

Different Responses of *Echinochloa crus-galli* and *Echinochloa colona* to Bispyric-sodium (Nominee™)

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ECHINOCHLOA crus-galli and *Echinochloa colona* mostly grown as weeds in different economic crops and cause losses in their yield. Flooding and crop rotation can not suppress these weeds. Herbicides are widely applied to control barnyard grasses. Bispyric-sodium was applied at FRD (Field Recommended Dose) on both species, the results of preliminary experiment indicated that *E. crus-galli* was susceptible to bispyric-sodium but *E. colona* was resistance. The confirmation experiment for *E. colona* resistance indicated that herbicide effect on the biomass of plants which caused reduction in fresh and dry weight. The photosynthetic pigments were affected by herbicide, which decreased by increasing the herbicide concentration. While anthocyanins content were increased to protect the leaves from the stress of photoinhibitory light fluxes by absorbing the excess photons. The soluble and insoluble sugar content was increased by increasing the concentration of herbicide. The transcript level of *ALS* in *E. colona* was modified by different concentrations of herbicide. The present study indicated that *ALS* expression level was responsive to herbicide treatment. This suggests that *ALS* is a target site of bispyric-sodium. But 50% FRD can be considered as sublethal dose which upregulated the expression of *ALS* by 4 folds.

Keywords: *ALS*, *Echinochloa*, Herbicide, Anthocyanine.

Introduction

The genus *Echinochloa* includes serious weeds in agriculture being the third and fourth most important weeds in the world (Holm et al., 1977). *Echinochloa colona*, a vigorous C4 annual species, is one of the world's most serious grass weeds in rice (Holm et al., 1991 and Rao et al., 2007). More than 60 countries have reported it as a weed problem in 35 crops, including rice (*Oryza sativa*), maize (*Zea mays* L.), sorghum (*Sorghum bicolor* L.), sugarcane (*Saccharum officinarum* L.), cotton (*Gossypium hirsutum* L.), and peanuts (*Arachis hypogea* L.) (Holm et al., 1991). This weed has been reported to occur in 24 countries in dry-seeded rice, in 12 countries in wet-seeded rice, and also in transplanted rice (Rao et al., 2007). *E. colona* is an example of crop mimicry because it closely resembles rice at the seedling stage and is sometimes unintentionally transplanted into fields together with the crop. *Echinochloa crus-galli* (L.) Beauv is a C4 weed that grows essentially in paddy fields, well known weed of this genus and is distributed worldwide. Yield losses caused by *Echinochloa* sp. invasion in rice can be very

severe and variable in relation to the cultivar and the duration of competition.

The importance of *Echinochloa* species in rice is increasing and this is due to the development of resistance to herbicides in several populations. Within the major herbicides applied in rice, resistances have been reported to bispyric-sodium, cyhalofop-butyl, molinate, propanil, and quinclorac (Fischer et al., 2000 and Busi et al., 2002). Several management practices, including intensive chemical control, are used to reduce *E. crus-galli* infestation in rice production systems in many countries (Gibson et al., 2003 and Hoagland et al., 2004). Intensive herbicide used on *E. colona* and *E. crus-galli* has already led to the evolution of herbicide resistance to propanil, quinclorac, azimsulfuron, and fenoxaprop-p-ethyl (Rao et al., 2007).

Bispyric-sodium, sodium 2, 6-bis [(4, 6-dimethoxy-2-pyrimidinyl) oxy] benzoate, is an herbicide used for rice farming. It is indicated for the post-emergence control of cyperaceous grasses and some dicotyledonous grasses

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DOI :10.21608/ejbo.2018.1417.1115

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and is sold commercially as a suspension concentrate under the name of Nominee. The herbicides bispyribac-sodium, penoxsulam and imazamox are acetolactate synthase (ALS; EC 2.2.1.6)-inhibiting herbicides and have a propensity rapidly to select for resistant weed accessions (Tranel & Wright, 2002). This is confirmed by the fact that 126 weed species have already developed resistant accessions to ALS-inhibiting herbicides (Tranel et al., 2012). In most cases, resistance is due to point mutations in one of the following codons: Ala122, Pro197, Ala205, Asp376, Arg377, Trp574, Ser653 and Gly654, numbered on the basis of the sequence of *Arabidopsis thaliana* (L.) Heyhn (Tranel et al., 2012). In most cases, resistance to ALS-inhibiting herbicides is caused by an altered ALS enzyme (Tranel & Wright, 2002).

Acetolactate synthase (ALS, EC 2.2.1.6; also referred to as acetohydroxyacid synthase) is the first common enzyme in the biosynthetic pathways leading to the branched-chain amino acids (isoleucine, leucine, and valine). ALS does not exist in mammals, and thus ALS-inhibiting herbicides are thought to be less toxic to mammals. Resistance to ALS-inhibiting herbicides in plants has in most cases been conferred by either single or double amino acid substitutions at a particular position in ALS (Kawai et al., 2007 and Okuzaki et al., 2007). ALS-inhibiting herbicides have become widely used in world agriculture. Consequently, there has been evolution of many ALS herbicide-resistant weed populations, with resistant biotypes of 110 weed species worldwide (Heap, 2011).

The knowledge of the response of *Echinochloa* populations to different herbicides may have several implications. This information, when coupled with the knowledge of the *Echinochloa* population structure at field or regional scale, is fundamental for the elaboration of appropriate weed management strategies, including the development of models capable to predict the overall weed control level achievable with a certain herbicide.

Materials and Methods

Preliminary experiment

Investigation the response of *E. crus-galli* and *E. colona* to Bispyribac-sodium (Nomineetm 0.015%) herbicide

Experimental design

The seeds of the two species *E. crus-galli* and *E. colona* were sown in a green house in June

2012. Greenhouse conditions were: 1550 $\mu\text{mole m}^{-2} \text{ s}^{-1}$ maximum light intensity, 28/19°C day/night temperature, 14 h photoperiod, 70% relative humidity, 4.97 m s⁻¹ wind speed. After one month, 10 seedlings of each species were transplanted into blocks 50×50 cm. The experimental design was a randomized complete block design with 3 replicates, 12 blocks represent all treatments 20 plants in each block (10 *E. crus-galli* and 10 *E. colona*). After 10 days of transplanting, the plants were treated with 50%, 100% and 200% field recommended dose (FRD) of nominee. The herbicide applications were made using sprayer. Nitrogen fertilizer was applied as urea (50 gm / Sq.m.) one week before herbicide application. The herbicide treatment was carried out as recommended by the manufacturer.

Harvesting of the plants

Seven days after treatment with herbicide, the plants were collected, placed in paper envelopes and oven-dried at 65°C for 48 h.

Biomass yield

Determination of fresh and dry weights and water contents: After recording the fresh weights of shoots, they were dried in oven at 80°C for 2 days and the dry weights were then recorded. The water contents were calculated on fresh weight basis. Three plant replicates were used for each treatment.

Confirmation experiment

The resistance of E. colona to Nominee

Seeds of *E. colona* were planted in the green house in July 2012. Greenhouse conditions were: 1550 $\mu\text{mole m}^{-2} \text{ s}^{-1}$ maximum light intensity, 28/19°C day/night temperature, 14 h photoperiod, 70% relative humidity, 4.97 m s⁻¹ wind speed. The experimental design was a randomized complete block design with 3 replicates, the block area 100 cm×100 cm. After 25 days, the plants were treated with 50%, 100%, 200% and 300% FRD of nominee. The herbicide applications were made using sprayer. Nitrogen fertilizer was applied as urea one week before herbicide application. The herbicide was applied as recommended by the manufacturer

Harvesting of the plant material

After Seven days of treatment with herbicide, plant leaves (the second leaf from the top) from 3 replicates of each treatment were collected at 10 AM. Frozen immediately in liquid nitrogen and stored at -80°C until used for subsequent biochemical and molecular analyses.

Biomass yield

Determination of fresh and dry weights and water contents: The fresh weight, dry weight and water content were determined as described in preliminary.

*Biochemical analysis**Estimation of photosynthetic pigments*

It was done according to the method described by Wellburn & Lichtenthaler (1984).

Determination of anthocyanins

According to the method adopted by Hoagland (1980).

Determination of protein content

The protein content of fresh plant material was measured according to the method of Bradford (1976).

*Molecular analysis**Quantification of gene expression*

Quantification of gene expression by semi-quantitative RT-PCR: Total RNA was extracted from about 50 mg frozen leaves using TRI-reagent (Sigma, UK) according to the manufacture's protocol. To prevent DNA contamination, the extracted RNA was treated with DNA-free kit (Ambion, UK) for 30 min

at 37°C. Poly A tail mRNA was then isolated by reacting 10 µl of RNA with 2 µl of oligo dT(18) and 3 µl RNase and DNase free H₂O for 5 min at 70°C and the reaction was terminated on ice for 2 min. The reverse transcription was conducted by using MMLV-reverse transcription kit according to the supplier's recommendations (Promega, UK). Primers for gene were designed to recognise conserved regions resulting from the alignment of the characterized gene in *E. colona*. The primers used for amplifying ALS and 18S rRNA are listed in Table 1. The PCR conditions were as follows: Initial denaturation at 94°C for 3 min, followed by 35 and 45 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 50 sec, the number of PCR cycles was optimized to show the maximal differences among samples within the linear phase of amplification. PCR products were resolved by electrophoresis on 1% agarose gels, stained with ethidium bromide in 1X TAE (Tris-acetic acid-EDTA) using Bio- Rad equipment and visualized and documented using Trans illuminator UViTec. The band volumes were measured by using Lab Image V 2.7.2 software. The measurement was normalized for equal 18S rRNA bands.

TABLE 1. Primer pairs used for amplification of ALS, primer pairs were designed according to the DNA sequence for the identified genes from *E. crus-galli*, *E. phyllopogon* and *Oryza sativa* to the data from: <http://www.ncbi.nlm.nih.gov>.

Serial no.	Forward primer 5' 3'	Reverse primer 5' 3'	Amplified fragment size (bp)
Primer I	GAACACGCCCATGAGTCTG	GGTACGGCCCTGGAGTCTCG	1105

Statistical analysis

Each measurement was repeated until consistent results were obtained with at least three independent measurements. In order to compare between samples one way ANOVA-LSD was performed using SPSS 18.0. and CoStat 6.311 (Carver & Nash, 2011).

Results*First experiment**Effect of herbicide on plant growth(E. crus-galli and E. colona)*

The negative effect of herbicide on the

fresh and dry weights of plants increased by increasing the concentration compared to the control. The Fresh weight was decreased by 75.7%, 93.87% and 96.5%, respectively at 50%, 100% and 200% in *E. crus-galli* and also in *E. colona* it decreased by 75%, 84% and 84.9%, respectively. The Dry weight also decreased by 78%, 86.3% and 90%, respectively at 50%, 100% and 200% in *E. crus-galli* and in *E. colona* it decreased by 75.5%, 71% and 70.6%, respectively (Fig. 1 and Fig. 2).

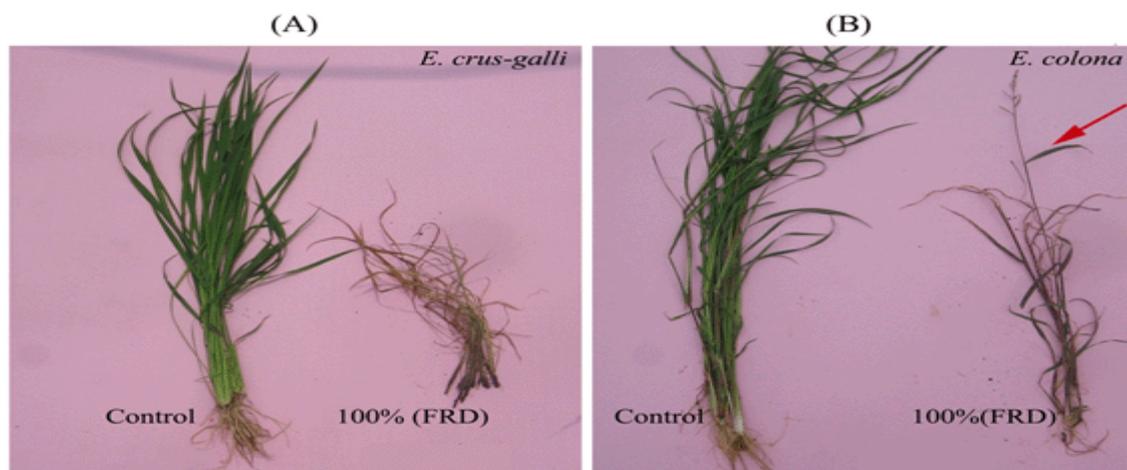


Fig. 1. Effect of Nominee (100% FRD) on the growth of *E. crus-galli* (A) and *E. colona* (B). Young leaf (→)

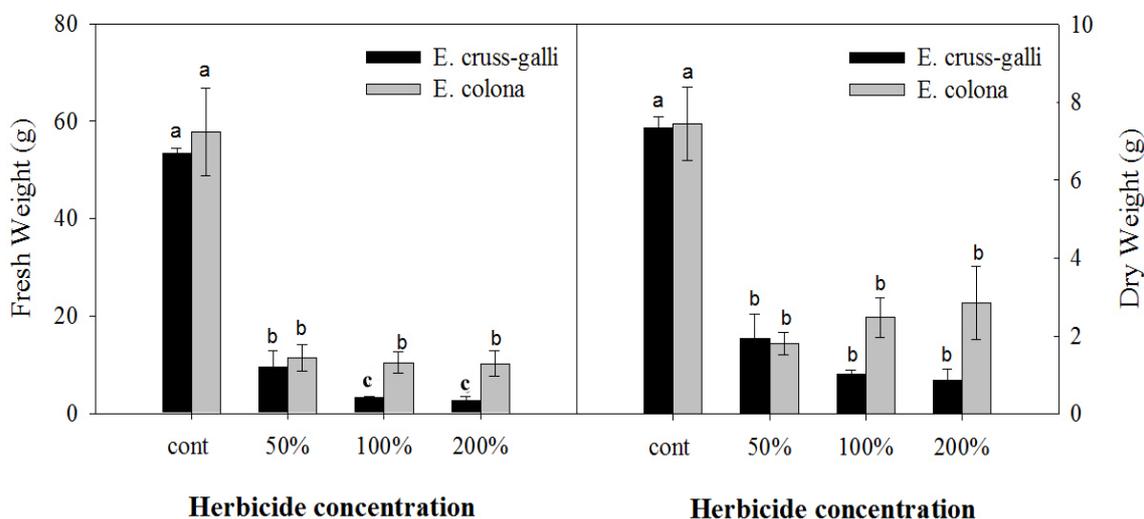


Fig.2. Effects of Nominee (50%, 100% and 200% FRD) on fresh weight and dry weight of *E. crus-galli* and *E. colona*. Bars are means \pm SE. Bars labeled with different letters are significantly different at $p < 0.05$.

Second experiment

Resistance of *E. colona* to Nominee

Biomass yield (fresh weight, dry weight and water content): The fresh and the dry weight was decreased by increasing the concentration of the applied herbicide compared to the control. Fresh weight was decreased by increasing the concentration of herbicide, at 50%, 100%, 200% and 300% by 80%, 90%, 90% and 92%, respectively (Fig. 3 and Fig.4 A).

Dry weight was decreased by increasing the concentration at 50%, 100%, 200% and 300% by 67.8%, 80.2%, 81.3% and 81.9%, respectively. Water content was slightly

variable at different herbicide concentrations, it was decreased compared to the control by increasing the concentration of herbicide at 50%, 100%, 200% and 300% by 7.8%, 11.9%, 10% and 16.8%, respectively (Fig. 4 B & C).

Physiological parameters

Photosynthetic pigments: Chlorophyll a was decreased by 25.5%, 36.6%, 43.8% and 67.4%, respectively at 50%, 100%, 200% and 300%, also carotenoids were decreased by 24%, 40%, 41.3 and 65.5%, respectively at 50%, 100%, 200% and 300%, but chlorophyll b was increased at 50% compared to the control then decreased by increasing the concentration up to 200% (Table 2).

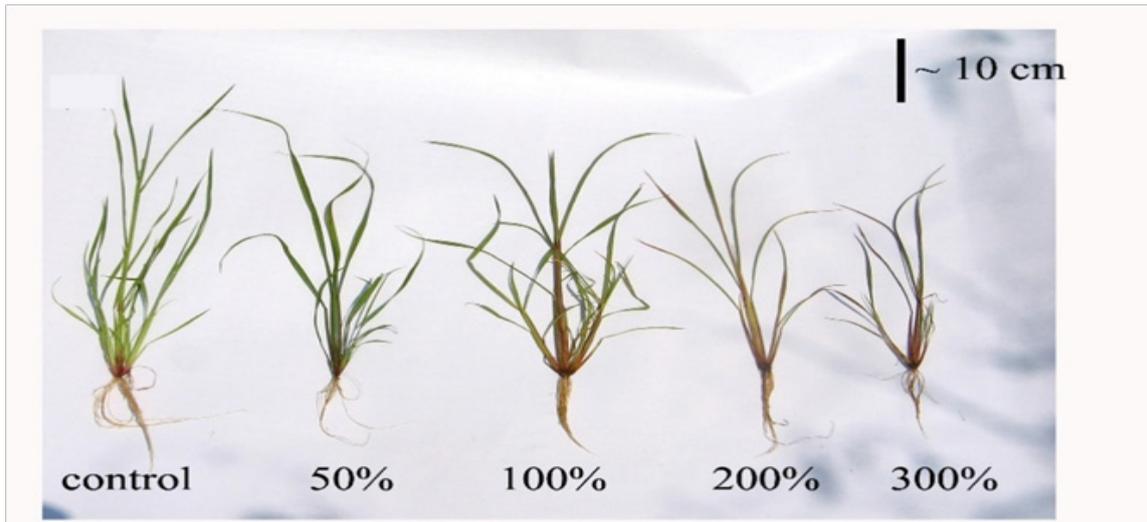


Fig.3. Effects of Nominee (50%, 100%, 200% and 300% FRD) on growth of *E. colona*, bar = 10cm.

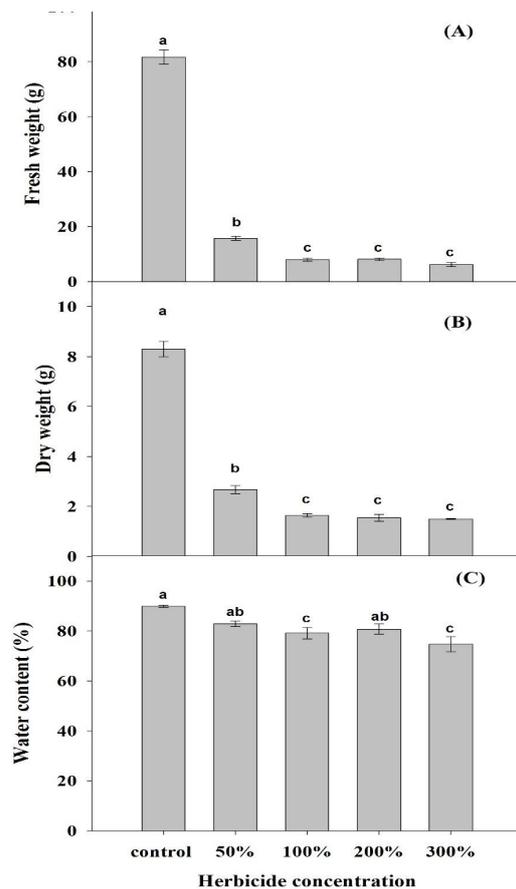


Fig. 4. Effects of Nominee (50%, 100%, 200% and 300% FRD) on fresh weight, dry weight and water content of *E. colona*. Data is mean \pm SE. Bars labeled with different letters are significantly different at $P < 0.05$.

Anthocyanins: The anthocyanins content were increased in comparing to the control by increasing the herbicide concentration, they were increased by 60.8%, 2 folds and 2.7 folds, respectively at 100%, 200% and 300% (Fig. 5).

Soluble and insoluble sugars : Both soluble and insoluble sugars content were affected according to the herbicide concentration. soluble sugars were increased compared to the control at 50%, 100%, 200% and 300% by 65.78%, 2.5 folds, 3 folds and 2.8 folds, respectively and insoluble sugars were increased at 100%, 200% and 300% by 36.7%, 72.2% and 18.5%, respectively. (Table 2).

Protein content: Protein content was affected with herbicide concentration, it significantly decreased by 18.4% at 300% FRD (Table 2).

Expression of acetolactate synthase (ALS) gene in response to Nominee treatments.

The transcript level of *ALS* in *E. colona* was modified by different concentrations of nominee. In absence of rice 7 days after treatment, it increased at 50% by 4 folds, then decreased at 100% by 93.7%, and was not detected in 200% and 300% (Fig.6).

TABLE 2 . The effects of Nominee (50%, 100%, 200% and 300% FRD) on chlorophyll A, chlorophyll B, carotenoid, soluble sugar, insoluble sugar and protein content of *E. colona* Data is mean±SE. Data in a column with different letters are significantly different at P<0.05.

Treatment	Physiological parameters					
	Chloro. A	Chloro. B	Carotenoids	Sol. sugar	Insol. sugar	Protein
Cont.	1.3±0.06 ^a	0.55±0.09	0.4±0.007 ^a	0.04±0.002 ^d	0.31±.003 ^c	5.9±0.07 ^d
50%	1.1±0.007 ^a	0.61±0.02	0.35±0.03 ^{ab}	0.14±.001 ^b	0.35±.002 ^b	7.9±0.19 ^{bc}
100%	0.7±0.01 ^b	0.43±0.01	0.3±0.009 ^{bc}	0.23±.004 ^a	0.37±.005 ^a	8.8±0.15 ^{ab}
200%	0.56±0.04 ^{bc}	0.37±0.03	0.24±0.005 ^{cd}	0.24±.003 ^a	0.38±.001 ^a	9.7±0.32 ^a
300%	0.18±0.02 ^c	0.07±0.002	0.17±0.002 ^c	0.11±.004 ^c	0.32±.003 ^c	7.18±0.23 ^{cd}
F	94.384***	12.559	45.747***	670.229***	92.209***	47.48***

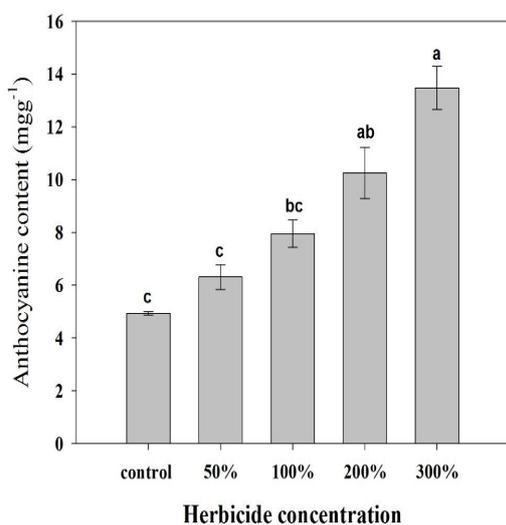


Fig.5. Effects of Nominee (50%, 100%, 200% and 300% FRD) on anthocyanins content of *E. colona*. Data is mean±SE. Bars labeled with different letters are significantly different at P<0.05.

Discussion

Herbicides have been used to enhance the productivity of crops by killing the weeds which compete with the growth of cultivated plants. In the present study, Nomineetm (bispyribac-sodium) was applied as herbicide and plant responses was studied on *E. crus-galli* and *E. colona*. A preliminary experiment indicated that the growth of *E. crus-galli* in terms of fresh and dry weight was reduced by 93.87% and 86.3%, respectively at 100% FRD. While *E. colona* growth in terms of fresh and dry weight was decreased at 100% FRD by 84% and 71%, respectively, but nonetheless, the plants were

still growing and young leaves were present (Fig. 1). Different responses to bispyribac-sodium were reported by Riar et al. (2012), who found that three *Echinochloa crus-galli* populations from rice fields in Arkansas (AR1 and AR2) and Mississippi (MS1) showed different responses, reduction in dry weight of AR2 was 99% (susceptible population), in resistant populations, the dry weight reduction was from 54% to 62% and from 71% to 73% in AR1 and MS1 populations, respectively. Kaloumenos et al. (2013) also indicated that bispyribac-sodium applied at the recommended rate reduced the fresh weight of nine susceptible *E. oryzicola* accessions by 83–100%. The present results suggest that *E. crus-galli* was susceptible to bispyribac-sodium but *E. colona* showed some resistance.

Investigation of the resistance of *E. colona* to Nominee

The dose response studies on plants treated with bispyribac-sodium confirmed that *E. colona* was resistant. Although the herbicide effect on the biomass of plants which caused reduction in fresh and dry. The fresh and dry weight of *E. colona*, was decreased by increasing herbicide concentration from 50% to 300% FRD (Fig. 3 and 4). Kaloumenos et al. (2013) reported that bispyribac-sodium applied at the recommended rate reduced the fresh weight of six *E. oryzicola* accessions by 63–77%. These accessions were labelled 'less Susceptible' and applied at 4 times the recommended rate reduced the fresh weight of two *E. oryzicola* accessions only by 16–33% and 12–31% respectively and these accessions were labeled 'Resistant'. Based on the present results, *E. colona* can be considered susceptible (fresh weight decreased by >80%).

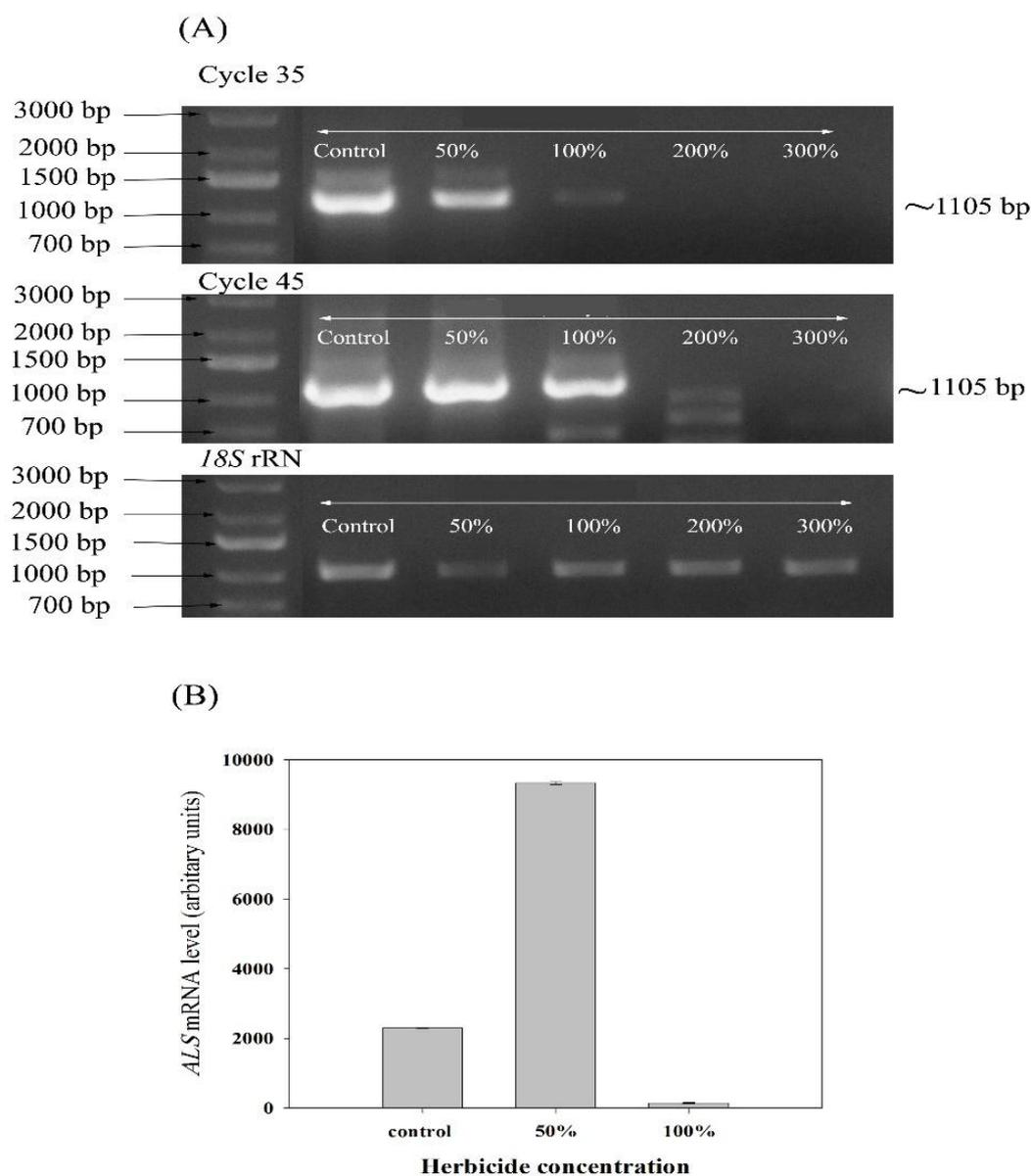


Fig. 6. Semi-quantitative RT-PCR of *E. colona* ALS under different concentrations of Nominee (50%, 100%, 200% and 300% FRD). (A): Ethidium bromide-stained gels after different cycles showing the growth of the bands at cycle 35 and at cycle 45 (saturated cycle). (B): Quantification of expression in terms of band volumes at cycle 35.

The photosynthetic pigments were affected by herbicide, where the chlorophyll a, chlorophyll b and carotenoid content was decreased by increasing the herbicide concentration from 1000 to 300% FRD (Table 2). Reduction in chlorophyll content may be due to its incorporation into the cell membrane function through physiological processes, such as depolarization of membrane potential (Wright, 1994). Reduction also might be due to the enhanced activity of 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 of chlorophyll and decreased its concentration (Hamza et al., 2012).

The anthocyanin content were increased by increasing the herbicide concentration from 100 to 300% FRD (Table 2). *E. colona* have anthocyanin pigment in leaves through their life span, or else they are induced and retained only after the plant has experienced stress (Chalker-

Scott, 1999). The function of anthocyanins in leaves is the photoprotection of chloroplasts; under saturated light, anthocyanins potentially mitigate photoinhibitory and photo-oxidative damage by absorbing a proportion of the photons surplus to the requirements of the light reactions of photosynthesis (Esteban et al., 2008 and Zeliou et al., 2009). This may be the case in the present study where, herbicide affects photosynthetic pigmentation which decreases. So anthocyanine pigments increased to protect the leaves from the stress of photoinhibitory light fluxes by absorbing the excess photons.

The soluble and insoluble sugar content was increased by increasing the concentration from 50 to 300% FRD (Table 2). Carbohydrate accumulation in leaves has been repeatedly reported after treatment with ALS inhibitors (Wittenbach & Abell, 1999). Zabalza et al. (2004) reported that decrease in sink strength seems to cause the carbohydrate accumulation in leaves. Plants with lowered sink strength (tuber excised) accumulated carbohydrate in the leaves and displayed a considerably reduced maximum photosynthetic rate (Basu et al., 1999). This agrees with the present results where accumulation of carbohydrates in leaves attached coincided with a decrease in plant growth rate.

The protein content was increased by increasing the concentration from 50 to 200% FRD then decreased by 18.4% at 300% FRD. Several biochemical and physiological effects have been described as a secondary consequence of the primary action of ALS inhibitors like decrease in protein levels (Sidari et al., 1998). It could propose that an increase in the protein content was detected because it measured in young leaves that may have been produced after application of herbicide. This view agrees with Rhodes et al. (1987) who suggested that decline in protein content was caused by an increase in the protein turnover.

Expression of acetolactate synthase (ALS)

The transcript level of ALS in *E. colona* was modified by different concentrations of bispyric-sodium (nominee), it increased at 50%, then decreased at 100%, and was not detected in 200% and 300% FRD. The present study indicated that ALS expression level was responsive to herbicide treatment. This suggests that ALS is a target site of bispyric-sodium. But 50% FRD can be considered as sub-lethal dose which upregulated the expression of ALS by 4 folds. Often, evolved ALS herbicide resistance in plants is target-site ALS based, endowed by one or more specific resistance-endowing point mutations in the ALS gene. Generally, ALS

resistance mutations at Pro-197 confer SU and TP resistance, mutations at Ala-122, Ala-205, Ser-653 or Gly-654 confer IMI resistance, and mutations at Asp-376, Arg-377 or Trp-574 confer broad spectrum resistance across ALS herbicides (Yu et al., 2012). So in the present results the resistance of *E. colona* to bispyric-sodium might be due to mutation in one of these points on ALS gene. Kaloumenos et al. (2013) reported that the sequence alignment comparison between the susceptible *E. crus-galli* and resistant *E. oryzicola* and putative resistant *E. oryzicola* accessions revealed a heterozygous mutation in resistant and putative resistant accessions at the second nucleotide of codon 574. The presence of Trp574Leu mutation in the 12 analysed *E. oryzicola* ALS sequences from resistant *E. oryzicola* supports the evidence that cross resistance to ALS-inhibiting herbicides was due to mutant ALS alleles encoding an amino-acid replacement at codon 574. So the resistance of *E. colona* to bispyric-sodium could be due to presence of Trp574Leu mutation.

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(Received 2 / 8 / 2017;
accepted 11 / 1 / 2018)

الإستجابات المختلفة لنباتى الدنبيه و أبوركبه لمبيد النوميى

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يعتبر نباتى الدنبيه و أبوركبه من الحشائش الضارة المؤثرة على نمو وإنتاجية بعض المحاصيل الإقتصاديه مثل الأرز والقطن ، لذا كانت مقاومة هذه النباتات ضرورية. فقمنا بعمل تجريبه أولية لمعاملة كلا النباتين بمبيد الحشائش نوميى لمعركة مدى إستجابتهما له ، فتبين أن نبات الدنبيه قد تم القضاء عليه بينما نبات أبوركبه أظهر مقاومه لهذا المبيد عند تركيزات أعلى من المقترحه عند الإستخدام. فقمنا بعمل تجريبه تأكيديه لمقاومة نبات أبو ركه لمبيد النوميى فتبين من نتائج الدراسة أنه بزيادة تركيز مبيد النوميى يقل الوزن الغض والجاف لنبات أبو ركه و كذلك يقل البناء الضوئى بينما يزداد صبغ الأنثوسيانين لحماية الأوراق عن طريق امتصاص كمية الفوتونات الزائدة. واتضح من الدراسه الجينيه لإنزيم ALS وهو الموقع المستهدف لهذا المبيد لأنه يلعب دورا رئيسيا فى إنتاج الأحماض الأمينيه طويلة السلسه وأن مستوى هذا الإنزيم يقل بزيادة المعامله و لكن عند تركيز 05% من التركيز المقترح تبين أن هذا التركيز يعتبر جرعه غير مميتة محفزة لإنتاج الإنزيم بمقدار أربعة أضعاف.