Mutation of Beauveria Bassiana Using Low-energy N^+ Implantation and Selection of a High Virulence Strain

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Abstract: Low-energy ion implantation technology was used to generate mutants of the high virulence *Beauveria bassiana* strain BbIII and bioassays were carried out on *Biston marginata*. The survival rate curve of *B. bassiana* to doses of N^+ showed a "saddle shape." The peak value 15×10^{14} ions·cm^{-2} was the optimal dose for mutagenesis. The growth characteristics of *B. bassiana*, such as colony morphology, sporulation, and spore germination rate, were affected by N^+ implantation. Three stains of *B. bassiana* were selected based on sporulation, chitinase, and Pr1 enzyme activity as the screening indices. The activities of chitinase and Pr1 enzyme in Bb III 22 were 0.230 and 0.137 (OD value), and these were almost twice that of the original strain. The Bb III 22 strain was the most virulent to *B. marginata* and the mortality was > 86.7% at a concentration of 10^7 spores·mL^{-1}. The log LD_{50} of the mutant strain spores against *B. marginata* was 5.1951 (4.5174~5.8416). The results indicate that low-energy N^+ implantation and mutation can be effective for increasing the virulence of *Beauveria bassiana*.

1 INTRODUCTION

Beauveria bassiana (Bals.) Vuillemin is an entomopathogenic fungus, many strains of which have been formulated for arthropod pest control (Castrillo *et al.*, 2003). Excessive use of chemical pesticides has increased the "3R (Resistance, Resurgengce, Residue)" problem. Alternative biocontrol agents, such as *B. bassiana*, remain effective for pest control and more attention should be given to their production and application (Li *et al.*, 2006; Fernandes *et al.*, 2008; Li *et al.*, 2009). Low-energy ion implantation is a mutation breeding technique invented in China (Yuan and Yu, 2003). Ion beams produce energy deposition, momentum transfer, and strong ionization in the target organisms. Indirect injury is produced by highly-reactive radicals. Low-energy ion implantation is an efficient, safe, and pollution-free mutagenic method. It has the advantages of a broad mutation spectrum, light ancillary damage, and induction of stable mutations.

Low-energy ion implantation has been used in rice, wheat, cotton, and tomatoes (Dai *et al*., 2007; Xin *et al*., 2007; Yu *et al*., 2007). The mutagenic effects of ion implantation Streptomyces are recognized, and it is now widely used in microbial selection (Li *et al*., 2011; Yuan *et al*., 2003; Song *et al*., 2004; Zhu *et al*., 2006). The avila neomycin-producing strain of Streptomyces was mutagenized by low-energy N^+ implantation and the resulting mutants had yields that were 41.4% to 47.2% greater than the parent strain (Zhu *et al*., 2006). However, the application of N^+ implantation in *B. biassana* has not been reported.

Since mutagenesis methods generate random mutations, it is difficult to target mutations to produce desired results. These methods require considerable screening work and this reduces the efficiency of mutation-based selection. Sporulation, Pr1 protease, and chitinase activity appear to be associated with microbial virulence (Peng *et al*., 2000; Feng, 1998). Use of these indicators as secondary screening methods for mutation breeding can improve screening efficiency. We studied the mutagenic effects of low-energy N^+ implantation on *B. bassiana* to determine the optimal mutagen dose. Several

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mutagenized strains were then selected for their higher virulence.

Camellia oleifera Abel (from Theaceae family) originated from China. It occurs in 18 provinces or municipalities in southern China, especially those south of Hunan and Guangxi Provinces. Tea oil (*Camellia oleifera* oil) is a high-quality edible oil (Lee *et al*., 2007) containing oleic acid, unsaturated fatty acids, and monounsaturated fatty acids. It is one of the four primary edible tree oils (Zhang *et al*., 2007). More than 100 insects and mites attack the roots, stems, leaves, and buds of *Camellia oleifera* in China. Of these, *Biston marginata* Shiraki (Lepidoptera: Geometridae) is a key pest that causes significant damage to tea oil plantations (Deng *et al*., 2013). The larvae feed mainly on tender leaves, which they skeletonize. *Beauveria bassiana* is an important biological control agent used in Integrated Pest Management systems because it is efficient, easy to apply, economical, and effective against many insect pests. We studied the the mutagenic effect of low-energy nitrogen ion implantation on *B. bassiana* and determined the best mutagenic dose. Our goal was to select a *B. bassiana* strain with superior virulence to pests of *Camellia oleifera*, specifically *B. marginata*, and to provide guidance for the effective use of this strain.

2 MATERIALS AND METHODS

2.1 Materials

B. bassiana Bb III was obtained from the Culture Preservation Center (Central South University of Forestry &Technology). SDY (Sabouraud Dextrose Agar with Yeast Extract) medium consisted of 10 $g \cdot L^{-1}$ peptone, 40 $g \cdot L^{-1}$ glucose, and 10 $g \cdot L^{-1}$ yeast extract. Chantui induction medium had 2 $g \cdot L^{-1}$ chantui powder (diameter<0.02), 0.2 $g \cdot L^{-1}$ KH_2PO_4, and 0.2 $g \cdot L^{-1}$ $MgSO_4$. Acetate buffer was made with 0.1 $mol \cdot L^{-1}$ sodium acetate (pH 5.0). Boric acid KOH buffer was composed of 0.8 $mol \cdot L^{-1}$ boric acid, adjusting the pH to 10.0 with KOH. DMAB solution, 1.0 g DMBA (P-dimethylaminobenzaldehyde), was dissolved in 90 mL of glacial acetic acid and 10 mL of hydrochloric acid. Suc-$(Ala)_2$-Pro-Phe-pNA, Tween-80 and Tris were purchased from Sigma-Aldrich Co. LLC, and the others were AR grade reagents made in China.

2.2 Methods

2.2.1 Preparation of Spore Suspension

The BbIII strain was added to a 9-cm Petri dish containing about 10 mL SDY medium and incubated at 25°C and relative humidity 69% for 14 d resulting in a large number of spores. the dish was then washed with 0.1% Tween-80 and the mycelium filtered by sterile cotton. The spore concentration was adjusted to approximately 10^6 $spores \cdot mL^{-1}$.

2.2.2 Low-energy N+ Implantation

The mutagenesis experiments were conducted using a LZD-1000 ion implanter at the Forest Cultivation Laboratory of Central South University of Forestry &Technology in Changsha, China. We added a uniform coating of 1 mL of the spore suspension to sterile Petri dishes (9 cm) for ion implantation. The target chamber vacuum was adjusted to 10^{-3} Pa and the ion implantation energy was 30 keV. We tested 9 doses: 3×10^{14}, 6×10^{14}, 90×10^{13}, 12×10^{14}, 15×10^{14}, 18×10^{14}, 21×10^{14}, 24×10^{14}, and 27×10^{14} ($ions \cdot cm^{-2}$). Non-irradiated Petri dishes in the target room were the controls (CK). The velum was washed with 1 mL sterile water after mutagenesis, coated uniformity in Petri dishes of SDY medium, incubated at 25°C, and counted after 5 d. All of the treatments were repeated three times, and a graph was made with the spore survival rate as the ordinate and implanting dose as the abscissa.

2.2.3 Determination of Sporulation, Survival Rate, and the Morphological Attributes of Mutant Strains

The Bb III strain was mutagenized by N^+ implantation and then washed down with 1 mL sterile water. The single-spore isolation method was performed based on the method of Zhang (Zhang and Zhang, 2009). We took the uniform 100 μL coating of bacterial suspension exposed to radiation in the SDY Petri dishes, incubated it at 25°C for 36 h, and picked a single conidia under a dissecting microscope. We continued to culture the selected stains and those with high levels of sporulation were transferred to SDA Petri dishes and saved. Details of colony morphology were recorded.

Mutant strains were inoculated into 50-mL flasks with SDY medium after activation by SDY tablet, and incubated at 25°C, 160 $r \cdot min^{-1}$ for 3 d. A 3-mL sample of these cultures was inoculated into a 9-cm

SDY Petri dish and incubated at 25°C for 15 d. Then, colony three holes (0.9-cm diam) were punched in the colony and placed in 0.1% Tween-80 and faltered with a high-speed disperser. Determination of sporulation was made with a hemocytometer Determination of spore survival rate was performed using the method of Hong (Hong *et al*., 2001).

2.2.4 Enzyme Induction and Activity Assay

A loopful of slant culture was inoculated into a 50-mL flask with 15 mL SDY medium and incubated at 25°C, 160 r·min^{-1} for 3 d. A 0.2-mL portion of the above seed cultures was inoculated into a 50-mL flask with 15 mL Chantui induction medium and cultured at 25°C, 160 r·min^{-1} for 96 h. The supernatant was centrifuged 14 000 r·min^{-1} for 5 min for three times and determined the activity of Pr1 protease.

Determination the activity of Pr1 protease was performed using the method of St. Leger (St Leger *et al*., 1987). A 30-μL of 0.04 mol·L^{-1} Tris-HCl buffer was added to 10 μL of the above supernatant and 10 μL of 1 mg·mL^{-1} Suc-(Ala)$_2$-Pro-Phe-pNA. The response at 28°C for 10 min and the activity of Pr1 enzyme were measured as the optical density at 405 nm using a UV spectrophotometer (UNICO Instruments Co., Ltd, China).

Preparation of colloidal chitin was performed using the method of Hsu (Hsu and Lookwood, 1975). Determination of chitinase enzyme activity was performed using the method of Mauch (Mauch *et al*., 1984), with slight modifications. A 100-μL sample of the above supernatant cultures was combined with 100 μL colloidal chitin and 350 μL acetate buffer, placed in a 28°C water bath for 2 h, and then centrifuged for 5 min at 1,000 r·min^{-1}. A 300-μL portion of the above supernatant was combined with 100 μL boric acid KOH buffer, and then mixed with 2.5 mL DMAB after 3 min of boiling water, followed by exposure to a 40°C water bath for 20 min. The activity of the chitinase enzyme was measured as the optical density at 540 nm using a UV spectrophotometer.

2.2.5 Toxicity Test of the Mutants against B. Marginata

We adjusted the spore concentration to 10^6, 10^7, and 10^8 spores·mL^{-1} with 0.1% Tween 80. The larvae that had been treated with the mutant strains at different doses were placed into glass vials (61 mm diameter × 87 mm height) with 30 larvae per box provisioned with *Camellia* leaves under laboratory conditions of 25°C and relative humidity 69% and a 12: 12 (L:D) photoperiod.

All of the bioassays were replicated three times with different mutant strain concentrations. Each replication of each concentration included 1 vial with 30 larvae. Mortality was determined after every 24 h, and larvae were considered dead if there was no movement when they were prodded.

2.3 Statistical Analysis

Data were analyzed using DPS (Data Processing System) version 9.05, and expressed as mean values ± SD. Student's *t* test was used for testing the significance of differences between treatments.

3 RESULTS

3.1 Low-energy N^+ Implantation Dose Selection

We implanted different doses of N^+ with 30 keV of energy, and the survival rate curve of Bb III is shown in Figure 1.

As the N^+ implantation dose increased the biomass declined. The survival rate was lowest at a dose of 9 × 10^{14} ions·cm^{-2}. With an increasing implantation dose, the survival rate increased slightly at 15 × 10^{14} ions·cm^{-2} and then declined. The survival rate curve of *B. bassiana* had a typical "saddle shape (Yuan and Yu, 2003)" (Figure 1). Since the strain had a relatively higher rate of good mutations when the implantation dose was 15 × 10^{14} ions·cm^{-2}, this dose was selected as optimum for mutagenesis, and its survival rate was 26%.

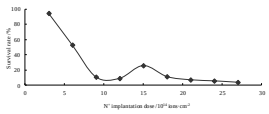


Figure 1: Viability of B. bassiana conidia at different dosages of irradiation with 30 keV N^+.

3.2 Sporulation and the Morphological Observation of Mutant Strains

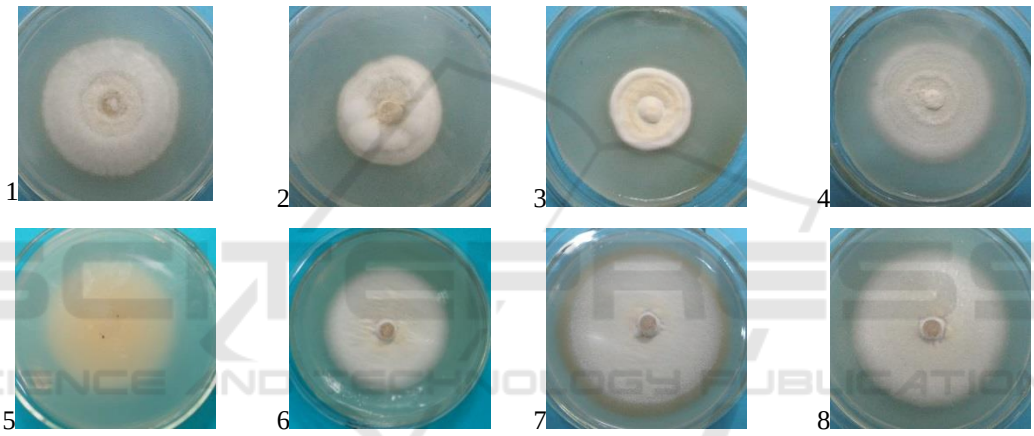
BbIII was subjected to ion implantation and six mutants were selected on the basis of mycelial growth rate and growth vigor (Table 1). The results showed that the colony morphology of *B. bassiana* was affected by N^+ implantation (Figure 2), and the sporulation of the six mutants was improved. The sporulation of Bb III 28 was 9.235 ± 0.023 (× 10^8

spores·cm^{-2}), but its germination rate was slightly lower than the original strain.

Table 1: Morphology and growth characteristics of different *B. bassiana* strains.

Isolate	Colony morphology	Days for sporulation (d)	Conidia amount (108 spores·cm-2)	Germination rate (%)
Bb III 05	White, flocculent	9	8.333±0.212b	91.23±0.21b
Bb III 13	Light yellow, powdery and convex	7	7.034±0.089c	68.23±0.09f
Bb III 22	White, flocculent concentric circles	7	7.340±0.112c	88.34±0.57d
Bb III 28	White, smooth concentric circles	7	9.235±0.023a	89.32±0.11c
Bb III 47	Hoar, flocculent	8	8.022±0.162b	92.63±0.30a
Bb III 83	White, flocculent and thick	7	7.218±0.067c	73.68±0.13e
Bb III (Control check)	White, flocculent	8	6.315±0.012d	92.59±0.31a

Note: Data in the table followed with different letters are significantly different at P=0.01.



1. Bb III 22; 2. Bb III 05; 3. Bb III 13; 4. Bb III 28; 5. Bb III 22; 6. Bb III 47; 7. Bb III 83; 8. Bb III (Control check).

Figure 2: Variation in the morphology of *B. bassiana* strains.

3.3 Enzyme Induction and Activity Assay

The activity levels of Pr1 enzyme were significantly higher (p=0.01) in Bb III 05, Bb III 13, and Bb III 22 compared with the parent strain, and the activity levels of chitinase enzyme were significantly higher (p=0.01) in Bb III 05, Bb III 22, and Bb III 28 compared to the parent strain (Table 2). Total enzyme activity levels in Bb III 22 were significantly higher than in all seven strains, and almost two times that of the parent strain.

3.4 Median Lethal Concentration (LD50) Determined by Bioassay

Contact toxicity of the 3 mutants to larvae using a glass-vial bioassay was determined. Mortality in all of the control groups was consistently less than 5%. The Bb III 22 strain was the most virulent to *B. Marginata*, with 86.7% mortality at a concentration of 10^7 spores·mL^{-1}. DPS was used to analyze the virulence regression equation and chi-square test (Table 3). Equation Chi-square values of mutants were 0.6003, 0.8132, and 0.2981. The *P* values were 0.7407, 0.6659, and 0.8615 (>0.05), which shows that the virulence of the regression equation is

suitable. Bb III 22 had the highest toxicity, and its logarithmic LD_{50} value was 5.1951 (4.5174~5.8416).

Table 2: Activity of chitinase and Pr1 enzyme in different *B. bassiana* strains.

Isolate	Pr1 enzyme activity (OD_{405nm})	Chitinase activity (OD_{540nm})
Bb III 05	0.140±0.015b	0.098±0.017b
Bb III 13	0.158±0.024b	0.053±0.009c
Bb III 22	0.230±0.017a	0.137±0.021a
Bb III 28	0.105±0.031bc	0.143±0.011a
Bb III 47	0.028±0.007d	0.085±0.013bc
Bb III 83	0.096±0.011bc	0.059±0.010c
Bb III (Control check)	0.078±0.013c	0.073±0.005bc

Note: Data in the table followed with different letters are significantly different at P=0.01.

Table 3: Equations of LC-P and Chi-test of *B. bassiana* mutants against *Biston marginata.*

Mutants	Regression equation	Correlation coefficient	Ψ	P	LD_{50}	95% Confidence limits
Bb III 05	Y=2.3382+0.4262x	0.9941	0.6003	0.7407	6.2458	5.5960-7.5760
Bb III 22	Y=2.2649+0.5265x	0.9643	0.8132	0.6659	5.1951	4.5174-5.8416
Bb III 28	Y=2.1543+0.4313x	0.9959	0.2981	0.8615	6.5979	5.8890-8.5143

4 DISCUSSION

Strains of BbIII were mutagenized by low-energy N^+ implantation. We implanted different doses of N^+ and the resulting survival rate curve of *B. bassiana* showed the typical "saddle shape." With 12×10^{14}, 15×10^{14}, and 18×10^{14} ions·cm^{-2} dose treatments, the mortality rate ranged between 70% and 80%. Since the mutagenesis microbial death rate ranged from 70% to 75%, the positive mutation rate tended to be higher. The positive mutation rate was very low and the suitable mutagenic dose was 15×10^{14} ions·cm^{-2}. Among the different mutant strains, colony morphology, sporulation time, sporulation number, and spore germination rate were significantly different. This indicated that low-energy ion implantation effected the physiology and biochemistry of *B. bassiana*.

The survival curve of the ion implantation treatments was saddle-shaped. With traditional physical and chemical mutagenesis methods, the survival curve is typically an index type or shoulder type (Song *et al*., 1999;Yuan and Yu, 2003; Yuan *et al*., 2003). Different combinations of ion number, energy, and dose could provide a large number of mutagenic conditions. The combined effects of the ion implantation treatment and their strong influence on cells has an advantage that traditional mutagenesis techniques cannot match.

B. bassiana is an important insect pathogenic fungus, and it has been widely used for the biological control of pests. We developed three strains of *B. bassiana* with high sporulation, high Pr1, and high chitinase activity using N^+ implantation mutation selection. These strains had high toxicity to *B. marginata*, an economically important pest of *Camellia*.

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