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# Potential of the entomopathogenic fungus *Beauveria bassiana* as an endophyte in cotton

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**Abstract**
Eight isolates of *Beauveria bassiana* were applied to cotton plant by four inoculation methods *viz*., seed immersion, seed coating, soil drenching and foliar application to establish *B. bassiana* as endophyte in cotton. One month later, the cotton plant leaves, stems and roots are sampled to evaluate endophytic fungal colonisation. Samples are cut into multiple sections, surface sterilized and incubated in media. The media is inspected every 2-3 days to observe fungal growth associated with plant sections and occurrence of *B. bassiana* recorded to estimate the extent of its endophytic colonisation. All the isolates were able to colonise the cotton plant. Among the four methods, plants inoculated by foliar application method recorded highest colonisation followed by soil drenching with no negative effect on plant growth or survival. *B. bassiana* isolate 8 was the best coloniser followed by *B. bassiana* 1 and *B. bassiana* 3. The result of colonization of *B. bassiana* as endophyte was revealed that, *B. bassiana* can able to establish as endophyte in cotton plants, causing no harmful effects and might provide an alternative method for biological control.

**Keywords:** Cotton, *Beauveria bassiana*, endophytes, colonisation, microbial control

## 1. Introduction
The indiscriminate use of pesticides and chemical fertilizers in agriculture has raised a number of ecological problems such as resistance development in plant pathogens and pests, environmental pollution and negative impacts on human health. In this context, beneficial microorganisms are now integrated as part of integrated pest management practices [1], which allow a significant reduction in the use of synthetic chemicals. The entomopathogenic fungi *Beauveria bassiana* is natural enemy of a wide range of insects [2] and have been extensively studied for biological control [3, 4].
Fungal entomopathogens can be used in multiple ways to protect crops against attacks of insect pests. In the last decade, several studies have shown that many entomopathogenic fungi also possess the potential to colonise plant tissues and thus to grow as an endophyte inside different plant species [5, 6]. Moreover, *B. bassiana* has been successfully established as an endophyte *via* an artificial application in a variety of crop plant species including maize, potato, cocoa, coffee, banana, date palm and sorghum [7]. Accordingly, endophytic establishment and entomopathogenic activity of *B. bassiana* in a given crop plant represents an alternative strategy for the application of this fungus in insect pest management programmes and has thus a high potential for development of new and sustainable crop protection strategies.
Enhanced endophytic expression of entomopathogen within the plant system is expected to be more advantageous than external application of bio-agents because of continuous presence and assured expression of the entomopathogens throughout the crop cycle and get protected from biotic and abiotic factors.

## 2. Materials and Methods
### 2.1 *B. bassiana* isolates inoculum preparation
Eight *B. bassiana* isolates (*B. bassiana* 1 to *B. bassiana* 8) were used in this experiment. The isolates *B. bassiana* 1 to *B. bassiana* 7 were isolated from Coffee berry borer, *Hamilton hampsei* infested with *B. bassiana* from different village of Dindigul district, Tamil Nadu, India and *B. bassiana* 8 was isolated from cotton leaf form Coimbatore. Isolated *B. bassiana* isolates were 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 containing antibiotics (0.1g penicillin, 0.2g streptomycin and 0.05g chlorotetracycline$^{-1}$ SDAY) in 55 mm diameter Petri dishes. The Petri dishes containing the *B. bassiana* were incubated for three weeks in the laboratory (22–30ºC, RH 65-70% and a photoperiod of 12:12 hrs). After three weeks from these cultures, *B. bassiana* suspension was prepared. The conidial concentration for each treatment were adjusted to 1 x$10^8$ conidia ml$^{-1}$. Germination test of conidia was done before inoculation of the cotton plants.

**2.2 Inoculation methods**

The pot culture experiments were conducted at the ICAR-Central Institute for Cotton Research, Regional Station, Coimbatore, Tamil Nadu, India during the period of 2015-16. To determine the potential of *B. bassiana* colonisation on cotton plants, *B. bassiana* was inoculated by four different methods: (1) Seed immersion (2) Seed coating (3) Foliar spray and (4) Soil drenching. The experiment was organized as a Completely Randomised Design with inoculum dose at $10^8$ conidia/ml. For each treatment, three replications were maintained and five plants per replication were used for experiment. The experiment was conducted a total of three times.

Suraj cotton variety seeds were used for these experiments. Seed coating was done by adding 1g of *B. bassiana* conidial suspension at $10^8$ concentration along with talc. Control seeds were coated with talc and deionsed water. For seed immersion inoculation, 50g of cotton seeds were immersed in 10 ml of a *B. bassiana* conidial suspension for 6 h. They were then dried on sterile tissue paper in a sterile laminar flow cabinet, sown in 15 cm dia plastic pots filled with a sterile soil and maintained in room temperature and a photoperiod of 12-12h L-D. Control seeds were immersed in a conidia-free solution of 0.01% Tween 80. For foliar spray, a hand sprayer was used to spray each seedling with 10 ml conidial suspension. Control plants were sprayed with a conidia-free solution of 0.01% Tween 80. For the soil drenching method, 10 ml conidial suspension of $10^8$ concentration was applied around the root zone of each seedling. In the control, sterile 0.1% Triton X-100 applied in the same way as in each treatment mentioned above. After inoculation, each plant was covered with a plastic bag for 24 hrs to maintain a high level of humidity.

**2.3 Evaluation for presence of *B. bassiana* in cotton tissues**

The colonisation of *B. bassiana* was evaluated by culturing method at one month after post inoculation. The inoculated plants were kept under conditions of room temperature and natural light conditions of 12:12 h and watered daily. Stems were cut off (about 5cm above the stem base) from the roots using a sterile blade. The plant parts were rinsed thrice in sterile deionized water. 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 (0.1g penicillin, 0.2g streptomycin sulphate, 0.25g chloramphenicol and 0.05g chlortetracycline$^{-1}$ SDAY) to prevent bacterial contamination. A total of 10 plants were evaluated for each treatment during the course of one time 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.

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 colonisation frequency data (expressed as percentages) were angular transformed. The transformed data were analysed using analysis of variance (ANOVA) performed with the software Version Infostat, 2001. For Scanning electron microscopy (SEM), young leaves inoculated with *B. bassiana* conidia were selected, cut into small pieces and then dehydrated in an ethanol series to 100% ethanol.

**3. Results**

**3.1 Colonisation of *B. bassiana* in cotton as an endophyte**

*B. bassiana* was able to endophytically colonise cotton plant in response to the demonstrated inoculation treatments. Placing plant parts of treated samples on medium showed emergence of *B. bassiana* by microscopic examination. *B. bassiana* was not detected in any of the control plant sections. All the inoculation techniques were successful in establishing *B. bassiana* as an endophyte, but differences observed in efficacy among the different inoculation techniques and different isolates and different plant parts.

The average percent colonization or colonization frequency varied among the endophytic *B. bassiana* isolates from 46.67% to 3.33%. The technique that resulted highest colonisation was the foliar spray, with 46.67% colonisation of leaves at thirty days post-inoculation. The highest colonization percent (46.67%) was recorded in *B. bassiana* isolate 8 followed by *B. bassiana* isolate 1 (43.33%). Irrespective of method of inoculation, among the eight *B. bassiana* isolates, the highest mean colonization percentage recorded as 19.59 in *B. bassiana* isolate 8 followed by *B. bassiana* isolate 1 as 19.17% (Table 1).

The inoculation method significantly affected the percent colonisation. Both foliar spray and soil drench methods resulted in endophytic colonization of 35% of the treated plants by *B. bassiana*. However, the extent of colonization depended on the plant part evaluated and the inoculation method used. Seed immersion method resulted with lowest mean colonisation percentage of 4%. Irrespective of inoculation methods and different isolates, leaves responded best with 16% mean colonisation and maximum of 21% colonisation by spray inoculation method. Stems also responded similarly to all inoculation methods with mean colonisation of nine percentage (Table 1).

Using Scanning Electron Microscopy on cotton seedlings inoculated with *B. bassiana*, conidial germination and hyphal growth were found to be associated with leaf. Germ tube formed from a conidium and hyphae of *B. bassiana* were observed also penetrating epithelial cells of cotton and ramifying through palisade parenchyma and mesophyll leaf tissues. Hyphae may gain access to the leaf interior through stomatal openings. The typical method of invasion is directly through the epidermal cell wall and into the leaf interior. Penetration of the epidermal cell wall shows that the plant cell wall is completely breached and the hyphae grow through the hole. Examination of hyphae inside the leaf shows that they grow through the air spaces between parenchyma cells. The fungus colonized parenchyma both intra- and intercellular (Fig. 1 & 2).

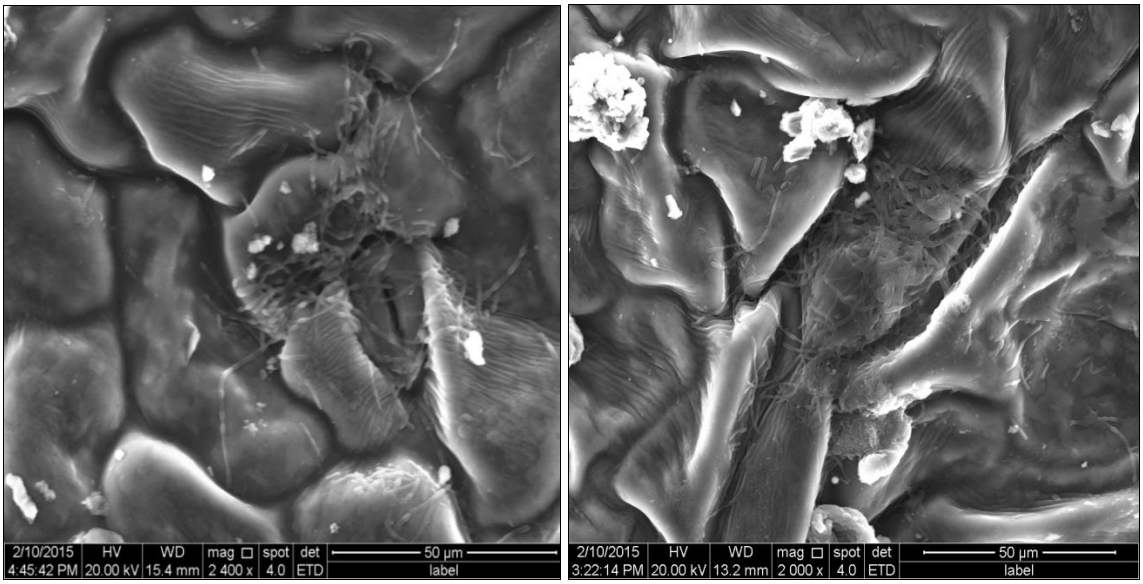


**Fig 1-2:** Scanning electron microscopy photograph showing *B. bassiana* colonisation on cotton leaf

**Table 1:** Efficacy of inoculation treatments on endophytic colonization by *Beauveria bassiana* on cotton plant (1 month after inoculation)

| Isolates | Per cent colonisation | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Seed coating | | Seed immersion | | Soil drenching | | Foliar spray | | Mean (Isolate) | Mean (Leaf) | Mean (Stem) |
| | Leaf | Stem | Leaf | Stem | Leaf | Stem | Leaf | Stem | | | |
| *Beauveria bassiana* 1 | 16.67 | 13.33 | 3.33 | 6.67 | 20.00 | 20.00 | 43.33 | 30.00 | 19.17 | 20.83 | 17.50 |
| *Beauveria bassiana* 2 | 13.33 | 6.67 | 3.33 | 3.33 | 20.00 | 13.33 | 30.00 | 13.33 | 12.92 | 16.67 | 9.17 |
| *Beauveria bassiana* 3 | 13.33 | 10.00 | 3.33 | 0.00 | 23.33 | 20.00 | 36.67 | 13.33 | 15.00 | 19.17 | 10.83 |
| *Beauveria bassiana* 4 | 20.00 | 10.00 | 6.67 | 3.33 | 20.00 | 13.33 | 33.33 | 16.67 | 15.42 | 20.00 | 10.83 |
| *Beauveria bassiana* 5 | 16.67 | 0.00 | 6.67 | 6.67 | 16.67 | 0.00 | 10.00 | 3.33 | 7.50 | 12.50 | 2.50 |
| *Beauveria bassiana* 6 | 6.67 | 3.33 | 0.00 | 3.33 | 10.00 | 3.33 | 20.00 | 6.67 | 6.67 | 9.17 | 4.17 |
| *Beauveria bassiana* 7 | 3.33 | 3.33 | 3.33 | 0.00 | 0.00 | 0.00 | 10.00 | 0.00 | 2.50 | 4.17 | 0.83 |
| *Beauveria bassiana* 8 | 16.67 | 13.33 | 6.67 | 6.67 | 30.00 | 16.67 | 46.67 | 20.00 | 19.59 | 25.00 | 14.17 |
| Mean (Method) | 10.42 | | 3.96 | | 14.17 | | 20.83 | | 12.35 | 15.94 | 8.75 |
| S.Ed | 1.402 | 1.659 | 2.348 | 2.099 | 1.022 | 1.215 | 1.016 | 1.330 | | | |
| CD (5%) | 2.971 | 3.517 | 4.978 | 4.450 | 2.166 | 2.576 | 2.154 | 2.82 | | | |

Replications-3, Samples/replication-10

## 4. Discussion

In the present study, at one month 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. Many factors can influence the outcome of an experiment to establish a fungal entomopathogen as an endophyte, includes crop species and the fungal entomopathogen isolate used, the concentration of the inoculum, the age of the plant during inoculations and inoculation methods.

This result indicates that foliar spray treatment with the spore suspension of *B. bassiana* is an effective method for achieving endophytic colonization of the entomopathogen in cotton. *B. bassiana* has been established as an endophyte in various plants by different methods of inoculation [8]. However, endophytic colonization of *B. bassiana*, depended upon the inoculation method, fungal isolate and plant species.

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. In the current study, *B. bassiana* colonization was differed among the plant parts isolated. The reason for higher colonization on leaves than stems is not clear but could reflect differences in microbial and physiological conditions in the different plant parts. Many endophytic fungi show a certain degree of tissue specificity because they are adapted to particular conditions present in a given organ [9-11]. Differential *B. bassiana* colonization on plant parts was equally demonstrated in corn (*Zea mays* L.) and cocoa (*Theobroma cacao* L.) [12-13]. 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. The reason for the lack of endophytic colonisation in seeds immersed with *B. bassiana* is not clear and requires further investigation. In soil drenching method, watering of plants might have led to loss of conidia through water filtration, reducing their chances of uptake by the roots. Instead, it appears that inoculation methods tend to favour a specific pattern of local colonization. In coffee, for example, foliar sprays favour leaf colonization whereas soil drenches favour root colonization.

*B. bassiana* isolate 8, isolated from cotton leaf, colonized plant tissues better than other isolates. Based on the origin of the strains and the results obtained in this study, we hypothesize that *B. bassiana* isolate 8 and *B. bassiana* 1 is well adapted to endophytically colonize cotton plants. Biswas *et al.*, 2013 [7] reported, the variation in colonization frequency among various *B. bassiana* strains may be due to their differential growth rate and endophytic adaptation. Ultimately, the choice of inoculation method should be guided by the intended location of the endophyte within a plant, presumably matching the niche of the target herbivore.

## 5. Conclusions

In conclusion, we provide evidence that important entomopathogenic fungi *B. bassiana* colonised living cotton plant and therefore may act as cotton endophytes. This can be an important ecological advantage for maintaining a natural *B. bassiana* inoculum in cotton plant. The success of artificial inoculation of *B. bassiana* as endophyte into cotton plants determines many future works, which should focus on the improvement of efficacy of endophytes against insects.

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