Entomopathogenic Characterization of *Beauveria bassiana* fungi against *Tetranychus kanzawai* (Kishida) (Tetranychidae: Acarina) Spider Mite by Its Region

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Abstract

The characteristics of six *Beauveria bassiana* isolates against *Tetranychus kanzawai* were investigated. The investigated aspects were *B. bassiana* isolates': (1) pathogenicity to *T. kanzawai*, (2) cellular characteristics, (3) infection process (4) phylogeny. The isolates were planted onto potato dextrose agar (PDA) in flat bottle flasks and incubated at certain room condition, i.e. 26±1°C temperature and 12-hour photoperiod, for a maximum of 21 days. The results showed that isolates’ phatogenicity level decreased in the following order: Bb6>Bb5>Bb4>Bb3>Bb1>Bb2. The surface of Bb4 and Bb5 isolates were yellowish-white, while Bb6 had a white color. The bottom of Bb4 had brown and white colors while both Bb5 and Bb6, on the other hand, had yellow color. All isolates had round shapes. Bb6 had the fastest sporulation growth of 3 days post inoculation while others grew approximately at 4 days post inoculation. The ITS region examination showed that three *B. bassiana* isolates were related, in which Bb4 and Bb5 (from Indonesia) were closely related and Bb6 (from Philippines) was distantly related.

Keywords: *Beauveria bassiana*, selection, cellular, infection, phylogeni characteristics of *T. kanzawai*, greenhouse

Introduction

Entomopathogenic fungi were among the first organisms to be used for the biological control of pests. More than 700 species of fungi from around 90 genera are pathogenic to insects. Most are found within the Class Deuteromycetes and Order Entomophorales (Zygomycetes). Some entomopathogenic fungi such as *Aschersonia aleyrodidis* (Webber) have restricted host ranges and infect only scale insects and whiteflies. Other fungal species within Hypomycetes have a wider host range, with individual isolates being more specific, such as *Metarhizium anisopliae* (Metschnikoff) Sorokin and *Beauveria bassiana* (Balsamo) Vuillemin. Numerous studies have been made on these fungal isolates which have been well characterized with respect to pathogenicity to several species of insects.

These generalist fungi have also been observed on mites and can be developed as potential biocontrol agents against major mite pests of agricultural crops (Chandler 1997) such as the spider mite, *Tetranychos urticae*.

Like other organisms, Acari may also be subjected to diseases usually caused by pathogenic microorganisms. Knowledge on pathogens of mites, however, is still fragmentary, in contrast to what is known about pathogens of insects. The rapid development of invertebrate pathology in the second half of the twentieth century has largely been due to the study of insect pathogens. Relatively few mite pathogens are known, despite the large number of
mite species. Approximately 48,000 species have hitherto been described and it is estimated that this number represents only 10% of the total number of mite species.

It is not surprising that mite pathogens have been studied less comprehensively because of their frequently small size which renders disease diagnosis often difficult and pathophysiological studies almost impossible. On the other hand, the often large reproductive potentials of mites make many acarine species ideal model organisms for detailed epidemiological investigations. Pathogens in populations of mite species often play a major role in the regulation of population size and population density in natural habitats. In several instances, populations of pest species in agricultural systems are kept below the economic threshold level by the occurrence of a disease. Such observations often provide impetus for further studies on the pathogen involved that may eventually lead to the successful application of a pathogen for the biological control of mite pest. In addition, the occurrence of disease may be undesirable, e.g., in the case of cultures of beneficial mites: several cases have been reported on the occurrence of disease in mass cultures of predatory mites. In many instances such observations have led to comprehensive studies on the mite pathogen involved. Interest in pathogens of mites is increasing. Van der Geest (2002) presents a survey of pathogenic fungi infecting plant-inhabiting mites.

Mites obtain their food in general by inserting their mouth parts into the integument of the host or plant tissue. It is therefore unlikely that mites would contract disease through the alimentary tract unless the vertebrate host or food plant is infected by a pathogen. As penetration through the mouth parts is hampered, the pathogen should follow other infection routes. A plausible infection route is through the integument. Fungi, in general, are capable to penetrate a mite (or other arthropod species) through the integument. Hence, it is not surprising that most mite pathogens are found among the fungi.

One of the earliest experiments in which a fungus was tested against a phytophagous mite was a field application of *B. bassiana* spores for the control of the two spotted spider mite *T. urticae* (Van der Geest, 2002). In this experiment conducted some *B. bassiana* from Indonesia and Philippines. Several methods have been used to describe the variation within a species of entomopathogenic and mycoparasitic fungi by morphological characteristic and Molecular identification techniques. (Samson, 1981) are needed in addition to the traditional morphological characteristics formally used to classify fungal species.

The RAPD characterization would be useful for proprietary reasons when fungal isolates are introduced into new ecosystems for pest control. It ensures a mean of detecting the introduced pathogen.

**Materials and Methods**

**Mass Rearing of the Red Spider Mites, *Tetranychus kanzawai***

**Maintenance of host plants**

Papaya fruits of the solo variety were bought from the public market. The seeds were collected, dried, planted in nursery plots using sterilized soil, fertilized using urea, and watered everyday. After the plants have reached 5 cm in height, they were transferred to plastic pots which contained 2 parts soil and 1 part cattle manure. Once the papaya plants in plastic containers reached 10 cm high, they were used as host plants for rearing mites.

**Rearing of *Tetranychus kanzawai***

A number of female adults of *T. kanzawai*, aged 24-36 hours after emergence from deutonymph stage, collected from papaya plants in the Institute of Plant Breeding were introduced into the papaya plants at the CPC greenhouse. The papaya plants were watered regularly and were also observed daily for growth of population of mites. Heavily damaged papaya plants were replaced with new healthy plants. Adult mites from previously reared generations were maintained and used for different experiment assays. Papaya leaves containing mites were transferred continuously to other papaya seedlings.

**Test fungi**

Fourteen isolates (Table 1) from the Philippines and Indonesia comprising six *Beauveria bassiana*,
isolates were evaluated in the present study. The isolates were plated onto potato dextrose agar (PDA) in flat bottle flasks and incubated at room condition at 26±1°C and a 12-hour photophase for a maximum of 21 days. The cycle was repeated for inoculation. The isolates were then stored in glass vials containing PDA culture medium.

Bioassay Tests for Virulence

Laboratory screening

Ten adult female mites, aged 24 to 36 hours after emergence from the stock colony were transferred on to abaxial surface of a 1.5 cm leaf disc in 2.5 cm petri dish with a brush. The females were classified visually by observation of the shape of the opisthosoma, which is round in females and funnel-shaped opisthosoma in males.

A series of 5 dilutions were prepared for each of the 14 fungal suspensions at 10⁴, 10⁵, 10⁶, 10⁷, and 10⁸ conidia mL⁻¹ concentrations. PBS served as control. These suspensions were transferred into a plastic sprayer. Spraying was done first on the control mites using 0.5 mL of suspension and then followed by the fungal suspensions starting with the lowest up to the highest concentration. Six trials were conducted using 10 mites per concentration, with 3 replications. Percent mortality was observed at 5 days post infection (DPI).

Morphological characterization of fungi

The observations covered color, mycelia structure, colony shape, growth rate, number of sporulation. Furthermore shape, conidia, and conidiophores were observed by taking a small amount of colony under binocular microscope and microcamera under 1000 x magnification.

Scanning Electron Microscope (SEM)

Specimens of four-day old infected female adult mites were examined by SEM. Infected mites were immersed overnight in 2.5 % glutaraldehyde in PBS, pH 7.0 and washed 3 times with PBS at 10 minute intervals for each wash. Washed samples were immersed in 1% osmium tetroxide (diluted in PBS) for 30 to 45 min. The osmium tetroxide treatment was followed by 3 washes of sterile water at 10-minute interval per wash and dehydrated at room temperature in a graded series of 25 %, 50%, 75% and 95% ethanol with 30-minute interval for each step. The final step was followed by 3 changes of absolute grade ethanol, with two changes at 30 minute interval and overnight for the last 100% ethanol change. After dehydration, the samples were transferred into mesh microcontainers flooded with 100% ethanol for critical point drying. Critical point drying was done for 45 minutes. The dried samples were mounted onto pin stubs with double-sided tape in different orientations and spitter coated with gold coating. Samples were examined and images were taken using Hitachi variable pressure SEM in high vacuum mode. The process of adhesion, germination and colonization was observed by light microscope and microcamera under 1000 x magnification from a small amount of colony taken from the flask.

Molecular Characterization

DNA

For each isolate grown on PDA, 10 mL of 0.75% PBS were added after which, incubated on shaker (150 rpm) at 37°C for 4 days. DNA extractions were done following the Jena
**Bioscience process**

One mL of PDB culture was transferred to 1.5 mL microtube. This was centrifuged at 15,000 g for 1 min after which the pellets were harvested. The cell pellets were resuspended in 300 µL Cell Resuspend Solution. 1.5 µL proteinase k solution was added and mixed by inverting several times. The solution was incubated at 55°C for 60 min and centrifuged again at 15,000 g for 1 min. The cell pellets were harvested and resuspended in 300 µL, at this time, cell lysis solution was formed.

Cell lysate solution added with protein precipitation solution was vortexed for 20 sec and centrifuged at 15,000 g for 3 min resulting to a tight pellet precipitated protein.

The supernatant was transferred to a clean 1.5 mL microtube containing 300 µL of isopropanol (>99%) mixed by inverting gently for 50 times. This was centrifuged at 15,000 g for 1 min. The DNA became visible as a pellet with white to light green color. The supernatant had to be discarded by draining the tube briefly on clean absorbent paper. DNA pellet was washed in 300 µL of 80% ethanol by inverting the tube several times then centrifuged at 15,000 g for 1 min and disposing ethanol. The DNA pellet was air-dried at room temperature for 10-15 minutes, once dried 75 µL DNA solution was added to the DNA pellet followed by 1.5 µL of RNase A. The sample was mixed by inverting the tube and incubated at 37°C for 60 min. Samples were incubated at 65°C for 60 min to hydrate DNA. The collected genome DNA was stored at 4°C.

**Random Amplified Polymorphic DNA Amplification**

The Internal Transcribe Spacer (ITS) region for 5.8S ribosomal DNA (rDNA) of *Beauveria* was amplified using four different types of primers. The pairing of the primer sets was based on the ITS region for 5.8s r DNA of the common fungi of *Beauveria bassiana* (ITS1-ITS2).

Amplification reactions were performed at a total volume of 20 ul consisting of 10 µL master mix, 6 µL ultra fresh sterile water, 2 µL DNA template, 1 µL primer a, and 1 µL primer b. The temperature was set using thermal cycling with an initial denaturation at 95°C for 5 min, followed by 35 cycles with denaturation at 94°C set for 1 min and 30 sec, annealing at 55°C for 2 min and extended at 72°C for 3 min and a final extension at 72°C for 5 min.

PCR product was separated by electrophoresis in 1.2% agarose gel mixed with cyber safe. Marker was pipetted with 50 bp ladder 10 µL. The PCR product was pipetted and mixed with DNA 10 µL and 2 µL loading dye. Electrophoresis was conducted at 450 mV, then at 400 mA for 45 min.

**DNA Sequencing**

A 15 µL amplified DNA sequence of the sample gene was placed on a 0.5 mL microtube for sequencing at Macrogen South Korea.

**Analysis of RAPD Profiles**

RAPD results were compared with RAPD amplification profiles by looking at the presence or absence of each band in each profile. Sequence homologous alignment and similarity were carried out using Blast Biological Software and multi-aligned with CLUSTALW Biological Software. Sequences were compared with published sequences of known fungi on the NCBI Gene Bank Database.

**Results and Discussion**

**Selection of the Most Pathogenic Isolates**

The pathogenicity of the three entomopathogenic fungi of the seven isolates from Philippines and Indonesia against the mite differed among each other. All isolates from *B. bassiana* and *P. lilacinus* infected the mite and sporulated outside its body. They demonstrated rapid external hyphal development and sporulation under moist condition. Initially, hyphal strands emerged from the anal region of the mite cadaver and then quickly covered the cadaver with profused hyphal growth followed by sporulation within 4 to 6 days. *B. bassiana* showed a whitish colour.

Six trials were conducted to determine the most pathogenic isolates for further characterization. Morbid mites observed at 5 days post infection (DPI) are presented in Table 2. In general, the five concentrations caused 50 to 100% mortality. Among the six *B. bassiana* isolates, the three most pathogenic isolates were Bb4, Bb5 and Bb6 (Table 2).
Table 2 LC₅₀ values (conidia/ml) of 6 entomopathogenic fungi at 5 days after application on T. kanzawai adult females

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Color Top</th>
<th>Color Bottom</th>
<th>Mycelia Structure</th>
<th>Colony Shape</th>
<th>Growth rate (mm)</th>
<th>Size (mm)</th>
<th>No. of days to sporulation</th>
<th>Shape of conidia</th>
<th>Size of conidia (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bb1</td>
<td>White</td>
<td>Brownish white</td>
<td>Thick and adpressed</td>
<td>Round</td>
<td>0.15</td>
<td>16</td>
<td>4</td>
<td>Globose</td>
<td>2 x 2.5</td>
</tr>
<tr>
<td>Bb2</td>
<td>White</td>
<td>Yellowish White</td>
<td>Thick and adpressed</td>
<td>Round</td>
<td>0.16</td>
<td>17</td>
<td>4</td>
<td>Sub-globose</td>
<td>2 x 3</td>
</tr>
<tr>
<td>Bb3</td>
<td>White</td>
<td>White</td>
<td>Thick and adpressed</td>
<td>Round</td>
<td>0.21</td>
<td>22</td>
<td>3</td>
<td>Globose</td>
<td>2 x 1.5</td>
</tr>
</tbody>
</table>

With regards to the performance variability of the different isolates, Sosa Gómez and Alves (1983) reported a high enzymatic activity in more virulent isolates of M. anisopliae from several Brazilian regions, and suggested that they are probably associated with the presence of enzymes that influence the penetration process of the fungus (St Leger et al., 1988; De La Rosa et al., 1997), as well as with toxins such as destruxins and beauvericin, present in M. anisopliae and B. bassiana respectively, which vary in different isolates (Roberts and St. Leger 2004). However, unlike insecticides, fungal infection takes 4-6 days after application to kill a mite. During this time the infected mite can cause serious damage to the crops (St. Leger et al., 1996).

In the study of Tamai (1997) on the mite T. urticae using Beauveria spp. isolates at a concentration of 5×10⁸ conidia mL⁻¹, mortality ranged from 5.5 to 100% in total. Similarly, Oliveira et al. (2002), working with B. bassiana isolates at 10⁸ conidia mL⁻¹ and the red mite Oligonychus yothersi (McGregor), recorded 77 to 98% mortality. On the other hand isolates of M. anisopliae, causes 12.0 to 45.0%, and LT₅₀ of 8.6 to 18.4 days.

Morphological Characterization

The description and characteristics of the colony and mycelia of the three fungal isolates on PDA medium are presented in Table 3.

The surface of B. bassiana isolates for Bb4 and Bb5 were yellowish white, while Bb6 had a white color. The bottom of Bb4 had brown and white colors while both Bb5 and Bb6, on the other hand, had yellow color. All isolates in colony had round shapes. Bb6 had a faster sporulation growth at 3 days post inoculation while others grew approximately at 4 days post inoculation (Table 3).

All three Beauveria species had white mycelia which turned to creamy as they mature. Bb4 and Bb5 had a velvety colony, while Bb6 had a floccose colony. The three isolates had aerial mycelium with hyaline and smooth wool-bearing conidiophores in dense, short and globose with apical denticulate rachi, giving a prominent zigzag appearance. Conidia of Bb4 and Bb6 were smooth and globose while Bb5 had broadly ellipsoidal shape (Figure 2 and 3).

Table 3 Cultural characteristic of the selected entomopathogenic fungi virulent to Tetranychus kanzawai.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Top</th>
<th>Bottom</th>
<th>Mycelia Structure</th>
<th>Colony Shape</th>
<th>Growth rate (mm)</th>
<th>Size (mm)</th>
<th>No. of days to sporulation</th>
<th>Shape of conidia</th>
<th>Size of conidia (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bb4</td>
<td>White</td>
<td>Brownish white</td>
<td>Thick and adpressed</td>
<td>Round</td>
<td>0.15</td>
<td>16</td>
<td>4</td>
<td>Globose</td>
<td>2 x 2.5</td>
</tr>
<tr>
<td>Bb5</td>
<td>White</td>
<td>Yellowish White</td>
<td>Thick and adpressed</td>
<td>Round</td>
<td>0.16</td>
<td>17</td>
<td>4</td>
<td>Sub-globose</td>
<td>2 x 3</td>
</tr>
<tr>
<td>Bb6</td>
<td>White</td>
<td>White</td>
<td>Thick and adpressed</td>
<td>Round</td>
<td>0.21</td>
<td>22</td>
<td>3</td>
<td>Globose</td>
<td>2 x 1.5</td>
</tr>
</tbody>
</table>
Infection of Beauveria bassiana to Tetranychus kanzawai

There are different characterization for the three isolates of *B. bassiana* to attack *T. kanzawai*. After application of *B. bassiana* conidia growth were found on the mite gnathosoma and anal regions, but some conidia were also saw on the leg. Germination tubes on the mite’s cuticle was growth observed during the penetration process (Figure 3b). It assumed due to production of enzymes by on the infection process.

The mites killed by *B. bassiana* had a white coloration which is a characteristic of oosporein activity common in insects infected by this fungus. It seems Bb6 was the most potential since had colonization on 24 hours.

Molecular Characterization

The development of PCR and the design of primers for the amplification of the various rDNA regions have considerably facilitated taxonomic studies of fungi. ITS sequences are generally constant, or show little variation within species but vary between species in a particular genus.

A PCR product of approximately 200 bp was obtained from five entomopathogenic isolates using...
Figure 2  Scanning electron micrograph of *Beauveria bassiana* Bb6 isolate infecting *Tetranychus kanzawai* at 4 days infection (1000 x).

Figure 3  Morphological characteristics of three *Beauveria bassiana* isolates. The conidia of (a) Bb4 (b), Conidiophore of Bb5 and (c) Conidiophore of Bb6 were observed under a scanning electron microscopy. The magnification and the scale bars are indicated at the lower part of the figures.
the ITS1-ITS2 primer pair. All the sizes of PCR products can be amplified with the primer pairs ITS1, ITS2, and the 5.8s rDNA gene and also with 50 bp of the 3' end of the 18s rDNA and 50 bp of the 5' end of the 28s rDNA.

Phylogram

The results of ITS sequencing suggest that the three isolates were very closely related. A very high homology in DNA sequence of Bb4, Bb5, and Bb6 was observed when compared with other B. bassiana kept in the GenBank.

Bb4, Bb5, Bb6 aligned with blast sequencing with B. bassiana in GenBank showed high similarity. Phylogenetic sequence analysis using different Beauveria species showed a close relationship of Bb4, Bb5 and Bb6 with B. bassiana ITS1 (Table 4).

The analyses indicated some intraspecific variations among B. bassiana isolates and clustering associated with geographical origin, even though Bb5 isolates and Bb 6 had closer similarity. Hence the isolates may be from casual associations rather than clear association between B. bassiana genotypes adapted to the pest. In observation of the clustering to pathogenenicity, Bb5 and Bb6 which caused higher mortality than Bb4 were placed in the same cluster.

Conclusions

All of the selected entomopathogenic isolates of T. kanzawai completed conidiogenesis at 96 hours post infection, indicating that these isolates are virulent to the test mite. All three Beauveria species had white mycelia which turned to creamy as they mature. Bb4 and Bb5 had a velvety colony, while Bb6 had a floccose colony. Three species had aerial mycelium with hyaline and smooth wool-bearing conidiophores in dense, short and globose with apical denticulate rachi, giving a prominent zigzag appearance. Conidia of Bb4 and Bb6 were smooth and globose while Bb5 had broadly ellipsoidal shape. Morphological and ITS-5.8s DNA characteristics confirmed that Bb4, Bb5 and Bb 6 were Beauveria bassiana.

Table 4 ITS DNA sequence homology of Beauveria bassiana Bb4, Bb5 and Bb6 isolates compared with other B. bassiana ITS sequences submitted in the GenBank.

<table>
<thead>
<tr>
<th>Fungi species</th>
<th>Base pair length</th>
<th>Description</th>
<th>% Score similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beauveria bassiana Isolate Bb5172</td>
<td>416</td>
<td>18S rRNA gene, partial sequence; ITS 1, 5.8S rRNA gene, and ITS 2, complete sequence; and 28S rRNA gene, partial sequence</td>
<td>49 52 59</td>
</tr>
<tr>
<td>Beauveria bassiana isolate 9 germana1</td>
<td>523</td>
<td>18S rRNA gene, partial sequence; ITS 1, 5.8S rRNA gene, and ITS 2, complete sequence; and 28S rRNA gene, partial sequence</td>
<td>45 48 55</td>
</tr>
<tr>
<td>Beauveria bassiana Strain b10</td>
<td>649</td>
<td>18S rRNA gene, partial sequence; ITS 1, 5.8S rRNA gene, and ITS 2, complete sequence; and 28S rRNA gene, partial sequence</td>
<td>49 52 59</td>
</tr>
<tr>
<td>Beauveria bassiana ITS 1</td>
<td>2073</td>
<td>18S rRNA gene, partial sequence; ITS 1, 5.8S rRNA gene, and ITS 2, complete sequence; and 28S rRNA gene, partial sequence</td>
<td>78 78 81</td>
</tr>
<tr>
<td>Beauveria sp Strain SJ2010</td>
<td>461</td>
<td>18S rRNA gene, partial sequence; ITS 1, 5.8S rRNA gene, and ITS 2, complete sequence; and 28S rRNA gene, partial sequence</td>
<td>54 49 51</td>
</tr>
<tr>
<td>Beauveria brongniartii MRCIF</td>
<td>438</td>
<td>18S rRNA gene, partial sequence; ITS 1, 5.8S rRNA gene, and ITS 2, complete sequence; and 28S rRNA gene, partial sequence</td>
<td>55 52 51</td>
</tr>
<tr>
<td>Beauveria brongniartii IMBST 13</td>
<td>499</td>
<td>18S rRNA gene, partial sequence; ITS 1, 5.8S rRNA gene, and ITS 2, complete sequence; and 28S rRNA gene, partial sequence</td>
<td>50 49 54</td>
</tr>
<tr>
<td>Beauveria brongniartii small sub unit ribosomal RNA</td>
<td>559</td>
<td>18S rRNA gene, partial sequence; ITS 1, 5.8S rRNA gene, and ITS 2, complete sequence; and 28S rRNA gene, partial sequence</td>
<td>45 48 50</td>
</tr>
</tbody>
</table>
Figure 4 Rooted phylogenetic tree of ITS-5.8s rDNA region of *Beauveria bassiana* samples, Bb4, Bb5, Bb6 compared to *Beauveria* sp, *B. brongniartii* and *B. bassiana*.

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**References**


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