BioControl (2012) 57:565–571
DOI 10.1007/s10526-011-9424-0

Establishment of the fungal entomopathogen *Beauveria bassiana* as a season long endophyte in jute (*Corchorus olitorius*) and its rapid detection using SCAR marker

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Received: 4 May 2011 / Accepted: 8 November 2011 / Published online: 17 November 2011

Abstract An experiment was conducted to introduce the entomopathogen *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) as an endophyte in jute (*Corchorus olitorius*), a bast fibre crop through seed treatment. Colonization of root, leaf, stem, capsule, and seed were assessed through plating on selective medium and PCR based detection using *B. bassiana* specific SCAR markers. Endophytic colonization was detected in all the plants grown from treated seeds, but all the plant parts were not colonized. Colonization was detected in leaves, stems, and green capsules but not in roots and seeds. The endophytic colonization was influenced by both plant part and sampling period. Colonization was greater in leaves (55.87%) compared to stems (12.53%) and capsules (42.44%). The percent colonization was higher in case of 60 days old plants (43.34%) than in 30 days (23.89%) and 120 days (35.39%) old plants. As *B. bassiana* has already been reported to be pathogenic on jute pests, namely semilooper (*Anomis sabulifera*) and bihar hairy caterpillar (*Spilosoma obliqua*), its season long endophytic colonization within jute plant suggests a novel approach of biological control of these pests through seed treatment with the entomopathogen.

Keywords *Beauveria bassiana* · Endophyte · Colonization · Jute · PCR

Handling Editor: Helen Roy

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Introduction

Biological control of crop pests using endophytic fungal entomopathogens viz., *Beauveria bassiana* (Balsamo) Vuillemin, *Lecanicillum lecanii* (Zimmermann) Zare et W. Gams, *Paecilomyces farinosus* (Holm ex S. F. Gray) Brown & Smith, *Paecilomyces varioti* Bainier etc. is receiving research attention in recent years (Vega et al. 2008). Among these *B. bassiana* is the most widely used entomopathogen. It has been reported to control many crop pests such as stem borers, beetles, aphids, mites, termites, white flies, mealy bugs, thrips etc. (Feng et al. 1994; Shah and Pell 2003). However, most of these biocontrol measures are based on exogenous application of *B. Bassiana* as spray formulations (Shah and Pell 2003). Endophytic expression of an entomopathogen within the plant system is expected to be more advantageous than external application of bioagents because of continued presence and assured expression of the entomopathogen

throughout the crop cycle. Moreover, it is cost effective as multiple foliar sprays are not required.

Colonization, proliferation and establishment as an endophyte within a host plant are the prerequisites for providing durable resistance through such an approach. *B. bassiana* has been recovered as an endophytic colonist from several plant species including maize (Bing and Lewis 1991; Wagner and Lewis 2000; Arnold and Lewis 2005), potato (Jones 1994), cocoa (Posada and Vega 2005), coffee (Posada et al. 2007), banana tissue cultures (Akello et al. 2007), date palm (Gómez-Vidal et al. 2006), and sorghum (Tefera and Vidal 2009). Gurulingappa et al. (2010) reported *B. bassiana* to be endophytic on corn, wheat, cotton, bean, tomato, and pumpkin. There are some reports of pest control by endophytic *B. bassiana*. Cherry et al. (2004) recorded suppression of stem borer *Sesamia calamistis* Hampson in maize by treating with endophytic *B. bassiana* isolate. Endophytic *B. bassiana* was also reported to reduce the survival of banana stem weevil, *Cosmopolites sordidus*, and the damage caused by it in tissue cultured banana plants (Akello et al. 2008). Vega et al. (2008) reported control of coffee berry borer, *Hypothenemus hampei* Ferrari by using endophytic *B. bassiana*.

However, no reports are yet available as to whether *B. bassiana* could be introduced as an endophyte in jute (Jew's mallow, tossa jute, *Corchorus olitorius* L.). Jute is the most important bast fibre crop of the world, which is mainly grown in the South East Asian countries like India, Bangladesh, Nepal, China, Indonesia, Thailand, Myanmar, and few South American countries. Jute fibre has wide applications in making ropes, sacks, bags, carpets, shoes, geo-textiles, jewellery, and home decorations. Tender jute leaves, which are rich in antioxidants, are also used as a leafy vegetable and in soups (Kundu 1956). Despite tough competition from synthetics, use of jute fibre is increasing because of its eco-friendly, biodegradable, and recyclable nature (Roy 2010).

Pest infestation is a major problem in jute cultivation and it is attacked by a number of pests of which two lepidopterans namely, jute semilooper, *Anomis sabulifera* Guen. and bihar hairy caterpillar, *Spilosoma obliqua* Walk. (Rahman and Khan 2007) are most important. *B. bassiana* has earlier been reported to be pathogenic on jute semilooper (Pandit and Som 1988). We have also confirmed the pathogenicity of *B. bassiana* to jute semilooper and bihar hairy caterpillar. *B. bassiana* caused characteristic mycosis on bihar hairy caterpillar. In the present study an attempt was made to introduce *B. bassiana* as an endophyte in jute as a first step towards the use of this entomopathogen for the biological control of jute pests in a novel way. Following this, we monitored the persistence of endophytic colonization of *B. bassiana* in different parts of jute plants during the crop growth period through cultivation and PCR based detection techniques.

Materials and methods

B. bassiana strain and its maintenance

Beauveria bassiana strain ITCC (Indian type culture collection) 4796, which had been isolated from flea beetle (*Nisotra orbiculata* Motsch.) on mesta (*Hibiscus cannabinus* L.) at Central Research Institute for Jute and Allied Fibres, Barrackpore, Kolkata, India was used in the experiment. The fungal culture was maintained at 25°C on potato dextrose agar (PDA). The conidia were harvested by scraping the surface of three-weeks-old fungal culture with a sterile camel hair brush into a 100 ml glass beaker containing 50 ml sterile distilled water. The conidial suspension was prepared by mixing the solution with a magnetic stirrer for 5 min. Then the conidia concentration was adjusted to the desired concentration of 1×10^8 conidia ml^{-1} using haemocytometer and a light microscope (40× magnification).

Seed inoculation and sowing

Jute seeds of the most popular variety JRO 524 were obtained for the experiment from Crop Improvement Division, Central Research Institute for Jute and Allied Fibres, Barrackpore, India. The seeds were surface sterilized by treating with 0.5% sodium hypochlorite for 2 min and then with 70% ethyl alcohol for 2 min. The seeds were then washed with sterile distilled water. For seed inoculation 10 g seeds were immersed in 10 ml of *B. bassiana* conidial suspension (1×10^8 conidia ml^{-1}) and kept overnight at room temperature. The inoculated seeds were dried by mixing with sterile garden soil. Then they were sown in earthen pots (15 cm diameter) filled with sterile soil (autoclaved at 121°C for 15 min). Non-treated seeds were also surface sterilized and soaked overnight in distilled water and sown similarly in

separate pots to serve as control. Each treatment was replicated three times. The pots were kept in the glass house and watered regularly. Five plants were maintained per pot. Nine pots had *B. bassiana* treated plants and three pots served as untreated control.

Colonization study on selective medium

Colonization of jute plant parts namely root, stem and leaf by *B. bassiana* was studied at 30 (initial crop growth stage), 60 (active crop growth stage), and 120 days (fibre harvest stage) after sowing of treated seeds. In 120 days old plants colonization in green capsules was also studied. Out of total 60 plants, randomly selected 20 plants (15 treated and five untreated control) were evaluated at each sampling period. During each sampling period the plants/plant parts were carefully removed and washed with double distilled water. Five leaves and five roots were randomly taken out from each plant. After 120 days of sowing, five green capsules were removed from each of the 20 plants. These parts were surface sterilized with 1% sodium hypochlorite for 3 min and then further rinsed with sterile distilled water. Then the plant parts were placed on a blotting paper in a laminar flow and were cut into pieces by a sterile blade. Root and stem pieces were about 4 mm long and leaf segments were about 4 mm^2 in size. From each leaf, stem, root, and green capsule five pieces (five replicates) were randomly taken and placed on *B. bassiana* selective medium (five segments per plate) containing 0.55 g l^{-1} dodine and 5 mg l^{-1} chlortetracycline (Chase et al. 1986).The other pieces served as corresponding tissue samples of each plant for DNA extraction and PCR. The plates were maintained in controlled growth chamber (25 ± 1°C). Observations were recorded daily on appearance of hyphal growth from plant segments. The presence or absence of fungal growth from the plant pieces were recorded on the seventh day and were expressed as percent colonization which was calculated using the formula: Percent colonization = number of plant segments colonized/ total number of plant segments × 100. Thus percent colonization was determined for different plant parts viz., leaf, stem etc. at 30, 60, and 120 days after sowing. The percent colonization data was transformed using arcsine transformation and were analysed using analysis of variance (ANOVA) following completely randomized design. Colonization data from root and fruit parts were not included in the test. Significant differences between means were determined with the Duncan's multiple range test.

DNA extraction from jute plant

DNA extraction technique was standardized to obtain both plant and endophytic *B. bassiana* DNA from the treated samples. DNA extracted from untreated plants served as control. Total DNA was extracted from root, stem, leaf, capsule, and seed of both *B. bassiana* treated as well as untreated jute (*C. olitorius*) plants of 30, 60, and 120 days of age. 300 mg tissue was taken from all plant parts viz. root, stem, leaf, green capsule, and seed. DNA was extracted by using Doyle and Doyle's (1990) CTAB method (100 mM Tris, 1.4 M NaCl, 20.0 mM EDTA) with following modifications. The sample was crushed by adding PVP (Sigma) which helps to dissolve the mucilage present in jute. The tissues were ground in CTAB and transferred to 500 µl 4 M NaCl which was kept at 60°C for 1 h with occasional stirring. Then equal volume of dichloromethane was added and centrifuged at 14,000 rpm for 15 min. The supernatant was carefully taken and 5 µl RNAse (5 mg ml^{-1}) was added and kept at 37°C for half an hour. After that an equal volume of dichloromethane was added followed by centrifugation at 17,000 rpm for 15 min. The supernatant was carefully taken out and was precipitated with 0.6 volume of ice cold isopropanol. The precipitate was again centrifuged at 14,000 rpm and the pellet was washed with 70% ethanol and dried at room temperature. Then the DNA pellet was dissolved in TE buffer (10 mM Tris, 1.0 mM EDTA) and stored at −20°C.

DNA extraction from *B. bassiana* mycelia

For isolation of DNA from *B. bassiana*, monoconodial culture was grown in PDA broth (pH 5.5) for seven days at 25 ± 1°C. The mycelia were filtered through Whatman No.1 filter paper. An amount of 500 mg mycelia was ground in liquid nitrogen and transferred to DNA extraction buffer (100 mM Tris, 1.4 M NaCl, 20.0 mM EDTA, 4% CTAB (Murray and Thompson 1980) and incubated at 60°C for 1 h with occasional stirring. Equal volume of dichloromethane was added followed by centrifugation at 14,000 rpm for 15 min. The supernatant was carefully taken out and was precipitated with 0.6 volume of ice cold isopropanol. The precipitate was again centrifuged at 14,000 rpm and the pellet was washed with 70%

ethanol and dried at room temperature. Then the DNA pellet was dissolved in TE buffer (10 mM Tris, 1.0 mM EDTA) and stored at −20°C.

PCR amplification and gel electrophoresis

All the representative tissue samples of both treated and untreated jute plants were assessed by PCR using *B. bassiana* specific SCAR primers. Root, stem, and leaf samples were taken from 30, 60, and 120 days old plants while green capsule and immature seed were taken from 120 days old plants. Initially, three SCAR primers viz. $SCA14_{445}$ (F 5′ TCTGTGCTGGCCCTT ATCG 3′, R 5′ TCTGTGCTGGGTACTGACGTG 3′), $SCA15_{441}$ (F 5′ TTCCGAACCCGGTTAAGAGAC 3′, R 5′TTCCGAACCCATCATCCTGC3′) and SCB 9_{677} (F 5′ TGGGGGACTCGC AAA CAG 3′ R 5′ TGGGGGACTCAC TCC ACG 3′) were used to amplify the target DNA sequence in *B. bassiana* following Castrillo et al. (2003). Among these primers $SCA15_{441}$ showed better amplification of *B. bassiana* DNA than by $SCA14_{445}$ and $SCB9_{677}$. Therefore, for detection of *B. bassiana* ITCC 4796 in jute plant parts further studies were carried out with $SCA15_{441}$.

The PCR mixture (25 µl) volume for SCAR assays consisted 10× expand high fidelity buffer (10 mM Tris–HCl, 50 mM KCl, pH 8.3) (Biolab) with 25 mM $MgCl_2$ (Biolab), 200 µl of each dNTP (Biolab), 05 µM of each primer (forward and reverse primer), 50 ng μl^{-1} genomic DNA and 5 U μl^{-1} Taq DNA polymerase. The amplification was carried out using a gradient thermocycler (BioRad, USA). The amplification profile was 2 min initial denaturation at 94°C, ten cycles of denaturation at 94°C for 15 s, annealing at 63°C for 30 s, and elongation at 72°C for 45 s with an additional 5 s for successive cycle, and a final elongation at 72°C for 7 min. PCR products were visualized in 1.6% agarose gel stained with ethidium bromide. The molecular weight of the amplified fragment size was calculated following Ling et al. (1987).

Results

Detection of endophytic colonization by culturing on selective medium

Colonization of jute plant parts was studied on PDA based selective medium. White hyphal growth was noticed with naked eyes to emerge from the tissue samples of the plants grown from *B. bassiana* treated seeds. But no such growth was found from the tissue samples of plants grown from untreated seeds. The hyphal growth was confirmed to be of *B. bassiana* by microscopic examination, showing sympodial development of single celled conidia on zig-zag rachis proliferating from flask shaped conidiogenous cells. It indicates that seed treatment with the spore suspension caused endophytic colonization of the fungus in those plant parts. All the plants grown from treated seeds were colonized, however not all the segments from all plants were colonized.

The percent colonization was found to vary with the plant age as well as plant parts. The highest colonization of 43.34% was recorded in 60 days old plants followed by 30 days (23.89%) and 120 days (35.39%) old plants. The influence of plant age on percent colonization was significant ($F_{2,11} = 223.67$; $P < 0.01$). Significant difference in percent colonization was also observed between leaf and stem segments ($F_{1,11} = 143.37$; $P < 0.01$). Colonization was greater from the leaf segments (55.87%) than from stem segments (12.53%). At 30 days after sowing, colonization of leaf segments was 37.39% whereas that of stem segments was 10.38%. The colonization percent increased when the plants were 60 days old for both leaf (68.98%) as well as stem (17.70%) segments. Although the endophyte could be detected in 120 days old plants also, the colonization was found to decline and the percent colonization of stem segments (9.53%) reduced much more (Fig. 1) than that of leaf segments (61.25%). No colonization of *B. bassiana* could be detected from roots and seeds of jute plants grown from treated seeds. However, colonization of *B. bassiana* was detected in the green capsules of 120 days old plants (42.44%).

Detection of endophytic colonization by PCR

Genomic DNA extracted from untreated jute plant failed to amplify any PCR product using SCAR primer $SCA15_{441}$, indicating this primer does not bind to any region of jute genome. PCR amplification of mixed DNA extracted from *B. bassiana* treated jute plants proved endophytic colonization of *B. bassiana* in jute plants. All the treated plant parts that responded to the colonization test also observed to be positive by PCR amplification. At 30 days, *B. bassiana* specific

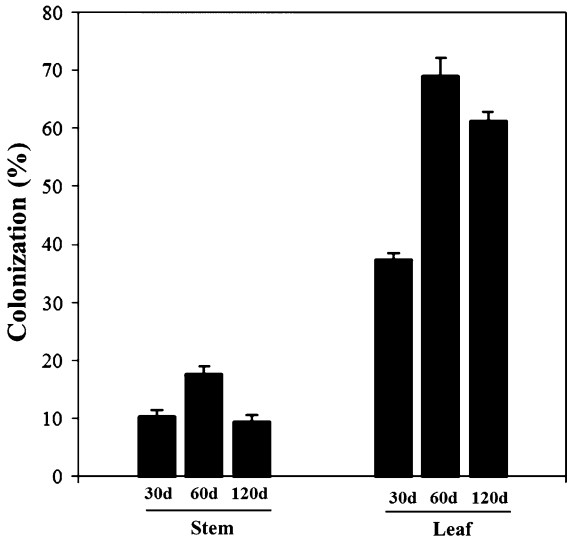

Fig. 1 Percent colonization of jute plant parts by *B. bassiana*. 30d, 60d, and 120d indicate colonization (%) in 30, 60, and 120 days old jute plants, respectively. Error bars indicate standard error of mean

amplicon (205 bp) was obtained from treated plants in stems and leaves using $SCA15_{441}$, but no amplification was observed from root DNA (Fig. 2a). This suggests that the endophytic strain could colonize in stem and leaves but not in roots, which was in accordance with results obtained from percent colonization on selective media at 30 days. At 60 days also, $SCA15_{441}$ amplified a 205 bp PCR product from stems and leaves of treated plants, but not from root (Fig. 2b). During the crop harvest stage (120 days), DNA samples from stem, leaf and green capsule produced same *B. bassiana* specific amplicon (205 bp). But in the PCR product of DNA samples from root and seed of

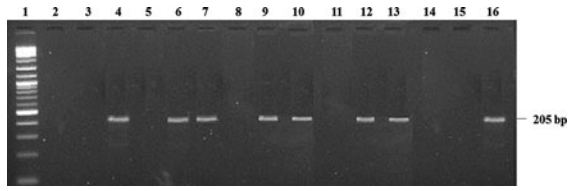

Fig. 3 PCR amplification of genomic DNA extracted from different plant parts of *B. bassiana* treated *C. olitorius* (120 days old). Lane 1—50 bp DNA ladder (BioLabs); Lane 2—DNA from *C. olitorius* root (untreated); Lane 3—DNA from *C. olitorius* root (from *B. bassiana* treated plant); Lane 5—DNA from *C. olitorius* stem (untreated); Lane 6—DNA from *C. olitorius* stem (from *B. bassiana* treated plant); Lane 8—DNA from *C. olitorius* leaf (untreated); Lane 9—DNA from *C. olitorius* leaf (from *B. bassiana* treated plant); Lane 11—DNA from *C. olitorius* capsule (untreated); Lane 12—DNA from *C. olitorius* capsule (from *B. bassiana* treated plant); Lane 14—DNA from *C. olitorius* immature seed (untreated); Lane 15—DNA from immature seed (from *B. bassiana* treated plant); Lanes 4, 7, 10, 13, and 16—*B. bassiana* DNA from pure culture

treated plants no amplification was observed (Fig. 3). In all the control (untreated) experiments no *B. bassiana* specific amplicon was present in PCR products. Thus endophytic colonization of *B. bassiana* was detected in stem, leaf, and green capsule but not in root and seed.

Discussion

Productivity of jute is severely hampered by insect-pests such as jute semilooper, *A. sabulifera*, bihar hairy caterpillar, *S. obliqua* etc. which may be controlled safely, if *B. bassiana* can be expressed in jute plant as endophyte. Earlier reports suggest that

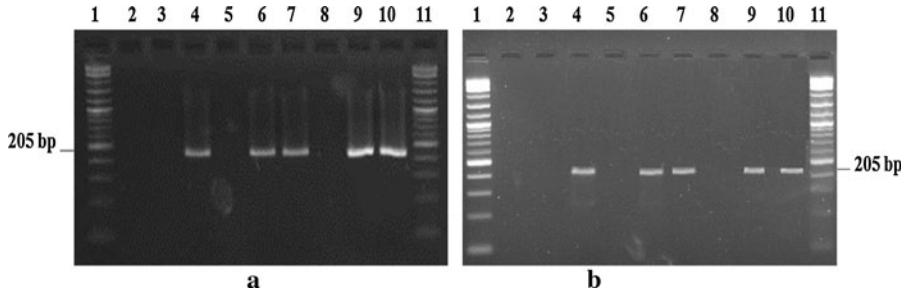

Fig. 2 PCR amplification of genomic DNA extracted from different plant parts of *B. bassiana* treated *C. olitorius*. **a** 30 days old plant; **b** 60 days old plant. Lanes 1, 11—50 bp DNA ladder (BioLabs); Lane 2—DNA from *C. olitorius* root (untreated); Lane 3—DNA from *C. olitorius* root (from *B. bassiana* treated plant); Lane 5—DNA from *C. olitorius* stem (untreated); Lane 6—DNA from *C. olitorius* stem (from *B. bassiana* treated plant); Lane 8—DNA from *C. olitorius* leaf (untreated); Lane 9—DNA from *C. olitorius* leaf (from *B. bassiana* treated plant); Lanes 4, 7 and 10—*B. bassiana* DNA from pure culture

jute pests can be controlled by *B. bassiana* (Pandit and Som 1988). In the present study we have established *B. bassiana* as an endophyte in tossa jute (*C. olitorius*) and monitored colonization of *B. bassiana* in different plant parts through culturing on selective media and PCR based detection. PCR based detection was found to be quick, reliable, and superior to detection by cultivation. The primer $SCA15_{441}$ does not amplify any fragment from jute genomic DNA but detects presence of endophytic *B. bassiana* which makes this SCAR primer robust and definitive for monitoring endophytic colonization of the entomopathogen in jute.

Our study shows that seed treatment with fungal spore suspension is an effective technique to induce endophytic expression of *B. bassiana* in tossa jute. The colonization of plant parts by *B. bassiana* was found to increase with plant age up to 60 days. These findings are in close conformity with the report of Posada and Vega (2005) in cocoa and of Tefera and Vidal (2009) in sorghum. However, percent colonization in 120 days old plants was lower than that in 60 days old plants and particularly in case of stem it was even lower than that in 30 days old plants. As jute is a bast fibre crop, reduction in stem colonization may be attributed to fibre formation in the phloem region, which might interfere with endophytic fungal growth or may be due to secondary cell wall thickening by lignin or other phenolic compound deposition in both xylem and phloem at the later stages of growth. At 120–125 days jute crop approaches harvestable maturity. This may be the reason for decline in endophytic colonization. The colonization was greater in leaves than in stems throughout the growth period, indicating better penetration and establishment of the fungus in leaves. However, we could not detect endophytic colonization of *B. bassiana* in roots of *C. olitorius* either through plating on selective media or PCR based detection method. Since PCR based detection methods are highly efficient it can be concluded that *B. bassiana* do not colonize in jute roots. Roots of *C. olitorius* contains some triterpenoids such as oxocorocin, urosolic acid, corosolic acid etc., which are known to have antimicrobial activities (Khan et al. 2006) and these acidic compounds might have inhibited colonization of *B. bassiana* in roots. Tefera and Vidal (2009) reported that sorghum leaves and stems are colonized by *B. bassiana* to a greater extent than root. Vega et al. (2008) reported the presence of the entompathogenic endophyte in coffee berry. Likewise, we could also detect *B. bassiana* as endophyte from the green capsules of jute.

Our study demonstrates that *B. bassiana* can translocate systemically throughout the plant tissues in jute and provides the basis for exploitation of the endophytic behaviour of this entomopathogen in controlling jute pests particularly, semilooper, and bihar hairy caterpillar. These pests have already been reported to be controlled by topical application of *B. bassiana* spore suspension (Pandit and Som 1988). We have discussed that the endophytic *B. bassiana* remains within the plant system throughout the crop growth period, however its colonization reaches maximum at around 60 days of age. Similarly, infestation of jute semilooper and bihar hairy caterpillar may occur at any stage of crop growth but the peak infestation is found near 60 days of age and this coincidence could be an important factor in efficient control of these pests by *B. bassiana*. Success of this novel approach of biological pest management depends on easy and ensured entry of suitable *B. bassiana* strain as an endophyte and its long term establishment in inoculated jute plants. This work is the first report of *B. bassiana* being established as a season long endophytic colonist in tossa jute (*C. olitorius*) as a first step towards that direction.

Acknowledgments We acknowledge the support provided by Prof. B. S. Mahapatra, Director, the Central Research Institute for Jute and Allied Fibres, Barrackpore, Kolkata, India. We also appreciate the comments of the two anonymous reviewers.

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Subrata Satpathy is an entomologist specialized in integrated pest management of vegetable and fibre crops. His current area of interest is pest control by entomopathogenic fungi and viruses.

Pratik Satya's research interest focuses on population genetic analysis of bacteria, fungi and plants using DNA markers. He is also working on host-pathogen interaction and development of host resistance.