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Enantioselective Effects of Imazapyr on Resistant *Arabidopsis thaliana* GH90

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Abstract

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The imidazolinone herbicide imazapyr is a chiral compound that targets acetolactate synthase (ALS). Imazapyr has been widely used for weed control since the early 1980s; however, some weed species are resistant to ALS-inhibiting herbicides. This study applied herbicide to the resistant mutant *Arabidopsis thaliana* GH90 to investigate the enantioselectivity of imazapyr. There was no observable effect on plant shoots exposed to (R,+)-imazapyr at a concentration of 0.01 mg L^{-1} or 1 mg L^{-1}; however, exposure to (R,+)-imazapyr at the same concentration turned the shoot stems and leaves purple and resulted in plants of smaller size. At a herbicide concentration of 0.1 mg L^{-1}, the total chlorophyll content was higher in samples exposed to (S,-)-imazapyr than in those exposed to rac- or (R,+)-imazapyr and did not differ from that of the controls. At a herbicide concentration of 1 mg L^{-1}, proline and malonaldehyde (MDA) content was proportional to the concentration of rac- and (R,+)-imazapyr as follows: (R,+)-imazapyr > rac-imazapyr > (S,-)-imazapyr. Overall, (R,+)-imazapyr was more stressful to mutant *A. thaliana* GH90 plants than was rac- and (S,-)-imazapyr. The effect of the herbicides on ascorbate peroxidase activity was in the following order: (R,+)-imazapyr > rac-imazapyr > (S,-)-imazapyr. Total superoxide dismutase activity did not increase with an increase in the concentration of (R,+)-imazapyr, due to damage caused to plant cells and a reduction in enzyme synthesis. The effect of (R,+)-Imazapyr on reducing ALS activity exceeded that of rac- or (S,-)-imazapyr. The structure of ALS reveals clues by which to elucidate the enantioselectivity of imazapyr to imazapyr-resistant *A. thaliana* GH90.

Key words: imazapyr, acetolactate synthase (ALS), herbicide resistance, protein structure

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Introduction

Up to 25% of the active ingredients in pesticides are chiral existing as mirror images of each other, referred to as enantiomers. The biological properties of enantiomers differ in terms of toxicity as a result of interactions with enzymes or other naturally occurring chiral molecules, such that one enantiomer may have the desired effect on a target species, while the other enantiomer does not [13]. Moreover, one or both enantiomers can have adverse effects on non-target species as well. Assessing enantiomer selectivity for exposure and effects is a crucial aspect of risk assessment.

Imidazolinone (IMI) herbicides have an asymmetric carbon atom (Fig. 1) [36], typically consisting of 2 enantiomers. They are sold as racemates and released into the environment as a mixture of the two 2 enantiomers at a 1:1 ratio. Due to their high selectivity, IMI herbicides have been widely adopted for crops pre- or post-emergence [35]. IMI herbicides function inhibit branched-chain amino acid synthesis by preventing the biosynthesis of acetolactate synthase (ALS) [32], which halts plant growth and eventually kills the plant.

The use of ALS-inhibiting herbicides (e.g., sulfonylurea and IMI) has led to resistant weed populations, which were first detected in 1987. Overall, 133 weeds species, including 50 monocot and 83 dicot species, currently present resistance to ALS-inhibiting herbicides [18]. As mentioned above, the herbicidal efficacy of imazapyr differs according the specifics of the enantiomers: (+)-imazapyr has a more severe effect on wild-type *Arabidopsis thaliana* than do (±)- and (-)-imazapyr. At present, researchers have yet to determine whether the stereo structure of chiral herbicides is involved in the pesticide resistance of these species. There has been relatively little research on the relationship between enantiomers and herbicide-resistant plant species.

In the current study, we investigated the mutant *A. thaliana*, GH90, which is resistant to imazapyr [17], as a target organism to determine whether imazapyr-resistance can be attributed to enantioselectivity at target and non-target sites. Affects were evaluated in terms of the physiologic features of *A. thaliana*, the activity of antioxidant enzymes, and ALS enzyme inhibition.

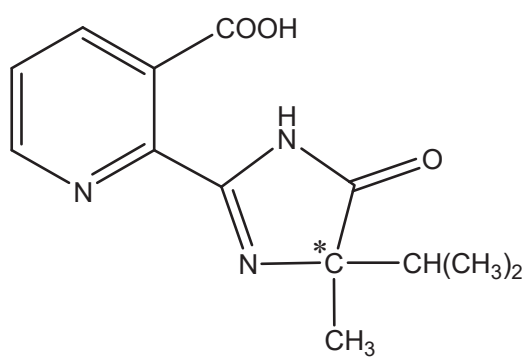

Fig. 1. Stereo molecular structures in the herbicide imazapyr. * denotes chiral center.

Materials and methods

1. Standard materials and reagents

Analytic standard racemate imazapyr (99.5% purity) was obtained from Merck (Germany). The stereo configuration of imazapyr is illustrated in Fig. 1. The other solvents used in this study were of analytical or high-performance liquid chromatography (HPLC) grade. Solvents including n-hexane, 2-propanol and acetonitrile were of HPLC grade and purchased from Merck KGaA (Darmstadt, Germany). Ultrapure water was obtained in the laboratory using a Milli-Q water purification system (Merck KGaA, Darmstadt, Germany). All other chemicals and solvents were of analytic grade from commercial sources. Pesticide stock solution was prepared by dissolving imazapyr in acetonitrile at a concentration of 1000 mg L^{-1} and then was stored at 4°C in the dark.

2. Preparation of imazapyr enantiomers

The separation of enantiomers was performed in accordance with the methods outlined in a previous study with some modifications [20]. Chiral separation was performed using a Hitachi LC-7400 series HPLC system (Hitachi, Tokyo) equipped with a Chiralcel OJ-H column (250 × 4.6 mmID, 5 μm, Daicel Chemical Industries, Japan) and UV detector operated at 254 nm. A mixture of n-hexane/2-propanol was used as the mobile phase with a flow rate of 1.5 mL min^{-1} to enable the injection of 20 μL for each analysis cycle. The column temperature was 25°C. Chromatographic data were acquired using an online integral data processing system (SISC, Taiwan). The specific rotation of imazapyr enantiomers was processed with using polarimeter, Model 341 LC (Perkin Elmer, USA) equipped with sodium lamp at wavelength of 589 nm as a light source. Optical rotation was calculated as $[\alpha]^{t}_{\lambda}=\alpha/(L\times C)$, where $[\alpha]^{t}_{\lambda}$ indicates the specific rotation under t °C and scanning wavelength is λ nm; α is the measured optical rotation; L is the length of the cell (dm), and C is the concentration of the analytes (mg L^{-1}). As previously described [37], (-)-imazapyr was identified as an S-form and (+)-isomer as an R-form. The purity and concentration of the recovered enantiomers were determined via HPLC with a C-18 column, and acetonitrile/phosphatic buffer (70/30, pH 3) was used as the mobile phase at a flow rate of 1 mL min^{-1}.

3. Preparation of plant material

Seeds of mutant *A. thaliana* GH90 (*csr1-2*/*csr1-2*) were obtained from the Arabidopsis Biological Resource Center (USA). The seeds were sterilized and then germinated on

Murashige and Skoog (MS) basal medium with Gamborg vitamins (Sigma Aldrich GmbH, Germany) [12], 2.2% (w/v) sucrose, and 1.0% (w/v) agar (BD, USA). The pH was adjusted to 5.7 before autoclaving.

Sterile Petri dishes (100 * 15-mm) containing 15-20 mL of MS solid medium were filled with 25 seeds and then sealed with parafilm M (Fisher Scientific, Inc.). After refrigeration at 4°C overnight for priming, the seeds were incubated in a growth chamber with light intensity of 50 μmol m^{-2} s^{-1} at 25°C under a 16-h light/8-h dark cycle. After 4 to 7 days, the most vital seeds underwent germination.

One week after germination, seedlings were transferred to a hydroponic system and incubated in a growth chamber. The hydroponic system contained 350 mL 1/4 MS liquid medium as a nutrient source and 12.5 g expanded clay covered with a thin layer of vermiculite as growth substrate. At 30 days after seeding, plants were ready for exposure to herbicides. Imazapyr enantiomer solutions were dissolved in acetone at a concentration of 0.05% (v/v) for the treatment and control groups. After 10-day exposure to imazapyr, cultures were prepared in triplicate to characterize the morphologic and physiologic features.

4. Morphology and physiology of *A. thaliana* GH90

The effect of imazapyr enantiomers on the mutant *A. thaliana* GH90 was evaluated in terms of morphologic and physiologic features after exposure to imazapyr or its enantiomers for 10 days. The plants were compared in terms of appearance, whereupon then the leaves were collected, weighed, and ground under liquid nitrogen cooling in homogenization medium containing 5 mL sodium phosphate buffer (50 mM, pH 6.8) using a mortar and pestle to produce leaf extract.

Chlorophyll extraction was performed in accordance with the methods outlined in a previous study with slight modifications [38]. Leaf extract (40 μL) was added to extraction solvent of 100% ethanol (960 μL) in a 1.5-mL microtube and stored in an incubator at 4 °C for 30 min. Samples were centrifuged at 1000 rpm for 15 min, whereupon the optical density was measured at 665 nm (A_{665}) and 649 nm (A_{649}) using a spectrophotometer. Total chlorophyll (μg) was calculated as follows:

$$\text{Total chlorophyll} = (6.1 \times A_{665}) - (20.04 \times A_{649})$$

Proline colorimetric determination with acidic ninhydrin reagent was performed as follows: 1 mL of leaf extract was placed in a microtube containing 0.5 mL of 9% sulfosalicylic acid [2]. The homogenate was centrifuged for 20 min at 3000 rpm. Ninhydrin reagent (1

mL each) was added to 0.5 mL of supernatant in a 10-mL glass tube. Closed test tubes containing the reaction mixture were held in a boiling water bath for 1 h, whereupon the reaction was terminated in an ice bath for 5 min. Proline was extracted using 4 mL toluene. Optical density was measured using a spectrophotometer at 520 nm (A_{520}). The proline concentration was determined from a standard curve and calculated on a fresh weight basis.

Lipid peroxidation in plant parts was estimated in terms of malondialdehyde (MDA) production using the thiobarbituric acid (TBA) method [19]. Leaf extract (1 mL) was placed in a microtube containing 0.5 mL of 0.5% trichloracetic acid. The homogenate was centrifuged at 9,000 rpm for 20 min at 20°C. The supernatant (1 mL) was mixed with 20% TCA (4 mL) containing 0.5% TBA and heated in a boiling water bath for 30 min and then rapidly cooled in an ice bath. The supernatant was centrifuged at 9,000 rpm for 10 min, whereupon the resulting supernatant was used to determine the MDA content. Optical density was measured using a spectrophotometer at 532 nm (A_{532}) and 600 nm (A_{600}).

5. ALS assay

ALS is the active site of imazapyr; therefore, we examined the effects of imazapyr and its enantiomers in inhibiting ALS activity. ALS was extracted from leaves as previously described, with slight modifications [4, 25, 33]. Briefly, 5 g of leaf extract was ground to a powder under liquid nitrogen cooling with 0.2 g polyvinylpyrrolidone and suspended in 5 ml enzyme extraction buffer (100 mM potassium phosphate, pH 7.5, containing 10 mM pyruvate, 5 mM $MgCl_2$, 5 mM EDTA, 100 mM flavin adenine dinucleotide [FAD] and 10% (v/v) glycerol). After centrifugation at 13,200 rpm for 20 min at 4°C, an equal volume of a saturated solution of ammonium sulfate was added to the supernatant fraction. The mixture was placed in an ice bath for 20 min and centrifuged at 13,200 rpm for 20 min. The resulting pellet was immediately redissolved in 2 mL dissolved buffer (50 mM potassium phosphate buffer, pH 7.5, containing 10 mM NaCl and 2 mM EDTA) to perform enzyme assays. The protein concentration in extracts was determined using the method proposed by Bradford (1976) in which bovine serum albumin is used for the standard curve [3].

The enzyme assay solution contained 100 mM pyruvate, 10 mM $MgCl_2$, 1 mM thiamine pyrophosphate, 10 μM FAD, 50 mM potassium phosphate buffer (pH 7, 300 μL), and 200 μL enzyme solution. Imazapyr and its enantiomers were dissolved in acetone in test tubes at concentrations of 0, 0.04, 0.2, 1, 5, and 25 mg L^{-1}. After the acetone evaporated, the enzyme assay mixture was added to the test tube for

incubation at 37°C for 1 h and then stopped using 30 μL of 6 N H_2SO_4. The reaction tubes were assayed for acetolactate via decarboxylation at 60°C for 15 min followed by the measurement of acetoin [37], wherein 350 μL each of 0.5% (w/v) creatine and 5% (w/v) a-naphthol was added to 2.5 N NaOH in test tubes and incubated at 60°C for 15 min. Absorbance was measured at 520 nm.

6. Extraction and determination of antioxidant enzymes

Leaf tissue was homogenized in 0.1 M sodium phosphate buffer (pH 6.8) under liquid nitrogen cooling. After centrifugation at 12,000 g for 20 min, the supernatant was used to characterize enzyme activity and protein content. Ascorbate peroxidase (APX) activity was determined as previously described [28]. One unit of catalase (CAT) enzymatic activity was defined as the quantity of enzyme that degraded 1 μmol H_2O_2 per minute [22]. Glutathione reductase (GR) activity was determined as previously described [11]. One unit of superoxide dismutase (SOD) was defined as the quantity of enzyme that inhibits by 50% the rate of nitro blue tetrazolium (NBT) reduction observed in a blank sample. Total SOD activity was determined as previously described [14].

7. Protein structure determination

The structure of mutant *A. thaliana* GH90–ALS complexes after treatment with imazapyr and its enantiomers was characterized in terms of modifications to protein coordinates in ALS–imazaquin complex [Protein Data Bank (PDB) entry 1Z8N] [26]. Figures were generated using the PyMOL Molecular Graphics System 1.6 [31] and WebLab Viewer (MSI, San Diego, CA).

8. Statistical analysis

Data analysis was performed using Microsoft Excel 2010. Results were analyzed using one-way ANOVA and the least significant differences test (LSD). $p<0.05$ was considered statistically significant.

Results and discussion

1. Enantioselective effects of imazapyr on the appearance of mutant *A. thaliana* GH90

The enantioselective effects of imazapyr on mutant *A. thaliana* GH90 were estimated using several indices (Fig. 2). shows the appearance of *A. thaliana* GH90 after exposure to imazapyr or its enantiomers at various concentrations for 10 days. The lowest

herbicide concentration (0.01 mg L^{-1}) had no observable effect on shoots; however, the shoots were smaller than those in the control group. Treatment with 0.1 mg L^{-1} rac- and (S,-)-imazapyr had no effect on appearance; however, treatment with 0.1 mg L^{-1} (R,+)-imazapyr caused the leaves to curl. After treatment with 1 mg L^{-1} rac- and (S,-)-imazapyr, old leaves turned reddish purple (data not shown). After treatment with 1 mg L^{-1} (R,+)-imazapyr, the stems and leaves became purple and the plants were smaller than those in the control and other treatment groups. Taken together, these results indicate that (R,+)-imazapyr can indeed damage imazapyr-resistant *A. thaliana* GH90. These results also revealed the enantioselectivity of imazapyr-resistant *A. thaliana*.

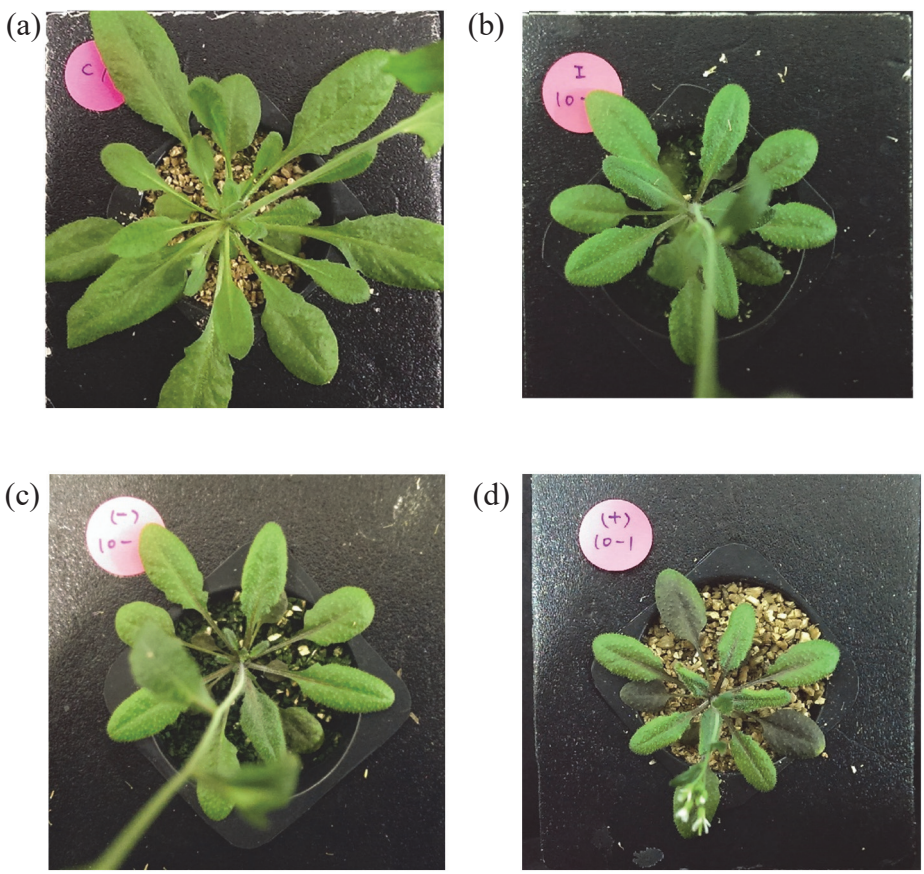

Fig. 2. Mutant *Arabidopsis thaliana* GH90 (30 days old) treated with 0.1 mg L^{-1} imazapyr or its enantiomers for 10 days. (a) Control mutant *A. thaliana* GH90; (b) rac-imazapyr; (c) (S,-)-imazapyr; (d) (R,+)-imazapyr.

2. Effect of imazapyr and its enantiomers on physiology of *A. thaliana* GH90

At an herbicide concentration of 0.01 mg L^{-1}, the total chlorophyll content was lower in samples exposed to (R,+)-imazapyr than in those exposed to rac-, (S,-)-imazapyr and the controls (Fig. 3a). At a herbicide concentration of 0.1 mg L^{-1}, the total chlorophyll content was higher in samples exposed to (S,-)-imazapyr than in those exposed to rac- and (R,+)-imazapyr treatment and no differ than in the controls. At a herbicide concentration of 1 mg L^{-1}, the total chlorophyll content was lower than in the control group, regardless of the treatment.

The biosynthesis of proline (a well-known osmo-protectant) is triggered in many plant species by drought and other forms of stress. Thus, proline concentration can be considered an index of stress in plants [7]. Sanada et al. (1995) reported that proline concentration should be carefully regulated in accordance with environmental conditions [30]. In the current study, proline content in most of the treatment groups was higher than in the control group, with the exception of (S,-)-imazapyr at concentrations of 0.01 and 0.1 mg L^{-1} (Fig. 3b). At a herbicide concentration of 0.01 mg L^{-1}, rac- and (R,+)-imazapyr respectively increased proline content to 0.074 and 0.116 mg L^{-1}, both of which were higher than the (S,-)-imazapyr

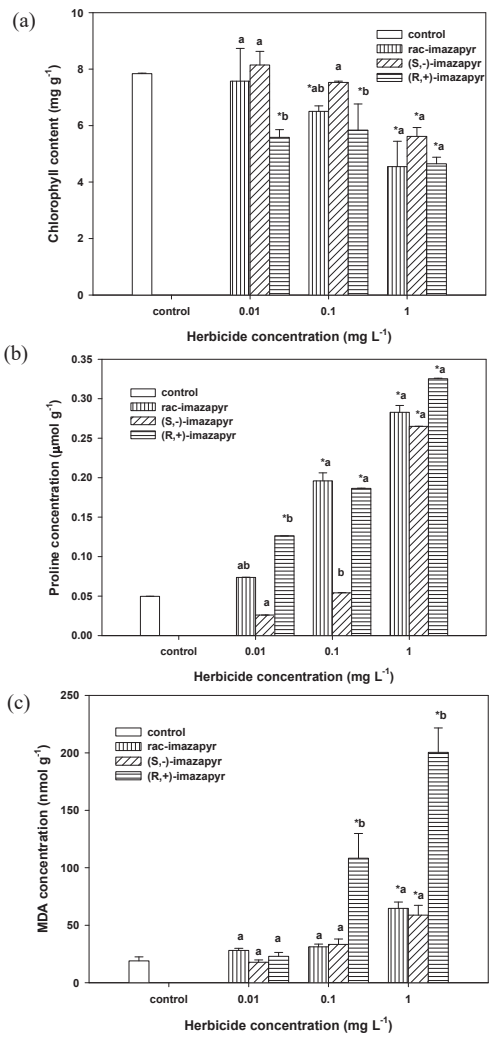

Fig. 3. Physiological analysis of *A. thaliana* GH 90 after 10 days' exposure to 0.01, 0.1,or 1 mg L^{-1} imazapyr or its enantiomers. (a) Total chlorophyll content; (b) proline concentration; (c) malonaldehyde (MDA) concentration. Data are mean ± SD. (n = 3). $^{*}p < 0.05$ compared with the control. ($^{a, b, c}$) Letters represent significant difference at $p < 0.05$ between 2 treatment groups at a given concentration. (Diagonal line bars, rac-imazapyr; horizontal line bars, (S,-)-imazapyr; vertical line bars, (R,+)-imazapyr; and solid bar, control).

treatment group and the control group. At a herbicide concentration of 0.1 mg L^{-1}, proline content was more than threefold higher in the rac- and (R,+)-imazapyr treatment group than in the (S,-)-imazapyr treatment group. At a herbicide concentration of 1 mg L^{-1}, proline content increased in the following order: (R,+)-imazapyr > rac-imazapyr > (S,-)-imazapyr treatment. Note however that the values in the treatment groups were not significantly higher than in the control group.

MDA is widely used as a marker of oxidative lipid injury, with the concentration varying as a function of biotic and abiotic stress [6]. Thus, MDA content is commonly used as an index indicating the level of stress in plants. At a herbicide concentration of 0.01 mg L^{-1}, we observed no difference in MDA production between any of the treatment groups and the control group (Fig. 3c). Under a higher herbicide concentration (0.1 mg L^{-1}), MDA production in the (R,+)-imazapyr treatment group was threefold higher than in the rac- and (S,-)-imazapyr treatment group. At a herbicide concentration of 1 mg L^{-1}, MDA production in the (R,+)-imazapyr treatment group was higher than in the rac- and (S,-)-imazapyr treatment groups (200.47 vs 64.65 and 58.78 nmol g^{-1}, respectively). Despite the fact that *A. thaliana* GH90 is resistant to imazapyr and the effects of imazapyr on GH90 were less pronounced than those on the wild type [20], we observed the herbicidal effects of (R,+)-imazapyr against the mutant *A. thaliana* GH90.

3. Effect of imazapyr and the corresponding enantiomers on antioxidant enzymes in mutant *A. thaliana* GH90

Plants induce antioxidant enzyme activity against oxidative stress. First, SOD catalyzes the dismutation of 2 molecules of superoxide into O_2 and H_2O_2. APX reduces H_2O_2 level in the ascorbate–glutathione cycle [1]. Finally, CATs transform H_2O_2 into H_2O and O_2 [23]. We measured the activity of APX, CAT and total SOD induced by imazapyr and its enantiomers in *A. thaliana* GH90 leaves (Table 1).

CAT activity did not show differ among all treatments except 0.1 and 1 mg L^{-1} (R,+)-imazapyr treatment. At a herbicide concentration of 0.01 mg L^{-1}, we did not observed any changes in APX activity; however, at a herbicide concentration of 0.1 mg L^{-1}, APX activity in the rac- and (R,+)-imazapyr treatment group was roughly two-fold higher than in the control group and APX activity in the (S,-)-imazapyr group was roughly threefold higher. At a herbicide concentration of 1 mg L^{-1}, we observed a significant increase in APX activity in the treatment groups, as follows: (R,+)-imazapyr > rac-imazapyr > (S,-)-imazapyr. Total SOD activity increased proportionally with the concentration

Table 1. Effects of 10-day exposure to imazapyr or its enantiomers on activity of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) in mutant *Arabidopsis thaliana* GH90 leaves

Herbicides	Concentration (mg L^{-1})	SOD	CAT	APX
		U mg $protein^{-1}$		
Control		13.38 ±2.22	0.136 ±0.006	0.546 ±0.063
(±)-imazapyr	0.01	17.96 ±4.02*	0.145 ±0.005	0.737 ±0.116
	0.1	18.31 ±5.75*	0.204 ±0.094	1.182 ±0.160*
	1	19.19 ±2.12**	0.206 ±0.108	2.244 ±0.264***
(-)-imazapyr	0.01	12.10 ±1.95	0.136 ±0.037	0.600 ±0.118
	0.1	14.28 ±2.43	0.141 ±0.041	0.910 ±0.146
	1	20.27 ±1.20**	0.151 ±0.006	1.757 ±0.149***
(+)-imazapyr	0.01	23.15 ±1.33***	0.154 ±0.025	0.924 ±0.092
	0.1	16.18 ±1.44	0.329 ±0.069***	1.992 ±0.636***
	1	12.64 ±1.54	0.796 ±0.038***	3.940 ±0.993***

Data are mean ± SD. (n = 3).

* $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$ compared with the control.

of rac- and (S,-)-imazapyr, but not (R,+)-imazapyr. Similar results have been observed in rice, wherein SOD activity decreased proportionally with salinity stress resulting in increased MDA activity in leaves. Thus, it appears that the stress-induced damage to plant cells was of sufficient severity to prevent enzyme synthesis [8].

The extent of damage to the leaf membrane was determined by measuring the amount of MDA produced when polyunsaturated fatty acids in the membrane underwent peroxidation. Under (R,+)-imazapyr treatment at concentrations of 0.1 or 1 mg L^{-1}, MDA production increased significantly (Fig. 3c). Thus, it appears that the oxidative stress induced by (R,+)-imazapyr on imazapyr-resistant *A. thaliana* exceeded that of the racemate or (S,-)-imazapyr. Note that oxidative stress led to increased MDA production and decreased SOD activity.

Abiotic stress, due to drought [34], salt [16], extreme temperature [24], UV [21], heavy metal contamination [15], reduced ozone levels [29], and exposure to polycyclic aromatic hydrocarbons (PAHs) can lead to the accumulation of reactive oxygen species in plants [5]. Few studies have discussed changes in antioxidant enzymes after resistant plants are exposed to herbicides. In a previous study, we revealed the enantioselectivity of imazapyr to wild-type *A. thaliana*. We also determined that the herbicidal effects of (R,+)-

imazapyr exceeded those of the racemate and S-isomer. In the current study, we confirmed that mutant *A. thaliana* GH90 is resistant to imazapyr [20]. Note however that the various stereo structures of imazapyr vary in their effects on mutant *A. thaliana*, wherein (R,+)-imazapyr appears to cause more damage.

4. Ability of imazapyr and its enantiomers to inhibit ALS in mutant *A. thaliana*

The mechanism underlying the inhibition of ALS by herbicides, such as sulfonylureas and IMIs, has previously been studied [9, 25, 27]. In the current study, ALS was extracted from mature leaves of mutant *A. thaliana* GH90 (*m*ALS) and *m*ALS activity was measured *in vitro* (Fig. 4). *m*ALS activity remained at > 90% of the control level under incubation with (S,-)-imazapyr at concentrations of 0 to 100 mg L^{-1}. Incubation rac-imazapyr at 0 to 5 mg L^{-1} did not inhibit *m*ALS activity. When the concentration of rac-imazapyr was increased from 5 to 100 mg L^{-1}, we observed a decrease in *m*ALS activity in a dose dependent manner. Incubation with rac-imazapyr at 100 mg L^{-1} reduced *m*ALS activity to 91% of the control level. Incubation with (R,+)-Imazapyr at 100 mg L^{-1} produced led to a pronounced reduction in *m*ALS activity to 73.1% of the control level.

In a previous study [20], we investigated the ability of imazapyr and its enantiomers to inhibit ALS in wild-type *A. thaliana*. The ALS inhibition ability of (R,+)-Imazapyr exceeded that of the racemate and (S,-)-imazapyr, even in imazapyr-resistant species. Note that *m*ALS inhibition corresponded to our physiologic and antioxidant enzymatic analysis of mutant *A. thaliana* GH90.

5. Imazapyr binding site of ALS

ALS is the target site of imazapyr; therefore, the inhibition of ALS by imazapyr is highly representative of enantioselective herbicidal activity. The structure of IMI molecules does not mimic the enzyme substrate, but rather

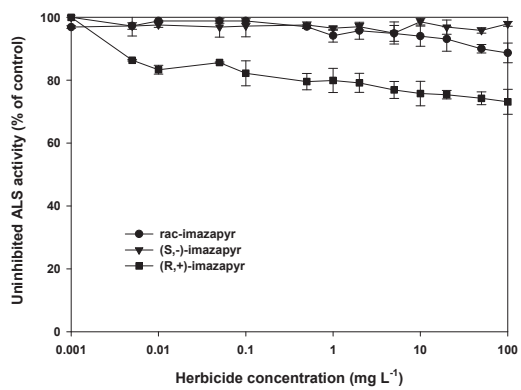

Fig. 4. Acetolactate synthase (ALS) activity in *A. thaliana* GH90 in response to imazapyr or its enantiomers (concentration on logarithmic x-axis). Data are mean ±SD of at least 3 independent determinations. ALS activity is presented as percentage activity in the absence of herbicide. Circle symbol: rac-imazapyr; inverted triangle symbol: (S,-)-imazapyr; square symbol: (R,+)-imazapyr.

inhibits ALS by blocking the channel through which access to the active site is gained [26]. In the current study, we modified the structure of *A. thaliana* ALS in complex with imazapyr as described by McCourt et al. (2006) (Fig. 5a) [20]. The imazapyr molecule fit the active sites of the channel and the isopropyl and methyl groups anchor the herbicide to the protein. The amino acid substitution for S653 of *m*ALS involves a single point mutation (Fig. 5b). If the substitution of asparagine for S653 narrowed the channel and impaired IMI binding, this would explain the resistance of *A. thaliana* GH90 to imazapyr; however, we found that (R,+)-imazapyr inhibited *m*ALS. In a previous study [20], two herbicide molecules were shown to bind to each subunit of ALS. In addition to the molecule within the channel leading to the active site, there is a second site located roughly 20Å from the active site. Figure 6 illustrates one possible binding of (R,+)-imazapyr to the second site (Fig. 6). If the (S,-)-isomer were positioned at this site, then unfavorable contacts could form between the isopropyl substituent of imazapyr and D397, S398 and A399. These residues would obstruct the space for isopropyl substituents and could expain why (R,+)-imazapyr inhibited ALS in *A. thaliana* GH90 in our experiments. Duggleby et al. (2003) observed that imidazolinones both activate and inhibit imidazolinone-resistant mutants of *Saccharomyces cerevisiae.*

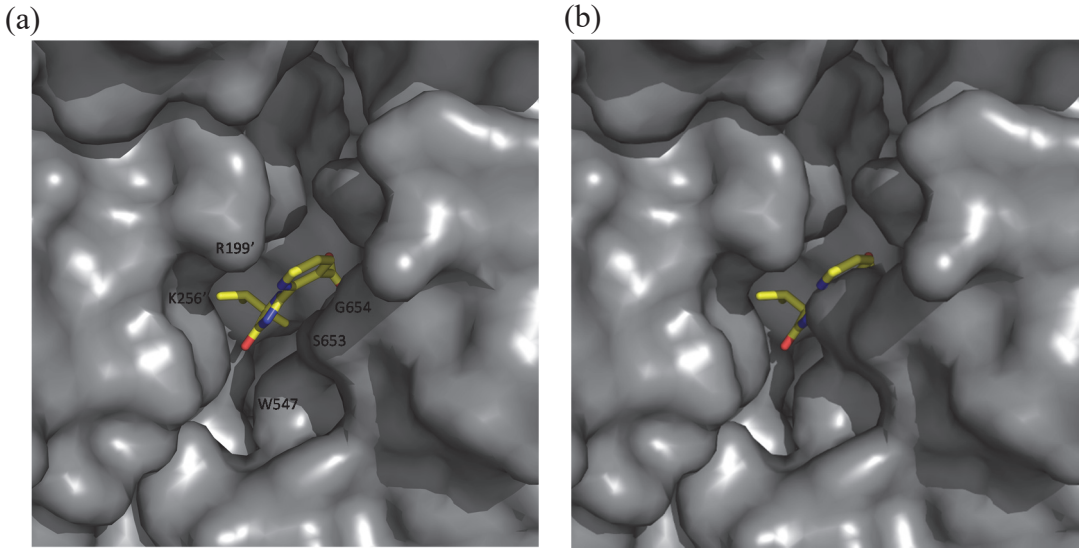

Fig. 5. Connolly surface and (R,+)-imazapyr blocking of the channel to active site associated with ALS. (a). ALS of wild-type *A. thaliana*; (b). ALS of mutant *A. thaliana* GH90. Imazapyr is presented as a stick model. Carbon is yellow; nitrogen, blue; and oxygen, red. The residue lining the channel is depicted as a gray surface. ' indicates residue from the neighboring subunit.

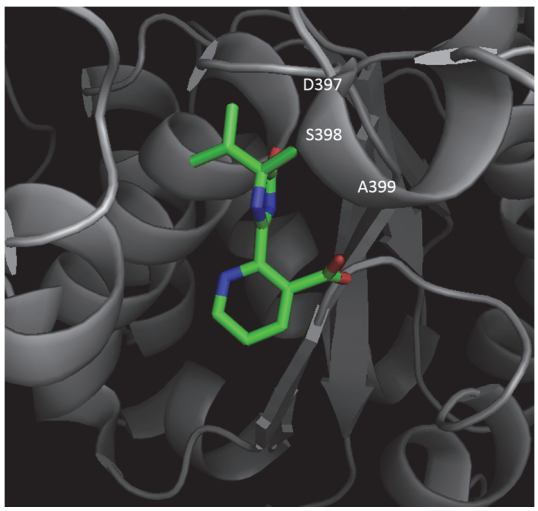

Fig. 6. Stereo conformation of (R,+)-imazapyr bound to the second site of ALS. Imazapyr is shown as a stick model. Carbon is green; nitrogen, blue; oxygen, red.

ALS [10], however, the imidazolinone-induced inhibition of ALS in yeast was weak. Nonetheless, the sensitivity of microbial ALS to imidazolinones is lower than to plant enzymes. Taken together, these observations suggest that the sensitivity of imazapyr-resistant ALS is enantioselective.

Conclusions

We determined that imazapyr-resistant *A. thaliana* GH90 can survive exposure to imazapyr; however, the damage caused by (R,+)-imazapyr to plants (in terms of appearance and physiology) exceeded that of the racemate or (S,-)-imazapyr. The effect of imazapyr and its enantiomers on antioxidant enzyme activity is an indication that some of the stereo structures of imazapyr adversely affect the functioning of plants at various levels. We also observed the imazapyr-induced enantioselective inhibition of *m*ALS. The inhibition of *m*ALS was more pronounced under exposure to (R,+)-imazapyr than to rac- or (S,-)-imazapyr. Accordingly, (R,+)-imazapyr had a more profound effect on mutant *A. thaliana* GH90 than did the (S,-)-isomer or racemate, including the resistant species. The enantioselectivity of chiral herbicides to target organisms is an important consideration in dealing with herbicide resistant weeds.

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Literature cited

1. Asada, K. 1992. Ascorbate peroxidase– a hydrogen peroxide-scavenging enzyme in plants. Physiol. Plant. 85: 235-241.
2. Bates, L. S.,Waldren, R. P., and Teare, I. D. 1973. Rapid determination of free proline for water-stress studies. Plant Soil 39: 205-207.
3. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram

quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

4. Chaleff, R. S., and Mauvais, C. J. 1984. Acetolactate synthase is the site of action of two sulfonylurea herbicides in higher plants. Science 224: 1443-1445.
5. Chen, B., and Yuan, M. 2012. Enhanced dissipation of polycyclic aromatic hydrocarbons in the presence of fresh plant residues and their extracts. Environ. Pollut. 161: 199-205.
6. Davey, M. W., Stals, E., Panis, B., Keulemans, J., Swennen, R. L. 2005. High-throughput determination of malondialdehyde in plant tissues. Anal. Biochem. 347: 201-207.
7. Delauney, A. J., and Verma, D. P. S. 1993. Proline biosynthesis and osmoregulation in plants. The Plant J. 4: 215-223.
8. Dionisio-Sese, M. L., and Tobita, S. 1998. Antioxidant responses of rice seedlings to salinity stress. Plant Sci.135: 1-9.
9. Duggleby, R. G., McCourt, J. A., and Guddat, L. W. 2008. Structure and mechanism of inhibition of plant acetohydroxyacid synthase. Plant Physiol. Biochem. 46: 309-324.
10. Duggleby, R. G., Pang, S. S., Yu, H., and Guddat, L. W. 2003. Systematic characterization of mutations in yeast acetohydroxyacid synthase. Interpretation of herbicide-resistance data. Eur. J. Biochem. 270: 2895-2904.
11. Foster, J. G., and Hess, J. L. 1980. Responses of superoxide dismutase and glutathione reductase activities in cotton leaf tissue exposed to an aamosphere enriched in oxygen. Plant Physiol. 66: 482-487.
12. Gamborg, O. L., MillerK, R. A., and Ojima, K. 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50: 151-158.
13. Garrison, A. W. 2006. Probing the enantioselectivity of chiral pesticides. Environ. Sci. Technol. 40: 16-23.
14. Giannopolitis, C. N., and Ries, S. K. 1977. Superoxide dismutases: I. Occurrence in higher plants. Plant Physiol. 59: 309-314.
15. Gorinova, N., Nedkovska, M., Todorovska, E., Simova-Stoilova, L., Stoyanova, Z., Georgieva, K., Demirevska-Kepova, K., Atanassov, A., and Herzig, R. 2007. Improved phytoaccumulation of cadmium by genetically modified tobacco plants (*Nicotiana tabacum* L.). Physiological and biochemical response of the transformants to cadmium toxicity. Environ. Pollut. 145: 161-170.
16. Gossett, D. R., Millhollon, E. P., and Lucas, M. C., 1994. Antioxidant response to NaCl stress in salt-tolerant and salt-sensitive cultivars of cotton. Crop Sci. 34: 706-714.
17. Haughn, G. W., and Somerville, C. R. 1990.

A mutation causing imidazolinone resistance maps to the *Csr1* locus of *Arabidopsis thaliana*. Plant Physiol. 92: 1081-1085.

18. Heap, I. 2013. The international survey of herbicide resistant weeds. Online. Internet. Retrieved from www.weedscience.org (Jun. 24, 2021).
19. Heath, R. L., and Packer, L. 1968. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys. 125: 189-198.
20. Hsiao, Y. L., Wang, Y. S., and Yen, J. H. 2014. Enantioselective effects of herbicide imazapyr on Arabidopsis thaliana. J. Environ. Sci. Health Part B: Pestic. Food Contam. Agric. Wastes 49: 646-653.
21. Julkunen-Tiitto, R., Häggman, H., Aphalo, P. J., Lavola, A., Tegelberg, R.,and Veteli, T. 2005. Growth and defense in deciduous trees and shrubs under UV-B. Environ. pollut. 137: 404-414.
22. Kato, M., and Shimizu, S. 1987. Chlorophyll metabolism in higher plants. VII. Chlorophyll degradation in senescing tobacco leaves; phenolic-dependent peroxidative degradation. Can. J. Bot. 65: 729-735.
23. Lin, C., and Kao, C. H. 2000. Effect of NaCl stress on H_2O_2 metabolism in rice leaves. Plant Growth Regul. 30: 151-155.
24. Mahan, J. R. 1994. Thermal dependence of glutathione reductase; thermal limitations on antioxidant protection in plants. Crop Sci. 34: 1550-1556.
25. Malkawi, H. I., Al-Quraan, N. A., and Owais, W. M. 2003. Acetolactate synthase activity and chlorsulfuron sensitivity of gamma-irradiated lentil (*Lens culinaris* Medik.) cultivars. J. Agric. Sci. 140: 83-91.
26. McCourt, J. A., Pang, S. S., King-Scott, J., Guddat, L. W., and Duggleby, R. G. 2006. Herbicide-binding sites revealed in the structure of plant acetohydroxyacid synthase. Proc. Natl. Acad. Sci. U S A. 103: 569-573.
27. Mourad, G., and King, J. 1992. Effect of four classes of herbicides on growth and acetolactate-synthase activity in several variants of *Arabidopsis thaliana*. Planta 188: 491-497.
28. Nakano, Y., and Asada, K. 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22: 867-880.
29. Pasqualini, S., Tedeschini, E., Frenguelli, G., Wopfner, N., Ferreira , F., D'Amato, G., and Ederli, L. 2011. Ozone affects pollen viability and NAD(P)H oxidase release from *Ambrosia artemisiifolia* pollen. Environ. Pollut. 159: 2823-2830.
30. Sanada, Y., Ueda, H., Kuribayashi, K., Andoh, T., Hayashi, F., Tamai, N., and Wada , K. 1995. Novel light-dark change of proline levels in halophyte (*Mesembryanthemum Crystallinum* L) and glycophytes (*Hordeum*

Vulgare L and *Triticum Aestivum* L) leaves and roots under salt stress. Plant Cell Physiol. 36: 965-970.

31. Schrodinger, LLC. 2010 The PyMOL molecular graphics system, Version 1.6. Schrödinger, San Carlos, CA, USA.
32. Shaner, D. L., Anderson, P. C., and Stidham, M. A.1984. Imidazolinones: potent inhibitors of acetohydroxyacid synthase. Plant Physiol. 76: 545-546.
33. Singh, B. K., Stidham , M. A., and Shaner, D. L. 1988. Assay of acetohydroxyacid synthase. Anal. Biochem 171: 173-179.
34. Smirnoff, N. 1993. The role of active oxygen in the response of plants to water deficit and desiccation. New Phytol. 125: 27-58.
35. Tomlin, C. D. S. 2006. The pesticide manual. A world compendium, 14th ed. British Crop Protection Council, Hampshire, UK. 1349 pp.
36. Wepplo, P. J. 1991. Chemical and physical properties of the imidazolinones. pp. 15-29. *In*: D. L. Shaner, S. L. O'Connor [eds], The Imidazolinone herbicides, CRC Press, USA. 290 pp.
37. Westerfield, W. W. 1945. A colorimetric determination of blood acetoin. J. Biol. Chem. 161: 495-502.
38. Wintermans, J. F. G. M., and De Mots, A. 1965. Spectrophotometric characteristics of chlorophylls a and b and their phenophytins in ethanol. Biochim. Biophys. Acta 109: 448-453.

抗依滅草阿拉伯芥突變品系 GH90 對依滅草之光學選擇性研究

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摘要

蕭鈺齡、顏瑞泓。2021。抗依滅草阿拉伯芥突變品系 GH90 對依滅草之光學選擇性研究。臺灣農藥科學　11：63-79。

咪唑啉酮類 (imidazolinone) 除草劑依滅草 (imazapyr)，為一具有對掌性 (chiral) 化學結構的農藥，市場上是以消旋體形式販售。而依滅草之作用位置為乙醯乳酸合成酶 (acetolactate synthase, ALS)，自 1980 年代初即廣泛被應用於田間雜草管理，由於長期使用，目前已有雜草對抑制乙醯乳酸合成酶的除草劑產生抗藥性的情形，而抗性雜草是否對依滅草不同光學活性成分具有光學活性的選擇性，即為本文主要探討目標。由結果顯示，抗依滅草的阿拉伯芥 (*arabidopsis thaliana*) 突變品系 GH90，在 0.01 mg L^{-1} 的各種除草劑依滅草處理下，皆未受影響；但在 0.1 mg L^{-1} 的處理下 (R,+)- 依滅草處理會造成葉部紫色植株矮小；而 (S,-)- 依滅草總葉綠素含量顯著高於外消旋 (rac)- 依滅草及 (R,+)- 依滅草，但與未以除草劑處理之對照組比較則無區別。GH90 阿拉伯芥中之脯氨酸及丙二醛含量隨著外消旋 (rac)- 依滅草及 (R,+)- 依滅草施用量增加而增加，影響效應則以 (R,+)- 依滅草>外消旋 (rac)- 依滅草> (S,-)- 依滅草，顯見 (R,+)- 依滅草對 GH90 阿拉伯芥造成較大生長壓力。抗壞血酸過氧化物酶活性以 (R,+)- 依滅草>外消旋-依滅草> (S,-)- 依滅草的順序增加。而總超氧化物歧化酶 (SOD) 活性則不隨 (R,+)- 依滅草煙濃度的增加而增加，則是因為 (R,+)- 依滅草已對植物細胞造成損傷而減少酶的合成。(R,+)- 依滅草比消旋 (rac)- 或 (S,-)- 依滅草更能降低 ALS 活性。由 GH90 阿拉伯芥 ALS 的結構亦可發現其對依滅草具有光學的選擇性。

關鍵詞：依滅草、乙醯乳酸合成酶、除草劑抗藥性、蛋白質結構

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