Potential Risks of Poaceous Plants as Infectious Sources of Rice Black-Streaked Dwarf Virus Transmitted by the Small Brown Planthopper, *Laodelphax striatellus*

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Abstract

The recent reemergence of rice black-streaked dwarf virus (RBSDV) has caused severe rice yield losses in several areas of East Asia. To identify the most important infectious sources of RBSDV, we compared the susceptibility of major poaceous plants to RBSDV infection and survival and the RBSDV acquisition efficiency of a vector insect, the small brown planthopper *Laodelphax striatellus*. RBSDV infection and survival rates of *L. striatellus* were significantly high in wheat (*Triticum aestivum* ‘Norin61’) and rice (*Oryza sativa* ‘Reiho’), indicating that these crops can be important sources of RBSDV. Our results also showed that RBSDV can complete its infection cycle between Italian ryegrass

(*Lolium multiflorum* ‘Hataaoba’) and *L. striatellus*. These results indicate that control of RBSDV and *L. striatellus* on winter-spring crops of wheat and Italian ryegrass may avoid an RBSDV epidemic on rice during the following summer. In addition to infections of wheat and Italian ryegrass, RBSDV infections were detected in *Avena fatua*, *Avena sterilis* subsp. *ludoviciana*, *Cynosurus echinatus*, *Festuca arundinacea*, *Festuca pratensis*, *Lolium perenne*, and *Vulpia myuros* var. *megalura*, although the infection efficiency varied.

Keywords: Fijivirus, long-distance migration, RT-qPCR,

Virus transmission as well as feeding injury by three rice planthoppers, the brown planthopper *Nilaparvata lugens* Stål, the white-backed planthopper *Sogatella furcifera* Horváth, and the small brown planthopper *Laodelphax striatellus* Fallén, are serious threats to stable rice production in East and Southeast Asia. Outbreaks of rice striped virus (RSV) transmitted by *L. striatellus* have been a threat to rice production in East Asian countries since around 2000 (Lee et al. 2008; Shiba et al. 2018; Wang et al. 2008). Southern rice black-streaked dwarf virus (SRBSDV) is transmitted exclusively by *S. furcifera* (Zhou et al. 2008) and has quickly expanded from southeast China to a wide distribution range from northern Vietnam to western Japan since its first detection in 2001 (Cuong et al. 2009; Matsukura et al. 2013). The epidemics of these viral diseases across a broad region of East and Southeast Asia are caused by long-distance migration of the vector planthoppers (Matsukura et al. 2017; Otuka et al. 2010). Development and improvement of the control strategies for the rice viruses mediated by the migratory planthoppers are urgent issues for stable rice production in East Asia.

Rice black-streaked dwarf virus (RBSDV) is an important rice virus belonging to the genus *Fijivirus*. This virus is transmitted by *L. striatellus*, *Unkanodes sapporonos* Matsumura, and *Unkanodes albifascia* Matsumura to several poaceous plants, including rice, maize, wheat, and barley (Shinkai 1962). RBSDV is transmitted by *L. striatellus* in a persistent, propagative manner (Hibino 1996), and it is close to maize rough dwarf virus in having common and/or similar characteristics in host range (Milne and Lovisolo 1977; Shinkai 1962), vector insects (Hibino 1996; Vidano 1970), and RNA sequences (Wang et al.

2003). After the serious epidemic of RBSDV in East Asia from the 1950s to 1970s (Hibino 1996), no serious outbreaks were reported until the end of the 1990s. During the last two decades, however, reemergence of RBSDV has occurred in several areas of East Asia. After the late 1990s, severe epidemics of RBSDV were reported in several areas of China (Bai et al. 2002). In South Korea, the range of RBSDV epidemics expanded after 2000 (Lee et al. 2005). Also, in Japan, RBSDV occurrence on rice began to increase in Saitama Prefecture after 2010.

The recent reemergence of RBSDV and RSV in East Asia is caused by increased occurrence of *L. striatellus* in this region. Both viruses are transmitted mainly by this planthopper to several poaceous plants, including rice, in a persistent-propagative manner (Hibino 1996); however, unlike RSV, RBSDV is not transmitted transovarially from adult female to offspring. Furthermore, typical symptoms of rice infected with these viruses are different. Rice plant infected with RSV shows yellowed and drooping leaves at late tillering stage. However, RBSDV-infected rice shows stunted growth with upheaving of leaf veins and twisting of leaf tips. These two viruses can be acquired by *L. striatellus* simultaneously, at least under laboratory conditions, although influence of the double infection on virus transmission efficiency is not clear (Li et al. 2010). Several ecological factors, such as the development of insecticide resistance in *L. striatellus* (Gao et al. 2008; Sanada-Morimura et al. 2011), efficient virus transfer along with long-distance migration of *L. striatellus* (Otuka et al. 2010; Sun et al. 2015), changes in cropping systems (Yoshida et al. 2014), and perhaps, recent global warming (Yamamura and Yokozawa 2002), are pointed out as promoting recent outbreaks of *L. striatellus* in East Asia.

Characterization of the RBSDV infection cycle, particularly between host plants and the vector *L. striatellus*, is important for evaluating the risk of RBSDV epidemics and establishing efficient control strategies for this viral disease. Several poaceous plants are known to be hosts of both RBSDV and *L. striatellus* (Mochida and Okada 1971; Shinkai 1962), yet the key infectious source of RBSDV leading to epidemics on rice has not yet been identified. In this study, we examined several biological traits related to the efficiency of RBSDV transmission to several poaceous plants under laboratory condition, which will provide the risk potentials of major poaceous crops and plants as infectious sources of this virus.

Materials and Methods

Virus and insect used. Symptomatic rice plants infected with RBSDV were collected in an experimental field at Saitama

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Agricultural Technology Research Center in Kumagaya City, Saitama Prefecture, Japan (36°10'09" N, 139°21'14" E) on 8 September 2017 and taken to the laboratory, where they were maintained in a phytotron at 23°C under a 16-h light/8-h dark photoperiod. RBSDV infection of each plant was confirmed with reverse transcription polymerase chain reaction (RT-PCR) followed by gel electrophoresis using two RBSDV-specific primer pairs: S9f1 and S9r1 for the partial sequence of the p9-1 gene and S10f3 and S10r3 for partial sequences of the outer capsid protein gene (Bai et al. 2002).

A laboratory-reared strain of *L. striatellus* originally collected in Ogori City, Fukuoka Prefecture, Japan (33°22'34" N, 130°32'29" E) on 8 October 2015 was used in this study. Only RSV-free *L. striatellus* confirmed by enzyme-linked immunosorbent assay (ELISA) (Omura et al. 1986) were used as progenitors of the strain to avoid the possible influence of RSV infestation on the acquisition and transmission of RBSDV in *L. striatellus*. The insects were mass reared on rice (*Oryza sativa* 'Reiho') at 25°C under a 16-h light/8-h dark photoperiod as described in a previous report (Sanada-Morimura et al. 2013).

Poaceous crop and plant hosts tested. Three poaceous crops (rice, wheat [*Triticum aestivum* 'Norin61'], and Italian ryegrass [*Lolium multiflorum* 'Hataaoba']) and 13 poaceous plants (*Agrostis alba*, *Avena fatua*, *Avena sterilis* subsp. *ludoviciana*, *Briza maxima*, *Cynosurus echinatus*, *Festuca arundinacea*, *Festuca pratensis*, *Lolium multiflorum* [wild type], *Lolium perenne*, *Poa annua*, *Poa pratensis*, *Vulpia myuros* var. *megalura*, and *Bromus catharticus*) were used in this investigation. Seeds of each species/cultivar were sown in a plastic dish (150-ml volume) with soil (Hinokunitokotsuchi) and maintained at 25°C and ~70% relative humidity under a 16-h light/8-h dark photoperiod. For *A. fatua* and *A. sterilis*, seeds were placed in a petri dish with filter paper and 12 ml of gibberellin solution (0.05% wt/vol) for 6 days before seeding to promote germination. After germination, each seed was transplanted into a 200-ml plastic cup containing soil. At 14 days after transplanting, young plants were used in the virus infection test described below.

RBSDV infection test. First and second instar nymphs of *L. striatellus* were used for the virus infection tests. Plants were individually covered by an acrylic cylinder with a nylon cloth placed over the top to provide ventilation for the insects and plants. Because *L. striatellus* requires an ~5-day acquisition period followed by a >20-day latent period to become viruliferous (Li et al. 2010; Miao et al. 2014), the plants were kept at 20°C during the virus acquisition period to suppress development of *L. striatellus*. At 14 days after release, when *L. striatellus* had grown to the old nymph and early adult stages, the viruliferous *L. striatellus* were transferred to 2-week-old poaceous plants for virus inoculation. The RBSDV acquisition rate of the viruliferous *L. striatellus* was determined the above-described RT-PCR method ($n = 40$ adults).

Viruliferous *L. striatellus* were released on the poaceous crops and plants at a density of two insects per plant. Plants were individually covered by an acrylic cylinder with a nylon cloth covering the top, and they were held for 46 days at 25°C under a 16-h light/8-h dark photoperiod. After the RBSDV inoculation period, RBSDV infection of each plant was examined using the reverse transcription quantitative polymerase chain reaction (RT-qPCR) method described below. Fifteen plants were tested for all species except *B. maxima* and *V. myuros* var. *megalura*, of which five plants were tested owing to low germination rates and/or poor development.

RBSDV detection with RT-qPCR. Presence of an outer capsid protein gene of RBSDV in each plant was examined with RT-qPCR, because this method is generally more sensitive than the other diagnosis methods, such as gel electrophoresis and ELISA. The primer pairs F3 (5'-CCC CAG AGA CTT TCC GAT AC-3') and B3 (5'-GGT CTT TAA GTT GCG TGA TGT-3') developed for RBSDV-specific detection with reverse transcription loop-mediated isothermal amplification (RT-LAMP) (Le et al. 2010) were used for our RT-qPCR method. Total RNA was extracted following a method described for the quantification of SRBSDV (Matsukura et al. 2013). Briefly, sampled leaves were weighed and then crushed in a mixture of 250 μ l of TriPure Isolation Reagent (Roche Diagnostics Co., Ltd.) and 50 μ l of chloroform. Purified total RNA from each

plant was dissolved in 50 μ l of diethylpyrocarbonate-treated water (Nippon Gene Co., Ltd.) and used as a template for RT-qPCR.

RT-qPCR was conducted with a Lightcycler 480FLM (Roche Diagnostics Co., Ltd.) using a One Step SYBR PrimeScript RT-PCR Kit II (Takara Bio Inc.). Each reaction contained 5 μ l of 2 $\times$ One Step SYBR RT-PCR Buffer 4, 0.4 μ l of PrimeScript 1step Enzyme Mix 2, 0.8 μ l of a mixture of F3 and B3 primers (10 μ M each), 2 μ l of template RNA, and 1.8 μ l of RNase-free dH₂O. Thermal conditions were 1 cycle of reverse transcription (42°C for 5 min and 95°C for 10 s), 35 cycles of amplification (95°C for 10 s, 60°C for 10 s, and 74°C for 15 s), 1 cycle of melting curve analysis (95°C for 5 s, 65°C for 1 min, and 97°C, with increment rates of 0.11°C per 1 s), and cooling (40°C for 30 s). Using a dilution series of RNA extracted from RBSDV-infected rice, the primer efficiency was estimated to be 1.928, with Cycle threshold (Ct) values ranging between 10 and 30. No proper melting curve was observed for samples with Ct values >30; therefore, samples with Ct values >30 were regarded as negative (i.e., no infection of RBSDV).

Nymph survival test on poaceous plants. The survival of *L. striatellus* nymphs on RBSDV-free poaceous plants was examined. Twenty first and second instar nymphs were released on each plant at 60 days after transplanting the germinated seeds. Each plant was covered by an acrylic cylinder with a nylon cloth covering the top and maintained at 23°C under a 16-h light/8-h dark photoperiod. At 10 days after releasing the nymphs, the number of surviving *L. striatellus* was counted. Of 16 poaceous plants used in the infection test, *A. fatua*, *F. pratensis*, *L. multiflorum*, *P. pratensis*, and *V. myuros* var. *megalura* were not used in this test because of poor development of these plants. Tests were replicated on five test plants of each species (i.e., 20 nymphs $\times$ five replicates).

RBSDV acquisition test from poaceous plants. Two plants each of RBSDV-infected rice, wheat, Italian ryegrass, and *L. multiflorum* (wild type) and one plant of RBSDV-infected *L. perenne* were used for the RBSDV acquisition test. These RBSDV-infected plants were those produced in the RBSDV infection test. Forty first and second instar nymphs of *L. striatellus* were released on individual plants covered by nylon cloth-covered acrylic cylinders and kept at 20°C for 5 days. After the virus acquisition period, nymphs were transferred to RBSDV-free rice seedlings and held at 20°C for 9 days. This additional incubation period on RBSDV-free rice avoided the high mortality on unfavorable host plants for *L. striatellus* and detection of RBSDV from the digestive tracts of nonviruliferous *L. striatellus*. The numbers of survived *L. striatellus* on each plant were from 6 to 26. After extracting total RNA from these survived *L. striatellus*, RBSDV acquisition was tested with the RT-qPCR method described above.

Statistical analyses. The RBSDV infection efficiency among poaceous plants and the RBSDV acquisition efficiency by *L. striatellus* among RBSDV-infected plants were tested with a χ^2 test followed by residual analysis as a post hoc test. Differences in survival rates of *L. striatellus* among poaceous plants were tested with analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test after arcsine transformation of the survival rates. All statistical analyses were performed with R version 3.3.2 (R Core Team 2016).

Results

Susceptibility to RBSDV in poaceous plants. The RBSDV acquisition efficiency of released *L. striatellus* in the infection test was 95% ($n = 40$), suggesting that at least one viruliferous nymph fed on most tested plants. The RBSDV infection efficiency significantly differed among the tested poaceous plants (χ^2 test, $\chi^2 = 55.19$, degree of freedom (df) = 15, $P < 0.001$). The subsequent residual analysis test showed significantly higher infection efficiency in rice, wheat, and *L. multiflorum*. No infection of RBSDV was observed on *A. alba*, *P. annua*, *P. pratensis*, *B. catharticus*, and *B. maxima* (Table 1). Wheat had the highest RBSDV infection efficiency.

Nymph survival on RBSDV-free poaceous plants. The survival rate of *L. striatellus* nymphs significantly differed among the 11 tested poaceous plants (ANOVA after arcsine transformation, $F = 21.60$, df = 10, $P < 0.001$); >80% of the nymphs survived on both rice and wheat, a level that is significantly higher than that on

the other plants (Table 1) (Tukey's HSD test after arcsine transformation, $\alpha = 0.05$). To the contrary, <10% of the nymphs survived on *B. maxima* and *A. sterilis* subsp. *ludoviciana*.

RBSDV acquisition efficiency of *L. striatellus* from poaceous plants. RBSDV acquisition efficiency after a 5-day virus acquisition period significantly differed among tested plants (χ^2 test, $\chi^2 = 34.82$, $df = 8$, $P < 0.001$); however, there were no clear trends in the virus acquisition efficiency among plant species/cultivars (Table 2).

Discussion

Both our virus infection test and nymphal survival test led to a common conclusion that rice and wheat are suitable host plants for both RBSDV and *L. striatellus* compared with the other poaceous

Table 1. Susceptibility to rice black-streaked dwarf virus (RBSDV) infection and survival of *Laodelphax striatellus* in poaceous crops and plants^w

Plants	RBSDV infection			<i>L. striatellus</i> survival ^x
	N	Percentage ^y		Percentage $\pm$ SD ^z
<i>Oryza sativa</i> 'Reiho'	15	46.7	H	82.0 $\pm$ 12.0 ab
<i>Triticum aestivum</i> 'Norin61'	15	60.0	H	94.0 $\pm$ 4.2 ab
<i>Lolium multiflorum</i> 'Hataaoba'	15	20.0		58.0 $\pm$ 12.0 bc
<i>Agrostis alba</i>	15	0.0	L	3.0 $\pm$ 6.7 e
<i>Avena fatua</i>	15	13.3		N.D.
<i>Avena sterilis</i> subsp. <i>ludoviciana</i>	15	20.0		54.0 $\pm$ 21.6 cd
<i>Briza maxima</i>	5	0.0		9.0 $\pm$ 7.4 de
<i>Cynosurus echinatus</i>	14	7.1		11.3 $\pm$ 10.3 de
<i>Festuca arundinacea</i>	15	13.3		24.0 $\pm$ 15.6 cde
<i>Festuca pratensis</i>	14	7.1		N.D.
<i>Lolium multiflorum</i>	15	53.3	H	N.D.
<i>Lolium perenne</i>	15	33.3		55.0 $\pm$ 23.2 bc
<i>Poa annua</i>	15	0.0	L	35.0 $\pm$ 16.2 cde
<i>Poa pratensis</i>	15	0.0	L	N.D.
<i>Vulpia myuros</i> var. <i>megalura</i>	5	20.0		N.D.
<i>Bromus catharticus</i>	15	0.0	L	45.0 $\pm$ 17.3 cd

^w N, number of plants tested for the presence of RBSDV. N.D., no data provided.

^x Percentage of survival of *L. striatellus* young nymphs after 10-day releasing on each plant.

^y Significantly high (H) and low (L) infection efficiencies (residual analysis, $\alpha = 0.05$) are indicated.

^z There is no significant difference between pairs with the same letter (a to e) within a column (Tukey's honestly significant difference test, $\alpha = 0.05$).

Table 2. Acquisition efficiency of rice black-streaked dwarf virus (RBSDV) by *Laodelphax striatellus* from poaceous crops and plants

Plants	RBSDV acquisition efficiency ^y	
	N	Number of viruliferous nymphs (%)
<i>Oryza sativa</i> 'Reiho'		
Plant 1	6	1 (16.7)
Plant 2	10	0 (0.0)
<i>Triticum aestivum</i> 'Norin61'		
Plant 1	16	1 (6.3)
Plant 2	17	11 (64.7) ^z
<i>Lolium multiflorum</i> 'Hataaoba'		
Plant 1	15	0 (0.0)
Plant 2	26	5 (19.2)
<i>Lolium multiflorum</i>		
Plant 1	24	6 (25.0)
Plant 2	12	0 (0.0)
<i>Lolium perenne</i>		
Plant 1	14	2 (14.3)

^y Virus acquisition rate of the first and second instars of *L. striatellus* nymphs by 5-day feeding on infected plants.

^z Significantly higher acquisition rate than the others (residual analysis, $\alpha = 0.05$).

plants (Table 1). Wheat is recognized as an important crop in the management of RSV, a virus also transmitted by *L. striatellus* on rice. Wheat is cropped from winter to spring in most areas of East Asia; therefore, it is a suitable host for both *L. striatellus* and RSV during the overwintering period and in spring. Viruliferous first and second generation *L. striatellus* multiply on wheat fields and migrate to young rice fields in the late spring to early summer, thereby transmitting RSV to rice (Shiba et al. 2018; Yoshida et al. 2014). Our results indicate that wheat is also a key infectious source of RBSDV, an observation that was also reported from field observations in China by Wang et al. (2009).

The RBSDV acquisition efficiency from wheat is higher than that of the other tested plants (Table 2). This high acquisition efficiency can promote successful RBSDV transmission from the overwintering generation to the first and second generations of *L. striatellus*. Because first and second generations of *L. striatellus* must acquire RBSDV from infected plants, whereas RSV is transmitted transovarially in *L. striatellus* (Hibino 1996), suppression of RBSDV infection of wheat by controlling *L. striatellus* and RBSDV in wheat fields should be an effective means to avoid RBSDV outbreaks in rice fields. Our results also showed that the efficiency varied among replicates of the same plants (Table 2), suggesting a possible influence of virus titer in infected plants on the acquisition efficiency as observed in RBSDV mediated by *S. furcifera* (Matsukura et al. 2015).

It is evident that the high infection efficiency of RBSDV and the survival rate of *L. striatellus* on rice (Table 1) are factors leading to outbreaks of RBSDV on rice. This finding does not always mean that rice is an important infectious source of RBSDV. As is known for other rice viruses, such as RSV (Shiba et al. 2018) and SRBSDV (Zhou et al. 2008), RBSDV infection occurs mainly on young rice before the tillering stage (Wang et al. 2009). Viruliferous *L. striatellus* produced on young rice can no longer spread the virus to new healthy rice in the same field, because the plants have developed enough to resist the RBSDV infection. Rice should be considered as an infectious source of RBSDV only when the rice cropping season varies among fields. Wang et al. (2009) found more severe epidemics of RBSDV on late-cropped rice cultivars belonging to *O. sativa* subsp. *japonica* than on earlier-cropped cultivars, *O. sativa* subsp. *indica*, although *japonica* cultivars are inherently susceptible to RBSDV compared with *indica* cultivars and *indica-japonica* hybrids. These investigators also found an increase in viruliferous *L. striatellus* from the overwintering generation on early-cropped fields compared with the second generation on late-cropped fields, indicating that large numbers of viruliferous *L. striatellus* were produced in early-cropped rice fields. The period for rice transplanting generally is synchronized among fields within most regions throughout East Asia, but in some cases, such as multiple rice cropping in Southeast Asia (Sakamoto et al. 2006) and mixed cropping of forage-food crops in East Asia (Nakano et al. 2008), transplantation times vary among fields. For these regions, control of *L. striatellus* in rice fields transplanted early is important to avoid RBSDV outbreaks during later plantings.

Italian ryegrass is a major forage grass grown from winter to spring in temperate East Asia (Ishii et al. 2005; Seo 2005). Our study confirmed that RBSDV can complete its infection cycle between Italian ryegrass and *L. striatellus* (Tables 1 and 2). Because farmers rarely practice pest control on forage crops in Japan, Italian ryegrass can become a source of several insect pests that attack poaceous crops. For example, two rice bugs, *Trigonotylus caelestialium* Kirkaldy and *Stenotus rubrovittatus* Matsumura, multiply on Italian ryegrass in spring and migrate to rice fields (Fujii et al. 2010; Nagasawa et al. 2012); maize orange leafhopper *Cicadulina bipunctata* Melchar feeds on Italian ryegrass from winter to spring before attacking maize in summer (Matsukura et al. 2009); and *L. striatellus* utilizes Italian ryegrass as a host plant from winter to spring (Yoshida et al. 2014). Additional studies on the dynamics of RBSDV and *L. striatellus* in Italian ryegrass fields are needed to clarify the potential risk of Italian ryegrass as an infectious source of RBSDV.

Several poaceous plants belonging to the tribes Oryzoideae, Panicoideae, and Pooideae are reported as host plants of RBSDV (Shinkai 1962). Our data support the previous findings that RBSDV

cannot infect *A. alba* and *B. catharticus* but can infect *L. perenne* (Shinkai 1962). *L. perenne* is a perennial plant closely related to Italian ryegrass (*L. multiflorum*), and it is often grown as a pasture grass in cold areas within temperate regions (Wilkins 1991). The persistency of RBSDV on *L. perenne* should be examined to determine if this plant is an important infectious source of RBSDV for several years after infected. Our data showed that RBSDV infects both *F. arundinacea* and *F. pratensis*, plants that were previously reported to be nonhosts of RBSDV (Shinkai 1962). This result is not surprising, because the genus *Festuca* is close to the genus *Lolium* in the phylogeny of the tribe Poeae (Gillespie et al. 2008). However, RBSDV infection on *P. annua* was reported by Shinkai (1962), although no RBSDV infection by this plant was observed in our study (Table 1). *P. annua* is an important winter-spring host for *L. striatellus* (Yoshida et al. 2014); therefore, this plant can be an important host of RBSDV.

Controlling vector insects is a general strategy in the management of plant viral diseases mediated by insects. Control of *L. striatellus* from winter to spring by removing host plants, such as residual rice and poaceous weeds, has been promoted in Japan for many years (Shiba et al. 2016; Yoshida et al. 2014). The relatively higher importance of winter-spring crops (i.e., wheat and Italian ryegrass) as infectious sources of RBSDV as implied in this study suggests a need for additional vector control in these crop fields. Application of effective insecticides for *L. striatellus* (Gao et al. 2008; Sanada-Morimura et al. 2011) would suppress densities of RBSDV-viruliferous insects and/or RBSDV-infected plants in wheat fields when concerned about an epidemic of RBSDV. Several RBSDV diagnostic methods, such as ELISA (Wang et al. 2006), RT-PCR (Bai et al. 2002), RT-LAMP (Le et al. 2010), and RT-qPCR (Zhang et al. 2013 and this study), will help to monitor potential RBSDV epidemics in winter-spring crops. There is no realistic way to control *L. striatellus* in Italian ryegrass fields at present, but the use of symbiotic fungal endophytes or other biocontrol agents on Italian ryegrass is a promising strategy for controlling these insect pests (Matsukura et al. 2012).

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