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Establishment of the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales) as an endophyte in cocoa seedlings (*Theobroma cacao*)

Francisco Posada
Fernando E. Vega[1]

Insect Biocontrol Laboratory, U.S. Department of Agriculture, Agricultural Research Service, Bldg. 011A, BARC-W, Beltsville, Maryland 20705

Abstract: The fungal entomopathogen *Beauveria bassiana* became established as an endophyte in in vitro-grown cocoa seedlings tested for up to 2 mo after inoculation to the radicle with *B. bassiana* suspensions. The fungus was recovered in culture from stems, leaves and roots. *B. bassiana* also was detected as an epiphyte 1 and 2 mo postinoculation. *Penicillium oxalicum* and five bacterial morphospecies also were detected, indicating that these were present as endophytes in the seed.

Key words: biological control, cacao, cocoa pod borer, *Conopomorpha*, entomopathogens, insect pathogens

INTRODUCTION

One possible pest management mechanism against the cocoa pod borer *Conopomorpha cramerella* (Snellen) (Lepidoptera: Gracillariidae), the most important insect pest attacking cocoa (*Theobroma cacao* L.) in Malaysia, Indonesia and the Philippines (Bradley 1986), involves the inoculation of cocoa plants with the fungal entomopathogen *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) in an attempt to establish the fungus as an endophyte (Arnold and Lewis 2005). Bing and Lewis (1991) have shown that *B. bassiana* can be introduced as an endophyte of *Zea mays*, where it causes mortality of the European corn borer, *Ostrinia nubilalis* (Hübner) (reviewed in Arnold and Lewis 2005). *B. bassiana* has been reported as an endophyte of maize (Arnold and Lewis 2005), potato (Jones 1994), tomato (Leckie 2002) and on *Theobroma gileri*, a relative of cocoa (Evans et al 2003). Recently endophytic *B. bassiana* has been isolated from coffee plants in Colombia; preliminary laboratory results demonstrate the possibility of inoculating coffee plants with *B. bassiana* using various methods (Posada and Vega unpubl data). Whether *B. bassiana* can persist as an endophyte in woody plant hosts, can spread within host tissues and can protect host plants against herbivores, such as the cocoa pod borer, has not been evaluated. Our ultimate goal is to determine whether cocoa plants inoculated with *B. bassiana* in the seedling stage can sustain the fungus in the field, and more importantly, whether *B. bassiana* can be detected in the pod, where it ideally would help control the cocoa pod borer.

The cocoa pod borer is believed to be endemic to Southeast Asia and to have shifted from rambutan (*Nephelium lappaceum* L.), pulasan (*N. mutabile* Blume) and nam-nam (*Cynometra cauliflora* L.) to cocoa, which was introduced from the American continent in the 16th century (Malaysian Plant Protection Society 1987).

Female cocoa pod borers can lay up to 100 eggs on the surface of the cocoa pod. Upon hatching, larvae bore into the pod where they feed on the pulp and placental tissue, which leads to deformed and clumped seeds and premature pod ripening, thus greatly reducing yield and quality (Malaysian Plant Protection Society 1987, Santoso et al 2004). Approximately 2 wk after hatching, larvae emerge from the pod to pupate on the leaf litter or directly on the pod, followed by adult emergence (Santoso et al 2004). Because these insects are small and larval development takes place inside the pod, they have proven to be extremely difficult to control and have caused yield losses of up to 80% (Day 1989). Various techniques have been used in an attempt to reduce pest incidence, including harvesting of all available pods to interrupt the life cycle (termed ''rampassen'', Wood et al 1992), chemical insecticides (Wardojo 1980, Day and Mumford 1994), pheromone-baited traps (Beevor et al 1993), biocontrol agents (Nagaraja et al 1986, Awang and Lee 1998) and bagging of cocoa pods (Vanialingam et al 1982). In this paper we describe the in vitro introduction of *B. bassiana* as an endophyte in cocoa seedlings, establishing an important first step in the possible use of this fungus as a biocontrol agent.

MATERIALS AND METHODS

Cocoa seeds removed from ripe pods were surface sterilized by submerging them in 10% bleach for 2 min, followed by submersion in 95% ethanol for 2 min and rinsed in sterile water three times. The seeds were placed on sterile filter paper moistened with sterile water and placed inside 100 ×

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[1] Corresponding author. E-mail: vegaf@ba.ars.usda.gov

15 mm Petri dishes. The dishes were sealed with parafilm and kept in an unlit growth chamber at 25 ± 2.0 C.

When the seeds had germinated and the roots were ca. 3 cm long, they were transferred to a laminar flow hood for inoculation with one of two isolates of *B. bassiana*: (i) Ivory Coast 5486 (IC-5486), isolated from an infected coffee berry borer (*Hypothenemus hampei* (Ferrari); Coleoptera: Curculionidae); or (ii) CS16-1, isolated as an endophyte from coffee berries in Colombia. Both isolates are highly pathogenic to the coffee berry borer (Posada and Vega 2005) and have been deposited in the Insect Biocontrol Laboratory fungal entomopathogens collection. Isolates were retrieved from single-spore stocks stored in 10% glycerol and grown on yeast-maltose agar (YMA; Sigma-Aldrich Co., St Louis, Missouri) to which a 0.1% stock antibiotic solution was added (YMA+3A). The antibiotic stock consisted of 0.02 g of each of three antibiotics (tetracycline, streptomycin and penicillin) dissolved in 10 mL sterile distilled water, followed by filter sterilization through a 0.2 µm filter (Nalgene Disposable Filterware, Nalge Nunc International, Rochester, New York); from this, 1 mL was added to each liter of medium. Spores were less than 30 d old when used. Before inoculation, spore germination rate was determined on 2.5% Noble agar (BD Difco™ Agar, Noble; Becton, Dickinson & Co., Franklin Lakes, New Jersey) as follows: a 15 µL aliquot from a 10^{-3} dilution was plated on the agar surface, spread with a sterile glass rod and incubated at 25 ± 2.0 C. At 24 h three groups of 100 spores were counted and the data were expressed as percentages.

To inoculate each individual seedling, 100 µL of a 1×10^7 mL^{-1} *B. bassiana* spore suspension were inoculated on the main radicle of each seedling with a pipette. The seeds then were placed inside individual 100 × 15 mm sterile Petri dishes and allowed to dry in the laminar flow hood before transferring to sterile 25 × 250 mm test tubes (Kimble Glass Inc., New Jersey) containing 20 mL of sterile 10% water agar (Difco Bacto Agar, Becton, Dickinson & Co., Franklin Lakes, New Jersey). Using sterile tweezers the germinated seeds were inserted individually in each tube making sure the radicle was inserted in the agar. Tubes were capped with sterile sponges overlaid with sterile aluminum foil and kept in the laboratory at ca. 12 : 12 photoperiod and ca. 25 ± 2.0 C.

Evaluations of *B. bassiana* colonization of cocoa tissues were carried out 30 and 60 d after spore inoculation. Twelve plants were used for each evaluation: four plants for each *B. bassiana* isolate and four for the control, which received 100 µL of sterile water at the moment of inoculation.

To determine the presence of epiphytes at each sampling period, leaves, stems and roots were removed from the seedling and placed in individual 50 mL sterile vials containing 20 mL of sterile water plus 0.1% Triton X-100 (Sigma Chemical Co., St Louis, Missouri). These suspensions were diluted to 10^{-4} aliquots from which 15 µL were plated on YMA+3A and spread with a sterile glass rod. For each suspension corresponding to one plant, two replicates were prepared and kept in the laboratory under ca. 12 : 12 photoperiod and ca. 25 ± 2.0 C. Four d after inoculation the colonies were counted and expressed as colony-forming units (CFU) per mL.

At all sampling times, the control was processed first, followed by CS16-1 and then IC-5486. To assess for the presence of endophytes tissues (roots, stems, leaves) were surface-disinfected by submersion in 0.5% sodium hypochlorite for 2 min, 70% ethanol for 2 min and then rinsed with sterile water (Arnold et al 2001). The tissues were dried on sterile paper towels and, after cutting off the edges to remove the dead tissue originating from the disinfection process, cut into 4 to 9 mm2 sections, six of which were placed in each of two Petri dishes containing YMA+3A. Tissues were examined four d later, and any fungal growth was subcultured onto individual plates containing YMA+3A for subsequent identification. To evaluate the quality of the surface sterilization method 10 mL of the water used to rinse the tissues after surface sterilization was taken and 15 µL aliquots of a 10^{-3} dilution were plated on YMA+3A and spread with a sterile glass rod. For each suspension two dishes were plated and after incubation for 4 d at 25 ± 2.0 C, colonies were counted and expressed as colony-forming units (CFU) per mL.

The experiment was organized as a completely randomized design with a factorial arrangement. The factors were the isolates (CS16-1, IC-5486 and the control), two sampling periods (1 and 2 mo after inoculation) and three types of tissues sampled (leaves, stem and root). Thus, at each sampling period, 432 subsamples of tissues were plated, based on the combination of two isolates and one control by four plants by three tissues (leaves, stems and roots) by two replicates for each tissue type and six subsamples plated on each Petri dish. The data obtained from the evaluation were expressed as colonization frequency using Fisher and Petrini's (1987) formula: colonization frequency = number of colonized segments divided by the total number of segments 100×. The data were square root-transformed and analyzed with ANOVA (SAS Institute, Inc. 2000).

RESULTS

Spore germination of *B. bassiana* isolates used to inoculate the radicles was >85%. Neither *B. bassiana* nor any other microorganism was ever observed in the water agar medium used to grow the seedlings. The water used to rinse tissues after sterilization yielded no microorganisms in either sampling period; thus any ensuing fungal growth from surface-sterilized tissues is inferred to have originated from internal plant tissues (i.e. as endophytes). The water used to wash nonsterilized tissues to determine whether epiphytes were present revealed the presence of *B. bassiana, Penicillium oxalicum* Currie & Thom (identified by S. Peterson [USDA, Peoria, Illinois] after sequencing ITS and partial large subunit rDNA (ID region), calmodulin gene and EF1α) and a bacterium (bacterial morphospecies 1). On average, 6.1×10^6 CFU mL^{-1} of *B. bassiana* for the plants inoculated with IC-5486 and 4.3×10^{-6} CFU mL^{-1} for CS16-1 were recovered as epiphytes, with no *B. bassiana* recovered from the controls (TABLE I). *P. oxalicum*

TABLE I. CFU's ml^{-1} of *B. bassiana*, *P. oxalicum* and bacterial morphospecies 1 isolated as an epiphyte from cocoa seedlings inoculated with *B. bassiana*. Data presented is combined for 1 and 2 mo post inoculation

Treatment	n	*B. bassiana*	*P. oxalicum*	Bacterial morpho sp. 1
Bb IC-5486	8	6.1×10^6	2.5×10^5	1.9×10^6
Bb CS16-1	8	4.3×10^6	0	0
Control	8	0	3.0×10^6	6.5×10^6

and bacterial morphospecies 1 were present in plants inoculated with *B. bassiana* IC-5486 and in the control (TABLE I). Based on the absence of these epiphytes in the pre-inoculation *B. bassiana* suspension and the demonstrated efficacy of our sterilization methods, these organisms must have originated as endophytes. The presence of *B. bassiana* as an epiphyte, based on conidiophores and hyphae on the surface of the nonradicle tissues, demonstrates an internal growth mechanism, entering through the radicle and emerging through the cotyledons, stems (FIG. 1) and leaves.

From the 432 subsamples plated for endophytes in the first mo postinoculation, 395 (91.4%) yielded fungi or bacteria, in contrast to 380 (88.0%) 2 mo post-inoculation. In addition to *B. bassiana, P. oxalicum* also was recovered; this fungus appears to be a cocoa endophyte possibly transmitted through the seed. Four unidentified bacteria were found in the first sampling period and two in the second. *B. bassiana* was isolated from 100% of the seedlings 1 and 2 mo postinoculation but never from the control.

The analysis of the colonization frequency for all pooled tissues by sampling period shows that *B. bassiana* was present in all evaluations. After 1 mo isolate CS16-1 was recovered from 21.5 ± 6.4% (mean ± standard error) of the tissue samples and isolate IC-5486 from 15.9 ± 4.8%. After 2 mo recovery for both

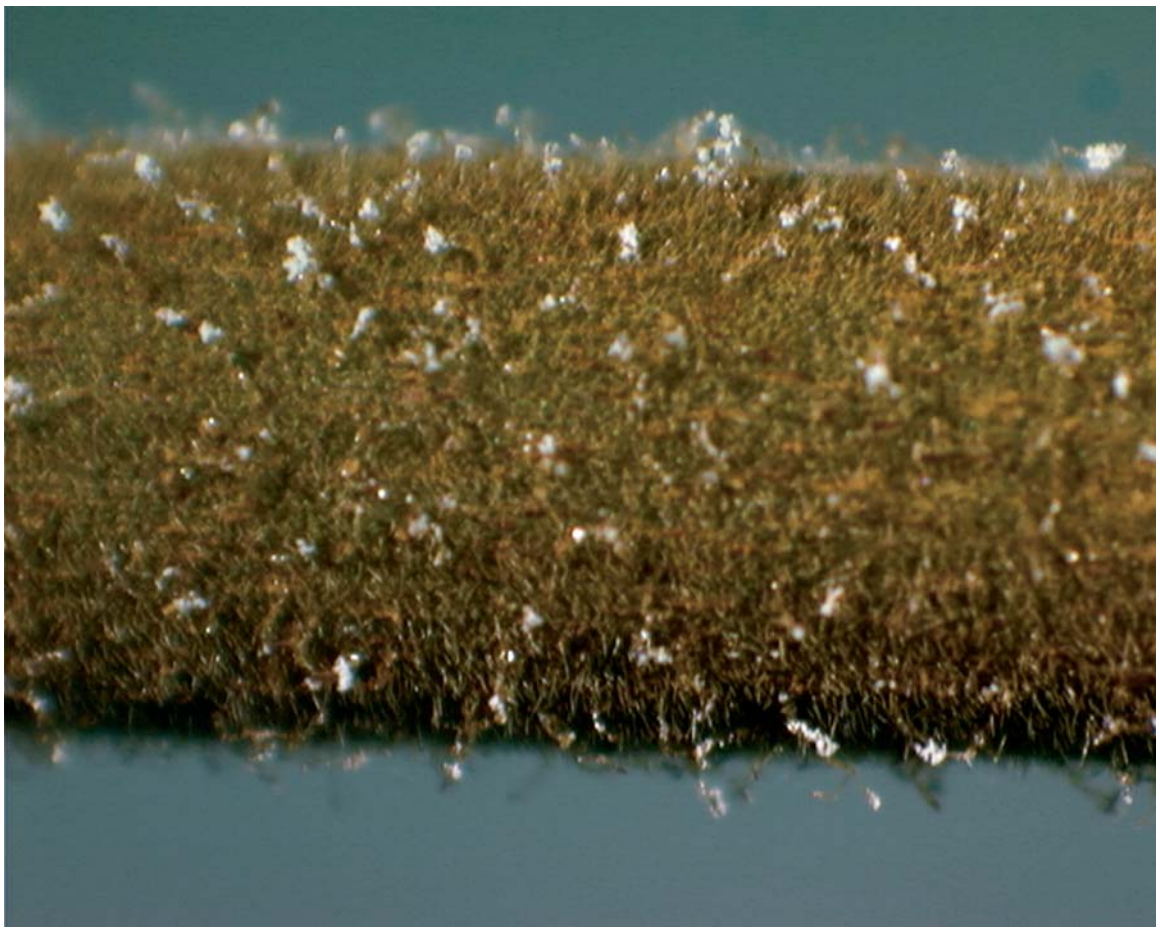

FIG. 1. *B. bassiana* growing as an epiphyte on cocoa stems 2 mo after inoculation on the radicles.

isolates increased to 62.5 ± 6.3% for CS16-1 and 53.5 ± 7.2% for IC-5486. The statistical analysis revealed significant differences for the interaction between isolates and sampling period (df = 2, 143, F = 12.7, $P < 0.001$).

Our results show that *B. bassiana* was present in all tissues sampled and exhibited a sharp increase from the first sampling period to the second. After 1 mo *B. bassiana* CS16-1 was isolated from 4.2 ± 2.7% of the leaves, in contrast to 37.5 ± 7.6% after 2 mo. For IC-5486 these numbers were 6.3 ± 4.4% after the first mo and 52.1 ± 14.6% after the second. CS16-1 colonization of stems in the first sampling period was 56.3 ± 10.9% in contrast to 68.8 ± 11.5% in the second. For IC-5486 colonization rates were 27.1 ± 8.9% for the first sampling period and 56.3 ± 10.9% for the second. The colonization rate of CS16-1 in the roots after 1 mo was 4.2 ± 4.2%, compared to 81.3 ± 7.3% after the second, while for IC-5486 the rate was 14.6 ± 9.7% after the first mo and 52.1 ± 13.5% after the second (FIG. 2). The statistical analysis for isolates by sampling period by tissues revealed significant differences (df = 4,143 F = 2.9, $P < 0.05$).

For *P. oxalicum* the statistical analysis of isolates by sampling period by tissues showed no significant differences (df = 4, 143, F = 0.63, $P > 0.05$). *P. oxalicum* was isolated from leaves, stems and roots and showed a tendency to reach higher values in the control than on the tissues that were colonized by *B. bassiana*. *P. oxalicum* also had a tendency to decrease from the first to the second sampling period in all treatments. The highest observed presence of *P. oxalicum* was obtained on the root tissues sampled after 1 mo, which decreased dramatically after the second mo (FIG. 2). The highest incidence for *P. oxalicum* was 89.6 ± 7.0% in root tissues inoculated with isolate CS16-1, followed by the control (87.5 ± 12.5%) and 64.6 ± 14.9% in root tissues inoculated with isolate IC-5486 (FIG. 2).

Based on visual characteristics, five types of bacterial morphospecies were isolated (four after 1 mo and 2 after the second). Bacterial morphospecies 1 and 2 were recovered in both sampling periods. The presence of bacterial morphospecies 1 was higher on the controls than in the plants inoculated with *B. bassiana*. It was present in all sampled tissues and for the treatments CS16-1, and IC5486 the highest values were found in the roots, followed by stems and leaves (FIG. 2). The statistical analysis of isolates by sampling period by tissues showed that there were no significant differences (df = 4, 143, F = 1.08, $P > 0.05$). The bacteria were present in all sampled tissues (leaves, stems and roots) and both fungi were isolated growing together with bacteria. No significant differences in colonization percentage were found between the bacterial morphospecies (FIG. 1).

DISCUSSION

The recovery of *B. bassiana* from cocoa tissues indicates that this fungus can become established as a cocoa endophyte when the radicles of seedlings are inoculated. Recovery from stems and leaves in addition to the roots also shows that *B. bassiana* can translocate throughout the plant tissues. The sharp increase in B. bassiana incidence in the second mo suggests that cocoa can serve as a suitable endophyte host for *B. bassiana*. The lack of any visual symptoms on the seedlings also would indicate that B. bassiana can colonize this plant without causing detriment to the host. Future studies will determine the fate of *B. bassiana* in seedlings transplanted in the field and its ability to become established within the cocoa pod. The finding that *B. bassiana* becomes an epiphyte introduces the possibility that the fungus might naturally recycle in the cocoa ecosystem. This can be an important ecological advantage for maintaining a natural *B. bassiana* inoculum.

The detection of *P. oxalicum* and five bacterial morphospecies indicate that they must be naturally occurring cocoa endophytes. The data indicate that there was a high incidence of *P. oxalicum* in the control along with several bacterial morphospecies. It was interesting to observe that plants challenged with *B. bassiana* had a lower incidence of *P. oxalicum* and bacteria, which may indicate that *B. bassiana* initiated an antagonistic response against these other organisms. When both fungi developed from tissues in the same plate an antagonist halo was observed.

Our results show that *B. bassiana* can become established in cocoa seedlings as an endophyte. We now need to determine whether *B. bassiana* will reach the pod and if so whether it will kill the cocoa pod borer. Phylogenetic studies show that the morphological species *B. bassiana* represents at least two unrelated species (Rehner and Buckley 2005). Further work will determine the phylogenetic position of the endophyte-forming isolates analyzed in this study.

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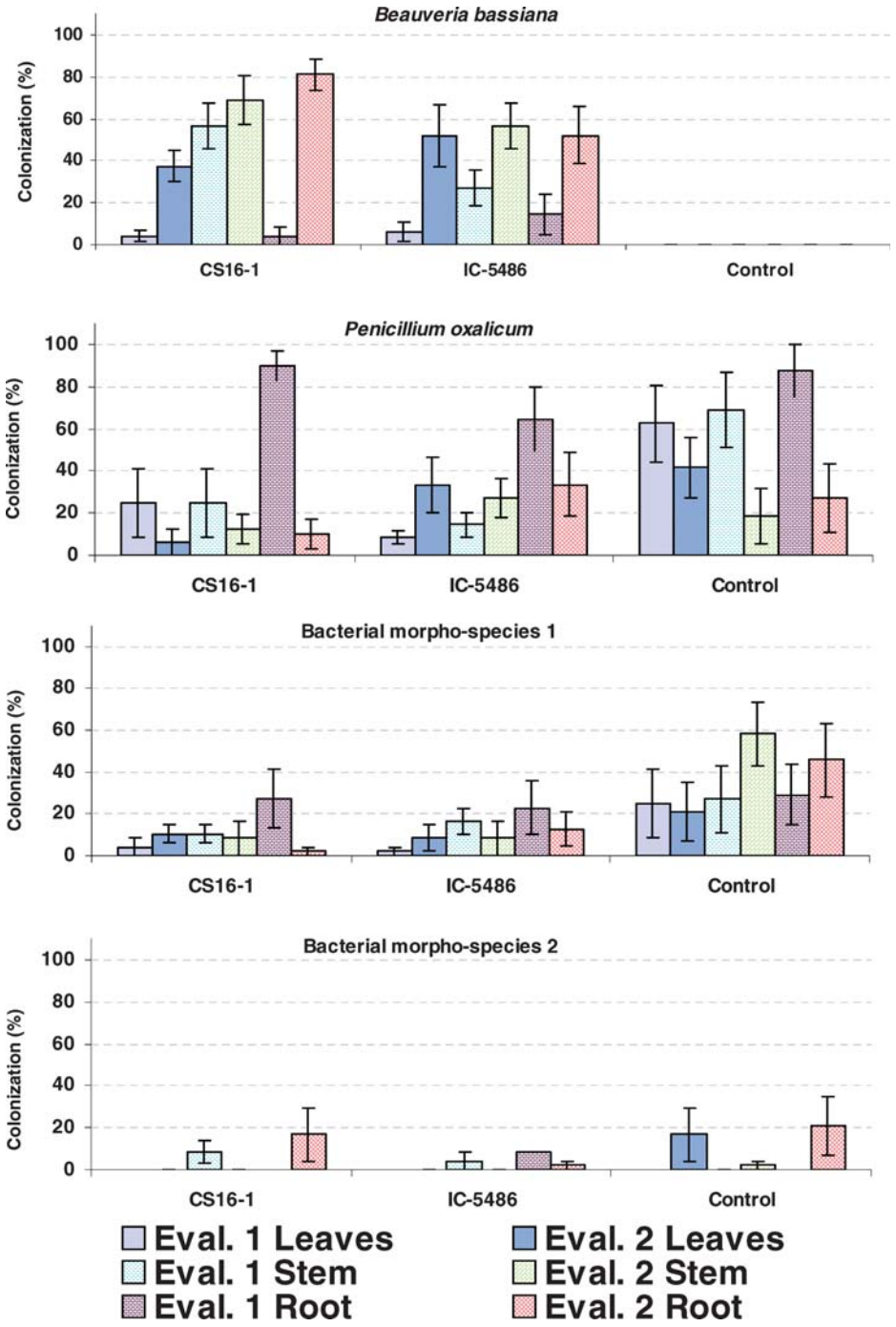

FIG. 2. Colonization frequency of *B. bassiana, P. oxalicum* and bacterial morphospecies 1 and 2 present in cocoa plants inoculated with *B. bassiana*. Bar represents the standard error for eight replicates.

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