Int.J.Curr.Microbiol.App.Sci (2017) *6*(6): 2506-2513

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 6 (2017) pp. 2506-2513**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2017.606.298

Establishment of *Beauveria bassiana* (Balsamo) Vuillemin as an Endophytein Cotton

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Keywords

Cotton, Entomopathogen, *Beauveria bassiana*, Endophytes, Colonization.

Article Info

Accepted:
29 May 2017
Available Online:
10 June 2017

A B S T R A C T

Entomopathogenic fungal bioagent, *Beauveria bassiana* is a potential target specific and eco friendly alternative for chemical control. *B. bassiana* despite being highly pathogenic to insects in the laboratory condition, it is less efficient in the field condition because, the external application of spray formulations of *B. bassiana* adversely get affected by abiotic factors. To reduce this limitation, endophytes have received considerable attention as a promising supplement or alternative to chemical control. This work aims to establish entomopathogen *B. bassiana* as endophyte into cotton plant system by artificial inoculation methods. *B. bassiana* was inoculated into cotton plant by four different methods *viz*., seed immersion, seed coating, foliar spray and soil drenching. The recovery of *B. bassiana* and other endophytic fungi was evaluated by culture methods at one month and two months of post inoculation. Among the different inoculation methods, the foliar application method recorded with highest colonization percentage. The *B. bassiana* was recovered from cotton plant parts even at two months after inoculation. In addition to *B. bassiana* recovery, the maximum of 157 numbers of other endophytic fungi were also obtained at two months post-inoculation, followed by 144 numbers at one month post inoculation. The results revealed that, *B. bassiana* can able to establish as endophyte in cotton plants.

Introduction

Cotton pest management has always been an immensely challenging task for entomologists. Continuous and indiscriminate use of insecticides for management of insects have increased the selection pressure and leading to resistance to insects. Therefore, alternate options of pest control are much awaited. The need of the hour is development of eco-friendly, microbe based insecticides which act differently from chemicals, thereby providing least chance to develop resistance. Among various microbial bioproducts, *Beauveria bassiana* popularly used. So far, prevailing microbial pesticides are being mainly used as foliar application (Ratna Kumari *et al.*, 2014) but its efficacy adversely affected by abiotic factors (Thompson *et al.*, 2006).

Endophytes have received increasing attention as a promising alternative to chemical control. Endophytic microorganisms reside asymptomatically within higher plants, inhabiting leaves, stems and roots without any

apparent harm to the plant (Jalgaonwala *et al.*, 2011). Endophytic fungi are important because they produce secondary metabolites with a range of potential uses in agriculture (Selim *et al.,* 2012). Furthermore, some endophytes protect plants from subsequent attack by insect pests and plant pathogens (Azevedo *et al.,* 2000). *B. bassiana* also exist as natural endophytes and can be introduced into plants using several artificial plant inoculation methods (Vega, 2008; Brownbridge *et al.*, 2012).

The use of *B. bassiana* as an artificial endophyte in cotton would potentially solve the constraints limiting its field application. Furthermore, once established as an endophyte, *B. bassiana* might offer the most suitable and season long protection against the insect pests of cotton. Hence, in the present investigation, the colonization efficiency of *B. bassiana* on the *Bt* and non-*Bt* cotton by different methods of inoculation with different concentration were compared. This study provides the baseline data for the further detailed studies.

Materials and Methods

B. bassiana inoculum preparation

The *B. bassiana* culture was obtained from National Bureau for Agriculturally Important Insects (NBAII) Bengaluru, Karnataka, India. The *B. bassiana* strain was subcultured on Sabouraud dextrose agar medium supplemented with yeast extract (SDAY) (10g peptone, 20g dextrose, 5g yeast extract and 15g agar^{-1} distilled water) and antibiotics (0.1g penicillin, 0.2g streptomycin and 0.05g chlortetracycline^{-1} SDAY) in 55 mm diameter Petri dishes. The Petri dishes containing the *B. bassiana* were incubated for three weeks in the laboratory. Conidia were harvested by gently scraping them off the surface of the dried medium using a sterile scalpel blade. Conidial concentration was determined by dissolving 0.1g conidial powder in 10 ml sterile deionized water containing 0.01% Tween 80 in a sterile 500 ml bottle. After vortexing for one minute serial dilutions were made, and the conidial concentration determined using an improved Neubauer haemocytometer. The conidial concentration for each treatment was adjusted to $1x10^6$, $1x10^7$ and $1x10^8$ conidia ml^{-1}. Germination test of conidia was done before inoculating in the plants.

Inoculation methods

Bunny *Bt* and respective non *Bt* seeds were used for these experiments. *B. bassiana* was inoculated by four different methods: (1) Seed immersion (2) Seed coating (3) Foliar spray and (4) Soil drenching. Twenty plants were used per inoculation.

For seed immersion treatment, 50g of seeds were immersed into 10 ml of *B. bassiana* conidial suspension of various concentrations for 6 hours. After that the inoculated seeds were dried on sterile tissue paper for 30 min and they were sown in 15cm diameter pots. Seeds soaked in sterile distilled water containing 0.01% Tween 80 were used as control. Seed coating was done by adding 1g of *B. bassiana* conidial suspension of various concentrations along with methyl cellulose coated with cotton seeds. For control seeds were coated with methyl cellulose alone. The foliar spray inoculation method was performed with a hand sprayer to inoculate each seedling with 10 ml conidial suspension of various concentrations at fifteen days after emergence of seedlings. The spray was directed mainly to the leaves but also incidentally coated the stems. To avoid conidial runoff to the soil, the soil top of each pot was covered with aluminium foils. For the soil drench inoculation method, 10 ml conidial suspension of various concentrations

was applied around the root zone of each seedling. In the control, sterile 0.01% Tween 80 applied in the same way as mentioned above. After inoculation, each plant was covered with a plastic bag for 24 hrs to maintain a high level of humidity. The inoculated plants were kept in room temperature and natural light conditions of 12:12 h and watered daily.

Evaluation for presence of *B. bassiana* in *Bt* and non-*Bt* cotton tissues

The recovery of *B. bassiana* and other endophytic fungi was evaluated by culture methods at one month and two months of post inoculation. Stems were cut off (about 5cm above the stem base) from the roots using a sterile blade. The leaves were randomly selected from the middle section of the seedling. Similarly, two parts of the stem were sampled, one towards the middle of the plant and the second one closer to the soil surface. The leaves were cut into 1 cm^2 sections, sterilized in a laminar airflow cabinet by dipping in 0.5% Sodium hypochlorite suspension for two minutes followed by dipping in 75% ethanol for 2 min. The tissues were dried on sterile paper towels and placed in 55 mm petri dishes containing SDAY. The medium was supplemented with antibiotics to prevent bacterial contamination. A total of 20 plants and 200 tissue subsamples were evaluated for each treatment during the course of one period of inoculation. The Petri dishes were incubated for four days at 25 ± 2^0 C, in the laboratory, after which all plant samples were visually examined for fungal outgrowth.

B. bassiana colony was characterized as based on white dense mycelia, becoming cream to pale yellow at the edge (Humber, 1997). Percentage colonization was calculated as number of samples exhibiting *B. bassiana* outgrowth per total number of samples, results are expressed as the percentage of plants positive for the presence of *B. bassiana* after inoculation. The number of fungal endophytes other than *B. bassiana* isolated from plants at one month and two months post-inoculation were also assessed and identified based on morphological character by service provided by Agharkar Institute, Pune, India.

Results and Discussion

Colonization of *B. bassiana* in non-*Bt* cotton as an endophyte

All fungal inoculation methods were effective in introducing *B. bassiana* into the plant, although at different levels of efficiency. When the data are combined for all inoculation methods, the total percent of plants that tested positive for *B. bassiana i.e.* colonisation of *B. bassiana* was 35%, 39.5% and 40% at concentrations of 10^6,10^7 and 10^8 respectively at one month post inoculation, 42.5%, 48.5% and 68% at concentrations of 10^6,10^7 and 10^8 respectively at two months post inoculation (Fig. 1).

At two months post inoculation, in foliar application method, highest colonization of 32% was observed in leaf samples followed by 21% in stem samples and 8 and 5.5% in stem and leaf samples respectively in soil drenching method at the concentration of 1x 10^8conidia ml^{-1}. Similarly at one month post inoculation, in foliar application method, the highest colonization of 23.5 and 14% was observed in leaf samples and stem samples respectively at 1x 10^8 conidia ml^{-1} concentration. Whereas, in the same inoculation method the colonization was 21 and 19.5% at 1x 10^7 conidia ml^{-1} concentrations in leaf and stem samples respectively (Fig. 1). In seed immersion and seed coating methods of inoculation there were no colonization was observed at 10^6 and

10^7 concentrations. But at 10^8concentration, it shows very low percentage of colonization *i.e.* less than one per cent. *B. bassiana* was not isolated from any of the samples from control plants.

In soil drenching method of inoculation, per cent colonization of *B. bassiana* was high in stem, than in leaves at both the sampling periods, whereas, in foliar application method the per cent colonization was high in leaf than in stem.

Irrespective of the inoculation methods, the total samples positive for *B. bassiana* colonization was 29% in leaves at 10^8 concentration followed by 23% in stem at 10^7 and 20.50% in stems at 10^6 concentrations at one month post inoculation. Similarly at two months post inoculation it was 39% at 10^8 in leaf samples followed by 30.50 % in stem, 28.50% in leaf at 10^7concentrations (Fig. 1).

In the present study, at two months post inoculation, *B. bassiana* was successfully re-isolated from the interior of stem and leaves of cotton plants, clearly indicating that cotton can serve as a suitable host for *B. bassiana* endophyte.

B. bassiana has been established as an endophyte in various plants by different methods of inoculation such as foliar sprays, radical dressing, root and rhizome immersion, seed coating and soil drenching (Parsa *et al.*, 2013).

It is speculated that exposure to high doses may increase colonization and persistence of endophytic *B. bassiana* in plant tissues. Recovery from stems and leaves also shows that *B. bassiana* can translocate throughout the plant tissues.

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.

Colonization of *B. bassiana* in *Bt* cotton as an endophyte

At two months post inoculation, in foliar application method highest colonization of 30.5% was observed in leaf samples followed by 19% in stem samples and 7 and 9% in stem and leaf samples respectively in soil drenching method at 10^8concentration. At one month post inoculation, in foliar application method the highest colonization of 20.5 and 12% was observed in leaf samples and stem samples respectively at 10^8concentration (Fig. 2).

The colonization of *B. bassiana* were observed in all the concentration tested (1x 10^6, 10^7 and 10^8) both in soil drenching and foliar application methods. In seed immersion and seed coating method there were no colonization was observed at 10^6 and 10^7 concentrations. But at 10^8 it shows very low colonization *i.e.* not more than two per cent. *B. bassiana* was not isolated from any of the samples from control plants. In soil drenching method of inoculation at both the sampling periods the per cent colonization was high in stem than in leaves whereas, in foliar application method the per cent colonization was high in leaf than stem (Fig. 2).

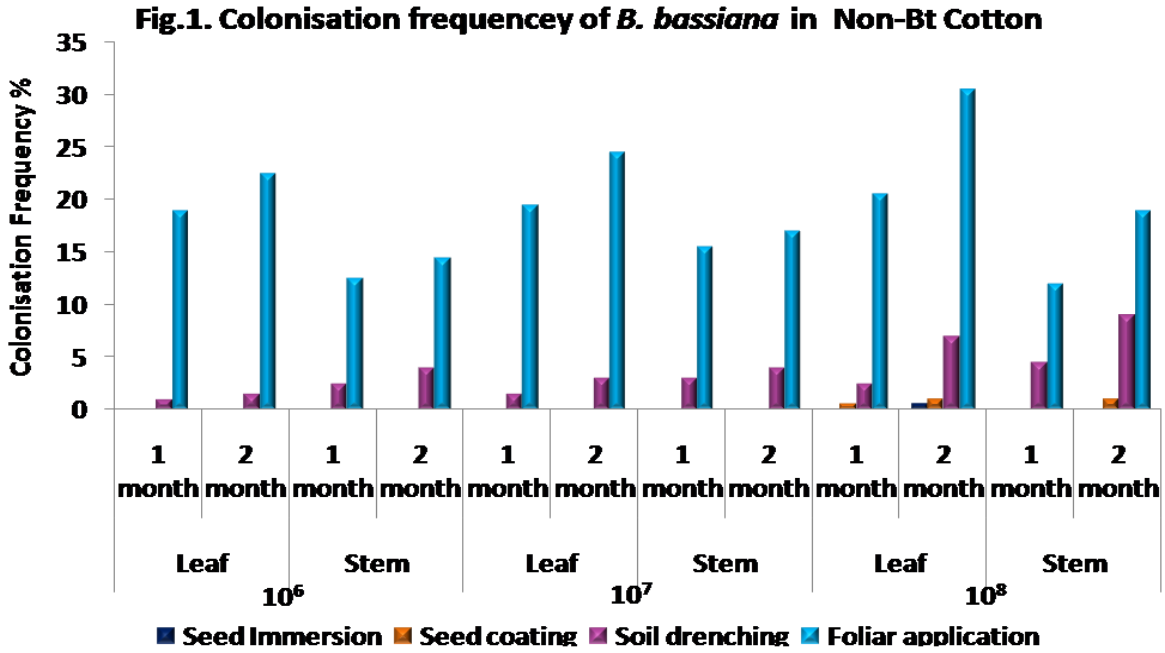
The colonisation of *B. bassiana* in Bt and non Bt cotton as endophyte was revealed that there were no significant differences observed regarding colonization. Similar trend were reported in corn by Lewis *et al.*, (2001).

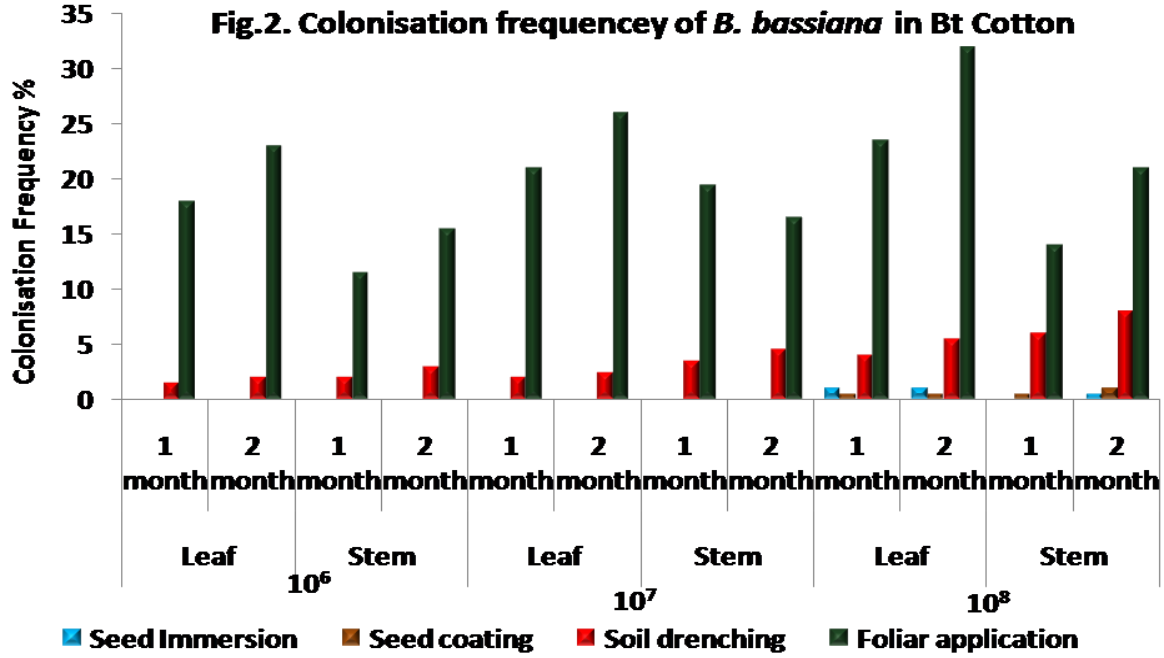
In the current study, *B. bassiana* colonization was differed among the plant parts isolated. Many endophytic fungi show a certain degree of tissue specificity because they are adapted to particular conditions present in a given organ.

Table.1 Endophytic fungi isolated from cotton plants at one and two months post inoculation

Isolates	n	One month post inoculation						n	Two months post inoculation					
		Leaf		Stem		Total			Leaf		Stem		Total	
		n	%	n	%	n	%		n	%	n	%	n	%
Fusarium solani	144	13	9.03	8	5.56	21	14.58	157	16	10.19	6	3.82	22	14.01
Fusarium oxysporum	144	12	8.33	6	4.17	18	12.50	157	14	8.92	7	4.46	21	13.38
Fusarium sp.	144	12	8.33	10	6.94	22	15.28	157	13	8.28	6	3.82	19	12.10
Penicillium piceum	144	7	4.86	5	3.47	12	8.33	157	9	5.73	5	3.18	14	8.92
Aspergillus flavus	144	11	7.64	5	3.47	16	11.11	157	11	7.01	7	4.46	18	11.46
Aspergillus terrus	144	10	6.94	4	2.78	14	9.72	157	9	5.73	6	3.82	15	9.55
Geotrichum candidum	144	2	1.39	1	0.69	3	2.08	157	2	1.27	0	0.00	2	1.27
Pestalotiopsis uvicola	144	3	2.08	0	0.00	3	2.08	157	2	1.27	1	0.64	3	1.91
Nigrospora sphaerica	144	3	2.08	1	0.69	4	2.78	157	2	1.27	0	0.00	2	1.27
Alternaria sp.	144	4	2.78	3	2.08	7	4.86	157	7	4.46	4	2.55	11	7.01
Chaetomium sp.	144	3	2.08	0	0.00	3	2.08	157	2	1.27	1	0.64	3	1.91
Paecilomyces sp.	144	2	1.39	0	0.00	2	1.39	157	2	1.27	0	0.00	2	1.27
Curvularia sp.	144	2	1.39	1	0.69	3	2.08	157	2	1.27	0	0.00	2	1.27
Cladosporium sp.	144	2	1.39	0	0.00	2	1.39	157	2	1.27	0	0.00	2	1.27
Phomopsis archeri	144	2	1.39	0	0.00	2	1.39	157	2	1.27	1	0.64	3	1.91
Phoma exigua	144	2	1.39	0	0.00	2	1.39	157	2	1.27	0	0.00	2	1.27
Acremonium sp.	144	2	1.39	0	0.00	2	1.39	157	1	0.64	0	0.00	1	0.64
Non sporulating hyphae	144	6	4.17	2	1.39	8	5.56	157	10	6.37	5	3.18	15	9.55
Total		98	68.05	46	31.93	144	99.99		108	68.8	49	31.2	157	100

Percentages are based on 144 positive fungal isolations at one month post-inoculation and 157 at two months post-inoculation.





Differential *B. bassiana* colonization on plant parts was demonstrated in corn and cocoa (Posada and Vega, 2005). In corn, the fungus was most frequently isolated from the internode below the primary ear and less frequently from the leaf collar at the primary ear. In cocoa, colonization rates in roots were higher than those in stems and leaves. The reason for the lack of endophytic colonization in seeds treated with *B. bassiana* is not clear and requires further investigation.

Fungal endophytes isolated from cotton plant

While isolation of *B. bassiana* from the 200 subsamples, other than *B. bassiana*, number of fungal endophytes present in the samples were recorded at one and two months post inoculation. At one month post inoculation, 144 isolates were recovered and identified as belonging to 17 species of fungus, one were non-sporulating hyphae whereas, at two month of post inoculation 157 isolates were recovered and belongs to 17 species of fungus (Table 1).

The most frequently isolated fungal species at one and two months post-inoculation were *Fusarium*, *Pencillium piceum*, *Aspergillus flavus* and *A. terrus*. Similar results were obtained in other endophytic studies also (Rajagopal and Suryanarayanan, 2000). Occurrence of non sporulating hyphae was observed both in leaves and stem samples at both the sampling period (Table 1). Occurrence of sterile mycelium as endophytes is not unusual (Bills, 1996).

At one month post inoculation, the highest number of endophytes recovered from leaves (98) followed by stems (46) similarly, at two months post-inoculation, the highest incidence of endophytes recovered from leaves (108) followed by stems (49) (Table 1). There are many fungi isolates occurred as endophytes in cotton also documented. McGee (2002) found that 13 fungal morpho species, including *Alternaria* spp., *Phomopsis* spp., and *Fusarium* spp. occurred as endophytes in leaves of cotton. Seventeen fungal genera, of which *Phoma*, *Alternaria*, *Fusarium*, *Botryo sphaeria*, *Dichomera* and *Phomopsis* were the common genera, were recovered from the stems of *Gossypium*. *Phoma* spp., *Fusarium* spp., and *Phomopsis* spp. are common fungal endophytes in both tropical and temperate climates (Schulz, 2005).

In conclusion, the results of this study indicated that the *B. bassiana* can form an endophytic relationship with cotton plants and foliar spray and soil drenching inoculation methods were the best method for the delivery of *B. bassiana* in to the cotton plants. As a result the *B. bassiana* inoculum load most likely remains stable in the cotton plant ever and protect the plant from herbivores. *B. bassiana* becomes an endophyte introduces the possibility that the fungus might naturally recycle in the cotton ecosystem. No significant differences were observed in colonization of *B. bassiana* as endophyte in *Bt* and non-*Bt* cotton plants. The success of artificial inoculation of *B. bassiana* as endophyte into cotton plants determines many future works. It should focus on fine tuning of methodology to optimise the long term establishment of *B. bassiana* on cotton plant and inoculated plants will be evaluated for its virulence against major pests of cotton.

Acknowledgement

The author is thankful to the Director and Project co-ordinator and Head of ICAR-Central Institute for Cotton Research, for providing necessary facilities during this study.

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How to cite this article:

Amutha, M. 2017. Establishment of *Beauveria bassiana* (Balsamo) Vuillemin as an Endophyte in Cotton. *Int.J.Curr.Microbiol.App.Sci.* 6(6): 2506-2513.
doi: https://doi.org/10.20546/ijcmas.2017.606.298