 31.3, 62.5, 125.0, 250.0 and 500.0 g a.i. ha⁻¹) using a compressor-pressurized belt-driven sprayer (R & D Sprayer, USA) equipped with an 8002E flat-fan nozzle (Spraying System Co., USA) to deliver a spray volume of 600 L ha⁻¹. All treatments were replicated three times and arranged in a completely randomized design. Above-ground fresh weight was assessed at 30 DAT.

Growth Pouch Test

To detect cyhalofop-butyl resistance in the three biotypes at the seed germination stage (Fig. 1), the growth pouch test was applied (Zhang et al. 2015). In the case of the selection of cyhalofop-butyl concentration range, initially a wide range of herbicide concentrations (0-204.8 mg a.i. L⁻¹) was tested with reference susceptible Suwon biotype. The dose-responses results showed no significant difference ($P < 0.05$) in the inhibition of root growth at 0.05-0.1 mg a.i. L⁻¹ concentration range, but high inhibition of root growth at 102.4-204.8 mg a.i. L⁻¹ range. At the 0.2-51.2 mg a.i. L⁻¹ concentration range, an appropriate dose-response was found in root growth. Therefore, 0.2-51.2 mg

a.i. L^{-1} concentration range was selected for growth pouch test. The seeds of three *E. crus-galli* biotypes were firstly germinated in petri dishes in the incubation room maintained at 33/25°C (day/night), and pre-germinated seeds were placed on the paper wick of an 18 cm × 16.5 cm growth pouch containing 5 mL of cyhalofop-butyl solutions at a range of cyhalofop-butyl concentrations (0, 0.2, 0.8, 3.2, 12.8 and 51.2 mg a.i. L^{-1}). The growth pouches were then kept in the incubation room at 33/25°C (day/night) with supplementary light. Dark condition was maintained for the roots by placing the growth pouches in a top-open paper box. Deionized water (2 mL) was added to the growth pouches daily to compensate for evaporation. The root length and plant fresh weight were measured at 6 DAT. The test consisted of three replicates in a completely randomized block design.

Trimmed Seedlings Test

Each biotype of Seosan-3, Seosan-5 and Suwon grown in pots was sampled at four different growth stages: 1) 4 leaf-1 tiller stage, 2) 1-2 tillers stage, 3) 2-3 tillers stage and 4) 3-4 tillers stage. Soil was washed off from the roots of the sampled plants, and the plants were further kept 24 h in water for plant recovery due to root damage. Before herbicide treatment, the sampled plants were trimmed to an approximately 9-cm shoot length and a 5-cm root length (Fig. 1). The same procedure for the selection of cyhalofop-butyl concentration range was conducted in trimmed seedlings test as did in the growth pouch test. For *E. crus-galli* tested at the 4 leaf-1 tiller stage and 1-2 and 2-3

tillers stages, trimmed shoots and roots were placed in 60-mL glass bottles (approximately 8-cm in height) containing a range of concentrations of cyhalofop-butyl (0, 0.8, 3.2, 12.8 and 51.2 mg a.i. L⁻¹). In the case of plants at the 3-4 tillers stage, treated doses were adjusted to the rates of 0, 0.8, 1.6, 3.2, 6.4 and 12.8 mg a.i. L⁻¹ due to its high sensitivity at 12.8-51.2 mg a.i. L⁻¹ cyhalofop-butyl concentration range. Bottles were then kept in an incubation room at 33/25°C (day/night) with supplementary light and were topped off with the same amount of deionized water (5 mL) every 2 days. The longest shoot extension and all of the new shoot fresh weights were measured after 7 DAT. The test consisted of three replicates in a randomized complete block design.

Trimmed Stem Node Test

The trimmed stem nodes of each biotype were used to detect cyhalofop-butyl resistance in *E. crus-galli* at the heading stage (Fig. 1). *Echinochloa crus-galli* tillers were separated from the main stem without removing leaf sheath and trimmed to approximately 10-cm in length including 1 node. To stimulate rooting, the trimmed stem node segments were completely soaked in water for 24 h, followed by a further 48 h dark treatment. The same procedure for the selection of discriminating cyhalofop-butyl concentration range was used as described for growth pouch and trimmed seedlings tests. The rooted stem nodes were then placed in 60-mL glass bottles containing a range of cyhalofop-butyl concentrations (0, 0.4, 1.6, 6.4 and 25.6 mg a.i. L⁻¹) and were kept in the incubation room at 33/25°C (day/night) with supplementary light. The newly

extended root length was calculated by measuring the longest root compared to that of the original root length. The fresh weight of the newly extended root was measured accordingly as well after 7 DAT. The test consisted of three replicates in a randomized complete block design.

Statistical Analyses

Initially, all data were subjected to analysis of variance (ANOVA). As the ANOVA showed significant herbicide dose treatment effects ($P < 0.05$), the data were then processed by non-linear regression analysis. The three-parameter log-logistic dose-response model (Streibig 1980) was used to fit the data for estimating the GR_{50} , a dose rate required for 50% reduction of plant growth with respect to the untreated control.

$$y = \frac{c}{1 + \left(\frac{x}{GR_{50}}\right)^b} \quad [1]$$

where y is the shoot (root) length or fresh weight expressed as a percentage of the untreated control, c is the upper limit, and b is the slope of the curve through GR_{50} . The R/S ratio was computed as GR_{50} (tested biotypes)/ GR_{50} (susceptible reference biotype). GR_{50} values from each tested stage for the three biotypes were considered to be statistically different when their respective R/S ratio differed from 1.0 at $\alpha=0.05$. Statistical difference in R/S ratios for each tested stage was compared using Fisher's protected LSD test at $\alpha=0.05$. In all tests, there was no evidence of a lack of fit of the

chosen model. The residual mean squares and R^2 were used to indicate the goodness of fit to the log-logistic dose-response model. All statistical analyses were conducted using Genstat 5 (Genstat Committee 1997).

RESULTS

Whole Plant Test

For the three biotypes (Seosan-3, Seosan-5 and Suwon) of *E. crus-galli* sprayed with cyhalofop-butyl, shoot fresh weight measured at 30 DAT was significantly reduced with increasing doses ($P < 0.05$) and well described by the log-logistic dose-response model ($R^2 \geq 0.988$; $P < 0.001$), allowing to estimate GR_{50} values for those three biotypes (Fig. 2). The Seosan-5 biotype showed the most resistance to cyhalofop-butyl compared with the other two biotypes, Seosan-3 and Suwon (reference). By comparing the GR_{50} values among the three biotypes, Seosan-5 (55.8 g a.i. ha⁻¹) was confirmed to be 3.6 times more resistant to cyhalofop-butyl than the susceptible reference Suwon biotype (15.2 g a.i. ha⁻¹). The GR_{50} value of Seosan-3 (16.8 g a.i. ha⁻¹) was similar to that of Suwon biotype (Table 1).

Diagnosis of Resistance at the Seed Germination Stage

The root length of those three biotypes was significantly ($P < 0.05$) reduced with increasing concentrations of cyhalofop-butyl (Fig. 3), but the case is not for fresh weight of seedlings (data not shown). A goodness of fit between root length data and the

log-logistic model was observed with $R^2 \geq 0.952$ and $P < 0.001$ (Fig. 3, Table 2). The GR_{50} values of resistant and susceptible *E. crus-galli* to cyhalofop-butyl in root length were 3.04, 1.01 and 0.89 mg a.i. L^{-1} for the Seosan-5, Seosan-3 and Suwon biotypes, respectively, resulting in an R/S ratio of 3.0 between the resistant Seosan-5 biotype and the susceptible reference Suwon biotype (Fig. 3, Table 2). Similar discrimination results between the growth pouch test (R/S ratio, 3.0) and whole plant test (R/S ratio, 3.6) validated that the growth pouch test can be applied to detect cyhalofop-butyl resistance in *E. crus-galli* by measuring the root length. However, the fresh weights of those three biotypes were not fitted to the model (data not shown), which therefore made it impossible to estimate the GR_{50} , indicating that fresh weight was not an appropriate parameter for detecting cyhalofop-butyl resistance in *E. crus-galli* at the germination stage using the growth pouch test.

Diagnosis of Resistance at the Juvenile Stage

For plants at the juvenile stage, from 4 leaf to 4 tiller stages, the growth statuses of resistant and susceptible Seosan-3, Seosan-5 and Suwon biotypes were all significantly ($P < 0.05$) affected by cyhalofop-butyl, though the resistant biotype (Seosan-5) showed the greatest tolerance to cyhalofop-butyl when compared with the Seosan-3 and susceptible reference Suwon biotypes (Figs. 4, 5). As shown in Tables 2 and 3, the log-logistic dose-response model provided an acceptable fit to both data of new shoot length ($R^2 \geq 0.926$; $P < 0.001$) and shoot fresh weight ($R^2 \geq 0.891$; $P < 0.001$), allowing to

estimate the GR_{50} for each case. For the test of *E. crus-galli* at the 4 leaf-1 tiller stage with cyhalofop-butyl, the GR_{50} values of Seosan-5 for its new shoot length and shoot fresh weight were 3.76 and 1.74 mg a.i. L^{-1} , respectively, whereas those of Seosan-3 and the susceptible reference Suwon biotypes were 1.51 and 1.50 mg a.i. L^{-1} for their new shoot lengths and 0.49 and 0.46 mg a.i. L^{-1} for shoot fresh weight, respectively. The R/S ratios of Seosan-5 in terms of new shoot length and shoot fresh weight were 2.5 and 3.6 times compared to the susceptible reference Suwon biotype. Seosan-3 was also susceptible to cyhalofop-butyl both in terms of its new shoot length and shoot fresh weight, similar to the reference Suwon biotype (Figs. 4A, 5A, Tables 2, 3).

A test of *E. crus-galli* at the 1-2 tillers stage showed that the GR_{50} values of Seosan-5 for its new shoot length and shoot fresh weight were 1.10 and 3.27 mg a.i. L^{-1} , respectively. The GR_{50} values of Seosan-3 and the susceptible reference Suwon biotype both for their new shoot length and shoot fresh weight were substantially lower than those for the resistant biotype (Tables 2, 3), whereas the GR_{50} values of Seosan-3 and the susceptible reference Suwon biotypes were 0.48 and 0.48 mg a.i. L^{-1} for their new shoot length and 1.14 and 1.11 mg a.i. L^{-1} for their shoot fresh weight, respectively, showing similar susceptibility to cyhalofop-butyl. The R/S ratios of Seosan-5 for its new shoot length and shoot fresh weight were 2.3 and 2.9 times that of the reference Suwon biotype, respectively (Figs. 4B, 5B, Tables 2, 3).

A test of *E. crus-galli* at the 2-3 tiller stage revealed that the GR_{50} values of Seosan-5 for its new shoot length and shoot fresh weight were 0.61 and 0.29 mg a.i. L^{-1} ,

respectively. In comparison, the GR_{50} values of Seosan-3 and Suwon biotypes were 0.20 and 0.22 mg a.i. L^{-1} for their new shoot length and 0.06 and 0.08 mg a.i. L^{-1} for their shoot fresh weight, respectively. The R/S ratios of Seosan-5 for its new shoot length and shoot fresh weight were 3.1 and 4.8 times greater than that of the reference Suwon biotype, respectively (Figs. 4C, 5C, Tables 2, 3).

A test of *E. crus-galli* at the 3-4 tiller stage showed that the GR_{50} values of Seosan-5 for its new shoot length and shoot fresh weight were 3.05 and 2.67 mg a.i. L^{-1} , respectively. Nevertheless, the GR_{50} values of Seosan-3 and Suwon were 1.57 and 1.61 mg a.i. L^{-1} for their new shoot length and 1.34 and 1.36 mg a.i. L^{-1} for their shoot fresh weight, respectively. The R/S ratios of Seosan-5 for its new shoot length and shoot fresh weight were 1.9 and 2.0 times greater than that of the reference Suwon biotype, respectively (Figs. 4D, 5D, Tables 2, 3).

The similarity in R/S ratios between these diagnostic (R/S ratios, ranging from 1.9-3.1 for new shoot length and 2.0-4.8 for new shoot fresh weight) and whole plant tests (R/S ratio, 3.6) indicates the suitability of these tests for detecting cyhalofop-butyl resistance in *E. crus-galli* at the juvenile stage (Tables 1-3).

Diagnosis of Resistance at the Heading Stage

Newly regrown *E. crus-galli* root lengths were significantly ($P < 0.05$) reduced by increasing concentrations of cyhalofop-butyl (Fig. 1), although newly regrown roots from the resistant biotype were much longer than those from the susceptible biotype

(Fig. 6A). The GR_{50} values of Seosan-5, Seosan-3 and Suwon for their new root extensions were 0.57, 0.17 and 0.14 mg a.i. L^{-1} , respectively. The R/S ratio of Seosan-5 for its new root extension was 4.1 times greater than that of the reference Suwon biotype (Fig. 6A, Table 2). The GR_{50} values of fresh weight were 8.65, 3.36 and 3.70 mg a.i. L^{-1} for the Seosan-5, Seosan-3 and Suwon biotypes, respectively. The R/S ratio of Seosan-5 was 2.6 times greater than that of the reference Suwon biotype (Fig. 6B, Table 3). Seosan-3 showed a similar susceptibility to cyhalofop-butyl both in terms of its new root length and root fresh weight compared to the Suwon biotypes (Fig. 6, Tables 2, 3). The similarity in R/S ratios between the stem node (R/S ratio, 4.1 in their new root extension and 2.6 in their fresh weight) and whole plant tests (R/S ratio, 3.6) also demonstrated that the stem node test can be applied to detect cyhalofop-butyl resistance in *E. crus-galli* at the heading stage (Tables 1-3).

DISCUSSION

Developing a herbicide resistance diagnostic method is necessary for evaluating the occurrence of resistance and management during the chemical control of weeds (Zhang et al. 2015). Generally, the whole plant test is widely used and involves growing plants from seeds collected from the suspect field and spraying them with herbicides applied either at a single discriminating dose, or a range of doses (HRAC 2009). Evaluations are usually made based on visual assessments of mortality, or measurements of fresh or dry weight of foliage. However, due to its intensive labor and considerable space requiring,

and time-consuming for resistance confirmation, it is still restricted to a specific set of circumstances. Although other more sophisticated diagnostic methods such as enzyme activities and DNA sequencing assays (Kaundun and Windass 2006; Delye et al. 2009) have been developed, due to the cost, technicality, feasibility and facility limitations, these methods are also difficult in practical application. Therefore, there is a need for developing novel herbicide resistance diagnostic methods with more rapid and reliable results, more convenient application and lower costs, etc. (Moss 1995).

The plant cutting-related herbicide diagnostic method is based on the ability of plants to readily regenerate new shoots from root or stem tissues following damage to the plants (Anderson et al. 2001). In practice, such vegetative regeneration produces a special problem for weed control resulting in herbicide failure. However, this regeneration-based method has been used for the diagnosis of herbicide resistance in several weed species. Growing tillers in herbicide media has been reported for the rapid diagnosis of herbicide resistance by Letouzé et al. (1997). Using trimmed tillers and stems, Kim et al. (2000) detected fenoxaprop- and propanil-resistant *E. colona* from the seedling to flowering stages. Recently, an assay that entailed growing *Lolium multiflorum* tillers in glyphosate solutions also showed the discrimination between glyphosate susceptible and resistant plants (Ghanizadeh et al. 2015). Moreover, regenerating cuttings by growing them in soil and following herbicide application was successfully used to identify the resistance of *Alopecurus myosuroides* or sulfonyleurea-resistant biotype weeds (Boutsalis 2001; Shuji et al. 2004). Moon et al.

(2009) diagnosed cyhalofop-butyl resistant *E. crus-galli* by observing the different growth responses between resistant and susceptible biotypes from cutting parts after 24 h herbicide treatment. All of these examples demonstrate that the plant cutting-based herbicide diagnostic method has great potential to detect herbicide resistance, regardless of the type of inhibitor resistance or weed species.

The highlight of the present study was the extension of the trimmed plant method to detect cyhalofop-butyl resistance in *E. crus-galli*, which currently is the most troublesome weed in Korean paddy fields. Additionally, with the application of the growth pouch test to detect resistance at the seed germination stage, the tests presented herein are robust and enabled to detect cyhalofop-butyl resistance in *E. crus-galli* at various growth stages covering the whole life cycle of plants (from seed germination to the heading stage).

For the detection of resistance at the seed germination stage, the petri dish assay (also known as seed-bioassay) with agar incorporating herbicide concentrations was normally used (Moss 1990; Murray et al. 1996). Based on the plant growth response (root or shoot) in herbicide media, the diagnostic process could be done within one week. Tal et al. (2000) reported a seed-bioassay to detect grass weeds resistant to ACCase inhibitor herbicides within 7 days. An agar-based Syngenta quick test has also been developed to detect glyphosate resistance in grass and broadleaf weeds species within two weeks (Kaundun et al. 2014). Although the seed-bioassay provided an effective and early-season resistant diagnosis in a short period of time, it has been difficult to exclude

ungerminated seeds due to dormancy, resulting in an underestimation of the germination rate. A previous study showed that *E. crus-galli* seeds had variation in germination rate in terms of age and storage conditions, indicating that the seed soaking bioassays may not be appropriate for diagnosing herbicide resistance in the species, such as *E. crus-galli*, with large physiological and morphological variability (Martinkova et al. 2006; Matzenbacher et al. 2013). Additionally, for root length measurements, because substrates incorporating agar restrict and obstruct root development, the results were unreliable and unconvincing. By contrast, the growth pouch test, root growth response to herbicides via a transparent growth pouch system, provided a reliable and accurate way to discriminate between resistant and susceptible *E. crus-galli* biotypes. The previous study using growth pouch method also reported diagnosis of ALS inhibitor resistance in *E. oryzicola* (Zhang et al. 2015). The discrimination using growth pouch test could be done within one week, which provided a quick method to detect herbicide resistance in *Echinochloa* spp. with accurate and consistent results, practical simplicity and lower costs compared with the whole plant test. This study suggested that the discriminatory cyhalofop-butyl concentration range of 12.8-51.2 mg a.i. L⁻¹, which is similar to the previous study of 25.0-50.0 mg a.i. L⁻¹ (Zhang et al. 2015), was effective to discriminate cyhalofop-butyl resistance in *E. crus-galli* at seed germination stage.

The juvenile stage test applied here could complement the detection of *E. crus-galli* resistance until a later growth stage. In similar studies of seedlings tests conducted for other species and herbicides, the successful discrimination between resistant and

susceptible biotypes was also reported. In studies with *Lolium* species, the resistance to glyphosate evaluated with seedlings grown in agar containing herbicide could be identified within two weeks (Kaundun et al. 2014). An assay for discrimination of glyphosate resistance in *Lolium multiflorum* was developed using tillers with roots (Ghanizadeh et al. 2015). Moreover, the resistance of *E. crus-galli* to imidazolinones was effectively discriminated using seedlings test after incubation at the herbicide concentrations (Matzenbacher et al. 2013). All these studies demonstrated the effectiveness of seedlings test for the rapid diagnosis of herbicide resistance in weed species. In present study, four different *E. crus-galli* stages of growth within the juvenile stage (4 leaf-1 tiller, 1-2 tillers, 2-3 tillers and 3-4 tillers stages) involve in the herbicide resistance discrimination based on the comparison of shoot extension length or fresh weight between resistant and susceptible biotypes, which represent a wider range of juvenile plant growth stages compared with 4 leaf-1 tiller and 2-3 tillers tests in a similar study conducted by Kim et al. (2000). Hence, this study would be more informative in terms of herbicide resistance in *E. crus-galli* at a specific juvenile growth stage. In practical applications, if *E. crus-galli* control failure occurs in the field throughout the season and the suspect sample is collected from the field for detection, timely results would be directed to the farmers within several days for advising them to use another herbicide with different modes of action or to respray the herbicide if the sample is confirmed to be susceptible. This study suggested that the discriminatory cyhalofop-butyl concentration range of 0.8-3.2 mg a.i. L⁻¹ was effective to discriminate

cyhalofop-butyl resistance in *E. crus-galli*. It should be noted that due to the difference in responses of herbicide concentrations at different growth stage of juvenile plants, the discriminatory concentration may need adjustment in practice. The trimmed stem node test further provided more feasible and convenient options for detecting herbicide resistance in *E. crus-galli* even at the heading stage from the fields where herbicide performance has been shown to be poor or a failure. Timely test results advise farmers to shift herbicides or work out weed management decisions for the following season. Kim et al. (2000) reported that propanil resistance in *E. colona* could be discriminated using the rooted stem. Evaluation was made according to measuring regrown shoot length from the rooted stem. However, the preliminary work with this technique in this study was not successful because there was no further shoot growth from the rooted stem found, which may be due to the differences in the species (physiological characters) or herbicides tested (modes of action). Interestingly, a satisfactory herbicide dose-response was found that with increasing herbicide concentration, the root length from rooted stem node was decreasing (Fig. 6A). Therefore, in this study, measuring the length of root from rooted stem node was adopted for discrimination of cyhalofop-butyl resistance in *E. crus-galli*. Additionally, the results on evaluation of new root fresh weight (Fig. 6B; Table 3) also provided another option for cyhalofop-butyl resistance discrimination in *E. crus-galli*. This study suggested the discriminatory cyhalofop-butyl concentration range of 0.4-1.6 mg a.i. L⁻¹ was effective to discriminate cyhalofop-butyl resistance in *E. crus-galli*. All diagnostic data obtained from whole plant and various

diagnostic tests demonstrated that these diagnostic tests could be applied to detect cyhalofop-butyl resistance in *E. crus-galli*.

In summary, the trimmed seedling and stem node tests together with the growth pouch test yielded reliable and consistent diagnostic results compared with the whole plant test, providing a quick and practical diagnosis for cyhalofop-butyl resistance in *E. crus-galli* at various growth stages throughout the season. In addition, the various applied tests required a short period (within a week) to discriminate herbicide resistance in *E. crus-galli* and therefore significantly shorten the diagnostic period compared to conventional whole plant tests (approximately 4 weeks). The present studies coupled with the previous successful discrimination of fenoxaprop-ethyl and propanil resistant-*E. colona* (Kim et al. 2000), imidazolinone-resistant *E. crus-galli* (Matzenbacher et al. 2013) and glyphosate-resistant *L. multiflorum* (Ghanizadeh et al. 2015), a conclusion can be drawn that the trimmed plants tests are capable of detecting resistance regardless of herbicides with different modes of action. The growth pouch test, in addition to identifying ACCase and ALS-inhibitors resistance, also has the potential for detecting other types of herbicide resistance currently evolving in *Echinochloa* (Zhang et al. 2015).

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Table 1. Summary of parameters estimated from the log-logistic model for shoot fresh weight of resistant (Seosan-5) and susceptible (Seosan-3 and Suwon) biotypes of *E. crus-galli* to cyhalofop-butyl tested at the 5 leaf stage in the whole plant test.

Biotypes	Parameters			R/S ^b	R ²	RMS ^c	P values
	<i>b</i>	<i>d</i>	GR ₅₀ ^a (g a.i. ha ⁻¹)				
Seosan-5	1.42 (0.10) ^d	100.3 (2.27)	55.8 (3.14)	3.6 A ^e	0.988	15.72	<0.001
Seosan-3	1.4 (0.21)	99.9 (2.16)	16.8 (2.44)	1.1 B	0.992	13.98	<0.001
Suwon	1.44 (0.19)	100.0 (1.73)	15.2 (2.12)	1.0 B	0.995	9.02	<0.001

^a GR₅₀ represents the dose required to inhibit the growth of *Echinochloa* by 50%.

^b R/S ratios were calculated by dividing GR₅₀ values of Seosan-5 and Seosan-3 biotypes to that of the Suwon biotype.

^c RMS represents the residual mean square.

^d Values in parentheses in each column are the standard errors.

^e Different letters within a column indicated statistical differences according to Fisher's protected LSD test at $\alpha=0.05$.

Table 2. Summary of parameters estimated from the log-logistic model for shoot (or root) length of resistant (Seosan-5) and susceptible (Seosan-3 and Suwon) biotypes of *E. crus-galli* to cyhalofop-butyl tested at various growth stages with different testing methods.

Testing methods	Growth stages	Biotypes	Growth response	Parameters			R/S ^b	R ²	RMS ^c	P values
				<i>b</i>	<i>d</i>	GR ₅₀ ^a (mg a.i. L ⁻¹)				
Growth pouch method	Germination stage	Seosan-5	Root length	0.53 (0.06) ^d	100.9 (3.6)	3.04 (0.68)	3.0 A ^e	0.952	42.23	<0.001
		Seosan-3		0.52 (0.05)	98.8 (3.5)	1.01 (0.23)	1.1 B	0.963	38.07	<0.001
		Suwon		0.52 (0.03)	99.6 (2.2)	0.89 (0.12)	1.0 B	0.986	14.68	<0.001
Trimmed seedling methods	4 leaf-1 tiller stage	Seosan-5	New shoot length	0.78 (0.11)	99.9 (4.45)	3.76 (0.78)	2.5 A	0.954	61.38	<0.001
		Seosan-3		0.80 (0.09)	100.3 (3.18)	1.51 (0.22)	1.0 B	0.979	30.48	<0.001
		Suwon		0.86 (0.09)	100.4 (3.27)	1.50 (0.21)	1.0 B	0.978	31.32	<0.001
	1-2 tiller stage	Seosan-5	New shoot length	0.50 (0.05)	99.8 (2.96)	1.10 (0.24)	2.3 A	0.978	26.26	<0.001
		Seosan-3		0.47 (0.05)	99.9 (2.33)	0.48 (0.11)	1.0 B	0.988	16.28	<0.001
		Suwon		0.46 (0.04)	99.9 (2.30)	0.48 (0.12)	1.0 B	0.988	15.82	<0.001
	2-3 tiller stage	Seosan-5	New shoot length	0.72 (0.06)	100.1 (2.15)	0.61 (0.08)	3.1 A	0.991	13.88	<0.001
		Seosan-3		0.53 (0.06)	100.0 (2.09)	0.20 (0.03)	0.9 B	0.992	13.12	<0.001
		Suwon		0.59 (0.07)	100.0 (2.02)	0.22 (0.06)	1.0 B	0.992	12.21	<0.001
	3-4 tiller stage	Seosan-5	New shoot length	1.32 (0.17)	98.1 (4.47)	3.05 (0.37)	1.9 A	0.947	65.81	<0.001
		Seosan-3		1.12 (0.08)	99.6 (2.70)	1.57 (0.12)	1.0 B	0.981	22.18	<0.001
		Suwon		1.14 (0.18)	99.1 (5.54)	1.61 (0.30)	1.0 B	0.926	93.86	<0.001
Stem node method	Heading stage	Seosan-5	New root length	0.53 (0.05)	100.3 (2.73)	0.57 (0.11)	4.1 A	0.981	22.45	<0.001
		Seosan-3		0.45 (0.04)	100.1 (1.66)	0.17 (0.04)	1.2 B	0.994	8.23	<0.001
		Suwon		0.44 (0.03)	100.1 (1.38)	0.14 (0.02)	1.0 B	0.996	5.68	<0.001

^a GR₅₀ represents the dose required to inhibit the growth of *Echinochloa* by 50%.
^b R/S ratios were calculated by dividing GR₅₀ values of Seosan-5 and Seosan-3 biotypes by that of the Suwon biotype.
^c RMS represents the residual mean square.
^d Values in parentheses in each column are the standard errors of GR₅₀.
^e Different letters within a column indicated statistical differences according to Fisher's protected LSD test at α=0.05.

Table 3. Summary of parameters estimated from the log-logistic model for shoot (or root) fresh weight of resistant (Seosan-5) and susceptible (Seosan-3 and Suwon) biotypes of *E. crus-galli* to cyhalofop-butyl tested at various growth stages with different testing methods.

Testing methods	Growth stages	Biotypes	Plant response		Parameters			R/S ^b	R ²	RMS ^c	P values
					b	d	GR ₅₀ ^a (mg a.i. L ⁻¹)				
Trimmed seedling methods	4 leaf-1 tiller stage	Seosan-5	New shoot	fresh	0.76 (0.18) ^d	100.0 (7.54)	1.74 (0.63)	3.6 A ^e	0.891	172.0	<0.001
		Seosan-3	weight		0.55 (0.11)	99.9 (4.28)	0.49 (0.19)	1.1 B	0.962	55.1	<0.001
		Suwon			0.56 (0.11)	100.0 (4.10)	0.46 (0.17)	1.0 B	0.966	50.4	<0.001
	1-2 tiller stage	Seosan-5	New shoot	fresh	0.57 (0.11)	99.1 (6.06)	3.27 (1.17)	2.9 A	0.908	111.3	<0.001
		Seosan-3	weight		0.64 (0.09)	99.9 (4.18)	1.14 (0.28)	1.0 B	0.962	52.61	<0.001
		Suwon			0.54 (0.09)	100.2 (4.56)	1.11 (0.35)	1.0 B	0.951	62.64	<0.001
	2-3 tiller stage	Seosan-5	New shoot	fresh	0.61 (0.08)	99.6 (2.31)	0.29 (0.07)	4.8 A	0.991	15.98	<0.001
		Seosan-3	weight		0.60 (0.06)	100.0 (0.91)	0.06 (0.02)	0.8 B	0.999	2.52	<0.001
		Suwon			0.51 (0.13)	100.1 (3.24)	0.08 (0.07)	1.0 B	0.981	31.58	<0.001
	3-4 tiller stage	Seosan-5	New shoot	fresh	1.63 (0.26)	97.6 (5.26)	2.67 (0.34)	2.0 A	0.939	94.65	<0.001
		Seosan-3	weight		1.46 (0.21)	98.8 (4.75)	1.34 (0.16)	1.0 B	0.955	68.51	<0.001
		Suwon			1.31 (0.23)	99.3 (5.97)	1.36 (0.21)	1.0 B	0.927	108.2	<0.001
Stem node method	Heading stage	Seosan-5	New root	fresh	0.54 (0.12)	97.1 (5.71)	8.65 (3.26)	2.6 A	0.926	109.2	<0.001
		Seosan-3	weight		0.40 (0.13)	98.5 (4.50)	3.36 (1.24)	0.9 B	0.952	64.42	<0.001
		Suwon			0.46 (0.08)	98.6 (5.12)	3.70 (1.35)	1.0 B	0.944	86.25	<0.001

^a GR₅₀ represents the dose required to inhibit the growth of *Echinochloa* by 50%.

^b R/S ratios were calculated by dividing GR₅₀ values of Seosan-5 and Seosan-3 biotypes by that of the Suwon biotype.

^c RMS represents the residual mean square.

^d Values in parentheses in each column are the standard errors of GR₅₀.

^e Different letters within a column indicated statistical differences according to Fisher's protected LSD test at $\alpha=0.05$.

Figures Legends

Fig. 1. Application of various diagnostic tests for diagnosing cyhalofop-butyl resistance in *E. crus-galli* at various stages of growth.

Fig. 2. Dose responses in shoot fresh weight of *E. crus-galli* (Seosan-5 (▼), Seosan-3 (▲), and Suwon (●)) to cyhalofop-butyl using the whole plant test.

Fig. 3. Dose responses in root length of *E. crus-galli* (Seosan-5 (▼), Seosan-3 (▲), and Suwon (●)) to cyhalofop-butyl tested at the seed germination stage using the growth pouch test.

Fig. 4. Dose responses in new shoot length of *E. crus-galli* (Seosan-5 (▼), Seosan-3 (▲), and Suwon (●)) to cyhalofop-butyl tested at the 4 leaf-1 tiller stage (A), 1-2 tillers stage (B), 2-3 tillers stage (C), and 3-4 tillers stage (D) using the trimmed seedling method.

Fig. 5. Dose responses in new shoot fresh weight of *E. crus-galli* (Seosan-5 (▼), Seosan-3 (▲), and Suwon (●)) to cyhalofop-butyl tested at the 4 leaf-1 tiller stage (A), 1-2 tillers stage (B), 2-3 tillers stage (C), and 3-4 tillers stage (D) using the trimmed seedling method.

Fig. 6. Dose responses in new root length (A) and fresh weight (B) of *E. crus-galli* (Seosan-5 (▼), Seosan-3 (▲), and Suwon (●)) to cyhalofop-butyl tested at the heading stage using the trimmed stem node method.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.