Scientific Research Publishing

Agricultural Sciences, 2023, 14, 1277-1291
https://www.scirp.org/journal/as
ISSN Online: 2156-8561
ISSN Print: 2156-8553

Different Susceptibility in the Two Sympatric Sweet Potato Weevils, *Cylas formicarius* and *Euscepes postfasciatus*, to the Entomopathogenic Fungus *Metarhizium anisopliae*

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How to cite this paper: Reddy, G.V.P., Shimizu, S. and Ichinose, K. (2023) Different Susceptibility in the Two Sympatric Sweet Potato Weevils, *Cylas formicarius* and *Euscepes postfasciatus*, to the Entomopathogenic Fungus *Metarhizium anisopliae*. *Agricultural Sciences*, **14**, 1277-1291.
https://doi.org/10.4236/as.2023.149086

Received: August 30, 2023
Accepted: September 11, 2023
Published: September 14, 2023

Abstract

Laboratory and field experiments were performed to evaluate the pathogenicity of an isolate of the entomopathogenic fungus *Metarhizium anisopliae* to the two sympatrically occurring weevil species, *Cylas formicarius* and *Euscepes postfasciatus*. In the laboratory bioassays, suspension of conidia, $\geq 10^6$ CFU/mL, caused mortalities > 80% on adults of both weevils in seven days after inoculation. It took longer time 20 days for grain formulation of the isolate adhered on rice grains of ≥ 5 g/m^2 (10^7 CFU/g) to attain similar mortalities of *E. postfasciatus*, but no evident mortality was obtained in *C. formicarius*. The grain formulation was thus less effective on *C. formicarius* than the suspension. Field trials were carried out over two years from 2013 to 2014, in which adults of *E. postfasciatus* were released two times during the field experiments for enhancement of damage on plants by this weevil, whereas the other weevil species was left to naturally invade the experimental plots by flying. The results of the experiments revealed in both years that two applications of the isolate in grain formulation, equivalent to 50 kg/hectare, sprayed manually over the ground surface reduced the infestation of plants and tuber damage by weevils of both species as much as the conventional chemical insecticide applications. The occurrences of weevils at harvest were not significantly different among treatments. The potential and possible uses of the fungus are discussed for the management of these two weevil species.

Keywords

Biological Control, Coleoptera, Entomopathogen, *Ipomoea batatas*, Microbe

1. Introduction

Two sweet potato weevil species, *Cylas formicarius* Fab. (Coleoptera: Brentidae) and *Euscepes postfasciatus* (Fairmaire) (Coleoptera: Curculionidae), seriously damage sweet potatoes in tropical and subtropical regions [1] [2] [3]. These weevils are controlled effectively by chemical insecticides, often with incorporation with cultural managements such as field sanitation, the use of earlier harvest, and tillage between lines and/or watering to avoid soil cracking [4] [5] [6]. However, since both weevils spend much of their life in plants, where they are fairly shielded from insecticides, these control measures often fail enough to reduce the weevil damage. Thus, the success of the control agents depends on how efficiently the agents can be delivered to these locations. In this regard, biological control agents may be considered as possible agents to control these weevils, if they move actively for the search of hosts and maintain or even increase their population by self-multiplication.

Some agents may be considered for the biological control of the weevils. Parasitoids, *Bracon* spp. (Hymenoptera: Braconidae), cause more than 30% mortality in immatures of *E. postfasciatus* [7]. Entomopathogenic nematodes (EPNs) substantially reduce the population of *C. formicarius* [8] [9] [10]. Both parasitoids and EPNs can parasitize immatures inhabiting inside both root and stem of the plant [10] [11], where effective doses of chemical insecticides are hard to deliver. Despite the advantage in the use of these agents, their uses are not common or even not realized yet, primarily due to the costs of both mass production and the application to this low-valued crop. In this point, weevil management may be more economically performed with entomopathogenic fungi (EPF) which can be mass-produced more quickly and less costly [3] [6]. One difficulty in the use of EPF is their immobility: they cannot reach weevils living in the plant by themselves and need other forces to be spread such as wind. Despite this deficiency, several authors report their efficient infestation on insect pests in field [12] [13]. The point is how the chance of encountering of EPF with prey can be increased, and the chance in turn is greatly affected by biological and environmental conditions. The effects may even vary among closely related strains of one EPF species [14] [15]. Depending on species or strains, their efficacy may differ among the pest species to target [16] [17]. Thus, such uncertainty would confine the availability of EPF for pest control, particularly aimed at controlling two or more species together.

The variation in the efficacy of control agents may thus make the agents less useful for the control of insect pests that occur sympatrically and cause similar damage on one crop. This could be the case for two sweet potato weevils, *C.*

formicarius and *E. postfasciatus*, on Pacific Islands [18] including southern Japan [19]. Although the lethal effects of EPF on *C. formicarius* have been reported [20] [21], the efficacy on the other weevil, *E. postfasciatus*, still remains to be studied. However, if a given EPF can effectively reduce the populations of both weevil species, the agent would be worth testing for its potential to control the weevil in the region.

In other our preliminary experiment, *B. bassiana* isolates that had been obtained from cadavers of *C. formicarius* were considered to be effective to control the weevil and possibly used for the other sympatric weevil, *E. postfasciatus*. However, this weevil was slightly infected by the isolates, whereas both weevils were effectively killed by an isolate of the *Metarhizium anisopliae* (Metchnikoff) (Ascomycota: Hypocreales), SMZ2000, collected by Shimizu in Kyoto in 2000. In this study, thus, the efficacy of the isolate was examined in laboratory and field on the two sympatric sweet potato weevil species, *C. formicarius* and *E. postfasciatus*. The isolate persists at 10^5 to 10^6 CFU/m^2 10 cm deep in soil for at least one year after application of 10^7 CFU/m^2 (Shimizu, personal observation). Given that sweet potato tubers are harvested in about six months after the planting of slips in Japan, the application of this isolate at 100 times more than the minimum dose required for sufficient efficacy would keep effectiveness throughout an entire sweet potato cultivation. The application dose of the isolate was based on this hypothetical calculation in this study. We discuss the possible use of the isolate in the management of the two weevils.

2. Materials and Methods

2.1. Laboratory Assessment of Mortality

Both *C. formicarius* and *E. postfasciatus* weevils used in this study were reared in an air-conditioned room at 25˚C ± 3˚C with natural light at Okinawa Prefectural Agriculture Research Center, southern Japan. All weevils of both species which were ≤7 d were used in this study.

Two formulations, suspension and granular, of the *M. anisopliae* isolate SMZ2000 were examined in laboratory experiments. This isolate is registered by the company, Arysta Co. Ltd. (Tokyo, Japan), as NITE BP-1113. The isolate collected by S. Shimizu was identified as *M. anisopliae* var *anisopliae* based on its morphological characteristics [22] [23]. Suspension formulation was prepared by Shimizu and CFU was determined [24] [25]. Conidia were dissolved in 0.5% Tween 80 (Kanto Chemical Co. Inc., Tokyo) and the suspension was adjusted at 10^7 CFU/ml. The suspension was sent to Okinawa, where it was diluted at tested densities with the same solution. Granular formulation was prepared as conidia adhered on rice grain at 10^7 CFU/g by Arysta, registered as the patent number 5,326,043 and 5,563,107. Since this study was carried out before the launch of the product in the market, the product used for this study was sent several times to Okinawa from Arysta within one month before experiments.

Suspensions were prepared for concentrations 0 (no fungi for control) and 10^3

to 10^7 CFU/ml with 0.5% Tween 80 solution. Weevils of both *C. formicarius* and *E. postfasciatus* that were selected randomly from the laboratory colonies were immersed for a few seconds soon after the preparation of the conidial suspensions. After the treatment, 10 adults of one species were randomly selected and placed in a transparent plastic cup (8 cm in diameter and 5.5 cm height), on the bottom of which moist sand was bedded 1 cm thick. The surface area of the sand was about 50.0 cm^2, at the center of which one slice of sweet potato 1 cm thick was supplied for food. All concentrations were examined for each species in six replicates.

For the tests of grain formulation, cups with 10 weevils ≤ 7 days old of each species were prepared similarly. Before the weevil introduction, the grain formulation was applied on the surface at the dose of 0, 0.0125, 0.0250 and 0.0500 g/cup, being equivalent to 0, 0.1, 1.0 and 2.0×10^7 CFU/m^2, respectively. The application dose for field suggested by Arysta corresponds to 0.0250 g/cup. Combinations of weevil species and application doses were examined in six replicates. All cups were maintained at 25˚C ± 0.2˚C. Dead weevils in each cup were counted once every day. The isolate which was detected on a weevil cadaver morphologically [22] was attributed to the death of the weevil throughout this study.

2.2. Experimental Design for Field Experiments

Field experiments were performed at Itoman on the Okinawa Island in southern Japan in 2013 to 2014. All plants were prepared from slips cut 30 cm top of sweet potato vines grown in a greenhouse and planted in late May. The field was irrigated two to three times per week in the first two weeks and thereafter as needed. Dead plants were replaced with new vines during the first one month after planting, and none replaced thereafter. Tubers were harvested in mid-November. Neither pesticides nor fungicides other than the control agents tested in this study were applied to the plants.

The first experiment was started with establishing two quadrats, each 25.6 by 7.2 m, in May 2013. These quadrats were separated 1.6 m from each other on a long side. In each quadrat, 12 plots (3.2 × 3.6 m each) were made, each being separated by1.2 m. In each plot, four planting ridges 3.6 m long were created at 0.8 m intervals and nine slips were planted on each ridge spaced at 0.4 m intervals. Thus, 36 slips were planted per plot, and six treatments were randomly assigned to each quadrat. Treatments were: 1) an untreated control, 2) conventional chemical insecticide applications, and 3 - 6) four treatments involved in application frequency and two application mode. In the conventional insecticide treatment, 69.1 g of fipronil (1.9 g/plant), being equivalent to 60 kg/ha, was applied on the ridges where to plant slips, and 69.1 g chlorpyriphos was applied on the ridges of each plot in 2 and 4.5 months after planting.

Two application frequencies of the fungus, *M. anisopliae*, were examined: once in 2 mo or twice in 2 and 4.5 mo after planting. The fungus was applied either over the ground surface (over-surface) or around the main stem of the

plants on the ground (around-stem). In each application, the grain formulation was applied 57.6 g/plot, based on the results of the laboratory experiments. The application of the grain was examined also in two: over the surface, or 1.6 g per plant (=57.6 g/36plants) around the stem. All treatments were finished in one day. To facilitate weevil infestation and damage on plants, 36 adults of *E. postfasciatus* taken from the laboratory stock were released at the center of each plot in 2 and 4.5 mo after planting. Both releases were carried out just before the treatments. The weevil release is explained later in detail. No *C. formicarius* weevils were released to avoid the expansion of this species into surrounding farms by flying, while the non-flying *E. postfasciatus* did not carry this risk.

In 2014, three quadrats (8.8 by 13.4 m) were established, separated by 1.6 m along a long side. In each quadrat, 12 plots (2.4 by 2.4 m) were made, spaced 1.6 m apart. In each plot, three planting ridges were made each 2.4 m long at 0.8 m intervals, and six slips were planted at 0.4 m intervals on each ridge, totally 18 slips per plot. Four treatments were randomly assigned to the 12 plots in the quadrats, giving three replicates to each treatment in each quadrat.

Considering the results in 2013, one application of the fungus was not examined in 2014. Thus, the treatments were reduced as: 1) untreated control, 2) the conventional insecticide uses as in 2013, 3) over-surface twice application of the grain formulation, and 4) two applications around stem. In the fourth treatment, the fungus was applied first just before planting in holes of about a 5 cm diameter in 15 cm depth to plant slips, 1.6 g grain/hole, and a slip was planted in the hole. The second application was undertaken around the stem 4.5 months after the planting. The timing and frequency of weevil release were the same as in 2013, but the numbers of released weevils were different: 90 adults of *E. postfasciatus* in 2 and 4.5 mo after planting. The number of released weevils per plants was increased in 2014, 5/plant, compared to that in 2013, 1/plant in 2013, with an expectation that the effects of treatments would have been more distinct. No *C. formicarius* weevils were released.

2.3. Data Collection at Harvest

In the laboratory experiment, mortality in each cup for all non-control treatment was calculated as the division of the number of dead weevils in each cup by the initial number of added weevils, 10. The mortality was transformed in square-root to approximate the data to a normal distribution and then compared among treatments by ANOVA. When significant differences were detected, means were compared between treatments by Tukey's tests.

Six plants randomly selected in each plot were collected for 2013 and five for 2014. When harvested, the plants were dissected to count infecting weevils. Tuber was defined as any depleted root ≥ 100 g. Each part was weighed to the nearest 0.1 g. Collected weevils were preserved in 70% ethanol for later species identification under a dissecting microscope. Tubers were individually weighed, and the proportion of those infected by each weevil species was calculated.

2.4. Data Analyses

The weight of tubers was log-transformed and compared between quadrats and treatments by ANOVA for randomized block design, in which each quadrat was treated as a block. The proportion of tubers infected by each weevil species was compared after arc-sine root transformation. The number of infecting weevils was square-root transformed for comparison. The difference in the means was compared by Tukey's HSD test for each measure at a probability of 0.05 for significance.

3. Results

3.1. Laboratory Assessment of Mortality

On the seventh day, suspension formulation of the *M. anisopliae* isolate SMZ2000 at $\geq 10^5$ CFU/ml caused mortalities > 70% in *C. formicarius* and at $\geq 10^4$ in *E. postfasciatus* (Figure 1). Significant differences in the mortality on this day were detected between concentrations in both weevils: $F_{5,12} = 34.61$, $P < 0.001$ and $F_{5,12} = 35.04$, $P < 0.001$, respectively. The mortality of *C. formicarius* was not significantly different among 0, 10^3 and 10^4 CFUs or among 10^5 to 10^7 CFUs (Tukey's HSD test, $P > 0.05$), but significantly different between these two groups (Tukey's HSD test at $P < 0.05$). *E. postfasciatus* did not show significantly different mortalities on this day between control and 10^3 CFU concentration and among higher concentrations ($P > 0.05$), but mortalities were significantly different between these two groups ($P < 0.05$).

It took 15 to 16 days to attain similar mortalities with grain formulation of 0.0250 g, equivalent to CFU 10^7 (Figure 2). The grain formulation at 0.0125 g achieved only low to moderate mortality in *E. postfasciatus*, and little to none in *C. formicarius* compared to the control. Weevil mortality in the treatment of grain formulation on the seventh day was not significantly different either in *C. formicarius* among doses ($F_{3,20} = 0.426$, $P = 0.737$) or in *E. postfasciatus* ($F_{3,20} = 3.000$, $P = 0.055$). No significantly different mortalities among CFUs were obtained in *C. formicarius* on the final day ($F_{3,20} = 2.067$, $P = 0.137$), but mortalities of *E. postfasciatus* on this day were significantly different ($F_{3,20} = 12.060$, $P < 0.001$). Since the mortality of this weevil at 0.025 g was not significantly different from the mortality at 0.050 g but both were significantly larger than mortalities at 0 and 0.0125 g, the dose 0.025 g was used for the grain formulation in the field experiments.

Probit analyses were undertaken for weevil mortalities with suspension formulation of the fungus on the seventh day revealed that probits of weevil mortalities (y) were significantly linearly regressed on the concentration of conidia on log-scale (x): $y = 1.17x - 5.60$ for *C. formicarius* ($r^2 = 0.951$, $F_{1,3} = 38.71$, $P = 0.025$); and $y = 1.57x - 5.62$ for *E. postfasciatus* ($r^2 = 0.935$, $F_{1,3} = 28.64$, $P = 0.033$). The regression gave an estimate of LD_{50} as $10^{4.78}$ and $10^{3.58}$ CFU/ml in these weevils, respectively.

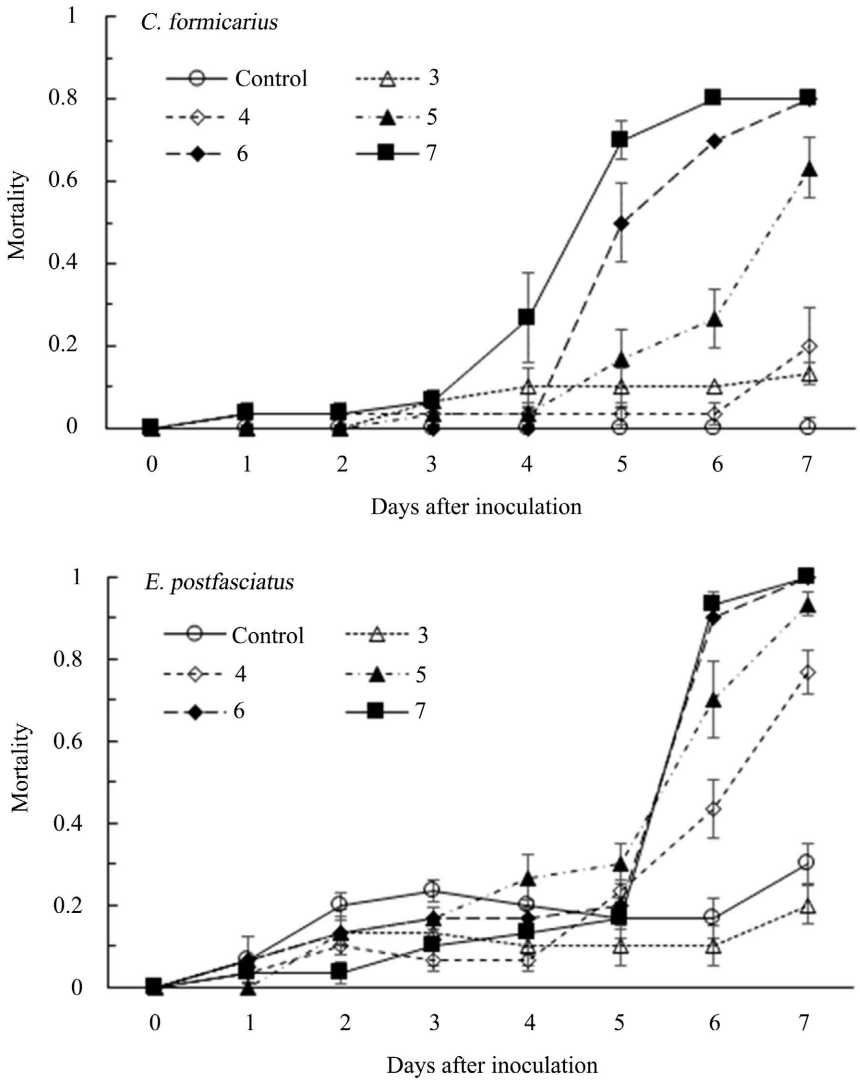

Figure 1. Mortalities of two sweet potato weevil species, *Cylas formicarius* and *Euscepes postfasciatus* caged with the isolate of *Metarizium anisopliae*, SMZ2000, in suspension formulation of different CFUs, which are indicated by numerals of the exponential of 10 in the panels.

3.2. Field Experiment in 2013

The means of the tuber numbers per plant lied between two to three in all treatments and weights of plant parts showed little variation, about 1.7 to 1.9 kg in 2013 (**Table 1**). Both were significantly different between quadrats ($F_{1,132}$ = 31.035 and $F_{1,132}$ = 10.942, respectively, $P < 0.001$ in both), but not among treatments ($F_{5,132}$ = 0.648 and $F_{5,132}$ = 0.238, respectively, $P > 0.05$ in both). The interaction of these two variables was not significant ($F_{5,132}$ = 0.675 and $F_{5,132}$ = 0.410, respectively, $P > 0.05$ in both). Both the proportion of tubers injured by *C. formicarius* and the number of this weevil per plant was significantly different between quadrats ($F_{1,132}$ = 7.828 and $F_{1,132}$ = 7.462, $P = 0.006$ and $P < 0.001$, respectively) and among treatments ($F_{5,132}$ = 7.828 and $F_{5,132}$ = 7.462, $P = 0.008$ and

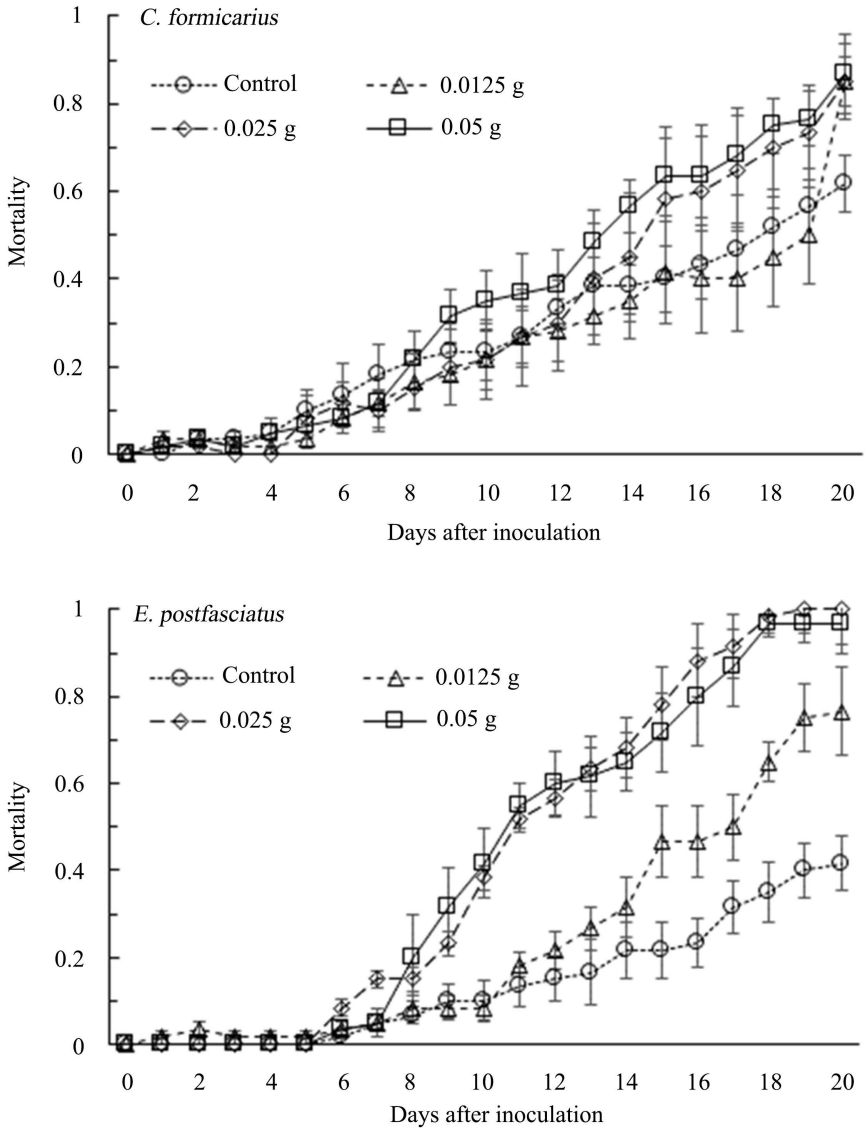

Figure 2. Mortalities of two sweet potato weevil species, *Cylas formicarius* and *Euscepes postfasciatus* caged with the isolate of *Metarizium anisopliae*, SMZ2000, in granular formulation of different doses, which are indicated in the panels.

$P < 0.001$, respectively), and the interaction of these variables was significant ($F_{5,132} = 7.828$ and $F_{5,132} = 5.961$, $P < 0.001$ and $P < 0.001$, respectively). No significant differences were detected in the proportion of injured tubers by *E. postfasciatus* between quadrats ($F_{5,132} = 0.497$, $P = 0.482$), among treatments ($F_{5,132} = 0.581$, $P = 0.714$) or in the interaction of these two variables ($F_{5,132} = 0.286$, $P = 0.920$).

3.3. Field Experiment in 2014

In this year, the means of the tuber numbers per plant were about two, being not so different from 2013, but the total tuber weight was half of 2013, about 0.8 kg (Table 1). Either of the measures were not significantly different between quadrats

Table 1. Per-plant means of measures in the field experiment in 2013.

Treat[1)]	Tuber	Tuber (kg)	Injured (kg)[2)]		Injured tuber (%)[2)]		Weevil[2)]	
	no	total	by C	by E	by C	by E	C	E
In 2013								
Cont	2.8 ± 0.3	1.62 ± 0.26	0.00 ± 0.00	0.03 ± 0.02	0.0 ± 0.0	2.7 ± 1.9	0.00 ± 0.00	4.42 ± 1.25
Conv	2.8 ± 0.4	1.87 ± 0.28	0.00 ± 0.00	0.26 ± 0.18	0.0 ± 0.0	4.8 ± 2.6	0.00 ± 0.00	2.04 ± 0.46
Fso1	2.3 ± 0.2	1.68 ± 0.17	0.00 ± 0.00	0.08 ± 0.03	0.0 ± 0.0	6.7 ± 2.9	0.42 ± 0.29	4.00 ± 1.03
Fso2	2.5 ± 0.3	1.94 ± 0.19	0.00 ± 0.00	0.04 ± 0.03	0.0 ± 0.0	1.6 ± 1.2	0.04 ± 0.03	3.25 ± 0.97
Fsf1	2.3 ± 0.4	1.73 ± 0.08	0.22 ± 0.13	0.03 ± 0.01	12.7 ± 7.7	5.6 ± 2.7	6.17 ± 4.16	2.50 ± 0.49
Fsf2	2.6 ± 0.3	1.73 ± 0.20	0.00 ± 0.00	0.00 ± 0.00	0.0 ± 0.0	0.0 ± 0.0	0.00 ± 0.00	0.92 ± 0.34
In 2014								
Cont	2.2 ± 0.3	0.84 ± 0.15	0.02 ± 0.02	0.25 ± 0.11	1.4 ± 1.3	22.0 ± 8.8	1.09 ± 0.75	3.27 ± 1.18
Conv	1.9 ± 0.2	0.79 ± 0.15	0.02 ± 0.00	0.13 ± 0.06	1.1 ± 1.0	11.7 ± 5.5	0.11 ± 0.06	2.40 ± 0.80
Fso2	1.9 ± 0.2	0.75 ± 0.16	0.00 ± 0.00	0.19 ± 0.08	0.0 ± 0.0	15.2 ± 4.1	1.07 ± 0.82	4.27 ± 1.58
Fsf2	2.4 ± 0.3	0.86 ± 0.13	0.00 ± 0.00	0.17 ± 0.07	0.0 ± 0.0	11.3 ± 3.9	0.33 ± 0.31	3.16 ± 1.42

[1)]Treatments applied were no weevil control agents (Cont), conventional weevil management with one application of fipronil before planting and two applications of chlorpyriphos (Conv), one or two applications of *Metarhizium anisopliae* in granular formulation on the ground around the main stem of sweet potato (Fso1 and Fso2, respectively), and one or two application of the fungus on the ground surface of the treated plots (Fsf1 and Fsf2, respectively). [2)]C and E indicate weevil species, *Cylas formicarius* and *Euscepes postfasciatus*, respectively.

($F_{1,172}$ = 0.006 and $F_{1,172}$ = 0.065, respectively, $P > 0.05$ in both) or among treatments ($F_{3,172}$ = 1.554 and $F_{3,172}$ = 0.326, respectively, $P > 0.05$ in both). The interaction of these two variables was not significant ($F_{3,172}$ = 1.607 and $F_{3,172}$ = 2.572, respectively, $P > 0.05$ in both). The proportion of tubers injured by *C. formicarius* was significantly different between quadrats but the number of this weevil per plant was not ($F_{1,172}$ = 4.122, P = 0.044 and $F_{1,172}$ = 0.334, $P > 0.05$, respectively). No significant differences were detected in these values among treatments ($F_{3,172}$ = 1.438 and $F_{3,172}$ = 1.422, $P > 0.05$ in both, respectively). The interaction of these variables was not significantly different in the former but significantly different in the latter ($F_{3,172}$ = 0.756, $P > 0.05$ and $F_{3,172}$ = 3.568, P = 0.015, respectively).

Both the proportion of tubers injured by *E. postfasciatus* and the number of this weevil per plant were significantly different between quadrats ($F_{1,172}$ = 4.122, P = 0.044 and $F_{1,172}$ = 4.410, P = 0.037, respectively). No significant differences were detected in these values among treatments ($F_{3,172}$ = 1.438 and $F_{3,172}$ = 0.772, $P > 0.05$ in both, respectively). The interaction of these variables was not significantly different in the former but significantly different in the latter ($F_{3,172}$ = 0.756 and $F_{3,172}$ = 0.844, $P > 0.05$ in both, respectively).

4. Discussion

In laboratory, *M. anisopliae* isolate, SMZ2000, in suspension attained mortalities ≥ 80% of both *C. formicarius* and *E. postfasciatus* in seven days after inoculation, whereas the grain formulation needed 20 days for equivalent effects. The results of the former weevil apparently contrast to the efficacy of *Beauveria bassiana* (Bals.-Criv.) Vuill., which shows high virulence to this species, causing high mortalities within several days after inoculation [12] [21] [25] [26]. The difference in the efficacy of these two fungi on the weevil suggests that *C. formicarius* is more susceptible to *B. bassiana* and less or more resistant to *M. anisopliae*. This corresponds to the results in [27], where better control for *C. formicarius* is reported in *B. bassiana* than in *M. anisopliae*. Our preliminary observations on *B. bassiana*, however, showed lower lethal effects on *E. postfasciatus*. Another possibility may be that *C. formicarius* in Okinawa could have been affected more with different strains or isolates of SMZ2000. Since the origin of this weevil lies in Asia [28], the weevil would have obtained the resistance to Asian isolates of the fungus. In contrary, since *E. postfasciatus* was evolved in the Caribbean region [3] and might have not acquired enough resistance to the fungus yet, it could have been more susceptible to SMZ2000. The difference in the susceptibility between these weevils is supported by the differences in both the coefficient of probit regressions and the LD_{50}. Fungi for the management of these weevil should be selected in accordance with the distribution of weevils as well as the dominance of weevil species in the area.

In field, *C. formicarius* occurred only occasionally in 2013, mostly in plots with one over-surface application and few or none plots with the other treatments. Since no *C. formicarius* weevils were released in this study, all the weevils of this species collected were those that had been invaded the area or were their descendants. Not significant but lower occurrences of this weevil species in the plots with insecticide or two over-surface applications in both 2013 and 2014 suggest that the two applications of the isolate over the surface controlled this weevil as much as the conventional insecticide management. Since *C. formicarius* weevils begin to invade sweet potato fields in two to three months after the planting of slips [29] [30], these results indicate that SMZ2000 applied in two months after the planting of slips could not have survived enough to control the weevil effectively by the harvesting of tubers, in four months after the application. In both years, on the other hand, *E. postfasciatus* weevils in the plots of two over-surface applications were as few as those in plots of the insecticide treatment. However, two around-stem applications of the fungus did not lower tuber damage by weevils than control with no applications. These results suggest that the isolate in this application mode was as efficacious on *E. postfasciatus* as the insecticides, if applied over the ground surface two times during the cultivation. Therefore, the two over-surface applications could have reduced weevils as effectively as the conventional insecticide application.

The efficacy of the isolate could be different between the over-surface and around-stem application modes according to the possibility that weevils would have been in contact with the fungus. In general, fungus is transferred to prey passively by other carrier organisms or by physical forces such as wind or water [31]. Thus, the weevils could have been in chances of infestation through directly passing over the fungus formulation, touching anything on which transported fungus existed, or even touching other weevils that had been already infected with the fungus. Fungus infection through behavioral contacts is known in *C. formicarius* [21] [32]. Both the low occurrences of this weevil and relatively immobile habit of *E. postfasciatus* suggest that weevils could be reduced in the fungus-applied plots through the direct touch of weevil on the fungus formulation most likely. This possibility may be explained also from the plots with the two surface applications. The wider the isolate had been spread over soil surface, the more chances the isolate would have had to infest insects. However, the isolate that was spread when the cover of the vegetation crown was thinner would have been more likely inactivated by solar radiation. This was the case at the first application of the fungus, leading the survival of conidia reduced substantially [31].

Nonetheless, the failure of the around-stem application failed in reducing weevil populations may be explained by behaviors of weevil to explore any materials to feed or reproduce. Weevils can reach the subterranean part of the plant to infest not only from main trunk but also via soil cracks [4] [6]. Places where cracks are created are not confined around the main stem. Cracks were found anywhere in the fields in this study. Fungus that had been applied around the main stem on the ground surface of ridge could have been little transported on places between ridges, where many cracks were observed. If this happed, weevils that reached roots through soil cracks on lower places would have been less infected with the isolate. Therefore, any application of the fungus before the sufficient growth of the crown would fail in controlling weevils effectively.

The yearly difference in yield may be attributed to field conditions, which could in turn have led weevils to infect sweet potato differently. Since it was first to plant sweet potatoes at the research site in 2013, infestation pressure by weevils was likely lower. With no insecticide applications in 2013, weevils surviving after the harvest would have chances to multiply on native host plants near the experimental farm. Higher infestation pressure in 2014 could have resulted in higher proportions of infested tubers, and the lack of fertilization during this study would have resulted in lower yields in 2014.

Considering the higher efficacy of *M. anisopliae* on *E. postfasciatus* in laboratory, this isolate would be a good candidate as a biological control agent for regions suffering predominantly from this species, such as South America [4]. Although laboratory experiments found the isolate to be more effective in suspension than in grain formulation, the latter is easier for the use in field. Two over-surface applications of grain formulation provided substantial reduction

both in the occurrences of this weevil in sweet potato plant and in tuber injuries by weevils. No distinct effects of the fungus on *C. formicarius* were not confirmed in the field experiments in the present study. However, this weevil occurred relatively few in both two years, but two applications of the fungus on the ground surface were likely to reduce its occurrences. These results suggest that the agent may be used for the reduction in tuber damage by these two weevils which are sympatrically distributed in such areas as eastern Pacific islands [19] [26] and the Caribbean region [33]. Therefore, the application mode can be practical as a biological control measure for sweet potato weevils, at least for *E. postfasciatus*.

5. Conclusion

The isolate of *M. anisopliae*, SMZ2000, in both suspension and granular formulation was tested for its lethal effects on two sympatric sweet potato species, *C. formicarius* and *E. postfasciatus* in laboratory. Mortalities on the seventh day were raised > 70% by the isolate in suspension at $\geq 10^5$ and $\geq 10^4$ CFU/mL in these weevils, respectively (**Figure 1**). On the other hand, it took the isolate 15 to 16 days to attain similar mortalities with grain formulation of 0.0250 g, equivalent to CFU 10^7. The grain formulation in less doses achieved only low to moderate mortalities in *E. postfasciatus* and little to none in *C. formicarius*. Probit analyses revealed significantly linearly regression of the weevil mortality (y) on the concentration of conidia in suspension formulation (x): $y = 1.17x - 5.60$ for *C. formicarius* and $y = 1.57x - 5.62$ for *E. postfasciatus*. The regressions gave an estimate of LD_{50} at $10^{4.78}$ and $10^{3.58}$ CFU/mL in the weevils, respectively. The efficacy of the isolate in granular formulation to control these weevils was evaluated in a sweet potato field for two years, in which the application frequency was compared for one or two times after the planting, and the two application modes, spray over the ground surface or on the ground only around the main stem of each sweet potato plant. These treatments were compared with no agents as control and two applications of chlorpyriphos as a conventional chemical management for the weevils in Japan. The most efficient treatments were the conventional chemical management and two applications of the isolate over the ground surface, both of which showed similar efficacies to reduce both the population of *E. postfasciatus* and the reduction of damaged tubers by these weevils in both years. Any treatments were not evaluated sufficiently for their efficacy on *C. formicarius* due to its relatively low occurrences. Based on these results, the isolate SMZ2000 can be considered to possess the potential as a biological control agent for the management of *E. postfasciatus*, whereas more field studies are needed to confirm its potential to control the other weevil, *C. formicarius*.

Acknowledgements

The authors thank Dr. Yamanaka, who supplied the fungus isolate, SMZ2000, and provided suggestions for the mode of the application.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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