BEAUVERIA BASSIANA CHARACTERIZATION AND EFFICACY GRASSHOPPER /*ANGARACRIS*/

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ABSTRACT

The B. bassiana is a fungusof many arthropods, including more than 200 species of insects and acaridae. When spores of the fungus come into contact with the body of an insect host, they germinate, enter the body, and grow inside, eventually killing the insect.

Two local strains, including B. bassiana-G07, which was isolated from grasshopper Oedaleusasiaticus, died on natural infection, and B. bassiana-G10, which was isolated from grasshopper Caliptomus abbreviates, died of soil borne infection, were detected and it was identified as species B. bassiana by PCR. SCAR primers OPB9 F/R677 and OPA15 F/R441 was specific to of B.bassiana

The highest infection rate by B. bassiana-G07 and mortality was observed in variants of both concentrations 2.1 x 108 conidia/ml, 2.1 x 109 conidia/ml; where mortality reached 86.3-100%.

KEY WORDS: *Beauveriabassiana*, fungi, grasshopper, PCR

INTRODUCTION

The origins of the microbial pest control date back to the early nineteenth century, when the Italian scientist Agostino Bassi spent more than 30 years studying white muscardine disease in silkworms (BombyxmoriL.). He identified Beauveriabassiana
(Bals.-Criv). Vuill., named in his honour, as the cause of the disease.The *B. bassiana* is a parasite of many arthropods, including more than 200 species of insects and acaridae. The disease caused by the fungus is called **white muscardine disease** [Fargues.J1997]. When spores of the fungus come into contact with the body of an insect host, they germinate, enter the body, and grow inside, eventually killing the insect.

Of approximately 750 species of fungi, two species such as *Beauveriabassiana (B. bassiana)* and *Metarhiziumanisopliae* are mostly used for controlling harmful insects and as of 2007 a total of 58 biological preparations were produced by using fungus *B. bassiana*, and are being broadly used for controlling harmful insects in rangelands, forests, crop fields and greenhouses [Marcos.R.de Faria. 2007].

Fungus *B. bassiana* has not been studied and biological preparation of this fungus is not used in our country, it has been important to investigate the possibility of isolation of this fungus species and applicability of such preparation for controlling harmful insects in our country.

MATERIALS AND METHODS

Fungal strains

Two local strains (Table 1) *of B. bassiana*stored at the Microbiological laboratory in the Plant protection research institute were taken into this study. Strains of *B.bassiana*isolated from infected grasshoppers collected in 2007 and in 2010 from pasture.

Table 1

Host of *B.bassiana* strains used in the experiments

№	Strains	Insect host	Family	Origin
1	*B.b-G07*	*Oedaleusasiaticus*	*Acrididae*	Mongolia
2	*B.b-G10*	*Caliptamus abbreviates*	*Acrididae*	Mongolia

Culturing the fungus;

From stocks, the culture was revived using the yeast extract peptone glucose agar (YPGA – with 2% glucose, 1% peptone, 1% yeast extract and 1.5% agar) slants.

Insect assays;

Grasshoppers /*Angaracris*/ were collected from pasture outside Ulaanbaatar city, 5 day prior to testing and held in plastic vented containers (20 x 15 x 10 cm) containing fresh quitch leaves. There were ten grasshoppers per container (15 containers). Four concentration assays (at 2.1 x 10^6 conidia/ml, 2.1 x 10^7 conidia/ml, 2.1 x 10^8 conidia/ml, 2.1 x 10^9 conidia/ml 0.1% Tween-80). A control, 0.1% Tween-80 suspension, was included in each assay. The containers were kept in the laboratory at room temperature /22±3/ under natural light conditions. Mortality counts were taken every day for 14 days post treatment. Dead insect were removed daily and stored at cold storage /- 20^0C/. Also Dead insect placed in Petri dishes covered with wet filtering paper for fungal emergence. Conidia from dead insects of each isolate were transferred separately to YPGA medium in a sterile condition. Light microscopic studies and colony form proved that recovered fungus is the same as inoculated fungus *B. bassiana*. Used Abbott's formula for control mortality in bioassays*DNA extraction*

Culturing the fungus;

Stock conidial suspension of *B. bassiana* strains *B.b–G07* and *B.b–G10* was inoculation at 1% into YPD (2% glucose, 1% peptone, 1% yeast extract). The culture was incubated (25 ± 0.5^0C) for 4 days on a rotary shaker at 150-180 rpm and harvested by centrifugation. Using Genomic DNA Purification Kit #K0512 (Fermentas, USA) for DNA extraction. They were maintained in a chamber at 25 ± 0.5^0C. The suspension of the conidia was swilled (solution 0.1% Tween-80) from 14 day old cultures for the experiments.

From culture.

200μl of harvested culture should be placed in a 1.5 ml micro centrifuge tube and resuspended in 200 μl of TE buffer

From grasshopper;

30 mg of grasshopper tissue should be pulverized, placed in a 1.5 ml microcentrifuge tube and resuspended in 200 μl of TE buffer. Mix 200 μl of sample with 400 μl of lysis solution and incubate at 65°C for 5 min. Immediately add 600 μl of chloroform, gently emulsify by inversion (3-5 times) and centrifuge the sample at 10,000 rpm for 2 min. Prepare precipitation solution by mixing 720 μl of sterile deionized water with 80 μl of supplied 10X concentrated Precipitation Solution. Transfer the upper aqueous phase containing DNA to a new tube and add 800 μl of freshly prepared precipitation solution, mix gently by several inversions at room temperature for 1-2 min and centrifuge at 10,000 rpm (~9400 x g) for 2 min.Remove supernatant completely (do not dry) and dissolve DNA pellet in 100 μl of NaCl solution by gentle vortexing.

Add 300 μl of cold ethanol, let the DNA precipitate (10 min at -20°C) and spin down (10,000 rpm (~9400 x g), 3-4 min). Remove the ethanol. Wash the pellet once with 70% cold ethanol and dissolve DNA in 100 μl of sterile deionized water by gentle vortexing.

Primers

Two SCAR primers (Table 2) were synthesized by the Sigma.

Table 2

Sequence of SCAR primers for *B.bassiana*

№	SCAR markers	Primers	Sequence (5' – 3')[d]
1	$SCA15_{441}$	OPA15 F_{441}	TTC CGA ACC CGG TTA AGA GAS
		OPA15 R_{441}	TTC CGA ACC CAT CAT CCT GC
2	$SCB9_{677}$	OPB9 F_{677}	TGG GGG ACT CGC AAA CAG
		OPB9 R_{677}	TGG GGG ACT CAC TCC ACG

PCR mixtureand conditions;

In total of 25 µl of mixture, containing 12.5 µl Dream tag (Fermentas,USA), 1 µl of each primer (forward and reverse primers), 3 µl template DNA, 7.5 µl ddH_2O.
The amplication profile was 2 min initial denaturation at 94^0C, 10 cycles of denaturation at 94^0C for 15 s, annealing at 63^0C for 30 s and elongation at 72^0C for 45 s; followed by 15 cycles of denaturation at 94^0C for 15 s, annealing at 63^0C for 30 s and elongation at 72^0C for 45 s, with an additional 5 s for each successive cycle; and a final elongation at 72^0C for 7 min. PCR products were harvested in 1% agarose gels stained with ethidium bromide.

RESULTS AND DISCUSSION

Insect assays

150 Grasshoppers /*Angaracris*/ were collected from pasture outside Ulaanbaatar city for use in this experiment. The efficacy of the the isolates of *B. bassiana* was examined and their virulence against grasshoppers tested (Table 3).

Table 3

Efficacy of strains of *B.bassiana* against grasshoppers *(Angaracris)* in laboratory

Strains	Concentration (conidia/ml)	Grasshoppers in days (Mean of four replicates)															Mortality % (by Abbott)
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
B.b-G07	2.1×10^6	10	10	10	10	10	10	9.6	9.3	9	8.3	7	5.3	4.6	4	4	45.2
	2.1×10^7	10	10	10	10	10	9.6	9.3	8.6	8	7	4.3	3.3	2.6	2.6	2.6	63.5
	2.1×10^8	10	10	10	10	10	8.6	8	7.6	6.6	4.3	2	1	1	1	1	86.3
	2.1×10^9	10	10	9.3	9	9	6.3	3.6	0.3	0	0	0	0	0	0	0	100
Control	0.1%Tween-80	10	10	10	10	10	10	10	9.3	9.3	8.6	8.6	8.3	7.6	7.3	7.3	

The experiments showed that isolated the local strain *B.b - G07* is high virulence for the Angaracris. The highest infection rate by fungi and mortality was observed in variants of both concentrations 2.1×10^8 conidia/ml, 2.1×10^9 conidia/ml; where mortality reached 86.3-100%.
Comparison of grasshopper mortality generally demonstrated the superiority of four concentrations and no significant difference was observed (Figure 1)

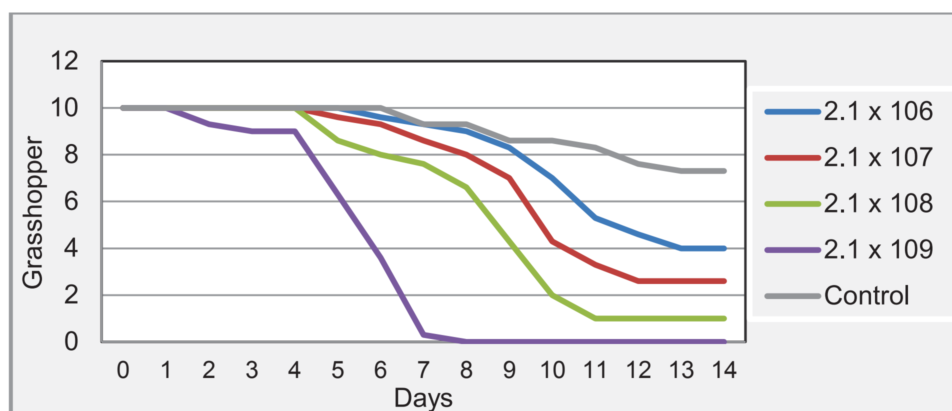


Figure 1.Mortality of grasshopper in test days

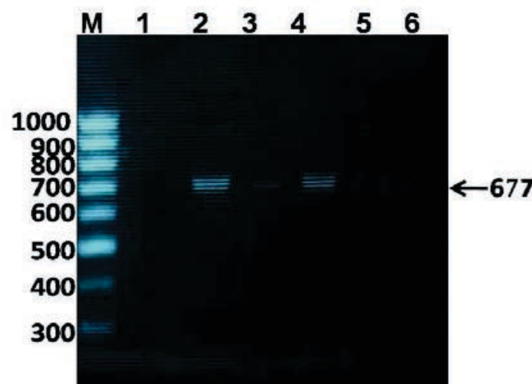
Dead insect for test time placed in Petri dishes covered with wet filtering paper for fungal emergence. Dishes were incubated at 25 ±0.5^{0}C in a incubator for 7 days.

Picture 1. Emergence of *B. bassiana* from dead *Angaracris*

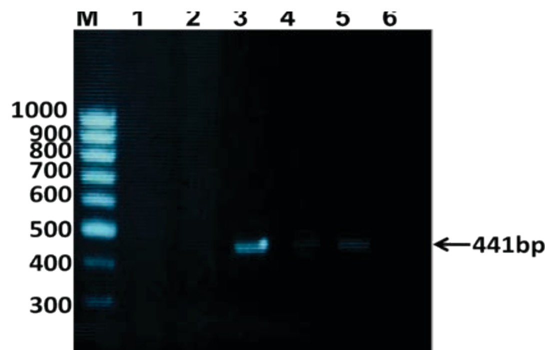
After host death and utilization of all its internal nutrients, fungus emerged from insect body and produced aerial mycelia and conidia on it (Picture 1).

Results of PCR

Using the above-described PCR screen protocol, PCR assays two strains of *B.bassiana /B.b-G07, B.b-G10/,* one isolate from infected grasshopper cadavers (pending experiment), soil and healthy grasshopper showed showed that SCAR primers OPB9 F/R_{677} and OPA15 F/R_{441} was specific to of *B.bassiana*(Picture 2, 3).



Picture 2. PCR products of DNA samples detectedwith *B.bassiana* – specific 677bp gene fragment (SCAR primer OPB9 F/R_{677}). M- Standartmarker (100bp); 1- Grasshopper ; 2- Strain *B.bassiana-G07;* 3- Isolate from infected grasshopper cadavers pending experiment of laboratory (*B.b-G07 treated);*4- Strain *B. bassiana-G10; 5.*Healthy grasshopper, 6-Soil;



Picture 3. PCR products of DNA samples detectedwith *B.bassiana* – specific 441bp gene fragment (SCAR primer OPA15 F/R_{441}). M- Standartmarker (100bp); 1- Grasshopper; 2-soil*;* 3- Strain *B.bassiana-G07; 4-*Isolate from infected grasshopper cadavers pending experiment of laboratory (*B.b-G07 treated);*5- Strain *B. bassiana-G10;6.*Healthy grasshopper

OPA-14 F/R_{445}, OPB9 F/R_{677} and OPA15 F/R_{441} SCAR primers were highly sensitive, capable of detecting 100pg *B.bassiana* GHA genomic DNA, and thus could be used to detect varying levels of the fungus in the field (Castrillo L.A.2003).

SUMMARY

1. Two local strains, including *B. bassiana-G07,* which was isolated from grasshopper *Oedaleusasiaticus*, died of natural infection, and *B. bassiana-G10*, which was isolated from grasshopper *Caliptomus abbreviates*, died of soil borne infection, were detected and it was identified as species *B. bassiana* by PCR. SCAR primers OPB9 F/R677 and OPA15 F/R441 was specific to of *B.bassiana*
2. The highest infection rate by *B. bassiana-G07* and mortality was observed in variants of both concentrations 2.1 x 108 conidia/ml, 2.1 x 109 conidia/ml; where mortality reached 86.3-100%.

REFERENCES

1. Castrillo L.A., Vandenberg J.D., Wraight S.P.Strain-specific detection of introduced *Beauveriabassiana* in agricultural fields by use of sequence-characterized amplified region markers. Journal of Invertebrate Pathology 82, 2003 75–83
2. Fargues.J, Goettel.M.S, Smits.N, Ouedraogo.A and Rougier.M 1997. Effect of temperature on vegetative growth of *Beauveriabassiana* isolates from different origins.Mycologia 89, 383-392
3. Joung-Eon Park, Gi-Young Kim and other Phylogenetic analysis of caterpillar fungi by comparing ITS 1-5.8S-ITS 2 ribosomal DNA sequences. Mycobiology 29(3): 121-131 (2001)
4. Marcos.R.de Faria, Stephen.P. Wraight. Mycoinsecticides and Mycoacaricides: Acomprehensive list with worldwide coverage and international classification of formulation types. ScienceDirect, Biological Control 43 (2007) 237-256.
5. Nicolai V. Meyling. Methods for isolation of entomopathogenic fungifrom the soil environment 2007.
6. Otgonjargal.Kh Results of the study on local strain of entomopathogenic fungi *Beauveriabassiana.* PhD thesis. 2011.
7. Pfeifer . Tom A, Khachatouriance.G. Isolation and characterization of DNA from Entomopathogen*Beuaveriabassiana.* Experimental mycology 13, 392-402 (1989)
8. Shinkafi. S.A and Sanusi.M.M. Biological control of locust and grasshoppers using some fungi. Scholars academic journal of biosciences. 2013; 1(3):90-95