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Characterization and virulence of *Beauveria bassiana* associated with auger beetle (*Sinoxylon anale*) infesting allspice (*Pimenta dioica*)

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ABSTRACT

The incidence of auger beetle, *Sinoxylon anale* Lesne (Bostrichidae: Coleoptera), a destructive pest of cosmopolitan occurrence is reported for the first time on allspice trees, *Pimenta dioica* (L.) Merr. in Kerala, India. The insects bored through the basal region of fresh twigs resulting in dieback symptoms. Morphological characterization and sequencing of a partially amplified fragment of the mitochondrial CO1 gene (696 bp) revealed the insect to be *Sinoxylon anale*. An entomopathogenic fungus was isolated from infected cadavers of *S. anale* that was identified as *Beauveria bassiana* (Bals.-Criv.) Vuill., sensu stricto (s.s.) (Ascomycota: Hypocreales) based on morphological and molecular studies. The partial sequences of the ITS, TUB, TEF and Bloc gene regions were sequenced. The fungus grew well in ambient room temperature conditions (28–32 ± 2 °C; 60–70% relative humidity) and the infection process on the insect was documented by scanning electron microscopy. Bioassay studies with the isolate indicated that the fungus was virulent against adult beetles as evidenced by the LC_{50} (3.6×10^6 conidia/ml) and ST_{50} values (6.8 days at a dose of 1×10^7 conidia/ml and 5.8 days at a dose of 1×10^8 conidia/ml, respectively). This is the first record of *B. bassiana* naturally infecting *S. anale* and the fungus holds promise to be developed as a mycoinsecticide.

1. Introduction

Augur beetles (Coleoptera: Bostrichidae) are economically important pests of agriculture, forestry and industrial wood products. Of the ~500 known species of the family Bostrichidae, a majority are polyphagous and a few are specialist feeders on specific host plants (Beaver et al., 2011; Price et al., 2011). Among the augur beetles, *Sinoxylon anale* Lesne, native to the Indo-Malaya and Palearctic ecozones has become cosmopolitan in distribution, mainly through transportation of infested wood materials (Price et al., 2011; Chase et al., 2012). The insect is reported to feed on more than 70 plant species in addition to wood industry products. The insect is not reported breeding on live plants, but the adults feed or hibernate by boring into live twigs of trees and saplings (Sittichaya et al., 2009). The maturation feeding on trees leads to dieback of shoots, which makes them more prone to wind breakage and secondary infection by pathogens (Liu et al., 2008; Sittichaya et al., 2009).

Allspice tree (*Pimenta dioica* (L.) Merr.) (Myrtaceae), an important spice crop valued for its fruits and oil, is widely used in different cuisines and also in the preparation of the liqueurs Chartreuse and Benedictine. Apart from its culinary value, it has anesthetic, analgesic, antiseptic, carminative, bactericidal, fungicidal, and antioxidant properties. It is widely cultivated in Jamaica, Cuba, Central America and India (Charles, 2013). Only very few insect pests have been recorded on these trees globally (Purseglove et al., 1981; Devasahayam, 2001). The control measures recommended for *S. anale* are mainly based on chemical pesticides however, insect borer pests are difficult to control with chemical pesticides due to their cryptic nature. Entomopathogenic fungi are natural enemies of a wide range of insect pests (Tanada and Kaya, 1993), and among them *Beauveria bassiana* (Bals.-Criv.) Vuill. has been developed into mycoinsecticide against many major insect pests (Faria and Wraight, 2007). Several studies have demonstrated the efficacy of different *B. bassiana* isolates against economically important bostrichid pests (Moino et al., 1998; Bourassa et al., 2001; Kassa et al., 2002; Mahdneshin et al., 2009). Identification of pathogenic strains of entomopathogenic fungi indigenous to an agroecosystem is also important for the development of mycoinsecticides (Cortez-Madrigal et al., 2003). However, to the best of our knowledge, there is no report on the natural occurrence of *B. bassiana* on bostrichid beetles.

Here we report for the first time the occurrence of *S. anale* on allspice trees; identified using morphological and molecular

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techniques using a partial CO1 gene sequence. Further, we report the occurrence of *S. anale* infected with an entomopathogenic fungus that was identified as *Beauveria bassiana* (Bals.-Criv.) Vuill., sensu stricto (s.s.) (Ascomycota: Hypocreales) using several techniques, which included morphological descriptions, scanning electron microscopy as well as molecular studies of partial sequences of five gene regions. Finally, bioassays and fungal growth studies were undertaken to assess the biological control efficacy of the fungus against this important insect pest.

2. Materials and methods

2.1. Insects

Adult live beetles (n = 10) collected from damaged shoots of 8–10 year old allspice trees grown at the research farm of ICAR-Indian Institute of Spices Research (IISR), Kozhikode, Kerala (11°17′59.81″N, 75°50′30.76″E) during July 2014 were used for identification. The morphological identity of the beetle was confirmed by the Identification Service Unit of National Pusa Collection, Division of Entomology, ICAR-Indian Agricultural Research Institute, New Delhi, India. The insect was identified based on the following key taxonomic characters: segmentation of the antenna, type of head, mouth parts, shape of the pronotum, visibility of the scutellum, elytral and leg characters. Voucher specimens were deposited in the National Pusa Collection at ICAR – Indian Agricultural Research Institute, New Delhi as 2117 CO720-725/14.

Field-collected insects (~250 nos.) maintained in the laboratory on fresh allspice twigs in plastic containers (4 l) secured with perforated plastic lids were used for laboratory bioassays. Insects were maintained at ambient room conditions (28–32 ± 2 °C, 60–70% relative humidity and a 12:12 h day:night photoperiod).

2.2. Amplification and sequencing of the mitochondrial cytochrome c oxidase subunit 1 (CO1)

For sequencing the CO1 gene, genomic DNA was extracted by grinding a whole insect using QIAmp® DNA Mini Kit (Qiagen) according to manufacturer's instructions from three insects separately. PCR amplification was performed in a 25 µl reaction volume containing 2.5 µl of 10× PCR buffer, 2.0 µl of dNTPs (2.5 mM each), 0.75 µl each of (20 µM) forward and reverse primer pairs: LEP-F1 and LEP-R1 (Hebert et al., 2004), 1 µl of genomic DNA (~10–20 ng), 0.2 µl of 5 units/µl *Taq* DNA polymerase (Sigma, USA) after making the remaining volume with PCR grade water (Himedia, India). The PCR conditions were as described by Hebert et al. (2004) with slight modifications to the annealing temperature profiles. Briefly, the profile consisted of one cycle of 1 min at 94 °C, six cycles of 1 min at 94 °C, 1 min and 30 s at 45 °C, and 1 min and 15 s at 72 °C, followed by 35 cycles of 1 min at 94 °C, 1 min and 30 s at 51 °C, and 1 min and 15 s at 72 °C, with a final step of 5 min at 72 °C. The products were confirmed for amplification by loading 5 µl of the PCR product on 0.8% w/v agarose gel, stained with ethidium bromide and visualized. The PCR products were sent to SciGenom Labs Private Ltd., Kochi, India and bi-directional sequencing was carried out in ABI 3730 XL DNA Analyzer using the same set of PCR primers. Trimmed sequences were manually edited using Bioedit software (Hall, 1999), checked for similarity and a sequence was deposited in GenBank with accession no. KT254591.

2.3. Isolation of the fungus

Cadavers (n = 2) of the beetle fully covered with fungal mycelia present in the bore tunnels of allspice shoots collected from the research farm of ICAR-IISR were used for isolation of the fungus. The cadavers were transferred to Oat Meal Agar (OMA)—CTAB medium (Posadas et al., 2012) in Petri dishes (17 × 100 mm) under aseptic conditions in a laminar air flow chamber. The plates were incubated at 26 ± 1 °C for 10 days in a biochemical oxygen demand (BOD) incubator and the fungus was further purified by sub-culturing. Single spore isolations were made from oat meal agar plates while sub culturing of the fungus on quarter strength Sabouraud's dextrose agar medium supplemented with yeast (SDAY) by arbitrarily selecting one colony forming unit (CFU) after plating a serially diluted conidia suspension of the fungus (Johny et al., 2012). An isolate of the fungus after confirming the identity was deposited in the Entomopathogenic Fungal Repository of ICAR-IISR with accession number IISR-EPF-04.

2.4. Testing the fungus against S. anale

Conidia from 2 week old fungal culture were harvested by washing with 3 ml of sterile 0.05% Triton-X 100 in water. The final conidial concentration in the suspension was adjusted to 5×10^8 conidia/ml by diluting with 0.05% Triton-X 100. Fifteen adult insects in groups were immersed in 2 ml of the conidia suspension for 30 s and transferred to a glass Petri dish (100 × 17 mm) lined at the bottom with a layer of cotton beneath a circular sterile filter paper of the same dimension of the Petri dish. An equal number of insects dipped in sterile 0.05% Triton-X 100 served as control. The experimental setup was maintained at ambient room conditions (28–32 ± 2 °C, 60–70% relative humidity and a 12:12 h day: night photoperiod) and replicated four times. The insects were fed with fresh allspice twigs cut into pieces and a moist cotton ball was placed inside the Petri dish to maintain humidity. The mortality of insects was recorded daily after 24 h of post treatment up to 6 days post inoculation (p.i.). Samples of insects showing signs of mycosis were observed under a light microscope to confirm the infectivity by the entomopathogen and the fungus was re-isolated from the dead insects.

The median lethal concentration (LC_{50}) of the fungus to the insect was tested at four conidial concentrations: 10^5, 10^6, 10^7 and 10^8 conidia/ml. For median survival time (ST_{50}) studies, conidial concentrations of 10^7 and 10^8 conidia/ml were used. The experimental setup was as described above. Ten healthy insects were used in each treatment that was replicated three times. Conidial suspensions were prepared separately for each replication. In control, the insects were exposed only to sterile 0.05% Triton® X-100 solution. Insect mortality was recorded at 24 h intervals for LC_{50} studies and at 12 h intervals for ST_{50} assays. Insect mortality due to fungal infection was recorded up to 10 days p.i. The bioassays were repeated twice with freshly prepared conidial suspensions to confirm the results.

2.5. Identification of the fungus

2.5.1. Morphological characterization and influence of temperature on growth

The fungus was identified based on macro- and micro-morphological characters (Humber, 2012) as well as using molecular tools. The conidial measurements were recorded using Leica DM 5000 B research microscope fitted with a digital camera.

Influence of temperature on the growth of the fungus was tested at four different temperatures: 20 ± 1 °C, 27 ± 1 °C, room temperature (28–32 ± 2 °C) and at 35 ± 1 °C. Agar plugs of 6 mm diameter obtained from the periphery of 2 week old culture were inoculated on Petri dishes (17 × 100 mm) containing quarter strength SDAY medium. There were five replications under each temperature condition and the colony growth was recorded 15 days after inoculation (DAI).

2.5.2. DNA extraction, PCR and sequencing

For molecular identification, DNA was extracted from mycelium and conidia (~500 mg) scraped from 2 week old culture grown on quarter strength SDAY medium using fungal genomic DNA extraction Kit (Chromous Biotech, India) following the manufacturer's instructions. PCRs were performed to amplify the ITS gene with primers ITS4 and ITS5 (White et al., 1990), partial β tubulin gene (TUB) with primers bt2a and bt2b (Glass and Donaldson, 1995), partial translation elongation factor 1α gene (TEF) with primers 983F and 2218R (Rehner and Buckley, 2005) and the intergenic Bloc region with primers B5.1F and B3.1R (Rehner et al., 2006). PCR conditions for ITS, TUB, TEF and Bloc were as described by Senthil Kumar et al. (2015a) and Rehner et al. (2006) with 10–20 ng of template DNA in volumes of 25 μl as described above with slight modifications to the annealing temperature profiles. Briefly, PCR conditions for ITS were: initial denaturation at 94 °C for 3 min, followed by 34 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and a final extension step at 72 °C for 10 min; for TUB: initial denaturation at 94 °C for 4 min, followed by 35 cycles at 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min, and a final extension step at 72 °C for 7 min; for TEF: initial denaturation at 94 °C for 4 min, followed by 37 cycles at 94 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min 30 s, and a final extension step at 72 °C for 7 min; for Bloc: initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min 30 s, and a final extension step at 72 °C for 10 min. The amplified bands were excised, purified using Sigma GelElute™ Gel Extraction Kit (Sigma, India) as per manufacturer's protocol and sequenced as described earlier. The sequences were deposited in GenBank with accession numbers KU363833–KU363836 and subjected to BLAST (Basic Local Alignment Search Tool) search to identify sequences deposited in GenBank that had significant homology.

2.5.3. Phylogenetic analysis

The ITS, Bloc and TEF sequences were multiple aligned with gene sequences of 12 reference *Beaveria* species (Rehner et al., 2011) retrieved from the GenBank using MUSCLE incorporated in MEGA 5.05 (Tamura et al., 2011). A partition homogeneity test was performed to assess incongruences among these three gene sets with PAUP* version 4.0a149 (Swofford, 2002). The test indicated no incongruence between the Bloc and ITS genes ($P = 0.11$), whereas the gene sets ITS, TEF and TEF, Bloc were incongruent ($P = 0.01$). The Bloc and ITS gene sequences were concatenated and an optimal evolutionary model was selected based on the lowest score value of Bayesian Information Criterion (BIC) available in MEGA 5.05. The maximum likelihood (ML) phylogenetic tree for Bloc + ITS was constructed using the obtained optimal evolutionary model, Tamura 3-parameter model plus Gamma (T92 + G) and for TEF sequences using Tamura-Nei model plus Gamma (T93 + G). *Isaria cicadae* Miq. (Ascomycota: Cordycipitaceae) was included as outgroup. The TUB sequence was aligned with published sequences of *B. bassiana* (Fitriana et al., 2015; Neelapu et al., 2009), *B. amorpha* (Höhn.) Minnis, Rehner & Humber and *B. brongniartii* (Sacc.) Petch (Neelapu et al., 2009) retrieved from GenBank and a ML tree was constructed using Kimura two-parameter model (Kimura, 1980) based on the score of BIC. *Cordyceps militaris* (L.) Fr. (Ascomycota: Clavicipitaceae) was used as outgroup. Gaps in alignment were treated as missing data and bootstrapping was performed with 1000 replicates.

2.6. Scanning electron microscopy (SEM)

For SEM studies, sporulated insect cadavers were dehydrated in a desiccator for 5 days at room temperature. The specimens were mounted on carbon stubs, gold sputtered for 20 s and directly viewed under a Scanning Electron Microscope (Hitachi SU6600) (Senthil Kumar et al., 2015a).

2.7. Statistical analysis

Data on colony growth were subjected to one-way ANOVA and multiple comparisons were undertaken using a least squares difference (LSD) post hoc test ($\alpha = 0.05$) by SAS® 9.3 software for statistical analysis (SAS, 2011). Per cent mortality of beetles during bioassay was subjected to Abbott's correction (Abbott, 1925). The median lethal concentration (LC_{50}) to kill the insects was determined as described by Finney (1962) using probit analysis by LdP Line software (Bakr, 2016) after adjusting the control mortality. Median survival time (ST_{50}) of the insects under each treatment and 95% confidence intervals were calculated based on Kaplan-Meier survival distribution functions and compared by log-rank test using the web-based program OASIS (Yang et al., 2011).

3. Results

3.1. Insect identification and sequencing of mitochondrial CO1 gene

Insects were found to bore into fresh allspice twigs through the basal region, resulting in drying of twigs from the tip presenting dieback symptom (Fig. 1a) with a circular bore hole in the shoots (Fig. 1b). The insect was identified as *Sinoxylon anale* Lesne (Bostrichidae: Coleoptera) based on taxonomic characters. The insect had a distinct hypognathous head, eyes were round or oval, prominent and posteriorly fitting tight against pronotum. Antennae three segmented with segments in the antennal club transverse or flabellate forming a strongly lamellate club. Mandibles short and powerful with their apices broadly rounded with a distinct labrum and simple palpi. Pronotum hood shaped, covering head from above without distinct lateral margins with its anterior

Fig. 1. (a) *Sinoxylon anale* infested twigs of allspice presenting a dieback symptom. (b) Presence of bore hole with extruding frass on an infested twig.

margin truncate/emarginate with asperites across middle and especially behind front angles with fine granules behind front margin and on posterior half, covered with short decumbent pubescence. Scutellum visible and elytra parallel sided, without distinct epipleura, with strong apical declivity bearing tubercles, spines and ridges and a pair of conical, slightly separated spines next to suture on the elytral declivity. Fore legs with conical coxae, their cavities open behind and hind legs with simple 5 segmented tarsi at least as long as their tibiae. Abdomen had 5 visible sternites and the basal sternite sub equal to second. An approximate 696 bp portion of the CO1 gene region was amplified and the sequence showed 82% similarity with a *Sinoxylon* sp. (JX412742) available in the GenBank database.

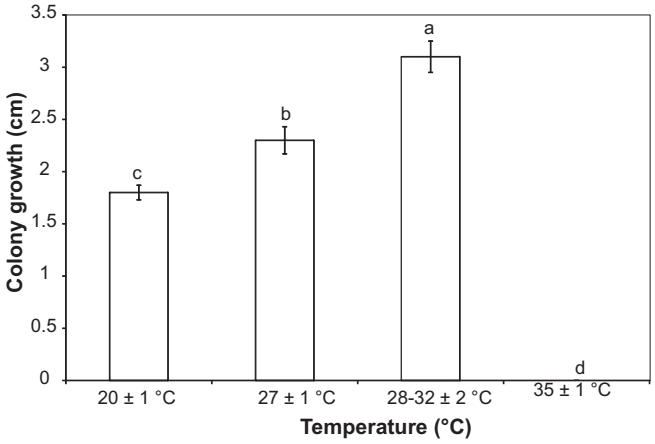

Fig. 2. Mean (±SE) colony diameter of *Beauveria bassiana* grown at different temperatures. Error bars represent SE error of five replicates. Bars represented by different letters are significantly different by LSD ($\alpha = 0.05$).

3.2. Bioassays

At the tested dose of 5×10^8 conidia/ml, 100% mortality was achieved within 5 days p.i. The insects that died in treatments were colonized by profusely growing mycelia with powdery conidia presenting a mummified appearance. However, insects that died in control, when plated on quarter strength SDAY did not show fungal growth and the average control mortality was only 5.0%. The LC_{50} value against adult stages of *S. anale* was 3.6×10^6 (upper 95% confidence interval (CI): 2.0×10^6; lower 95% CI: 6.5×10^6) conidia/ml. The ST_{50} of *S. anale* adults when treated at 1×10^7 conidia/ml was 6.8 days (±0.27) (upper 95% CI: 6.2 days; lower 95% CI: 7.3 days) and 5.8 days (±0.27) (upper 95% CI: 5.2 days; lower 95% CI: 6.3 days) at a dose of 1×10^8 conidia/ml, respectively. The ST_{50} values were significantly different from each other at the two doses tested (Log-rank test; $P < 0.05$).

3.3. Identification of the fungus

3.3.1. Morphological identification and influence of temperature on growth

The fungus was identified as *Beauveria bassiana* (Bals.-Criv.) Vuill., sensu stricto (s.s.) (Rehner et al., 2011) based on morphological characters. Colonies on quarter strength SDAY media appeared white and powdery with yellowish white reverse. Conidia were observed in a zigzag orientation on a sympodial rachis, a typical character of *B. bassiana*. The shape of the conidia was more or less globose and had an average length of 1.6 ± 0.03 µm (range 1.3–2.2 µm) and width of 1.3 ± 0.03 µm (range 0.9–1.8 µm) (n = 50).

Growth of the fungus was significantly influenced by temperature ($F = 153.4$; $P < 0.001$; $df = 3, 12$). At room temperature, the fungus reached an average diameter of 3.1 ± 0.15 cm (range

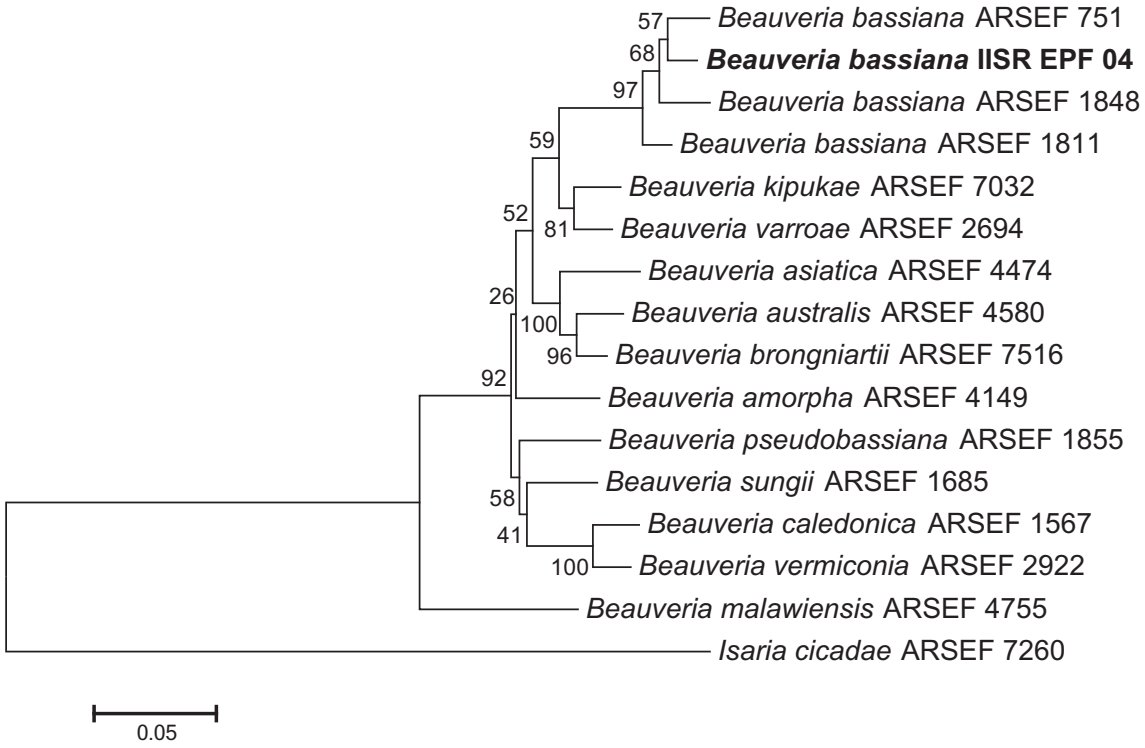

Fig. 3. Maximum likelihood (ML) tree based on concatenated nucleotide sequences of partial, Bloc and ITS gene sequences of different *Beauveria* spp. Numbers above or below the nodes indicate bootstrap values generated after 1000 replications using the Tamura 3-parameter model plus Gamma distributed with invariant sites (T92 + G) and the evolutionary distances are in the units of the number of base substitutions per site. Fungal species and sequences obtained from GenBank are shown with their strain numbers. *Isaria cicadae* was used as outgroup.

2.7–3.5 cm) 15 days post inoculation (p.i.). The mean colony diameter was significantly less at the other lower temperatures tested: 2.3 ± 0.13 cm and 1.8 ± 0.7 cm at 27 ± 1 °C and 20 ± 1 °C respectively. At the highest temperature tested (35 ± 1 °C), the fungus failed to grow even after 15 days p.i. (Fig. 2).

3.3.2. Molecular characterization

To further characterize the fungus, partial sequences of the ITS (545 bp); Bloc (1485 bp), TEF (875) and TUB (342 bp) regions were obtained. BLAST homology searches showed that the present isolate shared 100% similarity with several *B. bassiana* isolates for ITS and 99% similarity with several isolates of *B. bassiana* reported elsewhere for the Bloc and TEF regions. However, it showed 100% similarity with three strains of *B. bassiana* (AB830334, AB829899 and AB829898) for the TUB gene. Phylogenetic analysis with concatenated nucleotide sequences of Bloc and ITS (Fig. 3) and separately for TEF and TUB gene sequences (figures not shown) of *Beauveria* spp. showed that our isolate (IISR-EPF-04) clustered with other *B. bassiana* isolates with a bootstrap support of 97% inferred by maximum likelihood (ML) phylogeny supporting its morphological identity as *B. bassiana*.

3.4. SEM studies

SEM studies proved the infection and colonization of *B. bassiana* in *S. anale*. Conidia adhered to and germinated on the host cuticle (Fig. 4a). Intercalary appressoria and infective pegs (Fig. 4b) produced by mycelial hyphae were found to penetrate the body surface of the insect and signs of cuticle degradation were observed on the cuticle. The hyphae were found to emerge through the inter-segmental membranes (Fig. 4c). Ramification of mycelial network with conidial balls (Fig. 4d) bearing globose conidia arranged on a sympodial rachis typical to *B. bassiana* were found throughout the body surface presenting a mummified appearance of the host.

4. Discussion

We have recorded *S. anale* infesting allspice trees from India and also a naturally occurring entomopathogen infecting this pest. To the best of our knowledge, this pest is hitherto not reported on allspice. Affected plants showed symptoms of dieback with extrusion of frass through bore holes. Severely affected twigs showed a wilted appearance from a distance. More than 70% of the trees were found to be infested under field conditions (n = 26). In the laboratory, the beetles fed on fresh host plant twigs by making feeding tunnels, confirming their pest status. Only adult beetles were present in the bore tunnels and other developmental stages of the insect were absent confirming earlier reports that the adults can bore into live twigs of trees and saplings for maturation feeding (Argaman, 1987; Sittichaya et al., 2009).

We have isolated *Beauveria bassiana* (Bals.-Criv.) Vuill., sensu stricto (s.s.) (Rehner et al., 2011) from naturally infected *S. anale* and the fungus was identified based on morphological, molecular and SEM studies. The primary criterion for selecting an isolate towards developing it as a mycoinsecticide is based on its virulence as determined by laboratory bioassays (Reay et al., 2008). In our bioassay studies, the fungus was able to cause 100% mortality of the test insects, which is similar to the findings reported earlier

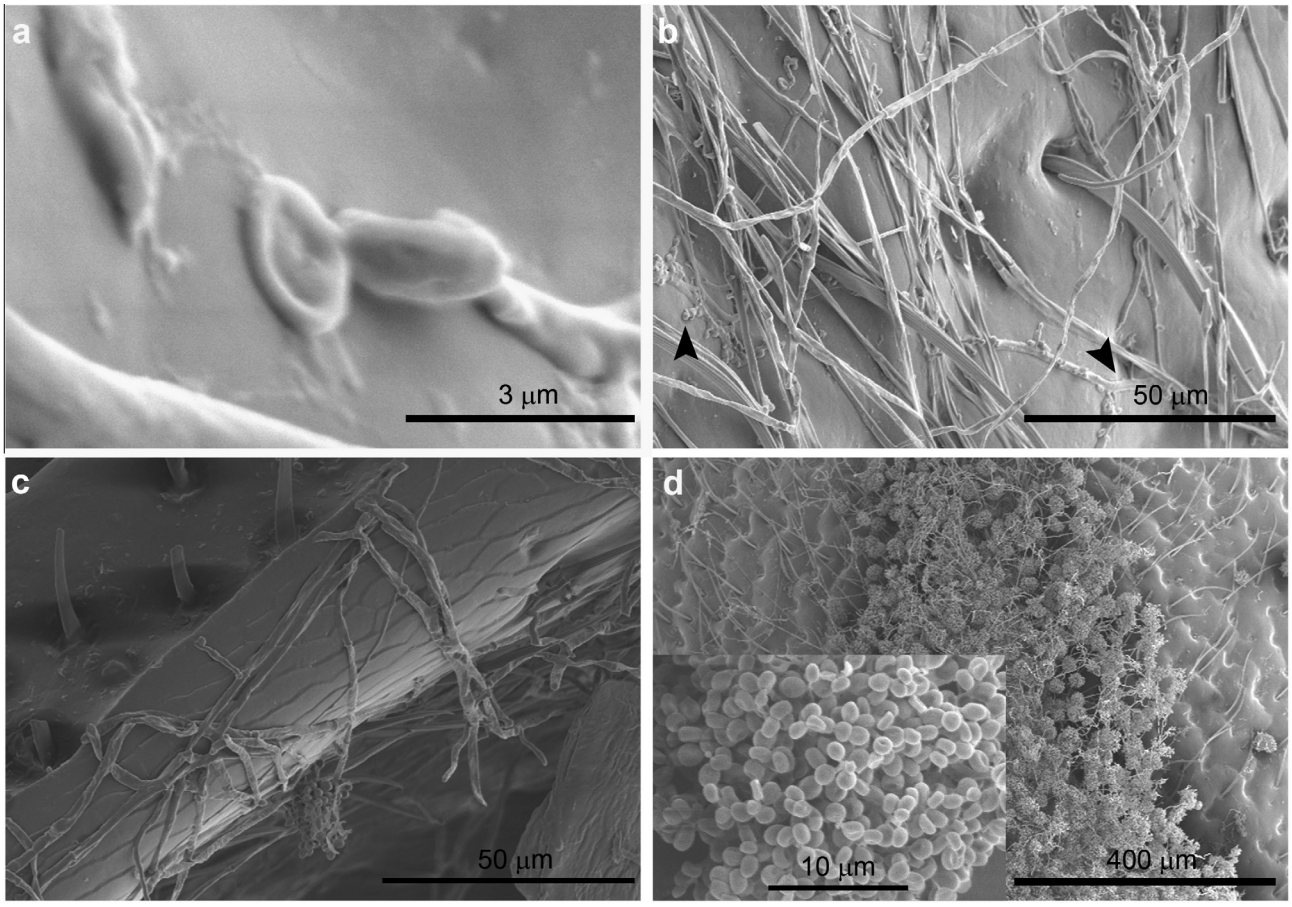

Fig. 4. Scanning electron micrographs showing infection of *Sinoxylon anale* by *Beauveria bassiana*. (a) Adhesion and germination of conidia on host cuticle. Bar = 3 μm. (b) Intercalary appressoria formation and infective pegs produced by hyphae (arrows indicate signs of cuticle degradation). (c) Emergence of hyphae through inter-segmental region. Bars = 50 μm in (b and c). (d) Mycelial network with conidial balls and (inset) close up of conidia arranged on a sympodial rachis. Bar = 400 μm and inset bar = 10 μm.

against other bostrichids, *Rhyzopertha dominica* (F.) (Moino et al., 1998) and *Prostephanus truncatus* (Horn) (Kassa et al., 2002) by *B. bassiana*. The present isolate was found to be virulent against adult beetles of *S. anale* as evidenced by the LC_{50} value of 3.6×10^6 conidia/ml. Mahdneshin et al. (2009) reported LC_{50} values in the range of 9.6×10^5 to 1.9×10^7 conidia/ml for different isolates of *B. bassiana* against *R. dominica*. The ST_{50} values of adult beetles when treated with *B. bassiana* ranged from 5.76 to 6.75 days, which is lower than the previous reported values for *R. dominica* (Mahdneshin et al., 2009) and slightly higher than those reported for *P. truncatus* (Kassa et al., 2002). However, it is difficult to make comparisons across insect species because different insects were found to respond differently to different fungal isolates as evidenced from earlier reports (Moino et al., 1998; Bourassa et al., 2001; Kassa et al., 2002; Mahdneshin et al., 2009).

Colony morphological characteristics and micro-morphological features of the present fungus agreed with the earlier described features of *B. bassiana* (Humber, 2012). Colonies were white, powdery and conidia were more or less globose in shape. In general, globose conidiogenous cells and production of conidia in sympodial succession on an indeterminate, denticulate rachis is the feature of the genus *Beauveria*. However, colony growth characteristics and shape and size of the conidia overlap extensively among *Beauveria* spp., making it difficult to differentiate them based on morphological characters alone (Rehner et al., 2011). Sequence data of conserved gene regions provide much deeper taxonomic analysis than morphological characters and also they are comparable across studies (Beris et al., 2013). Phylogenetic assignment of *Beauveria* isolates to a particular species can be accomplished by sequencing the partial ITS, TEF, intergenic Bloc (Rehner et al., 2006, 2011) and TUB (Neelapu et al., 2009) gene regions.

In our studies, molecular analysis of the gene regions proved to be a valuable tool in confirming the identity of the fungus. BLAST homology search for the sequence data generated for the four conserved gene regions (ITS, TEF, Bloc and TUB) of the present isolate showed high level of similarity with *B. bassiana* isolates reported elsewhere. Phylogenetic analysis with concatenated nucleotide sequences of Bloc and ITS for the present isolate and that of representative sequences published in Rehner et al. (2011) placed the fungus in the *B. bassiana* clade with high bootstrap support confirming its morphological identity. The TEF and TUB phylogram also supported its identity as *B. bassiana*.

Knowledge about the growth of a fungus in relation to temperature is a prerequisite for developing it as a mycoinsecticide (Yeo et al., 2003). Studies have indicated that growth of the entomopathogenic fungi was highly influenced by changes in temperature (Fargues et al., 1997; Devi et al., 2005). Our studies also showed that the growth of the fungus was significantly influenced by temperature. The fungus attained its maximum growth at room temperature, which had a cyclic temperature regime of 28–32 ± 2 °C during December. At a continuous temperature regime of 25–32 °C, *B. bassiana* germinated and grew rapidly (James et al., 1998) and at 32 ± 1 °C (8 h)/25 ± 1 °C (16 h), all isolates of *B. bassiana* showed more than 90% relative growth (Devi et al., 2005). *B. bassiana* isolates from the warmest regions of Japan exhibited highest growth rates at 30 °C, and tended to have higher optimal temperatures for mycelial growth than isolates from coldest regions, indicating that the optimal growth temperature can vary depending on geographic origin (Shimazu, 2004). The present isolate being from a tropical region supports the view of these earlier findings indicating a positive association between the optimal growth temperature and geographical origin. However, it also appears the converse association in some studies showing that no apparent relationship exists between climatic origin of the *B. bassiana* isolates and their ability to grow at higher temperatures (Fargues et al., 1997; Devi et al., 2005).

The infection process of an entomopathogenic fungus on its insect host could be studied by scanning electron microscopy (Vestergaard et al., 1999; Güerri-Agulló et al., 2010; Senthil Kumar et al., 2015a). In our studies, we observed adhesion and germination of conidia, penetration into the host, mycelial colonization on the body surface and sporulation. Initial attachment and germination of fungal conidia is based on hydrophobic interactions and permanent attachment is mediated through the secretion of mucilage (Boucias and Pendland, 1991). Appresoria formation and secretion of mucilaginous substances have been documented earlier in *B. bassiana* (Vestergaard et al., 1999). Signs of depression were seen at the site of penetration indicating degradation of the epicuticle, which could be due to secretion of cuticle degrading enzymes or mechanical pressure (Vestergaard et al., 1999; Senthil Kumar et al., 2015a). Towards the later stages of infection, the mycelia formed a network throughout the body bearing conidial balls indicating completion of life cycle in the host (Güerri-Agulló et al., 2010; Senthil Kumar et al., 2015a).

As the present trends in the selection of an isolate for microbial control of pests emphasize not only its virulence (Senthil Kumar et al., 2015b) but also its adaptability to the abiotic conditions at which the pest survives (Ibarra-Cortés et al., 2013), isolation of a *B. bassiana* isolate with high virulence and its ability to grow at higher temperatures holds promise to develop it as a mycoinsecticide against *S. anale*, a notorious pest of global importance, which causes severe economic damage to various crops and industrial produce. Further, entomopathogenic fungi are now known to have several newly understood traits like plant endophytes, plant disease antagonists, rhizosphere colonizers, and plant growth promoters (Lacey et al., 2015). Our future studies will aim at understanding these traits of the fungus and developing suitable delivery systems for management of this pest species.

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