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論　文（Original article）

Density dynamics of an entomopathogenic fungus, *Beauveria bassiana* introduced into fresh water

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Abstract

Density dynamics of *Beauveria bassiana* in fresh water were investigated to detect possible influences of this fungus on aquatic ecosystems. Conidia of *B. bassiana* were inoculated in non-sterilized lake water, sterilized lake water, non-sterilized distilled water, and sterilized distilled water in the laboratory, and their densities monitored. The conidia decreased sharply in all experimental waters over a short time. More than 90% of the live conidia were lost within 28 d, only 0.03% survived in the non-sterilized lake water. No germinating conidium was found in non-sterilized waters after 27 d, while they had been observed in sterilized waters up to 84 d after inoculation. Other microorganisms existing in natural waters might inhibit germination of this fungus and reduce the densities of live conidia. The result testified the difficulty of *B. bassiana* accidentally contaminating natural fresh waters.

Key words : density, entomopathogenic fungus, aquatic ecosystem, germination, conidia

Introduction

Beauveria bassiana (Balsamo) Vuillemin has been regarded as an efficient biocontrol agent against many aggressive insect pests including immigrant disaster pests, and we have been studying this fungus as a control agent of the Japanese pine sawyer, *Monochamus alternatus* which vectors the pinewood nematode, pathogen of pine wilt disease (Shimazu et al., 1995; Shimazu et al., 2002a; b; Shimazu and Sato, 2003; Shimazu 2004a; b). To utilize this fungus as a microbial insecticide, dynamics of the fungus after application should be considered since it could persist in certain niches for a relatively long time. Shimazu (2002a) and Wang et al. (2002) studied the density dynamics of *B. bassiana* in forests. *Beauveria bassiana* conidia applied in the environment may accidentally enter aquatic ecosystems. Investigating the proliferation of *B. bassiana* in water will produce a good understanding of its life cycle and its possible influence on aquatic lives when the fungus accidentally enters fresh water. Up to now, the density dynamics of *B. bassiana* in water have not been understood. Therefore, we experimentally inoculated *B. bassiana* conidia in fresh water, and the density dynamics of the fungus were investigated.

Materials and Methods

Fungal isolate

A strain of *B. bassiana* F-263 isolated from a larva of the Japanese pine sawyer, *Monochamus alternatus* collected in Kumamoto prefecture of Japan in 1980, was used for the experiment. The isolate shows a high virulence to *M. alternatus* (Shimazu and Kushida, 1983) and has been studied for its use for the control of pine wilt disease by killing this insect (Okitsu et al., 2005; Shimazu et al., 1992, 1995). The isolate has been preserved in the Forest and Forestry Product Research Institute, Japan.

Experimental water and inoculation

Lake water and distilled water were used in the present study to assess proliferation ability of *B. bassiana* in fresh water. The lake water was collected from Lake Kasumigaura, the second largest lake in Japan on 17 May 2005 and kept at room temperature.

On the next day, collected lake water was roughly filtered with filter paper (Advantec #2), and then its natural pH was measured with an electronic pH meter. A portion of the experimental water was sterilized using a 0.2 μm cellulose nitrate filter (Sartorius AG) in order to assess the influence of microbes or other organisms on dynamics of *B. bassiana* conidia. Altogether four kinds of experimental waters were thus prepared; sterilized distilled water, non-sterilized distilled water, sterilized lake water and non-sterilized lake water (natural lake water). Since the natural pH of the lake water was 8.0, the pH of the distilled water for the experiment was also adjusted to 8.0 with Na_2CO_3 solution.

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Preparation and inoculation of *B. bassiana* F-263

Beauveria bassiana F-263 was cultured on Sabouraud's dextrose agar with 1% yeast extract (abbreviation SDAY), at 25 ˚C for 3 weeks. The conidia were scraped from the culture with a sterile glass rod and suspended in sterilized water to obtain a conidial suspension. The suspension was filtered through a piece of tissue paper to remove conidial masses and mycelial fragments. The original concentration of the suspension was calculated using a hemocytometer.

This original high-density conidial suspension was added to each kind of experimental water at a final concentration at 1×10^6 conidia/ml. Two hundred milliliters of each experimental water containing conidia was poured into a 500 ml sterilized flask and capped with a Silicosen® culture plug (Shin-Etsu Polymer Co., Ltd.). Three replicates were prepared for each kind of water. All the flasks were statically incubated at 25˚C with an L/D cycle of 16/8.

Sampling

A small portion (1 ml) of each experimental water was sampled weekly, and diluted with sterile water to make a final concentration of approximately 1×10^3 conidia/ml. Onto a D0C2 selective medium plate (3 g Bactopeptone, 0.2 g $CuCl_2 \cdot {}_2H_2O$, 2 mg crystal violet, 15 g agar, 1000 ml distilled water, pH 10.0 with Na_2CO_3, developed by Shimazu and Sato, 1996), 0.1 ml of the diluted sample suspension was dropped and spread out with a sterilized glass rod. Three plates were prepared for one sample; altogether 9 plates were used for each experimental water sample. All the plates were incubated at 25˚C with continuous light for 6 d. The colony forming units (CFUs) on D0C2 plates were counted, and the colonies were identified. For identification of the colonies, 10 colonies were randomly transferred onto SDAY when more than 10 colonies appeared on a plate, and if less than 10, all of them were transferred, cultured and identified. The ratio of *B. bassiana* versus other species from SDAY cultures was used to estimate the true number of *B. bassiana* colonies. The day before sampling and culturing of the experimental waters, each water sample was also checked using a Thoma's hemocytometer to count the number of conidia and hyphae to investigate the rate of germinating conidia and to estimate the proper dilution ratio for culturing of the sampled water on the following day.

Results

Density dynamics of *B. bassiana*

All fungal cells of *B. bassiana* in four kinds of waters decreased sharply over 4 weeks (Fig. 1). Since the initial conidial densities of *B. bassiana* in four groups of water were not equal (one-way ANOVA, p=0.012), densities at each observation were compared with the initial densities of each flask. Decline of *B. bassiana* densities in the first week in non-sterilized lake water (72.62%) and non-sterilized distilled water (79.20%) were higher than those in sterilized lake water (52.65%) and in sterilized distilled water (25.01%). After 28d of survival, densities of *B. bassiana* in sterilized lake water, non-sterilized lake water, sterilized distilled water, and non-sterilized distilled water decreased by 91.92%, 99.97%, 93.94%, and 96.99%, respectively. The decline ratios of all treatments were over 90%, and the conidia in non-sterilized lake water lost their viability most rapidly. There was a significant difference in decline tendencies of the fungal density between sterilized and non-sterilized distilled waters (two-way Repeated-Measures ANOVA, p=0.0075 for waters and p<0.0001 for days × waters),

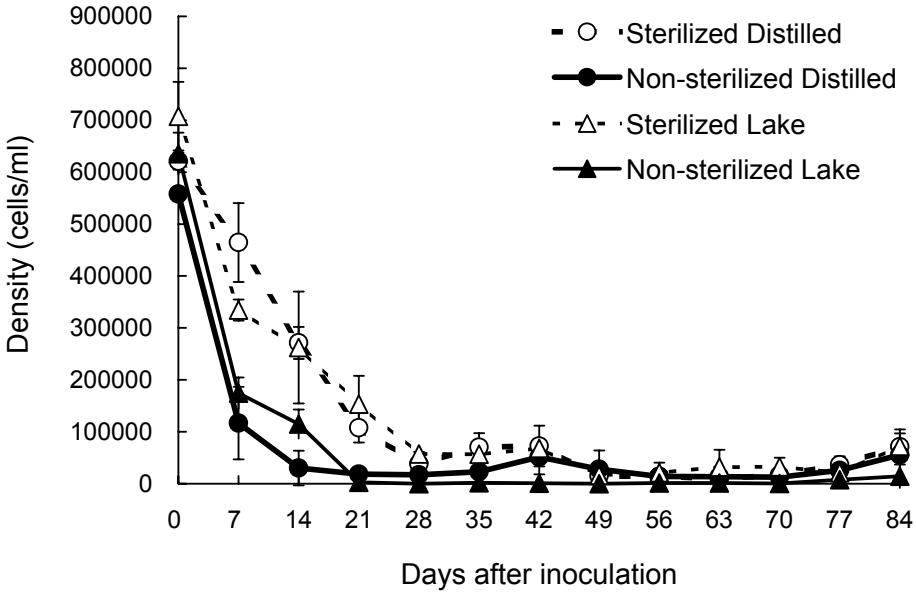

Fig. 1. Dynamics of densities of *Beauveria bassiana* in four kinds of waters by terms of CFUs. Vertical bars = SD.

and between sterilized and non-sterilized lake waters (two-way Repeated-Measures ANOVA, $p<0.0001$ for waters and $p<0.0001$ for days × waters).

Germination dynamics of *B. bassiana*

In the experimental waters, *B. bassiana* conidia produced germ tubes, hyphal bodies, and hyphae (Fig. 2). Newly reproduced conidia from hyphae or from hyphal bodies were also observed within a short time (Fig. 2). Among all the fungal cells, conidia with germ tubes, hyphal bodies, and hyphae were treated as germination. The newly reproduced conidia were obviously a consequence of germination, but they were not morphologically distinguishable from the ungerminated ones. In the first few weeks, germ tubes, hyphal bodies, and hyphae could be seen in all experimental waters, although their rates were not so high. Especially, visible cotton-like mycelia appeared and remained visible for a long time in the sterilized waters (Fig. 3), while such visible mycelia did not appear and

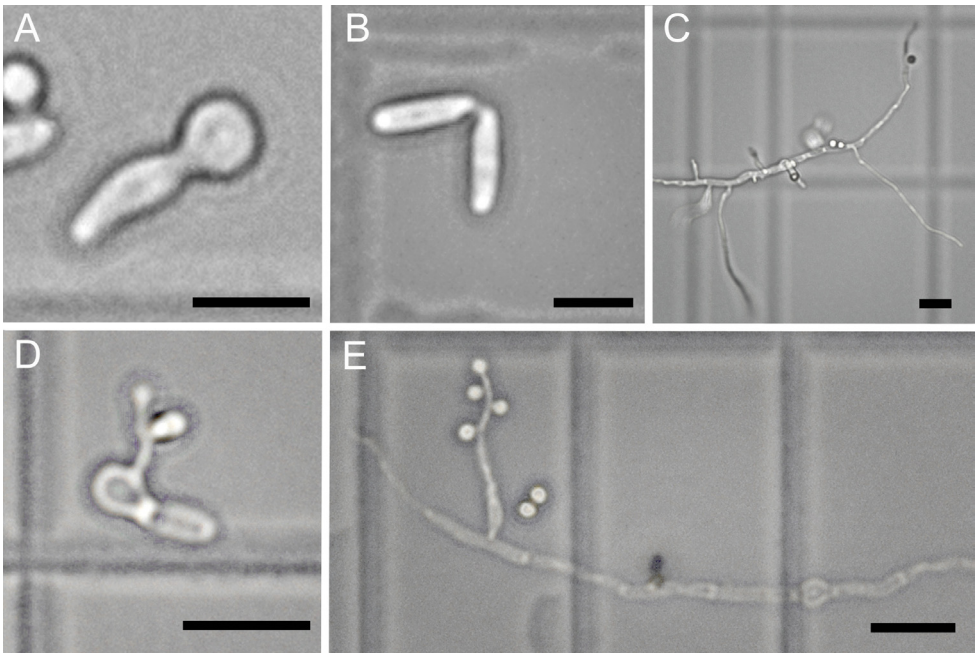

Fig. 2. Various morphologies as outcomes of germination of *Beauveria bassiana* conidia (A: elongation of germ tube, scale bar = 5 μm; B: hyphal bodies, scale bar = 5 μm; C: hyphae, scale bar = 10 μm; D: secondary reproduced conidia from a hyphal body, scale bar = 5 μm; E: secondary reproduced conidia from a hypha, scale bar = 10 μm).Fig. 1. Dynamics of densities of *Beauveria bassiana* in four kinds of waters by terms of CFUs. Vertical bars = SD.

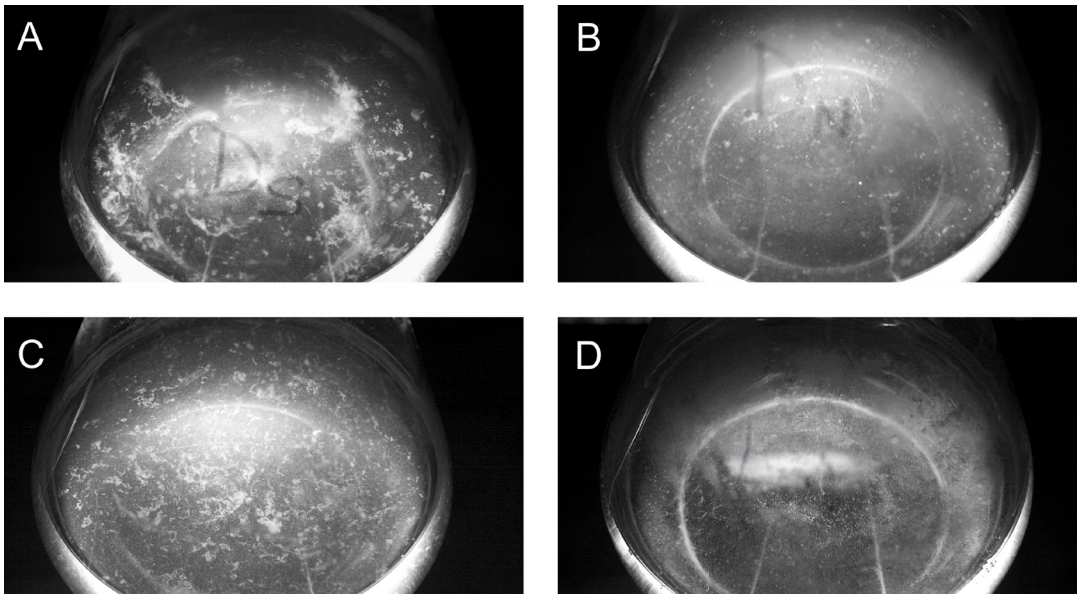

Fig. 3. Visible difference of the growth of mycelia as appeared in sterilized and non-sterilized groups. Some fuzzy cotton-like mycelia can be seen in sterilized waters (A: sterilized distilled water; B: non-sterilized distilled water; C: sterilized lake water; D: non-sterilized lake water).

the conidia were restrained from germination in both of the non-sterilized waters. The germination rates of *B. bassiana* conidia persisted in sterilized distilled water and sterilized lake water and ranged from 1.76% to 6.95%, and from 1.55% to 7.49%, respectively, within 48 d. In the next 5 weeks, the percentage of germinating conidia in the two groups rose and ranged from 10.45% to 18.08%, some higher than in the first half periods (Fig. 4). The germination rates in both of the non-sterilized waters were 0% after 27 d. Dynamics of germination rates in sterilized and non-sterilized distilled water differed significantly (two-way Repeated-Measures ANOVA, p=0.0072 for waters and p<0.0001 for days × waters). Those in sterilized and non-sterilized lake water also differed significantly (two-way Repeated-Measures ANOVA, p<0.0001 for waters and p<0.0001 for days × waters). On the other hand, those in non-sterilized distilled and lake water were not statistically different (two-way Repeated-Measures ANOVA, p=0.1855 for waters and p=0.2281 for days × waters respectively). Similarly, no statistical difference was found between those in sterilized distilled and lake water (two-way Repeated-Measures ANOVA, p=0.9856 for waters and p=0.3315 for days × waters respectively).

Discussion

Beauveria bassiana can infect many species of insects and can grow on artificial media or some kinds of soil (Li, 1988). It is believed that these hosts and survival niches supplied the necessary nutrients for growth (Smith & Grula, 1980). No report has been found studying whether *B. bassiana* could grow in water. The present study revealed that *B. bassiana* could germinate and extend hypha in sterilized waters, both in sterilized lake water and in sterilized distilled water. Just one-week after inoculation, visible long mycelia appeared in the two kinds of sterilized waters, but not in non-sterilized ones. The new mycelia showed degradation after 4 weeks survival; perhaps because of exhaustion of nutrients stored in the conidia. No visible mycelium appeared in non-sterilized waters, suggesting the influence of inhibition by other aquatic organisms.

The fungal densities in sterilized treatments were generally higher than in non-sterilized treatments. This phenomenon was especially conspicuous during the first one month of survival, but in the following several months, their densities were around 1 x 10^4 /ml except non-sterilized lake water in which the fungal density was almost 1/10 of that in other waters. Non-sterilized water must contain more microorganisms than distilled water, and they might keep the density of *B. bassiana* lower than in the other waters.

Beauveria bassiana persists in some niches including insect cadavers and soil (Wang et al., 2005). Wild *B. bassiana* could be isolated from natural soil (Doberski & Tribe, 1980; Shimazu et al., 2002a), and introduced populations of *B. bassiana* could keep a certain density for a long time in soil (Shimazu et al., 2002b; Wang et al., 2002). *Metarhizium anisopliae*, another important entomopathogenic fungus, could also survive for a long time in soil (Mikuni et al., 1982; Yaginuma, 1990). The present study revealed that *B. bassiana* could persist for relatively long periods (12 weeks) in sterilized waters, while in non-sterilized waters the fungus decreased sharply at the

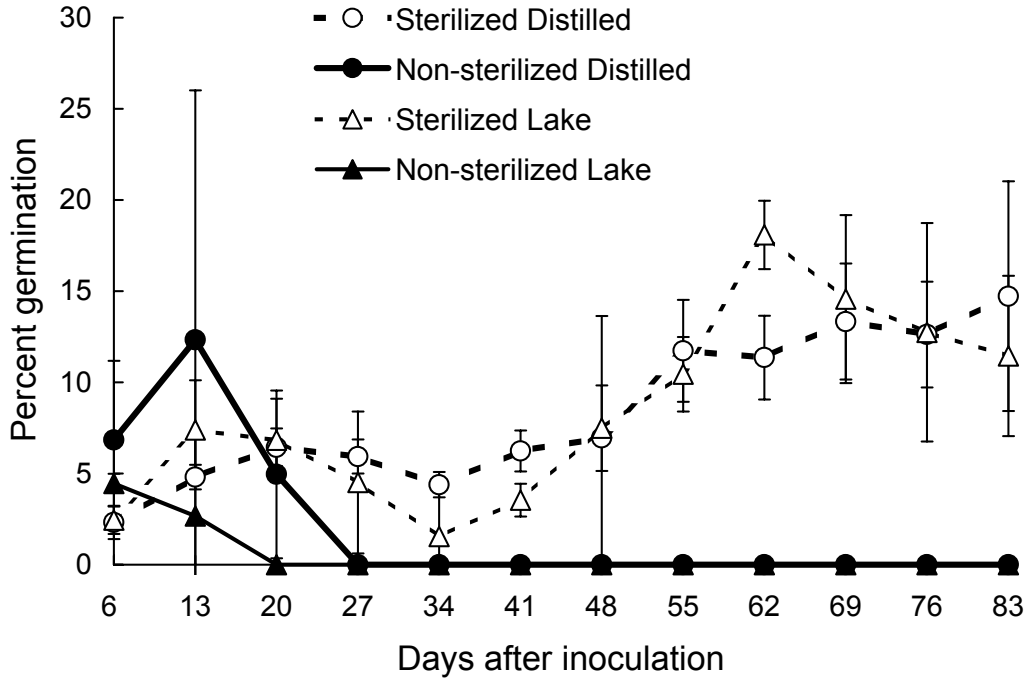

Fig. 4. Dynamics of conidial germination rates in four kinds of waters. Vertical bars = SD.

beginning and then remained at a low density.

Biotic factors in soil are disadvantageous to the growth of entomogenous fungi (Walstad et al., 1970; Pereira et al., 1993). Shimazu et al., (2002b) observed that *B. bassiana* conidia germinated in sterilized soil, but not in non-sterilized soil. Walstad et al. (1970) revealed that microbes in soil were detrimental to the growth and infection of *B. bassiana* to the pales weevil, *Hylobius pales*. Pereira et al. (1993) also found soil antagonism affecting the dose-response of workers of the red imported fire ant, *Solenopsis invicta*, to *B. bassiana* conidia. The half lethal concentration (LC50) increased from 1×10^2 conidia/g soil for sterilized soil to 2×10^9 conidia/g for non-sterilized soil. A similar result was found in water in the present experiment. In non-sterilized groups, germination of conidia was possibly inhibited by some microbes in water. Sterilization of water probably removed either microbial competition or inhibition to *B. bassiana*, and benefited the persistence of the fungus. Compared with soil, nutritional conditions in water are thought to be poorer. That may limit the germination of *B. bassiana* in sterilized water, and keep it at a low level. The conidia may quickly lose their viability when they exhausted their own stored nutrients.

Although *B. bassiana* has been known as a pathogen of many insect species, there have been few records from aquatic insects. Clark et al. (1968) reported *B. bassiana* conidia floated on the surface of the water and killed *Culex pipiens* larvae, but the virulence was not clarified. Miranpuri and Khachatourians (1991) found that both conidia and blastospores (=hyphal bodies) of this fungus had larvicidal activities against *Aedes aegypti* at concentrations of 4×10^8 cells/ml, although their infection route might not be percutaneous. The rice water weevil, Lissorhoptrus oryzophilus was highly susceptible to *B. bassiana* at a conidial concentration of 1×10^8/ml, and 5×10^{11}/m^2 of conidia were necessary to reduce the population density of the larvae and the pupae on rice plants to 30% of that of non-treated rice (Yoshizawa, 1998). Throughout those studies, numerous conidia are thought to have been needed to allow infection of aquatic insects compared with the susceptibility of *M. alternatus* larvae; whose LC_{50} to *B. bassiana* F-263 was 1.1×10^3/ml (Shimazu, 1994). Moreover, our isolate is expected to be used on dead pine logs in the forest and there will be a very low possibility for the conidia to mingle in water at high densities to infect aquatic insects.

B. bassiana F-263 does not seem to cause damage to aquatic insects for the following reasons when the fungus is applied to control *M. alternatus* in the forest; 1) large decrease of viable conidia in natural waters, 2) requirement of high dose to infect aquatic insects, and 3) low possibility of conidia to contaminate aquatic systems. Also, there is a concern about the infection of terrestrial insects by this fungus which may survive in water. However, this possibility should be far less than direct infection from applied conidia.

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昆虫病原菌 *Beauveria bassiana* の淡水中における密度動態

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要 旨

Beauveria bassiana が水域生態系に影響をおよぼす可能性を検討するため，この菌の淡水中での密度変動を調査した。室内で *B. bassiana* の分生子を無殺菌湖水，殺菌湖水，無殺菌蒸留水，殺菌蒸留水に入れ，その密度を追跡した。いずれの水の中でも分生子は短時間に急速に減少した。28 日以内に 90％以上の分生子が消失し，とくに無殺菌湖水中では 0.03％しか生き残らなかった。無殺菌の水の中では 27 日以降には発芽している分生子はみられなくなったが，殺菌した水の中では接種後 84 日までみられた。自然の水の中では，この菌は存在している他の微生物により発芽を阻害され，生きた分生子の密度が減少したと考えられた。この結果から，偶然 *B. bassiana* が自然の淡水系に混入しても，増殖することは困難なことが明らかになった。

キーワード：密度，昆虫病原菌，水域生態系，発芽，分生子

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