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Research Article

In planta colonisation of *Beauveria bassiana* in cotton plant and its effect against insect pests

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ABSTRACT: The aim of the present study was to evaluate the efficiency of artificial establishment of *Beauveria bassiana* as endophyte in cotton plant using different inoculation methods, and aimed to determine the effect of colonisation in cotton plant on key insect pests of cotton. Eight strains of *B. bassiana* isolated as endophytes were used in this experiment. The strains *B. bassiana* isolated as endophytes were concentrated at 1×10^8 conidia ml^{-1} and bioassays were conducted under laboratory conditions on *Aphis gossypii, Spodoptera litura* and *Pectinophora gossypiella.* These endophytic strains demonstrated high virulence against above mentioned insects. Different inoculation methods were used to establish *B. bassiana* as endophyte in cotton plants. Endophytic colonisation of *B. bassiana* was successful in cotton plant. *Beauveria bassiana* colonised plant infested with insect was monitored at different time intervals. Survival of the insect was affected considerably in the *B. bassiana* inoculated plant. The current study clearly indicated that strains of *B. bassiana* isolated as endophytes caused the mortality of *A. gosypii, S. litura* and *P. gossypiella* as an entomopathogen and also as an endophyte.

KEY WORDS: *Beauveria bassiana*, cotton, endophytes, microbial control

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INTRODUCTION

Endophyte mediated pest management is a novel area in agricultural pest management. It offers an eco-friendly management system by decreasing the use of pesticides, improving crop vigour and providing resistance against insect pests. Fungal entomopathogens are important regulators of insect populations with considerable potential as mycopesticides (Vega *et al.,* 2012). Only recently, fungal entomopathogens been shown to occur as endophytes, both naturally and in response to various inoculation methods (Vega *et al.,* 2008).

Beauveria bassiana (Vuillemin) is a common entomopathogen has been found naturally as endophytes in many crop plants and has been artificially inoculated in many crop plants (Vidal & Jaber, 2015; Jaber & Ownley, 2017). *B. bassiana* has been shown to reduce herbivores following their colonization in plants as endophytes (Jaber & Ownley, 2017). There are several studies that endophytic *B. bassiana* can reduce a pest damage to plants directly by mycosis or indirectly by inhibiting the insect development and reproduction (Gurulingappa *et al.,* 2010; Akello & Sikora, 2012; Batta, 2013; Biswas *et al.,* 2013; Mantzoukas *et al.,* 2015).

Antagonistic potential of endophytic fungi has been reported for a variety of different insects living inside plant tissues like stem-borers or root-boring weevil larvae (Akello *et al.,* 2009; Reddy *et al.,* 2009). The mechanisms involved in the control of arthropod pests using endophytes include antagonism, induction of plant host defenses, host plant tolerance, or a combination of these (Ownley *et al.,* 2010; Gomez-Vidal *et al.,* 2009; Porras-Alfaro and Bayman, 2011). Most of the well known defence systems evolved to fight against insect attack (Akello & Sikora, 2012; Allegrucci *et al.,* 2018) mostly involves antifeedant or toxic compounds that hamper the insect growth (McCormick *et al.,* 2016). A plant which was induced, transforms the metabolic compounds that hamper the feeding of insects (Hokkanen & Menzler-Hokkanen, 2017).

Application of *B. bassiana* as endophyte into cotton plant might have a good potential for a sustainable insect pest

control. Endophytes present in the cotton plants will serve as initial inoculums for insects start feeding on the plant; endophytic fungal propagules might persist in cotton plant parts and might thus infect progeny from any subsequent infestations by this insect. The current study was conducted to determine the strains of *B. bassiana* isolated as endophytes, can be artificially re-inoculated in to cotton plant to establish as endophytes and investigate the effects on insects pests of cotton using *in planta* bioassays.

MATERIALS AND METHODS

Beauveria bassiana 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, *Hypothenemus hampei* infested with *B. bassiana* from different villages of Dindigul district, Tamil Nadu, India and *B. bassiana* 8 was isolated from cotton plant at Coimbatore. Isolated *B. bassiana* isolates were subcultured on Sabouraud Dextrose Agar medium with Yeast extract (SDAY) containing antibiotics in 55 mm diameter Petri dishes. The Petri dishes containing the *B. bassiana* were incubated for three weeks in the laboratory (22–30°C, RH 65-70% and a photoperiod of 12:12 hrs). After three weeks, from these cultures of *B. bassiana* suspension was prepared. Conidial concentration was adjusted to $1x10^8$ conidia ml^{-1} and germination test of conidia carried out before inoculating the plants.

Evaluation of pathogenicity of *B. bassiana* isolates

Virulence of all *B. bassiana* isolates against insect pests was evaluated prior to the inoculation of the cotton plants. Conidial suspensions for experiments were obtained by scraping conidia from three weeks old cultures on medium into an aqueous solution of 0.02% Tween 80. The viable conidia containing $1x10^8$ was prepared and utilised for the bioassay experiment. For control distilled water was utilised. Pathogenicity was evaluated against major insect pests of cotton under laboratory condition. Second instar larvae of *Spodoptera litura, Pectinophora gossypiella* and *Aphis gossypii* were used in this experiment. Larvae of *S. litura* and *P. gossypiella* were dipped for 1 min into 15 ml of a conidial suspension of *B. bassiana*, as well as 15 ml of distilled water (control treatment). After 10 min of treatments, each larva was placed individually in a Petri dish (15 cm diameter) with a cotton leaf for *S. litura* and *A. gossypii* and artificial diet for *P. gossypiella* for feeding. The treatments were repeated four times and each treatment unit consists of 20 insects. Mortality of insects was observed every day. The experiment was arranged in completely randomized design (CRD). Results were analysed using Analysis of Variance followed by followed by Duncan's New Multiple Range Test (DNMRT).

In planta feeding experiment

Pot culture experiment was carried out to study the efficiency of the endophytic *B. bassiana* isolates against insect pests of cotton using *in planta* feeding trials. The pot culture experiments were conducted at the ICAR-Central Institute for Cotton Research, Regional Station, Coimbatore, Tamil Nadu, India. Eight *B. bassiana* endophytes were inoculated into cotton plant by four different methods *viz*., (1) Seed immersion, (2) Seed coating, (3) Foliar spray, and (4) Soil drenching. The experiment was laid as a Completely Randomised Block Design with inoculum dose at 10^8 conidia/ml. For each treatment, three replications were maintained and five plants per replication were used for the 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 one gram of *B. bassiana* conidial suspension at 10^8 concentrations along with talc were coated with cotton seeds. For control, seeds were coated with talc and deionsed water. For seed immersion inoculation, 50 g 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 h - 12 h Light-Day. 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 concentrations was applied in the region of root zone of seedling. In the control, sterile 0.01% Tween 80 was applied in the similar method as in each treatment mentioned above. After inoculation, every plant was enclosed with a plastic bag for 24 hours to sustain a high level of humidity. Seed immersion and seed coating method of inoculation carried out at the time of sowing, foliar spray and soil drenching method of inoculation carried out at fifteen days after sowing. Thirty days after treatment, inoculated plants were used for *In planta* feeding experiment.

Inoculated cotton plants were artificially infested with 15 numbers of *S. litura,* and *A. gossypii* per replication. During the bioassay, inoculated plants were kept individually in cages covered with Mylar film cages. Ten neonates of *P. gossypiella* were released per replication. Neonates of *P. gossypiella* released in young bolls and covered with cage to avoid escape of larvae. The percent mortality was observed at fifteen days after treatment. Dead insects were removed from the plants and incubated on SDAY medium in petridishes to stimulate the fungal growth. If signs of mycosis observed, microscopic observation were followed to confirm the *B. bassiana* infection.

Colonisation of *B. bassiana* in cotton plant

Beauveria bassiana inoculated plants were evaluated for the colonisation of *B. bassiana* by culturing method at one month after post inoculation. The plant parts after surface sterilisation were placed in 55 mm petri dishes containing SDAY. Petri dishes were incubated for four days at 25 ± 2^0 C in the laboratory, after that all plant part samples were visually examined for *B. bassiana* outgrowth.

Phylogenetic analysis of *B. bassiana* isolates

Molecular characterisation of eight *B. bassiana* isolates carried out by outsourcing to Chromous Biotech Private Limited, Bangalore. The genomic DNA was extracted from fresh mycelia. The PCR reactions were carried out in Eppendorf Mastercycler with cycling parameters. The PCR products were sequenced using respective primers. Sequences obtained from the respective primers (ITS5 and ITS4) were aligned and consensus sequences were generated and deposited in NCBI-GeneBank. Phylogenetic analyses were carried out for all the eight *B. bassiana* isolates using MEGA software.

RESULTS AND DISCUSSION

Pathogenicity of *B. bassiana* isolates

All the eight *B. bassiana* isolates have exhibited entomopathogenic activity with variation in degree of virulence. Pathogenicity of *B. bassiana* isolates showed that, among the eight isolates, Bb8 isolate is most virulent isolate; it caused mortality of *A. gossypii* and *S. litura* highest up to 75% and 73% mortality in *P. gossypiella*. Virulence of Bb4 isolates was low with an average mortality of 40% (Table 1) (Figure 1-3).

In planta feeding experiment

The aim of this study was to identify whether colonization of *B. bassiana* in plants influence the insects. The recovery of *B. bassiana* from parts of cotton plants indicates the colonisation of *B. bassiana* in cotton plant. *B. bassiana* could be successfully introduced as an endophyte through different inoculation methods. In the present study, *in planta* feeding bioassays clearly shows that all the three insects are susceptible towards the entomopathogenic fungus *B. bassiana*. Increase in mortality of *A. gossypii, S. litura* and *P. gossypiella* in *In planta* feeding experiment with endophytic *B. bassiana* inoculated cotton plants, which was significantly high across the treatments compared to the control. The variability in mortality was detected among the strains of *B. bassiana* and method of inoculation.

Significant differences were observed between mean percent mortality of *A. gossypii, S. litura* and *P. gossypiella* developing on *B. bassiana* inoculated plants and control plant. The mean mortality of *A. gossypii* ranged from 8.7% to 14.83%, maximum of 14.83% observed in *B. bassiana* 4 colonised plants. Average of 3.88 – 7.95 % mortality of *S. litura* was detected in *B. bassiana* colonised plants. For *P. gossypiella,* maximum mortality of 17.2% was recorded in *B. bassiana* 4 inoculated plants and minimum mortality of 8.3% was recorded in plants which was colonised by strain of *B. bassiana* 6. A total mean mortality in the control plants reached below one percent (Table 2). A comparison of mean percentage mortalities among different *B. bassiana* strains indicated significant variability. The infestation level was low in *B. bassiana* inoculated plants compared to control where extensive damage was observed. Development of all larval stages was affected significantly after feeding on plants

Table 1. Pathogenicity of endophytic *B. bassiana* isolates against insect pests of cotton

***Beauveria bassiana* isolates**	**Percent cumulative mortality**		
	P. gossypiella	***A. gossypii***	***S. litura***
	18 DAT	**9 DAT**	**21 DAT**
B. bassiana 1	53.33	71.11	60.00
B. bassiana 2	40.00	66.66	55.55
B. bassiana 3	42.22	68.88	62.22
B. bassiana 4	39.99	55.55	48.88
B. bassiana 5	45.55	68.88	66.66
B. bassiana 6	57.77	73.33	62.22
B. bassiana 7	62.22	64.44	71.11
B. bassiana 8	73.33	75.55	75.55
Control	6.66	4.44	8.88
S.Ed	**0.95**	**1.05**	**0.93**
CD (5%)	**1.99**	**2.20**	**1.95**

Fig. 1. *A. gossypii*

Fig. 2. *S. litura*

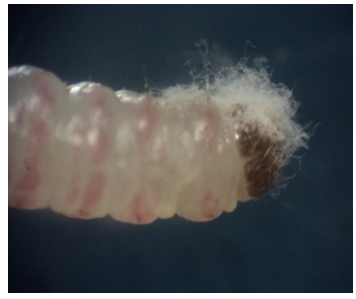

Fig. 3. *P. gossypiella*

Fig. 1-3. Pathogenicity of *B. bassiana* against insect pests of cotton

Table 2. *In planta* feeding experiment of *B. bassiana* against insect pests of cotton

Treatments	Percent cumulative mortality														
	A. gossypii **(15 DAT)**					***S. litura*** **(15 DAT)**					***P. gossypiella*** **(15 DAT)**				
	a	**b**	**c**	**d**	**e**	**a**	**b**	**c**	**d**	**e**	**a**	**b**	**c**	**d**	**e**
B. bassiana 1	9.6	7.4	8.9	8.9	8.7	6.7	5.2	4.4	5.9	5.55	14.4	15.6	13.3	16.7	15.0
B. bassiana 2	9.6	8.9	11.9	12.6	10.75	5.2	4.4	3.7	5.2	4.63	11.1	8.9	12.2	13.3	11.4
B. bassiana 3	12.6	14.1	13.3	15.6	13.9	4.4	3.7	3.0	4.4	3.88	10.0	7.8	7.8	11.1	9.2
B. bassiana 4	14.1	15.6	13.3	16.3	14.83	6.7	7.4	6.7	8.1	7.23	16.7	17.8	15.6	18.9	17.2
B.bassiana 5	10.4	8.9	9.6	10.4	9.83	5.2	3.7	4.4	6.7	5.00	11.1	7.8	8.9	11.1	9.7
B. bassiana 6	9.6	8.9	9.6	10.4	9.63	7.4	8.1	7.4	8.9	7.95	8.9	6.7	7.8	10.0	8.3
B. bassiana 7	11.9	11.1	9.6	12.6	11.3	6.7	4.4	4.4	7.4	5.73	13.3	11.1	8.9	14.4	11.9
B. bassiana 8	10.4	8.9	9.6	11.9	10.2	5.9	6.7	5.9	7.4	6.48	11.1	8.9	7.8	12.2	10.0
Control	3.0	0.0	0.0	0.0	0.75	1.5	0.0	0.0	0.0	0.38	3.3	0.0	0.0	0.0	0.8
Mean	10.13	9.31	9.53	10.97		5.52	4.84	4.43	6.00		11.10	9.40	9.14	11.97	
	SED	CD 5%				SED	CD (5%)				SED	CD 5%			
Treatment (T)	0.98	1.95	**			1.07	2.12	**			1.39	2.79	**		
Method (M)	0.65	1.29	NS			0.71	1.41	*			0.93	1.85	*		
TXM	1.95	3.89	NS			2.13	4.25	NS							

a. Seed coating, b. Seed immersion, c. Soil drench, d. Foliar spray, e. Mean.

which were treated with endophytic *B. bassiana.* Mycosis not observed on any of the dead insects during *in planta* bioassays. Fungal outgrowth of *B. bassiana* was evident on dead insects only after kept under incubation in laboratory condition. Whereas, none of the dead insects were collected from control plants detected for *B. bassiana* growth.

The method of inoculation of *B. bassiana* into cotton plant also significantly affected the mean percent mortality of insects. Highest mean mortality (10.97%) of *A. gossypii* was detected in plants colonised with *B. bassiana* by the method of foliar application. Similarly, maximum mean mortality of 6% and 11.97% was observed in *S. litura* and *P. gossypiella* respectively by the same method of inoculation. The order of efficiency in method of inoculation was foliar spray followed by seed coating and seed immersion and least was soil drench.

In the present investigation, results of *In vivo* and *In planta* bioassays shows that insects are significantly susceptible to the endophytic *B. bassiana*. Variation in insect mortality observed between different *B. bassiana* isolates in the bioassays. The differences in the virulence of *B. bassiana* isolates might be due to differences in physiological character of different isolates *viz*., germination of conidia, growth of colonies, sporulation and ability to produce enzymes and toxins (Mahdneshin *et al.,* 2009). Variation in insect mortality among *B. bassiana* isolates also reported by Kassa *et al.,*

(2002) who stated that different *B. bassiana* isolates had a different speed against *Sitophilus zeamais.*

A significant larval mortality of lepidopteran pests were obtained on endophyte colonised plants of Brassica, Sorghum or Solanum (Batta, 2013; Mantzoukas *et al.,* 2015; Qayyum *et al.,* 2015). Sanchez-Rodriguez *et al.,* (2018) reported about 57% mortality of cotton leafworm (*Spodoptera littoralis*) on the endophytic *B. bassiana* treated cotton leaves. Similarly, Resquin-Romero *et al.* (2016), reported, 25% to 46.7% mortality of *S. littoralis* larvae in alfalfa, melon and tomato plants. Rondot and Reineke (2016) recorded significant decline in infestation and growth of vine mealybug (*Planococcus ficus*) in grapevine (*V. vinifera*) with endophytic *B. bassiana*.

No mycosis was observed on any of the dead insects during *in planta* bioassays. The results are similar to the previous studies, no fungal outgrowth was discovered in cadavers of insects fed with endophyte colonized plants (Sanchez-Rodriguez *et al.,* 2018; Resquin-Romero *et al.,* 2016). The above results infer that when fungal outgrowth is absent, endophytic fungal entomopathogens may cause mortality of insects by secretion of mycotoxins which indicates indirect mechanism by antibiosis or by induced host plant resistance.

The reduction in survival, consumption of feed and prolongation of development duration was observed when insects fed on *B. bassiana* inoculated plants; concluding that *B. bassiana* effectively exhibited the insecticidal effect on the herbivores. The same result was obtained by Mutune *et al.* (2016) where feeding behaviour of *Ophiomyia* sp. was negatively affected. Similarly, Martinuz *et al.* (2012), Lopez *et al.* (2014) and Lopez and Sword (2015) performed choice experiments and found that aphids prefer feeding on uncolonized plants, endophytes colonized plants adversely affected growth and development of the insects. Many studies have also reported adverse effect on insect fitness due to the presence of endophytic fungi on hosts plants (Crawford *et al.,* 2010; Gurulingappa *et al.,* 2011; Hernawati *et al.,* 2011).

The stimulation of an indirect systemic response might be responsible for this change of behaviour in insects (Lopez and Sword, 2015). In this line, Shrivastava *et al.* (2015) confirmed that, elevated levels of terpenoids, which are considered secondary metabolites with anti herbivore properties recorded in plants inoculated with *B. bassiana.* These studies highlighted the significance of incorporating the use of entomopathogenic endophytes into IPM strategies for protecting plants from pests.

Colonisation of *B. bassiana* in cotton plant

Beauveria bassiana was able to endophytically colonise the cotton plant in response to the inoculation treatments. Placing *B. bassiana* inoculated cotton plant parts on medium showed emergence of *B. bassiana* by microscopic examination. *Beauveria bassiana* growth was not observed in any of the control plant sections. All the strains of *B. bassiana* were successful in establishing as an endophyte in cotton plant by different inoculation techniques irrespective of source of isolation, from which they had been isolated (Figure 4-9).

All the eight strains of *B. bassiana* tested have effectively colonised on the cotton plant and the significant variations were observed in the colonisation by different strains and also by different methods of inoculation (Amutha, 2018). The results of *in planta* bioassays revealed that insects were susceptible to *B. bassiana* colonised cotton plants. This result is in line with previous studies on endophytic establishment of *B. bassiana* in a variety of crop plants.

Different studies evolved different results in the efficacy of artificially introduced entomopathogenic fungi into plants using foliar spray, plant dipping, stem injection, seed coating and soil or root drenching (Lopez *et al.,* 2014; Qayyum *et al*., 2015; Rondot and Reineke, 2016; Jabber and Araj, 2018; Sanchez-Rodriguez *et al.,* 2018). Recent review by McKinnon *et al.* (2017), seed coating and foliar treatment have been used most frequently for inoculation of endophytes in bioassays.

Phylogenetic analysis of *B. bassiana* isolates

In this study, the relationships among the isolates were examined and represented as dendrogram by using UPGMA. The *Beauveria* isolates were divided into two main groups in the distance of 3.00 from the origin. Among the isolates, only one isolate (Bb8) isolates was under group II and all other seven isolates were grouped under group I. In group I, there were two sub-clusters namely A and B. Four isolates were grouped in sub-cluster A and three isolates in sub-cluster B (Figure 10). The dissimilarity index (DI) showed that the isolates Bb3 and Bb8 were distantly related with the maximum value of 8.992 and similarly the isolates Bb5 and Bb7 were closely related with low DI value of 2.217 (Table 3).

CONCLUSION

Beauveria bassiana has successfully established as endophyte in cotton plant after artificial inoculation in laboratory condition. *Beauveria bassiana* colonisation in plant

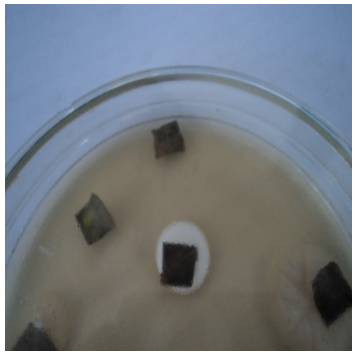

Fig. 4

Fig. 5

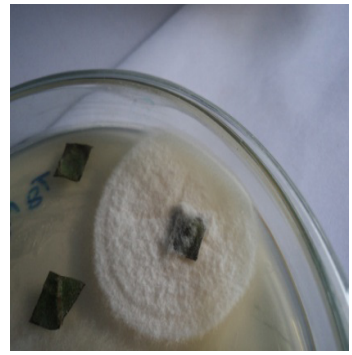

Fig. 6

Fig. 4-6. Colonisation of inoculated *B. bassiana* in cotton leaf parts

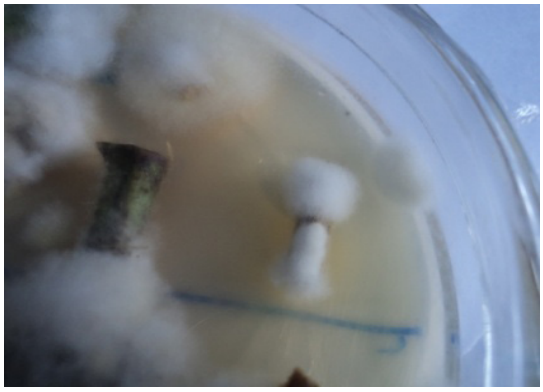

Fig. 7

Fig. 8

Fig. 7-8. Colonisation of inoculated *B. bassiana* in cotton stem parts

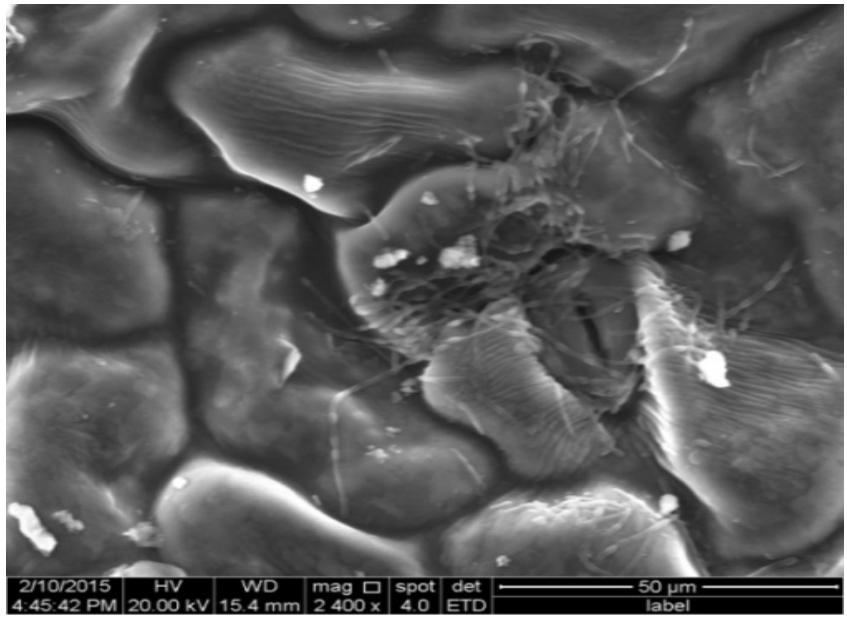

Fig. 9. Scanning electron microscopy photograph showing *B. bassiana* colonisation on cotton leaf

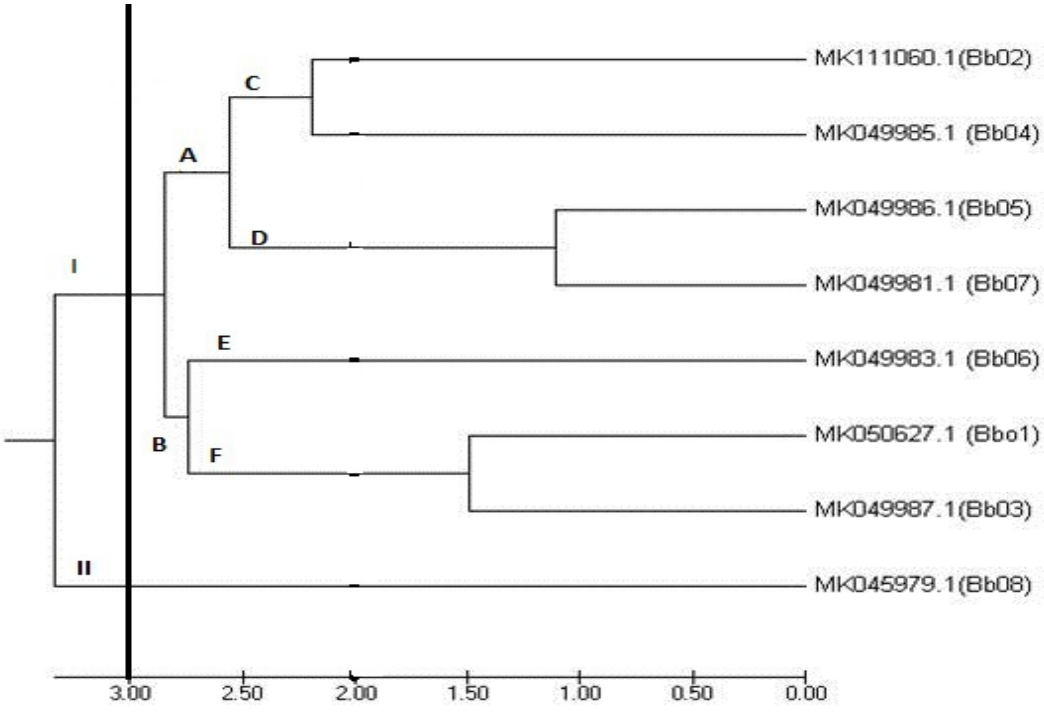

Fig. 10. Dendrogram of endophytic *B. bassiana* isolates using UPGMA

affected survival of the insects, reduced damage on colonised plants speculated that endophytic *B. bassiana* negatively influence the feeding and development of insect. *Beauveria bassiana* is not only directly acting as entomopathogen on insects, but is also able to provide protection for plants against insects as endophytes. This potential could be of great significance in ecofriendly pest management. Endophytic establishment and entomopathogenic activity of endophytic *B. bassiana* in a crop represents a novel approach in insect pest management programmes and has a high potential for development of new and sustainable crop protection strategies. This strategy may be a suitable tool for designing new biocontrol strategies for cotton plant.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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