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Physiological disorder of plants depending on clopyralid concentration in the soil and plant

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The influence of clopyralid in soil on plant growth was investigated over time using three plants. The order of clopyralid sensitivity was as follows: *Solanum lycopersicum* > *Solanum melongena* > *Momordica charantia*, especially physiological disorder of *S. lycopersicum* were rapidly expressed as various serious symptoms with increasing concentration of clopyralid. In contrast, the clopyralid concentration of above-ground part was in the following order: *M. charantia* > *S. lycopersicum*, *S. melongena*, which differed from the order of sensitivity to clopyralid. © Pesticide Science Society of Japan

Keywords: clopyralid, synthetic auxin, physiological disorder, above-ground plant concentration.

Introduction

Clopyralid (3,6-dichloro-2-pyridinecarboxylic acid), which is a growth regulator herbicide, is widely used for weed control in the cultivation of cereal and grass in the USA, Canada, and Australia, among other regions, although it is not used in Japan.¹⁾ It is discharged in the excrement of livestock that have eaten feed containing it. Because clopyralid is hardly degraded,²⁾ it remains in the compost produced from this excrement. Indeed, it has been reported that crops were damaged by the application of compost with residual clopyralid.³⁻⁵⁾ Although clopyralid is not registered for use in Japan, exposure to it has occurred in the country *via* compost made from the excrement of livestock that have eaten imported feed containing clopyralid.^{6,7)} Thus, crop damage caused by compost with residual clopyralid has become a problem in Japanese agriculture.⁸⁾

The sensitivity to clopyralid differs among plant species; Solanaceae, Fabaceae, and Asteraceae have higher sensitivity to it than other plant families.⁹⁾ For example, according to Kelley

et al.,¹⁰⁾ the leaves of *Glycine max* Merrill became cupped and crinkled after clopyralid was sprayed on them. Moreover, when 50 µg of clopyralid was applied to the cotyledons, the leaves of *Helianthus annuus* L. became epinastic after 4 hr, and the stem was curved after 8 hr.¹¹⁾ Injury to *Solanum tuberosum* L. was also observed upon the presence of 3 µg/kg-DW clopyralid in soil when this species was cultivated in soil mixed with *Mentha cardiaca* L. hay that had been treated with clopyralid.¹²⁾ Damage to *Trifolium pratense* L. due to clopyralid was also found to be more severe than that to *Pisum sativum* L. upon their cultivation in compost containing 10 and 50 µg/kg-DW clopyralid.¹³⁾ Although the damage due to clopyralid was investigated in several plant species having high sensitivity to that herbicide,¹⁴⁾ there are few reports on the influence of clopyralid concentration in soil on the time-dependent shoot morphogenesis in plants having different sensitivities to it. The concentrations of clopyralid in plants were investigated for plants that are relatively tolerant to it, such as *Secale cereale* L.¹⁵⁾ and Brassicaceae.¹⁶⁾ In contrast, the concentrations of clopyralid in clopyralid-sensitive plants such as Solanaceae remain unclear.

Against that background, this study was performed to determine the influence of clopyralid concentration in soil over time on physiological disorders in the initial growth of plants such as *S. lycopersicum*, *Solanum melongena* L., and *Momordica charantia* L. which differ in their sensitivity to clopyralid and have been reported to suffer damage by it in Japan.¹⁷⁾ In addition, the sensitivity of these plants to clopyralid was evaluated using the physiological disorder and biomass of above-ground part, followed by discussion of the relationship of these variables to the clopyralid concentration in the above-ground part.

Materials and Methods

1. Plant culture in test soil

Clopyralid (chemical purity 98.0%; Fujifilm Wako Pure Chemical, Osaka, Japan) was dissolved in acetone. This clopyralid solution was then added to uncontaminated compost, after which the acetone was evaporated at room temperature in a draft chamber. In the results obtained in the survey of compost, the concentrations of clopyralid in compost were the mean value of 23 µg/kg-DW in the range of <0.1 to 380 µg/kg-DW.⁷⁾ When these composts are added to soil at 1 ton per 10 are, and suppose that plow layer is 10 cm, the concentrations of clopyralid in soil is 10 cm are the mean value of 0.23 µg/kg-DW and the maximum value of 3.8 µg/kg-DW. Thus, the final concentrations of clopyralid in the compost were 0, 100, 500, and 2,500 µg/kg-DW. This compost was added to uncontaminated nursery soil (Nihon Hiryo Co., Ltd., Tokyo, Japan) and the final concentrations of clopyralid in the soil were 0, 1, 5, and 25 µg/kg-DW.

Plastic pots (500 mL) were filled with this prepared soil (297 g of nursery soil mixed with 3 g of prepared compost). We pre-

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pared the soil 1 day before transplanting the seedlings and placed the plastic pots in the dark in plant-cultivation conditions. Seeds of *S. lycopersicum* 'Aiko' (Sakata Seed Corp., Yokohama, Japan) and 'Chika' (Takii & Co., Ltd., Kyoto, Japan), *S. melongena* 'Senryou-2-gou' and 'Chikuyou' (Takii & Co., Ltd.), and *M. charantia* 'Abashi-goya' (Sakata Seed Corp.) were sown in nursery soil and germinated in a growth chamber (Koito Kogyo, Kanagawa, Japan) at 25:20°C for *S. lycopersicum* and *S. melongena*, and 25°C for *M. charantia* under a 12:12 hr light:dark cycle. At 14 days for *S. lycopersicum* and *S. melongena*, and at 10 days for *M. charantia*, the seedlings were transplanted into pots and raised under the same conditions as in germination for 28 days. We supplied water from the bottom of the pot to prevent the runoff of clopyralid, and the soil moisture was maintained at 60–80% water-holding capacity. Every 7 days after transplanting, we observed the morphology of the above-ground part. Twenty-eight days after transplanting, the above-ground parts were harvested. The fresh weights of the above-ground parts were measured for each sample and then cut finely, mixed, and divided into two subsamples. One subsample was dried at 70°C to measure the moisture content, and the other was used to measure the clopyralid content. These uptake experiments were conducted in quadruplicate.

2. Analysis of clopyralid concentration in plants

To extract clopyralid, 10 g of above-ground plant samples were homogenized with 125 mL of methanol:1 M NaOH (99:1) for 3 min. The extract was passed through a 0.8 µm glass-fiber filter and concentrated to 100 mL in a rotary evaporator. Ten milliliters of extract was washed through a solid-phase extraction cartridge (Oasis HLB 225 mg, Waters, Milford, MA, USA) and eluted with 10 mL of methanol:0.01 M NaOH (1:1). The eluate was concentrated to 5 mL in a rotary evaporator and then supplemented with 3 mL of 1 M HCl. The concentrated solution was washed through a solid-phase extraction cartridge (Oasis HLB 225 mg) with 10 mL of 0.1 M HCl and then washed with 5 mL of 0.1 M HCl:acetonitrile (9:1) and 5 mL of water, followed by elution with 4 mL of 0.0028% (w/v) ammonia water:acetonitrile (9:1). The eluate that was supplemented with 100 µL of 1 M NaOH was extracted twice with 2 mL of dichloromethane with shaking for 30 sec, followed by centrifugation at 740×g for 1 min and removal of the organic phase. The aqueous phase that was supplemented with 150 µL of 12 M H₂SO₄ was extracted three times with 2 mL of dichloromethane with shaking for 30 sec, followed by centrifugation at 740×g for 1 min, after which the extract was collected. The extract was then supplemented with 1 mL of *n*-hexane and passed through Na₂SO₄ for dehydration, followed by drying under a nitrogen stream. Next, the dried residue was dissolved in 200 µL of 0.1% formic acid by ultrasonication for 30 sec. The solution was subsequently centrifuged at 1,700×g for 1 min and passed through a 0.22-µm PVDF membrane. The clopyralid in the cleaned-up samples was measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS, ACQUITY UPLC-Quattro Micro API, Waters) with an

ACQUITY UPLC HSS TS (50 mm×φ2.1 mm, 1.8 µm particle size; Waters). The limits of quantification (LOQs) for clopyralid analysis were calculated in accordance with Japanese Industrial Standard (JIS) K 0312.¹⁸⁾ The LOQ of clopyralid in above-ground plant samples was 5 µg/kg-DW calculated from tenfold standard deviation obtained from 7 times analysis (from extraction to LC-MS/MS measurement). Clopyralid recovery tests were performed on pulverized samples of *S. lycopersicum* above-ground parts in triplicate, spiked at 10 and 250 µg/kg-DW. The recovery rate of clopyralid ranged from 97.6 to 116.5% for 10 µg/kg-DW and from 92.6 to 104.4% for 250 µg/kg-DW. Performance of the analytical method was described in Watanabe *et al.*¹⁹⁾

3. Statistical analyses

Statistical analyses were performed using SPSS 23 software (SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was performed followed by Dunnett's test for multiple comparisons to 0 µg/kg-DW or Tukey's multiple comparison test using a pairwise comparison matrix to determine which samples differed significantly.

Results and Discussion

We analyzed the relationship between the temporal expression of physiological disorders due to clopyralid in *S. lycopersicum* 'Aiko,' which was classified as a species with high sensitivity to clopyralid, and the concentrations of clopyralid in soil which were 0, 1, 5, and 25 µg/kg-DW (Fig. 1).

In *S. lycopersicum* cultivated in soil with 1 µg/kg-DW clopyralid, the edges of the upper leaves tended to harden beginning on the 21st day after transplanting (Fig. 1D), and the morphology of flowers and fruits indicated no developmental dysfunction in this experiment. However, in *S. lycopersicum* cultivated in soil with 5 µg/kg-DW clopyralid, the growth point was twisted on the seventh day after transplanting (Fig. 1B); subsequently, rugose leaves and tortuous rachis were observed from the 14th day after transplanting (Fig. 1C). In contrast, the morphology of flowers (Fig. 1E), such as their petals and sepals, indicated no developmental dysfunction. However, because the ovary was enlarged immediately after flowering, it was highly possible that the fruit was parthenocarpic. On the 28th day after transplanting, the fruit of plants grown with 5 µg/kg-DW clopyralid showed the deformity of being elongated compared to the fruit of those grown in soil without clopyralid (Fig. 1F). In *S. lycopersicum* cultivated in soil with 25 µg/kg-DW clopyralid, the growth point of the leaves exhibited difficulty developing from the third day after transplanting (Fig. 1A) and then showed a twisted deformity on the seventh day (Fig. 1B). *Solanum lycopersicum* has compound leaves composed of a number of leaflets. The leaflets and blades in the leaves higher than the fifth leaf could not develop properly, and these leaves developed remarkable abnormalities in soil with 25 µg/kg-DW clopyralid (Fig. 1C). The deformed leaves were those that differentiated after transplanting, so it was assumed that they were strongly influenced by clopyralid. On the 21st day after transplanting, the tissue of these de-

Fig. 1. The physiological disorder in *S. lycopersicum* 'Aiko' depending on the concentration of clopyralid in the soil. (A) Growth point of the leaves on the 3rd day after transplanting. (B) Growth point of the leaves on the 7th day. (C) Leaves on the 14th day. (D) The upper leaves on the 21st day. Arrows indicate the leaf edges. (E) Flowers. (F) Fruits. (G) Necrotic leaf. (H) Growth point of leaves on the 21st day grown with 25 µg/kg-DW clopyralid in the soil.

formed leaves developed partial necrosis (Fig. 1G), and in one plant, growth stopped altogether (Fig. 1H). The flowers did not bloom because they did not develop normally, with the petals and sepals being transformed into tubular structures (Fig. 1E). Additionally, in some plants, flower clusters did not develop properly.

Overall, the findings showed that the period when symptoms of physiological disorders appeared in *S. lycopersicum* 'Aiko' became earlier with increasing concentrations of clopyralid in the soil. In addition, the physiological disorders were rapidly expressed in the form of various serious injuries with increasing concentrations of clopyralid in the soil. The concentration gradient of auxin controls leaflet initiation in plants exhibiting compound leaves, such as *S. lycopersicum*.²⁰⁾ Moreover, forms of synthetic auxin, such as clopyralid, bind to auxin receptors and then activate the transcription of auxin-responsive genes.²¹⁾ This results in the abnormal development of compound leaves and fruit that is controlled by the auxin concentration gradient. Therefore, it was inferred that various serious symptoms, such as rugose leaves and parthenocarp, were expressed with increasing concentrations of clopyralid in the soil.

Clopyralid sensitivity and the influence of clopyralid concentration in the soil on initial growth were evaluated using morphological observation (Fig. 2A) and by determining the dry weight of the above-ground part of each plant (Fig. 2B). The images of *S. lycopersicum* 'Aiko' and 'Chika' and *S. melongena* 'Senryou-2-gou' and 'Chikuyou' are from the 21st day after transplanting, while the images of *M. charantia* 'Abashi-goya' are

from the 14th day after transplanting (Fig. 2A).

At 1 µg/kg-DW clopyralid in soil, abnormal morphology of the above-ground part was not observed in any of the *S. melongena* and *M. charantia* samples, and it was suggested that clopyralid at 1 µg/kg-DW in soil did not negatively affect initial growth in these plants. Although the dry weight of the above-ground part of *S. lycopersicum* did not significantly decrease, the edges of the upper leaves tended to harden in *S. lycopersicum* at 1 µg/kg-DW clopyralid in the soil. On the other hand, 5 µg/kg-DW clopyralid in the soil led to remarkable abnormalities of the above-ground plant morphology in *S. lycopersicum*, such as rugose leaves and tortuous rachis. In addition, the above-ground plant dry weight of *S. lycopersicum* decreased in association with 5 µg/kg-DW clopyralid in the soil, so it was concluded that the initial growth of *S. lycopersicum* is inhibited under these conditions. In *S. melongena*, slight waving or hardening of the leaf edges due to 5 µg/kg-DW clopyralid in the soil was observed. However, no abnormal development of leaves or decrease in the above-ground plant dry weight occurred in this species. Thus, the physiological disorder in *S. melongena* was not as severe as in *S. lycopersicum* upon exposure to 5 µg/kg-DW clopyralid in the soil. In contrast to the findings for *S. lycopersicum* and *S. melongena*, the above-ground plant morphology and dry weight of *M. charantia* were not affected by 5 µg/kg-DW clopyralid in the soil. At 25 µg/kg-DW clopyralid in soil, *S. lycopersicum* showed severely rugose leaves, and its leaflets and blades could not develop properly. In addition, the growth point of *S. lycopersicum* became deformed and partially necrotic, after which

Fig. 2. The sensitivity to clopyralid in each plant species depending on its concentration in the soil. (A) The physiological disorder in each plant. Images of *S. lycopersicum* and *S. melongena* are from the 21st day after transplanting, while those of *M. charantia* are from the 14th day after transplanting. (B) Dry weights of above-ground parts of plants 28 days after the start of treatment. Data were analyzed by one-way ANOVA, followed by Dunnett's test for multiple comparisons to 0 µg/kg-DW (* $p < 0.05$, ** $p < 0.01$).

growth stopped. Moreover, the above-ground plant dry weight of *S. lycopersicum* with 25 µg/kg-DW clopyralid decreased to approximately half that with 0 µg/kg-DW clopyralid in the soil. Therefore, it was determined that the presence of clopyralid at 25 µg/kg-DW in soil markedly affects the initial growth of *S. lycopersicum*. In *S. melongena*, the leaves exhibited cupping, and the above-ground plant dry weight also tended to decrease with 25 µg/kg-DW clopyralid in the soil. In contrast, although unexpanded leaves were slightly tortuous at 14 days after transplanting into the soil, *M. charantia* was generally not influenced by 25 µg/kg-DW clopyralid in the soil, since its leaves exhibited normal development thereafter.

These results indicate that the sensitivity of plants to clopyral-

id is in the following order: *S. lycopersicum* > *S. melongena* > *M. charantia*. It was concluded that the clopyralid concentrations in soil that can cause serious physiological disorders, such as specifically inhibiting initial growth, are more than 5 µg/kg-DW for *S. lycopersicum* and 25 µg/kg-DW for *S. melongena*. In addition, it is possible that the difference in sensitivity to clopyralid between *S. lycopersicum* and *S. melongena*, despite their both being from the Solanaceae family, is caused by their differences in leaf formation. Because the control of plant hormones involved in leaf initiation differs between *S. lycopersicum*, which has compound leaves, and *S. melongena*, which has single leaves,²²⁾ it was assumed that *S. lycopersicum* is more sensitive to clopyralid than *S. melongena*. Moreover, the affinity for auxin receptors is one

Table 1. Concentration of clopyralid in each above-ground part after 28 days of growth.

Plants	Clopyralid concentrations in soil		
	1 µg/kg-DW	5 µg/kg-DW	25 µg/kg-DW
Concentrations of clopyralid in above-ground part (µg/kg-DW)			
<i>S. lycopersicum</i> 'Aiko'	5.7±0.5 A	41.2±6.7 A	471.7±18.6 B
<i>S. lycopersicum</i> 'Chika'	<5.00 ^{a)} A	20.4±0.6 A	493.8±35.2 B
<i>S. melongena</i> 'Senryou-2-gou'	5.3±0.8 A	31.6±2.1 A	210.4±17.9 A
<i>S. melongena</i> 'Chikuyou'	5.9±0.3 A	29.7±1.6 A	209.7±13.7 A
<i>M. charantia</i> 'Abashi-goya'	23.6±0.8 B	129.6±3.5 B	662.2±16.0 C

Mean±standard error of the mean ($n=4$). Data were compared using one-way ANOVA followed by Tukey's multiple comparison test ($p < 0.001$). Within a column, means followed by the same letter are not significantly different. ^{a)} Under the quantification limit.

of the most important factors determining selectivity in the activation by synthetic auxins such as clopyralid, and plants have several auxin receptors that have different affinities for auxin.²³⁾ Therefore, although the auxin receptors that clopyralid has high affinity have unknown, differences in the sensitivity to clopyralid might be caused by the differences in auxin receptors among plant species.

The clopyralid concentrations in the above-ground parts of plants are shown in Table 1. Clopyralid was not detected in the above-ground parts upon cultivation in soil with 0 µg/kg-DW clopyralid (data not shown), so all of the clopyralid in the above-ground parts was considered to have translocated from the roots.

At 1 µg/kg-DW clopyralid in soil, the above-ground plant concentrations of clopyralid in *S. lycopersicum* and *S. melongena* were very low, whereas those in *M. charantia* were four times higher than in the other tested plants. In addition, the above-ground plant concentration of clopyralid in *M. charantia* was about three to six times higher than those in *S. lycopersicum* and *S. melongena* at 5 µg/kg-DW clopyralid in soil. At 25 µg/kg-DW clopyralid in soil, the above-ground plant concentrations of clopyralid in *S. melongena* were the lowest, those in *S. lycopersicum* were low, and those in *M. charantia* were the highest, as in the cases of cultivation with other concentrations of clopyralid in the soil. These results show that the concentrations of clopyralid in above-ground parts of tested plants did not necessarily correspond to the degree of physiological disorder in their above-ground parts.

In general, the uptake of organic chemicals including pesticides such as clopyralid by plants is influenced by the chemicals' properties, especially those that determine the *n*-octanol–water partition coefficient (K_{OW}).²⁴⁾ According to Dettenmaier *et al.*,²⁵⁾ organic chemicals with a $\log K_{OW} < 0$ have a high ability to translocate from root to shoot. Because the $\log K_{OW}$ value of clopyralid is -2.63 ,²⁶⁾ it was assumed that clopyralid can smoothly translocate from the root to the above-ground part of plants by transpiration. In addition, although Cucurbitaceae has a species-specific high translocation ability for high hydrophobic chemicals with a $\log K_{OW} > 5$, such as dieldrin, this ability could not be observed in organic chemicals with a $\log K_{OW} < 4$.^{27,28)} Therefore, it was inferred that *M. charantia* does not have a species-specific high translocation ability from the root to the above-ground part of the plant for clopyralid. Although *S. lycopersicum* and *S. melongena* were considered to have taken up clopyralid like *M. charantia*, the concentrations of clopyralid in the above-ground parts of these plants were low. This suggested that these two species might metabolize the clopyralid or accumulate it in the root to prevent transport to the above-ground parts.

In future work, it will be necessary to identify the auxin receptor(s) that has high affinity for clopyralid and the mechanisms of clopyralid metabolism in order to explain the differences in sensitivity and above-ground plant concentration of clopyralid among plant species. In addition, elucidation of the factors behind the differences in response to clopyralid among plant

species should contribute to preventing physiological disorders.

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