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Measuring chlorophyll fluorescence parameters for rapid detection of ametryn resistant junglerice [*Echinochloa colona* (L.) Link.]

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Abstract

Measuring chlorophyll fluorescence is a rapid and noninvasive technique for assaying photosynthetic apparatus status in plants. Chlorophyll fluorescence measurements were performed in greenhouse to characterize how the fluorescence induction curve (Kautsky curve) and its parameters were affected by ametryn (a PSII inhibiting herbicide) in R- and S-junglerice biotypes. The experiment was conducted in a completely randomized design with four replications. The maximum quantum efficiency of PSII photochemistry (F_v/F_m), the relative changes at J step (F_{vj}) and the area between the Kautsky curve and maximum fluorescence (F_m) (Area) of S-biotype decreased dramatically at much lower doses (100 g ai ha^{-1}) than in the R-biotype at 4 HAS. The R-biotype showed decrease in the fluorescence parameters only at high concentration of ametryn at the same time. There were positive significant correlation between F_{vj} taken at 4 HAS and fresh weight taken 28 DAS of the R- and S-biotypes as 0.71 and 0.86, respectively ($p \leq 0.01$). The chlorophyll fluorescence parameters are suitable and practical indicators for monitoring photosynthesis inhibition; also, they could be useful in detecting biotypes showing resistance to PSII-inhibiting herbicides.

Keywords: Kautsky curve; photosynthesis, PS II-inhibiting herbicides.
Abbreviations: Ametryn- N^2-ethyl-N^4-isopropyl-6-methylthio-1,3,5-triazine-2,4-diamine; DAP- Day after planting; DAS- Day after spraying; DAWE- Day after weed emergence; F_m- Maximum fluorescence; F_0- Minimum fluorescence; F_v- Variable fluorescence; F_{vj}- Variable fluorescence at J step; F_v/F_m- Maximum quantum efficiency of photosystem II photochemistry; HAS- Hour after spraying; PSII- Photosystem II; R- Resistant biotype; S- Susceptible biotype.

Introduction

Since the first report in 1970 on triazine-resistant weed, common groundsel (*Senecio vulgaris* L.), herbicide resistance has been reported to most herbicide classes among 210 Weed species (Heap, 2013; Ryan, 1970). Herbicide resistance results from the continuous selection for resistant individuals in field populations. Gradually, the numbers of resistant plants were increased and finally dominated in the population (Devine and Shukla, 2000). Triazine resistance occurred because of an alteration in the herbicide action. In oxygenic photosynthetic organisms, PSII, is one of the two large pigment–protein complexes, carry outs the light-driven water oxidation, leading to the evolution of proton and dioxygen (Kato et al., 2012). Ametryn, a triazine herbicide, is widely used for pre- and post-emergence control of broadleaf and some grass weeds in sugar cane (*Saccharum officinarum* L.) in Iran. It is systemic, xylem-translocated SPII inhibitor, mainly absorbed by both leaves and roots (Monaco et al., 2002). Its mode of action is through inhibiting photosynthesis in susceptible plants by blocking electron transfer from Q_A to Q_B through PSII, causing light energy to be dissipated as fluorescence (Norsworthy et al., 1998; Eleftherohorinos et al., 2000). Chlorophyll fluorescence can be used as an indirect measure of the penetration and detoxification of herbicides that inhibit PSII electron transport (Norsworthy et al., 1999). It has also been applied to study the recovery of crop and weed from herbicide treatment (Norsworthy et al., 1999; Abbaspoor and Streibig, 2007). Also, it could be applicable to determine if a compound has phytotoxic interactions (synergistic, antagonistic, or additive) toward a particular species when mixed with a photosynthetic inhibitor (Norsworthy et al., 1999). Finally, researcher would able to realize herbicide efficacy soon after treatment among biotypes in comparison untreated control plants. By measuring photosynthesis-related parameters we would realize herbicide efficacy before the visual symptoms appear in plants (Riethmuller-Haage et al., 2006). The analysis of the fluorescence induction curves are an important tool for quantifying herbicide effects (Klem et al., 2002). The advantage of this approach lies in its adaptability and the possibility of using a portable instrument with rapid collection of data and advanced software enabling statistical analysis (Klem et al., 2002). Chlorophyll fluorescence

Table 1. Summary of GR_{50} values for the fresh weight in dose-response of R- and S-junglerice biotypes at 4 WAS with ametryn.

Biotype	Lower limit	Upper limit	Slope	GR_{50} (g ai ha^{-1})	Lack of fit test (5%)	Resistance factor
R	-[a]	2.09 (± 0.06)	0.38 (± 0.04)	1799 (± 360.49)	0.61 (NS[b])	15.89
S	-	1.46 (± 0.05)	0.64 (± 0.07)	113.18 (± 16.94)	0.11 (NS)	

Data are means of two experiments ± SE, [a] Parameter not estimated. [b]NS: non-significant at the 5% level.

induction curve from a dark-adapted leaf consisted of steps that characterized by O, J, I and P (Abbaspoor and Streibig, 2007). The three phases are described as follows: the O-J phase which is the fluorescence level obtained when the PSII reaction centers are in the open state (capable of photochemistry since Q_A, the primary quinone acceptor of PSII, is in maximum oxidized state); the J-I phase is stage of the electron transfer from Q_A to Q_B; the I-P phase denotes to fluorescence quenching by the oxidized plastoquinone pool. Triazine herbicides inhibit photosynthesis in susceptible plants by blocking electron transfer from Q_A to Q_B. Therefore, it could be concluded that the J-I phase is an indicator of mode of action of triazine herbicide (Abbaspoor and Streibig, 2007; Baker and Rosenqvist, 2004). After immediate exposure to light, fluorescence rises to the minimal level of fluorescence, termed F_o level, which is equal to the O-J phase; The fluorescence then rises rapidly to reach a peak level, F_m (Baker and Rosenqvist, 2004). The chlorophyll F_0 (initial level of fluorescence) and F_m (maximum level of fluorescence) were used to calculate the F_V/F_m (difference between F_m and F_o is termed the variable fluorescence (F_v)) (Eleftherohorinos et al., 2000). Assumingly, the maximum quantum efficiency of PSII photochemistry (F_V/F_m) is more potent than other fluorescence parameters. Also, it could be used as a potential indicator to discern R- and S-biotypes in a rapid bioassay. A large number of researchers have used it for detecting resistant biotypes including common groundsel (*Senecio vulgaris* L.) (Ahrens et al., 1981), common ragweed (*Ambrosia artemisiifolia* L.) (Ahrens et al., 1981), barnyardgrass (*Echinochloa crus-galli* (L.) P. Beauv.) (Norsworthy et al., 1998 and 1999), *Amaranthus* spp. (Ahrens et al., 1981; Foes et al., 1998; Eleftherohorinos et al., 2000), common lambsquarters (*Chenopodium album* L.) (Ahrens et al., 1981; Eleftherohorinos et al., 2000), *Bromus* spp. (Ahrens et al., 1981; Park and Mallory-Smith, 2005; Menendez et al., 2007) and annual bluegrass (*Poa annua* L.) (Perry et al., 2012). Other popular parameters related to the chlorophyll fluorescence are the relative changes at the J step (F_{vj}) and the area between Kautsky curve and F_m (Area) (Christensen et al., 2003; Abbaspoor and streibig, 2005; Abbaspoor et al., 2006; Abbaspoor and streibig, 2007). The goals of the present study were to study ametryn effect on Kuatsky curve and detect the resistant and susceptible biotypes by chlorophyll fluorescence parameters.

Results and discussion

Dose-response studies

Parameters derived from dose response curves for R- and S-junglerice biotypes were described in Table 1. The ametryn rates required to reduce growth by 50% (GR_{50}) was less for the S-biotype than for the R-biotype. The GR_{50} values were 1799 and 113.18 for the R- and S-biotypes, respectively. The GR_{50} value of the R-biotype was 15.89 times more than for the S-biotype (Table 1). These results are in agreement with findings of Eleftherohorinos et al. (2000), Kim et al. (2000), Park and Mallory-Smith (2005) and Mallory-Smith et al. (2012).

Chlorophyll fluorescence studies

The Kautsky curves

Fig. 1 illustrates the shape of the Kautsky curves was affected significantly at 4 HAS at 100 and 1000 g ai ha^{-1} in the S-biotype, while these curves with distinctive OJIP steps were clearly observed in the R- biotype. Ametryn could severely affected the S-biotype and injuries were irreversible. Since JI phase corresponds to electron transfer from Q_A to Q_B and mode of action of ametryn in the S-plants is by blocking electron flow through PSII, causing its effect at J-I step. In addition, electron transportation from Q_A to Q_B at J-I step was affected from oxygen evolving complex (OEC); Therefore, exposing PSII by saturated light in dark adapted leaves resulting in flowing many electron toward PSII. Since the electron transport chain was damaged by PSII inhibiting herbicide; therefore, PSII increase chlorophyll fluorescence in order to vacate additional electrons (Stirbet and Govindjee, 2011). By increasing the dose of the ametryn OJIP steps of the Kautsky curve were omitted and turned in to straight lines in the S-biotypes (Fig. 1). Whilst, each step of the Kautsky curve that represents those photochemical events associated with PSII indicating no damage to the electron transfer chain in photosynthesis in the R-biotype (Fig. 1). These findings are in agreement with the response of white mustard (*Sinapis alba* L.) treated with bentazone, flurochloridone and glyphosate (Christensen et al., 2003), lambsquarters (*Chenopodium album* L.) treated with atrazine (Hiraki et al., 2003), wild mustard (*Sinapis arvensis* L.) treated with dicamba + 2, 4-D (Avarseji et al., 2012) and soybean (*Glycine max* (L.)) exposing with dark chilling (Van Heerden et al., 2003). Sahid et al. (2011) stated that propanil inhibited the quantum efficiency of the PSII in both the R- and S-biotypes after 2 h of incubation time. However, when the leaf disks were transferred and incubated in deionized water for 48 h, the quantum efficiency increased in the R-biotype but decreased in the S-biotype. In healthy dark adapted leaves all PSII centers were incomplete oxidation state. When a saturated light was glintted all PSII centers were completely reduced and maximum fluorescence at P step (F_m) were emitted in leaves. Fluorescence of treated leaves increase from the ground-state value (F_o) but it could not reach to its maximum value (F_m). Therefore, F_m was decreased and this reaction result in reduction in Area parameter. The chlorophyll fluorescence was decreased after P step (1000 ms) in the R-biotype while it was not occurred in the S-biotype. The lack of fluorescence decay after this time suggests PSII centers were damaged and PSII-mediated fluorescence quenching cannot be taken place (Fig. 1). In the presence of PSII inhibitors, excitation energy generated by p680 cannot be dissipated by normal electron flow further than Q^-_A, and so fluorescence yield is dramatically increased and activated oxygen species generated (Cobb and Reade, 2010). Finally, cell membranes and tissues disintegrate from electron transport chain reaction of free-radical attack (Cobb and Reade, 2010). This phenomenon was observed for many PSII inhibitors by a large number of researchers (Habash et al., 1985; Christensen et al., 2003; Hiraki et al., 2003; Van Heerden et al., 2003; Abbaspoor et al., 2006, Avarseji et al., 2012). Totally, changes in the shape of Kautsky curve in the

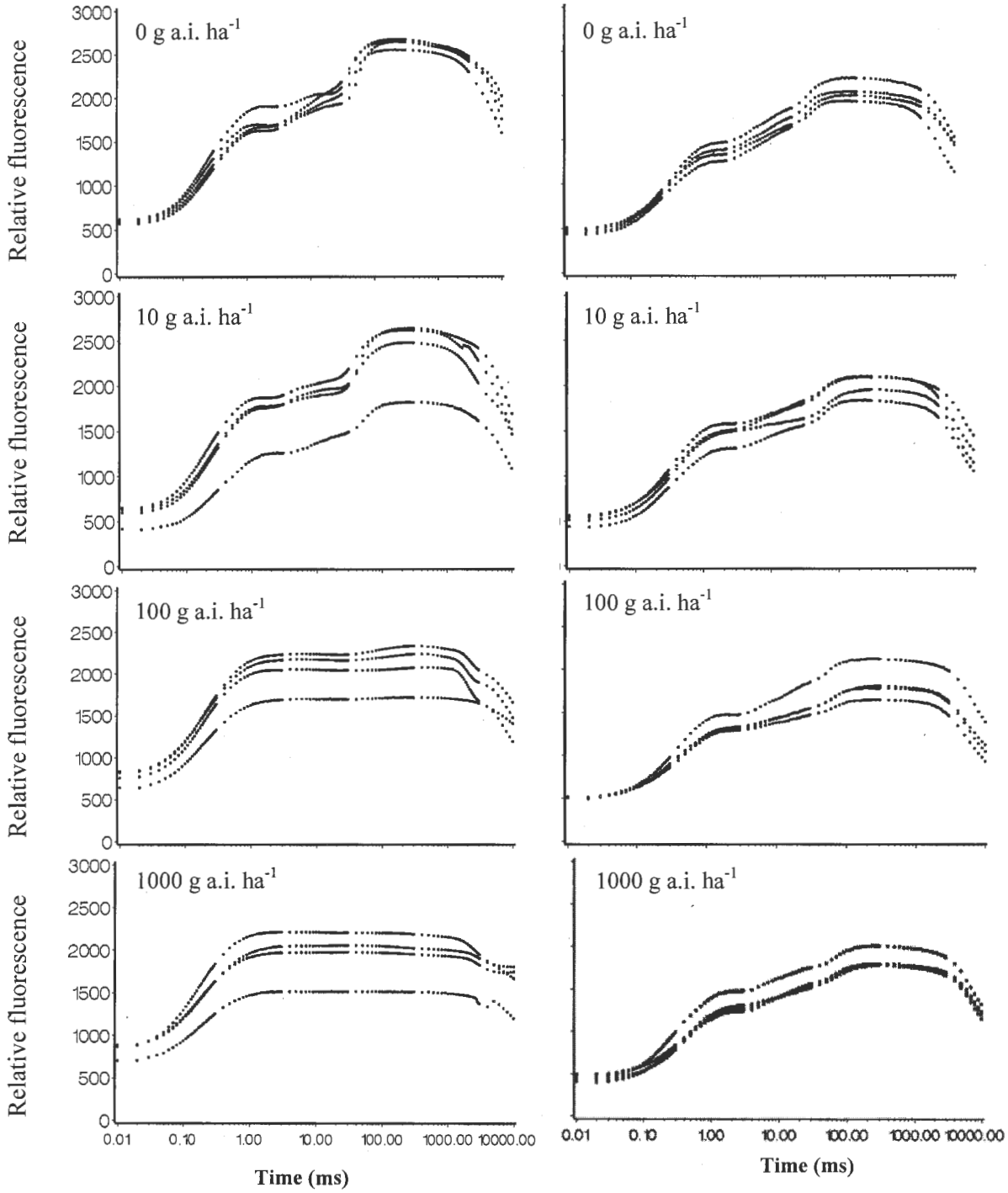

Fig 1. Effect of different doses of ametryn on the shape of Kautsky curves (10 s) at S- (left) and R (right) junglerice biotypes at 4 HAS. Each curve indicating one replication.

R-biotype were reversibly affected by ametryn while injuries were irreversibly in S-biotype (Fig. 1). Abbaspoor and Striebig (2007) reported parameters F_v/F_m and F_{vj} were irreversibly affected by desmedipham for black nightshade, whilst injuries were rapidly repaired by the sugar beet.

The fluorescence parameters

To describe changes in the shape of the Kautsky curves in Fig 1 three important fluorescence parameters such as F_v/F_m, Area and F_{vj} were plotted at the range of doses of ametryn at 4 HAS in R- and S- biotype and are shown in Fig. 2. The Fv/Fm, Area and F_{vj} were greatly decreased as the dose was increased in the S- biotype, while these parameters were not severely affected by the ametryn doses in the R-biotype (Fig. 2). F_v/F_m, is about 0.78-0.83 in healthy leaves, regardless of plant species (Abbaspoor and streibig, 2007; Stribet and govindjee, 2011). Therefore, The PSII reaction centers condition could be known by measuring parameter F_v/F_m. In untreated control leaves the values of F_v/F_m were approximately 0.80, but with increasing the dose of ametryn

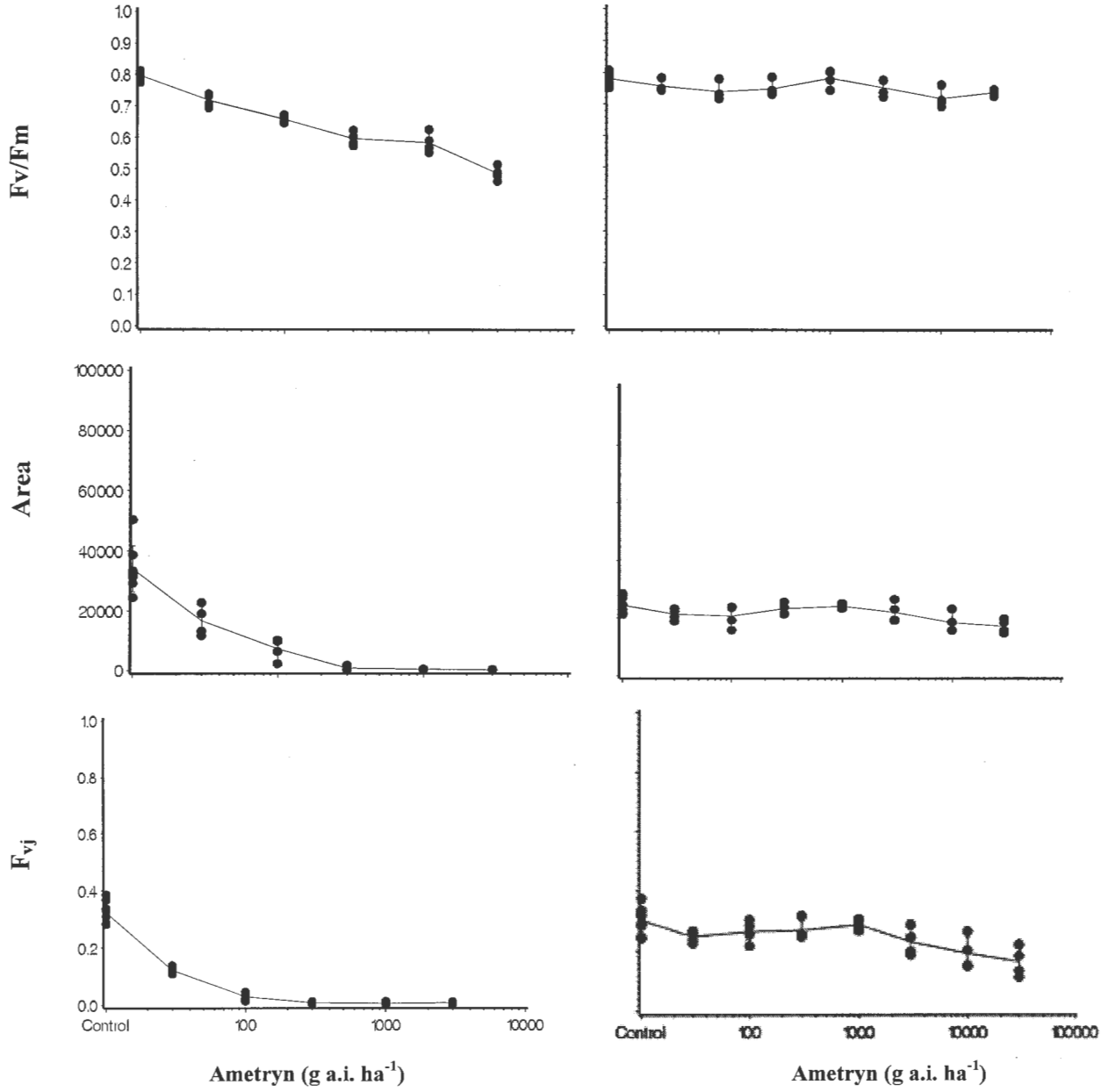

Fig 2. Maximum quantum efficiency of PSII photochemistry (Fv/Fm), Area and F_{vj} of the S- (left) and R (right) junglerice biotypes treated with ametryn at 4 HAS. Each point representing one replication. Vertical bars represent the standard errors of the mean values.

this value started to decrease in the S- biotype. Response of Area and F_{vj} to increasing the dose of ametryn were sensitive than F_v/F_m. These findings were according to results reported by Singh et al. (1997), Norsworthy et al. (1998), Eleftherohorinos et al. (2000), Hoagland et al. (2004) and Park and Mallory-Smith (2005) and Perry et al. (2012) who found that F_v/F_m parameter in R-biotypes of redroot amaranth (*Amaranthus retroflexus* L.), cheatgrass (*Bromus tectorum* L.), common lambsquarters, barnyardgarss, annual bluegrass, curlytop knotweed (*Polygonum lapathifolium* L.), black nightshade (*Solanum nigrum* L.) and common chickweed (*Stellaria media* L.) was not affected by any herbicide rates or time after treatments. As shown in Fig. 2, the Area of the R-biotype was more than S-biotype indicating more photochemistry capacity in R-biotype than S-biotype. This parameter decreased rapidly after ametryn application at often doses, as an application rate of 100 g ai ha^{-1} completely affected this parameter in the S-biotype (Fig. 2). Area in R-biotype, generally, was affected at higher doses of herbicides (Fig. 2). Korres et al. (2003) in a procedure adopted for the examination of winter wheat cultivar sensitivity to herbicide found the area above the fluorescence induction curve and the F_v/F_m parameters are appropriate for detection of differential herbicide response between wheat cultivars. As shown in Fig 2, the F_{vj} was sensitive than F_v/F_m and Area identifying it as a good and sensitive indicator of the condition of a plant's health and PSII functionality. The F_v/F_m values at 300, 1000 and 3000 g ai ha^{-1} in the S-biotype was about 0.6-0.5 whereas these values for F_{vj} were zero at these doses. Whilst, it was not observed significant difference

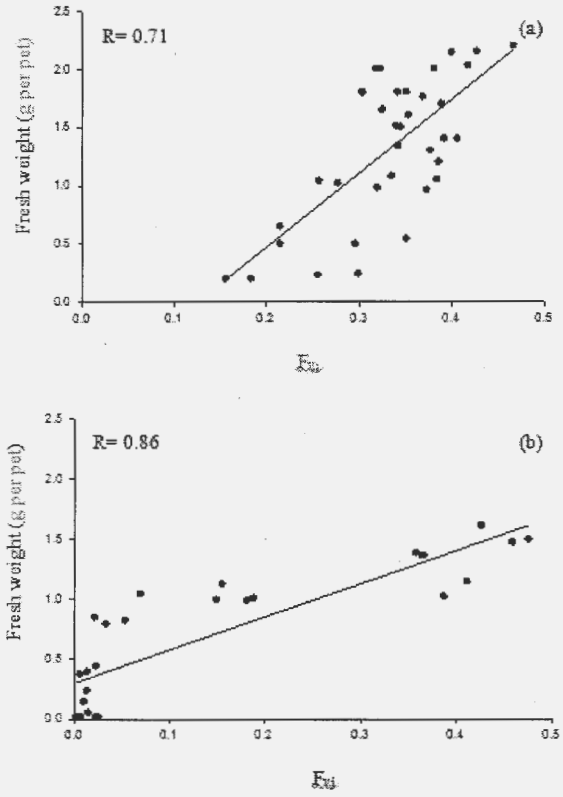

Fig 3. Relationship between fresh weight and F_{vj} at 4 HAS of ametryn in (a) R- and (b) S-junglerice biotypes ($p \leq 0.01$).

among R- biotype reactions as compared to untreated control treatment (0 g ai. ha^{-1}) (Fig. 2). Christensen et al. (2003), Abbaspoor and Streibig (2007) and Avarseji et al. (2011) have earlier suggested the use of F_{vj}. Studying chlorophyll fluorescence, emphasized presence mechanisms of herbicide resistance such as metabolism and target-site-based resistance. We found a modification in the target site that confers a single amino acid substitution from serine to glycine at residue 264 in the D1 protein (data not shown). These results are similar to those observed in target-site-based triazine resistant green bristlegrass (*Setaria viridis* (L.) P. Beauv.) and cheatgrass (De Prado et al., 2000; Park and Mallory-Smith, 2005). Sundby et al. (1993) concluded that the replacement of serine by glycine in the D1 protein in plants that were grown under high and intermediate light conditions has a direct effect on PSII function, which in turn causes increased photoinhibitory damage and increased rates of turnover of the D1 protein. The mutation causes two distinct effects. First, the mutation causes a direct effect on PSII photochemistry, which can be observed at all growth irradiances and causes an intrinsic lowering of photosynthetic efficiency of PSII which reduces light-limited but not light-saturated photosynthesis. Second, the altered PSII photochemistry also gives rise to an increased susceptibility to photoinhibition, which probably affects the crop yield at higher growth irradiances (Sundby et al., 1993). Since ametryn has changed the shape of Kuatsky curves at J step (F_{vj}), therefore, it may concluded that this parameter could explain dose-response curves and detect changes in electron transport chain (Christensen et al., 2003).

The relationship between fluorescence parameter and fresh weight

The relationship between F_{vj} of S- and R-biotypes taken at 4 HAS and fresh weight taken at 28 DAS is shown in Fig. 3. A linear relationship between F_{vj} parameter and fresh weight demonstrated significant correlation ($p \leq 0.01$). Other researchers, also, used relationship between F_{vj} parameter and dry weight in their experiments (Abbaspoor and Streibig, 2005; Christensen et al., 2003). The S-biotype response was more correlated than R-biotypes because of uniformity in the population. According to the test, the R-population may be composed of seeds slightly, moderately and highly resistant to triazine herbicides that affect the experimental results.

Materials and methods

Plant materials

The R- and S-seeds of junglerice (*Echinochloa colona* (L.) Link.) to ametryn (80% WP, Moshkfam Co., Iran) were collected from sugarcane fields that herbicide was failed to control it and adjacent areas that never been sprayed with herbicide of Karun Agro- Industry Inc. Company, Shoushtar, Iran in 2010 – 2011 growing season. These biotypes were named according to the biotype status and abbreviated as follows: R (resistant biotype) and S (susceptible biotype).

Dose-response studies

The seeds pre-germinated in petri dishes at 28 ± 2 ^{0}C in 16/8 hours (light/dark) photoperiod for 72 h. The pre-germinated seeds were sown in pots (with 10 cm diameter) containing loam: sand 2:1 mixture (v/v). Pots were transferred to a greenhouse and grown at 25 ^{0}C and 20 ^{0}C day and night temperature, respectively, with artificial light to provide a 16-h photoperiod. Pots were irrigated regularly to avoid any moisture stress. Ten DAP they were thinned to two seedlings per pot. Twenty DAWE, seedlings of the pots were subjected to ametryn application. Ametryn was sprayed at doses of 0, 10, 30,100, 300, 1000 and 3000 g ai ha^{-1} and 0, 10, 30,100, 300, 1000, 3000, 10000 and 30000 g ai ha^{-1} for S- and R-biotypes, respectively, when plants were at three to four leaf stages. Nonionic surfactant 0.25% (v/v) was applied with the herbicide at the time of spraying. The sprayer was calibrated to deliver 220 L ha^{-1} at pressure of 2 atm. The aboveground biomass was harvested 28 DAT and weighed.

Chlorophyll fluorescence studies

Chlorophyll fluorescence measurements were performed on dark-adapted leaves (30-min adaptation) at the same stage of development. Fluorescence emissions were measured using a portable chlorophyll fluorometer (Handy-PEA; Hansatech Instruments, King 's Lynn, Norfolk, UK), which emits light of 650 nm wavelength with an intensity of 3000 µmol photons m^{-2} s^{-1} for 10 s. The chlorophyll fluorescence measurements were taken 4 HAS for R- and S- biotypes. The measured parameters were F_v/F_m, the quantum yield of PSII $[(F_m-F_o) / F_m]$, F_{vj} the relative changes at the J step $[F_{vj}= (F_m-F_j) / F_m]$ and Area (area between Kautsky curve and F_m).

Statistical analysis

The experiment was conducted two times in a completely randomized design with four replications for each treatment and carried out in a greenhouse in Faculty of Agriculture,

Ferdowsi University of Mashhad, Iran in 2011. The data were analyzed using a nonlinear regression model and R software (drc add on packeges) (Knezevic et al., 2007), the three-parameter gompetrz (Eqn 1) was fitted to the data to describe the response of the biotypes to herbicide:

$$Y = d\exp\{-\exp\{b(\log(x) - e)\}\} \quad (1)$$

Where e is ED_{50}, the upper limit is d, the response when dose is zero, the parameter b denotes the relative slope around e and x is the dose. Kautsky curves and their parameters that obtained by the PEA Plus program, were analyzed by SAS software (9.2).

Conclusions

To manage herbicide resistant weeds it is necessary to understand the physiological and biochemical basis of resistance. Chlorophyll fluorescence is a highly sensitive and noninvasive tool which the effect of herbicide can be detected within a few hour of herbicide application especially for PSII inhibiting herbicides. Therefore, it has been used to monitor triazine resistance weeds in field and greenhouse. It does not depend on plant age and is able to determine resistance or susceptibility after only a few days after herbicide application. Based on the results, optimizing the evaluated parameters allows the use of chlorophyll fluorescence as a technique for detection and confirmation of resistant junglerice biotypes. Our results illustrated large differences between control and treated biotypes soon after application of the PSII inhibiting herbicide, ametryn. But if we want to choose a parameter which is best suited for field or green house assessment of herbicide efficacy, it could be the maximum quantum efficiency of PSII photochemistry (F_v/F_m). Whilst, parameter F_v/F_m, is about 0.78-0.83 in healthy leaves, therefore, the PSII reaction centers condition could be known by measuring this parameter. Therefore, researchers with comprising F_v/F_m parameter which was shown by fluorometer for treated biotypes able to monitoring photosynthesis inhibition by herbicides.

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