J. Pestic. Sci. **49**(2), 77–86 (2024)
DOI: 10.1584/jpestics.D23-036

JOURNAL OF PESTICIDE SCIENCE

Regular Article

Dimesulfazet, a novel rice paddy herbicide, is an inhibitor of very long-chain fatty acid biosynthesis

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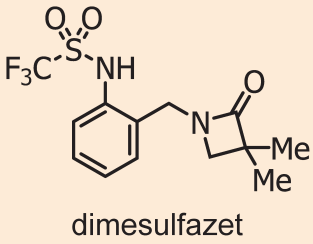
(Received July 27, 2023; Accepted February 5, 2024)

Supplementary material

Dimesulfazet can control annual and perennial sedges in rice paddies. Here we assessed its mode of action. We performed a phenotype assay of *Arabidopsis*, conducted a metabolomic analysis of *Echinochloa crus-galli*, and analyzed the endogenous concentration of very long-chain fatty acids (VLCFAs) in *Schoenoplectiella juncoides*. Dimesulfazet treatment caused curling and greening symptoms in the leaves and *fiddlehead*-like symptoms in the inflorescences of *Arabidopsis*. These symptoms were visually indistinguishable from those caused by flufenacet and benfuresate, which belong to Herbicide Resistance Action Committee (HRAC) Group 15. We performed GC-MS/MS analysis of primary metabolites and LC-MS analysis of lipids in the herbicide-treated *E. crus-galli*, followed by Orthogonal Partial Least Squares Discriminant Analysis clustering. The results showed that dimesulfazet belongs to the HRAC Group 15 cluster. The endogenous concentrations of C24:0, C26:0, and C28:0 decreased in dimesulfazet-treated plants as compared to those in the control. Overall, the mode of action of dimesulfazet involves the inhibition of VLCFA biosynthesis.



dimesulfazet

Keywords: metabolomics, endogenous concentration, Herbicide Resistance Action Committee, mode of action, phenotype assay.

Introduction

Dimesulfazet (2'-[(3,3-dimethyl-2-oxoazetidin-1-yl)methyl]-1,1,1-trifluoromethanesulfonanilide; code number: NC-653) is a novel herbicide used in rice paddy fields (Fig. 1). This herbicide was discovered in a study of trifluoromethansulfonanilide derivatives.[1)] Dimesulfazet at a field application rate of 150 g a.i./ha is highly efficient against Cyperaceae weeds, such as *Schoenoplectiella juncoides* (Roxb.), *Eleocharis kuroguwai* Ohwi, and *Bolboschoenus koshevnikovii* (Litv. ex Zinger) A.E.Kozhevn. Dimesulfazet selectivity was clearly observed between rice and *S. juncoides*, whose resistance to acetolactate synthase (ALS) inhibitors has been reported previously.[2,3)]

In plants, very long-chain fatty acids (VLCFAs) with more than 20 carbon atoms exhibit various functions. They are primarily involved in cuticle wax production.[4)] Some VLCFA biosynthesis inhibitors, such as flufenacet, have been identified; they inhibit several elongases in yeast cells.[5)] VLCFA biosynthesis inhibitors are categorized into Group 15 (ex-groups K3 and N) of Herbicide Resistance Action Committee (HRAC). In Japan, Group 15 herbicides, such as butachlor, pretilachlor, fentrazamide, and fenoxasulfone, are commonly used to control *Echinochloa oryzicola* var. in rice paddies. In contrast to VLCFA biosynthesis inhibitors, dimesulfazet has herbicidal effects on sedges. Although dimesulfazet and Group 15 herbicides show different weed spectra, the herbicidal symptoms of dimesulfazet are similar to those of Group 15 herbicides, which cause *fiddlehead*-like organ fusion.[6)] In addition, the trifluoromethanesulfonanilides mefluidide and perfluidon, whose structures are similar to that of dimesulfazet, are VLCFA biosynthesis inhibitors.[7)]

In the present study, we assessed the mode of action of dime-

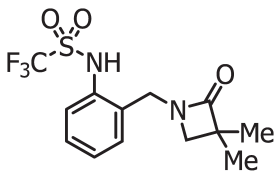


Fig. 1. Chemical structure of dimesulfazet (code number: NC-653).

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Published online April 27, 2024

sulfazet by examining the phenotype of *Arabidopsis*. We also studied the inhibitory effect of dimesulfazet, based not only on changes in metabolites using metabolomic approaches, but also on the endogenous concentrations of VLCFAs in *S. juncoides*.

Materials and methods

1. Chemical materials

Dimesulfazet, butachlor, esprocarb, and quizalofop-ethyl were synthesized by Chemical Research Laboratories (Nissan Chemical Corporation, Chiba, Japan) and were provided to Biological Research Laboratories (Nissan Chemical Corporation, Saitama, Japan). All other chemical compounds were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). For the phenotype assay, emulsifiable concentrate formulations at the desired concentrations were prepared by Nissan Chemical Corporation.

2. Plant materials

Arabidopsis thaliana ecotype Col-0 was purchased from Inplanta Innovation, Inc. The barnyard grass (*Echinochloa crus-galli* (L.) Beauv. var. *crus-galli*) and Japanese bulrush (*Schoenoplectiella juncoides* (Roxb.)) were collected from rice paddies in Shiraoka, Japan.

3. Phenotype assay

3.1. Cultivation

The sterilized diluvial soil was placed in plastic pots (5 cm long, 5 cm wide, and 8 cm deep). *Arabidopsis* seeds were sown in plastic pots. The seeds were allowed to germinate and grow in a growth chamber at 25°C for a light period of 16 hr and at 20°C for a dark period of 8 hr, with light intensities between 15,000 and 20,000 lx.

3.2. Herbicide treatment

The plants were treated with herbicides 6 weeks after sowing, when they had 8–10 true leaves with a rosette diameter of 6–9 cm. Each compound was prepared as an emulsifiable concentrate and diluted to the desired concentration with water containing a wetting agent (Tween20, at a final concentration of 1 mL/L). *Arabidopsis* plants in the stem elongation stage were sprayed with an equivalent of 1,000 L/ha test solution using a hand sprayer, AS-100 (Hitachi Industrial Equipment System, Tokyo, Japan). After the treatment, the plants were returned to the growth chamber. Dimesulfazet was sprayed at 50, 100, and 250 g/ha. Flufenacet was sprayed at 50 and 250 g/ha. Benfuresate was sprayed at 100 and 1,000 g/ha. There were two control groups in the experiment: (1) an untreated control group, and (2) a solvent control group treated with an emulsifiable concentrate formulation/water/Tween20 mixture.

3.3. Symptom evaluation

The plants were visually inspected for morphological abnormalities 7, 14, and 24 days after herbicide application.

4. Metabolomics

4.1. Cultivation

Seeds of *E. crus-galli* were soaked in water and stored in a refrigerator at 4°C for 1 week. Seeds were sown in alluvial soil in plastic pots (100 cm^2). Plants were grown in a greenhouse in Shiraoka, Japan.

4.2. Herbicide treatment

Echinochloa crus-galli plants were treated with the herbicides 14 days after sowing at the two-leaf stage. Each compound was prepared as a wettable powder and diluted to the desired concentration with water containing a wetting agent (Tween20, at a final concentration of 1 mL/L). Barnyard grass plants at the two-leaf stage were sprayed with an equivalent of 400 L/ha test solution using a hand sprayer. After the treatment, the plants were returned to the greenhouse. Dimesulfazet and benfuresate were sprayed at 600 and 1,200 g/ha. Butachlor was sprayed at 750 and 1,500 g/ha. Fentrazamide and cafenstrole were sprayed at 300 and 600 g/ha. Molinate was sprayed at 2,400 and 4,800 g/ha. Mefenacet and esprocarb were sprayed at 1,200 and 2,400 g/ha. Quizalofop-ethyl and cyhalofop-butyl groups were applied at concentrations of 100 and 200 g/ha, respectively.

4.3. Extraction of metabolites

Two days after the herbicide application, the shoots of the treated plants were cut and immediately frozen in liquid nitrogen. The shoot was homogenized in an extract solvent (methanol : chloroform : water = 3 : 1 : 1, 40-fold the fresh weight of shoot) using a FastPrep-24 beads homogenizer (MP Biomedicals, Santa Ana, USA) and centrifuged (9180 *g*, 10 min, 4°C) The supernatant was collected and subjected to mass spectrometry.

4.4. Derivatization and GC-MS/MS analysis

The plant extract (200 μL) was placed in a vial with an insert and the solvent was removed using centrifugal evaporator (EZ-2 Elite, Genevac, Ipswich, UK). To the residue, 40 μL of methoxymethane hydrochloride (20 mg/mL in pyridine) was added, and the sample was stirred at 30°C for 2 hr at 1200 rpm for methoxymation. Thereafter, 40 μL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide was added to the sample, which was then stirred at 37°C for 1 hr at 1200 rpm for trimethylsilylation and introduced into the GC-MS/MS system. For GC-MS/MS analysis, a Shimadzu GCMS-TQ8030 triple quadrupole gas chromatograph mass spectrometer (Shimadzu, Kyoto, Japan) was used, coupled to a BPX-5 column (30 m × 0.25 mm, 0.25 μm film thickness); helium was used as the carrier gas. Linear velocity was maintained at 39 cm/s. The vaporization chamber temperature was 280°C, and the column was heated from 60°C to 320°C at a rate of 10°C/min. One microliter of the experimental sample was injected at a split ratio of 5. The multiple reaction monitoring (MRM) method based on the Smart Metabolome Database (Shimadzu, Kyoto, Japan) was used.

4.5. Lipid analysis using LC-MS

Liquid chromatographic separation was performed using an Acquity UPLC CSH C18 column (100 mm × 2.1 mm, 1.7 μm; Waters, Milford, USA) attached to a Waters UPLC system. The metabolites prepared in phases A (acetonitrile/2-propanol = 1/9

with 10 mM ammonium formate and 0.1% formic acid) and B (acetonitrile/water=6/4 with 10 mM ammonium formate and 0.1% formic acid) were eluted from the column at a flow rate of 0.4 mL/min. The temperatures of the column and autosampler were maintained at 55°C and 10°C, respectively. The mobile phase started at 20% phase A, gradually increased to 99% phase A in 8 min, and maintained at 99% phase A for 6 min. Samples were injected randomly; the volume of sample injected was 10 μL. Electrospray ionization mass spectrometry (ESI-MS) was performed using a Waters SynaptG2-S mass spectrometer (Waters, Milford, USA). The desolvation gas and cone gas flow rates were set at 800 and 150 L/hr, respectively, and the gas temperature was 450°C. MS analysis was performed in scan mode with a mass scan range of 100–1500 *m*/*z*.

4.6. Data analysis

The metabolites identified by GC-MS/MS were annotated using the Smart Metabolite Database (v3; Shimadzu, Kyoto, Japan), and the annotated ions and their respective peak areas were used as the dataset for multivariate analysis. Metabolite peaks obtained from the LC-MS analysis were analyzed using Progenesis QI (Waters, Milford, USA), and all metabolite ions were checked against the Lipidblast database (Metabolomics Fiehn Lab, California, USA). Retention time, *m*/*z*, and normalized peak volumes of lipid ions were used as datasets for multivariate analysis. Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) was performed using SIMCA-14 (v14.1.0; Umetrics, Umeå, Sweden) with autoscaling. The reliability of the OPLS-DA model was verified using permutation tests. Metabolites contributing to the discrimination were determined based on the correlation coefficients in the S-plot of OPLS-DA.

5. Quantitative assay of fatty acids

5.1. Cultivation

The seeds of *S. juncoides* were soaked in water and stored in a refrigerator at 4°C for 1 week. Seeds were sown in alluvial soil in plastic pots (100 cm^2). Plants were grown in irrigated water at a depth of 4 cm in a greenhouse in Shiraoka, Japan.

5.2. Herbicide treatment

Schoenoplectiella juncoides plants were treated with herbicides 10 days after sowing at the two-leaf stage. Each compound was prepared as a wettable powder and diluted with water to the desired concentration. *Schoenoplectiella juncoides* plants at the two-leaf stage were treated with an equivalent of the test solution *via* drip application to the water surface. Dimesulfazet was applied at 150 and 300 g/ha. Butachlor was applied at 750 and 1,500 g/ha. Benfuresate was applied at 900 and 1,800 g/ha.

5.3. Extraction of fatty acids

At 3, 5, and 7 days after herbicide application, the shoots of the treated plants were cut and immediately frozen in liquid nitrogen. Shoots were homogenized in 25-fold methanol using a FastPrep-24 bead homogenizer. After adding a 25-fold volume of chloroform, the extract solution was homogenized and centrifuged (9186×*g*, 10 min, 4°C). Finally, the supernatant (400 μL) was collected and dried under reduced pressure.

5.4. Quantitative analysis of fatty acids

Standard solutions of each fatty acid methyl ester (a chain length of 12–28 carbons) were prepared in the concentration range of 10–3000 ng/mL *via* stepwise dilution of Supelco 37 Component FAME Mix (MERCK, Darmstadt, Germany), methyl cerotate (Merck), and methyl octacosanoate (Merck) in chloroform/acetonitrile at a 1 : 9 ratio. For the GC-MS analysis of fatty acids in plant extracts, lipid saponification and methylation were performed using a fatty acid methylation kit (Nacalai Tesque, Kyoto, Japan). To the fatty acids extraction residue, 175 μL of methylating reagents A and B was added, and the tubes were sealed and allowed to react overnight at 27°C under 1200 rpm. Thereafter, 175 μL of methylating reagent C was added and the reaction was carried out at 37°C for 20 min at 1,200 rpm. The extraction reagent included in the methylation kit (350 μL) was added and the mixture was stirred at 1,200 rpm for 5 min. Subsequently, the mixture was separated into two layers using centrifugation (9186×*g*, 20°C). The upper layer was collected and washed by adding 350 μL ultra-pure water and stirring. The samples were centrifuged again (9168×*g*), the upper layer was transferred to a vial with an insert, and the solvent was removed under reduced pressure using a centrifugal evaporator. The residue was dissolved in 70 μL of chloroform and then introduced into the GC-MS system. For GC-MS analysis, a Shimadzu GCMS-TQ8030 triple quadrupole gas chromatograph mass spectrometer (Shimadzu) coupled to a DB-5MS column (30 m×0.25 mm, I.D. 0.25 μm) was used. Helium was used as the carrier gas and the linear velocity was maintained at 39 cm/s. The temperatures of the inlet and ion source were 280 and 200°C, respectively, and the column was heated from 60 to 330°C at a rate of 15°C/min. The analysis was performed in EI+ mode at 70 eV and splitless injection was performed. Multiple reaction monitoring (MRM) was used to quantify fatty acids based on the Smart Metabolome Database (v3; Shimadzu, Kyoto, Japan).

Results

1. Phenotype assay

Seven days after treatment with dimesulfazet, flufenacet, or benfuresate, the plants started to shrink and presented with the greening of newly emerged leaves (Fig. 2). Although the control plants progressed to the stem elongation stage, the herbicide-treated plants showed delayed inflorescence development, especially under herbicide treatments at higher concentrations.

Fourteen days after treatment, the inflorescence of the herbicide-treated plants expanded. Inflorescence development was completely inhibited in some plants. After treatment with 250 g/ha dimesulfazet, none of the plants developed inflorescences, which was attributable to the herbicidal effect of dimesulfazet. The leaves of the treated plants showed abnormal morphology with shrinking, curling, and greening. Morphological abnormalities were also observed in the inflorescences, including curling and greening (Fig. 3). However, these symptoms were dissimilar to *the fiddlehead*-like symptoms caused by Group 15 herbicides 14 days after treatment.

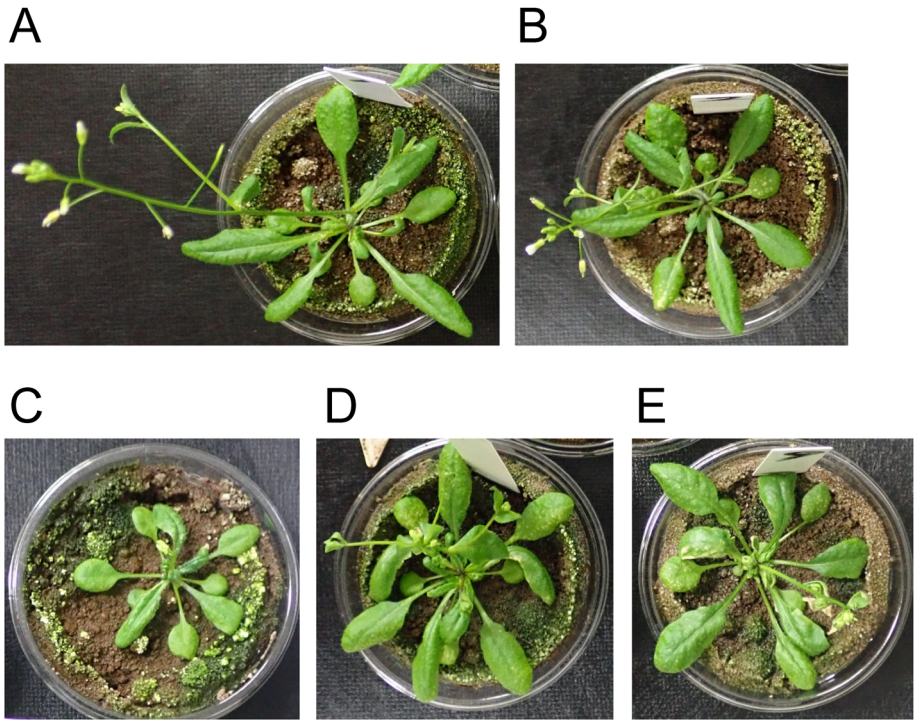

Fig. 2. Phenotypes of *Arabidopsis* at 7 days after dimesulfazet application. Dimesulfazet-, flufenacet-, and benfuresate-treated plants all showed shrinking and greening symptoms in newly emerged leaves. (A) Untreated control. (B) Solvent control. (C) Dimesulfazet 100 g/ha. (D) Flufenacet 250 g/ha. (E) Benfuresate 100 g/ha.

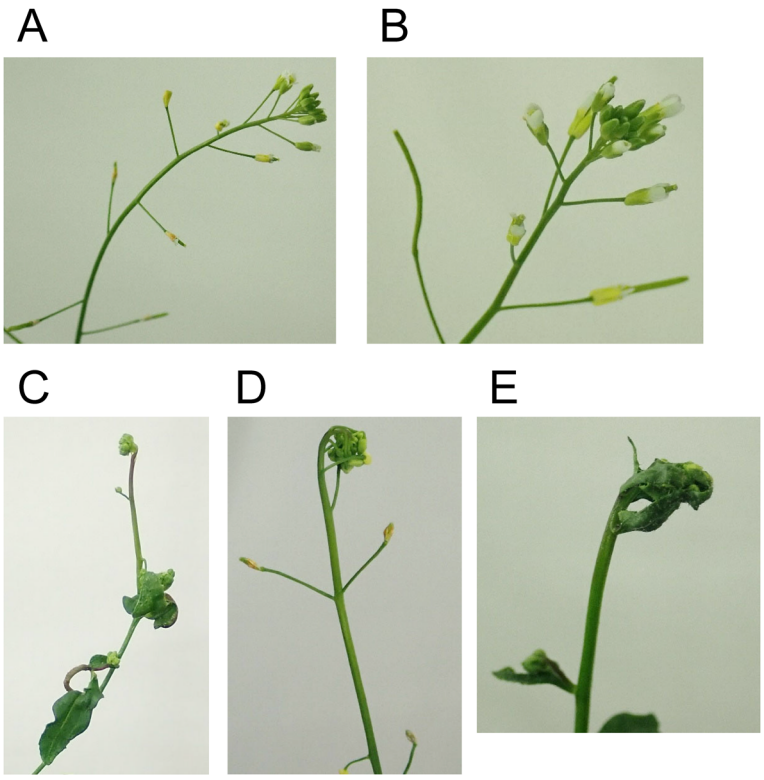

Fig. 3. Phenotypes of *Arabidopsis* at 14 days after dimesulfazet application. Dimesulfazet-, flufenacet-, and benfuresate-treated plants all showed shrinking and greening symptoms in inflorescences. (A) Untreated control. (B) Solvent control. (C) Dimesulfazet 100 g/ha. (D) Flufenacet 250 g/ha. (E) Benfuresate 100 g/ha.

At 24 days after treatment, the inflorescence length of the control plants was greater than 30 cm. Flufenacet- and benfuresate-treated plants exhibited *fiddlehead*-like symptoms (Fig. 4). Plants treated with 50 and 100 g/ha of dimesulfazet showed the same symptoms. However, plants treated with 250 g/ha dimesulfazet did not show *fiddlehead*-like symptoms because of the strong reduction in growth, as described above. A direct comparison of the herbicidal symptoms among dimesulfazet, flufenacet, and benfuresate indicated that the abnormal morphology was visually indistinguishable.

2. Metabolomics

We performed GC-MS/MS analysis of primary metabolites and LC-MS analysis of lipids to assess the mode of action of dimesulfazet. As most VLCFA inhibitors have herbicidal efficacy against *Echinochloa* spp., we selected *E. crus-galli* for metabolome analysis. In addition, benfuresate-treated *Arabidopsis* reportedly showed less dramatic changes in metabolites than other herbicide-treated plants.[8] To visually analyze the metabolomic changes under the herbicide-treated conditions, OPLS-DA was performed (Fig. 5).

Discriminant models were created using OPLS-DA for Group 1, Group 15, and the untreated control ($R^2X=0.602$, $R^2Y=0.894$, $Q^2=0.686$, Fig. 5A; $R^2X=0.808$, $R^2Y=0.91$, $Q^2=0.693$, Fig. 5C). Dimesulfazet was fitted to the score plot of this model as unclassified and was evaluated to determine which of the three groups was closer to dimesulfazet. Dimesulfazet plotted very close to Group 15, suggesting that it had an inhibitory effect on VLCFA biosynthesis. The validation plot demonstrated that the OPLS-DA model was valid, as all the corresponding permutated Q^2 and R^2 values to the left were significantly lower than the original Q^2 and R^2 values to the right, and the regression line had a negative intercept value on the Y-axis (Fig. 5B and 5D).

First, we created discriminant models using OPLS-DA for two groups, the untreated control and benfuresate, to verify the reliability of this experiment and selected metabolite ions that contributed to discrimination (Supplemental Fig. S1). The discriminant models were subjected to a permutation test to evaluate their validity. We identified some metabolites involved in the discrimination between the untreated control and benfuresate-treated plants (Supplemental Tables S1 and S2). As previously reported, asparagine and phenylalanine accumulated significantly in benfuresate-treated plants.[8] In contrast, glyceric acid and 2-ketoglutaric acid decreased.

Next, we created discriminant models using OPLS-DA for two groups, the untreated control and Group 15, to identify

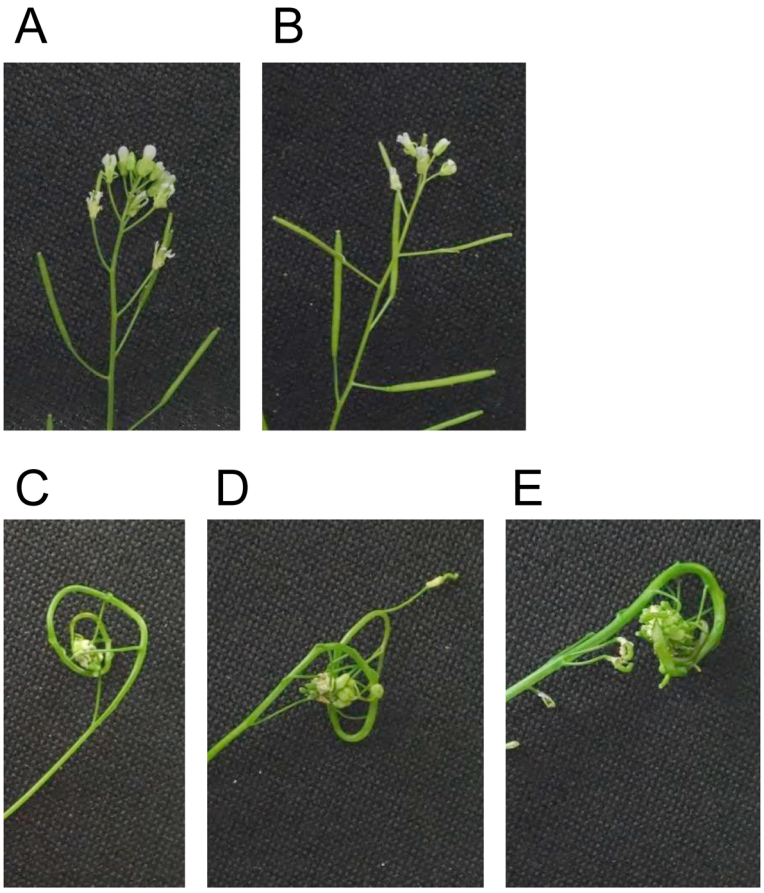

Fig. 4. Phenotypes of *Arabidopsis* at 24 days after dimesulfazet application. Dimesulfazet-, flufenacet-, and benfuresate-treated plants all showed *fiddlehead*-like symptoms. (A) Untreated control. (B) Solvent control. (C) Dimesulfazet 100 g/ha. (D) Flufenacet 250 g/ha. (E) Benfuresate 100 g/ha.

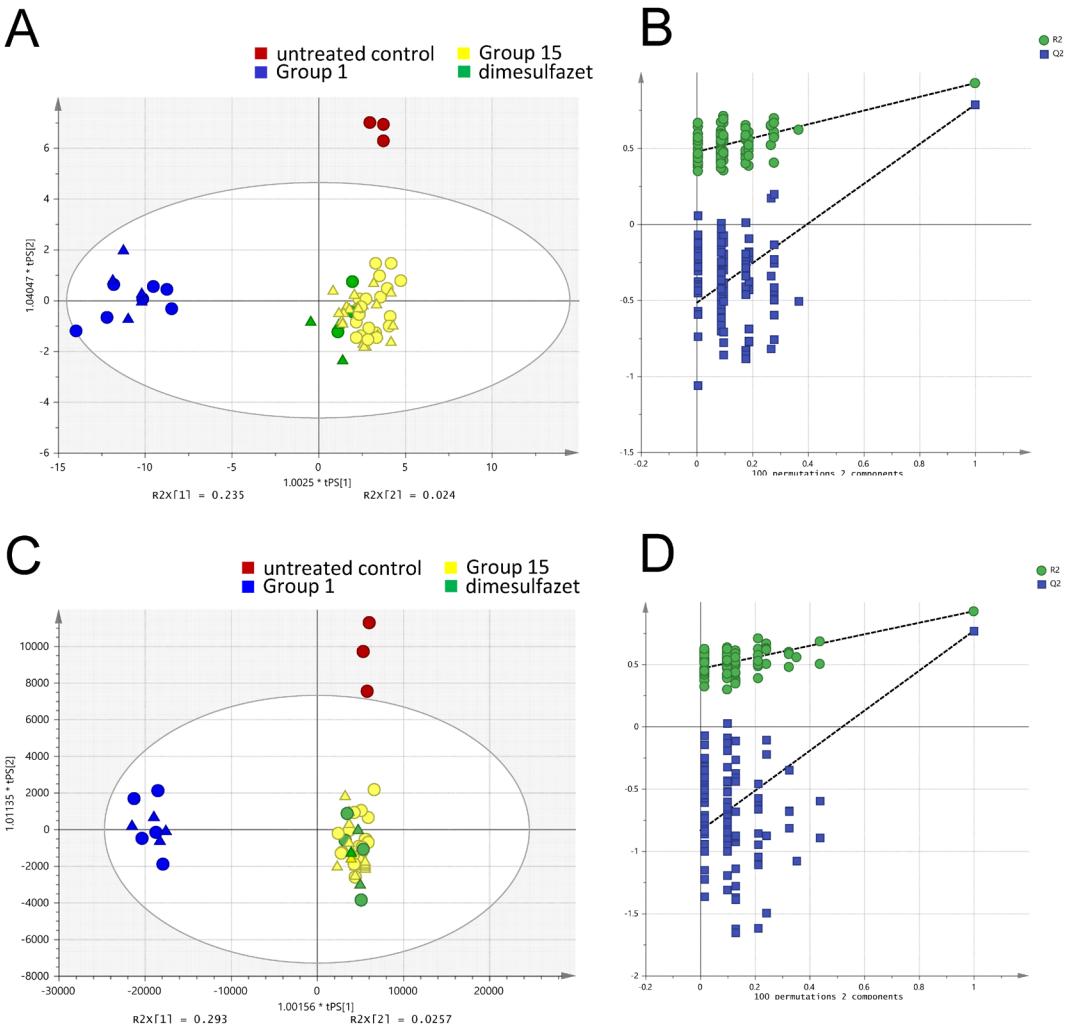

Fig. 5. Metabolome clusters of herbicides obtained using the OPLS-DA discriminant model. The yellow plot of Group 15 includes butachlor, mefenacet, fentrazamide, cafenstrole, molinate, benfuresate, and esprocarb in Fig. 5A and 5C. The blue plot of Group 1 includes quizalofop-ethyl and cyhalofop-butyl in Fig. 5A and 5C. Circle shows a lower rate of application and triangle shows a higher rate of application of each herbicide in Fig. 5A and 5C. (A) Metabolome clustering by GC-MS/MS. (B) Validation plot of the OPLS-DA model by GC-MS/MS. (C) Metabolome clustering by LC-MS. (D) Validation plot of the OPLS-DA model by LC-MS.

metabolites that fluctuated after treatment with VLCFA biosynthesis inhibitors, and selected metabolite ions that contributed to discrimination (Fig. 6). The discriminant models were subjected to a permutation test to evaluate their validity. We identified some metabolites that were involved in the discrimination between the untreated control and group 15. Inositol, allantoin, and specific amino acids such as glutamine, tyrosine, phenylalanine, and tryptophan accumulated significantly in Group 15 herbicide-treated plants (Table 1). Glyceric acid and 2-ketoglutaric acid levels decreased in Group 15 herbicide-treated plants (Table 2).

We then created discriminant models using OPLS-DA for two groups, the untreated control and dimesulfazet-treated groups, to examine their mode of action and selected metabolite ions that contribute to discrimination (Fig. 7). The discriminant models were subjected to a permutation test to evaluate their validity. We identified some metabolites that were involved in the discrimination between the untreated control and dimesulfazet-treated plants. Inositol, allantoin, and some organic acids accumulated significantly in the dimesulfazet-treated plants (Table 3). In addition, specific amino acids such as glutamine, asparagine, valine, threonine, tyrosine, and phenylalanine also increased in dimesulfazet-treated plants (Table 3). Glyceric acid, arabinose, and uridine levels decreased in the dimesulfazet-treated plants (Table 4).

Finally, we created discriminant models using OPLS-DA for two groups, Group 1 and Group 15, and selected the metabolite ions that contributed to discrimination (Supplemental Fig. S2). The discriminant models were subjected to a permutation test to evaluate their validity. We identified some metabolites that were involved in the discrimination between Groups 1 and 15. The specific amino acids such as glycine, phenylala-

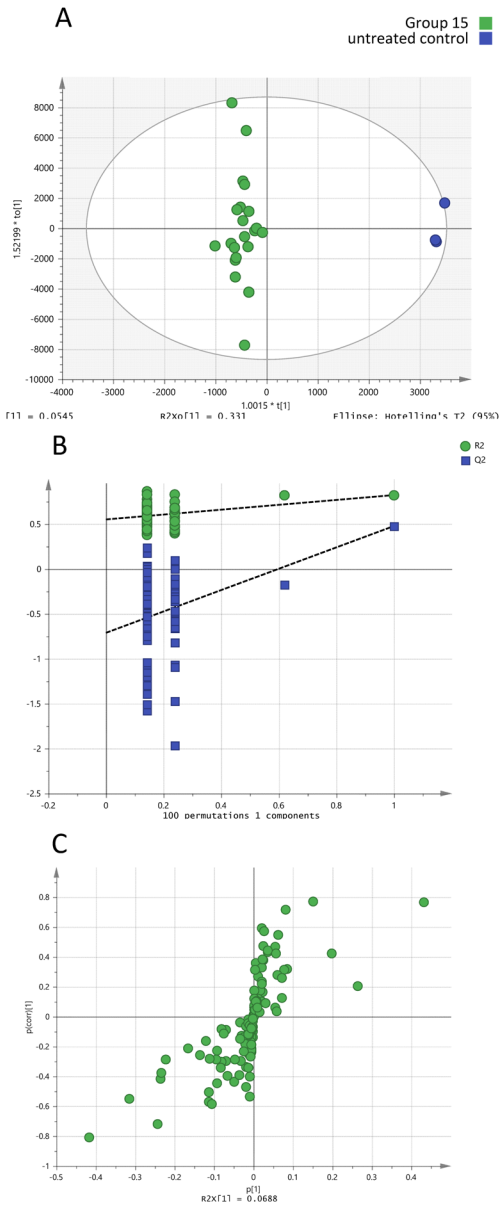

Fig. 6. OPLS-DA using GC-MS ions to discriminate the untreated control and Group 15. (A) Metabolome clustering by GC-MS/MS. The green plots of Group 15 includes butachlor, mefenacet, fentrazamide, cafenstrole, molinate, benfuresate, and esprocarb at a higher application rate. $R^2X=0.69$, $R^2Y=0.823$, $Q^2=0.479$. (B) Validation plot of the OPLS-DA model by GC-MS/MS. (C) S-plot derived from the OPLS-DA model, indicating biomarkers for the untreated control (upper right) and Group 15 (lower left). Magnitude (p[1], x axis) and reliability (p(corr), y axis) of GC/MS ions on this model were visualized.

nine, tyrosine, and tryptophan, and some organic acids, such as 3-hydroxypropionic acid, 3-aminopropanoic acid (*β*-alanine), and 3-aminoisobutyric acid, accumulated significantly in the Group 1 herbicide-treated plants (Supplemental Table S3 and S5). In addition, glyceric acid, 5-oxoproline, 2-ketoglutaric acid, and ribose accumulated in Group 15 herbicide-treated plants (Supplemental Table S4).

In the LC-MS lipid analysis, the annotation of detected ions using Lipidblast was also performed to identify the lipids contributing to the difference between Group 1 and Group 15. However, because data-dependent MS/MS measurements were not performed in this study, the annotation of lipid ions detected by LC-MS was not reliable and could not provide a clear indication of the lipid components involved in the differences between the two groups. To annotate the metabolites that contributed to the separation between these groups, it was necessary to obtain information on the fragment ions by MS/MS.

3. *Quantitative assay of fatty acids*

According to metabolomic analyses, the mode of action of dimesulfazet is predicted to involve inhibition of VLCFA biosynthesis. Therefore, we conducted a quantitative assay for fatty acids in the herbicide-treated *S. juncoides*. Each herbicide was administered in both practical and double doses in Japanese rice paddies. Because all herbicide treatments showed lethal efficacy against *S. juncoides*, a dose-response between the practical and double doses was not observed. We investigated the optimal time for the preparation of fatty acid samples. We found a similar tendency in the inhibitory effect on VLCFA biosynthesis among the herbicides 3, 5, and 7 days after herbicide application (data not shown).

Five days after herbicide application, the concentrations of C24:0, C26:0, and C28:0 fatty acids decreased in dimesulfazet-treated plants compared with those in the untreated control (Table 5 and Fig. 8). In contrast, the concentrations of C22:0, C24:0, C26:0, and C28:0 fatty acids decreased after butachlor treatment compared with those in the untreated control. In addition, the concentrations of C15:0 and C17:0 fatty acids, which are precursors of VLCFAs, increased considerably in all three herbicide treatments as compared to those in the untreated control.

Discussion

Our study elucidated the mode of action of dimesulfazet

Table 1. GC-MS ions with p[1]<−0.1 and p(corr)<−0.5 selected from Fig. 6C.

Annotated metabolites	p[1]	p(corr)[1]
Inositol	−0.417	−0.811
Glutamine	−0.315	−0.551
Tyrosine	−0.244	−0.720
Phenylalanine	−0.113	−0.569
Allantoin	−0.112	−0.504
Tryptophan	−0.106	−0.584

Table 2. GC-MS ions with p[1]>0.1 and p(corr)>0.5 selected from Fig. 6C.

Annotated metabolites	p[1]	p(corr)[1]
Glyceric acid	0.432	0.766
2-Ketoglutaricacid	0.152	0.769

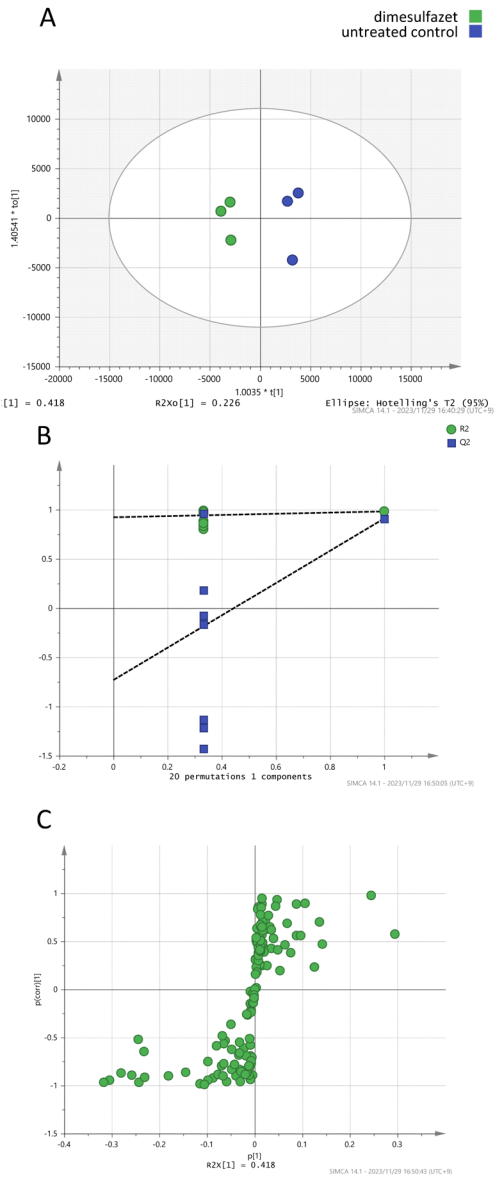

Fig. 7. OPLS-DA using GC-MS ions to discriminate the untreated control and dimesulfazet. (A) Metabolome clustering by GC-MS/MS. The green plots include dimesulfazet at a higher application rate. $R^2X=0.644$, $R2Y=0.982$, $Q^2=0.910$. (B) Validation plot of the OPLS-DA model by GC-MS/MS. (C) S-plot derived from the OPLS-DA model, indicating biomarkers for the untreated control (upper right) and dimesulfazet (lower left). Magnitude (p[1], x axis) and reliability (p(corr), y axis) of GC-MS ions on this model were visualized.

through a phenotypic assay in *Arabidopsis*, metabolomic analysis of *E. crus-galli*, and measurement of the endogenous concentrations of VLCFAs in *S. juncoides*.

Initially, we surmised that dimesulfazet may have an inhibitory effect on VLCFA biosynthesis because of its structural similarity to mefluidide and perfluidon.[5] Therefore, we performed a phenotype assay to confirm the morphological similarity after herbicide treatment between dimesulfazet and HRAC Group 15 herbicides. Flufenacet- or benfuresate-treated plants showed *fiddlehead*-like symptoms in *Arabidopsis*, similar to the findings of a previous study.[4] Dimesulfazet-treated plants also showed similar symptoms. Furthermore, all three herbicide-treated plants exhibited leaf greening. This finding indicates that the mode of action of dimesulfazet involves inhibition of VLCFA biosynthesis.

Metabolomic approaches have been commonly used in recent studies on the mode of action of pesticides.[8,9] Therefore, we applied a metabolomic approach to confirm the mode of action of dimesulfazet. In this study, we chose seven herbicides belonging to Group 15 of HRAC as inhibitors of VLCFA biosynthesis. In addition, we selected two ACCase inhibitors, quizalofop-ethyl and cyhalofop-butyl, in Group 1 of HRAC as negative controls, which inhibited the biosynthesis of C18 fatty acids from C16 fatty acids. These herbicides were applied to *E. crus-galli*, as most of the selected compounds showed high efficacy against this weed species. The metabolome clusters obtained using OPLS-DA revealed that the clusters in groups 1 and 15 were clearly separated. Dimesulfazet was categorized into a cluster of Group 15.

To identify the key metabolites involved in inhibiting VLCFA biosynthesis, we performed a series of discriminant analyses. The validity of this analysis was confirmed because asparagine and phenylalanine accumulated in benfuresate-treated plants, as previously reported.[8] In the metabolites involved in the discrimination of the untreated control and Group 15, we revealed that inositol and allantoin commonly increased and glyceric acid decreased following treatment with VLCFA biosynthesis inhibitors. The same metabolic changes in inositol, allantoin, and glyceric acid following treatment with dimesulfazet suggested an inhibitory effect of dimesulfazet on VLCFA biosynthesis. It

Table 3. GC-MS ions with $p[1]<-0.1$ and $p(corr)<-0.7$ selected from Fig. 7C.

Annotated metabolites	p[1]	p(corr)[1]
Glutamine	−0.317	−0.969
2-Aminoethanol	−0.305	−0.941
Asparagine	−0.281	−0.873
4-Aminobutyricacid	−0.258	−0.893
Valine	−0.242	−0.969
Inositol	−0.230	−0.915
Threonine	−0.181	−0.898
Tyrosine	−0.145	−0.865
Phenylalanine	−0.115	−0.981
Allantoin	−0.105	−0.987
3-Aminopropanoicacid	−0.098	−0.944
Adenine	−0.098	−0.751

Table 4. GC-MS ions with $p[1]>0.1$ and $p(corr)>0.7$ selected from Fig. 7C.

Annotated metabolites	p[1]	p(corr)[1]
Glyceric acid	0.245	0.978
Arabinose	0.136	0.702
Uridine	0.106	0.893

Table 5. Fatty acid concentrations of *S. juncoides* at 5 days after dimesulfazet application.

Fatty acid	Concentration (μg/g fresh weight)						
	Control	Dimesulfazet		Butachlor		Benfuresate	
		150 g/ha	300 g/ha	750 g/ha	1,500 g/ha	900 g/ha	1,800 g/ha
C14:0	2.81±0.24	3.16±0.39	3.16±0.39	3.16±0.38	3.80±0.51 *	3.20±0.35	3.96±0.75
C15:0	2.34±0.25	6.34±0.63 **	6.66±0.73 **	11.80±1.66 **	16.35±2.20 **	4.12±0.54 **	5.79±0.86 **
C16:0	526.77±53.93	605.63±20.03	622.45±45.69	619.75±73.42	612.12±87.07	670.86±94.27	768.26±62.46 **
C17:0	6.30±0.61	12.01±1.11 **	13.28±1.38 **	13.59±1.40 **	12.96±2.12 **	11.16±1.45 **	13.69±1.15 **
C18:0	220.13±25.19	212.60±14.84	231.63±7.15	239.48±28.04	232.28±30.77	241.39±40.66	282.18±31.27
C18:1	121.33±25.23	129.11±3.71	128.98±10.88	122.35±13.35	128.28±13.00	152.14±13.95	173.42±1.89
C18:3	1651.80±100.76	1886.40±71.31 *	1853.95±183.85	1869.69±98.21	1879.67±168.38	2040.12±162.74 *	2323.96±120.18 **
C20:0	6.99±0.72	8.61±0.29 *	8.76±0.63 *	6.85±0.38	6.48±0.59	8.70±0.87	9.34±0.86 *
C20:1	5.21±1.24	3.84±0.63	4.15±0.15	3.68±0.65	3.46±0.50	4.74±0.60	5.13±0.21
C22:0	15.41±1.19	17.33±0.37	15.56±0.79	12.01±0.48 *	11.55±0.78 **	17.90±0.92 *	18.51±1.92
C24:0	33.08±3.37	22.16±0.63 **	19.66±1.30 **	21.41±0.66 **	20.23±0.82 **	25.76±1.87 *	27.09±1.34 *
C26:0	9.22±0.78	7.13±0.24 *	6.32±0.21 **	6.83±0.48 *	6.52±0.33 **	8.27±0.97	8.28±0.37
C28:0	8.07±0.20	6.41±0.53 **	5.49±0.17 **	6.05±0.63 **	5.61±0.28 **	6.64±0.87	6.84±0.85

Data are expressed as the mean±S.D. of three independent experiments.
* Significantly different compared with the control, $p<0.05$ by Student's *t*-test.
** Significantly different compared with the control, $p<0.01$ by Student's *t*-test.

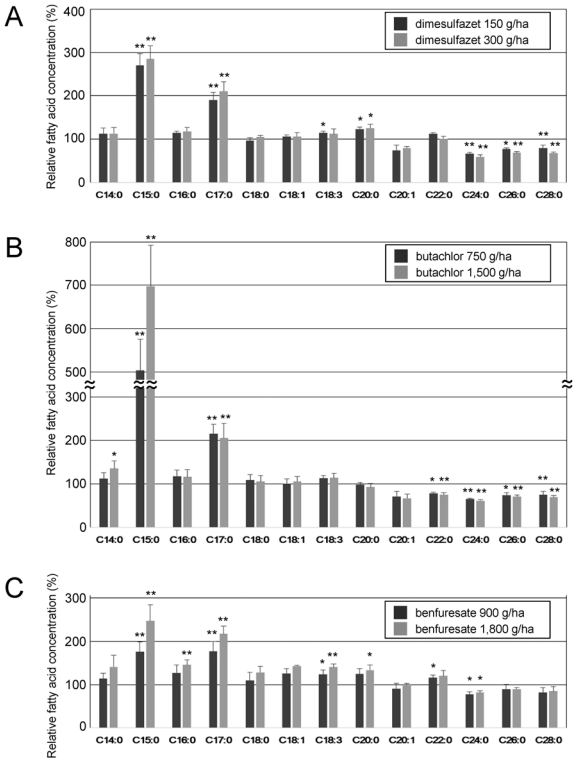

Fig. 8. Inhibitory effect of each herbicide on the biosynthesis of VLCFAs in *S. juncoides*. Each data set is expressed as the average and SD of three independent experiments. Single and double asterisks indicate significant differences compared with the control at $p<0.05$ and $p<0.01$ by Student's *t*-test, respectively.

has been reported that inositol and VLCFAs are precursors of sphingolipids.[10–13] It can be speculated that the accumulation of inositol is caused by an insufficient concentration of VLCFAs owing to treatment with VLCFA biosynthesis inhibitors. However, few studies have reported the relationship between the accumulation of allantoin or aromatic amino acids and treatment with inhibitors of VLCFA biosynthesis. Among the metabolites that are involved in the discrimination of Group 1 and Group 15, β-alanine and 3-hyrdoxypropionic acid were known as precursors of Coenzyme A (CoA).[14] It is suggested that the precursors of CoA accumulated due to reduced consumption of acetyl-CoA by inhibition of ACCase. These results strongly suggest that the mode of action of dimesulfazet involves the inhibition of VLCFA biosynthesis.

Finally, we confirmed the endogenous concentrations of fatty acids, including VLCFAs, which have more than 20 carbon atoms in their fatty acid structures. In the present study, herbicides were applied to *S. juncoides* because of their high efficacy against it. The endogenous concentrations of C15:0 and C17:0 drastically increased in dimesulfazet-, butachlor-, and benfuresate-treated plants compared with those in the untreated control. The same effect has been reported in pyroxasulfone-treated cultured rice cells and fenoxasulfone-treated barnyard millet cultured cells.[15,16] This indicates that Dimesulfazet has a similar mode of action as pyroxasulfone and fenoxasulfone, which are VLCFA biosynthesis inhibitors. The endogenous concentrations of C24:0, C26:0, and C28:0 decreased in dimesulfazet-treated plants as compared to those in the untreated controls. This finding indicates that dimesulfazet inhibits the elongation of C22:0 to C24:0. However, it is still unclear whether the elongation steps from C24:0 to C26:0 and from C26:0 to C28:0 are inhibited by dimesulfazet, because C24:0 is a precursor of these VLCFAs.

It has been previously reported that 3-ketoacyl-CoA synthases (KCS), such as CER60 (KCS5), CER6 (KCS6), and KCS17, produce saturated fatty acids with chain lengths longer than or

equal to 24 carbon atoms.[7] It indicates that dimesulfazet might inhibit one or some of these KCS enzymes. The endogenous concentration of C24:0 decreased in benfuresate-treated plants as compared to that in the untreated control. Although the decrease in the endogenous concentrations of C26:0 and C28:0 was not statistically significant in benfuresate-treated plants, the decrease in their VLCFA concentrations was similar to that in dimesulfazet-treated plants. This finding indicates that dimesulfazet and benfuresate may inhibit the same elongation steps involved in VLCFA biosynthesis. However, the endogenous concentrations of C22:0, C24:0, C26:0, and C28:0 were lower in butachlor-treated plants than in the untreated controls. The response to butachlor differed from that to dimesulfazet and benfuresate. This observation indicates that butachlor may inhibit different elongation steps involved in VLCFA biosynthesis. In the future, we aim to investigate the inhibitory activity of dimesulfazet against KCS enzymes, which are the target sites of VLCFAs biosynthesis inhibitors.[5,7]

In conclusion, the mode of action of dimesulfazet involves inhibition of VLCFA biosynthesis. Dimesulfazet can control various sedges in rice paddies, such as *S. juncoides*, *E. kuroguwai* and *B. koshevnikovii*. Currently, 4-hydroxyphenylpyruvate dioxygenase (4-HPPD) inhibitors such as benzobicyclone, tefuryltrione, and fenquinotrione, and fatty acid thioesterase (FAT) inhibitors such as bromobutide[17] are commonly used for *S. juncoides* control in Japanese rice paddies. Furthermore, perennial sedges, such as *E. kuroguwai* and *B. koshevnikovii*, are controlled using ALS inhibitors, such as metazosulfuron, propyrisulfuron and pyrimisulfan. Therefore, dimesulfazet, a VLCFA biosynthesis inhibitor, can be used to control Cyperaceae weeds in rice paddy fields. Dimesulfazet could be a novel agent for managing herbicide resistance of *S. juncoides* in rice production.

Declarations of interest

None

Electronic supplementary materials

The online version of this article contains supplementary material, which is available at https://www.jstage.jst.go.jp/browse/jpestics/

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