

Emergence of Glyphosate- and Glufosinate-resistant Italian Ryegrass (*Lolium multiflorum*) Populations in Japanese Pear Orchards in Japan and their Responses to Several Foliar-applied Herbicides

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Abstract

In this study, we aimed to evaluate the levels of resistance of *Lolium multiflorum* populations to glyphosate and glufosinate in Japanese pear orchards in Hamamatsu City, Shizuoka Prefecture, Japan, and determine the efficacy of several foliar-applied herbicides to control this weed during several growth stages. Bioassays using glyphosate-potassium (1.08-2.70 kg ai ha⁻¹) and glufosinate (0.60-1.00 kg ai ha⁻¹) were conducted, and the survival rates of *L. multiflorum* populations after foliar application were 11.1%-53.3% and 0.0%-5.6%, respectively. After treatments with several foliar-applied herbicides in the early growth stage (at a plant length of about 5 cm) with the maximum dosage, the survival rate of plants treated with glyphosate-potassium (38.9%) was not significantly different from that of plants receiving no treatment (100%). However, the survival rates of plants treated with quizalofop-ethyl (0.0%), glufosinate (17.8%), and glufosinate-P-sodium (18.9%) were significantly lower than that of plants receiving no treatment. In the middle growth stage (at a plant length of about 35 cm), although the survival rate of plants treated with quizalofop-ethyl (8.3%) was significantly lower than that of plants receiving no treatment (100%), the survival rates of other plants were not significantly different from that of plants receiving no treatment. These results indicate that *L. multiflorum* has developed resistance to glyphosate and glufosinate in the orchards, and suggest that the foliar application of quizalofop-ethyl (0.74 kg ai ha⁻¹) is particularly effective for the control of this weed.

Discipline: Crop Science

Additional key words: glyphosate, glufosinate, herbicide resistance, Japanese pear orchard, *Lolium multiflorum*

Introduction

Lolium multiflorum Lam. is an exotic winter annual weed and an anemophilous cross-pollinated species that has a significant negative impact on crop production in Japan (Asai & Yogo 2005). This weed has been naturalized in farmlands such as wheat fields, rice paddy levees, and Japanese pear orchards in Shizuoka Prefecture, Japan. In wheat fields, this weed causes huge economic losses in yield through competition with the crop (Suzuki et al. 2010), and is a host plant in rice paddy levees for rice bugs that cause pecky rice by sucking on the developing kernels and reduce rice quality (Higuchi

2010, Yasuda et al. 2013). It has become increasingly difficult to control this weed with glyphosate- and glufosinate-based herbicides, and the weed interferes with farming in Japanese pear orchards in Hamamatsu city, Shizuoka Prefecture, where these herbicides are repeatedly used every year. Therefore, *L. multiflorum* populations in the orchards may contain glyphosate- and glufosinate-resistant plants.

Resistance to both glyphosate and glufosinate has been confirmed in *Eleusine indica* (L.) Gaertn. in a vegetable farm and oil palm nursery in Malaysia (Jalaludin et al. 2015), *L. multiflorum* in hazelnut orchards in Oregon, USA (Avila-Garcia & Mallory-Smith 2011),

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L. multiflorum in vineyards and pear orchards in California, USA (Karn et al. 2018), and *L. multiflorum* and *Lolium perenne* in vineyards in New Zealand (Ghanizadeh et al. 2015). In Japan, resistance to glyphosate (Ichihara et al. 2016, Niinomi et al. 2013) and glufosinate (Ichihara et al. 2018) and multiple resistance to these herbicides (Ichihara et al. 2018) have been confirmed in *L. multiflorum* populations in the levees of rice paddies in Shizuoka Prefecture. These herbicides have also been generally used in orchards in Japan. However, it is unclear whether the weed has multiple resistance to these herbicides in orchards in Asia, including Japan. To develop effective management strategies for *L. multiflorum* populations containing glyphosate- and glufosinate-resistant plants in Japanese pear orchards, it is necessary to evaluate the levels of resistance to these herbicides.

The efficacy of several herbicides in controlling glyphosate-resistant *L. multiflorum* in rice paddy levees has been evaluated with a two-year study in Shizuoka Prefecture (Miyata et al. 2015). In the study, vegetation coverage of this weed in the levees in late May (spike emergence stage) was investigated after the foliar application of several herbicides in April. The vegetation coverage was high in the plot treated with glyphosate-potassium in both years (85% in the first year; 88% in the second year), whereas it was low in the plot treated with fluzifop-P (19% in the first year; 5% in the second year) and varied greatly in the plot treated with glufosinate (28% in the first year; 88% in the second year). Effective herbicides for *L. multiflorum* populations that may contain glyphosate- and glufosinate-resistant plants in Japanese pear orchards are unknown. It is particularly necessary to determine the efficacy of herbicides applied on foliage due to the general use of herbicides in Japanese pear orchards in Hamamatsu City. In this study, we investigated the levels of resistance to glyphosate and glufosinate in *L. multiflorum* populations in Japanese pear orchards in Hamamatsu City, and the efficacy of several foliage-applied herbicides that are registered (or likely to be registered) for use in pear orchards in Japan to control this weed during several growth stages.

Materials and methods

1. Levels of resistance to glyphosate and glufosinate

Resistance to glyphosate and glufosinate was investigated in three populations of *L. multiflorum* in Japanese pear orchards (field 1, field 2, and field 3) in the Hiraguchi area (34.80°N, 137.76°E), Hamakita ward, Hamamatsu City, Shizuoka Prefecture, Japan. In field 1, glyphosate-potassium was applied in April and June, and

glufosinate was applied in August every year. In fields 2 and 3, glyphosate-potassium was applied in March and November, and glufosinate-P-sodium was applied in July every year. The glyphosate-based herbicides have been used for at least 20 years in each field, and the glufosinate-based herbicides have been used for at least 20 years in field 1, and for six years in fields 2 and 3.

Mature seeds of *L. multiflorum* were collected from each field on June 23, 2017, and stored in paper envelopes at room temperature under dry conditions. In addition, seeds of Tachimusha® (CV; Snow Brand Seed Company, Sapporo, Japan), an *L. multiflorum* cultivar for livestock feed, were used as the susceptible control. All seeds were placed on moistened filter paper in 9-cm plastic petri dishes on September 25, 2017. After germination, the seedlings were individually transplanted to cell trays (4.2 × 4.2 cm and 4.5 cm in depth) filled with culture soil (Hochiaguriko, Omaezaki, Japan), and then grown in a greenhouse at Shizuoka Prefectural Research Institute of Agriculture and Forestry in Iwata City, Shizuoka Prefecture, Japan. When the plant length was about 15 cm (the 2.5-3.5 leaf stage) on October 24, bioassays for glyphosate-potassium and glufosinate were conducted. The seedlings were treated with glyphosate-potassium (Roundup Maxload®, Nissan Chemical, Tokyo, Japan) at rates of 0, 1.08, 1.62, 2.16, and 2.70 kg ai ha⁻¹, or glufosinate (Basta®, Bayer CropScience, Tokyo, Japan) at rates of 0, 0.60, 0.80, and 1.00 kg ai ha⁻¹. To understand the response of this weed to the herbicide dosage in practical use, dosages were set in the ranges specified as per the registration of these herbicides for use in pear cultivation in Japan. About 36 plants were tested per treatment, and all treatments had three replicates. The number of surviving plants was counted seven weeks after the treatment. The survival rate of the plants was calculated using the following equation:

$$S = (A/B) \times 100$$

where S is the survival rate, A is the number of surviving plants seven weeks after the treatment, and B is the number of plants before the treatment. The surviving plants were defined as the plants with new leaves. The greenhouse temperature was recorded during the investigation period using a TR-72U Thermo Recorder (T & D Corporation, Matsumoto, Japan) placed 2.3 m above the ground.

2. Efficacy of several foliar-applied herbicides

The seeds of *L. multiflorum* population in field 3 were used. On January 22, 2018, the seeds were sown in 1/5000 a Wagner pots filled with culture soil (Hochiaguriko, Omaezaki, Japan) and covered with 1-2 cm of soil. These pots were placed in a greenhouse at

Shizuoka Prefectural Research Institute of Agriculture and Forestry. The plants were treated with several foliar-applied herbicides when the plant length was about 5 cm (the 1.0-1.5 leaf stage) on February 22 (early growth stage), about 35 cm on March 30 (middle growth stage), and about 70 cm just before spike emergence on April 27 (late growth stage). We set up treatments of glyphosate-potassium (2.70 kg ai ha⁻¹), glufosinate (1.00 kg ai ha⁻¹), glufosinate-P-sodium (0.63 kg ai ha⁻¹), and quizalofop-ethyl (0.74 kg ai ha⁻¹), and all the treatments had three replicates. The major foliar-applied herbicides registered for pears in Japan (glyphosate-potassium, glufosinate, and glufosinate-P-sodium) were used in this study. Quizalofop-ethyl (predicted to be effective for the control of this weed and likely to be registered in the future) was also used. The dosages of the herbicides were set to the maximum at registration (or future registration) of these herbicides for pears in Japan. The number of plants per pot in the early, middle, and late stages of growth was 30, 20, and 15, respectively. The number of surviving plants was counted seven weeks (for treatment in the early and middle growth stages) or five weeks (for treatment in the late growth stage) after the treatment. The survival rate of the plants was calculated using the following equation:

$$S = (A/B) \times 100$$

where, S is the survival rate, A is the number of surviving plants seven or five weeks after the treatment, and B is the number of plants before the treatment. The surviving plants were defined as the plants with new leaves. The greenhouse temperature was recorded during the investigation period using a TR-72U Thermo Recorder placed 2.0 m above the ground.

3. Statistical analyses

Differences in the survival rates of the plants between each treatment or each field were tested using Tukey's honestly significant difference (HSD) test for multiple comparisons. All statistical analyses were performed with R (Version 2.15.1; R Development Core Team 2012). Before testing, the survival rates were arcsine-transformed in investigating the levels of resistance to glyphosate and glufosinate, and the numbers of surviving plants were log-transformed in investigating the efficacy of several foliar-applied herbicides.

Results

1. Levels of resistance to glyphosate and glufosinate

All plants of the susceptible cultivar were killed by the treatments with glyphosate-potassium or glufosinate, whereas a portion of plants of *L. multiflorum* populations in the Japanese pear orchards survived (Table 1). As the

dosage of glyphosate-potassium increased, the survival rates of plants in *L. multiflorum* populations in the orchards tended to decrease. The survival rates of plants treated with glyphosate-potassium at 1.08 kg ai ha⁻¹ were 38.0-53.3%, whereas those of the plants treated with glyphosate-potassium at 2.70 kg ai ha⁻¹ were 11.1-27.8%. Although the survival rates of the plants were not significantly different between each treatment of glufosinate in the range of 0.60 to 1.00 kg ai ha⁻¹ ($P > 0.05$), those of the plants treated with glufosinate at 1.00 kg ai ha⁻¹ tended to be the lowest (0.0-1.9%).

The survival rates of plants treated with glyphosate-potassium at 2.16 kg ai ha⁻¹ was higher in field 3 than in field 2 ($P < 0.05$), and the survival rates of plants treated with 2.70 kg ai ha⁻¹ were higher in field 3 than in both other fields ($P < 0.01$) (Fig. 1 (a)). However, the survival rates of plants treated with glufosinate at 0.60, 0.80, or 1.00 kg ai ha⁻¹ did not differ significantly ($P > 0.05$) (Fig. 1 (b)).

The average temperatures in the greenhouse and outdoors in Iwata City (data from the Japan Meteorological Agency) during the investigation period were 15.2°C and 12.0°C, respectively.

2. Efficacy of several foliar-applied herbicides

After the treatments with several foliar-applied herbicides in the early growth stage of *L. multiflorum*, the survival rates of plants treated with glufosinate (17.8%) and glufosinate-P-sodium (18.9%) were significantly lower than that of the plants receiving no treatment (100%) ($P < 0.01$), although the survival rate of plants treated with glyphosate-potassium (38.9%) did not differ significantly from that of the plants receiving no treatment ($P > 0.05$) (Fig. 2 (a)). Moreover, the survival rate of plants treated with quizalofop-ethyl (0.0%) was significantly lower than the survival rates of plants treated with glufosinate and glufosinate-P-sodium ($P < 0.01$).

After the plants were treated with the herbicides in the middle growth stage, the survival rates of plants treated with glyphosate-potassium (78.3%), glufosinate (98.3%), and glufosinate-P-sodium (86.7%) and that of the plants receiving no treatment (100%) were not significantly different ($P > 0.05$), whereas the survival rate of plants treated with quizalofop-ethyl (8.3%) was significantly lower than the survival rates of the other plants ($P < 0.01$) (Fig. 2 (b)).

After the plants were treated with herbicides in the late growth stage, the survival rates of plants treated with glyphosate-potassium (100%), glufosinate (100%), glufosinate-P-sodium (91.1%) and that of the plants receiving no treatment (97.8%) were not significantly different ($P > 0.05$), whereas the survival rate of plants

Table 1. Survival rates of the cultivar and 3 wild populations of *L. multiflorum* after foliar application of glyphosate-potassium or glufosinate

Cultivar or population	Glyphosate treatments (kg ai ha ⁻¹)	% Survival (\pm SE)	Cultivar or population	Glufosinate treatments (kg ai ha ⁻¹)	% Survival (\pm SE)
Tachimusha®	0.00	99.0 \pm 1.0 a	Tachimusha®	0.00	99.0 \pm 1.0 a
	1.08	0.0 \pm 0.0 b		0.60	0.0 \pm 0.0 b
	1.62	0.0 \pm 0.0 b		0.80	0.0 \pm 0.0 b
	2.16	0.0 \pm 0.0 b		1.00	0.0 \pm 0.0 b
	2.70	0.0 \pm 0.0 b			
Field 1	0.00	100.0 \pm 0.0 a	Field 1	0.00	100.0 \pm 0.0 a
	1.08	38.0 \pm 5.6 b		0.60	4.8 \pm 2.5 b
	1.62	37.8 \pm 2.3 b		0.80	1.9 \pm 1.9 b
	2.16	23.1 \pm 2.4 c		1.00	1.9 \pm 0.9 b
	2.70	11.1 \pm 1.6 d			
Field 2	0.00	100.0 \pm 0.0 a	Field 2	0.00	100.0 \pm 0.0 a
	1.08	53.3 \pm 4.1 b		0.60	1.0 \pm 1.0 b
	1.62	34.8 \pm 5.4 b		0.80	0.9 \pm 0.9 b
	2.16	13.0 \pm 4.0 c		1.00	0.0 \pm 0.0 b
	2.70	13.1 \pm 2.0 c			
Field 3	0.00	99.1 \pm 0.9 a	Field 3	0.00	99.1 \pm 0.9 a
	1.08	47.2 \pm 1.6 b		0.60	5.6 \pm 2.8 b
	1.62	45.7 \pm 7.3 bc		0.80	1.9 \pm 1.0 b
	2.16	28.7 \pm 0.9 bc		1.00	0.9 \pm 0.9 b
	2.70	27.8 \pm 1.6 c			

* Different letters indicate significant differences between treatments within each field or cultivar at $P < 0.05$, based on Tukey's honestly significant difference test.

treated with quizalofop-ethyl (64.4%) was significantly lower than the survival rates of the other plants ($P < 0.01$) (Fig. 2 (c)).

The respective average temperatures in the greenhouse and outdoors in Iwata city (data from the Japan Meteorological Agency) were 14.2°C and 12.0°C during the early growth stage, 19.1°C and 16.8°C during the middle growth stage, and 21.4°C and 19.1°C during late growth stage of the investigation.

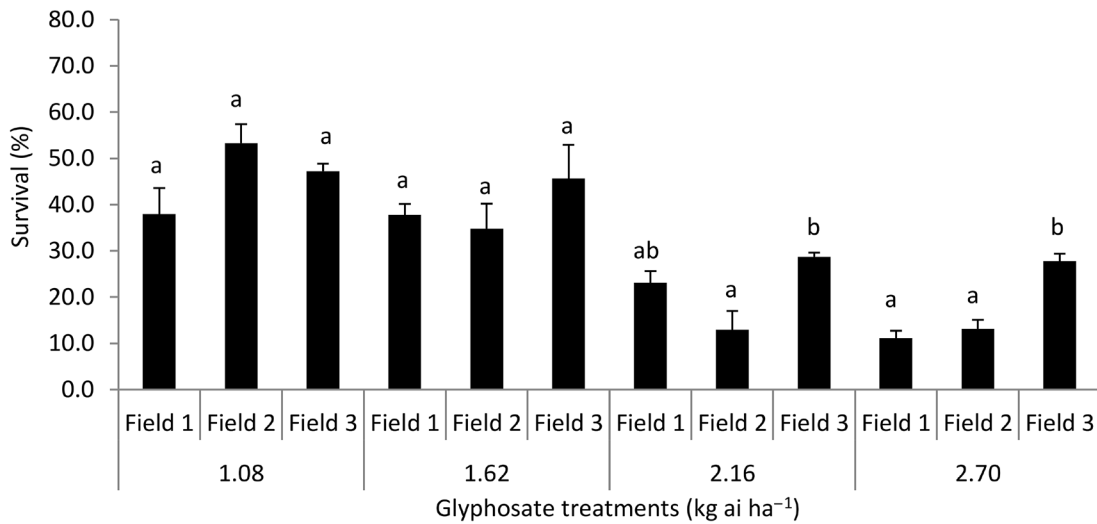
Discussion

1. Levels of resistance of *L. multiflorum* to glyphosate and glufosinate

This study revealed the levels of resistance of *L. multiflorum* populations in the Japanese pear orchards to glyphosate and glufosinate in Hamamatsu City, Shizuoka Prefecture, Japan. Some of the *L. multiflorum* plants in

the orchards survived after treatment with glyphosate-potassium or glufosinate (Table 1). Therefore, *L. multiflorum* populations in the orchards contain glyphosate- and glufosinate-resistant plants. The reason for the different levels of resistance of the individuals to glyphosate and glufosinate is that the weed is an anemophilous, self-sterile, cross-pollinated species. To the best of our knowledge, this is the first report on the emergence of weeds with multiple resistance to these herbicides in orchards in Asia, including Japan. In previous studies, resistance to both glyphosate and glufosinate due to repeated use of these herbicides has been reported in *Eleusine indica* in a vegetable farm and oil palm nursery in Malaysia (Jalaludin et al. 2015), *L. multiflorum* and *L. perenne* in vineyards in New Zealand (Ghanizadeh et al. 2015), and *L. multiflorum* in vineyards and pear orchards in California, USA (Karn et al. 2018). In the Japanese pear orchards in this study, glyphosate-

(a) Glyphosate application



(b) Glufosinate application

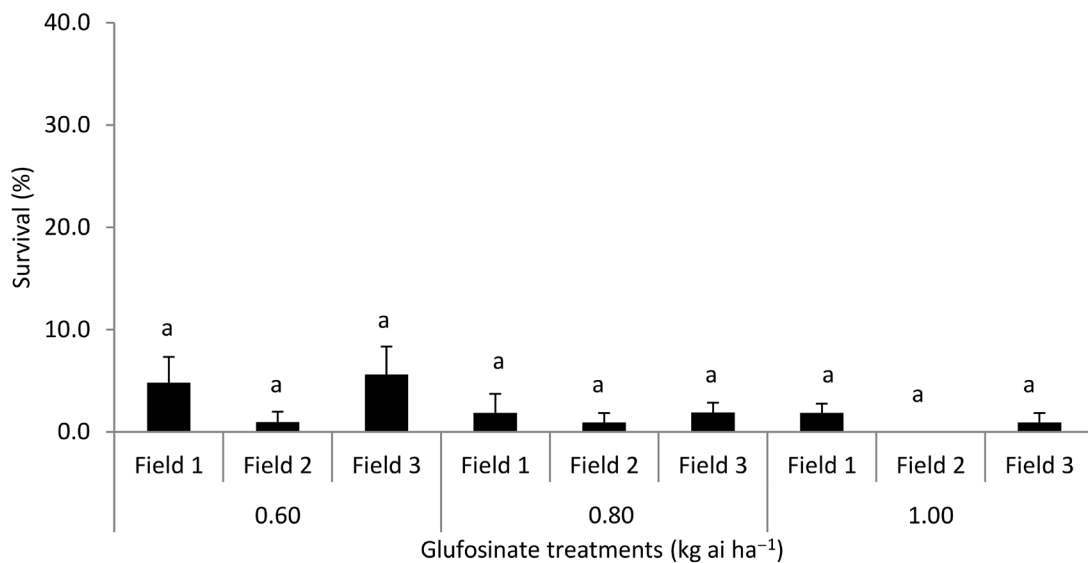


Fig. 1. Differences in survival rates of *L. multiflorum* plants after foliar application of (a) glyphosate-potassium or (b) glufosinate between fields within each treatment

Different letters indicate significant differences between fields within each treatment at $P < 0.05$, based on Tukey's honestly significant difference test. The vertical bars represent the standard errors of the means.

and glufosinate-based herbicides have been repeatedly used every year. *L. multiflorum* probably developed resistance to glyphosate and glufosinate due to the repeated use of these herbicides.

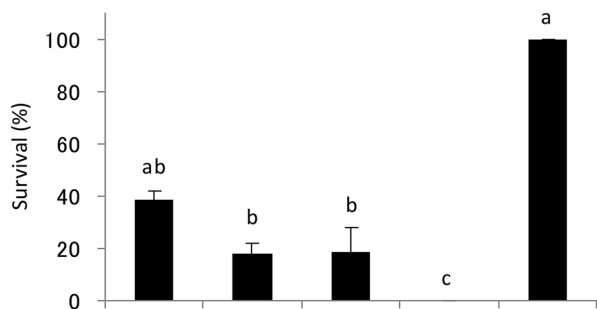
In a population of *L. multiflorum* found in rice paddy levees (34.78°N, 137.75°E) located about 2 km from the Japanese pear orchards, the survival rates of plants treated with glyphosate-potassium at 2.70 kg ai ha⁻¹ was

3.2% (Ichihara et al. 2016). The resistance genes of *L. multiflorum* can be widely dispersed via pollen movement (Busi et al. 2008, Ichihara et al. 2016). However, the Japanese pear orchards are surrounded by residential areas, vegetable fields, and trees; therefore, the populations in rice paddy levees and Japanese pear orchards are unlikely to be cross-pollinated. It is more likely that the glyphosate resistance of *L. multiflorum*

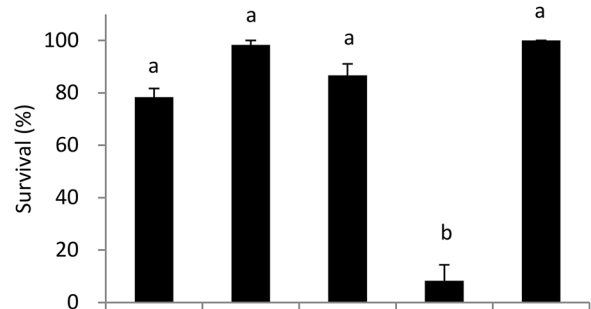
developed as a result of repeated use of glyphosate at each site.

The evolution of glufosinate resistance, when compared with that of glyphosate resistance, is considered to be at an early stage in *L. multiflorum* populations in the

(a) Early stage of growth



(b) Middle stage of growth



(c) Late stage of growth

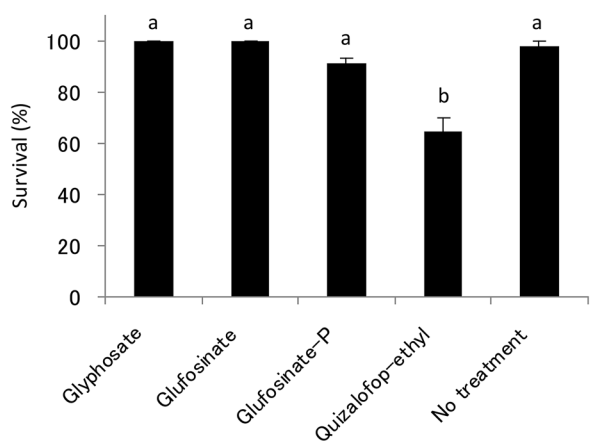


Fig. 2. Survival rates of *L. multiflorum* after treatment with foliar-applied herbicides in the (a) early, (b) middle, and (c) late stages of growth

The dosages of the herbicides are described in the main text. Means that are significantly different at $P < 0.05$, based on Tukey's honestly significant difference test, are identified using different letters. The vertical bars represent the standard errors of the means.

Japanese pear orchards. The survival rates of plants treated with glyphosate-potassium were 11.1-53.3%, which is in the range of the registration of this herbicide in pears in Japan, whereas those of the plants treated with glufosinate were 0.0-5.6%, which is in the range of the registration of this herbicide (Table 1). Selection pressure for the evolution of glufosinate resistance may be low because glufosinate-based herbicides are only used during summer, after the death of *L. multiflorum* in the Japanese pear orchards investigated in this study. However, the use of glufosinate can also be a selection pressure for the resistance as some after-ripened seeds of *L. multiflorum* on the soil surface can emerge in late July (Ichihara et al. 2009). It is necessary to monitor the levels of resistance of this weed to glyphosate and glufosinate in the Japanese pear orchards. Although the mechanism underlying glyphosate resistance is limited translocation of the herbicide in the resistant biotype of *L. multiflorum* in rice paddy levees in Shizuoka Prefecture (Kurata et al. 2018), the mechanism underlying glyphosate resistance in *L. multiflorum* in Japanese pear orchards is unclear. In the future, it is necessary to investigate the mechanism underlying the multiple resistance to glyphosate and glufosinate. Although the level of glufosinate resistance was not significantly different between the fields, the level of glyphosate resistance was significantly different between fields 2 and 3 (Fig. 1). The reason for this difference is unclear at this stage. According to an interview with the farmer, *L. multiflorum* apparently invaded both fields about 10 years ago, and the weed management strategy since then has been the same for both fields.

2. Efficacy of several foliar-applied herbicides

This study also revealed the efficacy of several foliar-applied herbicides for the control of *L. multiflorum* resistant to glyphosate and glufosinate in the Japanese pear orchards. In the early growth stage of *L. multiflorum*, the efficacies of both glufosinate and glufosinate-P-sodium were higher than that of glyphosate-potassium (Fig. 2 (a)). However, in the middle and late growth stages, the survival rates of plants treated with these three herbicides were not significantly different from those of plants receiving no treatment (Fig. 2 (b)-(c)). Meanwhile, the survival rates of plants treated with quizalofop-ethyl were significantly lower than those of the other plants at all three growth stages (Fig. 2 (a)-(c)). Quizalofop-ethyl was particularly effective in the early growth stage, when no plants (0%) survived this treatment. In this study, the growth conditions and temperatures were similar between the greenhouse and outdoors: the temperature difference was 2-3°C. Therefore, *L. multiflorum* that is

resistant to glyphosate and glufosinate may be effectively controlled using quizalofop-ethyl in Japanese pear orchards, once this herbicide is registered for use in pear orchards in Japan. In the future, it is necessary to report the efficacy of these foliar-applied herbicides and the appropriate dosage and application timing in fields, and evaluate the efficacy of a combination of herbicides applied to the soil.

Physical control by mowing during the flowering period of *L. multiflorum* would be an effective measure for controlling this weed resistant to glyphosate in rice paddy levees (Handayani et al. 2017). Handayani et al. (2017) indicated that mowing during the flowering period (early May to mid-May) resulted in reduced aboveground biomass, seed production, and soil seed bank as compared with mowing before the flowering (mid-April to late April) or glyphosate application. Therefore, mowing during the flowering period may also reduce *L. multiflorum* populations in the Japanese pear orchards. In the future, it is important to control *L. multiflorum* by integrating effective measures based on the ecology of this weed. To date, Japanese pear orchards where glyphosate- and glufosinate-resistant *L. multiflorum* grow have only been found in certain parts of Hamamatsu City. *L. multiflorum* is an anemophilous, self-sterile, cross-pollinated species, and the resistance genes may be widely dispersed by pollen movement (Busi et al. 2008, Ichihara et al. 2016). From now on, it will be very important to monitor the distribution of resistant *L. multiflorum* and the occurrence of resistance in all surrounding areas.

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