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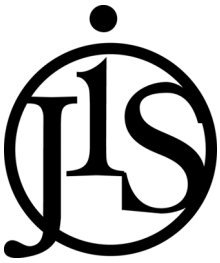

A new method to evaluate the biocontrol potential of single spore isolates of fungal entomopathogens

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Abstract

Fifty *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) strains isolated from the coffee berry borer were used to develop a novel screening method aimed at selecting strains with the highest biocontrol potential. The screening method is based on percent insect mortality, average survival time, mortality distribution, percent spore germination, fungal life cycle duration, and spore production on the insect. Based on these parameters, only 11 strains merited further study. The use of a sound scientific protocol for the selection of promising fungal entomopathogens should lead to more efficient use of time, labor, and financial resources in biological control programs.

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Introduction

The coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae), an insect endemic to Central Africa, is considered to be the most serious pest of coffee throughout the world, causing hundreds of millions of dollars in losses on a yearly basis. The insect has been reported in most coffee producing countries throughout the world and presents a tremendous management challenge due to its cryptic life cycle that occurs inside the coffee berry (Bustillo et al., 1998). Among the many biocontrol methods being explored for use against this insect, the fungal entomopathogen *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) remains one of the most promising. Pascalet (1939) was the first to record *B. bassiana* infections in *H. hampei* in Cameroon. Subsequently, the pathogenicity of *B. bassiana* against the coffee berry borer as a biocontrol agent has been studied in Brazil (Fernández et al., 1985), Honduras (Lazo, 1990), Mexico (Méndez, 1990), Colombia (Jiménez, 1992), Nicaragua (Barrios, 1992), Ecuador (Sponagel, 1994) and India (Haraprasad et al., 2001). These studies have been aimed at the development of a sustainable pest control method against the coffee berry borer that does not rely on chemical insecticides such as endosulfan, a product that has been banned in Colombia and to which the insect has developed resistance (Brun et al., 1989). Thus, there is a great interest in coffee producing countries in developing commercial formulations of *B. bassiana*.

The first step in the process leading to commercialization is to conduct standard bioassays to select the more promising isolates. Butt and Goettel (2000) reviewed the bioassays used for entomopathogenic fungi and outlined the different procedures to conduct the bioassays, which will depend upon the type of insect and fungi. In these bioassays, percent mortality and average survival time and, in a few cases, LC50 are the only parameters that have been taken into consideration for isolate evaluation.

Our objective was to develop a novel method for selecting fungal entomopathogens aimed as biocontrol agents against insects in general. This method involves the estimation of several parameters, including percent insect mortality, average survival time, mortality distribution, percent spore germination, fungal life cycle duration, and spore production on the insect. Measuring these parameters provides a better idea of which isolates would have higher potential for success in a biological control program; these isolates can then be used for scaling up production, formulation, and subsequent field application studies leading to commercialization.

Isolates

Fifty *B. bassiana* isolates originating from coffee berry borers collected in Africa, Central and South America were used (see Table 1 for strain identity). Most of the isolates were kindly provided by R. Humber, Curator of the U. S. Department of Agriculture, Agricultural Research Service, Collection of Entomopathogenic Fungi in Ithaca, New York. All isolates were individually applied to coffee berry borers using a Potter Tower (Burkard Manufacturing, www.burkard.co.uk). After sporulation on the insect cuticle, single spore cultures for each isolate were initiated on potato dextrose agar (Becton Dickinson, www.bd.com) and stored in 10% glycerol at -80° C.

Insects

Adult coffee berry borers reared on artificial diet (Villacorta, 1985; Portilla, 2000) were obtained from the Biological Control and Mass Rearing Research Unit (USDA, ARS, Stoneville, MS). All adult insects used in the bioassays were about 1 month old. Before they were used, to kill fungal spores present on the cuticle, insects were washed in a 0.5% sodium hypochlorite solution with 0.01% Triton X-100 (Sigma Chemical Co., www.sigmaaldrich.com) and gently shaken for two minutes. Afterwards, they were rinsed three times in sterile distilled water and dried in a container lined with sterile paper towels. They were then separated into small groups to be assigned to the treatments. No food was provided throughout the experiment to avoid problems with fungal contamination from the artificial diet and also because the insects burrow into the diet which would make it impossible to observe them. The insects can survive for extended periods of time without food (Posada, 1998).

Inoculation

To produce the spores necessary to run the bioassay the single spore isolates stored in 10% glycerol at -80° C were grown in Sabouraud's dextrose agar (Becton Dickinson Sparks, MD) and incubated at 25° C. All cultures were less than 30 days old when used in the bioassays. Spores were collected with a

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spatula and placed in sterile tubes containing 10 ml of sterile distilled water plus 0.01% Triton X-100 (Sigma Chemical Co., St. Louis, MO). Spore concentrations were adjusted to about $1x10^7$ spores ml^{-1} using a hemacytometer, which is the baseline concentration used to evaluate *B. bassiana* against coffee berry borers at the National Coffee Research Center (Cenicafé) in Colombia (González et al., 1993; Marin et al., 2000).

Spore germination was determined by plating a 10 µl low concentration *B. bassiana* aliquot on 2.5% Noble agar (Becton Dickinson Sparks, MD). The spore suspension was spread on the medium with a sterile spatula and incubated at 27° C. Germination was assessed only at 24 h by taking 3 samples of the media and placing them individually on slides, followed by counting 100 spores. Spores were deemed to have germinated when the longitude of the germ tube was longer than half the size of the spores. After finding that at 24 h some isolates had zero germination but caused 100% mortality, indicating germination had occurred post initial 24 h, a second germination test was done to examine spore germination at 24, 48 and 72 hours.

The coffee berry borers assigned to the treatments were dipped in 10 ml of a *B. bassiana* suspension with $1x10^7$ spores ml^{-1} plus 0.1% Triton X-100; control insects were dipped in sterile distilled water plus 0.1% Triton X-100 (our reasons for using dipping vs. spraying are presented in the Discussion). Insects were gently shaken for two minutes while dipped in their respective treatments and then placed in a sterile container from which they were taken individually with a paintbrush and placed in a vial containing two pieces of sterile Whatman #1 filter paper (2.1 cm diameter) moistened with 100 µl of sterile distilled water. The vials were closed with caps and sterile distilled water was added to the filter paper as needed. All treatments were incubated in the dark at 25° C in a growth chamber (Model E-36L, Percival Scientific, Inc., www.percival-scientific.com). Insect mortality was assessed on a daily basis.

Experimental Design

The experiment was conducted as a completely randomized design, with each isolate replicated 40 times and beetles individually placed in separate vials. For statistical purposes, mortality was analyzed by grouping the experimental units as 4 replicates with 10 beetles each (each beetle in a separate vial). Isolates were evaluated at different times (15, 15, 14 and 6 isolates each time) due to the same person being in charge of examining the insects and recording variables on a daily basis to reduce bias. All observations were concluded 15 to 17 days after treatments were applied.

Percent spore germination and percent insect mortality was analyzed using PROC MIXED (SAS, 2001). Average insect survival time was analyzed using survival analysis that includes all individuals (JMP SAS, 2000), in contrast to LT50 that only includes those that have died.

Our assessment of the saprophytic phase for *B. bassiana* isolates was based on the time it takes for four events to occur after the insect has died (Fig. 1). The first event is the number of days after death until mycelium is first observed on the insect corpse. The second event is number of days from the first formation of mycelium until the mycelium has reached a maximum coverage of the corpse. The third event is based on the time it takes from maximum mycelium coverage until conidiophores are first seen. The fourth and final event consists of the time it takes from first observation of conidiophores until spores begin to discharge. Observations were made every 24 h and in cases where a certain event seemed to be already advanced at the time of the observation, indicating it had been initiated shortly after the previous observation, the time to the specific event was back-estimated 12 h. The data for each event were analyzed using PROC MIXED (SAS, 2001).

Spore production was determined by randomly selecting 5 beetles (out of 40) within each treatment for which there was spore production. Each beetle was washed with 5 ml of sterile distilled water with 0.1% Triton X-100 and sonified for one minute. A diluted spore suspension was placed in three different hemacytometers and eight 16 square grids were counted in each one. The data were analyzed using a one-way analysis of variance (ANOVA proc mixed; SAS, 2001). In all tests, $P<0.05$ was considered significant.

Results

Spore germination at 24 h, for the first germination test, ranged from 0-95% (Table 1). There were significant differences in *B. bassiana* spore germination due to isolates ($F = 47.7$, d f= 103, $P = 0.001$). In the second germination test, there were significant differences in percent germination among the isolates at 24 hours ($F = 62.2$, $df = 49$, $P = 0.001$), 48 hours ($F = 62.2$, $df = 49$, $P= 0.001$),

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and 72 hours (F =39.1, df = 49, P = 0.001) and among the three evaluation times (F = 462.2, df = 2, P = 0.001).

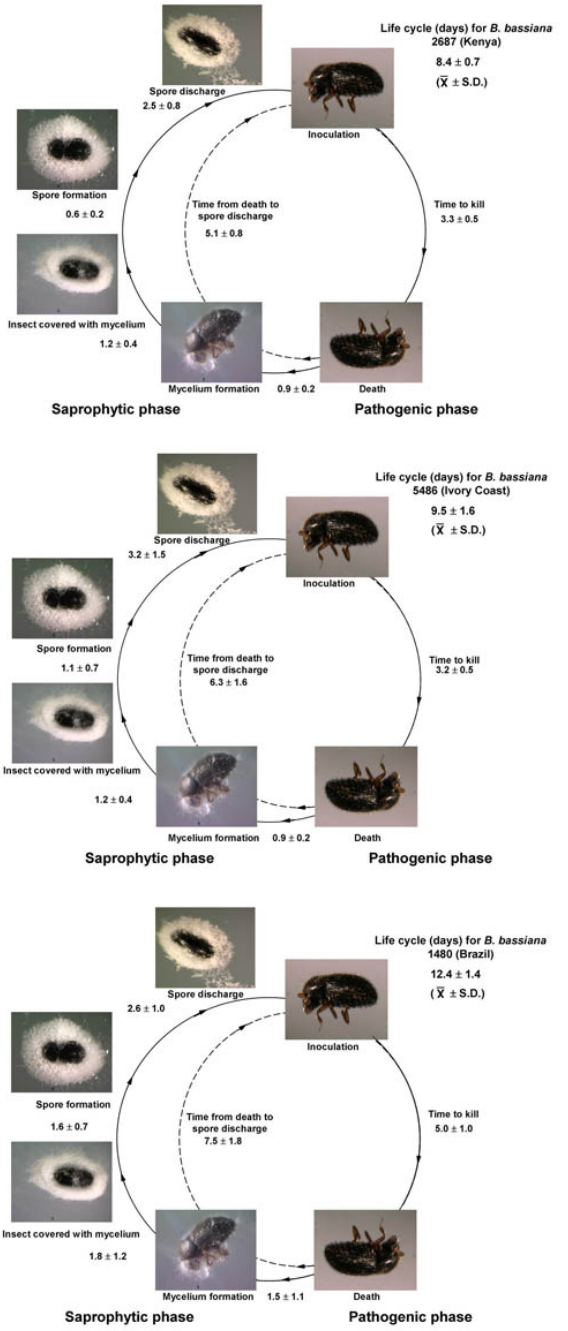

Fig. 1. Life cycle for three *Beauveria bassiana* single spore isolates on coffee berry borer, including short (top), medium (center) and long (bottom) life cycles.

The repetition of the germination at 24 h showed a similar tendency as the first germination test with some exceptions (Table 1). In the first 24 h germination test isolates Togo 2689 and Ivory Coast 5460 exhibited 0% germination while in the second test germination was 17% and 16%, respectively. Ivory Coast 5486, which had reached 36% germination at 24 h in the first test, had not germinated at 24 h in the second test. In the first test only two isolates (Togo 5451 and Mexico 3818) reached over 90% germination at 24 h, while in the second test only one showed over 90% germination (Togo 5458).

At 48 h all isolates had germinated (Table 1), and seven had reached over 90% germination, while three presented less than 50% germination. The other 40 isolates exhibited 65% - 89% germination. At 72 h, germination for 42 of the isolates tested was above 90%; however, none had reached 100% germination. Germination rates were not assessed post-96 h due to production of new spores after 48 h in the isolates exhibiting high germination rates. This production of new spores, if not taken into consideration, could result in an underestimate of germination if confused with the original spores or in an overestimation of germination if they have started to germinate. Thus, it is essential to be able to distinguish between the original spores and subsequent spore production; a careful examination of the mycelium and the presence of conidiogenous cells enable discrimination between original and new spores. Counting of spores at 48 and 72 h consumes inordinate amounts of time.

The spores in the first and second germination tests were handled the same way, counted using the same protocol, and kept under the same environmental conditions. The same medium was used and the source of cultures was the same (single spores maintained at -80°C.)

Spore suspensions of $1x10^7$ spores ml^{-1} caused high coffee berry borer mortality with 47 of the isolates reaching 100% mortality, two isolates causing 97.5% mortality and one causing 67.5% mortality (Table 1). There was no mortality in the controls due to fungal growth. There were significant differences in mortality due to isolates used (F = 5.7, df = 150, P = 0.001). Mortality distribution ranged from 3-11 days post application (Table 1). In 13 of the isolates tested there was a high mortality (up to 100%) within 2 days after the first insect died (Table 1). Average survival time ranged between 3.2

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Table 1. Parameters used for scoring of 50 Beauveria bassiana fungal isolates: % insect mortality, % spore germination, average survival time (days), mortality distribution (days), B. bassiana life cycle (days), and spore production (spores per insect).

Isolate	Mortality (%)	Germination (%) 1st test 24 h	2nd test 24 h	2nd test 48 h	2nd test 72 h	Average survival time (days)	Mortality distribution (days)	B. bassiana life cycle (days)	Spore production (spores insect-1)	Scoring 2
Kenya-2686	100 ± 0.0	75.2 ± 2.4	75.1 ± 3.2	88.1 ± 1.4	91.0 ± 1.9	3.3 ± 0.1	2	11.6 ± 2.0	$5.2x10^6 \pm 1.7 x10^6$	+-++--
Nicaragua-Managua-3443	100 ± 0.0	74.0 ± 8.7	64.9 ± 2.9	83.9 ± 3.1	91.1 ± 7.4	3.2 ± 0.1	2	12.9 ± 1.6	$1.3 x10^7 \pm 1.9 x10^6$	+-++--
Togo-Gbadi Gaodo -5447	100 ± 0.0	61.1 ± 5.1	45.5 ± 4.1	72.8 ± 7.4	97.3 ± 1.5	3.6 ± 0.1	2	8.6 ± 1.2	$7.8 x10^6 \pm 4.3 x10^6$	+-+---
Togo-Gbadi Gaodo-5450	100 ± 0.0	72.0 ± 1.9	56.9 ± 2.3	68.8 ± 6.6	97.7 ± 1.1	3.5 ± 0.1	2	8.3 ± 1.0	$9.2 x10^6 \pm 2.3 x10^6$	+-++--
Kenya-2687	100 ± 0.0	35.6 ± 7.5	50.2 ± 0.3	78.3 ± 5.1	88.2 ± 4.7	3.3 ± 0.1	2	8. ± 0.7	$2.5 x10^7 \pm 3.9 x10^6$	+-++-+
Nicaragua-Managua-3445	100 ± 0.0	30.2 ± 2.4	48.5 ± 2.5	93.5 ± 4.2	95.7 ± 2.4	3.4 ± 0.1	2	8.8 ± 0.8	$2.1 x10^7 \pm 3.5 x10^6$	+-++-+
I. Coast -Abengourou-5439	100 ± 0.0	87.2 ± 6.8	76.7 ± 5.6	86.1 ± 1.6	95.7 ± 3.0	3.2 ± 0.1	2	8.6 ± 1.1	$2.7 x10^6 \pm 7.0 x10^5$	++++--
Togo-Gbadi Gaodo-5451	100 ± 0.0	93.8 ± 2.7	78.8 ± 4.8	83.5 ± 3.4	95.8 ± 1.6	3.2 ± 0.1	2	9.3 ± 1.2	$6.5 x10^6 \pm 2.9 x10^6$	++++--
Togo-Gbadi Gaodo-5452	100 ± 0.0	42.6 ± 3.7	62.5 ± 2.1	83.0 ± 4.7	93.4 ± 2.8	3.5 ± 0.1	2	8.8 ± 0.7	$2.2 x10^7 \pm 3.8 x10^6$	+-++-+
Togo-Gbadi Gaodo-5454	100 ± 0.0	69.0 ± 3.5	70.7 ± 4.2	83.3 ± 4.1	93.9 ± 1.2	3.5 ± 0.1	2	8.1 ± 0.6	$1.0 x10^7 \pm 3.2 x10^6$	+-++-+
Cameroon-99057	100 ± 0.0	29.7 ± 3.6	52.9 ± 4.9	70.0 ± 1.8	97.1 ± 0.9	3.7 ± 0.1	2	9.0 ± 0.8	$1.7 x10^7 \pm 4.8 x10^6$	+-+--+
Cameroon-99059	100 ± 0.0	38.2 ± 1.6	43.9 ± 5.3	73.5 ± 2.6	96.5 ± 1.0	3.4 ± 0.1	2	8.2 ± 0.8	$1.8 x10^7 \pm 3.0 x10^6$	+-++-+
Togo-2691	100 ± 0.0	48.9 ± 3.5	43.6 ± 2.7	80.1 ± 3.8	83.7 ± 1.3	3.7 ± 0.1	2	10.7 ± 1.0	$1.4 x10^7 \pm 6.8 x10^6$	+-+--+
Togo-Gbadi Gaodo-5489	100 ± 0.0	30.6 ± 3.7	51.5 ± 7.5	91.4 ± 4.7	93.6 ± 1.6	3.5 ± 0.1	2	7.8 ± 0.7	$1.3 x10^7 \pm 3.1 x10^6$	+-++++
Cameroon-99070	100 ± 0.0	37.5 ± 0.6	49.7 ± 5.5	85.3 ± 4.1	98.2 ± 1.8	3.5 ± 0.1	2	8.1 ± 0.9	$1.6 x10^7 \pm 3.1 x10^6$	+-++-+
I. Coast -Abengourou-5437	100 ± 0.0	80.5 ± 0.0	76.5 ± 7.4	77.6 ± 1.6	92.9 ± 1.2	3.6 ± 0.1	3	9.4 ± 1.2	$7.3 x10^6 \pm 3.4 x10^6$	+-----
Togo-Gbadi Gaodo-5443	100 ± 0.0	48.0 ± 2 3	20.1 ± 4.4	64.8 ± 3.9	90.9 ± 1.2	4.1 ± 0.1	3	9.7 ± 1.3	$5.2 x10^6 \pm 2.2 x10^6$	+-----
Mexico-Oaxaca-3818	100 ± 0.0	90.9 ± 5.0	82.6 ± 2.2	86.3 ± 3.2	94.8 ± 1.4	3.9 ± 0.1	3	8.7 ± 1.1	$7.2 x10^6 \pm 1.9 x10^6$	++----
Togo-Gbadi Gaodo-5449	100 ± 0.0	56.4 ± 2.3	67.4 ± 2.2	91.2 ± 7.8	94.9 ± 1.3	3.9 ± 0.1	3	9.6 ± 1.1	$7.2 x10^6 \pm 3.1 x10^6$	+-----
Cameroon-99050	100 ± 0.0	55.2 ± 12.2	60.8 ± 9.0	72.8 ± 4.4	91.3 ± 1.2	4.5 ± 0.1	3	11.0 ± 1.7	$4.7 x10^6 \pm 3.7 x10^6$	+-----
Togo-2690	100 ± 0.0	38.7 ± 5.9	44.0 ± 9.1	85.3 ± 3.0	91.5 ± 2.7	3.3 ± 0.1	3	9.1 ± 0.9	$1.3 x10^7 \pm 5.2 x10^6$	+--+-+
Mexico-Oaxaca-3819	100 ± 0.0	35.1 ± 4.8	44.6 ± 1.1	70.2 ± 2.3	96.5 ± 1.9	3.7 ± 0.1	3	10.0 ± 1.1	$4.6 x10^6 \pm 2.0 x10^6$	+-----
I. Coast -Ayenoua-5483	100 ± 0.0	87.2 ± 2.7	81.5 ± 3.7	82.6 ± 6.7	92.5 ± 2.5	3.5 ± 0.1	3	8.4 ± 0.7	$2.1 x10^7 \pm 2.8 x10^6$	++-+-+
I. Coast -Bozi-5486	100 ± 0.0	36.3 ± 6.6	0.0 ± 0.0	10.0 ± 7.1	27.6 ± 6.4	3.2 ± 0.1	3	9.5 ± 1.6	$1.7 x10^7 \pm 2.6 x10^6$	+--+-+
Togo-Gbadi Gaodo-5455	100 ± 0.0	59.4 ± 8.7	76.3 ± 3.4	83.9 ± 3.6	93.4 ± 0.4	3.5 ± 0.1	3	7.8 ± 0.7	$1.7 x10^7 \pm 3.5 x10^6$	+--+++
Togo-Gbadi Gaodo-5458	100 ± 0.0	89.7 ± 1.1	91.6 ± 2.4	91.3 ± 1.1	93.9 ± 2.9	3.8 ± 0.1	3	8.5 ± 1.0	$9.2 x10^6 \pm 3.2 x10^6$	++----
I. Coast -Bozi-5487	100 ± 0.0	71.0 ± 7.3	66.1 ± 2.5	73.2 ± 0.4	73.3 ± 8.5	3.2 ± 0.1	3	8.1 ± 1.2	$9.4 x10^6 \pm 2.7 x10^6$	+--+--
Togo-Gbadi Gaodo-5490	100 ± 0.0	35.9 ± 3.1	36.8 ± 5.5	71.1 ± 3.3	94.3 ± 4.8	3.7 ± 0.1	3	7.7 ± 0.7	$1.5E+0^7 \pm 3.5 x10^6$	+---++
Mexico-2693	100 ± 0.0	59.3 ± 6.6	29.7 ± 4.2	32.6 ± 3.5	72.1 ± 1.9	3.8 ± 0.1	4	10.5 ± 1.7	$6.0 x10^6 \pm 3.4 x10^6$	+-----
I. Coast -Abengourou-5436	100 ± 0.0	82.6 ± 6.6	74.1 ± 6.7	78.8 ± 1.5	94.2 ± 0.9	3.9 ± 0.1	4	11.7 ± 1.6	$5.1 x10^6 \pm 2.9 x10^6$	+-----
Cameroon-99043	100 ± 0.0	58.4 ± 6.1	53.7 ± 4.5	70.0 ± 2.1	98.1 ± 1.0	4.0 ± 0.2	4	10.5 ± 1.1	$5.0 x10^6 \pm 2.2 x10^6$	+-----
Togo-2689	100 ± 0.0	0.0 ± 0.0	17.0 ± 2.0	89.1 ± 2.6	96.1 ± 1.0	7.8 ± 0.1	4	16.0 ± 0.0	$2.5 x10^6 \pm 1.5 x10^6$	+-----
I. Coast -Ba-5459	100 ± 0.0	55.2 ± 12.2	46.5 ± 6.7	75.2 ± 2.6	94.2 ± 2.9	3.8 ± 0.1	4	13.6 ± 2.3	$5.4 x10^6 \pm 1.9 x10^6$	+-----
Togo-Gbadi Gaodo-5448	100 ± 0.0	18.0 ± 2.5	81.1 ± 2.9	90.8 ± 2.1	94.1 ± 1.8	5.3 ± 0.1	4	9.8 ± 1.0	$4.3 x10^6 \pm 2.7 x10^6$	+-----
Cameroon-99055	100 ± 0.0	59.1 ± 8.7	60.8 ± 2.4	70.0 ± 1.7	95.5 ± 0.5	4.4 ± 0.1	4	11.6 ± 1.7	$7.3 x10^6 \pm 2.2 x10^6$	+-----
Kenya-2685	100 ± 0.0	76.4 ± 9.0	82.6 ± 3.7	86.4 ± 6.6	96.3 ± 2.5	5.1 ± 0.2	5	13.1 ± 1.3	$6.5 x10^6 \pm 1.5 x10^6$	+-----
Nicaragua-Managua-3440	100 ± 0.0	81.1 ± 2.2	67.3 ± 1.9	81.0 ± 2.0	97.7 ± 1.5	4.8 ± 0.2	5	12.0 ± 1.9	$5.7 x10^6 \pm 3.0 x10^6$	+-----
I. Coast -Bozi-5442	100 ± 0.0	74.9 ± 1.3	68.0 ± 6.1	70.6 ± 3.8	79.3 ± 2.2	5.5 ± 0.2	5	12.3 ± 1.4	$6.7 x10^6 \pm 4.1 x10^6$	+-----
Togo-Gbadi Gaodo-5445	100 ± 0.0	88.6 ± 7.8	67.4 ± 4.6	82.7 ± 2.5	96.4 ± 3.8	4.2 ± 0.3	5	9.4 ± 1.4	$6.9 x10^6 \pm 1.7 x10^6$	++-----
I. Coast -Toumbokri-5484	100 ± 0.0	82.6 ± 1.7	72.2 ± 4.4	79.4 ± 6.1	90.5 ± 3.5	4.6 ± 0.2	5	12.6 ± 1.7	$6.0 x10^6 \pm 1.4 x10^6$	+-----

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Isolate	Mortality (%)	Germination (%) 1st test 24 h	2nd test 24 h	2nd test 48 h	2nd test 72 h	Average survival time (days)	Mortality distribution (days)	B. bassiana life cycle (days)	Spore production (spores insect-1)	Scoring 2
I. Coast -Abengourou-5438	97.5 ± 15.8	81.2 ± 2.3	71.3 ± 5.0	73.8 ± 4.0	90.0 ± 2.2	4.3 ± 0.1	5	9	$8.3 \times 10^5 \pm 3.8 \times 10^5$	------
I. Coast -Bozi-5460	100 ± 0.0	0.0 ± 0	16.1 ± 1.5	49.1 ± 3.8	76.9 ± 7.6	5.6 ± 0.2	5	12.3 ± 1.7	$2.0 \times 10^6 \pm 1.3 \times 10^6$	+-----
Togo-Gbadi Gaodo-5453	97.5 ± 15.8	59.5 ± 3.7	64.3 ± 3.2	90.6 ± 4.5	94.9 ± 2.1	4.4 ± 0.2	5	5.6 ± 1.4	$1.6 \times 10^6 \pm 6.0 \times 10^5$	------
Cameroon-99046	100 ± 0.0	50.3 ± 4.7	76.9 ± 3.5	80.5 ± 1.5	93.4 ± 1.2	5.0 ± 0.3	6	12.3 ± 0.9	$7.0 \times 10^6 \pm 2.2 \times 10^6$	+-----
I. Coast -Ayenoua-5441	100 ± 0.0	75.1 ± 6.8	83.4 ± 1.4	86.1 ± 1.3	95.6 ± 1.6	4.7 ± 0.2	6	11.8 ± 1.3	$7.2 \times 10^6 \pm 2.1 \times 10^6$	+-----
Togo-Gbadi Gaodo-5444	100 ± 0.0	82.6 ± 3.9	62.5 ± 7.9	91.0 ± 2.0	95.2 ± 1.8	4.6 ± 0.3	6	11.0 ± 1.9	$1.9 \times 10^6 \pm 1.0 \times 10^6$	+-----
Cameroon-99048	100 ± 0.0	64.7 ± 4.3	58.3 ± 2.5	70.1 ± 0.6	95.1 ± 1.8	5.1 ± 0.3	6	12.0 ± 1.8	$6.9 \times 10^6 \pm 1.8 \times 10^6$	+-----
Brazil-1480	100 ± 0.0	50.4 ± 1.5	42.7 ± 2.3	75.4 ± 0.8	85.4 ± 4.3	5.1 ± 0.2	7	12.4 ± 1.4	$5.9 \times 10^6 \pm 1.4 \times 10^6$	+-----
Togo-2688	100 ± 0.0	76.3 ± 4.3	89.3 ± 3.6	90.0 ± 2.0	93.0 ± 3.1	5.5 ± 0.3	7	12.9 ± 1.5	$8.3 \times 10^6 \pm 3.2 \times 10^6$	+-----
I. Coast -Ayenoua-5482	67.5 ± 47.4	0.0 ± 0.0	23.5 ± 1.3	76.8 ± 1.0	93.4 ± 1.0	9.6 ± 0.3	9	15.1 ± 1.2	$4.7 \times 10^6 \pm 2.7 \times 10^6$	------

[1] Mortality distribution represents the number of days after which all insects were dead following the first recorded death.
[2] A "+" score was based on minimum values for each parameter as follows: mortality; 100 %; germination: > 85%; average survival time: ≤ 3.5 days; mortality distribution: ≤ 2 days; life cycle duration: ≤ 8 days; spore production: ≥ $1x10^7$ spores insect-1. The better the isolate, the higher the "+" score, with 6+ being optimal, 5+ good, 4+ average and below 4, unacceptable.

- 9.6 days (Table 1). Thirty-one isolates caused 100% coffee berry borer mortality within 3.2 and 4.0 days, while 17 took between 4 - 6 days; two isolates took longer than 6 days to kill the insects. The average survival time for the controls was 11.3 days (Table 1).

The life cycle of *B. bassiana*, which includes pathogenesis and saprogenesis (Fig. 1), was completed in 7.7 - 16.0 days, and 55.1% of the isolates completed their life cycle in less than 10 days (Table 2). The pathogenesis phase ranged between 3.1 to 9.1 days and 63.3% of the isolates completed the pathogenicity stage in less than four days. The saprogenesis life cycle ranged between 4.1 to 9.9 days and 49.0% of the isolates completed this step in less than six days (Table 1). For representative purposes, we selected three *B. bassiana* strains based on fast (Kenya 2687), medium (Ivory Cost 5486), and long (Brazil 1480) life cycles (Fig. 1). Some of the isolates caused rapid mortality shortly after exposure (Fig. 2) while others took much longer to kill the insect.

Fourteen isolates produced more than $1x10^7$ spores per beetle (Table 1). Other isolates produced between $1x10^6$ and $1x10^7$ spores per beetle. One isolate (Kenya 2686) produced $2.5 \times 10^7 \pm 0.7$ spores per beetle. There were significant differences in spore production ($F = 128.6$, $df = 1550$, $P = 0.001$).

Discussion

Various spore application methods can be used when conducting coffee berry borer bioassays with fungal entomopathogens, such as dipping the insects in the spore suspension and spraying either the berries or leaves for subsequent spore pickup by the insect, or spraying the insects directly (González et al., 1993; De la Rosa et al., 1997; Posada, 1998; Posada et al., 2002). We selected dipping for various reasons. First, spore concentrations are uniform and thus pathogenicity can be easily compared across isolates. Second, dipping provides a massive spore concentration to the insect and results in faster mortality than spraying. Previous studies have shown that coffee berry borer mortality caused by *B. bassiana* is just over three days using the dipping method (Fernández et al., 1985; González et al., 1993; Varela and Morales, 1996; De la Rosa et al., 1997; Marin et al., 2000), while spraying and exposing the insects to the droplet deposit can take more than 10 days (Posada, 1998; Posada et al., 2002). This has important implications when using the methodology for spore quality control due to the reduced time required for labor when assessing the effectiveness of the isolates. The dipping method also reduces labor when screening multiple fungal strains due to only one concentration being used in contrast to multiple doses that would result in enormous bioassay tests. We realize that this methodology does not mimic application methods

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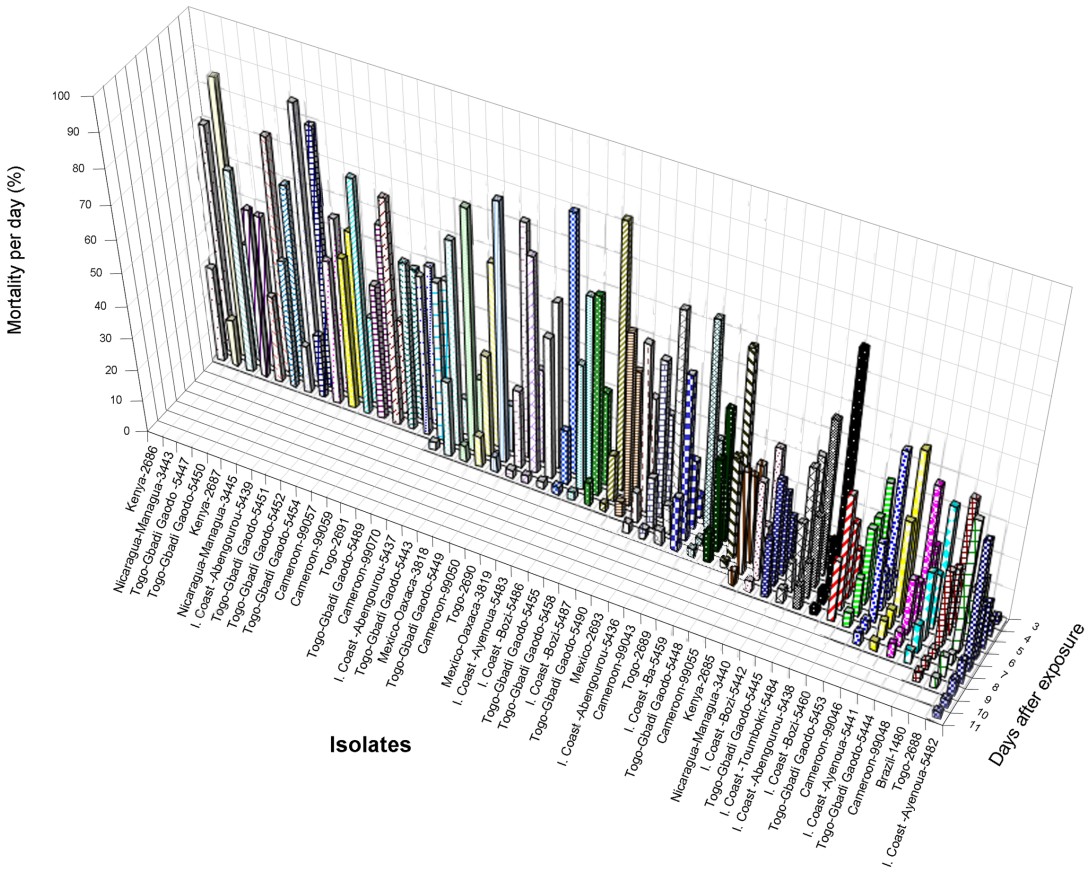

Fig. 2. Coffee berry borer percent mortality based on days after exposure to 50 different *Beauveria bassiana* fungal isolates. Notice how as one scans from left to right, some strains result in high mortality shortly after exposure, in contrast to others that take a long time to cause high mortality.

that would be used in the field, but it is a valuable tool for assessing what strains should be studied in more detail.

In the present study, there was no mortality in the controls due to fungal growth either by *B. bassiana* or fungal contaminants. This is a major improvement over other bioassay techniques used in the past (González et al., 1993) and is the result of using beetles reared in artificial diet in which the original parents are well disinfected.

The spore germination results indicate that even though the isolates came from the same host and were cultured as single spore isolates, they still respond differently. Spore germination at 24 h in the first test ranged from 0 to 95%, with isolates exhibiting no germination still causing 100% mortality, thus indicating that measuring germination at 24 h, which is what is usually done in bioassay tests (Goettel and Inglis, 1997; Legaspi et al., 2000; Kreutz et al., 2004), could be misleading in terms of assessing potential virulence for fungal pathogens. When compared to spores that germinate quickly, slow germinating spores are at a disadvantage due to them being exposed to potentially adverse conditions that might reduce their viability in the field. Spore germination rates are known to have very important consequences for insect infection (Vega et al., 1999 and references therein). Our results indicate that germination assessments at only 24 h might not yield the entire picture of fungal performance.

We also examined the life cycle of *B. bassiana* on the insect, which includes pathogenesis and saprogenesis phases. Pathogenesis begins with the formation of a germ tube, cuticle penetration and invasion throughout the insect followed by death. The saprogenesis phase begins after the insect has

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Table 2. Duration of the life cycle of *Beauveria bassiana* on the coffee berry borer (days) [1].

Isolate	FREQ	Inoculation to death	Death to start mycelium	Start mycelium to totally covered with mycelium	Mycelium covered to spore formation	Spore formation to spore discharge	Saprogenesis stages	Life cycle duration
Kenya-2686	40	3.6 ± 1.2	1.0 ± 0.2	1.7 ± 0.5	1.1 ± 0.6	4.2 ± 1.3	8.0 ± 1.4	11.6 ± 2.0
Nicaragua-Managua-3443	36	3.1 ± 0.4	1.1 ± 0.3	1.3 ± 0.5	2.7 ± 1.3	4.7 ± 1.7	9.7 ± 1.5	12.9 ± 1.6
Togo-Gbadi Gaodo 5447	40	3.6 ± 0.5	0.9 ± 0.2	0.9 ± 0.2	0.9 ± 0.4	2.4 ± 1.1	5.0 ± 1.1	8.6 ± 1.2
Togo-Gbadi Gaodo-5450	40	3.5 ± 0.5	0.9 ± 0.3	1.0 ± 0.3	0.8 ± 0.3	2.2 ± 0.9	4.8 ± 0.8	8.3 ± 1.0
Kenya-2687	40	3.3 ± 0.5	0.9 ± 0.2	1.2 ± 0.4	0.6 ± 0.2	2.5 ± 0.8	5.1 ± 0.8	8.4 ± 0.7
Nicaragua-Managua-3445	40	3.4 ± 0.5	0.9 ± 0.3	1.3 v 0.6	0.8 ± 0.3	2.5 ± 0.6	5.4 ± 0.7	8.8 ± 0.8
I. Coast -Abengourou-5439	39	3.2 ± 0.4	0.9 ± 0.3	1.4 ± 0.6	0.8 ± 0.3	2.4 ± 0.9	5.5 ± 1.1	8.6 ± 1.1
Togo-Gbadi Gaodo-5451	13	3.3 ± 0.5	1.0 ± 0.3	1.2 ± 0.4	0.7 ± 0.3	3.1 ± 1.0	6.0 ± 1.2	9.3 ± 1.2
Togo-Gbadi Gaodo-5452	40	3.5 ± 0.5	0.9 ± 0.3	1.3 ± 0.5	0.8 ± 0.4	2.5 ± 0.6	5.4 ± 0.8	8.8 ± 0.7
Togo-Gbadi Gaodo-5454	38	3.4 ± 0.5	0.9 ± 0.3	1.1 ± 0.4	0.7 ± 0.2	1.9 ± 0.5	4.6 ± 0.4	8.1 ± 0.6
Cameroon-99057	40	3.7 ± 0.5	0.9 ± 0.2	1.1 ± 0.4	0.8 ± 0.3	2.5 ± 0.8	5.2 ± 0.8	9.0 ± 0.8
Cameroon-99059	40	3.4 ± 0.5	1.0 ± 0.3	1.0 ± 0.1	0.7 ± 0.2	2.1 ± 0.6	4.7 ± 0.8	8.2 ± 0.8
Togo-2691	40	3.7 ± 0.5	0.8 ± 0.2	1.4 ± 0.5	1.1 ± 0.6	3.7 ± 0.8	7.0 ± 0.9	10.7 ± 1.0
Togo-Gbadi Gaodo-5489	40	3.5 ± 0.5	0.8 ± 0.2	1.1 ± 0.3	0.8 ± 0.4	1.6 ± 0.7	4.3 ± 0.6	7.8 ± 0.7
Cameroon-99070	40	3.5 ± 0.5	0.8 ± 0.3	1.0 ± 0.0	0.9 ± 0.2	2.0 ± 0.8	4.6 ± 0.8	8.1 ± 0.9
I. Coast -Abengourou-5437	40	3.6 ± 0.6	1.0 ± 0.3	1.4 ± 0.5	1.0 ± 0.7	2.5 ± 0.9	5.8 ± 1.3	9.4 ± 1.2
Togo-Gbadi Gaodo-5443	24	4.1 ± 0.6	1.1 ± 0.4	0.9 ± 0.3	0.8 ± 0.4	2.8 ± 1.4	5.6 ± 1.4	9.7 ± 1.3
Mexico-Oaxaca-3818	40	3.9 ± 0.5	0.7 ± 0.3	1.1 ± 0.5	1.1 ± 0.5	2.0 ± 0.9	4.8 ± 1.0	8.7 ± 1.1
Togo-Gbadi Gaodo-5449	36	4.5 ± 0.7	1.1 ± 0.5	0.9 ± 0.4	0.8 ± 0.3	2.3 ± 1.0	5.1 ± 1.1	9.6 ± 1.1
Cameroon-99050	40	3.9 ± 0.4	1.0 ± 0.2	1.4 ± 0.5	1.0 ± 0.6	3.8 ± 1.7	7.1 ± 1.6	11.0 ± 1.7
Togo-2690	36	3.3 ± 0.5	0.9 ± 0.2	1.7 ± 0.8	0.5 ± 0.1	2.7 ± 0.8	5.9 ± 0.9	9. 1 ± 0.9
Mexico-Oaxaca-3819	39	3.7 ± 0.5	1.0 ± 0.4	1.4 ± 0.6	0.8 ± 0.4	3.0 ± 1.0	6.3 ± 1.1	10. 0 ± 1.1
I. Coast -Ayenoua-5483	40	3.5 ± 0.6	0.9 ± 0.3	1.2 ± 0.4	0.7 ± 0.2	2.2 ± 0.7	4.9 ± 0.8	8.4 ± 0.7
I. Coast -Bozi-5486	40	3.2 ± 0.5	0.9 ± 0.2	1.2 ± 0.4	1.1 ± 0.7	3.2 ± 1.5	6.3 ± 1.6	9.5 ± 1.6
Togo-Gbadi Gaodo-5455	40	3.5 ± 0.6	0.9 ± 0.2	1.1 ± 0.3	0.6 ± 0.3	1.8 ± 0.5	4.4 ± 0.5	7.8 ± 0.7
Togo-Gbadi Gaodo-5458	37	3.8 ± 0.6	1.1 ± 0.4	1.4 ± 0.7	0.7 ± 0.2	1.6 ± 0.8	4.7 ± 1.0	8.5 ± 1.0
I. Coast -Bozi-5487	39	3.2 ± 0.4	0.9 ± 0.2	1.3 ± 0.6	1.0 ± 0.3	1.8 ± 0.9	5.0 ± 1.1	8.1 ± 1.2
Togo-Gbadi Gaodo-5490	40	3.7 ± 0.6	0.9 ± 0.2	1.0 ± 0.2	0.7 ± 0.2	1.5 ± 0.6	4.1 ± 0.6	7.7 ± 0.7
Mexico-2693	30	3.8 ± 0.7	1.0 ± 0.2	1.0 ± 0.3	1.1 ± 0.7	3.6 ± 1.5	6.7 ± 1.6	10.5 ± 1.7
I. Coast -Abengourou-5436	11	4.1 ± 0.5	1.3 ± 0.5	0.9 ± 0.6	1.8 ± 1.1	3.6 ± 1.0	7.6 ± 1.7	11.7 ± 1.6
Cameroon-99043	20	4.1 ± 0.7	1.0 ± 0.3	1.3 ± 0.5	1.4 ± 0.7	2.8 ± 0.9	6.4 ± 0.9	10.5 ± 1.1
Togo-2689	2	7.5 ± 0.7	1.0 ± 0.0	1.5 ± 0.7	1.0 ± 0.0	5.0 ± 0.0	8.5 ± 0.7	16.0 ± 0.0
I. Coast -Ba-5459	29	3.7 ± 0.5	1.0 ± 0.3	1.6 ± 0.7	1.9 ± 1.8	5.3 ± 2.2	9.9 ± 2.3	13.6 ± 2.3
Togo-Gbadi Gaodo-5448	39	5.3 ± 0.9	1.0 ± 0.2	0.8 ± 0.3	1.0 ± 0.6	1.7 ± 1.0	4.4 ± 1.0	9.8 ± 1.0
Cameroon-99055	38	4.3 ± 0.6	1.0 ± 0.3	1.1 ± 0.6	0.9 ± 0.5	4.2 ± 1.6	7.2 ± 1.6	11.6 ± 1.7
Kenya-2685	13	4.7 ± 1.0	0.9 ± 0.2	1.3 ± 0.5	3.3 ± 1.7	2.8 ± 1.2	8.4 ± 1.6	13.1 ± 1.3
Nicaragua-Managua-3440	26	4.8 ± 1.2	1.1 ± 0.5	2.0 ± 1.2	1.8 ± 1.1	2.2 ± 0.8	7.2 ± 1.9	12.0 ± 1.9
I. Coast -Bozi-5442	16	5.4 ± 1.0	1.3 ± 0.7	1.7 ± 1.0	1.5 ± 0.5	2.5 ± 1.1	7.0 ± 1.4	12.3 ± 1.4
Togo-Gbadi Gaodo-5445	17	4.0 ± 1.1	1.0 ± 0.3	1.1 ± 0.6	0.9 ± 0.2	2.5 ± 1.1	5.4 ± 1.1	9.4 ± 1.4
I. Coast -Toumbokri-5484	23	4.6 ± 0.9	1.0 ± 0.4	1.4 ± 0.7	1.5 ± 0.9	4.1 ± 1.5	8.0 ± 1.8	12.6 ± 1.7
I. Coast -Abengourou-5438	1	4. 0	2	1. 0	1	1	5	9.0
I. Coast -Bozi-5460	31	5.5 ± 1.2	1.2 ± 0.5	2.0 ± 0.7	1.2 ± 0.5	2.5 ± 1.3	6.9 ± 1.4	12.3 ± 1.7
Togo-Gbadi Gaodo-5453	24	4.1 ± 0.6	1.1 ± 0.4	0.9 ± 0.3	0.8 ± 0.4	2.8 ± 1.4	9.7 ± 1.3	5.6 ± 1.4
Cameroon-99046	21	4.8 ± 1.3	1.3 ± 0.6	1.5 ± 1.0	2.0 ± 1.2	2.6 ± 0.6	7.5 ± 1.0	12.3 ± 0.9
I. Coast -Ayenoua-5441	26	4.5 ± 0.9	1.0 ± 0.2	1.6 ± 1.1	1.6 ± 0.8	3.1 ± 1.2	7.3 ± 1.4	11.8 ± 1.3
Togo-Gbadi Gaodo-5444	15	4.0 ± 0.8	1.7 ± 0.7	1.9 ± 1.4	1.2 ± 0.7	2.3 ± 1.3	7.0 ± 2.1	11.0 ± 1.9
Cameroon-99048	20	5.0 ± 1.5	1.3 ± 0.6	1.2 ± 0.6	1.8 ± 0.9	2.8 ± 1.1	7.1 ± 1.2	12.0 ± 1.8
Brazil-1480	19	4.9 ± 1.0	1.5 ± 1.1	1.8 ± 1.2	1.6 ± 0.7	2.6 ± 1.0	7.5 ± 1.8	12.4 ± 1.4
Togo-2688	24	5.1 ± 1.4	1.8 ± 1.2	2.3 ± 1.3	1.4 ± 0.7	2.2 ± 1.3	7.7 ± 2.0	12.9 ± 1.5
I. Coast -Ayenoua-5482	15	9.1 ± 1.4	1.1 ± 0.4	1.2 ± 0.6	0.9 ± 0.4	2.9 ± 1.2	6.1 ± 1.0	15.1 ± 1.2

[1] Values presented for insects on which the entire *B. bassiana* life cycle was completed as indicated by "frequency."

died and has four steps: (1) mycelium begins to appear through the cuticle; (2) the mycelium covers most of the corpse; (3) spores form; and (4) spores are discharged. The isolates with a fast kill are usually selected for subsequent assessment and possible field use even though fast kill per se does not tell how long it takes for the fungus to cycle through the insect and what the subsequent level of spore production might be. If a low number of spores or no spores at all are produced on the cadaver, then a fast kill strain is not likely to increase the fungal inoculum load in the field thus requiring additional field applications which would result in increased pest control costs. Future studies should compare pest mortality across time in situations where fungal isolates result in high spore production in cadavers versus isolates that do not sporulate on the cadaver.

A spore production of $1x10^7$ spores per beetle with two-*B. bassiana* infected coffee berry borers per plant (a very conservative estimate) and 5000 plants per hectare would contribute $1x10^{11}$ spores per ha, which can be equal to what is applied in a commercial application. Thus, *B. bassiana* production on the cadaver could provide a fresh source of fungal inoculum directly in the coffee agroecosystem. Furthermore, the fungus is passing through the host, which is known to be necessary to maintain the virulence of the isolates (Tanada and Kaya, 1993; Lomer et al., 2001). Screening of fungal isolates to select promising biocontrol agents

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should consider possible recycling of the spores in the field,which might lead to new natural infections (Lomer et al., 2001). In addition, this information needs to be taken into account in order to select the more productive isolates in order to undertake fungal mass production.

Based on the parameters we measured, we developed a scoring system to rate the potential of the various *B. bassiana* strains used in the bioassays. In this scoring system a "+" was assigned when the isolate fulfilled minimum values for each parameter as follows: insect mortality of 100%; fungal spore germination > 85%; average insect survival time ≤ 3.5 days; insect mortality distribution ≤ 2 days; fungal life cycle duration ≤ 8 days; and spore production ≥ $1x10^7$ spores per insect. Thus, the higher the number of "+" signs, the better the isolate with six "+" being optimal; five "+" being good; four "+" average; and below four "+" unacceptable (Table 1). Of 50 strains we evaluated, only 11 show some potential as biocontrol agents. Thus, the protocol we have developed can be used to narrow down the number of strains to be assessed in more cumbersome or traditional spraying bioassay studies using various doses, and should result in a more efficient use of time and financial resources.

The low prices being paid to coffee growers throughout the world have resulted in a reduction of coffee pest management inputs, and thus the development of sustainable pest management strategies is a priority. Fungal entomopathogen isolates that provide a fast kill and produce a high number of spores in the insect cadaver can play an important role in causing natural epizootics, thus reducing coffee berry borer populations and leading to a more sustainable agricultural system (Lacey et al., 2001). Any investment in fungal entomopathogen production in coffee producing countries aimed at the coffee berry borer should be based on use of a strain with the highest potential for causing high mortality and for being recycled in coffee fields which, if growing under shade, have high humidity conditions favorable for *B. bassiana* (Staver et al., 2001).

An assessment of percent insect mortality and average survival time presents only a partial view of pathogenicity and does not provide adequate information to select the best fungal isolates. In this paper we present a novel method to evaluate candidate fungal entomopathogens based on six parameters: percent insect mortality, average survival time, mortality distribution, percent spore germination, fungal life cycle duration, and spore production. This information provides a more complete picture to determine which fungal isolates should be considered for subsequent mass production, formulation and possible commercialization. We have developed a screening method that should be useful in future studies aimed at assessing the potential use of fungal entomopathogens in insect pest management programs.

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