Accepted Manuscript

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PII: S0048-9697(19)32070-4
DOI: https://doi.org/10.1016/j.scitotenv.2019.05.058
Reference: STOTEN 32219

To appear in: *Science of the Total Environment*

Received date: 18 February 2019
Revised date: 5 May 2019
Accepted date: 5 May 2019

Please cite this article as: J. Xie, W. Tang, L. Zhao, et al., Enantioselectivity and allelopathy both have effects on the inhibition of napropamide on Echinochloa crus-galli, Science of the Total Environment, https://doi.org/10.1016/j.scitotenv.2019.05.058

Enantioselectivity and allelopathy both have effects on the inhibition of napropamide on *Echinochloa crus-galli*

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Abstract: Napropamide is a chiral acetamide herbicide commonly applied to control *Echinochloa crus-galli* in maize. The inhibition effect may be enantioselective for *Echinochloa crus-galli* and maize. It may also be affected by the potential allelopathy at field condition. To investigate this, we have examined the inhibition effect of napropamide on *Echinochloa crus-galli* mono-cultured or co-cultured with maize at field conditions. Our results on morphology, physiology, chlorophyll content and chlorophyll fluorescence suggest that *R*-napropamide has stronger inhibitory effect than *Rac*-napropamide and *S*-napropamide on *Echinochloa crus-galli*, while none of them affects maize. We found that both glutathione-*S*-transferase (GST) genes and oxidative enzymes (superoxide dismutase, malondialdehyde) played roles in the inhibition. Accumulations of napropamide in *Echinochloa crus-galli* were more prominent in roots than in shoots, and no enantioselectivity was found in medium dissipation. We have observed relative allelopathy when applying napropamide to *Echinochloa crus-galli* co-cultured with maize.The results warrant further field studies on the enantioselectivity and allelopathy of herbicides.

Key words: napropamide; *Echinochloa crus-galli*; environmentally realistic conditions; relative allelopathy; enantioselectivity

1. Introduction

Weed is one of the important limitations which causes negative interference to achieve high levels of crop productivity. According to United Nations Food and Agriculture Organization, weed causes approximately $95 billion losses in crop yields annually (Sukkel, 1990). *Echinochloa crus-galli* (*E.crus-galli*) is one of the world's most aggressive weeds (Randall, 2004). It can consume approximately 80% of the available soil nitrogen. Moreover, it can act as a host for mosaic virus diseases. To combat weeds such as *E.crus-galli*, herbicide is one of obvious choices.

Napropamide (*N, N*-diethyl-2-(1-naphthalenyloxy) propanamide) (Fig.1) is one of the most commonly applied selective systemic amide herbicides (Cycoń et al., 2013). It is widely used for controlling a number of monocotyledon and broadleaf weeds (Qi et al., 2015). Because napropamide is slightly soluble in water, it can be easily passed into the tissues (Biswas et al., 2007). For effective control of *E.crus-galli*, napropamide needs to be applied at application rate of 1500g ai ha^{-1}. Since napropamide has relatively long life-time in soils (dissipation half-life in soils ranges from 25 to 152 d) and significant toxicological properties to crops, weeds, microorganisms, and other living organs such as earthworm, there are significant concerns on its fate and transport (Cui and Yang, 2011; Guo et al., 2008, 2009; Zhang et al., 2010). Attributing to the single chiral C-atom in the napropamide structure, it has a pair of enantiomers including *R*-napropamide and *S*-napropamide. Although these enantiomers shared identical physiochemical properties in achiral environment, they can display remarkable differences in biochemical processes (Garrec and Jordan, 2004; Rudolf et al., 2002; Liu et al., 2005; Gu et al., 2008). Early studies suggest that enantioselectivity exists in herbicide toxicity for both target and non-target organisms (Qi et al., 2015; Xie et al., 2018). For example, *R*-napropamide's toxicity is stronger to *E. crus-galli* but weaker to algae

than that of *S*-napropamide (Xie et al., 2018). In addition, (-)-Napropamide is more toxic to soybean and cucumber than both racemate and (+)-napropamide (Qi et al., 2015). Although the exact mechanism has not been elucidated, napropamide may have an inhibitory effect on DNA, RNA , and/or protein synthesis (Di et al., 1988). Furthermore, degradation of napropamide enatiometers in soil, vegetables have been evaluated (Qi et al., 2014). Only slight stereoselective (the enantiomer fraction was 0.46) degradation has been observed in cabbage, and there was no enantioselectivity in napropamide degradation in tomato, cucumber, and grape. However, these reported results may not be applicable to field condition because i) dosage used in experiments was much higher than the concentrations in field conditions; ii) the mechanisms of enantioselectivity were not known; and iii) those early studies ignored the potential allelopathy when weeds and crops are growing together in the field. Thus, using experimental conditions that can closely mimick the natural fields is imperative in order to accurately determine the enantioselectivity of napropamide.

In this study, we have selected *E.crus-galli* and co-cultured maize as the target organisms for enantioselective analysis of napropamide at concentrations similar to the field relevant concentrations. Plant morphology, physiology, chlorophyll content, chlorophyll fluorescence, *glutathione-S-transferase* (GST) genes, and oxidative stress were investigated under both mono-cultured and co-cultured conditions. Moreover, environmental behavior of napropamide was evaluated through analyzing accumulation differences both in roots and shoots and residues in the medium. Relative allelopathy was tested and analyzed by comparing the phenomenons, behaviors and mechanisms under the two conditions.

2. Materials and Methods

2.1 Test chemicals

Napropamide (purity>85%) was obtained from Jiangsu Rudong pesticide factory (Rudong, China). The enantiomers with purity>99% were prepared by HPLC as described in our previous study (Xie et al., 2016). Working solutions of each compound were prepared in acetone immediately prior to the experiments. Chemicals and solvents utilized in the experiment were of LC/MS grade, obtained from Thermo Fisher Scientific (Shanghai, China). All the other reagents used in this study were of HPLC grade. The sorbents and analytical grade chemicals used for extraction and clean-up were purchased from Welchrom (Jinhua, China). Triphenyl phosphate (99.8%, GC) was used as internal standard and was purchased from Aladdin (Shanghai, China).

2.2 Plant materials and growth conditions

Mature seeds of *E.crus-galli* from over 300 individual plants were collected and combined in October (2015) from Fuyang District in Hangzhou, China (30.04N; 119.55E). The seeds were air dried and stored in paper bags at 4ºC for 3 months to break dormancy, and then stored at room temperature (20±5 ºC) until used. Based on preliminary germination and herbicide-screening experiments (data not shown), the seeds had 85% germination rate and were susceptible to napropamide. A waxy maize variety (Meiyu 3, Hainan Luchuan Seed Co., Ltd., Haikou, China) was selected as the crop material.

2.3 Herbicide treatment

Thirty seeds of *E.crus-galli* were sown in 9-cm-diameter plastic pots or mixed with 10 maize seeds in 15-cm-diameter pots at 0.5 cm depth. The pots were filled with potting medium (1:1:1:2 vegetable garden soil/compost/peat/dolomite, with pH at 6.3 and organic matter content at 13.7%) up to 4/5 volume. The working medium was free of napropamide. The two groups were treated at 0, 50.6, 101.3, 151.9, 202.5, 303.8 and 405.0 g ai ha^{-1} (lower than the field recommended dose) 24 h after sowing for *Rac*-, *R*- and *S*- napropamide,

respectively. A compressed air laboratory spray tower equipped with a Teejet TP6501E flat fan nozzle was used to deliver 450 L ha^{-1} at 0.28 MPa. After herbicide application, plants were cultivated in a screenhouse (a 8 m × 20 m chamber framed with 2-cm iron mesh) at CNRRI. The moisture of soil was maintained by adding water to trays under the pots throughout the experiment. Experiments were arranged in a randomized complete block factorial design with three replications for each herbicide dose and were performed twice. Three weeks after treatment, the germination rates, morphology and physiology of *E.crus-galli* and maize were determined for *Rac*-, *R*- and *S*- napropamide treatments, respectively. The shoot length, seedling height and aboveground fresh weight of *E. crus-galli* were expressed as a percentage of the untreated control to standardize comparisons between treatments. A non-linear four-parameter log-logistic curve (Equation 1) (Seefeldt et al., 1995) was used to fit these data by using Origin v.8.0. The effective dose of herbicide causing 50% growth reduction (GR_{50}) with respect to the untreated control was calculated for each herbicide. The model fitted was

$$y=C+(D-C)/\{1+\exp[b(\log x-\log GR_{50})]\}, \text{ Eq.(1)}$$

where *C* is the lower limit of the response, *D* is the upper limit of the response, x is the herbicide application dose, *b* is the slope of the curve through GR_{50}, and y is the response at the herbicide dose X.

For the 0, 50.6, 303.8 and 405.0 g ai ha^{-1} treatments in co-cultured, survived plants were carefully removed and rinsed in tap water. Plant height, above-ground biomass and root length were determined after drying with absorbent paper. For the determination of antioxidant system response, chlorophyll content, chlorophyll fluorescence, transcription of glutathione *S*-transferases (GSTs) genes in *E.crus-galli*, and enantioselective degradation and

enantiomeric transformation in medium, the plants or medium at 0, 101.3, 202.5g ai ha^{-1} treatments were chosen.

2.4 Analyses of chlorophyll in E.crus-galli

Chlorophyll content was extracted and measured according to our previous study (Xie et al., 2019). Calculations were completed from the absorption spectra using Arnon's method (Arnon et al., 1949) and expressed as mg g^{-1} dry weight.

2.5 Determination of Chlorophyll Fluorescence

After dark adaptation of the plants for more than 30 min, the chlorophyll fluorescence was determined with Technologica CFImager (Technologica, UK). The parameters were calculated and recorded.

2.6 GST genes

E.crus-galli (the whole plant) were separately grounded in liquid nitrogen using a ceramic mortar and pestle. Total RNA was extracted using a TaKaRa MiniBEST Plant RNA Extraction Kit (TaKaRa, Japan). Nucleic acid concentrations were measured spectrophotometrically at 260 nm, and the purity was tested by the ratio of 260/280 nm. First-strand cDNA was reverse transcribed from 1 μg of the total RNA using TaKaRa PrimeScript^{TM}RT Master Mix (Perfect Real Time) (TaKaRa, Japan) with Oligo dT as a primer. The PCR strategy was employed to create a fragment containing the complete coding region of *EcGSTZ1* and *EcGSTF1*. The primer pairs used for the *EcGST* genes followed Wang's report (Wang, 2013) and listed in Table S1. They were amplified from the cDNA using pairs of the primers by Premix TaqTM (ExTaqTM Version 2.0 plus dye). The integrity was tested by electrophoresis on a 1.2 % agarose formaldehyde gel. Real-time PCR was performed using a SYBR Premix Ex Taq^{TM} (Tli RNaseH Plus) (TaKaRa, Japan) with an Agilent Technologies Stratagene Mx3000P (Agilent, Japan). The amplification regime

consisted of an initial denaturation step of 95°C for 30s, followed by 40 cycles of 95°C for 5s and 60°C for 34s. *β*-Actin was used as a housekeeping gene to normalize the expression profiles (Qian et al., 2012). The relative gene expression among the treatment groups was quantified using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.7 Enzyme activity assay

E.crus-galli and maize (the whole plant, 0.05 g) were separately homogenized in ice-cold extraction buffer containing 0.01mol L^{-1} phosphate buffered saline (PBS) buffer (m/v=1/4). The homogenate was obtained with centrifugation at 4000 rpm (1800 g) for 10 min at 4°C. The supernatant was used as a crude extract for the assay of enzyme activities.

The activity of superoxide dismutase (SOD) and lipid peroxidation level reflected by malondialdehyde (MDA) were determined by kits (MSK, Wuhan, China) following the manufacturer's instructions.

2.8 Dissipation and Bioaccumulation experiment

Medium soil was sampled using a sterilized spatula, dried at room temperature, homogenized and sieved (2 mm). 5.0 g of dried homogenized medium and minced *E.crus-galli* (whole plant) sample was extracted separately and cleaned based on the QuEChERS method (He et al., 2015). A 1.0 ml supernatant sample was dried through a gentle stream of nitrogen, and triphenyl phosphate (TPP) used as an internal standard was added to give a final concentration of 20 μg L^{-1}. The residue was then adjusted to 1 ml with *n*-heptane and filtered through a 0.22 μm nylon syringe filter.

Napropamide enantiomers were determined by supercritical fluid chromatography system Acquity UPC^2 (Waters, Prague, Czech Republic) consisting of an Acquity UPC^2 binary solvent manager, Acquity UPC^2-FL sample manager, isocratic solvent manager and Xevo TQ-S detector. AMY1 column used for separation was coated by amylose tris (3, 5-

dimethylphenylcarbamate), and 150×3.0 mm i.d with 2.5 μm particles. The enantioseparation conditions were based on a previous study by Zhao (Zhao et al., 2018).

Blank samples were analyzed to evaluate the interference from the matrix. The linearity was determined by linear regression analysis of both standard solution and matrix-matched calibration curves (He et al., 2017). External calibration curves with six standard solutions between 1 and 60 μg L^{-1} of *R*-/*S*- napropamide were used in the calculations. Under these conditions, *S*-napropamide and *R*-napropamide eluted at 1.03 and 1.15 min, respectively. *Rac*-napropamide was found to yield an exact peak area ratio of 1:1 (*R*/*S*) in both standard solutions and soil extracts. The internal standard TPP eluted at 1.05 min. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on signal-to-noise (S/N) ratio of 3 and 10, respectively. The LOQs for *R*-and *S*-napropamide were found to be 0.5 μg g^{-1}, and the LODs were 0.2 μg g^{-1}. Recovery test was carried out by simultaneously adding *R*-/*S*- napropamide into blank samples (medium without napropamide) at three concentration levels (1, 3, 9 μg g^{-1}). The mean recoveries of the enantiomers ranged from 79.8% to 106.4%. The precision of the method for all chemicals was measured by three replicates.

S-napropamide eluted earlier than *R*-napropamide. Thus, the enantiomer fraction (EF) was used to measure the enantioselectivity of the bioaccumulation of napropamide (Eq.(2)).

$$EF=\text{concentration of } S/(R+S) \quad (2)$$

The EF values range from 0 to1, with EF=0.5 representing the racemic mixture.

2.9 Data analysis

Data obtained from screenhouse experiments were tested for normality and analysis of variance (ANOVA) was performed with the use of SPSS software (version 13.0, SPSS Inc., Chicago, IL). No interactions ($P > 0.05$) occurred between year and biotype for any of the

parameters. Therefore, data of the repeat experiments were pooled. The comparison of means was performed using Fisher's protected least significant difference (LSD) test, where the overall differences were significant ($P \leq 0.05$).

3. Results and Discussions

3.1 Seed bioassay

Seedling emergence of *E. crus-galli* occurred 4 days after sowing. Both *E. crus-galli* and maize reached 2-leaf-stage at the same time in the co-culture conditions. The *E. crus-galli* mono-cultured experiments showed that GR_{50} values of the seedling emergence were 202.5, 136.8 and 724.2 g a.i.ha^{-1} for *Rac*-, *R*- and *S*-napropamide, respectively (Fig. S1). However, the EC_{50} values turned to 177.4, 122.8 and 704.2 g a.i.ha^{-1} , respectively for co-cultured experiments. The slightly but not significantly shift in EC_{50} values in mixture suggested that the co-growth with maize may enhance the inhibition effects of napropamide. Thus, spraying napropamide after maize seedling can maximize weed control efficiency. The emergence percentage of maize was 93% to 100% for *R*ac, *R*- and *S*-napropamide treated does, therefore napropamide has no inhibition effect on the emergence rate of maize. The tolerance of plant species to herbicide is well known (Jablonkai, 2013), suggesting that maize be resistant to the napropamide.

3.2 Enantioselective effects of napropamide enantiomers on plant growth

Root and shoot lengths are usually used as indexes of growth because they are important agronomic traits that can be easily affected by environmental stress (Doncheva et al., 2005; Li et al., 2010). Growth inhibition of *E. crus-galli* by napropamide occurred in our experiments even at low concentration (50.6 g ai ha^{-1}). With increasing treatment concentrations, the extent of inhibition on root and shoot growth and on fresh weight changed from less than 20% (50.6 g ai ha^{-1}) to nearly 80% (405.0 g ai ha^{-1}, Fig. S2). The same effect

has also been observed on the enantiomers of napropamide. Furthermore, EC_{50} values suggests the inhibition on root biomass was more noticeable than on shoots, with 217.5g ai ha^{-1}(EC_{50}) for roots and 248.4 g ai ha^{-1}(EC_{50}) for shoots, respectively . This phenomenon was consistent with previous studies which reported that roots as the major absorptive organ for napropamide (Eshel et al., 2010; Gressel, 1978).

When *E. crus-galli* was mono-cultivated, application rate required for 50% inhibition of seedling root length, height and fresh weight for *R*-napropamide were 196.6, 119.2 and 225.2 g a.i. ha^{-1}, respectively. In contrast, *Rac*- (217.5, 248.4, 250.0 g ai ha^{-1}) and *S*-napropamide (249.8, 281.0, 278.7 g ai ha^{-1}, Table 1) require slightly higher application rates. For the selected three doses (50.6, 303.8, 405.0 g ai ha^{-1}) in the co-culture experiment, seedling root lengths of *E.crus-galli* treated by *S*-napropamide (6.1, 5.0, 4.3 cm) were higher than *Rac*- (4.2, 3.2,3.0) and *R*-napropamide (4.0, 3.1, 2.8 cm, Fig S3, a). There was no significant difference in seedling height and fresh weight of *E.crus-galli* for racemic and enantiomers of napropamide at 50.6 g a.i. ha^{-1}. However, at higher doses (303.8 and 405.0g a.i. ha^{-1}), seedling height and fresh weight of *E. crus-galli* treated with *S*-napropamide was higher than with *Rac*- and *R*-napropamide (Fig S3, b, c), and *R*-napropamide resulted in the highest reduction in seedling height and fresh weight. These results indicate that *R*-napropamide provides relatively higher degree of inhibition of the weed.

In the co-cultured experiments, the inhibition on *E. crus-galli* by racemic and enantiomers of napropamide was more pronounced (the detailed data were shown above) than in mono-cultured experiments. It might be due to enhanced inhibition effects by maize to napropamide on *E. crus-galli*. The observation can be explained by the allelopathy phenomenon in which certain plant can affect co-cultured plants. Previous studies have indicated that about 3-4% of the rice accessions throughout the world showed an allelopathic

potential towards weeds (Dilday et al., 1994). The enhanced inhibition effect in our co-cultured experiments clearly displayed the allelopathy between maize and napropamide on *E. crus-galli* which has not been reported before. This phenomenon needs to be further studied to reduce the napropamide dosage in order to promote sustainable agriculture practice (Fang et al., 2015).

3.3 Effects of napropamide enantiomers on chlorophyll content

Chlorophyll (Chl) content may change in response to severe environmental changes. At 101.3 g a.i.ha^{-1}, no significant differences were observed between the control and all treatments except a small increase total chlorophyll content by *R*-napropamide (0.48 to 0.56 mg g^{-1}). The small change in Chl content may be due to a greater effect of stress in leaf expansion than in Chl biosynthesis (Perveen et al., 2010), by which increase Chl content can be used as a strategy to resist external stress. At 202.5 g a.i.ha^{-1}, Chl content was significantly lower than that of control (0.48±0.04 mg g^{-1}). After three weeks' treatments, the total chlorophyll content decreased to 0.36±0.02 mg g^{-1} with *Rac*-napropamide, 0.27±0.027 mg g^{-1} with *R*-napropamide and 0.38±0.03 mg g^{-1} with *S*-napropamide. In the co-cultured experiments, total Chl contents did not decrease when compared to the control (0.44±0.03 mg g^{-1}) under *Rac*-napropamide (0.34±0.03 mg g^{-1}) and *S*-napropamide (0.37±0.05 mg g^{-1}), but decreased significantly under *R*-napropamide (0.20±0.01 mg g^{-1}) at 202.5 g a.i.ha^{-1}（Fig.2). This suggests that, the toxicity of *Rac*-napropamide and *S*-napropamide to *E. crus-galli* chlorophyll synthesis was lesser pronounced than that of *R*-napropamide. However, chlorophyll content of maize remains unchanged. This result suggests that the co-culture of maize in the field may enhance the effect of weed control.

3.4 Effects of napropamide enantiomers on chlorophyll fluorescence

Chlorophyll fluorescence reflects thylakoid membrane organization and photosynthetic function (Rimando et al., 1998). The maximum photochemical efficiency of photosystem II (PSII) in dark-adapted leaves (F_v/F_m; F_v, variable fluorescence; F_m, maximal fluorescence) was unaffected by low-concentration (101.5g a.i.ha^{-1}) of napropamide. At 202.5g a.i.ha^{-1}, however, the *R*-napropamide showed the stronger inhibitory effect than that of control. However, the difference in inhibitory effect was insignificant, F_v/F_m values were 0.816 and 0.795 for control and *R*-napropamide treated samples, respectively. However, the inhibitory effects are more pronounced for co-cultured experiments. The ratio of F_v/F_m decreased from 0.802 (control) to 0.775, 0.739 and 0.785 for *Rac*-, *R*- and *S*-napropamide, respectively (Fig.3). It is known that the F_v/F_m ratio for most C_3 plants ranges between 0.8 and 0.85, and a decrease in F_v/F_m value suggests that plants are under the stress (Björkman and Demmig, 1987). However, the difference in F_v/F_m ratio between control and treated samples are much smaller in our study compared to similar study(Kaiser et al., 2013). This maybe attributed to the fact that napropamide has more pronounced inhibition effect for roots than shoots (Eshel et al., 2010; Gressel, 1978). The changes in quantum yield of photosystem Ⅱ (φPSII) followed the same trend as F_v/F_m. In contrast to F_v/F_m and φPSII, the level of non-photochemical quenching (NPQ) was higher for napropamide racemate (1.601) and enantiomers (1.606 with *R* and 1.42 7 with *S*) than control (1.314) at 202.5g a.i.ha^{-1}. However, for co-cultural experiment, NPQ level was increased by 4.50%, 56.17% for *Rac*-and *R*-napropamide, respectively, while it was decreased by 24.51% for *S*-napropamide. The F_v/F_m ratio and NPQ level remained constant for maize, suggesting maize was unaffected by napropamide under tested concentrations.

3.5 Gene expression of GST in E. crus-galli

The expressions of the two GST genes were amplified after napropamide exposure. *EcGSTF1* (phi class) and *EcGSTZ1* (zeta class) belonging to specific classes of plant GSTs are differentially regulated in their response to stress. The expression of *EcGSTF1* in *E. crus-galli* (both in mono-cultured and co-cultured experiments) was not significantly influenced by *S*-napropamide at 202.5 g a.i.ha^{-1}. Moreover, real-time PCR suggests transcript level of *EcGSTF1* in *R*-napropamide treated *E.crus-galli* was higher than *Rac*-napropamide treated samples (Fig.4). The transcript level of *EcGSTF* for *R*-treated *E.crus-galli* was 1.94 and 1.10 times higher than those treated by *S*- and *Rac*-, respectively(in *E.crus-galli* exposure experiments). In the co-cultured case, enantioselectivity became more prominent. *EcGSTF1* expression in the *R*-treated *E.crus-galli* was 2.60 times higher than the *S*-treated samples. In addition, we have observed that napropamide exposure significantly increased the expression of *EcGSTZ1*. In the mono-cultured cases, the expression level has increased by 2.50, 2.94, and 1.95 times for *Rac, R*, and *S*-napropamide treatments, respectively, compared to that of the controlled. Those levels increased to 2.88, 4.11, and 2.14 times, respectively for co-cultured experiments. In fact, GST genes can both encode GST and promote the expression of GSTs in herbicide metabolism, which protects *E.crus-galli* from napropamide. Therefore, our results suggest that the *EcGST* genes might be associated with napropamide resistivity in *E. crus-galli* (Li et al., 2016).

3.6 Activities of oxidative-stress related enzymes

Oxidative stress, which results from the deleterious effects of reactive oxygen species (ROS), occurs when plants are exposed to contamination (Bowler et al., 1992). Antioxidative enzymes of SOD in plants play a major role in scavenging ROS that were produced under oxidative stress (Alscher and Hess, 1993). SOD activity of *E. crus-galli* is napropamide concentration dependent (Fig.5). For mono-culture experiments, insignificant change in SOD

activity was found between different treatments at 101.3 g a.i.ha^{-1}. In detail, compared with control, SOD activity was 122.67%,128.81%, and 120.21% for *Rac*-napropamide, *R*-napropamide, and *S*-napropamide, respectively. In contrast, the SOD activity showed significant differences between the treatments at 202.5 g a.i.ha^{-1}. SOD activities were 165.51%, 207.05%, and 133.71% of the control with *Rac*-, *R*- and *S*-napropamide, respectively. Similar trend in SOD activity of *E.crus-galli* has been observed for co-cultured experiments. More specifically, compared with control, SOD activities were up-regulated after napropamide exposure. In addition, *R*-napropmide has more pronounced effect on SOD activities than *S*- and *Rac*-napropamide. This result suggest that *R*-napropmide had the highest weed control ability among its antipode and the racemate. Moreover, *R*-/*Rac*-napropamide ratio and *R*-/*S*-napropamide ratios of SOD activity in *E.crus-galli* were1.38 and 1.77, which are higher than those of mono-cultured (1.25 and 1.54 for *R*-/*Rac*-napropamide ratio and *R*-/*S*-napropamide ratios of SOD activity). This suggests when co-cultured with maize, the enantioselectivity on SOD activity was more pronounced. As a byproduct of lipid peroxidation, MDA production is usually accompanied with the formation of ROS (Sun et al., 2016). Variation of MDA contents shared similar trend as SOD does for *E.crus-galli* (mono-culture and co-culture). However, comparing with SOD, the changes in MDA were subtler, and no significant signal was shown. The up-regulation of SOD and MDA suggest that they were necessary for antioxidative defense. Stereoselectivity in enzymes hydrolysis may lead to different behaviors of enantiomers in biotransformation, metabolism, and transportation in cells and organisms (Hu et al., 2010). As lipid peroxidation is linked to enzymatic activity of antioxidants (Soleimanzadeh et al., 2010), increase in MDA contents in *E.crus-galli* after napropamide treatments may be an indication that SOD was a critical factor in oxidative stress related damages (Liu et al., 2012). However, no differences in SOD and MDA levels

of maize were observed after napropamide exposure. This suggests that the oxidative damage was more obvious for *E.crus-galli* than maize.

3.7 Enantioselectivity in biodegradation and bioaccumulation of napropamide

To gain insights of napropamide's enantio-activity on *E.crus-galli*, concentrations of napropamide in weed tissues and potting medium were measured at the end of the study. We found that the roots accumulated more napropamide than shoots, which may be due to the direct exposure of roots to napropamide (Zhang et al., 2010). Our morphology test also confirms that roots were major adsorptive organ for napropamide.

We found that the dissipation of napropamide was slightly faster when higher concentration (202.5 g ai ha^{-1}) was applied (Fig.6). Table S2 suggests $C_{202.5}/C_{101.3}<2$. For mono-culture, the enantiomer fractions (EFs) of undegraded napropamide in weeds growth medium were 0.478 and 0.483 for 101.3, 202.5 g ai ha^{-1} treatments, respectively.The EFs were 0.472 with 405.0 g ai ha^{-1}in co-culture experiments. Since EFs were close to 0.5 at our experimental condition, the dissipation of napropamide maybe non-enantioselective. However, the steady EFs may also be contributed by the rapid racemization. To investigate a possible interconversion of enantiomers, further treatment with pure *R*-napropamide was tested. These experiments confirmed that, no *S*-napropamide was formed from the *R*-enantiomer. The opposite process was also performed, showing no *R*-napropamide occurrence (Buerge et al., 2015). The rate of dissipation of the enantiomers was similar to that of *Rac*-napropamide. The above results suggested that, conversion of enantiomers did not occur, and the degradation of napropamide was not enantioselective. Since enantioselectivity is generally attributed to a set of specific microbial processes, our finding suggests that in our experiments either the specific microorganisms was absent or the medium pH, organic carbon,

or medium texture were unsuitable for the activity of the specific microbial community (Lewis et al., 1999; Buerge et al., 2006; Jones et al., 2007).

3.8 Maize to napropamide disguised allelopathy

Allelopathy is a biological phenomenon that refers to the beneficial or harmful effects of one organism on another by influencing its growth, survival and reproduction through the release of chemicals into the environment (Rice, 1984). It plays an important role in natural and agricultural ecosystems (Jabran et al., 2015). Our data suggest that the inhibition effect of racemate napropamide and napropamide enantiomers on *E.crus-galli* co-cultured with maize was more apparent than mono-cultured *E.crus-galli*, suggesting a disguised relative allelopathic potential of maize to napropamide on *E.crus-galli*. Previous studies have reported that, plant secondary metabolites and plant gene clusters are often responsible for the biosynthesis of the allelopathic compounds (Khanh et al., 2010). In maize, there are gene clusters located on Chromosome 4 consisting of eight genes (BX1 to BX8), which can biosynthesize the allelopathic 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA). DIMBOA is a protective and allelopathic defense compound, which can significantly inhibit the growth of *E.crus-galli* (Frey et al., 2009).

Moreover, other than the metabolic products excreted from maize, other factors may contribute to the allelopathy between two plants, including light (shading) and soil conditions such as soil moisture, nutrients, and minerals. Thus, the relative allelopathy discovered by us is a preliminary allelopathy, more work need to be done to elucidate its mechanisms.

4. Conclusion

Our result on *E.crus-galli* morphology, physiology, chlorophyll content, chlorophyll fluorescence, GST genes, and oxidative stress under te simulated field conditions suggested napropamide could enantioselectively inhibit *E.crus-galli*. In addition, *R*-napropamide

showed stronger inhibitory effect than *Rac*-napropamide and *S*-napropamide. We found no enantioselective dissipation occurred in medium. The roots of *E.crus-galli* were more prone to napropamide accumulation than shoots. A disguised relative allelopathic potential of maize to napropamide on *E.crus-galli* has been observed.

Acknowledgements

The authors acknowledge financial supports from the National Natural Science Foundation of China (41806126, 21427815 AND 213320102007), A SPECIAL FUNDING FOR THE DEVELOPMENT OF SCIENCE AND TECHNOLOGY OF SHANGHAI OCEAN UNIVERSITY (A2-0203-00-100227), AND DOCTORAL SCIENTIFIC RESEARCH FOUNDATION OF SHANGHAI OCEAN UNIVERSITY (A2-0203-00-100351).

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Figure Legends

Figure 1 The structures of napropamide enantiomers

Figure 2 Chlorophyll content of *E.crus-galli* (mono-cultured and co-cultured) and maize in the medium.

Figure 3 Chlorophyll fluorescence parameter F_v/F_m content of *E.crus-galli* (mono-cultured and co-cultured) at 202.5 g a.i.ha^{-1}.

Figure 4 Changes in GST gene transcription in *E.crus-galli* after napropamide racemate and enantiomers treatments.

Figure 5 The activity of superoxide dismutase (SOD) and malondialdehyde content in *E.crus-galli* (mono-cultured and co-cultured) and maize after napropamide treatment (a) SOD in mono-cultured *E.crus-galli*; (b) in co-cultured *E.crus-galli*; (c) MDA in mono-cultured *E.crus-galli*;
(d) MDA in co-cultured *E.crus-galli*.

Figure 6 The SFC-MS/MS chromatograms for the analysis of the napropamide enantiomers, internal standard and heptanes (a) napropamide enantiomers; (b) internal standard, triphenyl phosphate (TPP); (c) heptanes

Table 1. Effect of *R*ac, *R*- and *S*-napropamide on seedling root length, seedling height and seedling fresh weight of *E. crus-galli* after 21 days in the screenhouse.

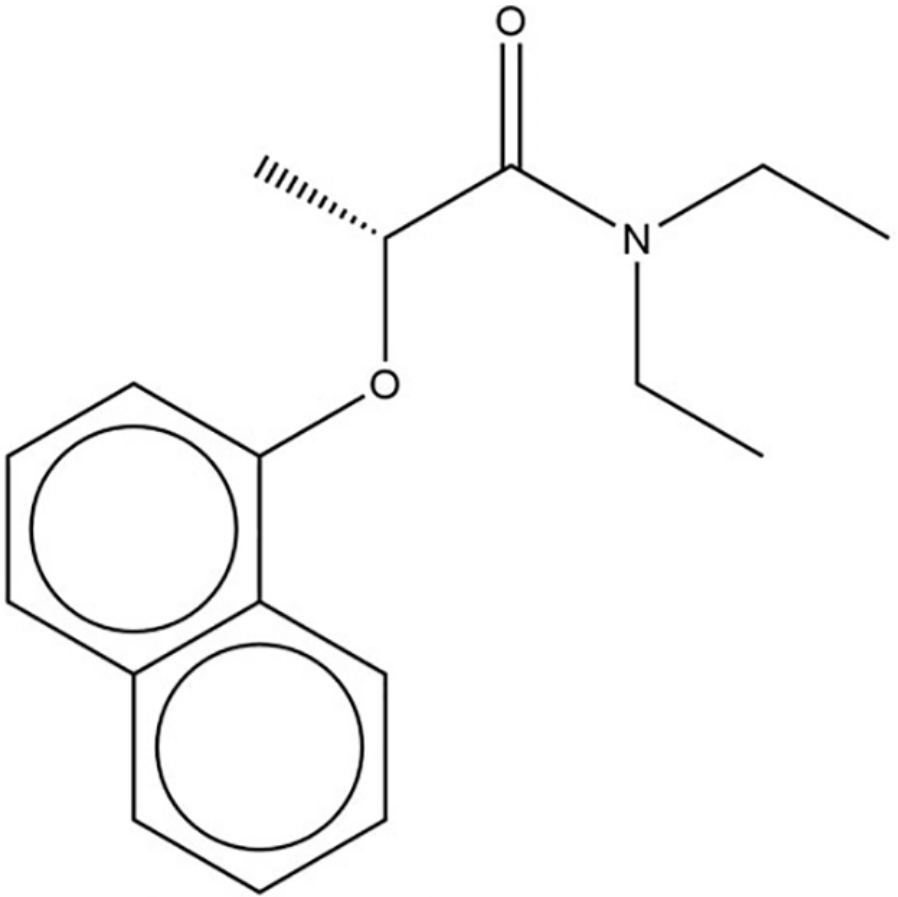
napropamide	GR_{50} [a] (g a.i. ha^{-1}) (S.E.[b])		
	Seedling root length	Seedling height	Seedling fresh weight
Rac-	232.3 (13.0) b	225.2 (3.0) b	250.0 (15.8) a
R-	196.6 (16.0) c	119.2 (18.4) c	225.2 (3.2) b
S-	411.4 (19.3) a	280.7 (19.9) a	278.7 (13.9) a

[a] GR_{50} refers to the herbicide rate required to decrease seedling root length, height and fresh weight by 50% compared to the untreated control. For each column, means followed by the same letter are not significantly different according to Fisher's protected LSD at $P \leq 0.05$.

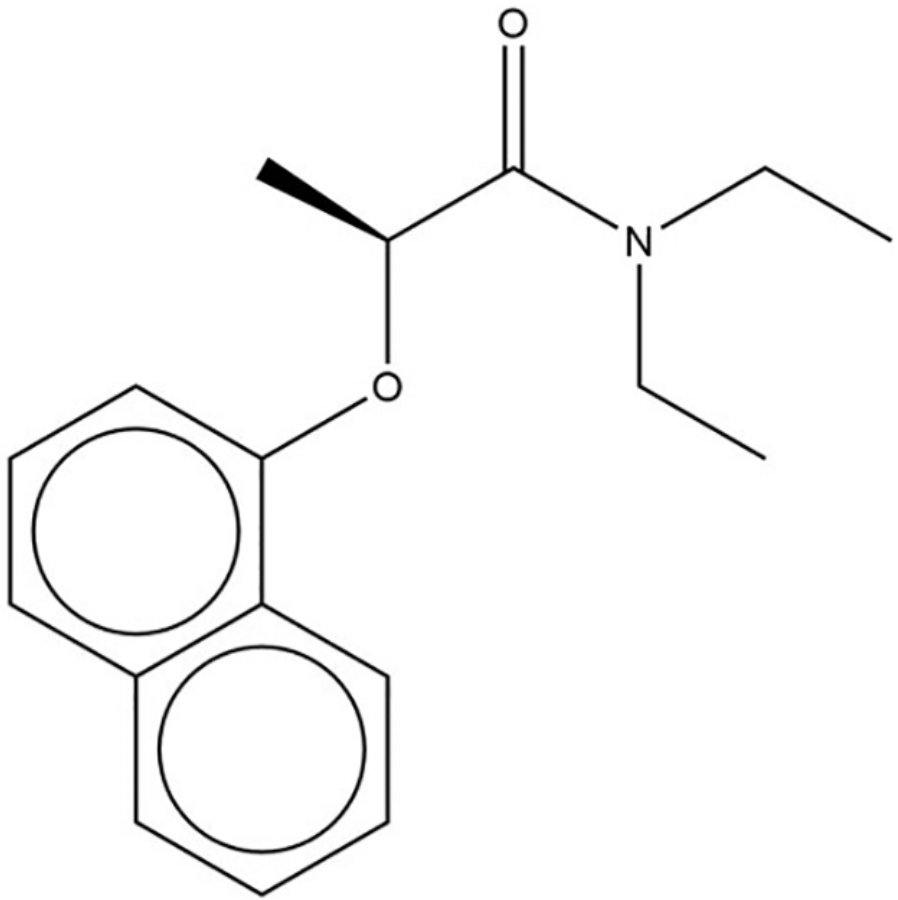
[b] Standard error.

Highlight

- During *E. crus-galli* and maize co-cultured experiments there was allelopathy
- In field conditions, enantioselective inhibition and behaviors of napropamide occurred
- Glutathione-*S*-transferase (GST) genes and oxidative enzymes play role in inhibition

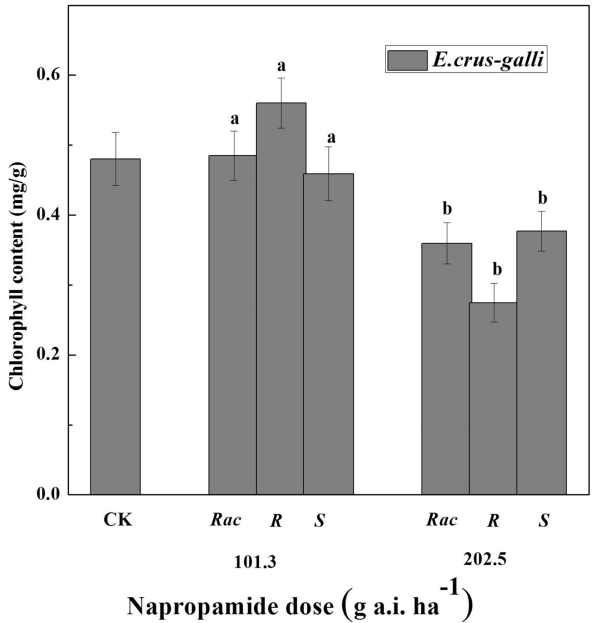


R-napropamide



S-napropamide

Figure 1


E.crus-galli
Chlorophyll content (mg/g)
0.0
0.2
0.4
0.6
a
a
a
b
b
b
CK
Rac
R
S
Rac
R
S
101.3
202.5
Napropamide dose (g a.i. ha-1)

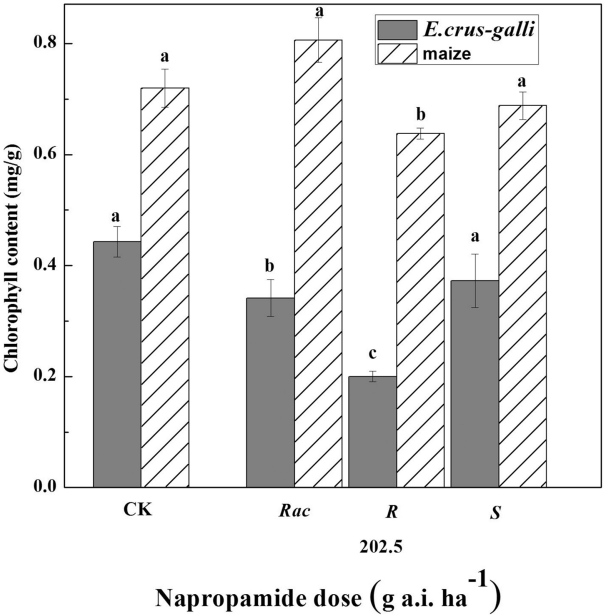

E.crus-galli
maize
Chlorophyll content (mg/g)
0.0
0.2
0.4
0.6
0.8
a
a
b
a
c
b
a
a
CK
Rac
R
S
202.5
Napropamide dose (g a.i. ha-1)

Figure 2

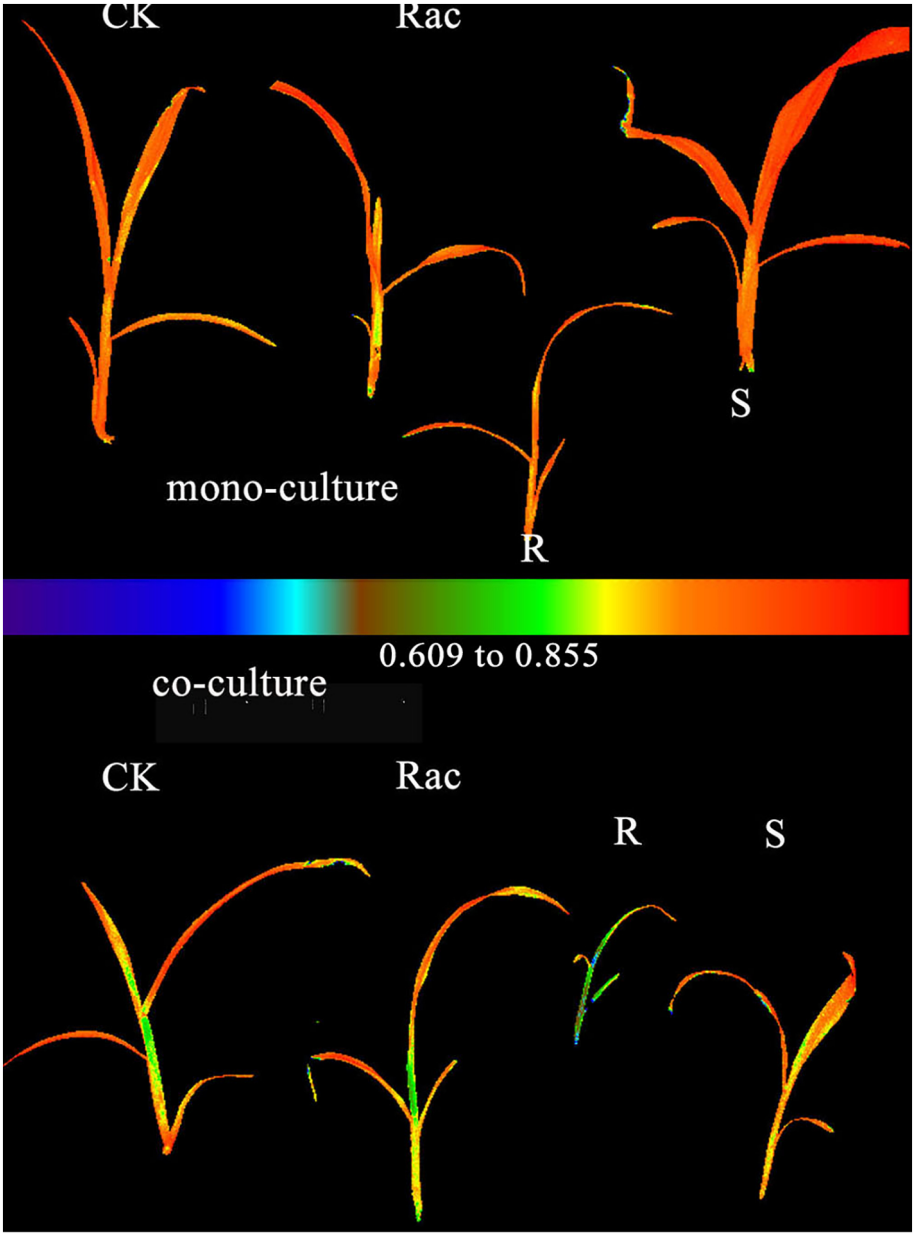
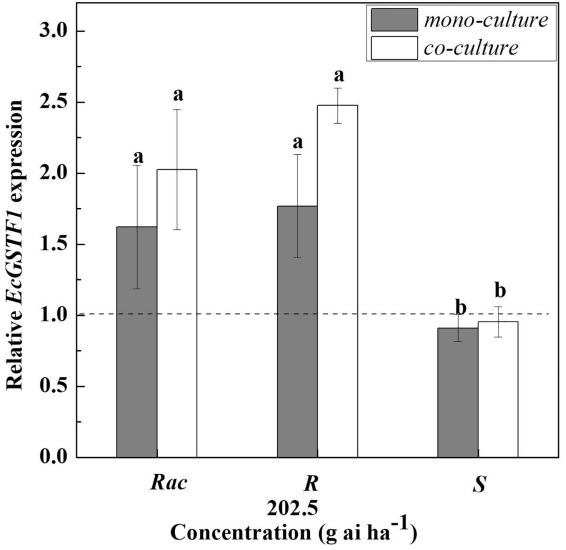


Figure 3


mono-culture
co-culture
Relative EcGSTF1 expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
a
a
b
b
Rac
R
S
202.5
Concentration (g ai ha-1)

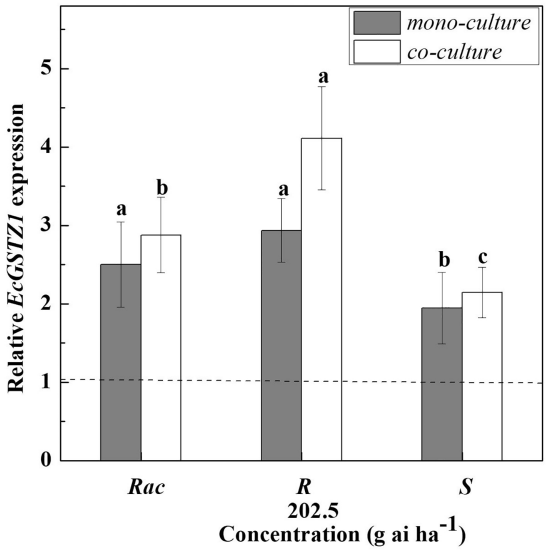

mono-culture
co-culture
Relative EcGSTZ1 expression
5
4
3
2
1
0
a
b
a
a
b
c
Rac
R
S
202.5
Concentration (g ai ha-1)

Figure 4

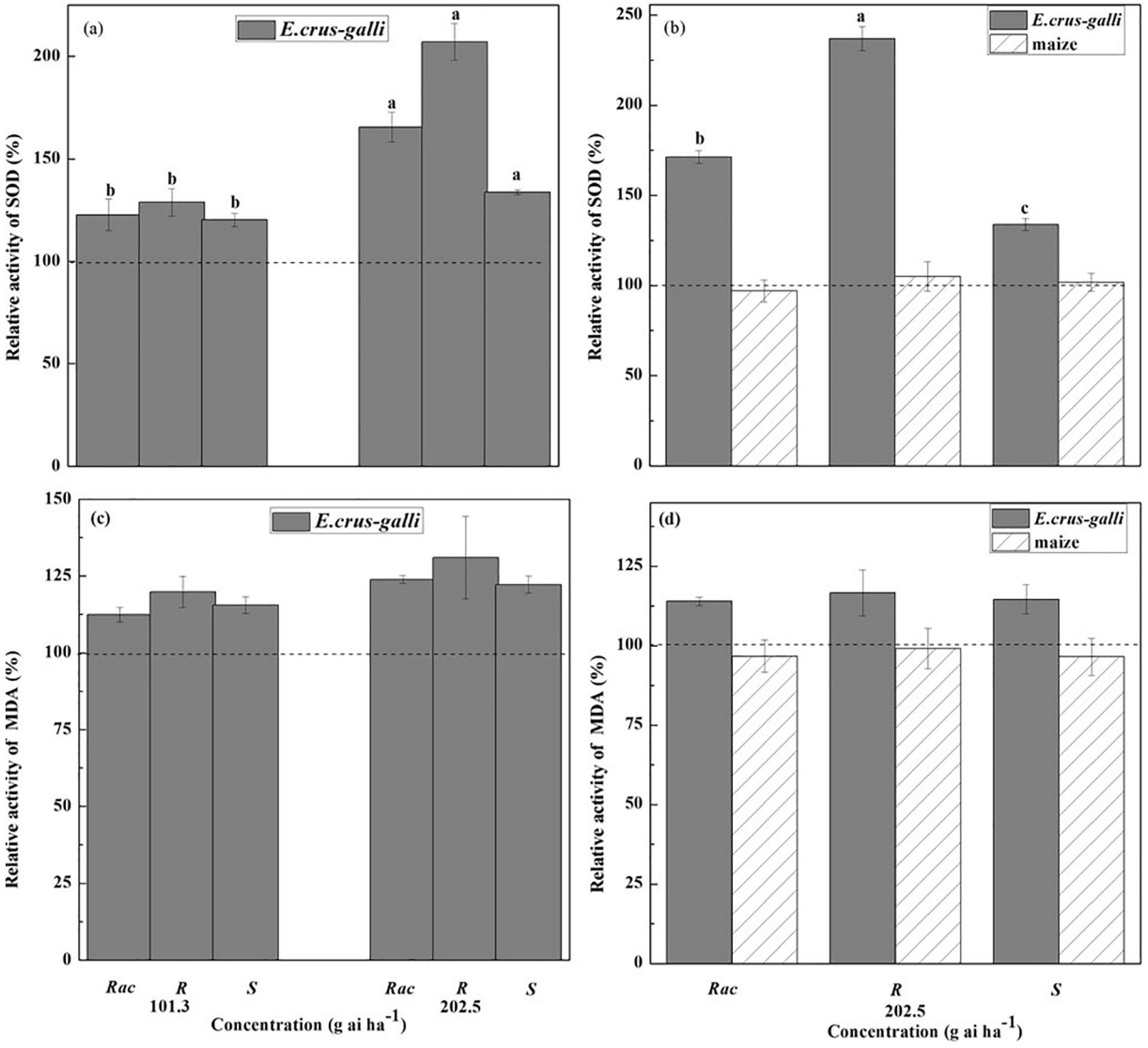

(a)
E.crus-galli
Relative activity of SOD (%)
b
b
b
a
a
a
(b)
E.crus-galli
maize
Relative activity of SOD (%)
b
a
c
(c)
E.crus-galli
Relative activity of MDA (%)
(d)
E.crus-galli
maize
Relative activity of MDA (%)
Rac
R
S
101.3
202.5
Concentration (g ai ha-1)

Figure 5

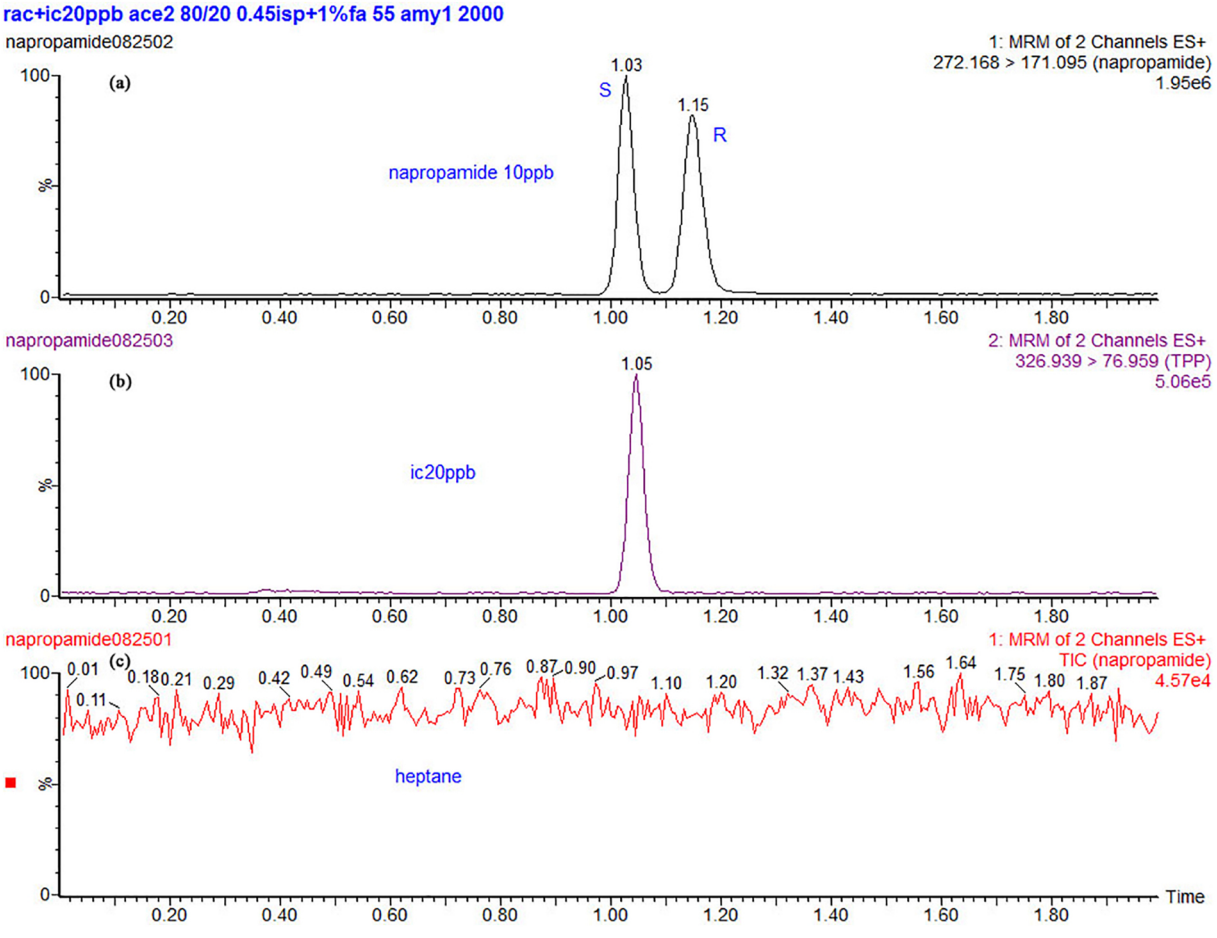
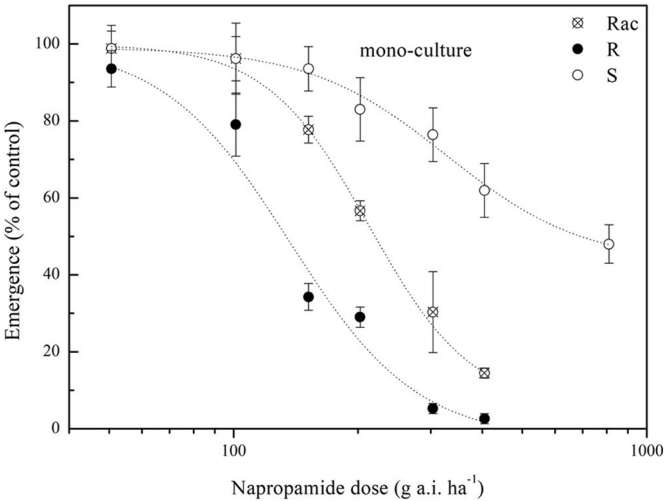

rac+ic20ppb ace2 80/20 0.45isp+1%fa 55 amy1 2000
napropamide082502
1: MRM of 2 Channels ES+
272.168 > 171.095 (napropamide)
1.95e6
(a)
1.03
S
1.15
R
napropamide 10ppb
napropamide082503
2: MRM of 2 Channels ES+
326.939 > 76.959 (TPP)
5.06e5
(b)
1.05
ic20ppb
napropamide082501
1: MRM of 2 Channels ES+
TIC (napropamide)
4.57e4
(c)
heptane
Time

Figure 6


mono-culture
Rac
R
S
Emergence (% of control)
Napropamide dose (g a.i. ha-1)
0
20
40
60
80
100
100
1000

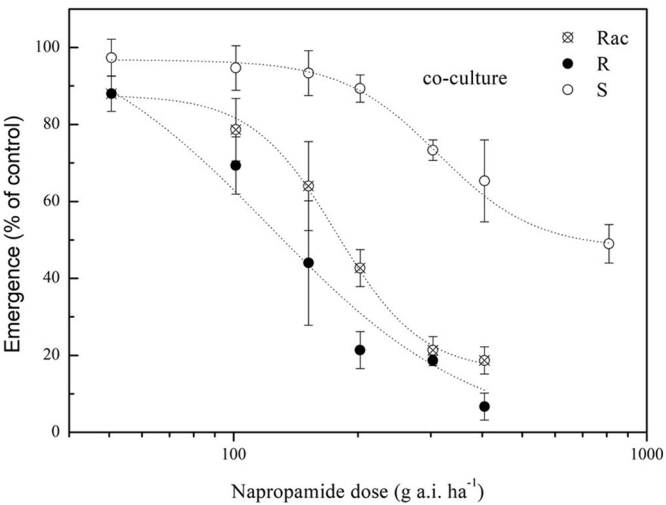

co-culture
Rac
R
S
Emergence (% of control)
Napropamide dose (g a.i. ha-1)
0
20
40
60
80
100
100
1000

Figure 7

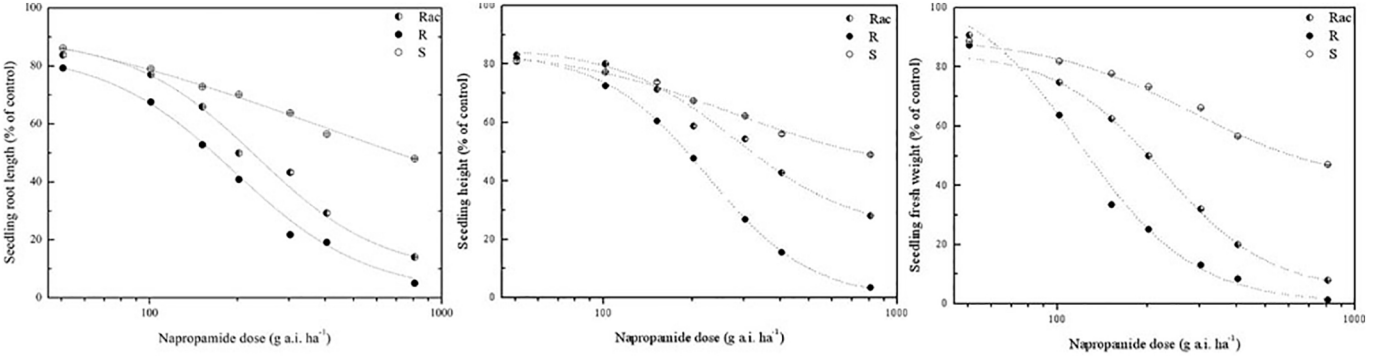

Seedling root length (% of control)
Seedling height (% of control)
Seedling fresh weight (% of control)
Napropamide dose (g a.i. ha-1)
Rac
R
S
0
20
40
60
80
100
1000

Figure 8

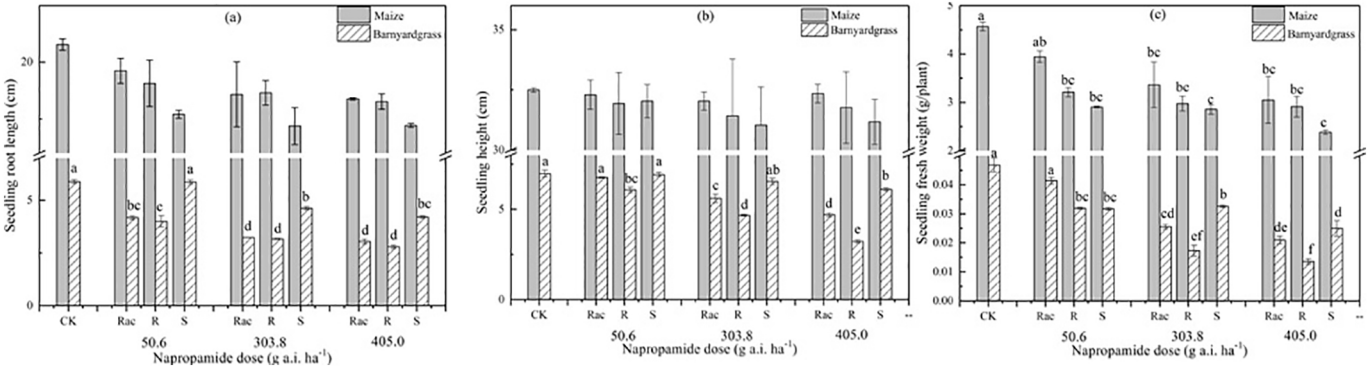

(a)
(b)
(c)
Maize
Barnyardgrass
Seedling root length (cm)
Seedling height (cm)
Seedling fresh weight (g/plant)
CK
Rac
R
S
50.6
303.8
405.0
Napropamide dose (g a.i. ha-1)

Figure 9