ECHINOCHLOA COLONUM RESISTANCE TO BISPYRIBAC-SODUIM IN EGYPT – OCCURRENCE AND IDENTIFICATION

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Abstract: Identification and mechanism of *Echinochloa colonum* (L.) resistance to bispyribac-soduim via physiological and anatomical differences between susceptible and resistant biotypes was investigated. The physiological and anatomical differences that were take into account were growth reduction, chlorophyll content reduction, protein analysis, lamina thickness and xylem vessel diameter in both susceptible and resistant biotypes of *E. colonum*. The results showed the growth reduction fifty (GR_{50}) of resistant biotype was 10.2 times higher than that of the susceptible biotype *E. colonum* treated with bispyribac-soduim. The chlorophyll content was highly reduced in the susceptible biotype relative to the resistant one of *E. colonum* treated with bispyribac-soduim. An anatomical test showed significant differences in the cytology of susceptible and resistant biotypes of *E. colonum* treated with bispyribac-soduim with respect to lamina thickness and xylem vessel diameter. Furthermore, leaf protein analysis showed significant differences between the susceptible and resistant biotypes of *E. colonum* in the number and the density of protein bands. The resistance of *E. colonum* to bispyribac-soduim may be due to the faster metabolism of bispyribac-soduim below the physiologically active concentration or the insensitivity of its target enzyme, (acetolactate synthase). These results implied the occurrence of *E. colonum* resistance to bispyribac-soduim in Egypt and provide conclusive evidence that a single resistance mechanism alone cannot explain insensitivity in *E. colonum* to bispyribac-soduim.

Key word: weed, chlorophyll, resistant, herbicide

INTRODUCTION

E. colonum (L.) is a severe competitor of rice and is one of the world's worst weeds; It is an alternate host of the fungus *Pyricularia oryzae*, which causes rice blast, the root-knot nematode *Meloidogyne incognita* (Kofoid and White) Chitwood, the yellow stem borer (*Scirpophaga incertulas*) and of the viruses that produce hoja blanca disease and tungro disease of rice (Naples and Kessler 2005). Yield losses caused by *Echinochloa* spp infestations in rice can be very severe and variable as far as the cultivar and the duration of competition are concerned. An infestation of *E. colonum* (L.) Link resulting from the sowing of about 40 viable seeds Fischer *et al*. (1997), yield losses ranged from 27 to 62%. These findings reflect the great risk of this weed on rice production in Egypt and worldwide.

Due to the great risk of this weed infestation; herbicide is becoming the most popular method of weed control in rice. While herbicide application certainly controls the weeds, experience shows that although herbicide use alleviates the problem of using labor for weeding, incorrect use of herbicides may bring about other environmental problems such as resistance to herbicides.

Weed resistance to herbicides concerns many sectors of the agricultural community: farmers, advisors, researchers, and the agrochemical industry in Egypt and worldwide. The fear exists that in an extreme case of resistance, farmers might lose a valuable chemical tool that had previously provided effective control of yield-reducing weeds. Resistance is often seen as a problem caused by a particular active ingredient. This is an over-simplification and a misconception. Resistance results from agronomic systems which have been developed to rely too heavily on herbicides as the sole method of weed control (WSSA 2007). Without monitoring and rapid detection of the resistance evolution, interpretation of its mechanism, and trying to find sustainable management strategies, the future usefulness of herbicides as a tool for weed control might be seriously jeopardized. The clearance of resistance mechanism to herbicides is considered the key step toward developing appropriate solutions to overcome this phenomenon. Resistance identification and mechanism evaluating the activity of target site enzymes have been reported before (Fischer *et al*. 2000; Osuna *et al*. 2002; Busi *et al*. 2004), however, characterizing the resistance mechanisms of weeds to herbicides by investigating the anatomical and physiological differences in susceptible and resistant biotypes considered a source of major concern, has not been studied before.

Bispyribac-sodium, a pyrimidinyl carboxy herbicide, is effective to control many annual and perennial grasses,

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sedges, and broad-leaved weeds in rice fields throughout the world. The mode of action has been considered as inhibition of acetolactate synthase (ALS-ase) in the biosynthetic pathway of three branched-chain amino acids (Shimizu 1997). However, resistance development affects the future use of this herbicide and other effective herbicides.

Therefore, this study attempted to identify the occurrence of *E. colonum* resistance against bispyribac-soduim by investigating the physiological (chlorophyll content, growth reduction and protein analysis) and anatomical differences between the susceptible and resistant biotypes of *E. colonum* treated with bispyribac-soduim.

MATERIALS AND METHODS

The used herbicide

Bispyribac-soduim with the trade name of Nominee (SL) 2% was obtained from Rice Weeds Research Department, Rice Research and Training Center, Sakah, and Kafrelsheikh, Egypt. This herbicide was applied at the rate of 40 cm^3 a.s. /hectare.

The tested weed

The susceptible biotype (SBT) of *E. colonum* to bispyribac-soduim (Obtained from Rice Weeds Research Department, Rice Research and Training Centre, Sakah, Kafrelsheikh).The resistant biotype (RBT) of *E. colonum*, used in the present experiment, was previously treated for several years with the tested herbicide by selection pressure and recorded resistance (Hamza 2009).

Soil used for the greenhouse experiment

The soil used for cultivation of the tested weed was collected from the upper 15 cm of the soil profile at the farm of Rice Research and Training Center Sakah, Kafrelsheikh. The collected soil was air dried, ground, and sieved to give the homogenous size.

Whole plant bioassay

Dose-response experiments were conducted at the greenhouse of the Agriculture Botany Department, Faculty of Agriculture, Kafrelsheikh University, Egypt. The soil used in this experiment was fertilized with nitrogen at rate a 360 kg/h of urea fertilizer (contain 46% nitrogen). Super phosphate fertilizer (phosphorus 15%) was added at a rate of 240 kg/ha before planting. Potassium was not added because the Egyptian soil is rich in this element. Germinated seeds of susceptible and resistant biotypes of *E. colonum* were planted in 30x30 cm plastic pots filled with soil. Emerged seedlings were thinned to four uniform and equally distant-spaced plants per pot. These experiments were conducted at average daily temperatures ranging from 22 to 31°C and at a 16-h day length. Pots were immersed with water up to 4 cm above the soil surface. The tested herbicide, bispyribac-soduim, was applied as a single application using a hand sprayer at the 4-leaf to 1-tiller stage of growth of the tested weed. The concentration levels used were 0.1, 0.5, 1 and 2 folds of bispyribac-soduim. After a forty-eight hour treatment, the plants were irrigated and water was raised up to 4 cm above the soil surface (Osuna *et al*. 2002). Experiments were done in a completely randomized design with six replications. Data were pooled and fitted to a log-logistic regression model (Streibig *et al*. 1993; Seefeldt *et al*. 1994) as shown in equation 1 below:

$$Y = c+\{(d-c)/[1+(x/g)^b]\} \quad (1)$$

where:
Y – the fresh weight of germinated seedling aboveground expressed as percentage of the untreated control,
c and d – the coefficients corresponding to the lower and upper asymptotes,
b – the slope of the line,
g (GR_{50}) – the herbicide rate at the point of inflection halfway between the upper (d) and lower (c) asymptotes,
x (independent variable) – the herbicide dose.

Regression analysis was conducted using the Sigma Plot statistical software version 10.0 (Osuna *et al*. 2002). The herbicide rate used to reduce plant growth by 50% relative to the untreated control (the growth reduction fifty – GR_{50}) was calculated for resistant and susceptible biotypes of *E.colonum*. R/S ratios were calculated as the GR_{50} of the (R) accession divided by the GR_{50} of the (S) accession.

Chlorophyll measurements

Chlorophyll content of resistant and susceptible *E. colonum* biotypes was determined after 14 days of treatment with bispyribac-soduim at the level applied in the real field conditions. Moreover, chlorophyll content of resistant treated and untreated biotype was re-measured after 21 days of treatment with bispyribac-soduim (recovery induced). Chlorophyll A, B and total were determined in *E. colonum* lamina using the spectrophotometer method described by Moran and Porath (1980). Data were subjected to statistical analysis of variance according to the method described by Gomez *et al*. (1984).

Anatomical test

The leaf specimens which included the midrib were taken after 14 days of treatment from the second leaf of the resistant and susceptible biotypes of *E. colonum* treated with bispyribac-soduim at the recommended dose level (1 fold). Specimens were fixed in a formalin, ethyl alcohol and acetic acid mixture (1:18:1 v/v). Then specimens were washed and dehydrated in an alcohol series. The dehyderated specimens were infiltrated and embedded in paraffin wax (52–54°C m.p.). The embedded specimens were sectioned using a rotary microtome (Leica RM 2125) to a thickness of 8–10 μm. Sections were mounted on slides and deparaffinized. Staining was accomplished with safranine and azur II (Gutmann 1995), cleared in xylol and mounted in canada balsam (Ruzin 1999). Ten reading from three slides were examined with an electric microscope (Lieca DM LS), and with a digital camera (Lieca DC 300), and then photographed. The anatomical manifestation was calculated using Lieca IM 1000 image manager software. Lieca software was calibrated using 1 cm stage micrometer scaled at 100 μm increment (Leitz Wetzler, Germany 604364) at a 4 and 10 X magnifications.

Protein analysis by electrophoresis SDS-PAGE

Leaves from susceptible and resistant biotypes of *E. colonum* were collected after 14 days of treatment with the recommended level of bispyribac-soduim. The collected leaves were stored in liquid nitrogen (–80°C) and transferred for determination to the Research Institute of Genetics Engineering, Menofia University, Egypt. The total protein was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Laemmli (1970).

Statistical analysis

Data from the experiments were statistically analyzed using one-way repeated measurement analysis of variance. Duncan's multiple range test was used to separate means using SAS software (Version 6.12, SAS Institute Inc., and Cary, USA).

RESULTS

***E. colonum* resistance to bispyribac-soduim**

A dose–response experiment was conducted on whole plants of *E. colonum* treated with bispyribac-soduim to detect its resistance level against this herbicide. The response of the susceptible and resistant biotypes to bispyribac-soduim was determined as reduction in the fresh weight of the treated plants relative to the control after 14 days of treatment. The results showed that, the rates of bispyribac-soduim required for 50% growth reduction were 2.5 and 25.5 gm a.s./ha for the susceptible and resistant biotypes of *E. colonum*, respectively (Table 1). Table 1 revealed that, the GR_{50} of *E. colonum* resistant biotype was 10.2 times higher than that required to obtain the same effect on the susceptible biotype.

Effect of tested herbicide on chlorophyll content of susceptible and resistant biotypes of *E. colonum*

The chlorophyll content of *E. colonum* was measured after 14 days of herbicide application to evaluate the physiological conditions of the susceptible and resistant biotypes. Table 2 showed the decrease in chlorophyll content after bispyribac-soduim application either in the resistant or susceptible biotypes relative to the untreated ones. However, the rate of reduction was higher in the susceptible biotype than in the resistant one of *E. colonum*. A very important action took place. Chlorophyll content of the resistant biotype treated with bispyribac-soduim increased again after 21 days of treatment relative to the same biotype after 14 days of treatment. This action was due to the re-growth of *E. colonum* leaves as shown in table 3.

Table 1. Effect of bispyribac-soduim on the susceptible and resistant biotypes of *E. colonum* expressed as the rates of the herbicide required for a 50% reduction of the aboveground biomass (GR_{50}) and estimated resistance ratio

Weed biotype	GR_{50} [gm a.s./ha]	b	c	d	R^2	R/S value	P value
Susceptible	2.5	0.53	0.00	98	0.99	–	< 0.05
Resistant	25.5	1.42	0.5	101	0.98	10.2	< 0.05

c – the mean response (fresh weight as percent of control) at a very high herbicide rate
d – the mean response (fresh weight as percent of control) at a zero herbicide rate
b – slope of the line
GR_{50} – herbicide rate to reduce plant growth by 50% relative to the untreated control
R^2 – the coefficient of determination
R/S ratio – the GR_{50} of the resistant biotype divided by the GR_{50} of the susceptible biotype
P-value – the probability of the obtained results
a.s. – active substance

Table 2. Chlorophyll content in susceptible and resistant biotypes of *E. colonum* after 14 days of treatment with bispyribac-soduim

Treatments	Chlorophyll pigments [mg/l]		
	A	B	total
Susceptible (the control)	3.079 a	1.181a	4.259 a
Susceptible + Bs*	0.426 d	0.031 d	0.456 d
Resistance (the control)	1.411 b	0.428 b	1.839 b
Resistance + Bs*	0.880 c	0.203 c	1.082 c

*Bs – bispyribac-soduim
a, b, c, d – indicate the significance and non-significance between means using Ducan's range test

Table 3. Chlorophyll contents in resistant, untreated, and resistant treated biotypes of *E. colonum* after 21 days of treatment with bispyribac-soduim

Treatments	Chlorophyll pigments [mg/l]		
	A	B	total
Resistance (the control)	1.013 a	0.560 a	1.573 a
Resistance + Bs*	0.980 b	0.443 b	1.423 b

*Bs – bispyribac-soduim
a, b, c, d – indicate the significance and non-significance between means using Ducan's range test

Anatomical differences between susceptible and resistant biotypes of *E. crus-galli* against bispyribac-soduim

The anatomical differences in the cytology of susceptible and resistant biotypes of *E. colonum* treated with bispyribac-soduim with respect to lamina thickness and xylem vessel diameter is presented in table 4 and figure 1. The results showed that SBT treated with bispyribac-soduim had less lamina thickness and tissues intensively stained with azur II compared with the untreated plants. The normal internal leaf structure of treated SBT is more difficult to identify, which may be due to cell death. Lamina thickness and xylem vessel diameter of treated biotype were reduced but the lowest value was caused by treated SBT. In contrast, leaf tissues in treated RBT seem to be normal and easily identified. Intensively stained cells with azur II, though, were noticed in some local lesions (areas), which may be due to cell death.

Data in table 5 and figure 2 indicated that lamina thickness increased to a level near to that of untreated RBT after 21 days of treatment relative to the same biotype after 14 days of treatment. Furthermore, data in table 5 and figure 2 shows that no differences in xylem vessel diameter were found between treated and untreated RBT after 21days of treatment, relative to the same biotype after 14 days of treatment.

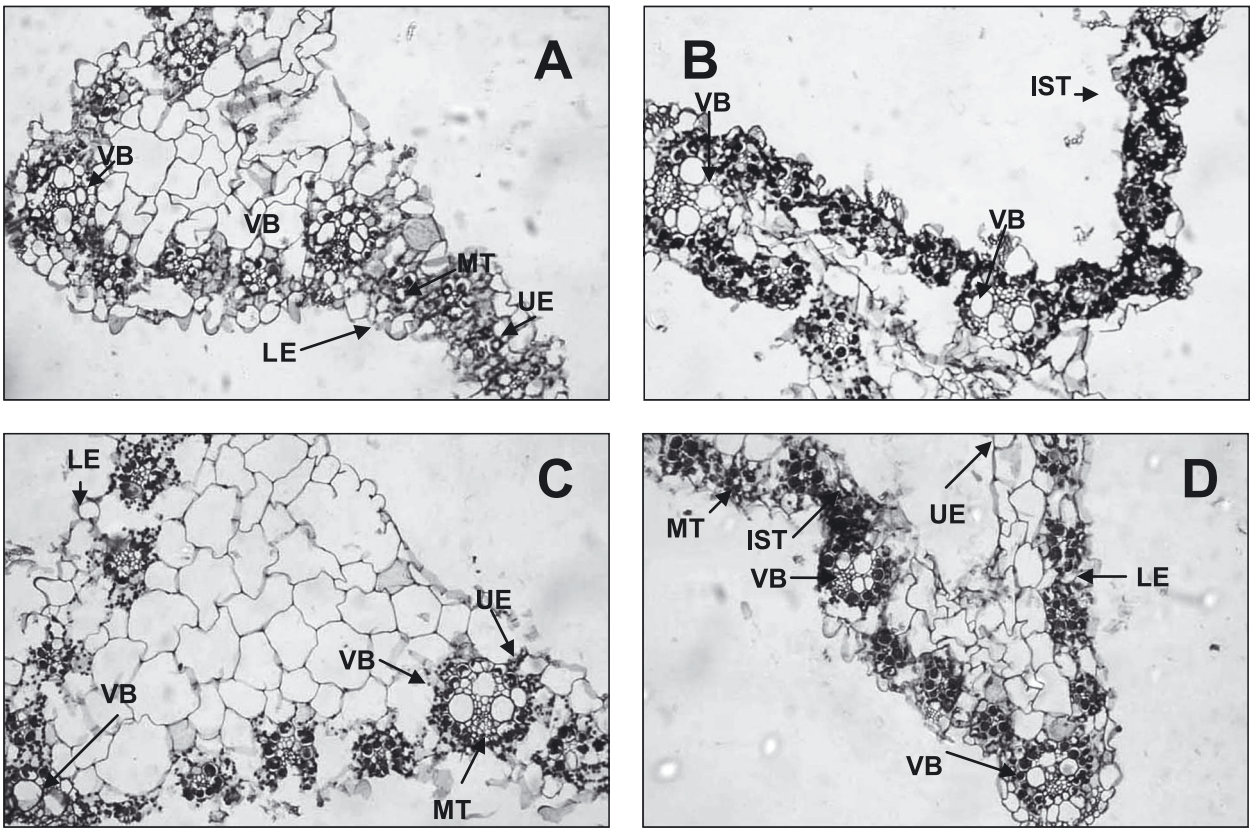

Fig. 1. Cross sections through the lamina of untreated susceptible biotype (A), treated susceptible biotype (B), untreated resistant biotype (C) and resistant treated biotype (D) of *E. colonum*
*upper epidermis (UE), lower epidermis (LE), parenchyma tissue (PT), mesophyll tissue (MT), motor cells (MC), vascular bundle (VB), intensive stained tissue (IST), (Bar = 500 μm)

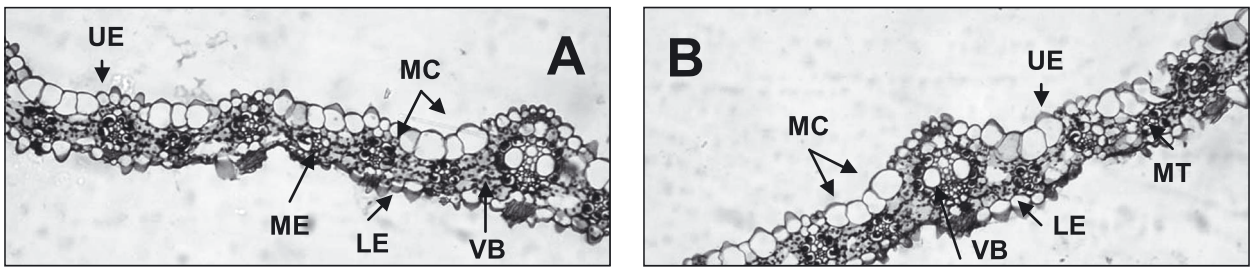

Fig. 2. Cross sections through the lamina of untreated resistant biotype (A) and treated resistant biotype (B) of *E. colonum*

Table 4. Some anatomical parameters in the two sensitive and resistant biotypes of *E. colonum i.e.*, lamina thickness, and vessel diameters as affected by foliar application of bispyribac-soduim

Treatments	Lamina thick [µm]	Xylem vessels diameter [µm]
susceptible (the control)	182.6 a	25 a
susceptible + Bs*	83 d	17 c
Resistance (the control)	149 b	25 a
Resistance + Bs*	116 c	20 b

*Bs – bispyribac-soduim

a, b, c, d – indicate the significance and non-significance between means using Ducan's range test

Table 5. Some anatomical parameters in untreated and treated though recovered, resistant biotype of *E. colonum i.e.*, lamina thickness and vessel diameters as affected by foliar application of bispyribac-soduim

Treatments	Lamina thick [µm]	Vessels diameter [µm]
Resistance (the control)	149 a	27 a
Resistance + Bs*	147 a	26 a

*Bs – bispyribac-soduim

a – indicate the significance and non-significance between means using Ducan's range test

Table 6. Molecular weight and density of protein in susceptible (1), and resistant (2) biotypes of *E.colonum* to bispyribac-soduim

Band Number	Lane Number	M. W.	Average (OD)	Lane Number	M. W.	Average (OD)
1	1	112.38	102.33	2	117.084	78.723
2	1	87.501	80.072	2	90.124	57.379
3	1	75.85	80.254	2	77.053	66.282
4	1	68.366	90.426	2	68.279	74.792
5	1	65.211	88.367	2	65.002	71.988
6	1	57.448	97.154	2	62.762	69.54
7	1	47.343	82.613	2	57.039	86.455
8	1	45.775	76.323	2	54.067	70.745
9	1	37.945	93.538	2	47.889	62.864
10	1	34.563	96.45	2	45.177	56.832
11	1	33.646	99.106	2	37.495	71.384
12	1	30.581	80.692	2	34.276	75.097
13	1	30.162	68.431	2	33.168	80.858
14	1	26.852	71.6	2	31.434	51.856
15	1	25.448	52.786	2	30.26	49.47
16	1	23.655	61.225	2	28.982	41.112
17	1	22.461	56.775	2	25.929	28.25
18	1	19.783	60.324	2	24.453	21.667
19	–	–	–	2	22.576	31.351
20	–	–	–	2	21.474	30.55

[1] M.W. – molecular weight

[2] OD – optical density

Leaf protein analysis in sensitive and resistant biotypes of *E. colonum*

Total leaf protein of both susceptible and resistant biotypes of *E. colonum* treated with bispyribac-soduim were extracted and analyzed as mentioned before, to confirm the resistance mechanism of *E. colonum* to bispyribac-soduim**.** The data in Table (6) shows that five bands with molecular weights of 19, 26, 75, 87 and 112 kilo Dalton (kDa) were presented in the susceptible biotype (lane 1) and absent in the resistant biotype (lane 2) of *E. colonum*. On the other hand, another seven bands with molecular weights of 21, 28, 31, 54, 77, 90 and 117 kDa were presented in the resistant biotype (lane 2) and absent in the susceptible biotype (lane 1) of *E. colonum*. In addition, there were eight bands with molecular weights of 68, 65, 45, 34, 33, 30, 25 and 22 kDa presented with high density in the resistant biotype (lane 2) compared to the susceptible biotype (lane 1) of *E. colonum* (Table 6). Finally, there were 20 total bands exhibited in lane 2 (resistant biotype) compared to 18 bands detected in lane 1, the susceptible biotype of *E. colonum* (Table 6).

DISCUSSION

The resistance of *E. colonum* to bispyribac-soduim (ALS inhibitor) was identified in this study and confirmed the occurrence of *E. colonum* resistance to bispyribac-soduim in Egypt. This finding had been reported previously outside Egypt (Fischer *et al*. 2000; Osuna *et al*. 2002; Ruiz Santaella *et al*. 2003b: Yun *et al*. 2005; Castor

and Alex 2006). The results of this study also implied that the physiological and anatomical dereferences as well as growth reduction help to identify the occurrence of resistant weed.

Chlorophyll content has been known as a typical parameter for evaluating the physiological conditions. The reduction in chlorophyll content of *E. colonum* after foliar application of bispyribac-soduim with different concentrations is in agreement with the findings of Lycan and Hart (2005), who reported that application of bispyribac-soduim as AlS-inhibitor for controlling different weeds leads to injury symptoms in the form of chlorosis (reduction in the chlorophyll content). Chlorophyll's reduction mechanism may be due to the enhanced activity of the chlorophyll degrading enzyme chlorophyllase and/or disruption of the fine structure of chloroplast, and instability of chloroplast or pigment-protein complex, which leads to oxidation and a decreased concentration of chlorophyll.

Bispyribac-sodium known as acetolactate synthase (ALS) inhibitor, is involved in biosynthesis of the branched – chain amino acids (Tranel and Wright 2002; Zhou *et al*. 2007). Therefore, the application of bispyribac-sodium against *E.colonum* should reduce biosynthesis of amino acids which subsequently inhibits protein synthesis, and growth, and finally causes cell and plant death (WSSA 2007). Also, the formation of protein, vital for cell division, may be disrupted and growth retardation may be induced as a result of cell division inhibition.

The data of protein analysis showed differences between the susceptible and resistant biotypes of *E. colonum.* This data implied that there was different gene (s) expression between the two biotypes of the weed, where some of them promoted while novel proteins were induced (Hamza 2009).

In this study, there were anatomical differences between resistant and susceptible biotypes of *E. colonum* treated with bispyribac-soduim with respect to leaf lamina thickness and xylem vessel diameter. Moreover, after 21 days of treatment, lamina thickness of treated RBT was increased up to that of the untreated RBT. After this time, no differences in xylem vessel diameter were found between treated and untreated RBT of *E. colonum*. All these anatomical differences were in agreement with the chlorophyll content and growth reduction fifty (GR_{50}) data for both susceptible and resistant biotypes of *E. colonum* treated with bispyribac-soduim. Reduction in leaf lamina thickness in sensitive biotype of *E. colonum* treated with bispyribac-soduim was a reflection of the decrease of mesophyll cells. This decrease in the number of mesophyll cells may be attributed to the inhibition of cell division or cell enlargement. This reduction in mesophyll cells either by inhibition in cell division or cell enlargement may be due to the disruption of amino acid biosynthesis, and subsequently of protein content.

The results also showed that the reduction in chlorophyll content, fresh weight, leaf lamina thickness and xylem vessel diameter in resistant biotype treated with bispyribac-soduim was lower than that of susceptible biotype of *E. colonum*. Moreover, the results indicated that the chlorophyll content, leaf lamina thickness and xylem vessel diameter of the treated resistant biotype of *E. colonum* again increased more than the untreated one, after 21days of bispyribac-soduim application.

The possible mechanism of lower reduction or reincrease of these parameters, in the resistant biotype of *E. colonum* relative to the susceptible one, may be due to the low reduction of amino acids which have gone through biosynthesis because of the enhanced degradation by monooxygenases (Osuna *et al*. 2002; Yun *et al*. 2005). Another possible mechanism may be through the insensitivity of the target enzyme (acetolatae sysnthase) in resistant biotype to bispyribac-sodium.

Therefore, the resistance mechanisms of *E. colonum* to the bispyribac-soduim may be conferred by two proposed mechanisms. Firstly, the mechanism may be due to an alteration in protein of the target site enzyme (acetolactate synthase), which is likely the mechanism that confers resistance of *E. colonum* to the bispyribac-sodium (Kuk *et al*. 2002; Osuna *et al*. 2002). The alteration or changes in the protein of the acetolactate synthase enzyme (the target of bispyribac-soduim) in the resistant biotype compared to the susceptible one, induced low affinity of bispyribac-soduim herbicide to bind with the target enzyme. Thus, the enzyme became insensitive to the herbicide. Similarly, the relatively high dose of growth reduction fifty, low reduction in chlorophyll content, anatomical differences, and protein analysis in the resistant biotype of *E. colonum* provide additional support for this proposed mechanism of resistance.

The second mechanism of *E. colonum* resistant to bispyribac-soduim may be due to the relatively faster degradation of bispyribac-sodium through enhanced herbicide degradation by monooxygenases (Osuna *et al*. 2002; Yun *et al*. 2005). Even if the plant is treated with a rate close to the lethal dose, the treated plants are still alive. These plants are likely to re-grow when the phytotoxic compound is degraded below the physiologically active concentration that may be due to enhanced bispyribac-sodium degradation by monooxygenases. Both proposed mechanisms of *E. colonum* to bispyribac-soduim and other ALSase inhibitors have been reported before for another weed (Osuna *et al*. 2002), however, against *E. colonum* based on physio-anatomical differences; this is considered to be the first report.

CONCLUSIONS

There were significant differences between susceptible and resistant biotypes of *E. colonum* treated with bispyribac-soduim with respect to chlorophyll content, growth reduction, protein analysis and cytology (lamina thickness and xylem vessel diameter). These differences pointed out the occurrence of *E. colonum* resistance to bispyribac-soduim in Egypt and assumed that the resistance mechanism could be explained either by target site insensitivity or by an enhanced rate of bispyribac-soduim metabolism.

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