Density dynamics of the entomopathogenic fungus, *Beauveria bassiana* Vuillemin (Deuteromycotina: Hyphomycetes) introduced into forest soil, and its influence on other soil microorganisms

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Abstract

*Beauveria bassiana* conidia were mixed into the forest soil, and the density dynamics of soil microorganisms were investigated using selective media to clarify the impact of *B. bassiana* application on them. The density of bacteria was between $1 \times 10^6$ and $10^8$ CFU/g, and that of actinomycetes was around $1 \times 10^6$ to $10^7$ CFU/g regardless of the addition of *B. bassiana*. Densities of general fungi and of *B. bassiana* in the non-treatment plot were always approximately $1 \times 10^5$ CFU/g and $1 \times 10^2$ CFU/g, respectively. On the other hand, densities of both general fungi and *B. bassiana* in the treatment plot increased to 3 to $5 \times 10^7$ CFU/g immediately after the mixing of *B. bassiana*. Densities gradually decreased to 1/10 the density after 12 months. Microscopic observations revealed that *B. bassiana* conidia do not germinate in non-sterilized soil, but they do germinate in sterilized soil. Densities of fungi other than *B. bassiana* could not be measured in the treatment plot. However, they were not thought to be affected by mixing of *B. bassiana*, because the metabolism of *B. bassiana* seemed to be very inert.

Key words: *Beauveria bassiana*, bacteria, actinomycetes, fungi, density

INTRODUCTION

*Beauveria bassiana* (Balsamo) Vuillemin isolate F-263 is a strong pathogen of *Monochamus alternatus* Hope (Coleoptera: Cerambycidae) (Shimazu and Kushida, 1983). We have been studying utilization of this isolate as a microbial control agent of *M. alternatus* (Enda et al., 1989; Kinuura et al., 1992; Shimazu et al., 1992, 1995). In order to utilize an insect pathogen for practical control of insect pests, registration as a microbial insecticide is necessary. In Japan, a series of data following the national guidelines (MAFF, 1997) must be submitted to register a microbial insecticide. Movement of the microbial agent in the environment and impacts on densities of soil microorganisms are criteria required for registration of a microbial agent. In this national guideline, the microbial agent must be applied at ten times the normal use, and densities of bacteria, actinomycetes and general fungi in soil must be investigated for 3 months.

Life cycles of entomopathogenic fungi including *B. bassiana* are not well known. For example, the status of *B. bassiana* or other entomogenous Hyphomycetes apart from host insects are unknown. Soil is generally thought to be one of the places that harbors such fungi during periods without a host insect, however, studies on movement of entomopathogenic fungi in soil have been limited.

In the present study, we mixed *B. bassiana* conidia in forest soils, and measured the densities of soil microorganisms and the mixed *B. bassiana* itself using selective media. We also observed the *B. bassiana* conidia buried in the soil by microscope to clarify its behavior in soil.

MATERIALS AND METHODS

Throughout the following experiments, *Beauveria bassiana* F-263 from a larva of *M. alternatus* in Kumamoto, Japan was employed. For mass production of conidia, the fungus was cultured in Sabouraud’s saccharose broth with 1% yeast extract in a shaker-incubator for 5 days at 25°C. The resulting culture was mixed at a ratio of 1:5 with Sabouraud’s saccharose agar with 1% yeast extract before the agar solidified. The mixture was then poured into plastic boxes ($23 \times 16.5 \times 4$ cm). Those
boxes were kept at 25°C for 3 weeks, air-dried, and then the conidia were harvested using a brush.

**Densities of microorganisms in soil mixed with Beauveria bassiana.** Two Pinus densiflora stands and a Pinus hybrid (P. thunbergii × Pinus massoniana) stand located in Chiyoda, Ibaraki, Japan, were used for the experiments. The soil types in each experimental stand based on the Forest Soil Division (1976) were moderately moist brown forest soil, water contents (Hasegawa, 1997) were 0.31 to 0.47, and pHs (KCl) (Kamewada, 1997) were 3.8 to 4.6. Artificial introduction of B. bassiana had never been made in these forest stands prior to our study. Two 1 m² plots (treatment and non-treatment) were established in each stand. Application of B. bassiana conidia and sampling of soil were carried out according to the guidelines for evaluation of microbial pesticide safety (MAFF, 1997): that is, a 10-fold density of conidia was applied to a 1 m² plot, four soil specimens were collected from each plot to count densities of bacteria, actinomycetes, and fungi, and the experiments were replicated three times.

On 17 September 1998, the treatment plots were tilled to a depth of 30 cm, and \(1.4 \times 10^{12}\) of B. bassiana conidia were mixed into the soil. The non-treatment plots were only tilled to this same depth.

Soil samples were collected immediately before, immediately after, 1 d and 11 d after the mixing, and monthly afterward for a total of 15 months. Four soil samples under the litter were randomly taken from each plot in individual plastic bags. The soil samples were suspended in an aqueous solution of Tween 80 (300 ppm) at a rate of 2.5 g per 50 ml in a 100 ml flask, and then strongly shaken using a test-tube-mixer for 1 min. Each suspension was diluted 10, 10², 10³, and 10⁴ times. Aerobic bacteria and actinomycetes growing on neutral medium were considered as representative of the bacteria and actinomycetes populations. For selective isolations of bacteria, actinomycetes, general fungi and B. bassiana, media shown in Table 1 were prepared in 9 cm-plastic petri dishes. Onto each petri dish with selective media, 0.2 ml of the soil suspension was dropped, and spread using a

<table>
<thead>
<tr>
<th>Target microorganism</th>
<th>Bacteria</th>
<th>Actinomycetes</th>
<th>Fungi</th>
<th>B. bassiana</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ingredients</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Peptone</td>
<td></td>
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<tr>
<td>Casein</td>
<td></td>
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<tr>
<td>Yeast extract</td>
<td>1 g</td>
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<tr>
<td>Glucose</td>
<td>1 g</td>
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<td></td>
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<tr>
<td>Soluble starch</td>
<td></td>
<td>10 g</td>
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<tr>
<td>CuCl₂</td>
<td></td>
<td></td>
<td>0.2 g</td>
<td></td>
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<tr>
<td>KH₂PO₄</td>
<td>0.3 g</td>
<td>0.5 g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.2 g</td>
<td></td>
<td>1 g</td>
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<tr>
<td>MgSO₄·7H₂O</td>
<td>0.2 g</td>
<td></td>
<td>0.5 g</td>
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<tr>
<td>Rose Bengal</td>
<td></td>
<td></td>
<td>33 mg</td>
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<tr>
<td>Crystal violet</td>
<td></td>
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<td></td>
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<tr>
<td>Agar</td>
<td>15 g</td>
<td>15 g</td>
<td>20 g</td>
<td>15 g</td>
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<tr>
<td>Distilled water</td>
<td>1,000 ml</td>
<td>1,000 ml</td>
<td>1,000 ml</td>
<td>1,000 ml</td>
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<tr>
<td>pH</td>
<td>6.8</td>
<td>7.0–7.5</td>
<td>6.8</td>
<td>10</td>
</tr>
<tr>
<td><strong>Culture days at 25°C</strong></td>
<td>3</td>
<td>7</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

\(\text{a Kato (1992).} \)
\(\text{b Miyashita (1992).} \)
\(\text{c Nitta (1992).} \)
\(\text{d Shimazu and Sato (1996).} \)
sterilized glass bar. Petri dishes were incubated at 25°C for the periods shown in Table 1 for each medium, and the numbers of colonies obtained were counted. Among the colonies on D0C2 medium, the numbers of those resembling B. bassiana were counted. If these were less than 10, all the colonies counted were transferred onto fresh Sabouraud’s dextrose medium with yeast extract (abbreviation SDY), and if they were more than 10, 10 randomly selected colonies were transferred onto SDY. The cultures on SDY were incubated at 25°C under fluorescent lamps to allow formation of conidia, and were then identified as B. bassiana by microscopic examination. The ratio of B. bassiana to other fungal species from each SDY culture was used to estimate the true number of B. bassiana.

A two-way repeated-measures ANOVA test was used to determine the significance of mean differences of densities between the treatment plot and the non-treatment plot and among days after mixing of B. bassiana.

Observation of germination of Beauveria bassiana conidia. In order to observe B. bassiana conidia in the soil or other substances, the method developed by Mikuni et al. (1982) was modified and used. Fresh dry conidia of B. bassiana were spread on the surfaces of sterilized cover slips using absorbent cotton. Soil samples for this experiment were taken from the A-layer in a P. densiflora forest in the Forestry and Forest Products Research Institute at Kukisaki, Ibaraki, and large impurities in the soil were eliminated by screening.

The cover slips with B. bassiana conidia were treated with moisture in the following ways to check for the ability to germinate without culture media: 1) burying the cover slips in non-treated soil in a 9 cm petri dish without controlling the moisture; 2) burying them in saturated non-treated soil with water in a 9 cm petri dish; 3) placing them in a 9 cm petri dish with a moistened filter paper; 4) placing the surfaces of cover slips with conidia directly against a moistened filter paper; and 5) placing the surfaces of cover slips with conidia against water agar. Three replicates were prepared for each treatment, and all petri dishes were placed at 25°C in the dark. Three days later, the cover slips were recovered and examined using a microscope. More than a hundred conidia on each cover slip were examined classified as germinated, inflated, or non-germinated. Inflated conidia were thought to be in an early stage of germination.

The following experiment was carried out to check the effect of sterilization of the soil on the germination of B. bassiana conidia. Twelve cover slips with B. bassiana conidia on their surfaces were buried in non-treated soil and sterilized soil (autoclaved for 2 h) in 9 cm petri dishes. Three cover slips were recovered from each soil 1, 2, 4, and 7 d later and observed using a microscope. More than a hundred conidia on each cover slip were classified as germinated, inflated, or non-germinated.

RESULTS
Densities of microorganisms in soil mixed with Beauveria bassiana

Regardless of whether or not B. bassiana was mixed in the soil, the densities of bacteria were between $1 \times 10^6$ and $10^8$ CFU/g for up to 1 year (Fig. 1). Densities of actinomycetes were between $10^6$ and $10^7$ CFU/g in both treatment and non-treatment plots. No statistical differences of bacterial densities between treatment and non-treatment ($p=0.848$ for the treatment and $p=0.923$ for time×the treatment interaction) and of actinomycetes densities ($p=0.724$ for the treatment and $p=0.566$ for time×the treatment interaction) were found. There were temporal changes in densities of bacteria and actinomycetes (both $p<0.01$ for time), and densities of bacteria at 124 d and actinomycetes at 11 d after mixing were lower than those on the other days. Since both densities in the treatment plot and non-treatment plot were equally lower on those days, these lower densities were thought to have no relation to the mixing of B. bassiana.

Densities of general fungi in the non-treatment plot were approximately $1 \times 10^5$ CFU/g throughout the investigation period (Fig. 1). Although the density in the treatment plot before the mixing of B. bassiana was also approximately $1 \times 10^5$ CFU/g, it increased markedly to $3\sim5 \times 10^7$ CFU/g after the mixing. The density gradually decreased as time proceeded, and became $2 \times 10^6$ after 365 d, which was still ten times higher than that of the non-treatment plot. Four hundred fifty-six days after the mixing, the density of general fungi decreased to 2.8% of that at the time of the mixing, however,
densities of general fungi in the treatment plot were always greater than that in the non-treatment plot \((p<0.01\) for both the treatment and time×the treatment interaction).

Density of \(B.\ bassiana\) in the non-treatment plot was around \(1\times10^2\) CFU/g during the investigation (Fig. 1). This indicates the natural distribution of wild \(B.\ bassiana\) at these sites. On the other hand, the density of \(B.\ bassiana\) in the treatment plot increased markedly from \(1\times10^2\) to \(3-5\times10^7\) CFU/g after the mixing of this fungus. It gradually decreased to less than \(10^6\) CFU/g after 1 year, which was 3.5% of the density at the time of mixing. Finally, 456 d after mixing, the \(B.\ bassiana\) density became 0.04% of that at the moment of mixing. However, the \(B.\ bassiana\) densities were always greater than those in the non-treatment plot \((p<0.01\) for both the treatment and time×the treatment interaction), and 80 times as large as that in the non-treatment plot.

Germination of \(Beauveria bassiana\) conidia

Some germinating conidia of \(B.\ bassiana\) were seen after 3 d of moistening conidia with various materials, even though they did not come in direct contact with any culture medium (Fig. 2). In the figure, the ratios of three replicates were averaged. Notably, when conidia were in direct contact with moistened filter paper, germination rates reached 34%, and if inflated conidia were included, germination was approximately 90%. Some conidia placed in the moistened air or on water agar also inflated and germinated. However, those buried in non-sterilized soil inflated only a little, and none produced germ tubes.

In sterilized soil, some conidia germinated and more than half were inflated after even 1 d (Fig. 3). In the figure, rates of three replicates were averaged. The germination rate increased as time proceeded, and more than 60% of conidia had germinated by day 4. When including the inflated conid-
ia, this was more than 97% on day 4. Secondary conidia were produced on the germ tubes afterward and those were not distinguishable from the initial conidia in appearance; therefore, the germination rate in the sterilized soil on day 7 was lower than that on day 2 or day 4. On the other hand, germinated conidia were never seen in the non-sterilized soil, and less than 10% of conidia in non-sterilized soil ever became inflated (Figs. 3 and 4). This phenomenon of inactivity in non-sterilized soil continued at least 7 d after the burial.

**DISCUSSION**

Densities of bacteria and actinomycetes in *B. bassiana* treatment and non-treatment plots showed the same temporal pattern. These results indicate that *B. bassiana* introduced into soil does not affect densities of bacteria and actinomycetes. On the other hand, the densities of general fungi were greatly increased by mixing *B. bassiana*. Densities of general fungi in the treatment plot before mixing and in the non-treatment plot were $1 \times 10^5$ CFU/g, while the number of *B. bassiana* conidia mixed into soil was $1.4 \times 10^{12}$ per 0.3 m$^3$ (1 m$\times$1 m, 0.3 m deep). These units are in different dimensions and cannot be compared directly. However, the number of *B. bassiana* mixed was much greater than the number of general fungi recovered. Therefore, the density of general fungi after mixing was thought to be composed principally of *B. bassiana*, because *B. bassiana* grows normally on Martin’s medium, the selective medium for fungi. In other words, it can be said that fungi other than *B. bassiana* were masked by *B. bassiana* and could not be counted in the treatment plot after mixing this fungus. A selective medium which inhibits only *B. bassiana* and does not affect the other fungi would be necessary to clarify the effect of *B. bassiana* on the other soil fungi. However, we know of no such medium.

Since we hypothesize that the density of general fungi is principally composed of the *B. bassiana* that was added to the soil, the densities of general fungi and *B. bassiana* were almost the same until 11 months after mixing. However, the densities of *B. bassiana* at 12 and 15 months were less than those of general fungi. Most of the isolated fungi on Martin’s medium for general fungi were *B. bassiana*. In agreement, the density of *B. bassiana* counted on Martin’s medium was greater than that on D0C2 medium. As discussed later, when *B. bassiana* conidia were introduced to non-sterilized soil, they were thought to remain in the conidial state and not germinate. Therefore, we hypothesize that *B. bassiana* conidia may survive in a conidial state and maintain their ability to grow on Martin’s medium, but lose their tolerance against D0C2 medium around 12 months.

Huber (1958) stated *B. bassiana* conidia did not germinate when mixed in fresh soil, although they germinated in sterilized soil. Our observations revealed the same phenomenon. In sterilized soil, *B. bassiana* conidia germinated and produced secondary conidia, while in non-sterilized soil, they did...
not germinate. Since *B. bassiana* conidia could germinate when they were kept with moistened filter paper, moistened air and water agar, they seem to be able to germinate under some moistened conditions without nutrients. Thus, the non-sterilized soil appears to inhibit germination of *B. bassiana*. Clerk (1969) discovered an inhibitory effect of soil extracts on germination of *B. bassiana* conidia. This effect was reduced by autoclaving and by filtering the extract. As an explanation for this phenomenon, he suggested the association with bacteria and other microorganisms in the soil. Lingg and Donaldson (1981) considered temperature and soil moisture content were important for the survival of *B. bassiana*. Kögler and Zimmerman (1986) mixed *B. bassiana* into soils of pine forests. In this sense, *B. bassiana* has often been isolated from soil in the present study, it was almost always isolated from soil samples in the non-treatment pine forests. In this sense, *B. bassiana* may be called a soil borne fungus. However, its behavior in soil was exceedingly different from that of *M. anisopliae*. Conidia of *B. bassiana* introduced into soil did not germinate in the field, and are thought to remain in the soil as conidia. Müller-Kögler and Zimmerman (1986) mixed *B. bassiana* conidia in soil at a rate of $10^6$ to $10^7/g$, and found that the number of conidia was reduced by 1/100 to 1/1,000 one year later. This reduction rate was especially rapid after 9 months. In our results, conidia of *B. bassiana* mixed into soils of pine forests also decreased gradually, and few remained 456 d after the mixing. This suggested that the introduced conidia did not germinate in the field and died a natural death as imgerminated conidia. While conidia are inert in soil, their metabolic activity is thought to be very low. Therefore, mixing *B. bassiana* conidia into soil does not seem to affect the other fungi, although densities of fungi other than *B. bassiana* could not be measured in the experiment. Since we could not exactly measure the densities of general fungi without growth of *B. bassiana*, we can only speculate the effect of *B. bassiana* on other fungi by observing *B. bassiana* conidia in soil.

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