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ORIGINAL ARTICLE

Endophytic Ability of Different Isolates of Entomopathogenic Fungi *Beauveria bassiana* (Balsamo) Vuillemin in Stem and Leaf Tissues of Maize (*Zea mays* L.)

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Abstract The present study was conducted to examine the ability of six promising indigenous isolates of *Beauveria bassiana* (NBAII-Bb-5a, 7, 14, 19, 23 and 45) as an endophyte in maize stem and leaf tissues. Maize seedlings (var. Nithyashree) were inoculated with conidial suspensions and were examined for endophytic establishment in leaf and stems at different intervals during 15–90 days after treatment. All six isolates showed colonization in stem and leaf tissues with varying abilities of colonization and persistence. The mean percent colonization ranged from 7.41 to 20.37 % in older stem tissues and 3.70 to 21.29 % in young stem tissues and in leaf, it ranged from 6.46 to 27.78 % in older leaf tissues and 11.11 to 26.85 % in young leaf tissues. Among six isolates tested, Bb-23 isolate recorded the maximum mean colonization in older stem (20.37 %), older leaf (27.78 %) and in young stem (21.29 %). Bb-5a isolate showed maximum mean colonization in young leaf tissues (26.85 %). Persistence of inoculated fungal isolates decreased with increase in age of the plant. No physical symptoms of damage were observed in any of the *B. bassiana* treated plants. No colonization of *B. bassiana* was observed in the untreated control maize plants. The results obtained in plating and PCR techniques were similar with regard to the confirmation of endophytic establishment of *B. bassiana*. This study indicated the possibility of using *B. bassiana* as an endophyte in maize for management of maize stem borer, *Chilo partellus*.

Keywords Entomopathogenic fungi · *Beauveria bassiana* · Endophyte · Maize

Introduction

Maize is an important cereal crop cultivated all over the world and it is the third major crop in India [1]. Maize has great importance for grain and fodder purpose and it is also used for production of oil, alcohol, acetic, lactic acid, glucose, starches for edible and laundry purpose, adhesives and methanol. Around 140 insect pests infest maize, among them ten pest species cause severe yield loss. *Chilo partellus* (Swinhoe) is one of the most important pests causing yield loss in maize and it mainly attacks the crop during *kharif* season [2]. The pest management strategies against this borer pest were focused mainly on the use of chemical insecticides. Control of borer pests by chemical insecticides is extremely difficult because of its cryptic life cycle, expensive and has adverse effects on environment as well as human health. Hence there is a need for development of an alternate, safe protection technology using endophytic entomofungal pathogens. In recent years, endophytic strains of *Beauveria bassiana* have been used for management of insect pests like, European corn borer, *Ostrinia nubilalis* in maize [3] and banana weevil *Cosmopolites sordidus* [4]. Endophytic colonization of entomopathogenic fungi within the plant system has more advantageous than external application because it gives season long protection against pests and it is cost effective.

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In India, the entomopathogenic fungus, *B. bassiana* has not been exploited as an endophyte in maize for stem borer (*C. partellus*) management. Six indigenous isolates of *B. bassiana* were identified as promising against *C. partellus* in the laboratory bioassay studies [5]. The present study was undertaken to determine the ability of these six promising isolates of *B. bassiana* to establish as an endophytes in maize.

Materials and Methods

A glasshouse experiment was conducted at National Bureau of Agricultural Insect Resources (NBAIR), Bengaluru, India to examine the endophytic ability of different isolates of entomopathogenic fungus *B. bassiana* in stem and leaf tissues of maize.

Seeds of the maize (*Zea may* L.) cultivar Nithyashree were obtained from University of Agricultural Sciences, Bengaluru, Karnataka. Maize seeds were surface sterilized with sodium hypochlorite (3 %) for 2 min, then with ethanol (70 %) for 2 min followed by two washing with sterile distilled water. The surface sterilized seeds were dried and then sown in plastic pots (15 cm diameter) filled with sterile soil (autoclaved at 121 °C for 20 min). The pots were kept at 21–22 °C, 60–80 % RH in the glasshouse and watered frequently. Three replications (Ten plants per replication) were maintained for each treatment.

The six indigenous isolates of *B. bassiana* (NBAII-Bb-5a, 7, 14, 19, 23 and 45) used in this study were initially isolated from different insect cadavers and soil samples from different geographical regions of India (Table 1). These fungal cultures were maintained on Sabouraud's Dextrose Yeast Agar (SDYA) (Dextrose 40 g, Mycological peptone 10 g, yeast extract 5 g, agar 20 g in 1000 ml of distilled water) slants at −20 °C at the culture repository of ICAR-NBAIR, Bengaluru until further use. Inoculums for succeeding studies were always prepared from the original culture.

Each isolate was grown on sterilized rice for 15 days at 25 ± 1 °C. The conidial suspension of each isolate required for the experiment was prepared by suspending 1 g of 15 days old conidiated rice in sterile distilled water with 0.1 % Tween 80. Conidial suspension was filtrated through three layered muslin cloth to get hyphal-free conidial suspension. The conidial concentration in the suspension was adjusted to 1×10^8 conidia/ml using Neubauer's improved haemocytometer under light microscope.

Plant Inoculation

The conidial suspension of each isolate was sprayed on the maize seedling (1×10^8 conidia/ml; 5 ml/seedling) at 15 and 30 days after germination. The control plants were sprayed with sterile distilled water with 0.01 % Tween 80. Top of the each pot was covered with aluminum foil to avoid conidial contact with soil in the pot [6].

Studies on Endophytic Establishment

Endophytic colonization of the six isolates of *B. bassiana* in maize was studied at 15, 30, 45, 60, 75 and 90 days after treatment. Three plants were randomly selected at each sampling period from each isolate. Plants were uprooted from pots and washed thoroughly with running tap water. From each plant, two older leaves (emerged before spraying) and two younger leaves (emerged after spraying) and two pieces of older stem (emerged before spraying) and two pieces of growing tip of the stem (emerged after spraying) were collected for endophytic colonization studies.

By Plating Technique

The leaf and stem samples were first surface sterilized with sodium hypochlorite (1 %) for 5 min and then with ethanol (70 % v/v) for 30 s. The surface sterilized samples were washed three times in sterilized distilled water for a minute each. The samples then, were cut into 5 mm pieces and transferred aseptically into petri dishes containing SDYA medium with chloramphenicol (100 mg/ml). The final

Table 1 Details of isolates of *B. bassiana* used in the study

Sl no.	Isolates code	Source	Location	Genbank accession number
1	NBAII-Bb5a	*Hypothenemus hampei* (coffee berry borer)	Madikeri, Karnataka	JF837134
2	NBAII-Bb7	*Plocaederus ferrugines* (cashew root and stem borer)	Puttur, Karnataka	JF837097
3	NBAII-Bb14	Unknown insect	Doddaballapura, Karnataka	JF837092
4	NBAII-Bb19	Banana rhizosphere soil	Trichy, Tamil Nadu	KC121555
5	NBAII-Bb23	*Maruca testulalis* (legume pod borer)	Karaikal, Puducherry	JF837082
6	NBAII-Bb45	Carrot rhizosphere soil	Nedugula, Tamil Nadu	JF837094

washed water was also plated (0.1 ml) on SDYA plates to check the effectiveness of surface sterilization. The plates were maintained at 25 ± 1 °C in a biological oxygen demand (BOD) incubator. The fungal growth from the plated bits of stem and leaf were examined under microscope for confirmation of *B. bassiana* growth. Percent colonization of each isolate in the older and young leaf/stem bits at different sampling times was calculated based on the number bits yielding *B. bassiana* and total number of bits plated. The percent colonization data were arcsine transformed and were analyzed by two-way ANOVA (analysis of variance) following completely randomized design [7].

By PCR Technique

Genomic DNA of *B. bassiana* was extracted from surface sterilized young and adult leaf and stem bits of both treated as well as untreated maize plants. DNA was extracted according to the instructor manual of CTAB (Cetyl Trimethyl Ammonium Bromide) method [7]. 300 mg of surface sterilized samples were grinded in pre-warmed CTAB extraction buffer and kept for incubation at 65 °C for 60–90 min in water bath with occasional stirring. After that allow the samples to cool, 5 ml of chloroform: isoamylalcohol (24:1 ratio) mixture was added. The samples were centrifuged at 7000 rpm for 15 min at 20 °C. The supernatant was carefully taken into new test tube containing 25 µl RNase (5 mg/ml) and kept for incubation at room temperature for 30 min. To this 6 ml of isopropanol was added and centrifuged at 7000 rpm for 10 min at 4 °C. Discard the supernatant, 8 ml of cold CTAB wash buffer was added into pellet and kept for 20 min at room temperature. The samples were centrifuged at 7000 rpm for 3 min at 4 °C. Discarded the supernatant, then washed the pellet with 70 % cold ethanol and centrifuged at 3000 rpm for 5 min at 4 °C. The supernatant was discarded, dried the pellet at room temperature. Finally the DNA pellet was dissolved in 100 µl of (10 mM Tris–HCl + 0.1 mM EDTA at pH 8.0) TE buffer and stored at −20 °C.

Endophytic *B. bassiana* DNA was amplified by PCR by using *B. bassiana* specific SCAR (sequence-characterized amplified region) primer ($SCA15_{441}$ (F 5′ TTCCGAACCC GGTTAAGAGAC 3′, R 5′ TTCCGAACCCATCATCCT GC 3′) [7]. Final volume of PCR mixture (50 µl) consisting of 50 ng of fungal genomic DNA, 50 pmol each of SCAR primers, 1.25 mM for each of dATP, dGTP, dCTP, dTTP, 2.5 units of Taq DNA polymerase, 5 µl of polymerase buffer, 2.5 Mm $MgCl_2$ and sterile water to makeup 50 µl of total volume. The PCR amplifications program was consisting of initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, extension at 72 °C for 3 min, and final extension at 72 °C for 10 min and store at 4 °C. Quantarus Thermal cycler was used to carry out the cycles. PCR product was visualized in 1.4 % agarose gel with ethidium bromide. The molecular weight of the amplified DNA fragment size was calculated according to Ling et al. [8].

Results

By Plating Technique

White hyphal growth was noticed from both older and young stem/leaf bits of the treated maize plants (Fig. 1). Microscopic examination of this white hyphal growth the bits showed typical conidiophores, phialides and conidia of *B. bassiana* indicating the endophytic colonization of the fungus in stem and leaf tissues of maize. But no such *B. bassiana* growth was found from the stem/leaf bits of the un-treated control maize plants (Fig. 1).

The results of the plating technique of stem and leaf are presented in Tables 2 and 3 respectively. All six isolates showed colonization in older and young stem/leaf tissues of the maize with varying per cent colonization and persistence during 15–90 DAT. The mean percent colonization of the six isolates ranged from 7.41 to 20.37 % in older stem tissues and 3.70–21.29 % in young stem tissues (Table 2). In leaf, the mean percent colonization ranged from 6.46 to 27.78 % in older leaf tissues and 11.11–26.85 % in young leaf tissues (Table 3). The results indicated that the isolates of *B. bassiana* colonized both older and younger stem/leaf tissues irrespective of whether the plant part received the spraying of *B. bassiana* or not. Among all the isolates tested, Bb-23 isolate recorded the maximum mean colonization in older stem (20.37 %) and in young stem (21.29 %), followed by Bb-45 (12.96 and 11.11 %), Bb-7 (12.02 and 16.67), Bb-14 (11.11 and 3.70), Bb-5a (10.18 and 12.04) and Bb-19 (7.41 and 9.26) in older and young stem tissues respectively (Table 2). With regard leaf, Bb-23 isolate showed the mean colonization of 27.78 % in older leaf and 24.07 % in young leaf and other isolates, Bb-45, Bb-5a, Bb-7, Bb-14 and Bb-19 showed 23.14 and 18.52, 22.22 and 26.85, 20.37 and 13.89, 14.81 and 11.11 and 6.46 and 12.04 % colonization in older and young leaf tissues respectively (Table 3). The untreated control samples of leaf and stem did not show colonization of any of the *B. bassiana* isolates.

Bb-5a isolate showed continuous colonization in older stem and leaf tissues up to 45 DAT, whereas in young stem and leaf it showed colonization up to 75 and 60 DAT respectively. Bb-7 isolate showed colonization up to 45 and 75 DAT in older and young stem tissues respectively, whereas in older and young leaf tissues it showed colonization up to 60 DAT. Bb-14 isolate persisted up to 45 and

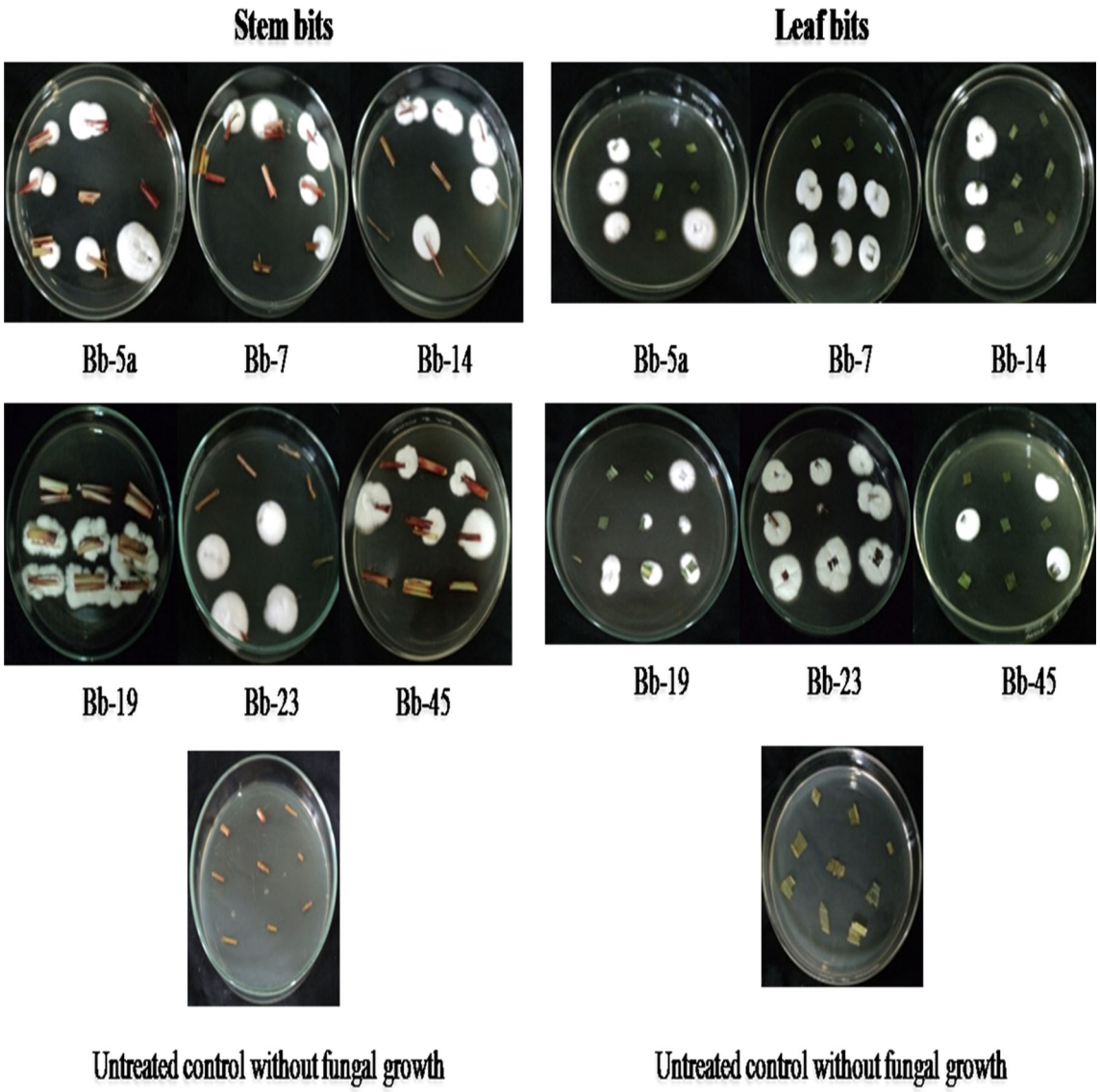

Fig. 1 *B. bassiana* growth (white fugal growth) from the treated stem and leaf bits

30 DAT in older and young stem tissues respectively, whereas in older and young leaf tissues it persistence up to 45 and 60 DAT, respectively. Bb-19 isolate persistence up to 45 DAT in older and young stem tissues, in older and young leaf tissues it persistence up to 45 and 60 DAT. Bb-23 isolates showed continuous colonization up to 60 DAT in both older and young stem and leaf tissues. Bb-45 showed colonization up to 60 DAT in older and young stem tissues, whereas in older and young leaf it showed colonization up to 60 and 90 DAT.

Bb-5a isolate has recorded significantly higher percent colonization in older stem tissues (38.88 %) at 30 DAT and in older leaf tissues (61.01 %) and in young leaf tissues (77.77 %) at 15 and 45 DAT. Bb-7 isolate showed higher percent colonization in older and young stem tissues (61.01 and 66.66 % respectively) at 45 DAT. Bb-14 isolate showed higher percent colonization in older stem tissues (27.77 and 38.88 %) at 30 and 45 DAT respectively, whereas in older leaf tissues it showed higher percent colonization (55.55 %) at 30 DAT. Bb-19 isolate showed higher percent colonization at 30 DAT in both older and young stem tissues (27.77 and 33.33 % respectively). Bb-23 isolate showed higher percent colonization at 30 and 45 DAT in older (50.00 and 49.99 % respectively) and young stem tissues (33.33 and 66.66 % respectively), whereas in leaf it showed higher percent colonization at 30 and 45 DAT in older leaf tissues (77.77 %) and at 45 DAT in young leaf tissues (88.88 %). Bb-45 isolate showed higher percent colonization at 60 DAT in both older stem tissues (33.33 %) and young stem tissues (44.44 %). It was found

Table 2 Percentage colonization of *B. bassiana* in older/young stem tissues of maize

Isolates	Days after treatment stem													
	Older stem tissues							Young stem tissues						
	15	30	45	60	75	90	Mean	15	30	45	60	75	90	Mean
Bb5a	5.55b	38.88a	16.66b	0.00	0.00	0.00	10.18A	11.11b	22.22b	22.22b	5.55b	11.11b	0.00	12.04A
Bb7	5.55b	5.55b	61.01a	0.00	0.00	0.00	12.02A	11.11b	11.11b	66.66a	0.00	11.11b	0.00	16.67A
Bb14	0.00	27.77a	38.88a	0.00	0.00	0.00	11.11A	0.00	22.22b	0.00	0.00	0.00	0.00	3.70B
Bb19	0.00	27.77a	16.66b	0.00	0.00	0.00	7.41A	0.00	33.33a	22.22b	0.00	0.00	0.00	9.26A
Bb23	11.11b	50.00a	49.99a	11.11b	0.00	0.00	20.37A	11.11b	33.33a	66.66a	16.66b	0.00	0.00	21.29A
Bb45	22.21b	22.21b	0.00	33.33a	0.00	0.00	12.96A	0.00	22.22b	0.00	44.44a	0.00	0.00	11.11A
Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Values in columns followed by the different letter (a, b, c) are significantly different with each other according to LSD ($P < 0.01$)

Values in columns followed by the different letter (A, B) are significantly different with each other according to LSD ($P < 0.01$)

Table 3 Percentage colonization of *B. bassiana* in older/young leaf tissues of maize

Isolates	Days after treatment													
	Older leaf tissues							Young leaf tissues						
	15	30	45	60	75	90	Mean	15	30	45	60	75	90	Mean
Bb5a	55.55a	38.88b	38.88b	0.00	0.00	0.00	22.22A	55.55a	22.22b	77.77a	5.55c	0.00	0.00	26.85A
Bb7	16.66b	44.44b	16.66b	44.44b	0.00	0.00	20.37A	0.00	22.22b	11.11c	49.99b	0.00	0.00	13.89A
Bb14	16.66b	55.55a	16.66b	0.00	0.00	0.00	14.81B	0.00	44.44b	11.11c	11.10c	0.00	0.00	11.11A
Bb19	0.00	27.77b	11.01c	0.00	0.00	0.00	6.46B	0.00	22.22b	44.44b	5.55c	0.00	0.00	12.04A
Bb23	0.00	77.77a	77.77a	11.11c	0.00	0.00	27.78A	0.00	44.44b	88.88a	11.11c	0.00	0.00	24.07A
Bb45	33.33b	38.88b	22.21b	44.44b	0.00	0.00	23.14A	22.22b	33.33b	33.33b	0.00	11.11c	11.11c	18.52A
Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Values in columns followed by the different letter (a, b, c) are significantly different with each other according to LSD ($P < 0.01$)

Values in columns followed by the different letter (A, B) are significantly different with each other according to LSD ($P < 0.01$)

that, the persistence of inoculated fungi decreased with the increase in age of the plant (Tables 2, 3). The plants treated with *B. bassiana* isolates did not show any pathogenic symptoms.

By PCR Technique

B. bassiana specific SCAR primer $SCA15_{441}$ amplified the genomic DNA extracted from *B. bassiana* treated maize leaf and stem tissues (from both older and young tissue samples) and it failed to amplify the DNA extracted from untreated control leaf and stem tissues. This indicated that, the SCAR primer does not bind to any other genomic DNA except for the genomic DNA of the entomopathogenic fungus, *B. bassiana*. During the sampling periods (at 15, 30, 45, 60, 75 and 90 DAT), *B. bassiana* specific amplicon (450 pb) was obtained from leaf and stem tissues (from both older and young tissue samples) of treated plants using $SCA15_{441}$ (Figs. 2, 3). The plant tissues which showed *B. bassiana* colonization in plating technique were confirmed by PCR amplification.

Discussion

The current study indicated that, all six indigenous isolates of *B. bassiana* were able to colonize in stem and leaf tissues of maize, when applied as spore suspension by foliar spray. Since the untreated control maize plants did not yield any *B. bassiana* growth in both plating and PCR methods, it is assumed that *B. bassiana* got established as endophyte in the treated maize plants by artificial inoculation. The present studies are congruent with Bing and Lewis, 1991 [9] who described endophytic colonization of *B. bassiana* in corn.

The six isolates tested showed considerable variations in their ability to colonize and persist in stem and leaf tissues of maize. Among six isolates tested, Bb-23 isolate recorded the maximum mean colonization in older stem (20.37 %),

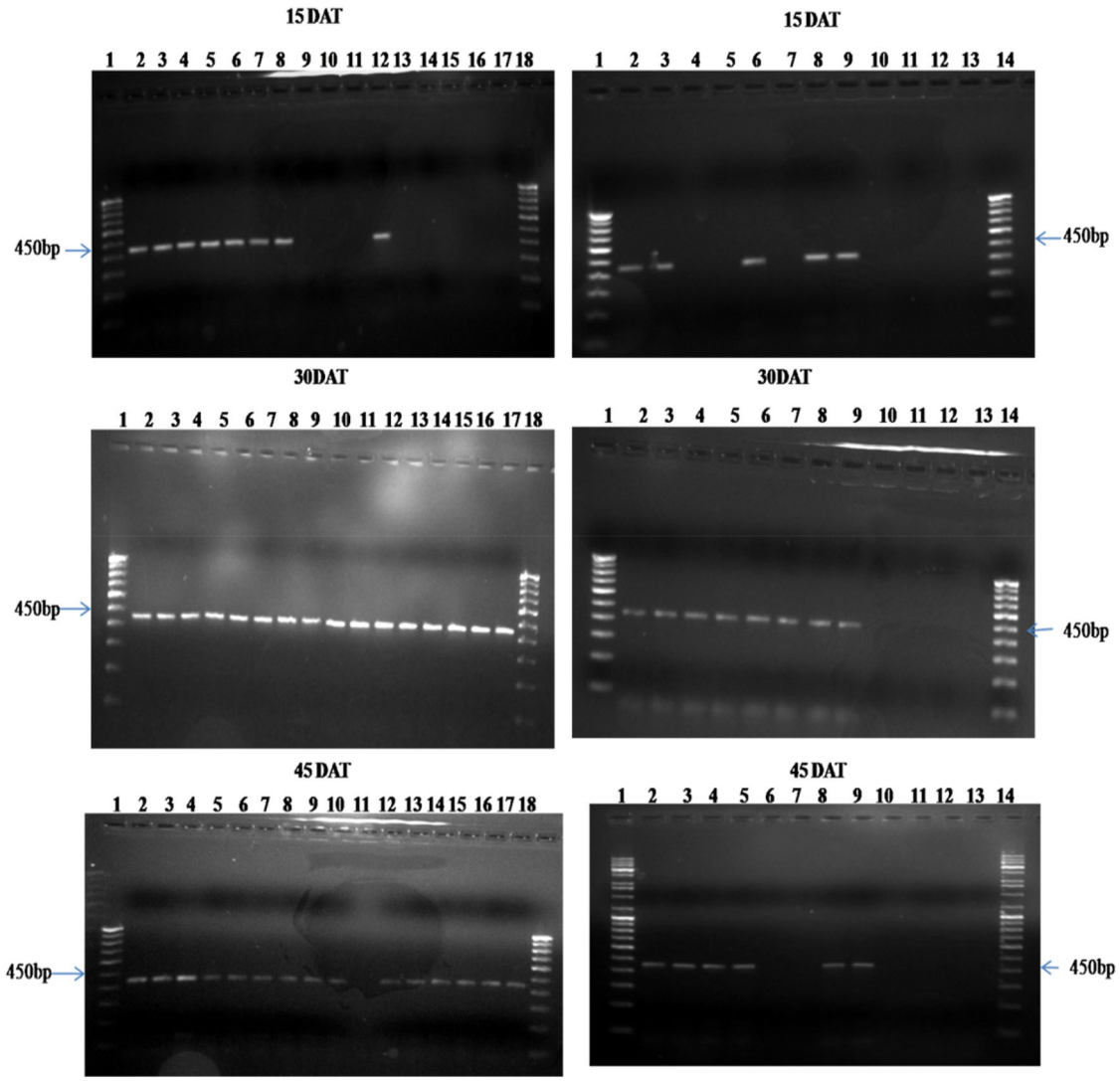

Fig. 2 PCR amplification of genomic DNA extracted from older and young stem and leaf tissues of *B. bassiana* treated maize. *Lanes 1–18*: *1* 100 bp ladder, *2* Bb5a older stem, *3* Bb5a young stem, *4* Bb5a older leaf, *5* Bb5a young leaf, *6* Bb7 older stem, *7* Bb7 young stem, *8* Bb7 older leaf, *9* Bb7 young leaf, *10* Bb14 older stem, *11* Bb14 young stem, *12* Bb14 older leaf, *13* Bb14 young leaf, *14* Bb19 older stem, *15* Bb19 young stem, *16* Bb19 older leaf, *17* Bb19 young leaf, *18* 100 bp ladder. *Lanes 1–14*: *1* 100 bp ladder, *2* Bb23 older stem, *3* Bb23 young stem, *4* Bb23 older leaf, *5* Bb23 young leaf, *6* Bb45 older stem, *7* Bb45 young stem, *8* Bb45 older leaf, *9* Bb45 young leaf, *10* control older stem, *11* control young stem, *12* control older leaf, *13* control young leaf, *14* 100 bp ladder

older leaf (27.78 %) and in young stem (21.29 %) and Bb-5a isolate in young leaf tissues (26.85 %). The maximum persistence of endophytic colonization in young leaf tissues was observed up to 90DAT with Bb-45 isolate and in the older leaf tissues up to 60DAT with Bb-7, Bb-23 and Bb-45 isolates. In young stem tissues, persistence up to 75 DAT was observed with Bb-7 and Bb-5a isolates and in older stem tissues, persistence up to 60 DAT was observed with Bb-23 and Bb-45 isolates. The endophytic colonization of the isolates may be depended on their ability to adjust to the nitch area of stem and leaf tissues of the host plant and the reason for higher colonization of particular isolate in particular plant part could be caused by diverse microbial and physiological environment present inside the plant parts. Several endophytic fungi show a certain degree of tissue specificity because they are adapted to particular conditions present inside the plant tissues [10].

In general, all isolates showed higher percent colonization in maize stem and leaf at 30–45 days of crop age which coincides with stem borer (*C. partellus*) infestation [11]. This coincidence could be a key factor for management of borer pest by endophytically established *B. bassiana*. Persistence of inoculated fungal isolates decreased with increase in age of the plant. It might be due to non availability of nutrients to the fungus at maturation period, competition between the inoculated fungus and

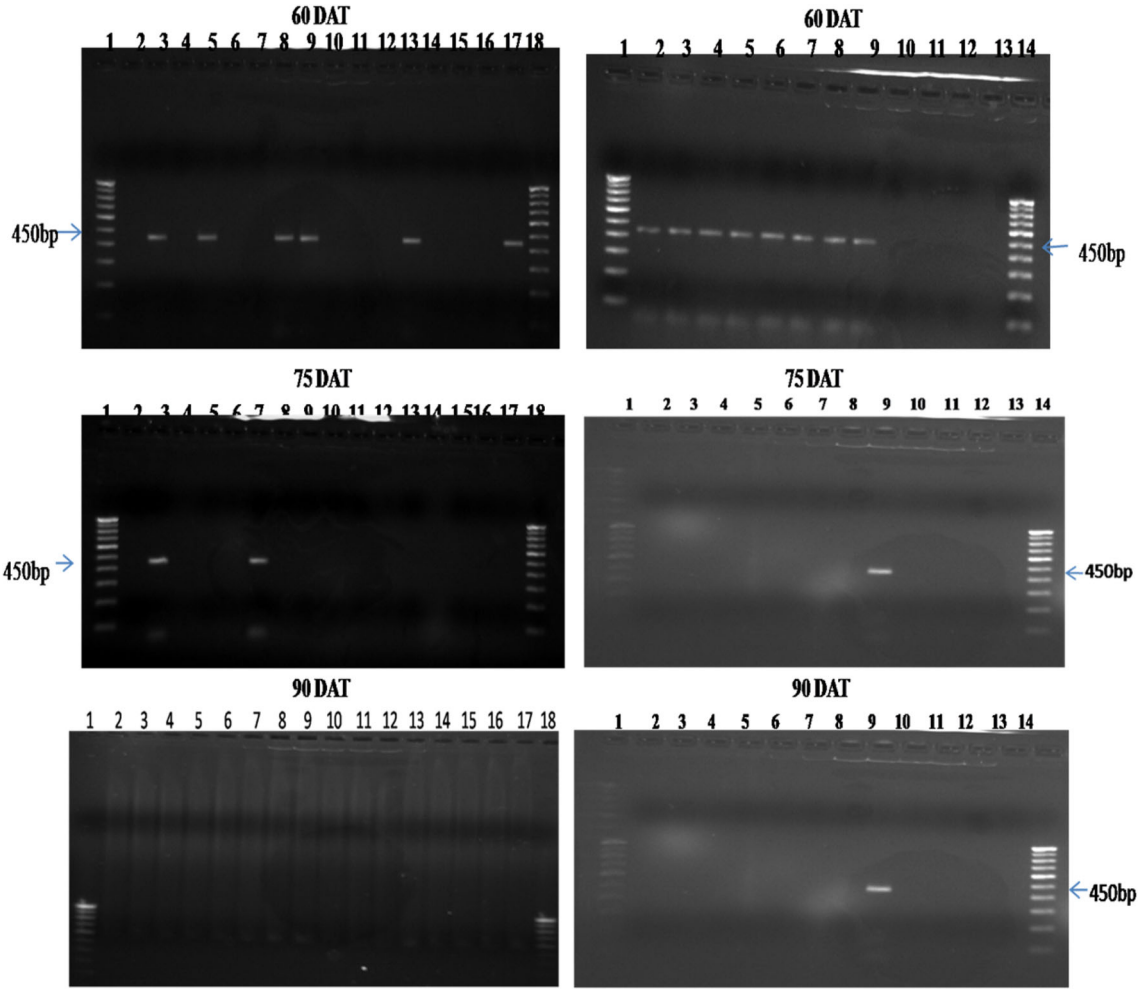

Fig. 3 PCR amplification of genomic DNA extracted from older and young stem and leaf tissues of *B. bassiana* treated maize. *Lanes 1–18*: *1* 100 bp ladder, *2* Bb5a older stem, *3* Bb5a young stem, *4* Bb5a older leaf, *5* Bb5a young leaf, *6* Bb7 older stem, *7* Bb7 young stem, *8* Bb7 older leaf, *9* Bb7 young leaf, *10* Bb14 older stem, *11* Bb14 young stem, *12* Bb14 older leaf, *13* Bb14 young leaf, *14* Bb19 older stem, *15* Bb19 young stem, *16* Bb19 older leaf, *17* Bb19 young leaf, *18* 100 bp ladder. *Lanes 1–14*: *1* 100 bp ladder, *2* Bb23 older stem, *3* Bb23 young stem, *4* Bb23 older leaf, *5* Bb23 young leaf, *6* Bb45 older stem, *7* Bb45 young stem, *8* Bb45 older leaf, *9* Bb45 young leaf, *10* control older stem, *11* control young stem, *12* control older leaf, *13* control young leaf, *14* 100 bp ladder

other natural endophytes and varying host response at different stages of plant [7].

Our study showed that in the fungal treated maize plants, *B. bassiana* can be detected in both old and young stem/leaf tissues, irrespective of the plant part receiving *B. bassiana* spray or not. This indicates the endophytic spread of the *B. bassiana* isolates in the maize plant. Wagner and Lewis, 2000 [12] reported passive movement of *B. bassiana* in the corn plant through xylem vessels. Such *B. bassiana* isolates with systemic properties can fit into the Integrated Pest Management (IPM) of maize stem borer. Further studies are required in this direction.

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