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Effects of fungal culture filtrates of *Verticillium lecanii* (*Lecanicillium* spp.) hybrid strains on *Heterodera glycines* eggs and juveniles

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Abstract

Many nematode-antagonistic fungi produce secondary metabolites and enzymes that demonstrate toxicity against plant-parasitic nematodes. The objective of this study was to evaluate the effects of fungal culture filtrates of *Verticillium lecanii* hybrid strains on mature eggs, embryonated eggs (eggs fertilized but without development of juveniles), and second-stage juveniles (J2) of *Heterodera glycines* and to compare these effects with those of their parental strains. The fungal culture filtrates of certain hybrid strains inhibited egg hatch of mature eggs. Furthermore, the fungal culture filtrates of two hybrid strains, AaF23 and AaF42, exhibited high toxicity against embryonated eggs of *H. glycines*. However, most of the fungal culture filtrates of *V. lecanii* did not inactivate J2. These results suggested that enzymes or other active compounds produced by the fungal culture filtrates of *V. lecanii* exhibit activity against specific stages in the *H. glycines* life cycle. In addition, based on a visual assessment of the morphological changes in eggs caused by filtrates of each strain, there were differences between the hybrid strains and their respective parental strains with regard to the active substances produced by *V. lecanii* against the embryonated eggs. As a result of promoting recombination of whole genomes via protoplast fusion, several hybrid strains may have enhanced production of active substances that are different from those produced by their

parental strains. It was concluded that natural substances produced by *V. lecanii* are one of the important factors involved in the suppression of *H. glycines* damage.

Keywords: Egg hatch*;* Embryonated egg; Fungal culture filtrate; *Heterodera glycines*; Hybrid strain; Protoplast fusion; *Verticillium lecanii*; Second-stage juvenile

1. Introduction

Verticillium lecanii (A. Zimmermann) Viegas has been investigated as a potentially useful biological control agent (BCA) against soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe (Meyer and Meyer, 1995; Meyer and Meyer, 1996). It is known that *V. lecanii* colonizes the cyst nematodes *H. glycines, Heterodera schachtii, Heterodera avenae* and *Globodera pallida*, and the root-knot nematodes *Meloidogyne incognita* (Hanssler and Hermanns, 1981; Gintis et al., 1983; Meyer et al., 1990; Olivares-Bernabeu and Lopez-Llorca, 2002; Uziel and Sikora, 1992; Eapen et al., 2005). A potential method of enhancing activity of this species against SCN is through production of hybrid strains. Aiuchi et al. (2004) performed protoplast fusion among three strains of *V. lecanii* and obtained 174 hybrid strains. Our previous study demonstrated that, when compared with their parental strains, some of these hybrid *V. lecanii* strains suppressed damage on soybean plants and reduced the density of SCN in greenhouse pot tests, thereby suggesting that these hybrid strains have potential as biological control agents against SCN (Shinya et al., 2007).

Consequently, further studies are being conducted to improve and ensure the activity of these strains by investigating the mechanisms responsible for the suppression of plant

damage and of SCN populations. One area under investigation is production of nematotoxic compounds by selected *V. lecanii* strains. Many nematode-antagonistic fungi produce secondary metabolites and enzymes that demonstrate toxicity against plant-parasitic nematodes (Chen et al., 2000; Meyer et al., 2004; Nitao et al., 1999; Nitao et al., 2001; Nitao et al., 2002; Anke et al., 1995; Hallmann and Sikora, 1996; Bonants et al., 1995), and it was hypothesized that *V. lecanii* produced a natural substance that could affect egg viability (Meyer and Wergin, 1998). While it has been reported that certain insecticidal secondary metabolites or enzymes are produced by *V. lecanii* (Kanaoka et al., 1978; Soman et al., 2001; Wang et al., 2005; Butt, 2002; Claydon and Grove, 1982; Jackson et al., 1985; Liu et al., 2003), secondary metabolites or enzymes with nematode-antagonistic properties are not as well studied. Therefore, the purpose of this study is to evaluate the toxic effects of fungal culture filtrates of hybrid strains of *V. lecanii* on eggs and juveniles of SCN, and to compare the toxicity of the hybrid strains with that of their parental strains.

2. Materials and methods

2. 1. Fungal strains

Twelve strains of *V. lecanii* were used for this experiment. Two of these strains (Vertalec® and Mycotal®) have been used for commercial purposes as biological control agents (Koppert UK Ltd. Wadhurst, East Sussex, UK) against pest insects (Hall, 1984). A third strain, B-2, was isolated from green peach aphids at Obihiro. The remaining nine strains were obtained by protoplast fusion (Aiuchi et al., 2004) (Table 1).

All twelve strains were cultured in potato dextrose broth (PDB; Difco Laboratories, Detroit, USA) or Czapek-Dox broth. Erlenmeyer flasks (50 ml) containing 40 ml of liquid medium were each inoculated with a 1 cm^2 block from a 2-week-old fungal colony growing on potato dextrose agar (PDA; Difco Laboratories, Detroit, USA). All the strains were grown in stationary culture for 4 weeks at 25°C, and the fungal biomass was removed by centrifugation (3000 rpm/1000g for 5 min) and passed through a 0.45-µm filter. This procedure remained identical in all the experiments. The pH of each culture filtrate was recorded (Table 2).

2. 2. Effect of fungal culture filtrates on hatch of mature eggs

SCN used for this experiment was cultured on soybean, *Glycine max* (L.) Merr. cv. Kurosengoku, (planted at the National Agricultural Research Center for Hokkaido Region, Shinsei, Japan). The SCN-infested soil was taken from the field and maintained at 4°C for 6 months. The cysts in the soil were washed with a vigorously flowing stream of tap water passing through a 710-µm-pore sieve onto a 250-µm-pore sieve, collected in tap water, and then centrifuged. Light brown to dark brown cysts were picked and crushed to release the eggs. The eggs were separated from the debris by passing through a sterile 64-µm-pore sieve onto a sterile 35-µm-pore sieve, and were then placed into 0.5% NaOCl for 2 min. The eggs were rinsed 5 times with 100 ml sterile distilled water in a sterile beaker.

Egg suspensions (0.1 ml) were placed into wells of a 24-well tissue culture plate (ca.100 eggs/well) and combined with 0.9 ml of fungal culture filtrates at various dilutions (diluted to 1/2, 1/4, 1/10, or 1/100 of the original concentration). PDB and Czapek-Dox broth without fungal inoculum were used as controls. Six replicates of each dilution were prepared. For 2 weeks, the eggs were preserved in a dark environment at 27°C. The numbers of second-stage juveniles (J2) hatched on days 4, 7, and 14 were counted using an inverted light microscope, and the percentages of hatched

eggs were calculated as follows: number of hatched J2/number of eggs originally placed in the well × 100 (Meyer et al., 2004).

2. 3. Effect of fungal culture filtrates on the development of embryonated eggs

Four strains of *V. lecanii* (AaF23, AaF42, Vertalec®, and Mycotal®) were cultured in PDB and fungal culture filtrates were prepared. *Heterodera glycines* was cultured on soybean, *Glycine max* (L.) Merr. cv. Kurosengoku, planted in a greenhouse. Pale yellow to yellow females were obtained by carefully washing the soybean roots (10 weeks old) and rhizosphere soil over a 710-µm-pore sieve onto a 250-µm-pore sieve. These females were collected and crushed with forceps and a needle to release the eggs. At this point, mature eggs containing first-stage juveniles (J1) and J2 were carefully removed using a Pasteur pipet and discarded. The embryonated eggs were collected and separated from the debris by washing them through a sterile 64-µm-pore sieve onto a sterile 35-µm-pore sieve, and were placed in 0.5% NaOCl for 1.5 min. The term "embryonated eggs'' is used to describe the eggs fertilized but without development of J1 or J2 in this paper. In addition, the eggs were rinsed 5 times with 100 ml sterile distilled water in a sterile beaker.

Egg suspensions (0.1 ml) were placed into wells of a 24-well tissue culture plate (ca.50 eggs/well) and combined with 0.9 ml of undiluted fungal culture filtrates. Uninoculated PDB was used as the control. All assays were conducted with 6 replicate wells. For 2 weeks, the eggs were preserved in a dark environment at 25°C. Eggs were classified as abnormal eggs (including eggs with vacuoles, discolored eggs, and floating eggs) or normal eggs (eggs during normal development, mature eggs containing J2, and hatched eggs) using an inverted light microscope at 1, 3, 5, 7, 10, and 14 days. Furthermore, the abnormal eggs were classified into eggs with vacuoles, dark discolored eggs, and floating eggs at 14 days and the percentages of each type of abnormal egg was recorded for each fungal strain.

2. 4. Effect of fungal culture filtrates on J2 motility

Five strains of *V. lecanii* (AaF23, AaF42, AaF80, Vertalec®, and Mycotal®) were used in this study. They were cultured in PDB and fungal culture filtrates were prepared. *Heterodera glycines* were cultured using the same procedures as described earlier for collecting pale yellow to yellow females. However, the dark brown cysts were isolated from the soil in a pot using the same procedures described for isolation

146 from the field soil. The cysts were soaked in distilled water at 28°C for 10 days and then
147 crushed to release the eggs. The eggs were separated from the debris by passing through
148 a sterile 64-µm-pore sieve onto a sterile 35-µm-pore sieve. The J2 that had already
149 hatched during the 10-day incubation were carefully removed at that time. The eggs
150 were then placed in 0.5% NaOCl solution for 2 min and rinsed 5 times with 100 ml
151 sterile distilled water in a sterile beaker. These eggs were suspended in distilled water
152 and incubated at 25°C. The J2 that hatched within 2 days were collected, and a J2
153 suspension containing 500 J2/ml of sterile water was prepared. J2 suspensions (0.1 ml)
154 were placed into wells of a 24-well tissue culture plate (ca. 50 J2/well) containing 0.9
155 ml of undiluted fungal culture filtrates or a PDB control. The treatments were replicated
156 in 4 wells. The plates were maintained at 25°C. The effect of fungal culture filtrates on
157 the viability of J2 was determined after 1, 24, 72, and 168 hours using a method that
158 was almost identical to that described by Chen and Dickson (2000). To summarize, 100
159 µl of 1N NaOH was added to each well, and the J2 response was observed within 5
160 minutes using an inverted light microscope. The response time was limited to 5 minutes
161 because during the preliminary examination it was observed that some J2 began to react
162 in approximately 4 minutes. J2 reactions that began after 5 minutes were not observed.
163 J2 that responded to the addition of NaOH within 5 minutes were considered alive,

whereas those that did not respond were considered inactive or dead. Percentages of inactive or dead J2 were determined. This experiment was repeated, and the results of the repeated trial were combined for analysis.

2. 5. Statistical analysis

The data were subjected to analyses of variance (ANOVA). The treatment means were compared by the Tukey's HSD test when the *F*-tests were statistically significant at $P < 0.05$. In addition, chi-square for independence test was used to determine the interaction between the strains and breakdown of abnormal eggs.

3. Results

3. 1. Effect of fungal culture filtrates on hatch of mature eggs

Fungal culture filtrates of PDB showed various effects on egg hatch. At dilutions of 1/4, 1/10, and 1/100 PDB, the fungal culture filtrates of five strains (AaF23, AaF42, AaF49, AaF80, and AaF103) significantly ($P < 0.01$) inhibited egg hatch when

compared with that observed in the controls (Table 3). On the other hand, three strains (Vertalec®, Mycotal®, and B-2) did not inhibit egg hatch at any dilution. None of the fungal culture filtrates of the Czapek-Dox broth showed any inhibitory effect on egg hatch (date not shown). There was a wide variation in the pH of the fungal culture filtrates in this study (Table 2). However, there was no relation between the pH value and the rate of egg hatch.

3. 2. Effect of fungal culture filtrates on the development of embryonated eggs

The fungal culture filtrates of two hybrid strains, AaF23 and AaF42, demonstrated toxicity against the embryonated SCN eggs (Figs. 1 and 2). Strain AaF42 demonstrated the greatest toxicity against the embryonated eggs when compared with the other treatments after day 3. On day 1, no significant difference was observed ($P > 0.05$). On day 3, AaF42 treatment significantly ($P < 0.01$) increased the abnormal eggs when compared with the other treatments. After the 3rd day, the fungal culture filtrates of the two hybrid strains demonstrated greater toxicity against the embryonated eggs than that observed in their parental strains, Vertalec® and Mycotal®. In all the treatments, the number of abnormal eggs gradually increased from the 3rd day to the 10th

day. Toward the 14th day, there were hardly any increases in the percentages of abnormal eggs. On day 14, the fungal culture filtrate of AaF42 killed 65.1% of the embryonated eggs. On the other hand, 30.2% of embryonated eggs in AaF42 filtrate had matured by day 14. The rest of the eggs were still immature but not abnormal in the form. The fungal culture filtrates of AaF23 killed 58.4% of the embryonated eggs and 31.0% had matured by day 14. The fungal culture filtrates of Vertalec® and Mycotal® killed 52.0% and 39.1% of the embryonated eggs, respectively, on day 14. In the PDB control, 73.9% of the eggs were mature on day 14, although 23.4% were abnormal.

There was also an interaction between the fungal culture filtrates and breakdown of the abnormal eggs on day 14 ($X^2 = 369.6$; $P < 0.001$) (Fig. 3). Breakdown of eggs in filtrates from the fungal strains AaF23 and AaF42 exhibited similar patterns, with vacuoles appearing in 89.6% and 94.8% of abnormal eggs, respectively. In the fungal culture filtrate of Vertalec®, 33.3% of the abnormal eggs were floating. In contrast, 98.6% of the abnormal eggs treated with the fungal culture filtrate of Mycotal® were dark discolored eggs, and vacuoles were hardly observed (1.4%) in the abnormal eggs.

3. 3. Effect of fungal culture filtrates on J2 motility

218

219 After an exposure period of 168 h, the fungal culture filtrate of AaF23
220 significantly ($P < 0.01$) inhibited J2 motility compared with the other treatments (Table
221 4). However, only 20.7% of J2 remained inactive. Moreover, no significant effect on J2
222 motility was observed at the other times.

223

224 **4. Discussion**

225

226 Four *V. lecanii* hybrid strains (AaF23, AaF42, AaF80, and AaF103) that
227 suppressed SCN populations in a previous greenhouse test (Shinya et al., 2007) were
228 found in this study to produce compounds that significantly inhibited hatch of mature
229 SCN eggs. Conversely, culture filtrates of three parental *V. lecanii* strains that hardly
230 suppressed SCN populations in a previous greenhouse test (Vertalec®, Mycotal®, and
231 B-2) did not cause any inhibition of egg hatch. This would suggest that the natural
232 substances produced by *V. lecanii* are one of the important factors in suppression of
233 SCN numbers. There were no relationships between the pH values of fungal culture
234 filtrates and the effects on egg hatch, indicating that the differences in the pH values
235 might not have directly affected egg hatch. Pike et al. (2002) demonstrated that pH was

not an emergence signal for SCN, and the present study supports their results. Production of active compounds is influenced by culture medium (Cayrol et al., 1989) and this appeared to be the case with *V. lecanii.* While activity was obtained from some PDB cultures, culture filtrates from Czapek-Dox broth did not affect egg hatch, although this result may partly be attributed to the low egg hatch rate in this broth.

Enzymes or other active compounds produced by *V. lecanii* cultures in this study tended to be more effective against eggs in early developmental stages than against mature eggs containing J1 or J2, and showed little activity against hatched J2. Culture filtrates from the Vertalec® and Mycotal® parental strains significantly increased the percentages of abnormal embryonated eggs compared to PDB controls, even though filtrates from these strains did not affect mature egg hatch. In addition, culture filtrates of the hybrid strains AaF23 and AaF42 exhibited high toxicity against the embryonated SCN eggs. Only the culture filtrate from AaF23, decreased activity of SCN J2, and this effect was not strong. These results suggested that the toxic effect is specific for certain stages of the SCN life cycle. Other studies have demonstrated this type of stage-specific response. For example, Bonants et al. (1995) reported that culture filtrates of *Paecilomyces lilacinus* had a deleterious effect on the immature eggs of *Meloidogyne hapla.* They suggested that an extracellular protease in the filtrate

254 played an important role in this effect, and that the immature eggs were extremely
255 vulnerable, while J2 motility of *M. hapla* was not visibly influenced by treatment with
256 this culture filtrate. It is likely that enzymes produced by the culture filtrates of *V.*
257 *lecanii* similarly affected SCN eggs, and further studies are necessary to identify the
258 active compounds. It has been demonstrated that *V. lecanii* produces chitinases and
259 proteases (St. Leger et al., 1997; Lu et al., 2005).

260 The varying effects among *V. lecanii* strains on morphological breakdown of
261 the embryonated SCN eggs indicates that there are differences in the active substances
262 produced by the different strains. Prabavathy et al. (2006) demonstrated that hybrid
263 strains of *Trichoderma harzianum* produced by protoplast fusion exhibited enhanced
264 chitinase activity. Therefore, producing the hybrid strains AaF23 and AaF42 by
265 promoting recombination of whole genomes via protoplast fusion might have facilitated
266 the production of active substances that are different from those produced by their
267 parental strains. Hence, protoplast fusion technique would be a useful tool for
268 improving activity of active substances against plant-parasitic nematodes. Further
269 studies are required to facilitate a better understanding of the relationship between
270 active substances produced by *V. lecanii* in liquid medium and the toxicity of *V. lecanii*
271 against SCN in the soil.

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276 **References**

277

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372 **Figure Legends**

373

374 Fig. 1. Percentages of abnormal eggs following incubation of embryonated *Heterodera*
375 *glycines* eggs (eggs fertilized but without development of J1 or J2) in culture filtrates
376 from strains of *Verticillium lecanii*. The values indicate means ± standard deviation of 6
377 replicates. The different letters associated with each point for the given days indicate
378 significant differences ($P < 0.01$, Tukey's test). No significant difference was observed
379 at day 1 ($P > 0.05$, Tukey's test).

380

381 Fig. 2. Effect of fungal culture filtrates on the development of embryonated eggs. (a)
382 Mature eggs containing J2 in the control well after 10-day incubation. (b) Abnormal
383 eggs treated with fungal culture filtrates of AaF23 after 10-day incubation

384

385 Fig. 3. Morphological breakdown of abnormal embryonated *Heterodera glycines* eggs
386 (eggs fertilized but without development of J1 or J2) after 14 days incubation in fungal
387 culture filtrates from strains of *Verticillium lecanii*. The values indicate means ±
388 standard deviation of 6 replicates. Interaction between the strains and the breakdown of
389 abnormal eggs was determined by chi-square for independence test ($P < 0.001$).

390 Table 1. *Verticillium lecanii* strains used in this experiment, corresponding parental
391 combinations, and evaluation of antagonism to *Heterodera glycines*, the soybean cyst
392 nematode (SCN)

Strains	Combinations of each parent [a]	Evaluation of antagonism to SCN [b]
AaF11	Vertalec × Mycotal	±
AaF17	Vertalec × Mycotal	++
AaF23	Vertalec × Mycotal	+
AaF42	Vertalec × Mycotal	++
AaF49	Vertalec × Mycotal	±
AaF80	Vertalec × Mycotal	+
AaF103	Vertalec × Mycotal	+
BbF17	Vertalec × Mycotal	±
2aF26	Mycotal × B-2	±
Vertalec	-	-
Mycotal	-	-
B-2	-	-

393

394 [a] Combination of each parent according to Aiuchi et al. (2004).

395 [b] ++: very good, +: good, ±: fair, −: poor, according to Shinya et al (2007). The
396 evaluation of antagonism to SCN is based on the suppression of soybean plants damage
397 and of SCN population in greenhouse pot tests.

398 Table 2. The pH values of fungal culture filtrates from *Verticillium lecanii* strains

399 grown in potato dextrose broth (PDB) or Czapek-Dox broth at 25°C for one month

Strains	Czapek-Dox broth	PDB
AaF11	4.91	5.32
AaF17	4.49	5.35
AaF23	4.62	4.99
AaF42	4.71	5.03
AaF49	4.83	4.92
AaF80	4.37	5.07
AaF103	4.51	5.23
BbF17	5.53	5.71
2aF26	7.23	6.62
Vertalec	4.67	5.34
Mycotal	5.32	6.58
B-2	3.99	4.54
Control	7.28	5.14

400

401 Table 3. Percentages of mature *Heterodera glycines* eggs hatching after 2 weeks in
402 different dilutions of culture filtrates from *Verticllium lecanii* strains, and in potato
403 dextrose broth (PDB) controls

Strains	Percentage egg hatch (%, mean ± SD)							
	Dilutions of the fungal culture filtrates							
	1/2		1/4		1/10		1/100	
AaF23	3.2 ± 1.9	a	3.4 ± 1.5	a	6.1 ± 3.4	a	19.6 ± 6.5	ab
AaF42	5.0 ± 4.5	a	4.0 ± 3.1	a	10.2 ± 4.3	abc	23.0 ± 16.5	ab
AaF11	5.9 ± 2.8	a	8.0 ± 0.7	ab	25.5 ± 4.3	cde	47.0 ± 5.5	cd
AaF49	5.9 ± 6.6	a	4.8 ± 5.4	a	9.6 ± 4.5	ab	34.0 ± 6.0	bc
AaF103	6.3 ± 7.1	a	4.5 ± 3.0	a	12.9 ± 7.6	abcd	26.8 ± 8.4	ab
AaF80	6.6 ± 7.2	a	2.9 ± 1.5	a	8.2 ± 4.9	ab	16.6 ± 5.4	a
AaF17	9.2 ± 6.5	ab	8.7 ± 2.6	ab	17.3 ± 8.0	abcd	54.6 ± 3.9	d
BbF17	9.6 ± 5.7	ab	7.3 ± 1.5	ab	22.0 ± 5.3	bcd	55.0 ± 6.9	d
Mycotal	19.7 ± 3.0	b	17.1 ± 12.3	bc	25.6 ± 5.9	cde	45.1 ± 4.5	cd
Vertalec	20.5 ± 5.7	b	23.1 ± 4.2	c	38.1 ± 12.8	e	48.8 ± 7.1	cd
B-2	20.6 ± 8.4	b	23.3 ± 5.0	c	27.2 ± 8.6	de	48.4 ± 4.3	cd
2aF26	21.1 ± 6.6	b	9.4 ± 2.3	ab	22.7 ± 8.7	bcde	49.0 ± 4.0	cd
Control	14.9 ± 4.7	ab	23.8 ± 6.4	c	33.9 ± 9.1	e	55.6 ± 7.8	d

404

405 The values indicate means ± standard deviation of 6 replicates. The different letters in
406 the same column indicate significant difference ($P < 0.01$, Tukey's test).

Table 4. Percentages of inactive second-stage juveniles of *Heterodera glycines* after incubation in culture filtrates from *Verticillium lecanii* strains

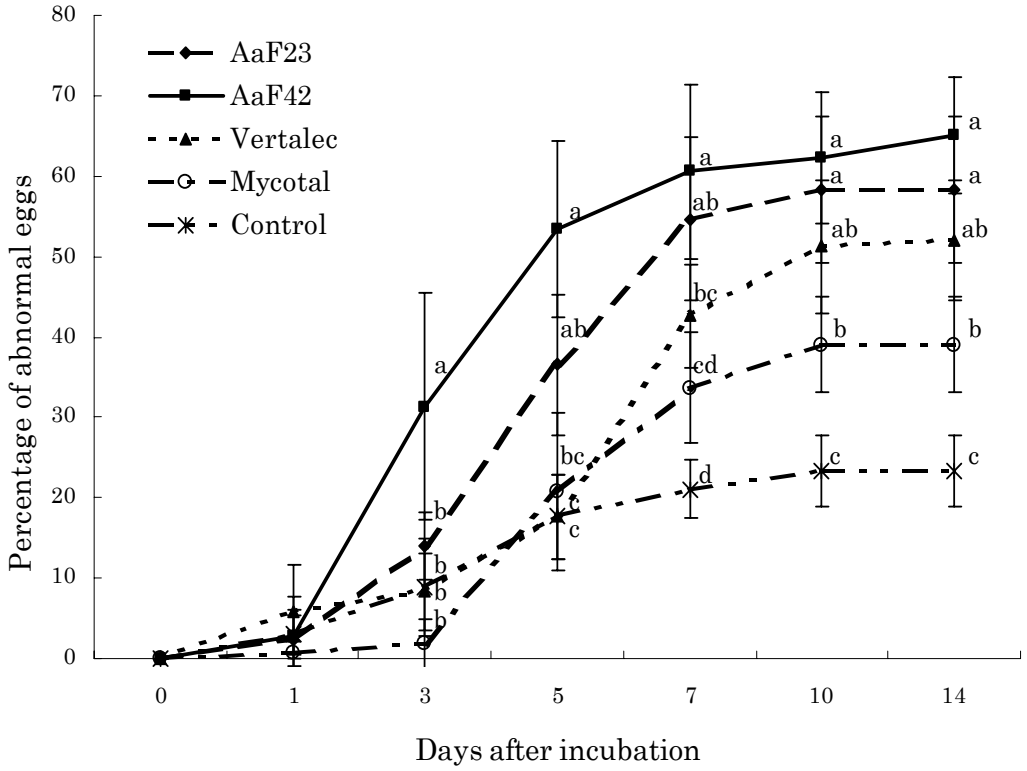
Strains	Incubation period (hours)			
	1	24	72	168
AaF23	0.5 ± 0.9 NS	1.5 ± 1.9 NS	1.5 ± 2.5 NS	20.7 ± 11.0 a
AaF42	0.4 ± 0.8 NS	0.3 ± 0.6 NS	2.5 ± 2.7 NS	3.7 ± 2.8 b
AaF80	0.8 ± 1.2 NS	1.1 ± 1.6 NS	1.7 ± 2.4 NS	2.0 ± 2.3 b
Vertalec	0.4 ± 1.0 NS	0.7 ± 1.5 NS	2.9 ± 2.9 NS	5.2 ± 3.6 b
Mycotal	1.6 ± 2.2 NS	2.1 ± 2.0 NS	3.1 ± 2.5 NS	4.4 ± 4.0 b
Control	0.1 ± 0.2 NS	0.5 ± 0.7 NS	1.0 ± 0.9 NS	1.1 ± 1.0 b

The values indicate means ± standard deviation of 8 replicates. The different letters in the same column indicate significant difference ($P < 0.01$, Tukey's test).

NS: not significant.

413 Fig. 1.

414



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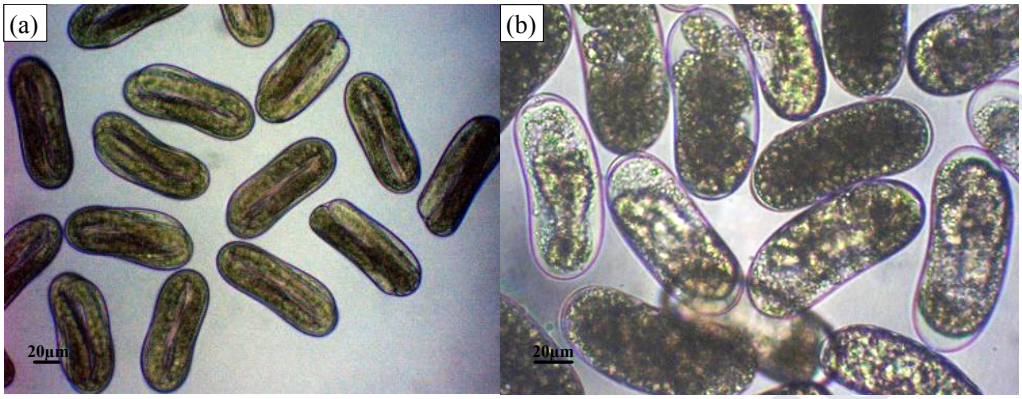
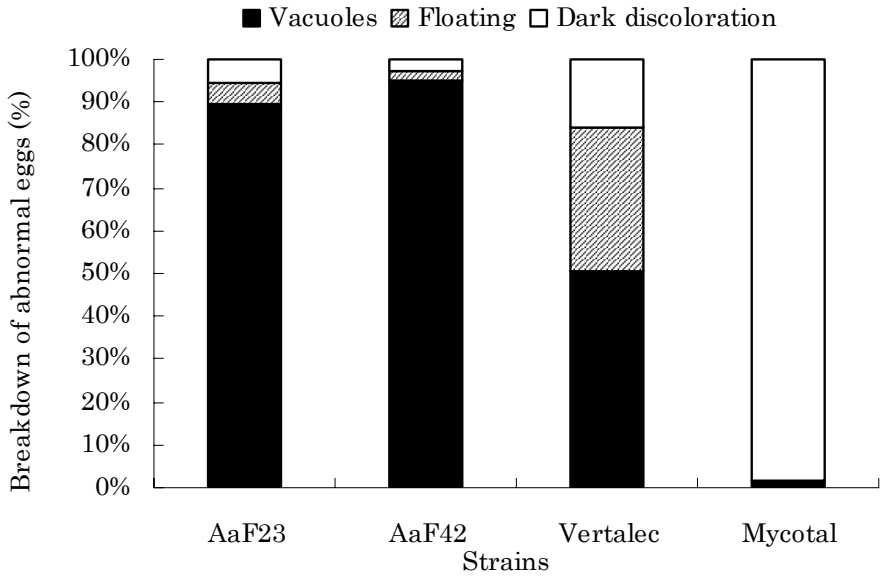
423 Fig. 2.



424 Fig. 3.



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