ENDOPHYTIC FUNGI FOR THE MANAGEMENT OF SPOTTED POD BORER, *Maruca vitrata* Fab. (LEPIDOPTERA: CRAMBIDAE) IN COWPEA

By

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(2016-21-012)

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VELLANIKKARA, THRISSUR – 680 656

2022

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THESIS

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requirement for the degree of

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DEPARTMENT OF AGRICULTURAL ENTOMOLOGY
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VELLANIKKARA, THRISSUR – 680 656
2022

DECLARATION

I, Smitha Revi (2016-21-012) hereby declare that the thesis entitled **'Endophytic fungi for the management of spotted pod borer, *Maruca vitrata* Fab. (Lepidoptera: Crambidae) in cowpea'** is a *bonafide* record of research work done by me during the course of research and this thesis has not previously formed the basis for the award to me any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Place: Vellanikkara

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CERTIFICATE

Certified that this thesis entitled **'Endophytic fungi for the management of spotted pod borer, *Maruca vitrata* Fab. (Lepidoptera: Crambidae) in cowpea'** is a *bonafide* record of research work done independently by **Mrs. Smitha Revi (2016-21-012)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

Place: Vellanikkara
Date: 09-12-22

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LIST OF ACRONYMS USED

Sl. No.	Symbols	Acronyms
1	@	At the rate of
2	%	Per cent
3	CD	Critical difference
4	cm	Centimeter
5	Fig.	Figure
6	hrs	Hours
7	*i.e.*	That is
8	ml	Millilitre
9	Mg	Milligram
10	G	Gram
11	g^{-1}	Per gram
12	DAS	Days after sowing
13	No.	Number
14	ha	Hectare
15	CRD	Completely randomised design
16	m	Metre
17	m^2	Square metre
18	mm	Millimetre
19	NS	Non-significant
20	*viz*.,	Namely
21	^oC	Degree Celsius

22	*Cv*	Cultivar
23	Cfu	Colony forming units
24	ITS	Internal transcribed spacer
25	rDNA	Ribosomal DNA
26	Sp. or spp.	Species (Singular and Plural)
27	L	Litre
28	µ	Micro
29	EPF	Entomopathogenic fungi
30	EEF	Endophytic entomopathogenic fungi
31	*et al.*	And others
32	KAU	Kerala Agricultural University
33	MSL	Mean Sea Level
34	Bp	Base pairs

Introduction

1. INTRODUCTION

Cowpea, [*Vigna unguiculata* (L.) Walp.], commonly known in India as *lobia* is one of the important *kharif* pulse crops grown in the country. It is cultivated as grain legume, vegetable, forage crop and green manure. Globally, cowpea is grown in an area of about 12.5 million ha, with a production of 8.16 million tonnes (FAOSTAT, 2019). In India, it is cultivated in an area of 3.9 million ha, with a production of 2.21 million tonnes (Mandal *et al*., 2009).

Cowpea is a popular legume in Kerala, and it is cultivated in an area of 5803 ha with a production of 35846 tonnes (DES, 2020). Cowpea is rich in proteins, vitamins and essential micronutrients and hence is often known as vegetable meat (Hussain and Basahy 1998; Pereira *et al.*, 2014; Goncalves *et al.,* 2016).

Infestation by the spotted pod borer, *Maruca vitrata* Fab. (Lepidoptera: Crambidae) is a major constraint in the production of legumes because of its wide host range, distribution and destructiveness (Shanower *et al*., 1999). The larvae initially damage flower buds, flowers, tender pods and tender leaf axils by webbing them together. This feeding habit protects the larvae from natural enemies, adverse environmental conditions and chemical sprays (Sharma, 1998). In cowpea, the loss due to pod borer infestation varies from 20.0 to 60.0 per cent, often reaching up to 80.0 per cent in severe cases (Singh *et al.*, 1990). Application of pesticides can hardly be recommended in cowpea as the pods are harvested at alternate days.

Biological control of *M. vitrata* using entomopathogenic microorganisms has received considerable attention in recent times. A number of microbes have been evaluated against the pest with varying results. However, the time lag between application and effective regulation of pest population as well as the overriding influence of environment on their efficacy tend to limit their utility.

The discovery that many of these entomopathogens, including the white muscardine fungus *Beauveria bassiana* (Balsamo) Vuillemin, enjoy endosymbiotic relationships with plants, which have opened up the possibility of engineering such associations for crop protection against insects. A growing number of information indicates the potential of these organisms to endophytically colonise an array of plants and confer protection for a much longer duration.

Endophytic entomopathogens colonise different plant parts and infect insects attacking those parts. In addition, they also act as plant disease antagonists, plant growth promoters and rhizosphere colonizers (Vega *et al*., 2009). Utilization of entomopathogens as endophytes confer several advantages such as season long protection, cost effectiveness and environment neutrality as compared to conventional approaches (Renuka *et al.*, 2016).

In this context, the study entitled "Endophytic fungi for the management of spotted pod borer, *Maruca vitrata* Fab. (Lepidoptera: Crambidae) in cowpea" was unertaken with the following objectives:

(i) Collection, isolation and characterization of endophytic entomopathogenic fungi in cowpea

(ii) Standardization of method of inoculation of endophytic entomopathogenic fungi in cowpea

(iii) Evaluation of selected endophytic fungi for the management of the spotted pod borer, *Maruca vitrata.*

Review of Literature

2. REVIEW OF LITERATURE

Biological control using endophytic entomopathogens has been shown to reduce pest attack and improve plant growth. Utilization of such entomopathogenic endophytes also confers advantages such as seed to seed protection, cost effectiveness, ease of adoption and safety to non target organisms. The literature pertaining to the investigation entitled "Endophytic fungi for the management of spotted pod borer, *Maruca vitrata* Fab. (Lepidoptera: Crambidae) in cowpea" is reviewed hereunder.

2.1 ENDOPHYTES

The German scientist, de Bary (1866) coined the term endophyte for organisms that live inside plant tissues. The word 'endophyte' was derived from two Greek words 'endon' (within) and 'phyte' (plant). There are several definitions of endophytes. According to Petrini (1991), "endophytes are all organisms inhabiting plant organs, which at some time in their life can colonize internal plant tissues without causing apparent harm to the host". Endophytes are ubiquitous in nature, as they have been isolated from almost every plant species (Stone *et al*., 2000).

A number of fungi and bacteria enjoy endophytic association of a symbiotic nature with plants. These endophytes have established close association with their plant hosts over the course of evolution. This association with the host may vary from symbiotic to bordering on pathogenic (Clay and Schardl, 2002). The colonisation of endophytes with specific host tissues has been recorded by Gautam and Avasthi (2009). Some endophytes may have a mutualistic interaction with one host species, but not with another. At the same time, some species associate with a wide range of hosts (Hardoim *et al*., 2015).

Association of mycorrhizal fungi with the roots of their host plants are designated as endophytic by some authors. Some may act in a similar way to mycorrhiza and may improve the growth and nutrient uptake of plants, while others may increase resistance of

foliage to insect herbivores (Jallow *et al*., 2004; Newsham, 2011). Brundrett (2006) distinguished endophytic ineractions from mycorrhizal ones, as those which possess synchronized plant-fungus association and nutrient transfer at specialized interfaces.

2.2 OCCURRENCE AND BIODIVERSITY OF FUNGAL ENDOPHYTES

Biodiversity of fungal endophytes is enormous, especially in tropical and temperate rainforests. Dreyfuss and Chapela (1994) predicted that there may be at least one million species of endophytic fungi residing in plants.

Endophytic fungi are highly diverse, polyphyletic and primarily ascomycetous fungi occurring within asymptomatic photosynthetic tissues of plants. They occur in all major lineages and communities of land plants from the arctics to the tropics and from agricultural fields to tropical forests (Arnold, 2007). Arnold (2008) opined that the fungal endophytes are colonizing nearly 300,000 land plant species, with each plant host having one or more of these fungi.

Endophytes have been detected in diverse group of plants including palms (Taylor *et al*., 1999; Frohlich *et al*., 2000), trees (Gonthier *et al*., 2006) and dates (Gomez-Vidal *et al*., 2006).

Occurrence of endophytic fungi in agriculturally important crops such as bananas (Pocasangre *et al*., 2000; Cao *et al*., 2002), tomatoes, soybean (Larran *et al*., 2001), wheat (Larran *et al*., 2002), haricot beans (Behie *et al*., 2015) and maize (Ramanujam *et al*., 2017) have also been reported.

Fungal endophytes mainly consist of members of the phylum Ascomycota, some taxa of Basidiomycota, Zygomycota and Oomycota (Zheng and Jiang, 1995; Sinclair and Cerkauskas, 1996; Rajamanikyam *et al*., 2017).

2.3 OCCURRENCE AND RELATIONSHIP OF ENDOPHYTES WITHIN HOST PLANT TISSUES

Endophytic relationships are considered to be very intimate and therefore very specific. However, a wide range of variability is still discernable in terms of host range, type of tissues colonized and level of persistence.

Host specific fungal endophytes are restricted to a single host or related species and do not occur in other unrelated plant hosts in the same habitat (Holliday, 1998). However, evidence suggests that such host specificity might be limited in endophytic fungi (Khiralla *et al*., 2016).

Petrini (1991) used two different terms *viz*., expression specificity and establishment specificity, to categorize the relationship between endophytes and host plants. Establishment specificity refers to an endophyte colonizing selected host plant species, while expression specificity denotes the colonisation of several host plants by a given fungus.

Endophytic relationships have also been classified as host-preference and host-selectivity. Host-preference is a phenomenon when one fungal endophyte species may form relationship with two related host plant species, but shows a preference for one particular host. The term host selectivity is used to indicate the occurrence of a fungus on a particular host (Paulus *et al*., 2006).

Tissue specificity in colonisation has also been reported by several authors. Significantly higher degree of colonisation in leaf segments as against stem segments of medicinal plants like *Callicarpa tomentosa*, *Lobelia nicotinifolia and Alstonia scholaris* was reported by Raviraja (2005). Bagchi and Banerjee (2013) studied the tissue specific colonisation by fungal endophytes like *Penicillium* sp., *Pestalotiopsis* sp. and *Aspergillus* sp. in *Bauhinia vahlii*. They isolated the fungi from leaf, petiole and stem and observed

that the colonisation frequency of endophytic fungi is much higher in petiole (86.67%) as against leaf (70.67%) and stem (77.33%).

Entomopathogenic fungi also have been known to exhibit preferential localization. Behie *et al.* (2015) conducted field and laboratory studies to assess the preferential localization of endophytic entomopathogenic fungi, *Metarhizium robertsii* and *Beauveria bassiana* within plant tissues of *Phaseolus vulgaris*. In laboratory studies, *M. robertsii* was restricted to the roots of plants while *B. bassiana* was found throughout the plant. In the field study that followed, root colonisation by *B. bassiana* was 74.30 per cent, while colonisation of stems and leaves was 13.80 per cent and that of hypocotyle was 11.90 per cent out of a total of 514 plant samples analysed. However, out of the 730 plant samples colonized by *M. robertsi*, 99.20 per cent was in roots.

2.4 CLASSIFICATION OF FUNGAL ENDOPHYTES

Fungal endophytes are broadly classified into two major categories *viz*., clavicipitaceous and non-clavicipitaceous, based on differences in host range, taxonomy, tissue specificity, transmission pattern and ecological function (Carroll, 1988; Petrini, 1991; Schulz and Boyle 2005; Rodriguez *et al*., 2009; Gautam and Avasthi, 2009). Clavicipitaceous endophytes (C-endophytes) inhabited grasses while non-clavicipitaceous endophytes (NC-endophytes) were associated with vascular and non-vascular plant species (Bamisile *et al*., 2018).

2.4.1 Class I or clavicipitaceous endophytes (C- endophytes)

Clavicipitaceae is a family of fungi (Hypocreales: Ascomycota) that include symbiotic and free living species associated with insects, grasses and sedges (Bacon and White, 2000). C-endophytes have life styles ranging from parasitism to mutualism (Rodriguez *et al*., 2009). Guerin (1898) cited by Rodriguez *et al*. (2009) reported that clavicipitaceous endophytes of grasses were first observed by European scientists in the

late 19th century in the seeds of *Lolium arvense, L. temulentum*, *L. remotum* and *L. linicolum*.

From their earliest discovery, Bacon *et al*. (1977) linked the fungal endophyte *Neotyphodium* (=*Acremonium*) *coenophialum* to the widespread incidence of 'summer syndrome' toxicosis in cattle grazing *Neotyphodium* colonized tall fescue pastures (*Festuca arundinacea*) Screb.

C- endophytes form symbiosis almost exclusively with grass hosts *viz*., cool-season C3 grasses and warm-season C4 grasses. Grass endophytes colonize their hosts systemically (except the roots) and several species were transmitted vertically by seeds to the next host generation. Schulz and Boyle (2005) also observed that C-endophytes (balansiaceous endophytes) were vertically transmitted through seeds from one generation to another, within all the above-ground plant parts of grasses and sedges.

Mycelia of clavicipitaceous endophytes occured in intercellular spaces of culms, rhizomes, leaf sheaths and could also be present on the surface of leaf blades (White *et al.,* 1996; Dugan *et al*., 2002). Rodriguez *et al*. (2009) reported that C-endophytes colonize shoots and show systemic intercellular infections throughout the entire life cycle of the host plant.

Funk *et al.* (1983) reported less damage by sod webworms (*Crambus* spp.) to endophyte infected perennial ryegrass than to uninfected plants. Prestidge *et al.* (1985) reported the enhanced resistance in endophyte infected perennial ryegrass to the Argentine stem weevil (*Listronotus bonariensis*) in New Zealand.

Khiralla *et al*. (2017) documented the effects of colonisation by clavicipitaceous endophytes on host plant as insect deterrence against herbivory by nematodes, insects and mammals, disease resistance and abiotic stress resistance.

Endophyte infected tall fescue has been widely reported as more resistant to a variety of insects than uninfected tall fescue. The larvae infesting infected plants typically had lower survival rates, increased larval duration, and lower pupal mass than larvae on uninfected grasses (Latch *et al*., 1985; Siegel *et al.,* 1989). Schmidt (1986) cited by Cheplick and Clay (1988) stated that *Epichloe typhina* infected tall fescue, *Dactylis glomerata* showed resistance to cutworm (*Agrotis segetum*) larvae which resulted in reduced survival of the latter.

C-endophytes are characterized by the production of toxic secondary metabolites and include species that are insect pathogens (White *et al*., 1996).

Gallagher *et al.* (1982) reported that the C-endophytic fungi had the potential to control many pests due to their ability to produce certain alkaloids. Clay (1991) reported grass endophytes enhanced host fitness by the production of alkaloids that inhibited insect herbivory and stimulated plant growth. In addition to protection from herbivore damage, alkaloids also provided disease resistance and stress tolerance (Purahong and Hyde 2011).

2.4.2 Class II or Non-clavicipitaceous endophytes (NC- endophytes)

Non-clavicipitalean endophytes represent a broad range of species from several families of ascomycetes and occur in all plant species including grasses **(**Sieber *et al.*, 1988). Most fungal endophyte species in this class belong to Ascomycetes, with a minority of Basidiomycetes (Rungjindamai *et al*., 2008; Khiralla *et al.*, 2017). NC - endophytes have life styles ranging from saprotrophic to mutualism (Rodriguez *et al.*, 2009).

Class II endophytes include the hyperdiverse fungal endophytes are seen associated with woody and herbaceous angiosperms, conifers, and in biomes ranging from tropical forests to boreal and polar communities (Carroll and Carroll, 1978; Petrini, 1986; Stone, 1988), seedless vascular plants, above-ground tissues of nonvascular plants and leaves of tropical trees (Frohlich and Hyde, 1999; Gamboa and Bayman, 2001).

Stone (1987) had reported that host colonisation by NC - endophytes is non-systemic and is restricted to disjunctive, endophytic microthalli which may consist of only a few cells. This was supported by Hyde and Soytong (2008), who observed that NC - endophytes form asystemic symbiosis with all species of nonvascular and vascular plants and establish mutualism within shoots, roots and rhizomes. They are commonly transmitted horizontally, infecting hosts through spores that are water, air or soil-borne (Rodriguez *et al*., 2009).

Eventhough NC - endophytes are treated as a single group, Rodriguez *et al*. (2009), classified the NC - endophytes into four distinct functional groups. The fungal endophytes were grouped into four classes, *viz.*, 1, 2, 3 and 4 based on life history traits and phylogeny data. While Class 1 endophytes formed host specific clavicipitaceous fungi that were transmitted vertically through seeds, Class 2, 3 and 4 groups comprised of non- clavicipitaceous endophytes as already stated.

Class 2 endophytes colonize the stems, roots, leaves or in other words, the whole plant. They are highly diverse and consist of species from Ascomycota and Basidiomycota. They can be vertically or horizontally transmitted. Class 3 endophytes restrict their colonisation to above-ground plant tissues, forming localized infections. This group is also extremely diverse, horizontally transmitted and comprise of species from Basidiomycetes. Class 4 endophytes are found in the rhizosphere and shows high biodiversity.

NC - endophytes enhance host growth and yield by improved mineral nutrient uptake, water uptake and utilization of organic nutrient pool. Class II endophytes also confer beneficial effects like abiotic stress tolerance (Redman *et al*., 2002; Marquez *et al*., 2007), increased biomass production (Tudzynski and Sharon, 2002) and protection from insect pests and fungal pathogens to their host plants (Vu *et al*., 2006; Campanile *et al*., 2007). They include common entomopathogens such as *B. bassiana*, *Lecanicillium*

lecanii and *M. anisopliae* which cause mortality of insect pests when *in planta*, antagonize plant pathogens and promote plant growth (Vega *et al*., 2009).

Fungal endophytes have also been classified into different groups based on various criteria such as host range, mode of reproduction, part of plant colonized, mode of transmission, source of nutrition, and ability to express symptoms in host plant. Detailed classification of endophytic fungi based on various criteria has been documented by Gautam and Avasthi (2009).

2.5 COLONISATION BY FUNGAL ENDOPHYTES

2.5.1 Initial infection by fungal endophytes

The initial steps of host infection by fungal endophytes are similar to that of any fungal plant pathogen and involve recognition, germination and penetration (Gao *et al*., 2010).

As the first step, spores attach themselves preferentially to host surface in response to specific chemical or physical host signals (Viret *et al*., 1994). Wagner and Lewis (2000), who were the first to describe the penetration and growth of an entomopathogenic fungus, *B. bassiana* into plant and observed that recognition and binding by the endophyte on to the host plant surface is often mediated by lectin-like molecules. Several scientists have reported that the secondary metabolites produced by the host plant were found to act as signal molecules for recognition. Arabinogalactan proteins (AGPs) have been reported as having a definite role in root colonisation by endophytes where it works as a repellent or an attractant for microbes and in the development of infection structures (Nguema-Onad *et al*., 2013). According to Chagas *et al.* (2017) root exudates from plants including sugars, phenols, amino acids, organic acids, and other secondary metabolites selectively invite the mutualistic microbes, particularly the endophytes. A carotenoid derived protein, strigolactone (SL), for instance,

secreted by roots of *Arabidopsis thaliana* was found to act as a signal molecule for endophytic colonisation of *Mucor* sp. (Rozpa-dek *et al*., 2018).

Host recognition is followed by entry into the host cell. Thines *et al.* (2000) explained that most fungal endophytes produce a series of exoenzymes which soften the cuticle and epidermal cell wall to ease penetration of the thread like infection hyphae. Alternatively, an appressorium is developed to overcome the resistance offered by the plant cuticle through mechanical force.

2.5.2 Colonisation of plants by fungal endophytes

Inside the plant, the endophytic fungi may spread through the plant systemically (Quesada-Moraga *et al*., 2006; Gurulingappa *et al*., 2010) or may remain localized (Yan *et al*., 2015).The mode of establishment and duration of presence of endophytic fungi in plants varies among different host plant-endophyte combinations (Powell *et al*., 2009; Brownbridge *et al*., 2012).

In some cases, endophytes may be retained within plants for considerable amounts of time, as in case of *B. bassiana* retained for as long as eight months in coffee plants (Posada *et al*., 2007) and nine months in *Pinus radiata* (Brownbridge *et al*., 2012).

2.5.2.1 Systemic colonisation

Re-isolation of a systemic fungus from plant parts distant from the site of inoculation indicates the systemic colonisation and confirms the ability of the fungal strains to move through interconnected vascular tissues to colonise entire plant (Wagner and Lewis, 2000).

Akello *et al.* (2007) conducted three screenhouse experiments to determine the endophytic potential of different strains of *B. bassiana* in tissue cultured banana plants (*Musa* spp.). The plantlets were dipped in spore suspension of the fungal strains before planting. It was observed that all the three strains were able to colonise roots, pseudostem

bases and rhizomes. The best performing strain, namely, G41, recorded up to 79.5, 68.0 and 41.0 per cent colonisation of rhizome, roots and pseudostem bases respectively.

Posada *et al*. (2007) inoculated coffee plants with *B. bassiana* and re-ioslated the fungus. The protection of plants that tested positive for *B. bassiana* was 30.6, 5.5, 2.7 and 0.0 per cent after two, four, six and eight months respectively.

Jaber and Enkerli (2017) tested different trains of *B. brongniartii* (2843, BIPESCO2), *B. bassiana* (NATURALIS) and *M. brunneum* (BIPESCO5) as foliar spray in broad bean and reisolated them from all plant parts at seven and 14 days post inoculation.

2.5.2.2 Colonisation in localised plant parts

Wearn *et al*. (2012) studied three herbaceous perennial plants *viz*., *Cirsium arvense*, *Plantago lanceolata* and *Rumex acetosa* to understand whether endophytic fungi had any specificity with regard to plant parts for colonisation. Colonisation was higher in roots when compared to shoots for all the three plant species. Among the different plants, roots of *C. arvense* harboured the highest number of endophytes with an overall mean of 6.5 species per plant, followed by roots of *P. lanceolata* and *R. acetosa* with an identical number of 4.0 species per plant.

2.6 MECHANISMS OF ENDOPHYTE MEDIATED HERBIVORE PROTECTION

Endophyte mediated protection against herbivory involved feeding deterrence, antibiosis or changes in metabolism of the plant and thus hosts plant quality rather than a direct fungal infection of the insect pests (Vega *et al*., 2008).

The mechanisms by which herbivores can be negatively affected by C-endophytes have been studied in different grass species and could involve antixenosis or antibiosis or a combination of the two. Clay (1988) reported that the antibiosis and

antifeedant properties of endophyte infected grass plants could reduce insect population growth rate.

These are largely mediated by induced production of secondary metabolites by the plant (Clay, 1996) or by the production of secondary metabolites by the endophytes themselves (Jaber and Vidal, 2010; Gurulingappa *et al*., 2011).

Clay and Schardl (2002) reported the harmful effects of metabolites by endophytic fungi on insect herbivores. Fungal endophytes produce biologically active compounds like alkaloids, steroids, paxillines and loliterms that provide protection from herbivores and promote plant growth. These organisms are therefore being increasingly valued as microorganisms that could produce beneficial secondary metabolites for agricultural and biotechnological use (Thalavaipandian *et al*., 2011).

Bultman *et al.* (2004) had reported that fungal endophytes confirmed plant resistance by production of mycotoxins as in case of tall fescue plants where colonized fungal endophytes provided constitutive resistance against herbivore damage through alkaloid production. Inoculation of banana rhizome with *B. bassiana* isolate resulted in colonistaion and exhibited higher mortality of banana rhizome borer larvae and mycosis of adults (Quesada-Moraga *et al*., 2009). Most of the above studies have attributed the reduction in the damage by insect herbivores to the accumulation of the mycotoxins in plant tissues (Gurulingappa *et al*., 2011).

Larvae of *Ostrinia nubilalis* exhibited reduced tunnelling in both sorghum and corn plants inoculated with *B. bassiana*. Absence of mycosed larvae indicated that fungal colonisation had an indirect effect on larval performance (Akello *et al*., 2008). Similarly, inoculation of opium poppy with endophytic *B. bassiana* reduced larval abundance of the gall wasp, *Iraella luteipes* up to 73.0 per cent, although without any mycosis (Quesada-Moraga *et al*., 2009).

Dugassa-Gobena *et al*. (1998) and Raps and Vidal (1998) reported that endophytes could alter the phytosterol profiles of host plants, and thus depriving of insects of these hormonal precursors.

One of the mechanisms behind endophyte-mediated plant protection from herbivores is through induction of a systemic response in the host plant induced by the fungus. This leads to increased production of superoxides, thus conferring resistance to insect feeding (Schardl *et al*., 2007; Hartley and Gange, 2009).

Estrada *et al*. (2013) observed that plants colonised with endophytes had relatively higher cellulose content, higher leaf lamina density and greater toughness, which in turn resulted in reduced herbivory rates, specifically by leaf-cutting ants.

2.7 ENDOPHYTIC ENTOMOPATHOGENIC FUNGI

Entomopathogenic fungi are mainly found amongst the Zygomycetes (*Entomophthorales*) and Ascomycetes (*Clavicipitales*, *Hypocreales*, Hyphomycetous anamporphs) and among these, endophytes have so far been reported from only Acsomycetes.

A number of entomopathogenic fungi such as *Beauveria bassiana* (Balsamo) Vuillemin, *Verticillium* (=*Lecanicillium* or *Akanthomyces*) *lecanii* (Zimm.) *Paecilomyces farinosus* (Holmsk.) Brown & Smith (=*Isaria farinosa*) (Bills and Polishook, 1991), and *Paecilomyces* sp. (Cao *et al*., 2002; Tian *et al*., 2004) have been reported as capable of endophytic associations with plants.

Vega *et al.* (2008), who conducted a survey of fungal endophytes in coffee plants from Colombia, Hawaii, Mexico, and Puerto Rico revealed the presence of 16 endophytes belonging to different genera of fungal entomopathogens, including *Beauveria*, *Paecilomyces*, *Acremonium*, *Cladosporium* and *Clonostachys*.

2.7.1 *Beauveria bassiana*

Beauveria bassiana (Ascomycota: Hypocreales), anamorph form of *Cordyceps bassiana* in the family Clavicipitaceae, has been reported as capable of colonising an array of plants, both naturally and artificially (Vega, 2008; Parsa *et al*., 2013), and confer protection against insect pests and plant pathogens (Jaber, 2015).

Beauveria bassiana has been reported as an endophyte in number of plants such as maize (Vakili, 1990; Bing and Lewis, 1991; Cherry *et al*., 2004; Arnold and Lewis, 2005), potato, cotton (Jones, 1994), tomato (Ownley *et al*., 2004; Powell *et al*., 2009; Gurulingappa *et al*., 2010), poppy (Quesada-Moraga *et al*., 2006), date palm (Gomez-Vidal *et al*., 2006), banana (Akello *et al*., 2007), coffee (Posada *et al.*, 2007), pine (Brownbridge *et al*., 2012) and jute (Biswas *et al.*, 2013).

Renuka *et al*. (2016) examined the endophytic ability of indigenous isolates of *B. bassiana* (NBAII Bb-5a, 7, 14, 19, 23 and 45) in maize stem and leaf tissues. All isolates showed colonisation in stem and leaf tissues to varying extents when maize plants (*var*. Nithyashree) were inoculated with conidial suspensions of different strains. The isolate Bb-23 showed the highest mean colonisation in older leaves (27.78 %), older stems (20.37 %), and in young stems (21.29 %), while the isolate Bb-5a recorded highest mean colonisation in young leaf tissues (26.85 %). Persistence of inoculated isolates decreased with increase in age of the plant. The results obtained in plating and PCR assays were consistent with the above observation on endophytic colonisation of *B. bassiana*.

2.7.2 *Purpureocillium lilacinum*

The genus *Paecilomyces* has been reported as an endophyte in barley and other plants (Schulz *et al*., 1998). Recent evidence indicates that *Purpureocillium lilacinum* (=*P. lilacinus*) can be an entomopathogen with potential for use in the biocontrol of insect pests (Fiedler and Sosnowaska, 2007; Imoulan, 2011; Wakil *et al*., 2012).

Endophytic *P. lilacinum,* isolated from cotton, was pathogenic to insects infesting cotton (Ek-Ramos *et al.*, 2013). The same strain was also observed to parasitize the eggs of root-knot nematode in lab assay and negatively affected the nematode reproduction as an endophyte in *in planta* assays (Zhou *et al*., 2017).

Lopez and Sword (2015) reported that both *B. bassiana* and *P. lilacinum* enhanced the growth of cultivated cotton and also reduced the survival of cotton boll worm, *Helicoverpa zea*. Control boll worms lived for a longer mean duration of 20 days on uninoculated cotton than on those colonized by either *B. bassiana* (14 days) or *P. lilacinum* (16.6 days).

2.8 INOCULATION METHODS

Plants can be protected from herbivorous insect pests by artificial inoculation of entomopathogenic fungal endophytes in the plant systems. Several artificial plant inoculation methods can be adopted for introducing fungal endophytes into plants (Vega, 2008; Brownbridge *et al*., 2012).

Endophytes like *Isaria farinosa, Acremonium* spp. (Cherry *et al*., 2004; Orole and Adejumo, 2009; Vega *et al*., 2009) *Hypocrea lixii, Fusarium oxysporum, Gibberella moniliformis* and *Trichoderma asperellum* (Akello and Sikora, 2012; Martinuz *et al*., 2012; Akutse *et al*., 2013) were reported to have been re-isolated from colonized host plant parts after artificial inoculation.

Colonisation of nearly 30 plant species with fungal endophytic entomopathogens such as *B. bassiana*, *L. lecanii*, *P. lilacinum*, *M. anisopliae*, *Metarhizium brunneum* and *Metarhizium robertsii* through different methods have been attempted. Different inoculation techniques such as seed soaking, seed dressing, foliar sprays, radical dressing, soil application of spore suspension and fungal plugs *etc.* have been used for colonizing the fungal entomopathogens. Among these, the most important and practically feasible methods include seed soaking, foliar application and soil application (Vega, 2018).

2.8.1 Soil application

Soil inoculation has been carried out for successful colonisation of *B. bassiana* in banana (Akello *et al.,* 2007), sorghum (Tefera and Vidal, 2009) and pine seedlings (Brownbridge *et al*., 2012).

Greenfield *et al.* (2016) achieved colonization by entomopathogenic fungi, *M. anisopliae* and *B. bassiana* in cassava roots by drenching the soil with conidial suspension. Colonisation by *B. bassiana*, at 84.0 per cent was higher when plants were sampled at seven to nine days post inoculation compared to 40.0 per cent at 47 to 49 days post inoculation. However, colonisation by *M. anisopliae* remained constant at 80.0 per cent for the same period.

Metarhizium brunneum and *B. bassiana* were similarly inoculated in sweet pepper by drenching the plant root zone with 15 ml of spore suspension containing 10^7 conidia ml^{-1}. Both the fungi successfully colonized the roots, stems and leaves at 7 and 17 days post inoculation (Jaber and Araj, 2017).

2.8.2 Seed treatment

Seed treatment has been successfully adopted as a method for endophytic colonisation in cotton (Ownley *et al*., 2008), sorghum (Tefera and Vidal, 2009) and poppy (Quesada-Moraga *et al*., 2009).

Akutse *et al*. (2013) attempted seed inoculation of *Phaseolus vulgaris* and *Vicia faba* with 10 isolates belonging to six genera of endophytic fungi. It was observed that while *Beauveria*, *Fusarium, Gibberella*, *Trichoderma* and *Hypocrea* colonized both the plant species, *Metarhizium* did not colonize either of the the plants. Isolates of *B. bassiana*, GILU3 and S4SU1 colonised only the roots of *V. faba*. Successful colonisation by *B. bassiana* in different crops like cotton (Lopez *et al*., 2014), beans (Gathage *et al*.,

2016) and faba bean (Jaber and Enkerli, 2017) through seed soaking has also been reported.

Pena-pena *et al.* (2015) soaked corn seeds in spore suspension of *Metarhizium pingshaense* and reported positive endophytic recovery from plants. Sanchez-Rodriguez *et al.* (2017) evaluated three inoculation methods *ie*., soil treatment, seed inoculation and leaf spraying in wheat plant to assess the extent of colonisation of *B. bassiana*. Seed inoculation was significantly superior to other treatments with 44.60 per cent colonisation and followed by soil treatment with 33.80 per cent. Meanwhile, the highest re-isolation from leaves was observed in the leaf spraying, seed dressing and soil treatment registering 86.00, 16.00 and 11.30 per cent respectively.

2.8.3 Foliar application

Gurulingappa *et al*. (2010) were able to establish both *L. lecanii* and *B. bassiana* in bean, cotton, tomato, wheat, pumpkin and corn plants when these crops were inoculated *via* foliar application. However, recovery rates in almost all cases significantly reduced by the third week after inoculation.

Foliar inoculation of *Beauveria ochroleuca* and *B. bassiana* on artichoke resulted in successful colonization to the extent of 84.00 and 78.00 per cent respectively. The inoculated entomopathogenic fungi were isolated from 78.00 and 56.00 per cent of new leaves inoculated with *B. ochroleuca* and *B. bassiana* respectively at 10 days post inoculation (Guesmi-Jouini *et al*., 2014).

Resquín-Romero *et al*. (2016) sprayed leaves of melon, alfalfa and tomato plants with suspensions of different strains of *B. bassiana* and *M. brunneum* in order to ascertain whether the foliar application of a fungal suspension could produce a temporal colonisation of the plants. All the fungal strains were able to successfully colonize the leaves, stems, and roots of three host plants to the extent of around 40.00 per cent which was constant for the evaluation period in all the three crops. This was significant since it

was the first report where foliar application of the inoculum resulted in the colonisation of the entire plant, including the roots.

2.9 CONFIRMATION OF ENDOPHYTIC FUNGAL COLONISATION IN PLANTS

Colonisation by endophytic entomopathogenic fungi in different plants can be confirmed using different methods such as re-isolation and molecular techniques.

2.9.1 Re-isolation

Re-isolation is the most common technique to confirm colonisation by endophytes. Posada *et al*. (2007) inoculated coffee plants with *B. bassiana* to evaluate the establishment inside the plant and it was confirmed by re-isolation. Recovery from sites distant from the point of application indicated that the fungus had the potential to move throughout the plant. The extent of plants that tested positive for *B. bassiana* was 30.60 per cent at two months, 5.50 per cent at four months, 2.70 per cent at 6 months and nil at 8 months.

Guesmi-Jouini *et al.* (2014) used leaf spray inoculation method in artichoke plants to colonise *B. bassiana* and *Bionectria ochroleuca*. The inoculated entomopathogenic fungi were re-isolated from leaves, though with significant differences between *B. bassiana* (56.0%) and *B. ochroleuca* (78.0%).

Renuka *et al*. (2016) applied indigenous isolates of *B. bassiana* (NBAII Bb-45, 23, 19, 14, 7 and 5a) on maize leaves. The colonized isolates were re-isolated from plant parts after surface sterilization. All isolates showed colonisation in stem and leaf tissues with Bb-23 isolate recording the highest mean colonisation of 27.78, 21.29 and 20.37 per cent in older leaves, older stems and young stems respectively. Meanwhile, Bb-5a isolate showed higher mean colonisation of 26.85 per cent in young leaf tissues. The above results were in conformity with that of PCR analysis.

Re-isolation was also used to confirm colonisation of coffee plants by endophytic entomopathogen *B. bassiana* applied as foliar spray, stem injection and soil drenching. The fungus was reisolated from stems, roots and leaves at two, four and six months after inoculation. All inoculation methods were effective in introducing the isolate into the plant. At two months after inoculation, colonisation was detected at the rate of 58.30, 25.00 and 8.30 per cent in stem injection, soil drenching and foliar application methods respectively. At four months post-inoculation, isolate was detected in 16.70 per cent of plants, and at six months, it was recorded only in 8.30 per cent of plants in the stem injection method (Posada *et al.*, 2007).

2.9.2 Molecular techniques

Molecular biology tools have been standardized and are increasingly being utilized for confirmation of plant colonisation by endophytic entomopathogens. Landa *et al.* (2013) standardized two-step nested and quantitative PCR assay to confirm the endophytic colonisation by a *B. bassiana* strain in opium poppy. Species specific primers were designed and that can be used in quantitative and conventional PCR for detection of *B. bassiana* in plant. The combination of the designed *Bb* forward and reverse primer set with the universal ITS1-F/ITS4-R primer set in a two-step nested-PCR approach allowed the amplification of DNA.

Lohse *et al.* (2015) extracted DNA from surface sterilized plant samples of oilseed rape and colonisation of fungus in treated plant samples was assessed by PCR assay using *B. bassiana* specific primers.

Renuka *et al.* (2016) confirmed the endophytic ability of different isolates of *B. bassiana* in stem and leaf tissues of maize, *Zea mays* through reisolation and PCR technique. Genomic DNA of fungus was extracted from different parts of both treated and untreated plants. SCAR (sequence characterized amplified region) primer was designed and used for amplifying the endophytic *B. bassiana* DNA. *B. bassiana* specific amplicons

were obtained from stem and leaf tissues of treated plants. Colonisation in the plant tissues, observed through plating methods could be confirmed by PCR amplification.

Rondot and Reineke (2016) confirmed the colonisation of two strains of *B. bassiana viz*., ATCC 74040 and GHA in grapevine plants by strain specific PCR analysis. DNA from endophytic fungus showed respective strain-specific peaks after amplification with *B. bassiana* microsatellite primers. Amplicons of strain specific primer pairs Ba01, Ba12 and Ba13 showed peaks at 117 bp, 222 bp and 168 bp for strain GHA and 117 bp, 231 bp and 216 bp for strain ATCC 74040 respectively.

Gautam *et al.* (2016) proved the endophytic potential of *B. bassiana* in cauliflower plants through SCAR marker assay. PCR amplification of total genomic DNA obtained from the treated plants yielded clear, discrete and consistent banding patterns corresponding to *B. bassiana*.

2.10 EFFICACY OF ENDOPHYTIC ENTOMOPATHOGENIC FUNGI IN INSECT PEST MANAGEMENT

Bing and Lewis (1991) reported that *B. bassiana* isolate ARSEF 3113 significantly reduced tunnelling by *Ostrinia nubilalis* in maize following injection of 4.55 x 10^7 conidia per plant. The fungus was found to move systemically from the site of injection and was re-isolated from 95.00 per cent of injected plants at havest. Stem tunnelling was reduced by 17.60 per cent relative to untreated plants.

Endophytic fungi have been described as plant mutualists and their presence in plant tissue has been referred to as an adaptive protection against herbivorous insects (White *et al*., 2003).

Jallow *et al.* (2004) reported drastic negative effects on larvae of *Helicoverpa armigera* Hubner reared on tomato plants infected with *Acremonium strictum* W. Gams. Larvae reared on endophyte treated plants showed significant reduction in growth rate,

suppressed moulting, prolonged development time and had smaller pupae. The emerged adult moths were less fecund compared to larvae reared on untreated plants. In glasshouse bioassays, larvae were allowed to feed freely on treated plants and only 20.00 per cent survived compared to 54.50 per cent on untreated plants.

Cherry *et al.* (2004) evaluated African isolates of *B. bassiana* in maize to test for their ability to provide protection against larvae of the stem borer, *Sesamia calamistis* Hampson and reported that the topical application of conidia to leaf axils led to a reduction in dead heart frequency by 20.00 per cent.

Ramanujam *et al.* (2007) conducted field study to assess the efficacy of endophytic isolates of *B. bassiana* (Bb-23, Bb-5a and Bb-45) and *M. anisopliae* (Ma-35) against maize stem borer (*Chilo partellus* Swinoe) and reported that all the isolates suppressed damage by the pest. Significantly lower extent of stem tunneling (3.98 - 4.36 cm plant^{-1}), dead heart incidence and (9.5 - 12.71 %) and fewer exit holes (2.59 - 2.79 no. plant^{-1}) were observed in the treated crop compared to untreated control which showed 8.01 cm/plant of stem tunneling, 21.38 per cent dead hearts and 6.99 exit holes/plant. Among the four isolates tested, Ma-35 isolate of *M. anisopliae* and Bb-5a isolate of *B. bassiana* were significantly superior to other strains in lowering the stem tunneling, incidence of dead hearts and exit holes with higher yields of 70.00 and 72.00 tonnes ha^{-1} respectively.

Beauveria bassiana colonized banana plants significantly reduced the survival of the larvae of banana weevil, *Cosmopolites sordidus* by as much as 23.5 to 88.9 per cent. About 42.00 to 86.70 per cent reduction in plant damage was also reported (Akello *et al*., 2008). *Papaver somniferum* L. plants were protected from the hymenopteran pest, *Iraella luteipes* by inoculation of endophytic strain of *B. bassiana* (Quesada-Moraga *et al*., 2009).

Endophytic insect pathogenic fungi such as *B. bassiana, M. anisopliae* and *L. lecanii* have been demonstrated to effectively control insect pests and reduce insect pest

damage in crops. Fungal endophytes with entomopathogenic capabilities can be utilized to enhance host plant tolerance to insects (Vega *et al*., 2009).

Gurulingappa *et al*. (2010) evaluated the protective effects of *L. lecanii*, *B. bassiana*, and *Aspergillus parasiticus* in six crop plants *ie*., cotton (*var*. Sicala V2), wheat (*var*. Morocco), bean (*var*. Coles prolific), corn (*var*. Honey sweet), tomato (*var.* Grosse lisse), and pumpkin (*var.* Queensland blue) against *Chortoicetes terminifera* (Orthoptera: Acrididae) and *Aphis gossypii*. Feeding by *A. gossypii* on *B. bassiana* and *L. lecanii* colonized cotton leaves affected the reproduction rate of aphids. Similarly, consumption of wheat leaves colonized by either *A. parasiticus* or *B. bassiana* slowed down the growth of *C. terminifera* nymphs.

Akello and Sikora (2012) inoculated entomopathogenic endophytes (*M. anisopliae* and *B. bassiana*) on faba plants by seed treatment and colonisation of the roots was found to be over 95.00 per cent after one month. Colonisation had a negative influence on the reproduction of two important aphid pests of the plant, *viz.*, *Aphis fabae* and *Acyrthosiphon pisum*.

Akutse *et al*. (2013) evaluated three fungal isolates belonging to the genus *Beauveria*, two isolates of *Fusarium* and one isoate each of *Hypocrea*, *Metarhizium*, *Gibberella* and *Trichoderma* in the laboratory to assess their efficacy against pea leafminer (*Liriomyza huidobrensis*) in *Vicia faba* and *Phaseolus vulgaris*. Both the host plants were colonized by isolates of *Beauveria* (ICIPE279), *Gibberella*, *Fusarium*, *Hypocrea* and *Trichoderma*. *Beauveria* isolates G1LU3 and S4SU1 colonized stem, roots and leaves of *P. vulgaris* but was confined to the stem and roots of *V. faba*. All the colonized fungal isolates were pathogenic to *L. huidobrensis* and caused 100.0 per cent mortality. However, *Hypocrea* was superior to other fungal isolates in reducing the mean number of pupae, longevity of the progeny and adult longevity with values of 80.00, 11.20 days and 3.80 days as against 387.0, 17.8 days and 9.9 days in control. Adult

emergence was significantly reduced in case of treatments involving *Beauveria* (38.00%) and *Hypocrea* (21.40%) compared to the control (82.90%).

Qayyum *et al.* (2015) evaluated an isolate of *B. bassiana* (WG-40) from wild tomato plant along with two isolates (WG-14 and WG-19) from soil for their ability to colonise tomato plants and infect *Helicoverpa armigera* larvae. Detached leaf bioassay showed isolate WG-40 to the most pathogenic causing the highest extent of mortality. Pupation as well as emergence of adults was adversely affected by the fungal infection.

Mantzoukas *et al*. (2015) reported that endophytic *Metarhizium robertsii* and *B. bassiana* caused protection of sweet sorghum from the damage of *Sesamia nonagrioides* under natural environmental conditions without affecting plant physiology and growth.

Rondot and Reineke (2016) carried out field and greenhouse experiments to optimize endophytic establishment of *B. bassiana* in seven–week-old potted plants as well as in mature grapevine plants. They used two different commercialized *B. bassiana* strains, ATCC 74040 and GHA for foliar inoculation. Survival of *B. bassiana* inside leaf tissues of potted plants was evident for a minimum of 21 days after inoculation, irrespective of the strain used. Bioassay was conducted using surface sterilized leaves and it was found that the endophytic *B. bassiana* reduced infestation rate and growth of vine mealybug, *Planococcus ficus*. *B. bassiana* was detected up to five weeks post inoculation in mature plants, resulting in significantly lower number of 2.25 leaf hoppers per plant as compared to 4.75 per plant in control.

Sanchez-Rodriguez *et al*. (2017) inoculated wheat plants with *B. bassiana* by different methods such as soil treatment, seed inoculation and foliar application against cotton leafworm (*Spodoptera littoralis*). Mortality of larvae reared on *B. bassiana* colonized plants ranged from 30.0 per cent in seed treatment to 57.0 per cent in foliar inoculation, as against no mortality in untreated control.

Pachoute *et al.* (2021) conducted to study the colonization of cowpea plants by *B. bassiana* and its effect on the feeding performance and survival of leaf beetle, *Cerotoma arcuata*. Colonization by *B. bassiana* was recorded in the plant parts such as stems (63.89%), roots (45.83%), and leaves (25.0%) of the cowpea plant. The Kaplan–Meier survival analysis showed that the fungal colonization negatively affected the survival of the insect in the leaf disc assays.

2.11 INFLUENCE OF ENDOPHYTIC ENTOMOPATHOGENIC FUNGI ON PLANT GROWTH AND YIELD

Endophytes often play an important role in maintaining the health of plants, as they can protect the host plant against abiotic and biotic stresses and enhance plant growth and yield (Vega *et al*., 2008; Lata *et al*., 2018).

It has been reported that endophytic fungi can promote host nutrient uptake and stress tolerance to different abiotic factors and provide resistance to pathogens and insect pests with the production of various alkaloids (Schardl *et al*., 2004). This is often mediated through production of bioactive compounds by the endophytes themselves or through induction of the host plant to produce secondary metabolites that promote plant growth and help them adapt better to the surrounding environment (Das and Varma, 2009).

Both clavicipitaceous and non-clavicipitaceous endophytes including *B. bassiana*, *L. lecanii, M. anisoplae* and *Isaria* (=*Paecilomyces*) spp. can have negative effects on insect pests when *in planta* and promote plant growth (Clay, 1991; Ownley *et al*., 2004; Vega *et al.,* 2009).

Many scientists have investigated the potential role of endophytic fungal entomopathogens to act as plant growth promoters (Kabaluk and Ericsson, 2007; Garcia *et al*., 2011; Sasan and Bidochka, 2012; Lopez and Sword, 2015).

Soil inoculation with *B. bassiana* significantly increased shoot and root weight of wheat plants sampled 20 days after inoculation as compared to control plants (Gurulingappa *et al*., 2010). Garcia *et al.* (2011) reported that soil inoculation with *M. anisopliae* significantly increased plant height, root length, root and shoot dry weight of 28-day-old tomato plants when compared with control plants. Similarly, cotton seeds were inoculated with *B. bassiana* and *P. lilacinum* increased cotton dry biomass and number of nodes (Lopez and Sword, 2015).

Lopez and Sword (2015) reported that the entomopathogen, *B. bassiana* stimulated the growth of *Gossypium hirsutum* by suppressing the population of *Helicoverpa zea* and by helping to transfer nutrients from the soil into the roots of the host plant.

Jaber and Enkerli (2017) conducted experiment to test the extent of colonisation on *V. faba* plants by *B. brongniartii* (BIPESCO2 and 2843) and *M. brunneum* (BIPESCO5) by comparing them with an endophytic strain of *B. bassiana* (NATURALIS®). Foliar inoculation of plants with the tested strains increased plant height, leaf pair number, fresh shoot weight and root weight at seven and 14 days post inoculation.

Several authors reported the influence of fungal endophytes on yield and yield attributing parameters in different crops. Rice *et al*. (1990) conducted field experiments to investigate the effects of endophyte infection on seed production and associated traits in tall fescue. They used endophyte-infected and uninfected clones of same genotype and found that endophyte infection resulted in 60.00 and 33.00 per cent more seeds per plant, 79.00 and 32.00 per cent more total seed by weight, 20.00 and 34.00 per cent more panicles per plant, and 32.00 and 4.00 per cent more seeds per panicle, respectively.

Sanchez-Rodriguez *et al.* (2017) applied *B. bassiana* on bread wheat plants using three inoculation methods *ie.,* soil treatment, seed inoculation and leaf spraying. In

addition to the negative effect on cotton leafworm (*Spodoptera littoralis*), the fungus also enhanced spike production and increased the grain yield by about 40.0 per cent.

Diaz-Gonzalez *et al.* (2020) inoculated the fungal endophyte, *Colletotrichum tofieldiae* strain Ct0861 in maize (*Zea mays* L.) and tomato (*Solanum lycopersicum* L.) and showed an increase in yield by 12.0 to 20.0 per cent in both tomato and maize.

Materials and Methods

3. MATERIALS AND METHODS

The present study on "Endophytic fungi for the management of spotted pod borer, *Maruca vitrata* Fab. (Lepidoptera: Crambidae) in cowpea" was undertaken in the Department of Agricultural Entomology, College of Agriculture, Vellanikkara, Thrissur during 2016-2019. The details of materials used and the methods adopted for the conduct of study are elucidated here.

3.1 ISOLATION OF ENDOPHYTIC FUNGI FROM COWPEA PLANTS

A sampling survey was conducted in four different districts of the state to isolate fungal endophytes from cowpea plants. Isolation of endophytic fungi from selected cultivars was also attempted.

3.1.1 Survey and collection of plant samples from different geographical areas of Kerala state

Purposive sampling surveys were conducted in the major cowpea growing areas of Kozhikode, Thrissur, Kottayam and Thiruvananthapuram districts of Kerala (Plate 1, Table 1, Fig. 1, 2, 3, 4 and 5). Plant samples were collected from ten organically managed plots in each district at reproductive stage for the isolation of endophytic fungi. Disease free cowpea plants were selected after close examination. Selected plants, along with roots, stems, flowers, leaves and pods were pulled out and cleaned by washing to remove adhered soil particles. Samples of different parts were collected in labeled polythene covers. Moist cotton was wrapped around the root system to avoid drying. The collected samples were brought to laboratory for isolation of fungi.

Table 1. Details of survey conducted at four districts of Kerala

Sl. No.	Location code	Location	Latitude	Longitude
Thiruvananthapuram				
1	TVM1	Vellayani	8º25'39.1"N	76º59'14.4"E
2	TVM2	Poonkulam	8º25'31.1"N	76º58'17.1"E
3	TVM3	Vavvamoola	8º25'10.7"N	77º00'13.0"E
4	TVM4	Paruthippara	8º31'53.4"N	76º56'46.4"E
5	TVM5	Vattappara	8º35'08.9"N	76º56'51.5"E
6	TVM6	Thekkada	8º37'49.1"N	76º57'13.3"E
7	TVM7	Chellamkodu	8º36'33.1"N	76º58'54.5"E
8	TVM8	Kuttichal	8º33'33.5"N	77º06'12.3"E
9	TVM9	Panavoor	8º38'13.8"N	76º59'47.5"E
10	TVM10	Kulakkode	8º34'10.7"N	77º02'57.6"E
Kottayam				
11	KTM1	Kumaranalloor	9º37'05.9"N	76º31'42.0"E
12	KTM2	Kumaranalloor	9º36'55.8"N	76º31'57.9"E
13	KTM3	Ettumanoor	9º40'08.0"N	76º33'23.8"E
14	KTM4	Manjoor	9º43'46.8"N	76º31'13.7"E
15	KTM5	Pampadi	9º34'14.9"N	76º38'32.0"E
16	KTM6	Marangattupalli	9º44'53.6"N	76º36'32.4"E
17	KTM7	Kollappalli	9º76'47.2"N	76º70'12.3"E
18	KTM8	Ezhachery	9º45'50.6"N	76º41'51.7"E
19	KTM9	Moonnani	9º42'43.7"N	76º42'07.9"E
20	KTM10	Vallichira	9º43'14.9"N	76º38'47.3"E
Thrissur				
21	TSR1	Chelakkara	10º38'08.3"N	76º21'55.3"E
22	TSR2	Chelakkara	10º40'21.9"N	76º21'23.3"E
23	TSR3	Elanadu	10º36'34.9"N	76º23'34.2"E
24	TSR4	Chuvannamannu	10º34'50.0"N	76º21'36.1"E
25	TSR5	Kannara	10º32'26.8"N	76º19'04.6"E
26	TSR6	Puthenchira	10º16'05.1"N	76º14'44.3"E
27	TSR7	Chirakkekode	10º33'53.3"N	76º17'14.3"E
28	TSR8	Vellanikkara	10º33'30.6"N	76º17'02.3"E
29	TSR9	Ramavarmapuram	10º33'42.2"N	76º14'39.1"E
30	TSR10	Kashumavumoola	10º32'32.2"N	76º16'19.8"E
Kozhikode				
31	KZD1	Kunnamangalam	11º17'50.0'N	75º53"45.6"E
32	KZD2	Kunnamangalam	11º18'10.2"N	75º52'39.4"E
33	KZD3	Edakkadu	11º18'27.8"N	75º46'30.4"E
34	KZD4	Kozhikode	11º15'15.9"N	75º46'17.1"E
35	KZD5	Mayanad	11º16'21.0"N	75º50'53.3"E
36	KZD6	Chaliyam	11º08'43.5"N	75º49'21.4"E
37	KZD7	Kannancheri	11º25'14.5"N	75º85'97.6"E
38	KZD8	Puthupanam	11º35'03.2"N	75º35'41.3"E
39	KZD9	Mayyannur	11º37'01.7"N	75º36'52.0"E
40	KZD10	Mannur	11º09'05.2"N	75º49'58.3"E

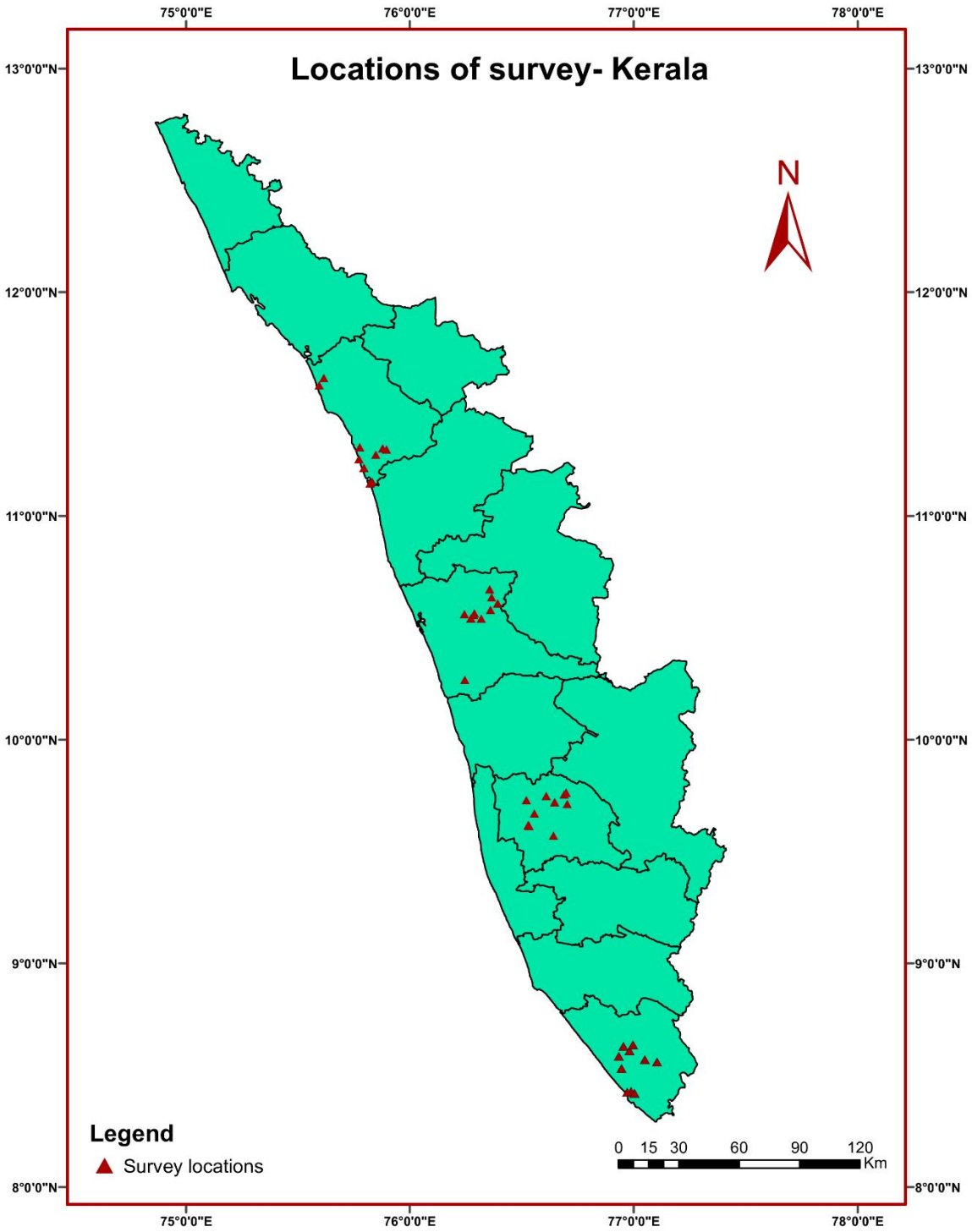


Fig. 1. Locations of survey conducted in Kerala

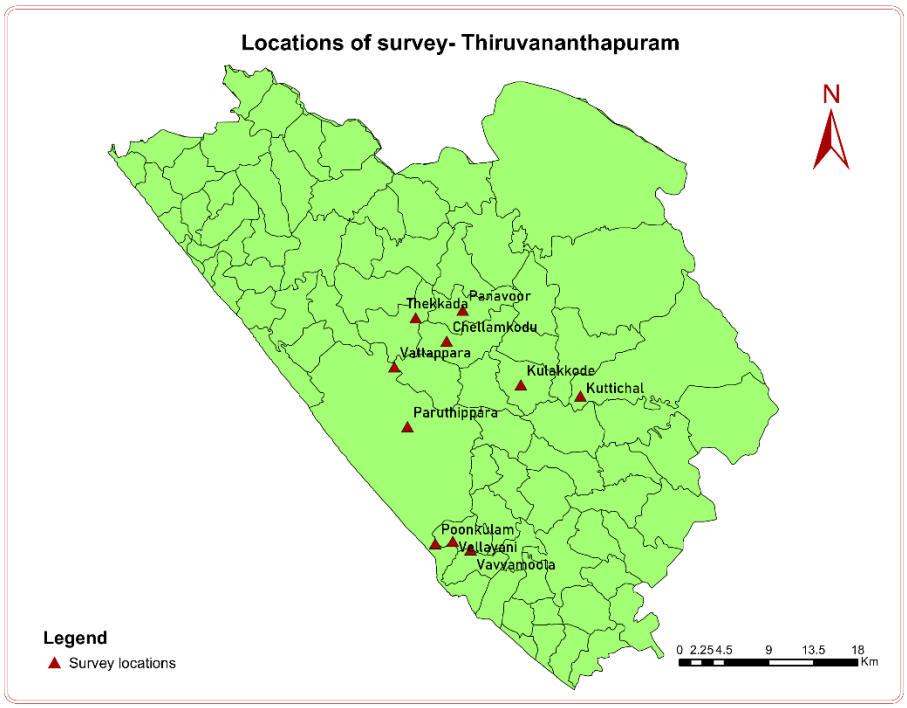


Fig. 2. Locations of survey conducted in Thiruvananthapuram district

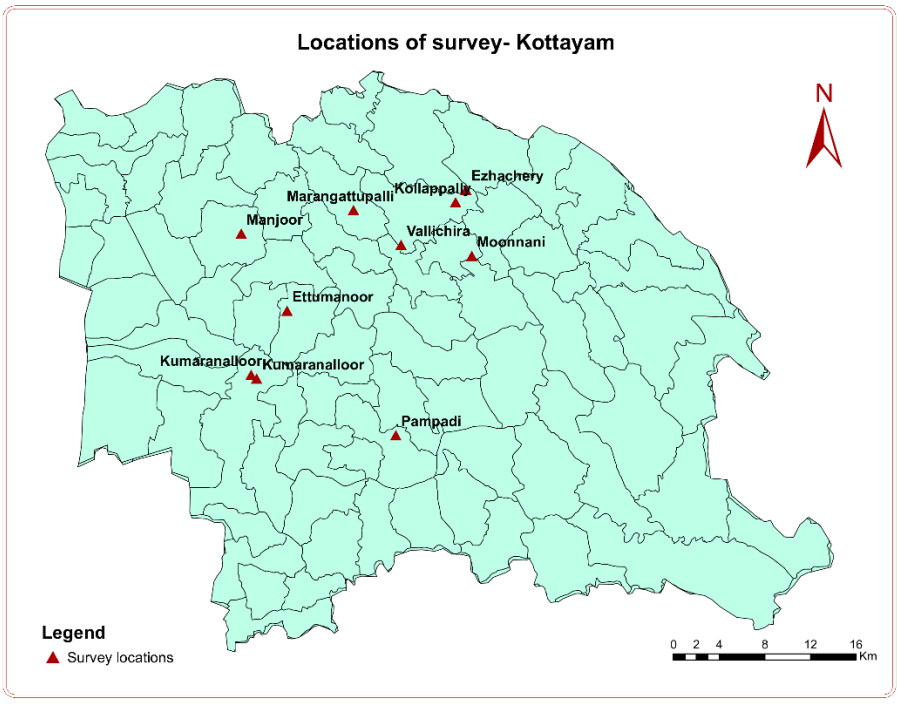


Fig. 3. Locations of survey conducted in Kottayam district

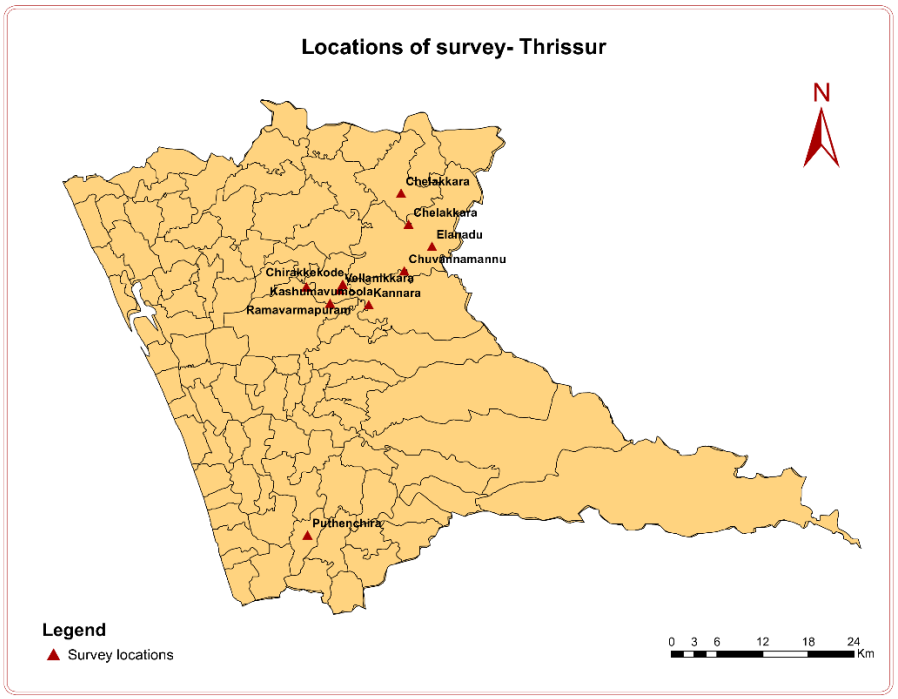


Fig. 4. Locations of survey conducted in Thrissur district

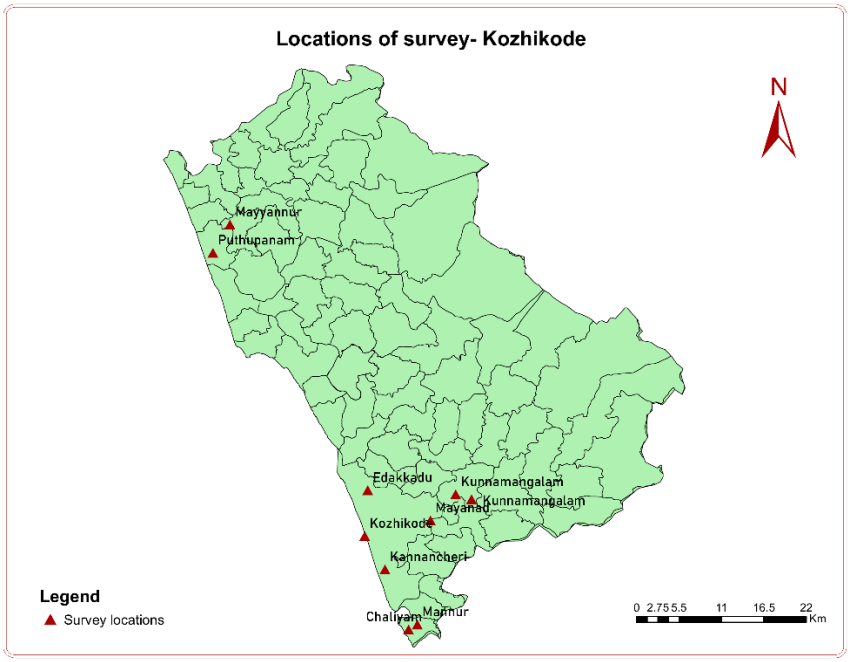


Fig. 5. Locations of survey conducted in Kozhikode district

Plate 1 . Collection of plant samples during survey

Plate 2. View of the experimental plot comprising of selected accessions of cowpea

Hridya

Palakkadan thandan payar

Kanakamony

Sreya

KBC -2

Mysore local

Anaswara

Lola

Bhagyalakshmi

Plate 3. Cowpea accessions selected for isolation of fungal endophytes

Table 2. List of cowpea accessions used in the study

Sl. No.	Accessions	Source
Resistant		
1	Hridya	Kerala Agricultural University, Thrissur
2	Palakkadan thandan payar	VFPCK, Thiruvananthapuram
3	Kanakamony	Kerala Agricultural University, Thrissur
Moderately resistant		
4	Sreya	Kerala Agricultural University, Thrissur
5	KBC – 2	UAS, Bengaluru
6	Mysore Local	IIHR, Bengaluru
Highly susceptible		
7	Anaswara	Kerala Agricultural University, Thrissur
8	Lola	Kerala Agricultural University, Thrissur
9	Bhagyalakshmy	Kerala Agricultural University, Thrissur
		(Beegum, 2015)

3.1.2 Collection of plant samples from different accessions of cowpea

Seeds of nine accessions of cowpea with different levels of resistance to spotted pod borer were collected from different sources and were sown in pots (Plates 2 and 3) at College of Agriculture, Vellanikkara, Kerala Agricultural University, Thrissur district (10º32'52.8"N latitude and 76º16'43.9"E longitude; 40 m above MSL) for isolating the fungal endophytes (Table 2)**.** Plant samples for isolating the fungal endophytes were collected during reproductive phase previously explained.

3.1.3 Isolation of endophytic fungi from plant samples of cowpea

The procedure followed for isolation of fungal endophytes from plant samples is explained below (Plate 4).

3.1.3.1 Surface sterilization of plant material

The samples were washed in running tap water to remove adhered soil particles. The plant parts were cut into three cm pieces and were washed four times using sterile distilled water. The plant parts were then transferred to a Petri dish containing sodium hypochlorite (4 % v/v) using sterilized forceps and were soaked for three minutes for

surface sterilization. The samples were again rinsed four times in sterile distilled water to remove traces of sodium hypochlorite and dried on sterile tissue paper under laminar air flow to ensure complete drying (Ahmad *et al*., 2000; Kjer *et al.,* 2010; Tenguria *et al*., 2012). Surface sterilization was ensured through spread plate and imprint methods as described in 3.1.3.1.3.

3.1.3.2 Inoculation of plant segments onto medium

The surface disinfected plant parts were then made into 6 mm long small pieces using a sterilized scalpel. They were carefully transferred onto previously prepared potato dextrose agar (PDA) plates supplemented with streptomycin (100 mg L^{-1}) and chloramphenicol (100 mg L^{-1}) at the rate of six pieces per plate. Antibiotics were filter-sterilized through a sterile PVDF syringe driven membrane filter with 0.22 mm pore size and 25 mm diameter (Himedia – SF-10-12X30N0) (Plate 5) before use. The plates were incubated at 28ºC under darkness (BOD incubator) for 14 days. In addition to PDA, Oatmeal Agar with CTAB was also used to isolate endophytic fungal isolates (Kumar *et al*., 2015; Appendix V).

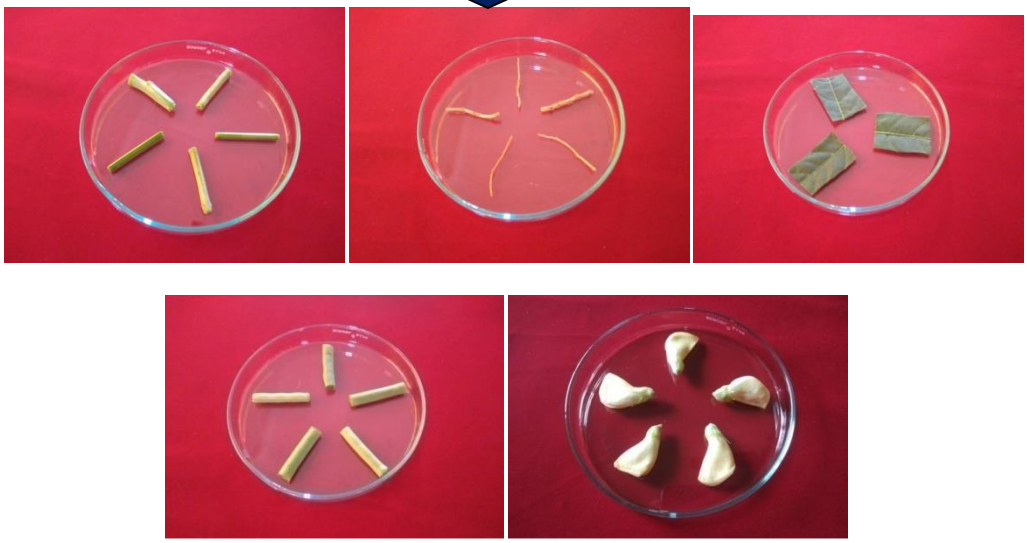
3.1.3.3 Confirmation of surface sterilization of plant parts

Plant tissue imprints were obtained prior to plating (imprint method) and the last rinse water was also plated out (spread plate method) to assess the effectiveness of surface sterilization (Schulz *et al*., 1998). Both these sets of plates were incubated at 28ºC for 14 days for observing the epiphytic fungal growth (Tenguria and Ferodiya, 2015). Absence of any fungal growth on the medium after incubation indicated the effectiveness of sterilization procedure.

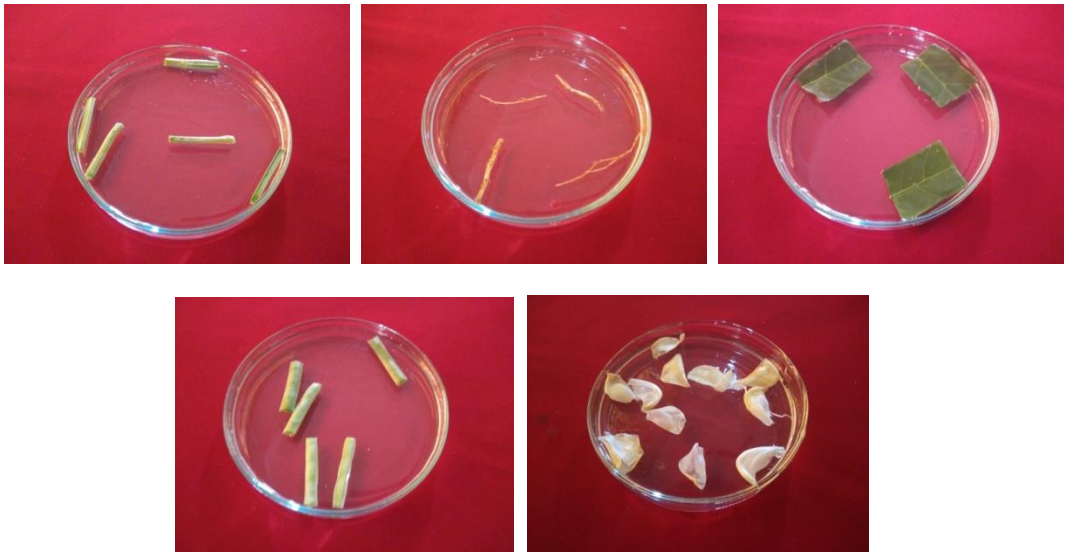
3.1.4 Purification and maintenance of isolates

Plates with plant fragments were periodically examined for fungal growth ensuing from the edges of the fragments. Most of the fungal growth was initiated within 10 days

Washing the plant samples with running tap water



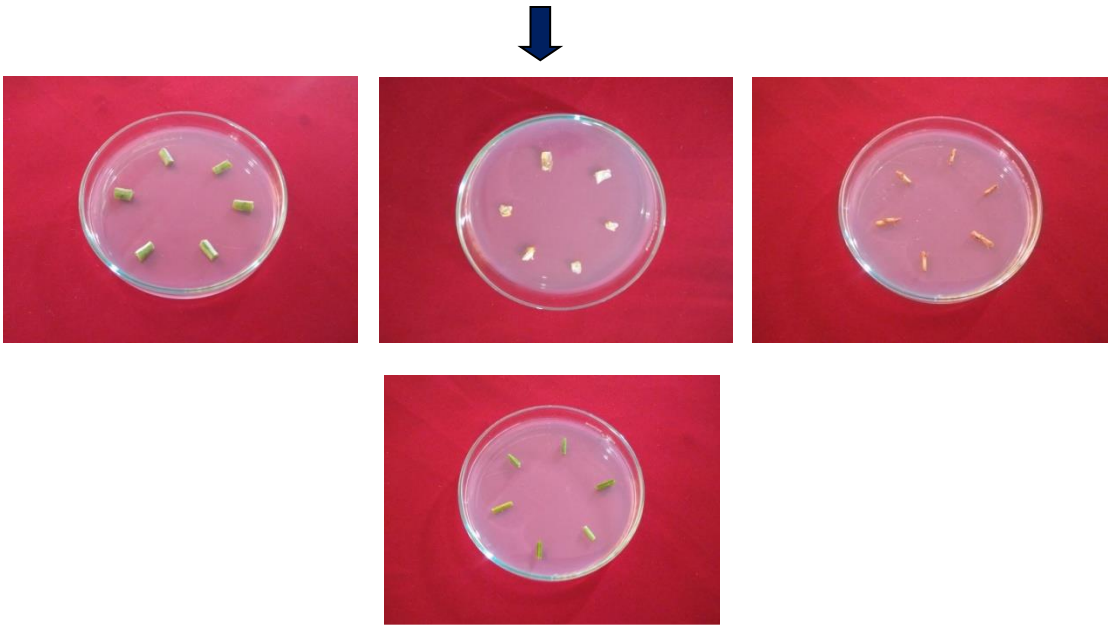
Cutting the plant samples to small pieces before surface sterilisation



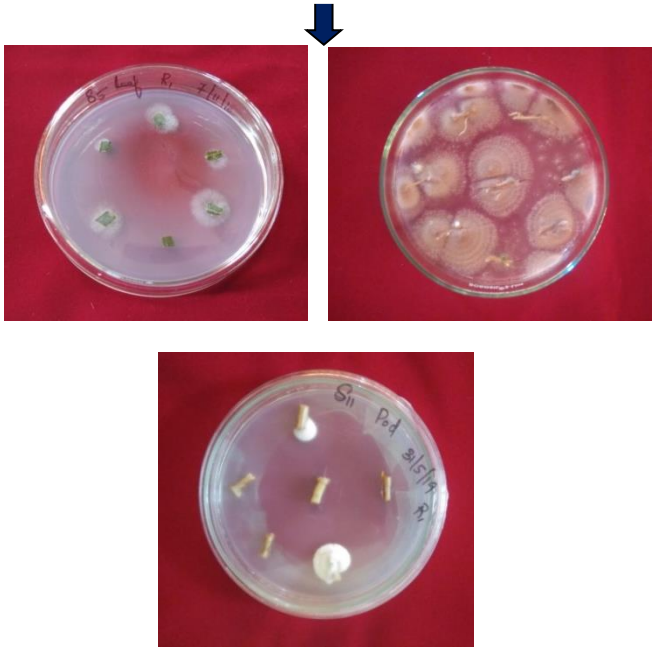
Surface sterilizing the plant pieces as per standard procedure

Plate 4. Different steps involved in isolation of fungal endophytes

Contd…

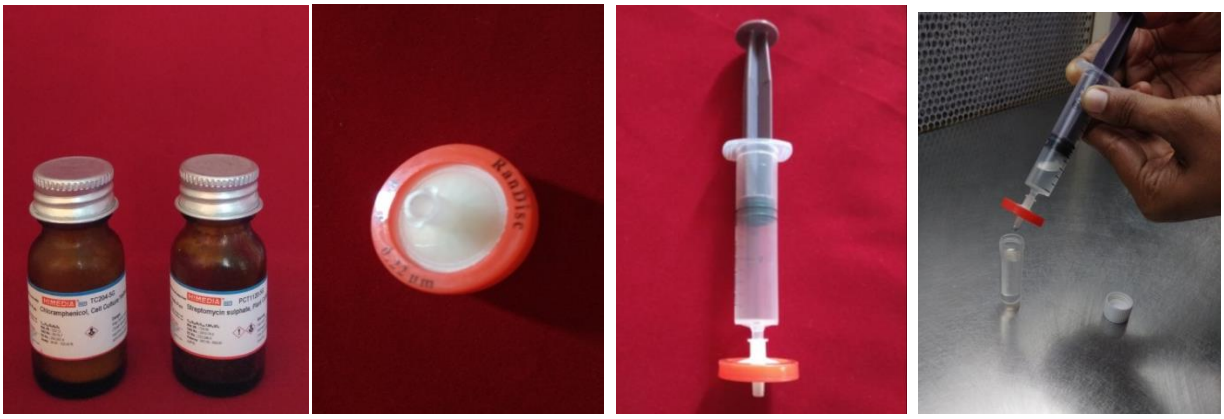


Inoculating plant segments onto medium



Growth of fungal endophytes from edges of plant segments

Plate 4. (Contd..) Different steps involved in isolation of fungal endophytes



Antibiotics PVDF filter Syringe with filter Filter sterilization

Plate 5. Filter sterilization of antibiotics

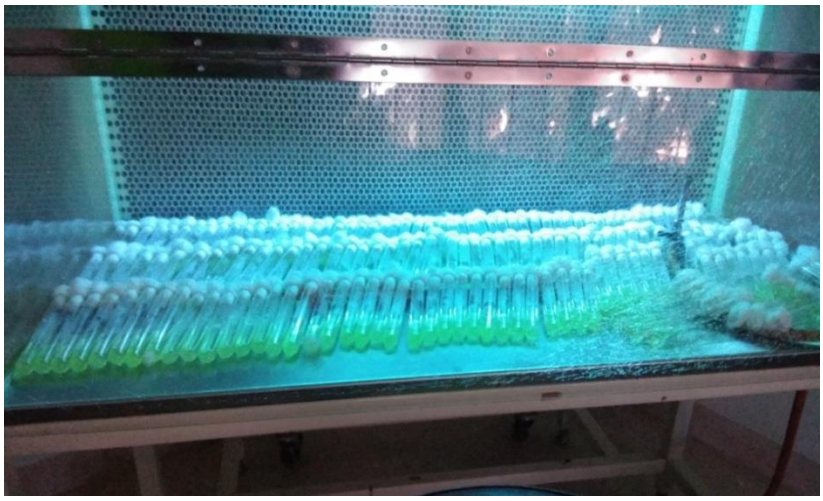


Plate 6. Media slants for preserving pure cultures

of inoculation. Such fungal growths were considered 'endophytic,' and were periodically picked and transferred to PDA plates. It was serially sub-cultured onto fresh PDA plate using hyphal tip method to obtain monosporic cultures as described by Suryanarayanan *et al*., 2002.

The cultures were then preserved in PDA slants at 4 ºC. Isolate numbers were given to fungal endophytic isolates to discriminate them and preserved in refrigerator (Plate 6). The stored cultures were subcultured periodically to maintain the viability.

Observations such as number of isolates from different locations and accessions were recorded and these isolates were used for further biological studies.

3.1.5 Analysis of fungal endophytes in cowpea by scanning electron microscopy (SEM)

Scanning electron microscopic photographs of plant samples were taken after 48 h of incubation on PDA. The plant specimens were fixed using 2.5 per cent gluteraldehyde in 0.1 M phospahate buffer for 18 h at 37 ºC and then washed five times in phosphate buffer. The specimens were dehydrated by passing through graded ethanol series (25.0, 50.0, 70.0, 80.0, 90.0 and 100.0%) for about 15 to 20 minutes. After drying, samples were mounted on metal stubs and covered with gold layer (120 seconds, 240 V, 10 mA, 10 Pa pressure) using a metallic covering (sputtering) apparatus. The metal covered plant parts were analysed in a SEM emission field at 5 kV and 7 mm distance. A metal coater (Quorum) and SEM (Tescan Vega Z-LMU) were employed for the histological analysis. The specimens were quickly examined at low magnification and after focusing, the photographs were taken at high magnification (John and Mathew, 2017). Facility available at Central Instrumentation Laboratory, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala was utilized for SEM (Plate 7).

3.2 EVALUATION OF BIOEFFICACY OF ENDOPHYTIC FUNGAL ISOLATES

The endophytic fungal isolates obtained from cowpea plants were evaluated for their entomopathogenicity. Endophytic isolates identified as pathogenic to the test insect, *Galleria mellonella* were further screened against the target pest, *Maruca vitrata.* For this *G. mellonella* and *M. vitrata* were maintained in the insectary of AICRP on BCCP as given below.

3.2.1 Preliminary bioassay using larvae of greater wax moth, *Galleria mellonella*

Preliminary bioassay to assess the entomopathogenicity of isolated fungi was carried out using third instar larvae of greater wax moth, *G. mellonella.*

3.2.1.1 Mass rearing of *G. mellonella* using semi-synthetic diet

A semisynthetic diet was prepared and used for rearing of *G. mellonella* larvae in the laboratory (Table 3). Male and female moths were released into plastic bottles and fed with five per cent honey solution containing vitamin E. Mouth of bottles were covered with muslin cloth for proper aeration. Strips of folded papers were suspended from the top of the bottle for ovipoistion. Eggs were regularly collected from the bottle and kept in plastic basins. Hatched larvae were transferred to a jar containing semisynthetic diet prepared as per standard procedure (Plate 8).

Table 3. Composition of semi-synthetic diet for rearing *Galleria mellonella*

Sl. No.	Ingredient	Quantity
1	Corn flakes	200 g
2	Wheat flour	100 g
3	Wheat bran	100 g
4	Milk powder	100 g
5	Honey	100 ml
6	Glycerol	100 ml
7	Dry yeast	30 g

Preparation of plant samples for SEM

Sputtering apparatus

Scanning Electron Microscope

Plate 7. Set up for scanning electron microscopy studies

Semi synthetic diet for *G. mellonella*

Placing eggs of *G. mellonella*

Larvae of *G. mellonella*

Adults of *G. mellonella*

Plate 8. Rearing of *Galleria mellonella*

3.2.1.2 Preparation of spore suspension

All fungal isolates were inoculated onto PDA plates under sterile conditions and incubated at 28°C for 14 days in complete darkness by keeping the plates in BOD incubator. Stock spore suspensions of each fungus were prepared under sterile condition by adding sterile distilled water containing Tween 80 (0.01% v/v) on 14-day-old sporulating cultures in Petri plates and gently scraping the surface with a sterile spatula (Quesada-Moraga *et al*., 2009; Lopez and Sword, 2015). The resulting mycelia and spores were then filtered through three layers of sterile muslin cloth into a sterile beaker containing sterile distilled water along with Tween 80 (0.01% v/v) to remove medium and mycelia (Sasidharan and Varma, 2005) (Plate 9a). The suspension was then homogenised with a vortex mixer for three minutes to get a uniform suspension (Plate 9b). Spore concentration for each fungal isolate was assessed under the microscope using an improved Neubauer haemocytometer (Lomer and Lomer, 1996).

$$\text{Number of conidia per ml} = \frac{X \times 400 \times 10 \times 1000 \times D}{Y}$$

Where, X - Average number of conidia per small square
400 - Number of small squares counted
10 - Depth factor
1000 - Conversion factor from mm^3 to cm^3
D - Dilution factor
Y - Number of small squares checked

The final concentration was adjusted to 1×10^8 conidia ml^{-1} by adding sterile distilled water using the formula of Parsa *et al.* (2013).

$$\text{Final volume} = \frac{\text{Stock volume} \times \text{Stock concentration}}{1 \times 10^8}$$

Spore suspension of low concentration was adjusted to desired concentration by centrifugation and decanting the excess water. Centrifugation was done at 12000 rpm for 25 minutes at 10ºC using a cooling centrifuge (Thermo Fisher Scientific™). The facility available at Toxicology laboratory, Dept. of Agricultural Entomology, College of Agriculture, Vellanikkara was utililized for this purpose. Fresh spore suspensions were prepared for each bioassay and were used on the same day.

3.2.1.3 Bioassay using greater wax moth, *Galleria mellonella*

Preliminary bioassay using fungal endophytes was carried out in the laboratory through contact toxicity method using neonate third instar larvae of *G. mellonella* (Ali-Shtayeh *et al*., 2003). Third instar larvae of *G. mellonella* were dipped into each fungal spore suspension of 1 x 10^8 spores ml^{-1} for ten seconds. The larvae were then placed on moisturized sterile tissue papers in sterile Petri plates and sides were covered with parafilm to avoid contamination. The Petri plates were then incubated in the dark at 28ºC.

In addition to the above, pathogenicity was also confirmed by topical application method and agar bit method. In topical application method, the larvae were sprayed with spore suspension of 1 x 10^8 spores ml^{-1} using an atomiser. After treatment, the larvae were fed with the semisynthetic diet. In agar bit method, agar bits from 14 day old cultures were placed in sterile glass tubes and larva was released into the tube individually. The tubes were subsequently plugged with sterile cotton and larvae were allowed to crawl on the mycelial mat for one hour. Then transferred to sterilised Petri plates lined with moistened sterile tissue paper. Petri plates were observed daily and the dead larvae were kept in Petri plates lined with wet tissue paper for observing external growth of the fungi (humid chamber) (Plate 10 and 11).

The dead larvae with fungal growth were surface sterilized in aseptic condition using four per cent sodium hypochlorite for three minutes and three washes of sterile distilled water. After the surface sterilization, the cadavers were placed on sterile tissue paper to remove adhered water and they were transferred to Petri plaes containing PDA

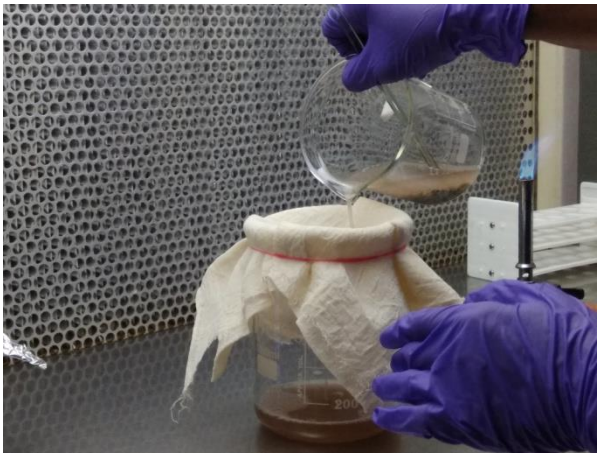


Plate 9a. Sieving the spore suspension through tree layered sterile muslin cloth

Plate 9b. Vortex mixing to get uniform suspension

Plate 9. Preparation of spore suspension

Plate 10. Experimental set up for bioassay

Plate 11. Fungal growth on infected cadaver in humid chamber

amended with sterilized antibiotics. The Petri plates were incubated at 28ºC in a biological oxygen demand (BOD) incubator. Petri plates were regularly observed for fungal out growths from cadavers. The fungi were further purified by sub-culturing for identification. The reisolated fungi were compared with the original isolates and confirmed by cultural and morphological characterization.

3.2.2 Bioassay against spotted pod borer, *Maruca vitrata*

After preliminary bioassay, virulence bioassay was carried out in the laboratory to confirm the pathogenicity of selected fungal endophytic isolates on the target pest, *M. vitrata*. *M. vitrata* was mass reared in the laboratory using natural and semi-synthetic diet.

3.2.2.1 Mass rearing of *M. vitrata*

Mass rearing of *M. vitrata* was standardized to maintain sufficient population in the laboratory. The initial culture was obtained by collecting larvae from nearby fields. The larvae were mass reared in partitioned transparent plastic boxes. The feed of larvae were replaced daily. Pupae were transferred to plastic jars and the tops of jars were covered with muslin cloth and tied firmly with rubber band.

An oviposition cage was designed or egg laying by adult moths (Plate 12a). A metal frame comprising of three iron rings connected by iron rods was constructed and covered with black cloth. Emerged moths were transferred to this cage for mating. A cotton swab soaked in honey solution (5.0% v/v) fortified with vitamin E was suspended from the top of cage as feed for adults. The swab soaked in honey solution was changed daily. A shallow container with water was kept at the bottom portion of the cage to maintain humidity inside. A twig of cowpea along with tender buds and pods were kept in 250 ml conical flask containing water to facilitate egg laying. The mouth of conical flask was closed with cotton to prevent moths falling into the water. Eggs laid on the sides of black cloth were collected using camel brush and transferred to partitioned plastic boxes for rearing. Buds in conical flask were also regularly collected and kept in the rearing

box. First instar larvae were fed with buds and flowers and late instars with pods or semi-synthetic diet.

3.2.2.1.1 Mass rearing of *M. vitrata* using semi-synthetic diet

On scarcity of organic cowpea pods, larvae were fed with semi-synthetic diet as described by Sreelakshmi (2014). Ingredients used for the preparation of diet are given in Table 4. The ingredients in fraction A of diet were mixed separately in a blender for three minutes. The agar from fraction B was added to 80 ml distilled water, boiled to dissolve the agar and then allowed to cool to 60 ºC. The molten agar was poured to the prepared fraction A in a blender and mixed thoroughly for three minutes. Formaldehyde was added to this mixture and blended further for 1.5 minutes. The whole ingredients were transferred into sterilized Petri plates and left overnight for solidification (Plate 12b). When properly solidified, the diet was kept under refrigerated condition for further use. Diet in Petri plates were cut into small pieces of 2 cm x 2 cm size and kept inside the partitioned rearing trough to feed larvae.

Table 4. Composition of semi-synthetic diet for rearing *Maruca vitrata*

Sl. No.	Ingredient	Quantity
	Fraction A	
1	Water	48.00 ml
2	Yeast	1.60 g
3	Ascorbic acid	0.44 g
4	Cowpea seed powder	14.00 g
5	Vitamin E	0.16 g
	Fraction B	
6	Agar	2.04 g
7	Water	80.00 ml
8	Formaldehyde	0.40 ml

Plate 12a. Rearing cage for spotted pod borer

Plate 12b. Artificial diet for spotted pod borer

Plate 12c. Partitioned tray for rearing

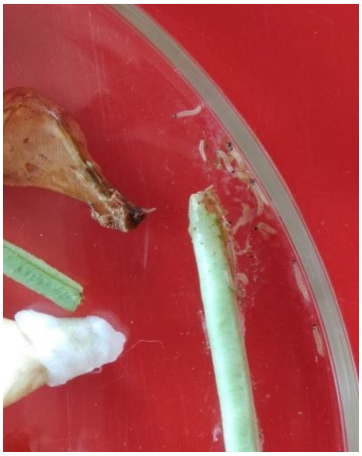


Plate 12d. Newly hatched larvae of spotted pod borer

Plate 12. Rearing of spotted pod borer in the laboratory

3.2.2.1.2 Mass rearing of *M. vitrata* on natural diet

Fresh cowpea flowers offered as feed to the neonates hatched in the laboratory. Late instar larvae were fed with cowpea pods of five centimetre length (Plate 12c and 12d). The cut ends of pods were covered with moist cotton to avoid fast drying. Dried pods were periodically removed and larvae were carefully transferred to fresh cowpea pods using camel hair brush. The top of trough was covered with muslin cloth to provide proper aeration.

3.2.2.2 Virulence bioassay using spotted pod borer, *M. vitrata*

The fungal isolates proved entomopathogenicity in the previous experiment were used for confirming the pathogenicity against the cowpea pod borer. The preparation of spore suspension and bioassay was carried out as explained in 3.2.1.2 and 3.2.1.3. Fungal isolates were re-isolated from infected cadavers and compared with the original isolates. The reisolated fungi were used further to prove Koch's postulates.

3.3 CHARACTERIZATION OF ENDOPHYTIC ENTOMOPATHOGENIC FUNGAL ISOLATES

Monosporic cultures of fungal isolates preserved at refrigerated condition were used for further studies. Endophytic fungal isolates which proved positive in entomopathogenicity assays were subjected to morphological, cultural and molecular characterization.

3.3.1 Morphological and cultural characterization

The colony morphology and cultural characters of endophytic entomopathogenic fungal isolates were studied (Kumar *et al*., 2015). Morphological identification for each fungal isolate was confirmed with the aid of the standard keys.

3.3.1.1 Morphological characterization

Morphological characters of endophytic entomopathogenic fungal isolates, such as spore size, shape, and arrangement of conidiophore were studied by slide culture technique.

Slide cultures were prepared from two weeks old pure cultures. Size and shape of conidia and characters of conidiophores were studied under different magnifications using a compound microscope. Measurements of 30 conidia were taken under 1000 X magnification. The photomicrographs of the slide cultures were taken with the facility available at AICRP on BCCP, College of Agriculture and College of Forestry, Vellanikkara.

3.3.1.2 Cultural characterization

Mycelial discs of 9 mm diameter were taken using a cork borer and placed at the centre of Petri plates containing standard media (Kumar *et al*., 2015). The Petri plates were then incubated in a BOD incubator at 28ºC for 14 days under dark condition to observe the cultural characters.

3.3.2 Molecular characterization and phylogenetic analysis

BLASTn and phylogenetic analyses were done to confirm the identity of endophytic entomopathogenic fungal isolates.

3.3.2.1 Molecular characterization

Molecular characterization of the isolated fungi was done through sequencing of the ITS of 5.8S rDNA conserved region. The ITS regions of the isolates were amplified using the universal primers *ie*., ITS-1F and ITS-4R primers (Zarrin *et al*., 2016).

Target region	Primer name	Direction	Sequence (5' → 3')
ITS	ITS-1F	Forward	TCCGTAGGTGAACCTGCGG
	ITS-4R	Reverse	TCCTCCGCTTATTGATATGC

DNA isolation and ITS sequencing were carried out at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram. Forward and reverse sequences of isolates were assembled to contig sequences using CAP3 sequence assembly software (PRABI-Doua) (Huang and Madan, 1999). Nucleotide BLAST analysis was done with the assembled sequences. The sequences of isolated fungi were then compared with the captured sequences from the nucleotide database of National Centre for Biotechnology Information (NCBI) by BLASTn analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Based on the results, rDNA sequences of isolates were submitted at NCBI BANKIT to obtain the GenBank accession numbers.

3.3.2.2 Phylogenetic analysis

Phylogenetic analysis was performed to assess the phylogenetic relationship between the endophytic entomopathogenic fungi and closely related strains. Nucleotide sequences of closely related and unrelated strains were downloaded from NCBI database (GenBank). Phylogenetic tree showing sequence variability and homology was generated using the softwares MAFFT and MEGA 7.0 (Kumar *et al*., 2016). The sequences were aligned using MAFFT and phylogenetic analysis was performed using MEGA 7.0. Neighbour joining method was used for inferring the evolutionary history (Saltou and Nei, 1987).

3.4 PLANT PATHOGENICITY TEST

The plant pathogenicity of the endophytic entomopathogenic fungi obtained in the study was evaluated against two weeks old potted cowpea plants (*cv*. Anaswara) through three different methods such as foliar application, agar block technique and soil application. Fungi were cultured on PDA media for 14 days at 28°C and the conidia were

collected in sterile water containing 0.01 per cent Tween 80. The suspension was sieved through three layers of sterile muslin cloth to remove the mycelial clumps. The spore suspension of isolates was adjusted to 7×10^6 conidia/ml (Sunitha, 1996; Aswinkumar, 2016).

In foliar application method, five ml of spore suspension was sprayed uniformly on leaves using a small hand sprayer (Plate 13a). Soil inoculation was carried out by pouring 250 ml of spore suspension around each seedling (Plate 13c). Agar blocks of 9 mm size from culture in Petri plate were used for the agar block inoculation method. Pin prick injuries were made on the leaves using sterile needle and agar blocks were placed over the punctures (Plate 13b). Moist cotton wools were placed over the agar blocks to provide humidity. Plants inoculated with agar block and foliar applications were covered with polyethylene cover to ensure humidity for spore germination. Covers were removed from the pots at 24 h post inoculation.

Untreated potted plants were kept as control and five replicates were maintained for each treatment. In each replicate two plants were maintained. Safety of fungal isolates to cowpea plants was recorded for a period of three months as described by Mishra and Dhar (2005).

3.5 EVALUATION OF BIOEFFICACY OF ENDOPHYTIC ENTOMOPATHOGENIC FUNGAL ISOLATES

The bioefficacy of isolates of endophytic entomopathogenic fungi obtained in the previous experiment was evaluated along with potential entomopathogen, *Beauveria bassiana*. The culture (NBAII Bb 5a) was obtained from NBAIR, Bengaluru. Evaluation was done to select the best fungal isolates for further studies. Contact toxicity bioassay technique was followed for evaluating the efficacy of isolates as explained in 3.2.1.3. The experiment was laid out in a completely randomized design with three replications. Each replication constituted of ten uniform sized 0-24 h old second instar larvae. Spore suspension having a concentration of 1×10^9 spores ml^{-1} was prepared and then diluted to

Plate 13a. Foliar application

Plate 13b. Agar block method

Plate 13c. Soil application

Plate 14. Serial dilution for sterility check of potting mixture

concentrations of 10^8, 10^7, 10^6 and 10^5 spores ml^{-1} for the test. A negative control was also maintained, wherein the larvae were treated only with sterile distilled water containing Tween 80 (0.01 % v/v). Treated larvae were fed with fresh and clean cowpea pods and maintained at ambient conditions. Pods were regularly replaced with fresh ones at an interval of 24 h.

Number of dead and mycosed larvae was recorded daily upto ten days. The dead larvae were removed and kept in humid chamber to allow outgrowth of fungus. Fungi were reisolated from the infected cadavers following standard procedure as explained in the preliminary screening assay. Data on per cent mortality were corrected with Abbott's formula (Abbott, 1925).

$$\text{Corrected mortality} = \frac{\text{(Treatment mortality - Control mortality)} \times 100}{\text{(100 - Control mortality)}}$$

The corrected data were then statistically analysed by analysis of variance (ANOVA) and the means were separated by DMRT.

3.6 STANDARDIZATION OF THE INOCULATION TECHNIQUE FOR ENDOPHYTIC COLONISATION

Two superior strains of entomopathogenic fungal isolates identified in the contact toxicity bioassay were used for standardizing the inoculation technique for endophytic colonisation in plants. The experiments were carried out in a polyhouse of 200 m^2 of Department of Agricultural Entomology, College of Agriculture, Vellanikkara.

Three different methods of inoculation *viz*., seed, soil and foliar inoculation were evaluated for identifying the best method for colonisation of entomopathogenic fungi. The evaluation was carried out through a pot culture experiment using the semi trailing cowpea variety, Anaswara. The experiment was laid out in a completely randomised

design (CRD) with nine treatments and three replications, with ten plants constituting one replication. The treatment details are as follows,

T_1: *Beauveria bassiana* (NBAII Bb5a) as seed inoculation
T_2: *B. bassiana* (NBAII Bb5a) as soil application
T_3: *B. bassiana* (NBAII Bb5a) as foliar application
T_4: *Purpureocillium lilacinum* (EEF 4) as seed inoculation
T_5: *P. lilacinum* (EEF 4) as soil application
T_6: *P. lilacinum* (EEF 4) as foliar application
T_7: Control 1 (Seed inoculation)
T_8: Control 2 (Soil application)
T_9: Control 3 (Foliar application)

3.6.1 Preparation of planting medium

The potting substrate was prepared by mixing sand, soil and powdered cowdung in the ratio of 1:1:1, and sterilized by autoclaving the mixture at 121°C and 15 lbs pressure for three consecutive days for eliminating foreign fungal spores. To confirm the effectiveness of sterilization, 1 cm^3 of sterilized potting mixture sample was suspended in nine millilitres of sterile 0.01 per cent Tween 80 (Plate 14), vortexed for 3 min, and 100 microlitres of 10^{-1} and 10^{-2} dilutions of this suspension was plated onto PDA plates amended with chloramphenicol and streptomycin (100 mg L^{-1} each). Plates were incubated at 28°C in darkness and were observed for any fungal growth (Parsa *et al*., 2016) to ensure that there were no fungal spores in any of the samples. Pots and labels used for the experiment were treated with surface disinfectants and air dried before use.

3.6.2 Cowpea seed sterilisation

Cowpea seeds (*cv.* Anaswara) for the experiment were obtained from the Department of Vegetable Science, College of Agriculture, Vellanikkara. They were surface sterilised by suspending in 70.0 per cent ethanol for three minutes with agitation,

followed by suspending in two per cent sodium hypochlorite (NaOCl) for three minutes and finally rinsing in sterile distilled water three times (Plate 15). Samples taken from the third rinse water (100 microlitres) were plated onto PDA media to confirm the efficiency of the surface sterilization procedure (Lopez and Sword, 2015). After surface sterilization, seeds were transferred into sterile Petri plates and air dried in a laminar flow hood for approximately one hour (Parsa *et al*., 2016).

3.6.3 Different methods of plant inoculation

Different methods of plant inoculation techniques such as seed inoculation, soil application and foliar application was followed for colonisation of cowpea plants by the selected endophytic entomopathogenic fungal isolates.

3.6.3.1 Seed treatment

Surface sterilized seeds were soaked overnight in spore suspension of $1x10^8$ spores ml^{-1} concentration containing Tween 80 (0.01 % v/v). The remaining seeds were soaked in sterile distilled water containing Tween 80 (0.01 % v/v) for control and other two treatments (Plate 16). The inoculated seeds were then dried by keeping them in sterile Petri dishes for 20 minutes under aseptic condition and sown in pots (Lopez and Sword, 2015) at the rate of two seeds per pot. Thinning was done seven days after sowing.

3.6.3.2 Soil application

Pots filled with potting mixture were irrigated to field capacity with sterile distilled water 24 h before inoculation. Sowing and thinning were carried out as described above. Plants were inoculated by adding 10 ml of the spore suspension at the base of each plant fourteen days after sowing (Plate 17) (Posada *et al*., 2007). The control plants were treated with 10 ml of sterile distilled water containing Tween 80 (0.01 % v/v).

3.6.3.3 Foliar application

Cowpea plants were raised as above. Fourteen day old plants were inoculated by spraying the leaves with five millilitres of spore suspension mixed with 0.01 % Tween 80 using a plastic hand sprayer (Plate 18). The control plants were treated with five ml of sterile distilled water containing Tween 80 (0.01 % v/v). A thick paper board covered with aluminium foil was held just below the leaves during spraying to prevent spray fluid impinging on other parts of plant. In addition, the soil surface in each pot was covered with aluminium foil during spraying to avoid spray runoff onto the soil. The inoculated plants were covered with polyethylene covers for 24 h to maintain high level of humidity and facilitate foliar invasion as described by Posada *et al*. (2007) (Plate 19).

The plants were grown in a polyhouse under ambient conditions with natural photoperiod for the duration of the experiment (Plate 20). Plants were irrigated using sterile water regularly and not fertilized throughout the experiment (Plate 21).

3.6.4 Confirmation of plant colonisation

Plant colonisation by endophytic fungi following different methods of inoculation was assessed by re-isolation and molecular method.

3.6.4.1 Re-isolation

Colony characters of isolated fungi were observed for confirming the identity of re-isolated fungi. Re-isolation of different endophytes from roots, stems, leaves and pods was carried out as explained in 3.1.3. PDA was used for isolating *P. lilacinum*, while SDAY medium was used for isolating *B. bassiana* from colonised plant parts (Renuka *et al*., 2016). Destructive sampling was done thrice at 30, 55 and 80 days after sowing, representing the initial, active growth and pod bearing stages. Three separate cowpea plants per treatment were randomly selected (Guesmi-Jouini *et al.*, 2014). From each plant, three old leaves, three young leaves, three pieces of old stem, three pieces of young

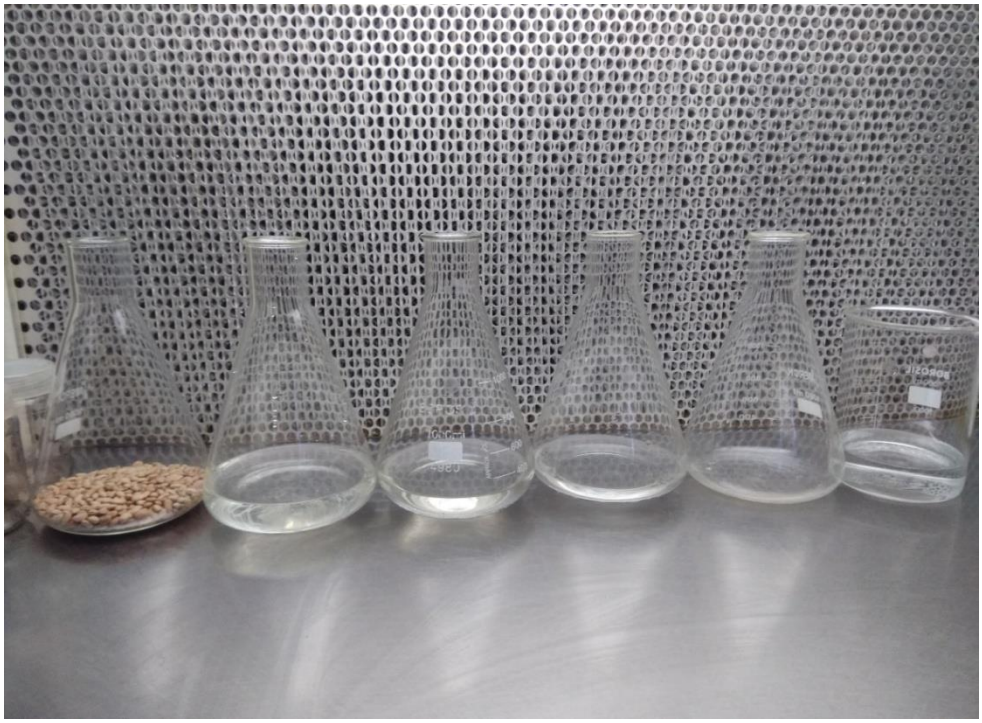


Plate 15. Sterilization of cowpea seeds

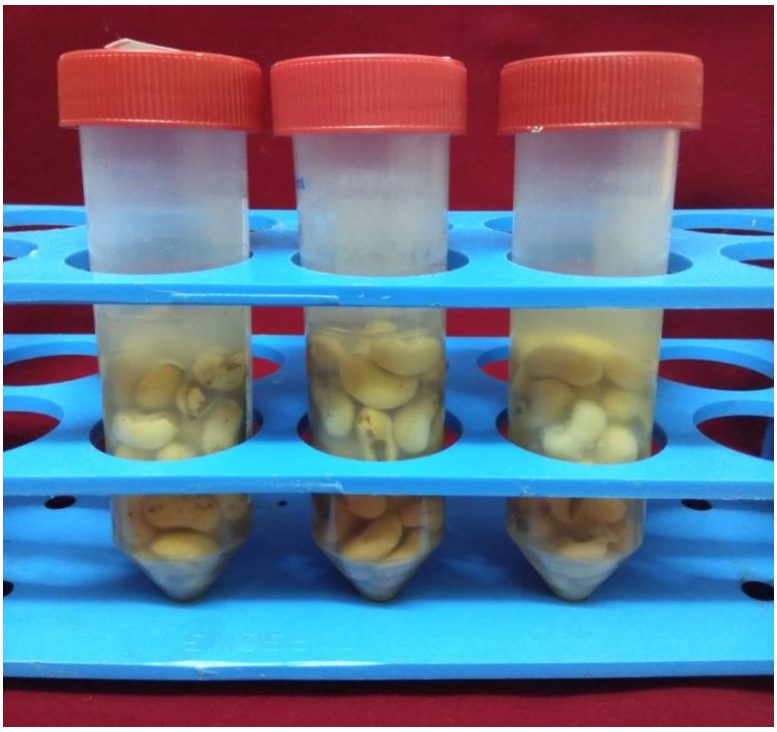


Plate 16. Seed inoculation with spore suspension

Plate 17. Soil application of spore suspension

Plate 18. Foliar application of spore suspension

Plate 19. Treated plants covered with polythene covers to maintain humidity

Plate 20a. View of the polyhouse experiment at 20 days after sowing

Plate 20b. View of the polyhouse experiment at 80 days after sowing

Plate 20. View of the polyhouse experiment

Plate 21. Irrigating the potted plants in polyhouse using sterile water

Plate 22. Field view of the experimental plot

stem and root samples were taken to elucidate the pattern of colonisation. In addition, pod samples were also taken from each plant at fruiting stage to assess colonisation. Surface sterility check was carried out as explained in 3.1.3.1.3.

After inoculation, Petri plates were incubated in BOD incubator at 28ºC in dark. The presence of endophytic fungi from the edges of plant pieces was observed and recorded regularly. Fungal colonies obtained were characterized based on morphological keys (Booth, 1971; Luangsa-Ard *et al*., 2011). Fungal structures were observed under microscope for morphological confirmation.

Colonisation of different plant parts by the respective inoculated fungus was calculated following the standard formula (Fisher and Petrini, 1987).

$$\text{Colonisation} = \frac{\text{Number of sampled plant tissue showing fungal outgrowth}}{\text{Total number of plated plant tissue samples}} \times 100$$

The data were arc sin transformed to stabilize the variances and were subjected to analysis of variance (ANOVA) and the means were separated by DMRT.

3.6.4.2 Molecular technique

The reisolated fungal isolates were subjected to PCR technique to confirm plant colonisation by respective isolates. The molecular work was carried out at Rajiv Gandhi Centre for Biotechnology, Thiruvanathapuram. ITS region of fungal isolates was amplified as explained in 3.4.2. The PCR products of fungal endophytes were analysed in 1.2 per cent agarose gel containing 0.5 µg/ml ethidium bromide. PCR products in gel were visualized under UV transilluminator and were compared with that of original samples.

3.6.5 Effect of fungal colonisation on growth and yield parameters under polyhouse condition

The effect of the fungal colonisation on plant growth was studied by measuring important growth and yield parameters.

3.6.5.1 Growth parameters

Growth parameters of colonized plants were taken at three different stages of crop growth.

3.6.5.1.1 Total number of leaves

Number of fully opened leaves in the plants was counted and the mean was recorded**.**

3.6.5.1.2 Plant height

The height of individual plant was measured in centimeter (cm) from base of the plant to tip of main stem**.**

3.6.5.2 Pod yield per plant

Pods from individual plants were picked as and when matured, weighed and total green pod yield was expressed in g plant^{-1}.

The recorded data were subjected to ANOVA and the analysed data were averaged and comparison between the treatment means was performed with DMRT.

3.7 EFFECT OF COLONISATION ON GROWTH, YIELD AND INFESTATION OF SPOTTED POD BORER IN COWPEA UNDER FIELD CONDITION

A pot culture experiment to assess the effect of endophytic entomopathogenic fungi against spotted pod borer on cowpea (*cv.* Anaswara) was conducted at Krishi

Vigyan Kendra, Kerala Agricultural University, Thrissur (Plate 22). The experiment was laid out in a completely randomized design with nine treatments and three replications as explained in previous polyhouse experiment.

3.7.1 Effect of fungal colonisation on growth parameters of cowpea under field condition

The effect of the fungal colonisation on plant growth under field condition was studied by measuring important growth parameters as explained in 3.6.1.4.

3.7.2 Effect of fungal colonisation on yield parameters under field condition

Pods from individual plants were picked as and when matured, weighed and mean green pod yield and marketable pod yield was expressed in g plant^{-1}.

3.7.3 Effect of fungal colonisation on infestation by spotted pod borer

The pods were harvested and presence of bore holes and larval frass on pods were used as an indication of pod borer damage. Based on this, the harvested pods were sorted and observations were made on number of pod borer infested and uninfested pods. Dropped flowers were collected from the field, sorted and observations were made on pod borer infested and uninfested flowers.

The number of total and infested pods and flowers per plant was recorded and per cent infestation was worked out using the following formula,

$$\text{Per cent infestation on pods/flowers} = \frac{\text{No. of damaged pods/flowers}}{\text{Total no. of pods/flowers produced}} \times 100$$

Data on growth, yield and per cent infestation by spotted pod borer were analysed by Analysis of Variance and the means were separated by DMRT.

3.8 EFFECT OF ENDOPHYTIC ENTOMOPATHOGENIC FUNGI ON INFESTATION BY SPOTTED POD BORER

A pot culture experiment was conducted to compare the efficacy of selected endophytic entomopathogenic fungi with a diamide insecticide *viz.*, flubendiamide against spotted pod borer on cowpea. The experiment was laid out in a completely randomized design with three treatments and seven replications at Krishi Vigyan Kendra, Kerala Agricultural University, Thrissur using the variety Anaswara (Plate 23).

The details of treatments are as follows,

T_1 : Foliar application of *Beauveria bassiana*

T_2 : Flubendiamide 20 WG @ 50 g a.i. ha^{-1}

T_3 : Untreated check

Foliar inoculation of *B. bassiana* was done at 14 days after sowing. Flubendiamide was applied when pod borer incidence was above economic threshold level. Two sprays were given at fortnightly interval.

3.8.1 Effect of endophytic entomopathogenic fungi on infestation by spotted pod borer

Observations such as total number of flowers, number of infested flowers, total number of pods ands number of infested pods were recorded. Mean per cent pod and flower infestation was worked out using the recorded data as explained in the previous experiment.

3.8.2 Effect of endophytic entomopathogenic fungi on yield of cowpea

The effect of endophytic entomopathogenic fungi on yield parameters such as total weight of pods and weight of marketable pods was observed.

Plate 23. Field view of the second experimental plot

The recorded data were subjected to analysis of variance (ANOVA) and the analysed data were averaged and comparison between the treatment means was performed with Duncan's Multiple Range Test (DMRT).

Results

4. RESULTS

The present study entitled "Endophytic fungi for the management of spotted pod borer, *Maruca vitrata* Fab. (Lepidoptera: Crambidae) in cowpea" was carried out in the Department of Agricultural Entomology, College of Agriculture, Vellanikkara, Thrissur, Kerala during 2016-2019. The experimental results obtained during the investigation are presented below**.**

4.1 ISOLATION OF ENDOPHYTIC FUNGI FROM COWPEA PLANTS

A sampling survey was conducted in different districts to isolate fungal endophytes from cowpea plants. Isolation of endophytic fungi from different cultivars grown in pots was also attempted. The results of isolation are given below.

4.1.1 Survey and isolation of endophytes from different geographical areas of the state

Purposive sampling surveys were carried out in the major cowpea growing areas of Kozhikode, Thrissur, Kottayam and Thiruvananthapuram districts of Kerala. Plant samples were collected from ten organically managed plots in each district at reproductive stage of the crop for isolation of endophytic fungi. Fungal endophytes were isolated from the plant samples as per standard protocol (Plate 24, 25 and Tables 5 to 9).

4.1.1.1 Survey in Kozhikode district

A total of 60 endophytic fungi were isolated from plant samples collected from Kozhikode district. Out of these, 32 were from roots, 12 from stems, eight from leaves and eight from pods. Highest extent of 53.34 per cent colonisation was observed in roots, followed by stems, leaves and pods with 20.0 and 13.33 per cent respectively. No fungal isolates were obtained from flower samples (Table 5).

Table 5. Details of fungal endophytes obtained from Kozhikode district

Location code*	Plant part					Number of isolates
	Root	Stem	Leaf	Flower	Pod	
KZD1	1	1	-	-		2
KZD2	1	1	1	-		3
KZD3	3	1		-	1	5
KZD4	1	2	1	-		4
KZD5	2	3		-	3	8
KZD6	11		2	-	3	16
KZD7	2			-	1	3
KZD8	8	3	2	-		13
KZD9	-	-	1	-		1
KZD10	3	1	1	-		5
Total	32	12	8	-	8	60
Proportion (%)	53.34	20.00	13.33	-	13.33	

*Location codes are given in Table 1

Table 6. Details of fungal isolates obtained from Thrissur district

Location code*	Plant part					Number of isolates
	Root	Stem	Leaf	Flower	Pod	
TSR1	3	1	1		1	6
TSR2	3				1	4
TSR3	2			2		4
TSR4			1		3	4
TSR5	5	3			6	14
TSR6	2	3	1			6
TSR7	2	1	3		2	8
TSR8	8	3	2		1	14
TSR9	5	7	1			13
TSR10	6	10	1	2	3	22
Total	36	28	10	4	17	95
Proportion (%)	37.89	29.47	10.52	4.22	17.90	

*Location codes are given in Table 1

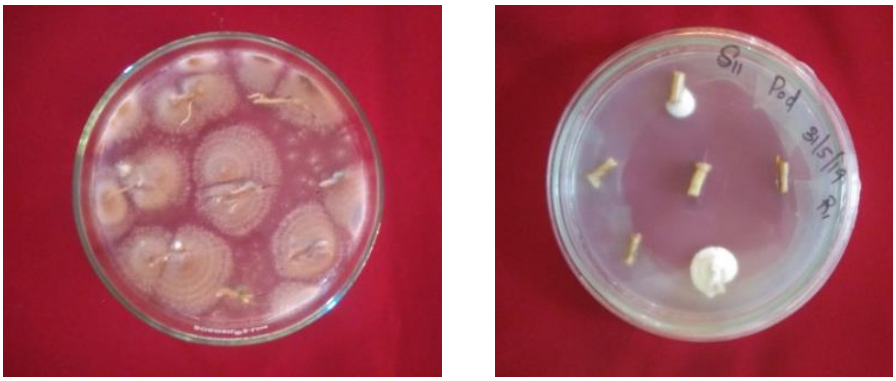


Plate 24. Growth of fungal endophytes from the cut end of roots onto the media

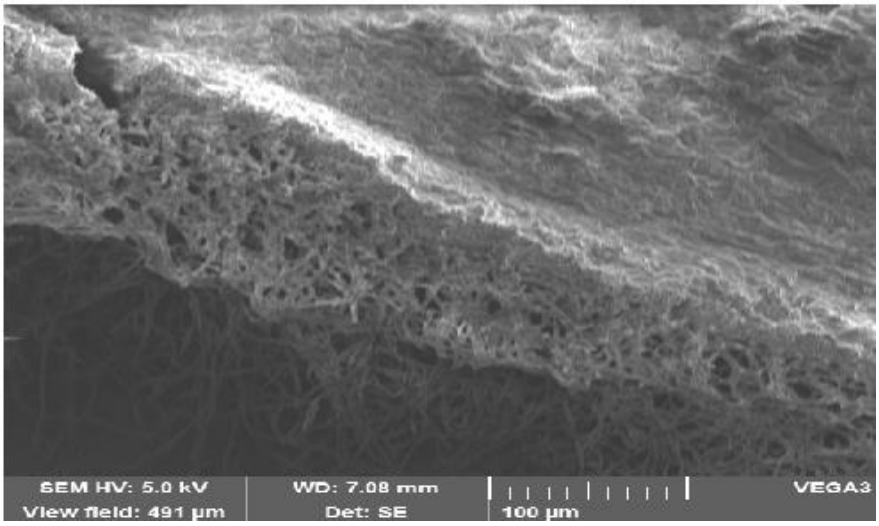


Plate 25. SEM image of ensuing endophytes from cut end of leaf

4.1.1.2 Survey in Thrissur district

Survey in Thrissur district (Table 6) yielded 95 endophytes out of which 36 isolates (37.89%) were obtained from roots while 28 (29.47%) were from stems. Pods, leaves and flowers yielded 17, 10 and 4 isolates each, equivalent to 17.90, 10.52 and 4.22 per cent respectively.

4.1.1.3 Survey in Kottayam district

A total of 57 fungal endophytes were collected during the survey in Kottayam district (Table 7). As in case of Kozhikode and Thrissur districts, the highest number of 24 isolates (42.11%) was obtained from roots. The number of isolates from stems, leaves and pods were 16, 10 and 7 representing 28.07, 17.54 and 12.28 per cent respectively. No isolates were obtained from flowers.

4.1.1.4 Survey in Thiruvananthapuram district

Endophytic fungal isolates obtained from ten different locations in Thiruvananthapuram district is illustrated in Table 8. Root endophytes at 47.82 per cent once again were the most encountered among the 23 isolates. This was followed by seven isolates from stems (30.43%) and three isolates from leaves (13.05%). Only one endophyte each was isolated from pod and flower samples.

A total of 235 endophytic fungal isolates were obtained from the plant samples collected from 40 locations. This comprised of 103 isolates from roots, 63 from stems, 31 from leaves, 33 from pods and five from flowers (Table 9). The highest occurrence of fungal endophytes, at 43.83 per cent was in roots, followed by stems with 26.81 per cent. Among the four districts, the highest number of 95 isolates was obtained from Thrissur district, followed by 60 from Kozhikode, 57 from Kottayam district and 23 from Thiruvananthapuram district.

Table 7. Details of fungal isolates obtained from Kottayam district

Location code*	Plant part					Number of isolates
	Root	Stem	Leaf	Flower	Pod	
KTM1	2	1	2		1	6
KTM2	1				1	2
KTM3	1		1			2
KTM4	1				1	2
KTM5		1			1	2
KTM6	8	5	1		2	16
KTM7			2		1	3
KTM8	4	7	3			14
KTM9	4	1	1			6
KT1M0	3	1				4
Total	24	16	10		7	57
Proportion (%)	42.11	28.07	17.54	-	12.28	

*Location codes are given in Table 1

Table 8. Details of fungal isolates obtained from Thiruvananthapuram district

Location code*	Plant part					Number of isolates
	Root	Stem	Leaf	Flower	Pod	
TVM1	1	1	1			3
TVM2	1	1				2
TVM3	1	1	1			3
TVM4	3			1		4
TVM5		2				2
TVM6	2					2
TVM7		1	1			2
TVM8	1	1				2
TVM9	1				1	2
TVM10	1					1
Total	11	7	3	1	1	23
Proportion (%)	47.82	30.43	13.05	4.35	4.35	

*Location codes are given in Table 1

Table 9. Details of fungal isolates obtained from different locations

Districts	Plant parts					Number of isolates
	Root	Stem	Leaf	Flower	Pod	
Kozhikode	32	12	8		8	60
Thrissur	36	28	10	4	17	95
Kottayam	24	16	10		7	57
Thiruvananthapuram	11	7	3	1	1	23
Total	103	63	31	5	33	235
Proportion (%)	43.83	26.81	13.19	2.13	14.04	

Table 10. Details of fungal isolates from different accessions of cowpea

Accessions	Endophytic fungal isolates from different plant parts					Number of isolates
	Root	Stem	Leaf	Flower	Pod	
Hridya			2		2	4
Palakkadan thandan payar		2		3	2	7
Kanakamony	1				2	3
Sreya	1		2			3
KBC2		2	1			3
Mysore local				1		1
Bhagyalakshmi	2			1	2	5
Anaswara		1	3	1		5
Lola			1			1
Total	4	5	9	6	8	32
Proportion (%)	12.50	15.63	28.12	18.75	25.00	

4.1.2 Isolation of endophytic fungi from different accessions of cowpea

Nine accessions of cowpea with different levels of resistance were screened for colonisation by entomopathogenic endophytes. All the nine accessions were found to harbor endophytic fungi. Among the nine accessions, Palakkadan thandan payar yielded the highest number of seven endophytic fungi, followed by Lola as well as Mysore local yielded a single endophytic fungal isolate each. Bhagyalakshmi and Anaswara harbored five isolates each, while Hridya was colonised by four isolates. Kanakamony, Sreya and KBC 2 were colonised by three endophytes each.

The occurrence of the endophytes varied in different host tissues. In contrast to the results of survey, the leaves of cowpea plant harbored nine endophytic fungi, while pods, stems, flowers and roots had eight, six, five and four number of endophytes respectively (Table 10).

4.2 EVALUATING THE BIOEFFICACY OF ENDOPHYTIC FUNGAL ISOLATES

The 267endophytic fungal isolates from cowpea plants were subjected to preliminary screening in the laboratory to assess their pathogenicity against insects, as described previously. Endophytic isolates identified as pathogenic to the test insect, *Galleria mellonella* were further screened against the target pest, *M. vitrata.*

4.2.1 Preliminary bioassay using larvae of greater wax moth, *Galleria mellonella*

The preliminary bioassay was carried out using 0-24 h old third instar larvae of *G. mellonella*. Dead larvae were removed from the Petri plate daily and kept in humid chamber for external growth of fungi. The entomopathogens were reisolated from the infected cadavers and compared with the original isolate of the fungus. Three isolates *viz*., EEF 1, EEF 4 and EEF 64 were found to be pathogenic to larvae of *G. mellonella* and selected for carrying out bioassay against the target pest, *M. vitrata*.

4.2.2. Bioassay against spotted pod borer, *Maruca vitrata*

After preliminary bioassay, virulence bioassay was carried out in the laboratory to confirm the pathogenicity of selected fungal endophytic isolates on the target pest, *M. vitrata*. All the three isolates (EEF 1, EEF 4 and EEF 64) were found to be pathogenic to the target pest, *M. vitrata*. These isolates were re-isolated from the infected cadavers of spotted pod borer (Plate 26) and compared with that of original isolates. Pathogenicity of all the isolates was established by proving the Koch's postulate. The entomopathogenic isolates were subjected to morphological and molecular characterization to confirm the identity.

Plate 26a. Growth of *Fusarium oxysporum* (EEF 1) from infected cadaver

Plate 26b. Growth of *Purpureocillium lilacinum* (EEF 4) from infected cadaver

Plate 26c. Growth of *Purpureocillium lilacinum* (EEF 64) from infected cadaver

Plate 26.Growth of fungal isolates from cadavers on media (upper and lower view of plates)

4.3 CHARACTERIZATION OF ENDOPHYTIC ENTOMOPATHOGENIC FUNGAL ISOLATES

The three out of the 267 isolates that had caused mortality to target pest, *M. vitrata* were subjected to further morphological and molecular characterization.

4.3.1 Morphological and cultural characterization

The morphological and cultural characters of potential EEF were studied and are presented in Table 11, and Plates 27 and 28. The identity of the three isolates was confirmed as *Fusarium oxysporum* (EEF1) and *Purpureocillium lilacinum* (EEF 4 and EEF 64) with the aid of standard descriptors. The cultural and morphological characters of fungal isolates are detailed hereunder.

4.3.1.1 *Fusarium oxysporum*

An endophytic entomopathogenic fungal isolate (EEF 1) identified as *F. oxysporum* was obtained from fresh cowpea pod during the survey in Kollappalli of Kottayam district (Table 11 and 12).

Colony characters

The colony of *F. oxysporum* (EEF 1) was off white in colour during initial growth, which later changed to peach with a purple tinge. Growth of the fungus was fast and complete mycelial growth in PDA plate was attained by seventh day. Fungus had floccose outgrowth and reverse side of plate was buff (Plate 27a).

Micro morphology

Fusarium oxysporum (EEF 1) produced two types of conida *ie*., microconidia and macroconidia. Microconidia were 0-1 septate, oval or cylindrical in shape and smooth walled. Size of the microconidia varied from 4.0-6.1 x 2.1-3.41 µm with average size of

5.48 x 2.99 µm. Macroconidia were 3-5 septate, fusoid to subulate and pointed at both ends. Macroconidia had mean dimensions of 25.81 x 3.37 µm within a range of 24.25-27.22 x 3.22-3.54 µm.

Hyphae were branched to unbranched, smooth walled and guttulate. Phialides were simple to polyphialidic and varied in shape and size (Plate 28a). EEF 1 was identified as *F. oxysporum* based on the descriptions made by Booth (1971).

4.3.1.2 *Purpureocillium lilacinum*

Two isolates of *Purpureocillium lilacinum* (EEF 4 and EEF 64) were obtained during the surveys. While isolate EEF 4 was obtained from cowpea stem at Marangattupalli in Kottayam district, EEF 64 was isolated from root of cowpea at Kunnamangalam in Kozhikode district (Table 11 and 12).

Colony characters

Colonies of EEF 4 showed moderate growth on PDA medium. Colonies had lilac shade (Plate 27b). Colony characters of EEF 64 were similar to that of EEF 4 (Plate 27c).

Micro morphology

Conidiophores of EEF 4 were rough walled, erect and mostly arising from the horizontal mycelium. Stalks were 3-4 µm wide with densely clustered metulae and phialides. Phialides consisted of a swollen basal part tapering into a thin distinct neck. Spores were formed from the ends of phialides in long chains. Spores were ellipsoidal to fusiform and smooth walled to slightly roughened. Size of conidia ranged from 2.5–3 × 2.0-2.2 µm with an average of 2.76 x 2.11 µm. Chlamydopsores were absent (Plate 28b).

Conidial characters of EEF 64 were similar to that of EEF 4. Size of conidia of isolate, EEF 64 ranged between 2.5–3.1 × 2.0-2.3 µm. Average size of conidium of EEF

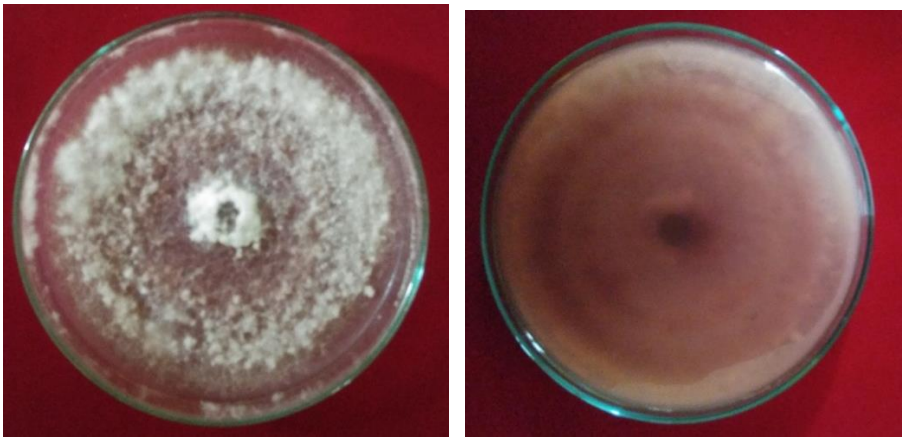


Plate 27a. Cultural characters of *Fusarium oxysporum* (EEF- 1)

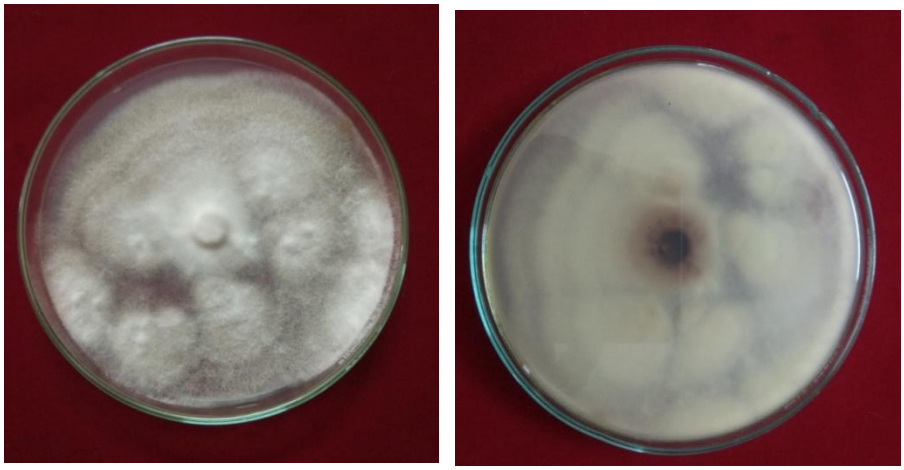


Plate 27b. Cultural characters of *Purpureocillium lilacinum* (EEF- 4)

Plate 27c. Cultural characters of *Purpureocillium lilacinum* (EEF- 64)

Plate 27. Cultural characters of endophytic entomopathogenic fungal isolates

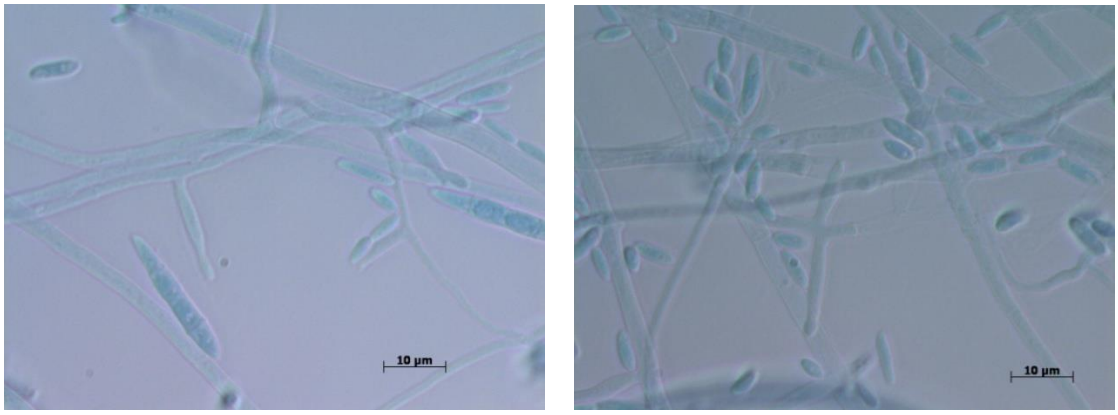


Plate 28a. Macro and microconidia of *Fusarium oxysporum* (EEF 1) (1000 X)

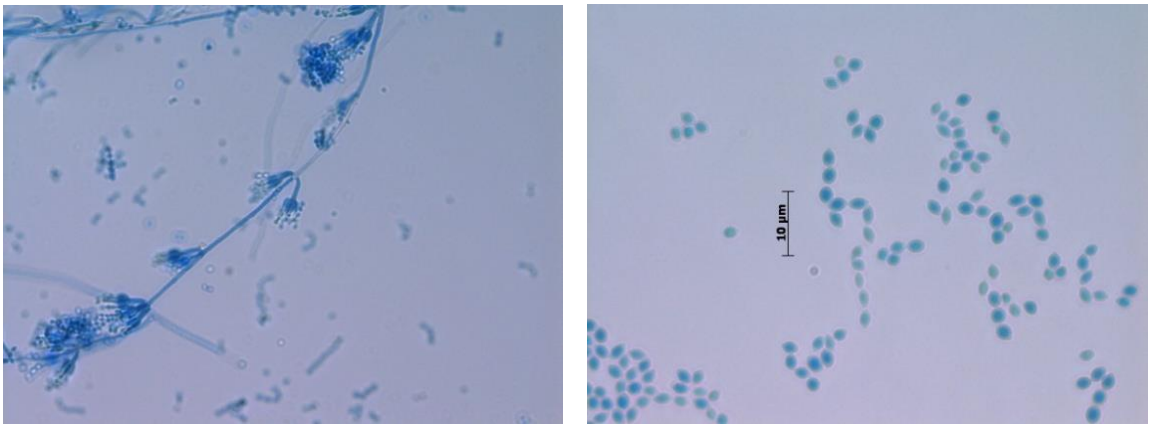


Plate 28b. *Purpureocillium lilacinum* (EEF 4) (400 X and 1000 X)

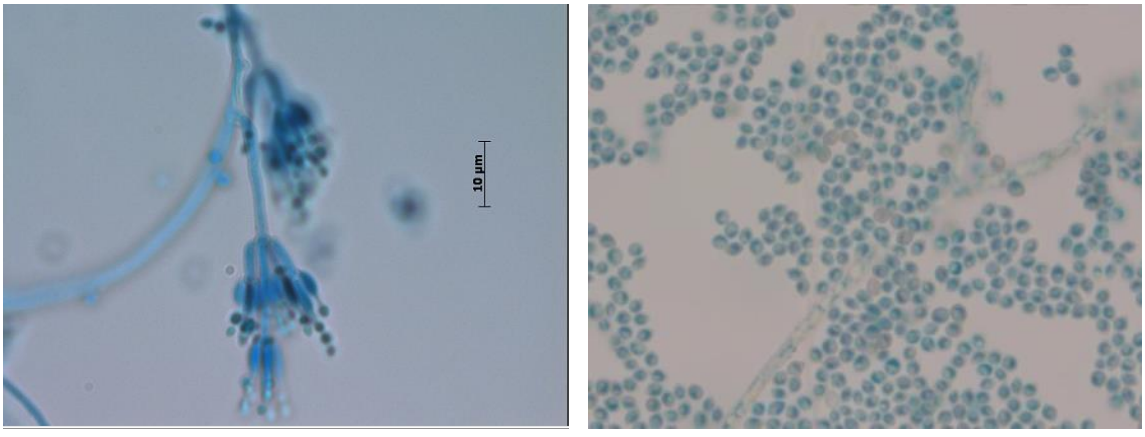


Plate 28c. *Purpureocillium lilacinum* (EEF 64) (1000 X)

Plate 28. Photomicrographs of endophytic fungal isolates

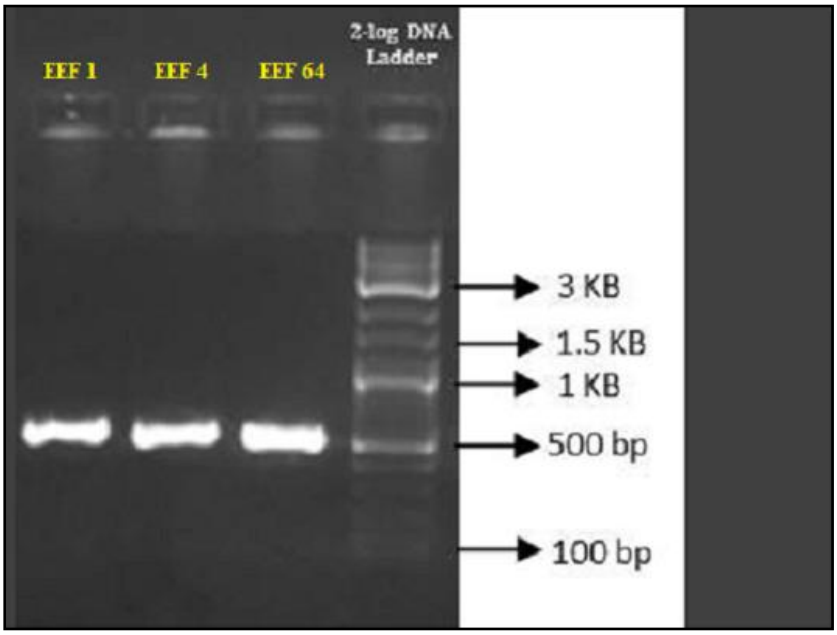


Fig. 6. Gel profile of endophytic entomopathogenic fungal isolates (Lane 1: EEF 1; Lane 2: EEF 4; Lane 3: EEF 64; Lane 4: 3 Kb ladder)

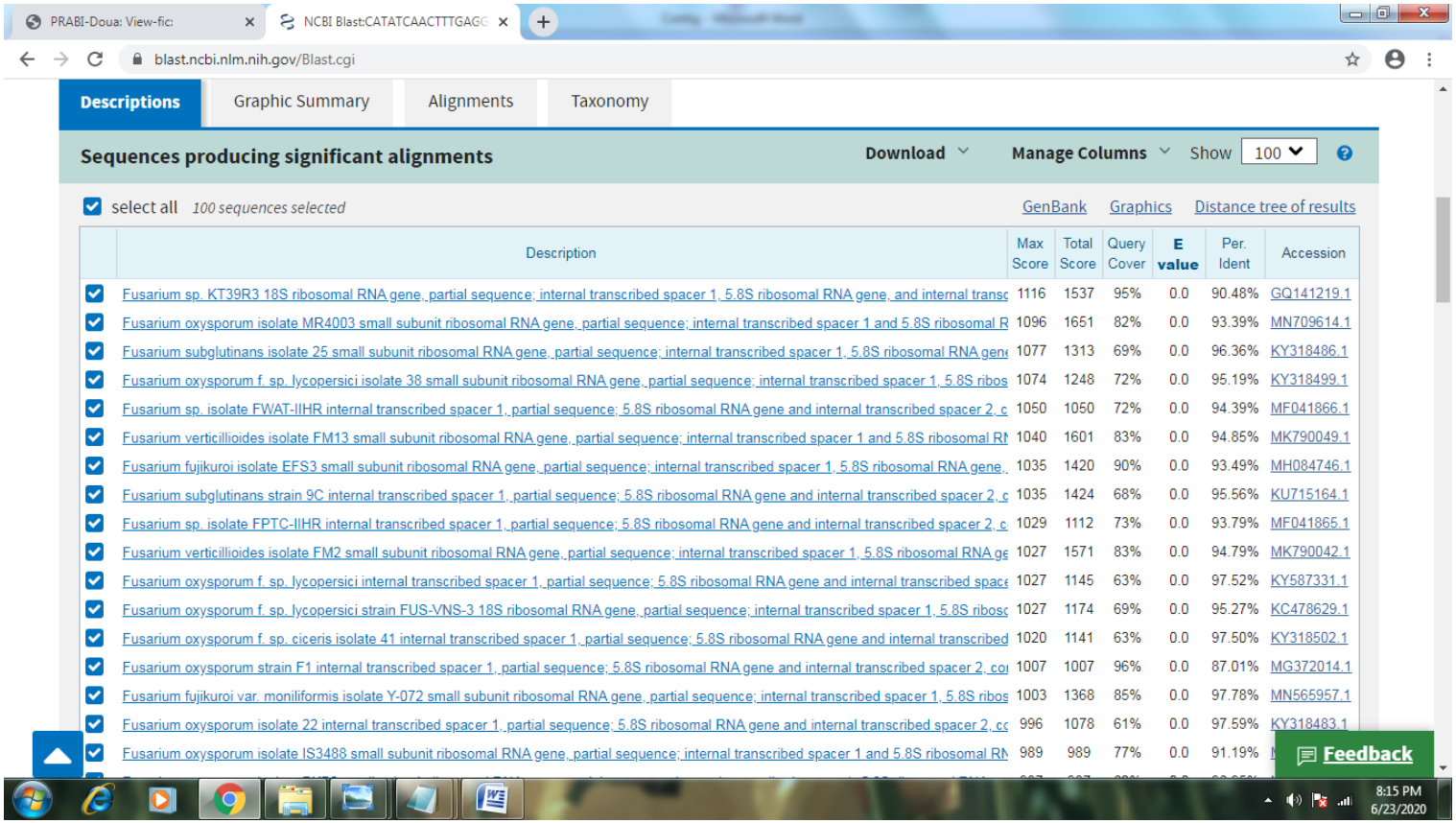


Fig. 7. BLASTn analysis of isolate of *Fusarium oxysporum* (EEF 1)

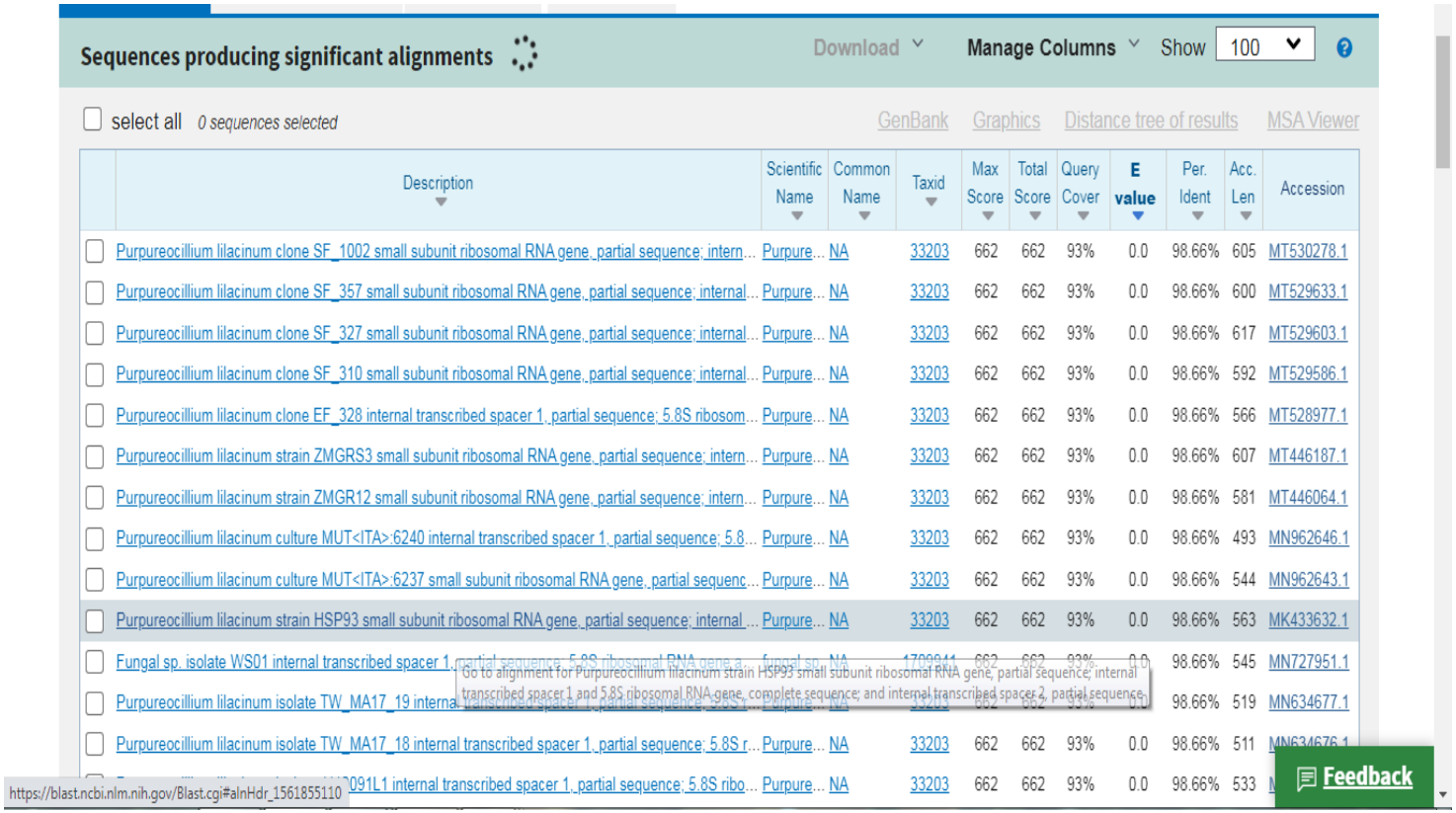


Fig. 8. BLASTn analysis of isolate of *Purpureocillium lilacinum* (EEF 4)

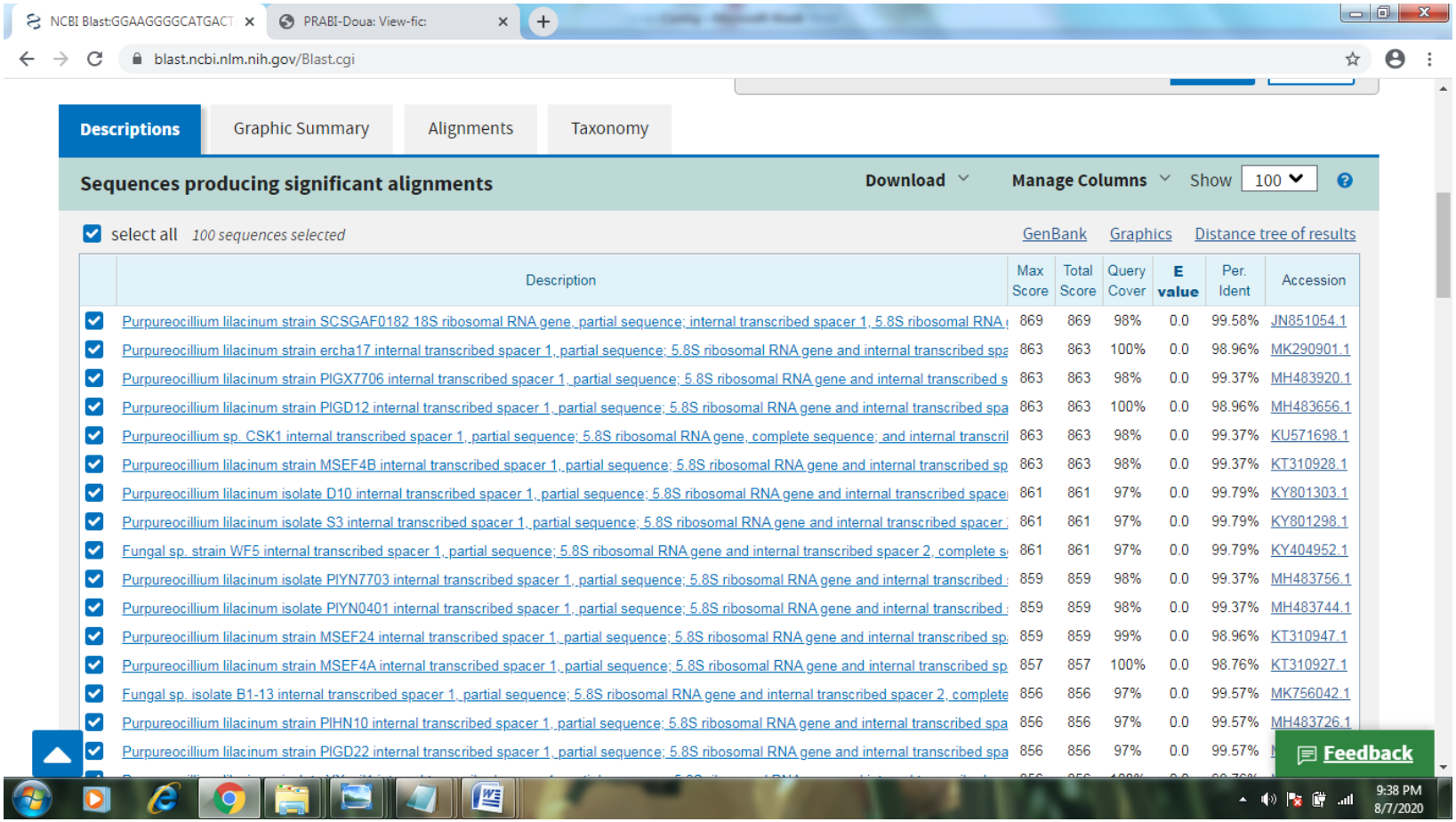


Fig. 9. BLASTn analysis of isolate of *Purpureocillium lilacinum* (EEF 64)

64 was 2.79 x 2.20 µm (Plate 28c). Two isolates of *P. lilacinum* were identified based on the descriptions made by Luangsa-Ard *et al.* (2011).

4.3.2 Molecular characterization and phylogenetic analysis

The endophytic entomopathogenic fungal isolates from cowpea plants were subjected to molecular characterization and phylogenetic analysis to confirm the identity of isolates.

4.3.2.1 Molecular characterization of fungal isolates

The sequences of the three endophytic entomopathogenic fungal isolates were analysed in BLASTn programme of NCBI to know the nucleotide homology of each fungal isolate (Fig. 6, 7, 8 and 9). After BLASTn analysis, the sequences of all isolates were submitted at NCBI Bankit and accession numbers were obtained to all isolates (Table 12). The results of BLASTn analysis and Bankit submission are detailed hereunder.

Table 11. Cultural and morphological characteristics of endophytic entomopathogenic fungal isolates

Sl. No.	Isolates	Macromorphology on PDA medium at 28ºC	Micromorphology		
			Shape and size of conidia	Arrangement of conidiophores and conidia	Size of conidia (µm)*
1.	EEF 1	White or peach with a purple tinge, floccose, fast growing and reverse buff.	Microconidia:0-1 septate, abundant, oval shaped or cylindrical, variable in size, straight to curved, smooth walled. Size: 4.0-6.1 x 2.1-3.41 µm Macroconidia: 3-5 septate, fusoid to subulate and pointed at both ends. Size: 24.25-27.22 x 3.22-3.54 µm.	Hyphae branched to unbranched, smooth walled, guttulate. Phialides simple to polyphialidic, variable in shape and size.	Macroconidia – 25.81 x 3.37 Microconidia – 5.48 x 2.99
2.	EEF 4	Moderate growth, lilac shaded and reverse vinaceous.	Ellipsoidal to fusiform, smooth walled to slightly roughened, 2.5–3 × 2.0-2.2µm Chlamydospore absent and conidia lilac coloured.	Conidiophores erect, mostly arising from the horizontal mycelium, rough walled, and stalks 3-4 µm wide, with densely clustered metulae and phialides. Phialides consist of a swollen basal part, tapering into a thin distinct neck. Spores are formed from the ends of phialides in long chains.	2.76 x 2.11
3.	EEF 64	Vinaceous shade and reverse vinaceous with moderate growth.	Ellipsoidal to fusiform, smooth walled to slightly roughened, 2.5–3.1 × 2.0-2.3 µm Chlamydospore absent.Conidialilac coloured.	Conidiophores erect, arising from the horizontal mycelium, rough walled and stalks 3-4 µm wide, with densely clustered metulae and phialides. Phialide and spore characters same as that of EEF 4.	2.79 x 2.20

*Mean of 30 observations

Table 12. Endophytic entomopathogenic fungal isolates: locations and accession numbers

Sl. No.	Culture code	Organism	Location and GPS co-ordinates	Plant part	GenBank accession number
1	EEF 1	*Fusarium oxysporum*	Kollappalli, Kottayam (9º76'47.2"N, 76º70'12.3"E)	Pod	MW221822
2	EEF 4	*Purpureocillium lilacinum*	Marangattupalli, Kottayam (9º44'53.6"N, 76º36'32.4"E)	Stem	MZ223467
3	EEF 64	*Purpureocillium lilacinum*	Kunnamangalam, Kozhikode (11º18'10.2"N, 75º52'39.4"E)	Root	MZ223384

4.3.2.1.1 Molecular characterization of *Fusarium oxysporum* (EEF 1)

The ITS sequencing of the isolate yielded a 553 base pair (bp) sequence as given below.

CTTCCGTAGGTGAACCTGCGGAGGGATCATTACCGAGTTTACAACTCCCAAACCCCTGTG
AACATACCACTTGTTGCCTCGGCGGATCAGCCCGCTCCCGGTAAAACGGGACGGCCCGCC
AGAGGACCCCTAAACTCTGTTTCTATATGTAACTTCTGAGTAAAACCATAAATAAATCAA
AACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATA
AGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGC
CAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCACAGCTTGGTG
TTGGGACTCGCGTTAATTCGCGTTCCCCAAATTGATTGGCGGTCACGTCGAGCTTCCATA
GCGTAGTAGTAAAACCCTCGTTACTGGTAATCGTCGCGGCCACGCCGTTAAACCCCAACT
TCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGC
GGAGGAA

BLASTn analysis showed that nucleotide sequence of the isolate EEF 1 had 90.48 per cent identity with 95.0 per cent query coverage to the sequences of *Fusarium* sp. KT39R3 (GQ141219). It showed 93.39 per cent identity with 82.0 per cent query coverage to the sequences of *Fusarium* isolate MR4003 (MN709614) (Fig. 7). Accession number obtained for isolate EEF 1 after NCBI Bankit submission was MW221822 (Table 12).

4.3.2.1.2 Molecular characterization of isolates of *Purpureocillium lilacinum*

The ITS sequencing of the isolate EEF 4 yielded a 454 base pair (bp) sequence as given below.

CGAGGTGTGTGCTACTACGCAGGGGAGGCTGCGGCGGGGTCGCCACTGCATTTCGGGGGC
GGCTGGTGTGCCGTCCCCCAACACCGAGGCCCCCGGGGGGGCTCGAGGGTTGAAATGACG
CTCGAACAGGCATGCCCGCCAGAATGCTGGCGGGCGCAATGTGCGTTCAAAGATTCAATG
ATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCA
GAACCAAAAGATCCGTTGTTGAAAGTTTTGATTCATTTGTTTTTGCTTGTGCAACTCAGAGA
AGAAATTCCGCCCGATGGGCGTAATGCAAGAGAGTTTGGGGTCCCTGCGGCGGGCGCCTG

GGTCCGGCGCCGGCGCGGGGGCCGGCGGCCGGGGCGTTCCCGCCGAGTCAACTGAGGTAA
GTTCACAGTTGCACGTGAGTTTTTTCCTCCCCA

Nucleotide sequences captured from NCBI database compared to the isolate of *P. lilacinum* (EEF 4) showed 98.66 per cent identity with 93.00 per cent query coverage to the sequences of *P. lilacinum* clone SF_1002 (MT530278) (Fig. 8). Accession number obtained for isolate EEF 4 was MZ223467 (Table 12).

The ITS sequencing of the isolate EEF 64 yielded a 550 base pair (bp) sequence as given below.

GGAAGGGGCATGACTACATGATCCGAGGTCAACTCTAAGAAAGTTGGGCGTTTTACGGCG
GGGCATCGGAGGTACACTCCCAACCCACATGTGACCTTACCTCAGTTGCCTCGGCGGGAA
CGCCCCGGCCGCCGGCCCCCGCGCCGGCGCCGGACCCAGGCGCCCGCCGCAGGGACCCCA
AACTCTCTTGCATTACGCCCAGCGGGCGGAATTTCTTCTCTGAGTTGCACAAGCAAAAAC
AAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGC
GAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAC
ATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGAGC
CCCCCCGGGGGCCTCGGTGTTGGGGGACGGCACACCAGCCGCCCCCGAAATGCAGTGGCG
ACCCCGCCGCAGCCTCCCCTGCGTAGTAGCACACACCTCGCACCGGAGCGCGGAGGCGGT
CA

Nucleotide sequences obtained from NCBI database after BLASTn were compared to the sequence of *P. lilacinum* isolate (EEF 64). EEF 64 showed 99.58 per cent homology with 98.00 per cent query coverage to the sequences of *P. lilacinum* strain SCSGAF0182 (JN851054). It showed 98.86 per cent identity with 100.00 per cent query coverage to the sequences of *P. lilacinum* strain ercha 17 (MK290901) (Fig. 9). Accession number obtained for isolate EEF 64 was MZ223384 (Table 12).

4.3.2.2 Phylogenetic analysis

Phylogenetic analysis was carried out to confirm the molecular identity of isolates. Phylogenetic analysis involved nucleotide sequences of *F. oxysporum* (EEF 1 –

MW221822) and *P. lilacinum* (EEF 4 – MZ223467 & EEF 64 – MZ223384) in the study and nucleotide sequences of *F. oxysporum* (MN709614), *P. lilacinum* (JN851054), *L. fusisporum* (MH859538 and MW303984), *Xylaria hypoxylon* (AY327489) and *Haplocilium sinense* (EF469022) available in the NCBI database. The phylogenetic tree was built from original dataset with the aid of Neighbor-Joining method. Grouping of sequences was supported by a bootsrap value of 1000. The Phylogram consisted of two clusters where *Haplocilium sinense* belonging to one cluster and others in cluster 2 (Fig. 10).

The phylogenetic analysis of the ITS gene region of *P. lilacinum* and *F. oxysporum* was congruent with the taxonomy of the same group, showing the relationship among species as reported in previous works (Forsyth *et al*., 2006; Luangsa-Ard *et al*., 2011).

4.4 PLANT PATHOGENICITY TEST

Fungal isolates were subjected to plant pathogenicity test on cowpea variety, Anaswara. Foliar application, agar block and soil inoculation methods were followed for the test. Plants were observed for the development of disease symptoms (Table 13). None of the treatments including *F. oxyporum* treated plants showed any symptoms of fungal infection, with the treated plants surviving up to three months after inoculation without any symptoms. However, *F. oxysporum* was not proceeded further, as the fungus was a potential plant pathogen as well.

4.5 EVALUATION OF BIOEFFICACY OF ENTOMOPATHOGENIC ENDOPHYTIC FUNGAL ISOLATES

The two endophytic entomopathogenic isolates of *P. lilacinum* obtained in the previous experiment were evaluated along with NBAIR culture of *Beauveria bassiana* (NBAII Bb 5a) for their bioefficacy against second instar larvae of *M. vitrata* through contact toxicity bioassay. Spore suspensions of five different concentrations *viz*., 10^5, 10^6,

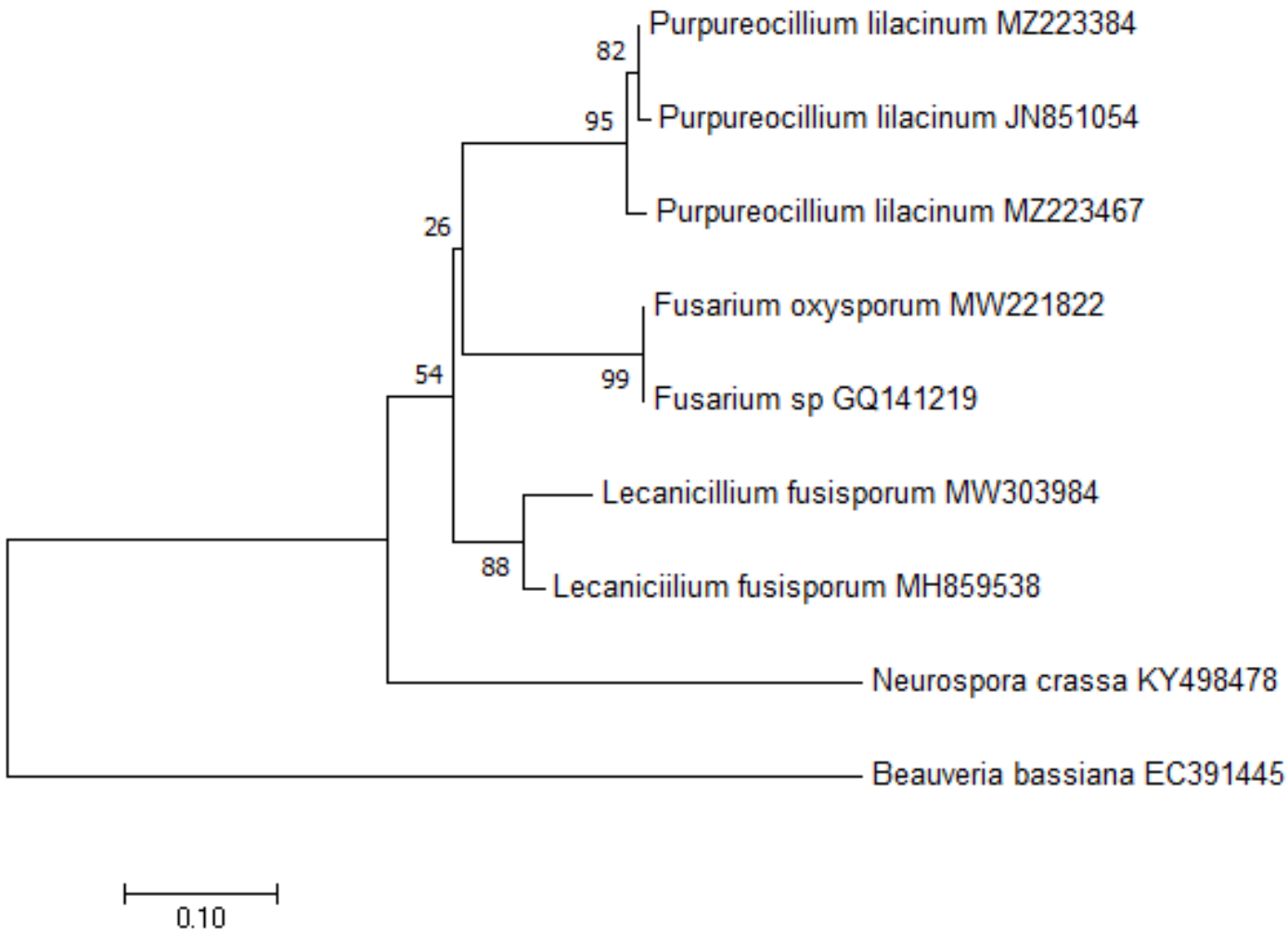


Fig. 10. Phylogram for isolates of endophytic entomopathogenic fungi

10^7, 10^8 and 10^9 spores ml^{-1} were used for the bioefficacy test. From the data collected, per cent mortality of larvae was calculated and was corrected using Abbott's formula (Table 14).

Two days after treatment, *B. bassiana* NBAIR strain, applied at the rate of 10^9 spores ml^{-1} recorded the highest mortality of 20.0 per cent and was on par with the same fungus applied at 10^8 spores ml^{-1}, with 16.67 per cent mortality. The above two treatments were significantly superior to all other treatments.

The isolate EEF 4 recorded the highest mortality of 10.00 per cent at a spore concentration of 10^9 spores ml^{-1}. EEF 64 failed to cause any mortality at lower concentrations and registered mortality of 3.33 per cent at the highest concentration of 10^9 spores ml^{-1}.

Beauveria bassiana (NBAIR strain) applied at the rate of 10^9 spores ml^{-1} again induced the highest mortality of 50.00 per cent four days after treatment, which was significantly superior to the remaining treatments. The above fungus, applied at the rate of 10^5, 10^6, 10^7 and 10^8 spores ml^{-1} recorded 6.67, 16.67, 33.33 and 36.67 per cent mortality respectively for the corresponding period.

Purpureocillium lilacinum (EEF 4) applied at the rate of 10^5, 10^6, 10^7, 10^8 and 10^9 resulted in 6.67, 10.00, 10.00, 13.33 and 26.67 per cent mortality respectively, while the corresponding values for *P. liacinum* (EEF 64) were 3.33, 6.67, 10.00, 16.67 and 26.67 per cent.

Beauveria bassiana (NBAIR strain) applied at the rate of 10^9 spores ml^{-1} once again recorded the highest mortality of 66.67 per cent six days after application, which was on par with the mortality values of 53.33 per cent induced by the fungus applied at the rate of 10^8 and 10^7 spores ml^{-1}, as well as the 50.00 per cent mortality by *P. lilacinum* (EEF 4) applied at the rate of 10^9 spores ml^{-1}. *P. lilacinum* (EEF 4) recorded 10.00 to 50.00 per cent mortality at concentrations ranging from 10^5 to 10^9 spores ml^{-1} whereas *P.*

lilacinum (EEF 64) registered mortality ranging from 6.67 to 43.33 per cent at different concentrations.

Eight days after treatment, application of *B. bassiana* (NBAIR strain) at the rate of 10^5, 10^6, 10^7, 10^8 and 10^9spores ml^{-1} resulted in 23.47, 40.23, 53.67, 57.04 and 67.14 per cent mortality respectively. *B. bassiana* (NBAIR strain) applied at the rate of 10^9 spores ml^{-1} was on par with the same strain applied at the rate of 10^8 spores ml^{-1}. *B. bassiana* applied at 10^7 spores ml^{-1} with mortality of 53.67 per cent, was on par with *P. lilacinum* EEF 4 and EEF 64 both applied at the rate of 10^9 spores ml^{-1} with 50.33 and 47.00 per cent mortality respectively. All other treatments resulted in significantly lower mortality values.

Ten days after treatment, *B. bassiana* (NBAIR strain) applied at the rate of 10^5, 10^6, 10^7, 10^8 and 10^9spores ml^{-1} registered 23.58, 43.77, 57.23, 63.98 and 70.71 per cent mortality. *P. lilacinum* (EEF 4) induced 10.07, 20.20, 43.77, 50.50 and 57.23 per cent mortality, while the isolate (EEF 64) recorded 10.10, 16.83, 33.67, 47.13 and 50.50 per cent mortality at 10^5, 10^6, 10^7, 10^8 and 10^9 spores ml^{-1} respectively. *B. bassiana* (NBAIR strain) applied at the rate of 10^8 and 10^9 spores ml^{-1} were significantly superior to all other treatments.

4.6 STANDARDISATION OF THE INOCULATION TECHNIQUE FOR ENDOPHYTIC COLONISATION

The best two organisms in the bioefficacy test *viz*., *B. bassiana* (NBAIR strain) and *P. lilacinum* (EEF 4) were used for standardizing the inoculation technique for endophytic colonisation in cowpea plant. Three different methods of inoculation *viz*., seed, soil and foliar inoculation were evaluated for identifying the best method for colonisation of entomopathogenic fungi in cowpea plants. Colonisation was confirmed by two detection methods *viz*., re-isolation and molecular technique.

Table 13. Plant pathogenic test of fungal isolates by different inoculation methods

Fungal isolates	Foliar application method	Agar block method	Soil inoculation method
	*Disease symptoms	*Disease symptoms	*Disease symptoms
Fusarium oxysporum (EEF 1)	-	-	-
Purpureocillium lilacinum (EEF 4)	-	-	-
Purpureocillium lilacinum (EEF 64)	-	-	-
Untreated control	-	-	-

*Presence of disease symptoms (+), Absence of disease symptoms (-)

Table 14. Bioefficacy of fungal isolates against second instar larvae of *Maruca vitrata*

Fungal isolates	Dose (Spores ml^{-1})	Mortality of insects (%)				
		2^{nd} day	4^{th} day	6^{th} day	8^{th} day	10^{th} day
B. bassiana (NBAIR strain)	1×10^5	0.00^{d}	6.67^{de}	23.33^{ghi}	23.47^{ghi}	23.58^{f}
	1×10^6	6.67^{bc}	16.67^{cd}	36.67^{def}	40.23^{def}	43.77^{d}
	1×10^7	10.00^{b}	33.33^{b}	53.33^{b}	53.67^{bc}	57.23^{bc}
	1×10^8	16.67^{a}	36.67^{b}	53.33^{b}	57.04^{ab}	63.98^{ab}
	1×10^9	20.00^{a}	50.00^{a}	66.67^{a}	67.14^{a}	70.71^{a}
P. lilacinum (EEF 4)	1×10^5	0.00^{d}	6.67^{de}	10.00^{ijk}	10.07^{j}	10.07^{g}
	1×10^6	0.00^{d}	10.00^{de}	16.67^{hijk}	20.13^{hij}	20.20^{f}
	1×10^7	3.33^{cd}	10.00^{de}	20.00^{ghij}	33.53^{efg}	43.77^{d}
	1×10^8	6.67^{bc}	13.33^{de}	40.00^{cde}	43.60^{cde}	50.50^{d}
	1×10^9	10.00^{b}	26.67^{bc}	50.00^{bc}	50.33^{bcd}	57.23^{bc}
P. lilacinum (EEF 64)	1×10^5	0.00^{d}	3.33^{e}	6.67^{k}	10.03^{j}	10.10^{g}
	1×10^6	0.00^{d}	6.67^{de}	13.33^{ijk}	13.40^{ij}	16.83^{fg}
	1×10^7	0.00^{d}	10.00^{de}	26.67^{fgh}	30.17^{fgh}	33.67^{e}
	1×10^8	0.00^{d}	16.67^{cd}	30.00^{efg}	43.60^{cde}	47.13^{d}
	1×10^9	3.33^{cd}	26.67^{bc}	43.33^{bcd}	47.00^{bcd}	50.50^{d}
CD (P=0.05)		5.558	10.248	11.658	11.322	7.941

Treatment means in the same column by common superscript are not significantly different

4.6.1 Re-isolation

After plating, Petri plates were incubated in BOD incubator at 28ºC in dark. Fungal structures were observed under microscope for morphological confirmation as explained in 3.6.1.3.1 (Plate 28b and 29).

4.6.1.1 Colonisation of fungal endophytes in stem tissues of cowpea

The results of colonisation of two fungal isolates *viz*., *B. bassiana* (NBAIR strain) and *P. lilacinum* (EEF 4) in younger and older stem tissues of cowpea plants are illustrated in Tables 15 and 16.

Inoculation of *B. bassiana* through seed and leaves resulted in 8.33 and 24.08 per cent mean colonization of young stem tissues respectively. While no colonization was observed in case of soil inoculation.

Purpureocillium lilacinum (EEF 4) showed relatively greater colonisation of young stems irrespective of the method of colonisation. Mean colonisation values were 11.73, 13.89 and 62.04 per cent when the isolate was applied through seed, soil and leaves respectively with foliar inoculation proving to be significantly superior to all the treatments (Table 15).

Colonisation by *B. bassiana* averaged from 1.85 to 10.19 per cent in older stem tissues, with the highest value being recorded in seed inoculation (Table 16).

The mean colonisation by *P. lilacinum* in old stem tissues ranged from 18.83 to 40.74 per cent with all the values being on par with each other. The highest colonisation in 55.56 per cent of samples was observed 55 days after sowing both in case of seed and foliar inoculation.

Colonisation of stem tissues was the highest at 55 DAS for both *B. bassiana* and *P. lilacinum* regardless of the method of inoculation and age of the stem tissues.

Table 15. Colonisation of fungal isolates in young stem tissues of cowpea

Treatments		Colonisation (%)			
		30 DAS	55 DAS	80 DAS	Mean
T_1	*Beauveria bassiana* (NBAIR strain) as seed inoculation	5.56 (8.81)^{c}	11.11 (12.54)^{b}	8.33 (10.78)^{bc}	8.33 (10.78)^{cd}
T_2	*Beauveria bassiana* (NBAIR strain) as soil application	0.00 (1.17)^{c}	0.00 (1.17)^{b}	0.00 (1.17)^{c}	0.00 (1.17)^{d}
T_3	*Beauveria bassiana* (NBAIR strain) as foliar application	36.11 (35.60)^{ab}	11.11 (15.99)^{b}	25.00 (30.00)^{b}	24.08 (28.88)^{b}
T_4	*Purpureocillium lilacinum* (EEF 4) as seed inoculation	7.41 (12.97)^{c}	11.11 (12.54)^{b}	16.67 (20.18)^{bc}	11.73 (19.79)^{bc}
T_5	*Purpureocillium lilacinum* (EEF 4) as soil application	11.11 (15.99)^{bc}	22.22 (27.82)^{b}	8.33 (10.78)^{bc}	13.89 (21.61)^{bc}
T_6	*Purpureocillium lilacinum* (EEF 4) as foliar application	44.44 (41.75)^{a}	75.00 (65.77)^{a}	66.67 (54.74)^{a}	62.04 (52.15)^{a}
T_7	Control 1 (Seed inoculation)	0.00 (1.17)^{c}	0.00 (1.17)^{b}	0.00 (1.17)^{c}	0.00 (1.17)^{d}
T_8	Control 2 (Soil application)	0.00 (1.17)^{c}	0.00 (1.17)^{b}	0.00 (1.17)^{c}	0.00 (1.17)^{d}
T_9	Control 3 (Foliar application)	0.00 (1.17)^{c}	0.00 (1.17)^{b}	0.00 (1.17)^{c}	0.00 (1.17)^{d}
	CD (P=0.05)	19.94	27.96	19.37	14.39

*DAS – days after sowing; Figures in parentheses are arc sin transformed values
In a column, mean followed by a common letter are not significantly different by DMRT (P=0.05)

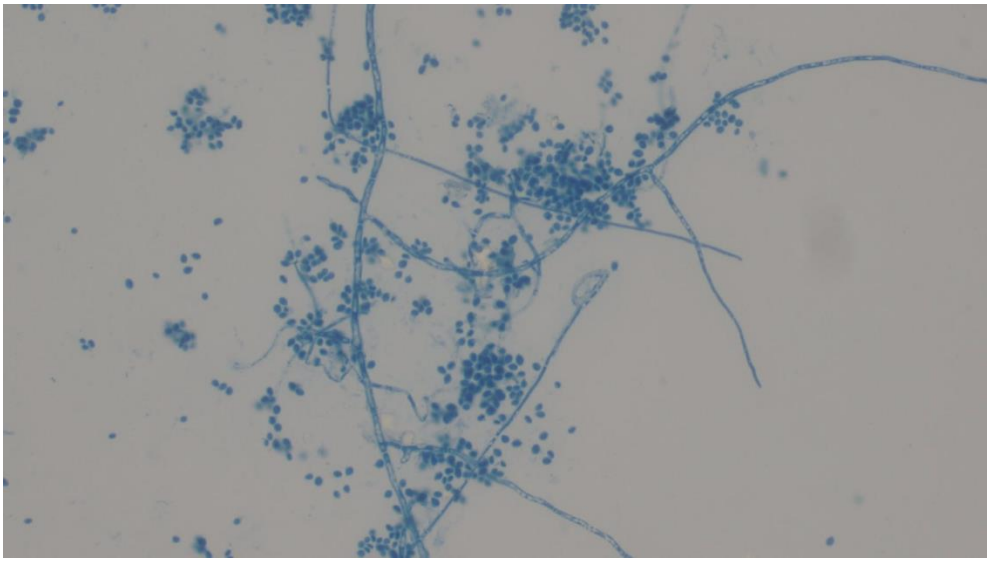


Plate 29. Photomicrograph of *Beauveria bassiana* (NBAIR strain) (400 X)

Plate 30. Growth of *Beauveria bassiana* (NBAIR strain) on SDAY

Table 16. Colonisation of fungal isolates in old stem tissues of cowpea

Treatments		Colonisation (%)			
		30 DAS	55 DAS	80 DAS	Mean
T_1	*Beauveria bassiana* (NBAIR strain) as seed inoculation	8.34 (16.79)	11.11 (16.46)	11.11 (16.46)^{b}	10.19 (17.79)^{bc}
T_2	*Beauveria bassiana* (NBAIR strain) as soil application	5.56 (8.81)	0.00 (1.17)	0.00 (1.17)^{c}	1.85 (5.33)^{cd}
T_3	*Beauveria bassiana* (NBAIR strain) as foliar application	11.11 (16.46)	16.67 (20.18)	0.00 (1.17)^{c}	9.26 (17.12)^{bc}
T_4	*Purpureocillium lilacinum* (EEF 4) as seed inoculation	18.52 (21.60)	55.56 (48.25)	16.67 (24.10)^{b}	30.25 (31.69)^{ab}
T_5	*Purpureocillium lilacinum* (EEF 4) as soil application	8.34 (14.02)	27.78 (26.67)	20.37 (22.75)^{b}	18.83 (24.58)^{ab}
T_6	*Purpureocillium lilacinum* (EEF 4) as foliar application	16.67 (20.18)	55.56 (52.64)	50.00 (45.00)^{a}	40.74 (39.57)^{a}
T_7	Control 1 (Seed inoculation)	0.00 (1.17)	0.00 (1.17)	0.00 (1.17)^{c}	0.00 (1.17)^{d}
T_8	Control 2 (Soil application)	0.00 (1.17)	0.00 (1.17)	0.00 (1.17)^{c}	0.00 (1.17)^{d}
T_9	Control 3 (Foliar application)	0.00 (1.17)	0.00 (1.17)	0.00 (1.17)^{c}	0.00 (1.17)^{d}
	CD (P=0.05)	NS	NS	15.20	15.89

*DAS – days after sowing; Figures in parentheses are arc sin transformed values
In a column, mean followed by a common letter are not significantly different by DMRT (P=0.05)

Table 17. Colonisation of fungal isolates in young leaf tissues of cowpea

Treatments		Colonisation (%)			
		30 DAS	55 DAS	80 DAS	Mean
T_1	*Beauveria bassiana* (NBAIR strain) as seed inoculation	8.34 (16.79)^{ab}	8.33 (14.01)^{bc}	5.56 (11.58)^{bc}	7.41 (15.58)^{b}
T_2	*Beauveria bassiana* (NBAIR strain) as soil application	0.00 (1.17)^{b}	0.00 (1.17)^{c}	0.00 (1.17)^{c}	0.00 (1.17)^{c}
T_3	*Beauveria bassiana* (NBAIR strain) as foliar application	13.89 (18.42)^{a}	5.56 (8.81)^{c}	22.22 (28.03)^{a}	13.89 (21.61)^{ab}
T_4	*Purpureocillium lilacinum* (EEF 4) as seed inoculation	14.82 (19.03)^{a}	42.59 (40.60)a	14.82 (19.03)^{ab}	24.08 (27.78)^{a}
T_5	*Purpureocillium lilacinum* (EEF 4) as soil application	25.01 (29.11)^{a}	11.11 (16.46)^{ab}	11.11 (16.46)^{ab}	15.74 (23.36)^{ab}
T_6	*Purpureocillium lilacinum* (EEF 4) as foliar application	19.45 (25.60)^{a}	33.34 (34.00)^{ab}	16.67 (24.10)^{ab}	23.15 (28.28)^{a}
T_7	Control 1 (Seed inoculation)	0.00 (1.17)^{b}	0.00 (1.17)^{c}	0.00 (1.17)^{c}	0.00 (1.17)^{c}
T_8	Control 2 (Soil application)	0.00 (1.17)^{b}	0.00 (1.17)^{c}	0.00 (1.17)^{c}	0.00 (1.17)^{d}
T_9	Control 3 (Foliar application)	0.00 (1.17)^{b}	0.00 (1.17)^{c}	0.00 (1.17)^{c}	0.00 (1.17)^{d}
	CD (P=0.05)	16.98	23.03	15.13	11.63

*DAS – days after sowing; Figures in parentheses are arc sin transformed values
In a column, mean followed by a common letter are not significantly different by DMRT (P=0.05)

Table 18. Colonisation of fungal isolates in old leaf tissues of cowpea

Treatments		Colonisation (%)			
		30 DAS	55 DAS	80 DAS	Mean
T_1	*Beauveria bassiana* (NBAIR strain) as seed inoculation	1.85 (5.33)^{b}	16.67 (20.18)^{bc}	2.78 (6.38)^{c}	7.10 (13.02)^{bcd}
T_2	*Beauveria bassiana* (NBAIR strain) as soil application	5.56 (8.81)^{b}	0.00 (1.17)^{c}	0.00 (1.17)^{c}	1.85 (5.33)^{cd}
T_3	*Beauveria bassiana* (NBAIR strain) as foliar application	14.82 (14.72)^{b}	11.11 (16.46)^{bc}	5.56 (8.81)^{c}	10.50 (15.14)^{bc}
T_4	*Purpureocillium lilacinum* (EEF 4) as seed inoculation	11.11 (19.47)^{ab}	16.67 (23.58)^{b}	16.67 (24.10)^{b}	14.82 (22.57)^{b}
T_5	*Purpureocillium lilacinum* (EEF 4) as soil application	0.00 (1.17)^{b}	0.00 (1.17)^{c}	0.00 (1.17)^{c}	0.00 (1.17)^{d}
T_6	*Purpureocillium lilacinum* (EEF 4) as foliar application	33.33 (34.9)^{a}	61.11 (51.97)^{a}	33.33 (35.26)^{a}	42.59 (40.69)^{a}
T_7	Control 1 (Seed inoculation)	0.00 (1.17)^{b}	0.00 (1.17)^{c}	0.00 (1.17)^{c}	0.00 (1.17)^{d}
T_8	Control 2 (Soil application)	0.00 (1.17)^{b}	0.00 (1.17)^{c}	0.00 (1.17)^{c}	0.00 (1.17)^{d}
T_9	Control 3 (Foliar application)	0.00 (1.17)^{b}	0.00 (1.17)^{c}	0.00 (1.17)^{c}	0.00 (1.17)^{d}
	CD (P=0.05)	19.72	22.06	10.60	13.42

*DAS – days after sowing; Figures in parentheses are arc sin transformed values
In a column, mean followed by a common letter are not significantly different by DMRT (P=0.05)

4.6.1.2 Colonisation of fungal endophytes in leaf tissues of cowpea

The results of colonisation of fungal isolates *viz*., *B. bassiana* (NBAIR strain) and *P. lilacinum* (EEF 4) in younger and older leaf tissues of cowpea plants are presented in Tables 17 and 18.

In case of young leaf tissues of cowpea, no colonisation resulted through soil application of *B. bassiana*. Mean colonisation was 7.41 per cent in case of seed inoculation and was on par with the 13.89 per cent recorded in case of foliar application.

Colonisation of young leaves by *P. lilacinum* was comparatively higher for all the three inoculation methods. Mean colonisation values were 24.08, 15.74 and 23.15 per cent for seed, soil and foliar inoculation respectively, the values being on par with each other.

In case of older leaves, mean colonisation by *B. bassiana* isolate ranged between 1.85 per cent in case of soil inoculation and 10.50 per cent in case of foliar inoculation, the treatments being at par. *P. lilacinum* (EEF 4) failed to colonize older leaves when applied through soil. Seed inoculation resulted in mean colonisation of 14.82 per cent while foliar inoculation led to the highest colonisation of 42.59 per cent, which was significantly superior to other treatments.

4.6.1.3 Colonisation of fungal endophytes in root tissues of cowpea

Colonisation by the two fungal isolates *viz*., *B. bassiana* (NBAIR strain) and *P. lilacinum* (EEF 4) in root tissues of cowpea plants are given in Table 19.

Mean colonisation by *B. bassiana* in roots ranged from 3.40 to 9.26 per cent with soil inoculation resulting in the highest colonisation (9.26%), followed by foliar inoculation (8.64%). Both treatments were at par. Seed inoculation registered the lowest extent of colonization (3.40%).

Purpureocillium lilacinum colonized 20.37, 29.01 and 31.48 per cent of roots through seed, soil and foliar inoculation methods respectively, the latter two treatments being at par.

4.6.1.4 Colonisation of fungal endophytes in pod tissues of cowpea

Mean colonisation of pods by the two EEFs *viz*., *B. bassiana* (NBAIR strain) and *P. lilacinum* (EEF 4) are given in Table 19.

Colonisation by *B. bassiana* in pods, when inoculated through soil and leaves averaged 25.93 and 31.48 per cent respectively, the values being on par with each other. Soil inoculation resulted in lowest extent of endophytic establishment in pods (1.85%).

Mean colonisation by *P. lilacinum*, at 27.78 per cent, was the lowest in case of soil inoculation. Seed and foliar inoculation resulted in colonisation of 68.52 and 38.89 per cent respectively, all the three treatments differing significantly from each other.

4.6.2 Molecular technique

The reisolated fumgal isolates were subjected to PCR technique to confirm plant colonization by respective isolates. Amplification of ITS region was done and the PCR products of re-isolated fungi were compared with that of original samples. The gel profile of PCR products confirmed the colonization by *B. bassiana* and *P. lilacinum* in different parts of cowpea plant at different stages of crop growth (Plates 31 to 36).

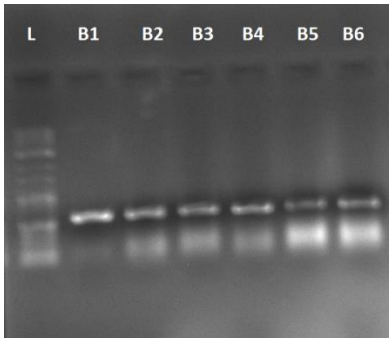
4.6.3 Effect of fungal colonisation on growth and yield of cowpea under polyhouse condition

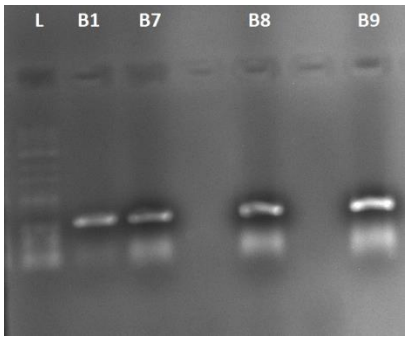
The effect of colonisation by EEF on plant growth under polyhouse conditions was studied by measuring the height as well the number of leaves. Observations were taken at three different stages of crop growth *i.e*., at 40, 60 and 80 DAS (Table 20 and 21).

Table 19. Colonisation of fungal isolates in root and pod tissues of cowpea

Treatments		Colonisation (%)				
		Roots				Pod
		30 DAS	55 DAS	80 DAS	Mean	
T_1	*Beauveria bassiana* (NBAIR strain) as seed inoculation	1.85 (5.33)^{b}	2.78 (6.38)^{c}	5.56 (13.64)^{c}	3.40 (10.14)^{d}	25.93 (30.27)^{c}
T_2	*Beauveria bassiana* (NBAIR strain) as soil application	9.26 (14.91)^{ab}	1.85 (5.33)^{c}	16.67 ((24.10)^{ab}	9.26 (17.53)^{c}	1.85 (5.33)^{d}
T_3	*Beauveria bassiana* (NBAIR strain) as foliar application	1.85 (5.33)^{b}	16.67 (23.19)^{b}	7.41 (13.37)^{c}	8.64 (16.36)^{cd}	31.48 (33.64)^{bc}
T_4	*Purpureocillium lilacinum* (EEF 4) as seed inoculation	20.37 (26.67)^{a}	29.63 (32.57)^{ab}	11.11 (19.47)^{bc}	20.37 (26.72)^{b}	68.52 (56.01)^{a}
T_5	*Purpureocillium lilacinum* (EEF 4) as soil application	31.48 (29.33)^{a}	38.89 (38.47)^{ab}	16.67 (24.10)^{ab}	29.01 (32.1)^{ab}	27.78 (31.81)^{c}
T_6	*Purpureocillium lilacinum* (EEF 4) as foliar application	18.52 (25.23)^{a}	51.85 (45.87)^{a}	24.07 ((29.35)^{a}	31.48 (34.09)^{a}	38.89 (41.84)^{b}
T_7	Control 1 (Seed inoculation)	0.00 (1.17)^{b}	0.00 (1.17)^{c}	0.00 (1.17)^{c}	0.00 (1.17)^{e}	0.00 (1.17)^{d}
T_8	Control 2 (Soil application)	0.00 (1.17)^{b}	0.00 (1.17)^{c}	0.00 (1.17)^{c}	0.00 (1.17)^{e}	0.00 (1.17)^{d}
T_9	Control 3 (Foliar application)	0.00 (1.17)^{b}	0.00 (1.17)^{c}	0.00 (1.17)^{c}	0.00 (1.17)^{e}	0.00 (1.17)^{d}
	CD (P=0.05)	19.35	15.73	7.13	7.36	9.27

*DAS – days after sowing; Figures in parentheses are arc sin transformed values
In a column, mean followed by a common letter are not significantly different by DMRT (P=0.05)





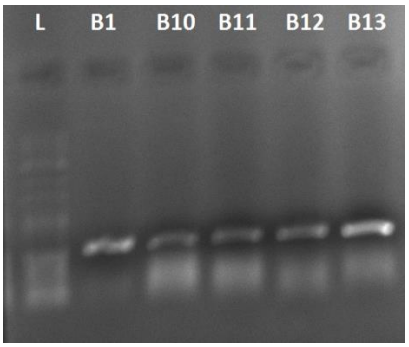
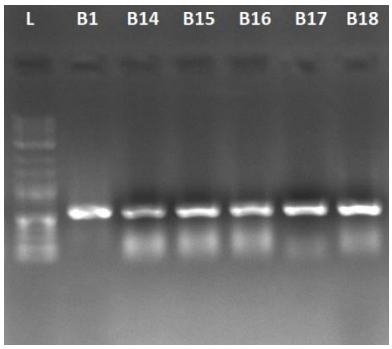
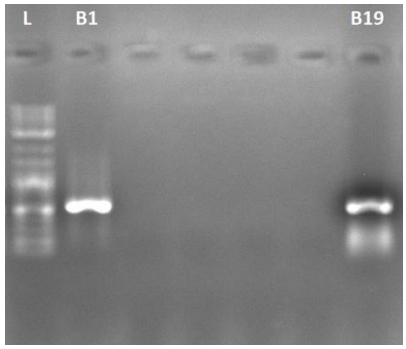


Plate 31a. Seed inoculation Plate 31b. Soil application Plate 31c. Foliar application

Plate 31. Gel profile of PCR amplified genomic DNA extracted from re-isolated fungal isolate of *Beauveria bassiana* from seed, soil and foliar application treatment at 30 DAS. Lanes 1-7: 1 3kb ladder, 2 Bb original isolate, 3 Bb old stem, 4 Bb young stem, 5 Bb old leaf, 6 Bb young leaf, 7 Bb root





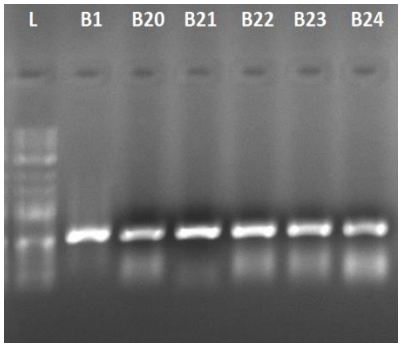
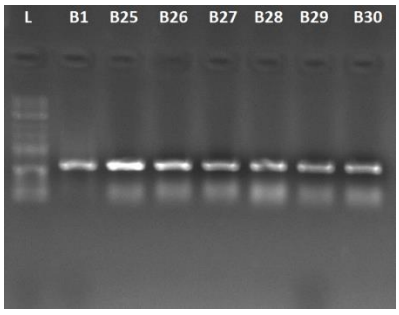
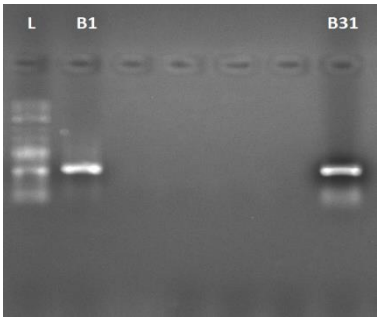


Plate 32a. Seed inoculation Plate 32b. Soil application Plate 32c. Foliar application

Plate 32. Gel profile of PCR amplified genomic DNA extracted from re-isolated fungal isolate of *Beauveria bassiana* from seed, soil and foliar application treatment at 55 DAS. Lanes 1-7: 1 3 kb ladder, 2 Bb original isolate, 3 Bb old stem, 4 Bb young stem, 5 Bb old leaf, 6 Bb young leaf, 7 Bb root





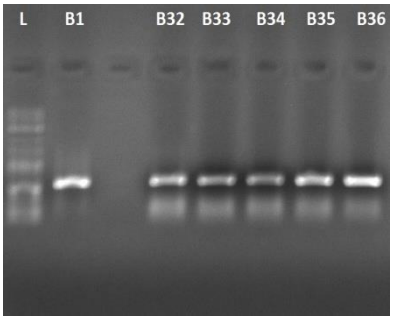


Plate 33a. Seed inoculation Plate 33b. Soil application Plate 33c. Foliar application

Plate 33. Gel profile of PCR amplified genomic DNA extracted from re-isolated fungal isolate of *Beauveria bassiana* from seed, soil and foliar application treatment at 80 DAS. Lanes 1-8: 1 3 kb ladder, 2 Bb original isolate, 3 Bb old stem, 4 Bb young stem, 5 Bb old leaf, 6 Bb young leaf, 7 Bb root, 8Bb pod

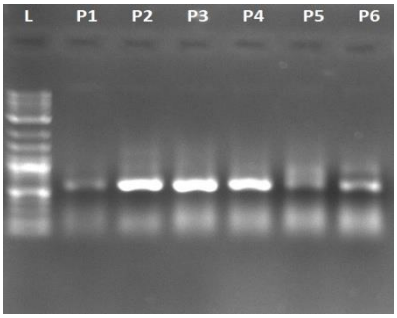


Plate 34a. Seed inoculation

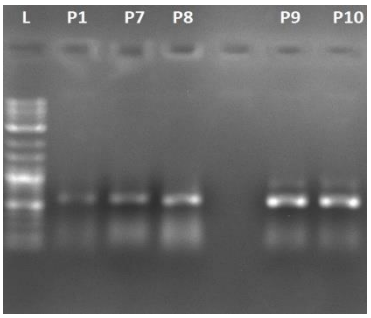


Plate 34b. Soil application

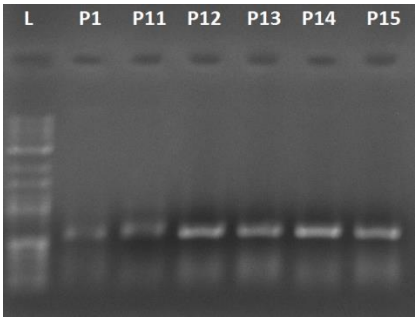


Plate 34c. Foliar application

Plate 34. Gel profile of PCR amplified genomic DNA extracted from re-isolated fungal isolate *Purpureocillium lilacinum* (EEF4) from seed, soil and foliar application treatment at 30 DAS. Lanes 1-7: 1 3 kb ladder, 2 EEF4 original isolate, 3 EEF4 old stem, 4 EEF4 young stem, 5 EEF4 old leaf, 6 EEF4 young leaf, 7 EEF4 root

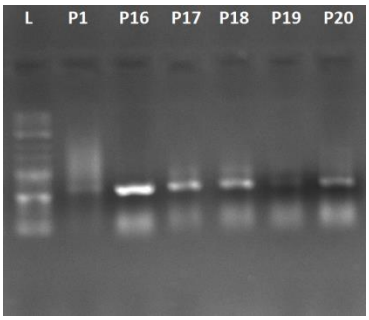


Plate 35a. Seed inoculation

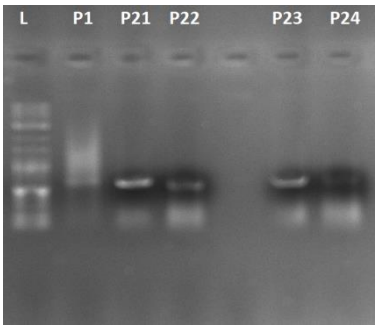


Plate 35b. Soil application

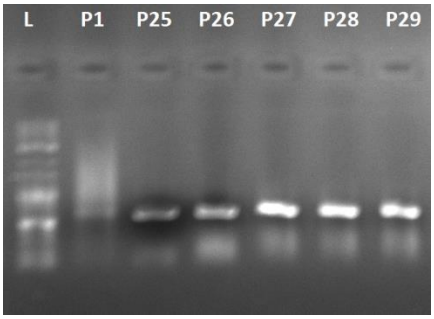


Plate 35c. Foliar application

Plate 35. Gel profile of PCR amplified genomic DNA extracted from re-isolated fungal isolate *Purpureocillium lilacinum* (EEF4) from seed, soil and foliar application treatment at 55 DAS. Lanes 1-7: 1 3 kb ladder, 2 EEF4 original isolate, 3 EEF4 old stem, 4 EEF4 young stem, 5 EEF4 old leaf, 6 EEF4 young leaf, 7 EEF4 root

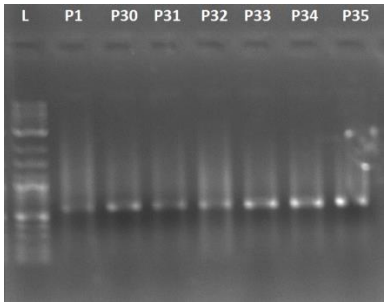


Plate 36a. Seed inoculation

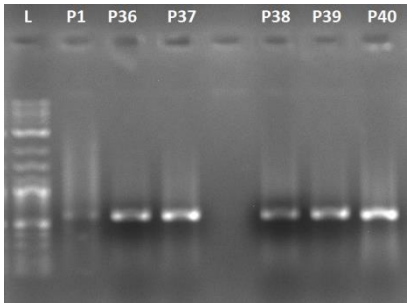


Plate 36b. Soil application

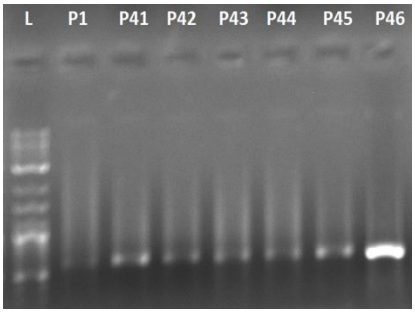


Plate 36c. Foliar application

Plate 36. Gel profile of PCR amplified genomic DNA extracted from re-isolated fungal isolate *Purpureocillium lilacinum* (EEF4) from seed, soil and foliar application treatment at 80 DAS. Lanes 1-8: 1 3 kb ladder, 2 EEF4 original isolate, 3 EEF4 old stem, 4 EEF4 young stem, 5 EEF4 old leaf, 6 EEF4 young leaf, 7 EEF4 root, 8 EEF4 pod

4.6.3.1 Total number of leaves

At 40 days after sowing cowpea plants inoculated with *B. bassiana* through seed inoculation recorded the highest mean number of 39.93 leaves. It was on par with plants inoculated with *B. bassiana* as foliar application with a mean number of leaves, 35.20, but was significantly superior to the remaining treatments with mean values ranging from 23.20 to 30.93 (Table 20).

At 60 days after sowing, *B. bassiana* inoculated through seed and foliar inoculations once again recorded the highest mean number of 54.93 and 50.20 leaves respectively and were on par. The former treatment was, however, significantly superior to all other treatments. *P. lilacinum* registered 45.80, 43.93 and 41.80 number of leaves on an average when inoculate through soil, seed and leaves respectively, the treatments being on par with each other.

At 80 days after sowing, there was no significant difference between the treatments in terms of mean number of leaves. However, the highest value was recorded in case of foliar application with *P. lilacinum* (70.20), followed by the same applied as soil application, with 70.13 leaves on an average.

4.6.3.2 Plant height

Influence of different treatments was found to be non-significant with respect to height of the plant at different stages of plant growth (Table 21). At 40 days after sowing, height of plant ranged from 77.37 cm in control 3 to 121.33 cm in plants inoculated with *P. lilacinum* as soil application. At 60 days after sowing, the plant height ranged between 162.20 cm and 184.87 cm, while at 80 days after sowing, it varied from 185.20 to 221.33 cm.

Table 20. Effect of fungal colonisation on number of leaves of cowpea under polyhouse condition

Treatments		Number of leaves		
		40 DAS	60 DAS	80 DAS
T_1	*Beauveria bassiana* (NBAIR strain) as seed inoculation	39.93[a]	54.93[a]	68.53
T_2	*Beauveria bassiana* (NBAIR strain) as soil application	30.93[bc]	45.93[bc]	56.40
T_3	*Beauveria bassiana* (NBAIR strain) as foliar application	35.20[ab]	50.20[ab]	65.87
T_4	*Purpureocillium lilacinum* (EEF 4) as seed inoculation	28.93[bcd]	43.93[bcd]	61.80
T_5	*Purpureocillium lilacinum* (EEF 4) as soil application	30.87[bc]	45.80[bc]	70.13
T_6	*Purpureocillium lilacinum* (EEF 4) as foliar application	28.20[bcd]	41.80[cd]	70.20
T_7	Control 1 (Seed inoculation)	27.47[cd]	40.27[cd]	63.60
T_8	Control 2 (Soil application)	23.20[d]	38.20[d]	64.17
T_9	Control 3 (Foliar application)	25.47[cd]	40.47[cd]	60.73
	CD (P=0.05)	7.126	7.097	NS

*DAS – days after sowing

In a column, mean followed by a common letter are not significantly different by DMRT (P=0.05)

Table 21. Effect of fungal colonisation on height of cowpea under polyhouse condition

Treatments		Height (cm)		
		40DAS	60 DAS	80 DAS
T_1	*Beauveria bassiana* (NBAIR strain) as seed inoculation	85.20	162.20	185.20
T_2	*Beauveria bassiana* (NBAIR strain) as soil application	105.73	184.87	205.67
T_3	*Beauveria bassiana* (NBAIR strain) as foliar application	112.87	177.47	212.87
T_4	*Purpureocillium lilacinum* (EEF 4) as seed inoculation	97.40	169.07	197.40
T_5	*Purpureocillium lilacinum* (EEF 4) as soil application	121.33	184.87	221.33
T_6	*Purpureocillium lilacinum* (EEF 4) as foliar application	109.33	184.53	205.07
T_7	Control 1 (Seed inoculation)	86.63	184.73	210.07
T_8	Control 2 (Soil application)	98.57	179.53	198.33
T_9	Control 3 (Foliar application)	77.37	184.87	189.40
	CD (P=0.05)	NS	NS	NS

*DAS – days after sowing

In a column, mean followed by a common letter are not significantly different by DMRT (P=0.05)

4.6.3.3 Pod yield per plant

The treatments varied significantly with respect to mean yield per plant (Table 22). *B. bassiana*, inoculated through seed, leaves and soil yielded 171.20, 157.70 and 150.00g per plant respectively and were on par with each other. *P. lilacinum* recorded the highest yield of 142.40g when applied as foliar inoculation, which, however, was on par with all other treatments except *B. bassiana* as seed inoculation and control treatment for foliar inoculation (115.20g).

4.7 EFFECT OF FUNGAL COLONISATION ON GROWTH, YIELD AND INFESTATION OF SPOTTED POD BORER IN COWPEA UNDER FIELD CONDITIONS

After standardizing the method of inoculation through polyhouse studies, the effect of fungal colonisation on spotted pod borer infestation was assessed through a pot culture experiment under field conditions. In addition, the effect of inoculation with endophytes on growth and yield parameters were also studied. The observations included number of leaves, height of plant, mean pod yield, marketable pod yield and mean infestation by pod borer.

4.7.1 Effect of fungal colonisation on growth of cowpea under field condition

The results of effect of fungal colonisation on growth parameters such as number of leaves and height of plant is presented in tables 23 and 24.

4.7.1.1 Total number of leaves

The mean number of leaves showed significant variation only at 40 days after sowing. The highest mean number of 68.73 leaves was observed in the plants treated with *B. bassiana* as seed inoculation, followed by *P. lilacinum* as seed inoculation having 60.53 leaves on an average, both being on par with each other. The remaining treatments

were on par with each other including untreated control plants. No significant variation was observed among the treatments at 60 and 80 days after sowing (Table 23).

Table 22. Effect of fungal colonisation on yield of cowpea under polyhouse condition

Treatments		Yield (g plant^{-1})
T_1	*Beauveria bassiana* (NBAIR strain) as seed inoculation	171.20^{a}
T_2	*Beauveria bassiana* (NBAIR strain) as soil application	150.00^{abc}
T_3	*Beauveria bassiana* (NBAIR strain) as foliar application	157.70^{ab}
T_4	*Purpureocillium lilacinum* (EEF 4) as seed inoculation	138.33^{bcd}
T_5	*Purpureocillium lilacinum* (EEF 4) as soil application	134.13^{bcd}
T_6	*Purpureocillium lilacinum* (EEF 4) as foliar application	142.40^{bc}
T_7	Control 1 (Seed inoculation)	135.33^{bcd}
T_8	Control 2 (Soil application)	126.00^{cd}
T_9	Control 3 (Foliar application)	115.20^{d}
	CD (P=0.05)	25.728

In a column, mean followed by a common letter are not significantly different by DMRT (P=0.05)

4.7.1.2 Plant height

The effect of fungal colonisation on height of cowpea plant is illustrated in Table 24. Forty days after sowing, inoculation with endophytes resulted in comparable increase in mean plant height with values ranging from 44.73 to 48.40 cm, except foliar inoculation with *B. bassiana*, recording 39.67 number of leaves.

At 60 DAS, no significant difference was registered between different treatments with mean plant height ranging from 55.07 cm in ccontrol 3 to 86.80 cm in case of *P. lilacinum* as soil application.

Eighty days after sowing, *P. lilacinum* as soil inoculation recorded the highest value of 120.67 cm, which was on par with foliar application of the same having a mean value of 113.33 cm. Meanwhile, *B. bassiana* recorded 93.00 cm, 91.00 cm and 90.00 cm when applied as foliar, soil and seed inoculation respectively. These values were on par with control 2 (88.13 cm), control 1 (85.93 cm) and control 3 (80.60 cm).

4.7.2 Effect of fungal colonisation on yield parameters under field condition

The results of effect of fungal colonisation on yield parameters such as total and marketable pod yield is presented in Table 25.

4.7.2.1 Total pod yield per plant

The mean yield per plant varied significantly among the treatments. Both *B. bassiana* as well as *P. lilacinum* recorded comparable yields of 163.33 to 173.67g per plant irrespective of method of inoculation and were on par with each other except *B. bassiana* inoculated as soil application, which had significantly lower mean yield of 132.87g plant^{-1}. The lowest mean yield of 121.0 g plant^{-1} was recorded in control 1, which was on par with 132.87g, 140.20g and 140.67g by *B. bassiana* as soil application, control 2 and control 3.

4.7.2.2 Marketable pod yield per plant

The marketable pod yield per plant was also significantly different among the different treatments. *B. bassiana* applied as foliar application resulted in the highest marketable pod yield of 152.83g, which was on par with *P. lilacinum* as foliar application (149.33g), *B. bassiana* as seed inoculation (147.00g), *Pl* as soil application (144.67g) and *P. lilacinum* as seed application (137.67g). Control treatments had comparable market yield with values and were on par with *B. bassiana* as soil application (109.67g).

4.7.3 Effect of fungal colonisation on infestation by spotted pod borer

The results of effect of fungal colonisation on infestation of spotted pod borer on pods and flowers are illustrated in Table 26.

4.7.3.1 Infestation on flowers

Inoculation by endophytes did not have any significant effect on infestation of flowers by spotted pod borer. Infestation varied from 7.72 to 10.61 per cent among the treatments, the lowest value being recorded by *B*. *bassiana* as foliar application. Control 1 recorded the highest incidence of 10.61 per cent.

4.7.3.2 Infestation on pods

Infestation by the pod borer on pods varied significantly among different treatments. *B. bassiana* as foliar application recorded the lowest infestation of 12.53 per cent and was followed by *P. lilacinum* as foliar application with 14.66 per cent infestation. The above treatments were on par with other treatments involving endophytes. Highest infestation was recorded in control 3 (25.44%), which was on par with control 1 (22.07%), control 2 (20.38%) and *B. bassiana* as soil application (20.71%).

4.8 EFFECT OF ENDOPHYTIC COLONISATION BY *Beauveria bassiana* ON INFESTATION BY SPOTTED POD BORER

Based on the results of the aforementioned experiments, foliar application of *B. bassiana* was selected as the best treatment and was carried forward to a field trial along with Flubendiamide as insecticidal check. Observations of the study are presented in Tables 27 and 28.

Table 23. Effect of fungal colonisation on number of leaves of cowpea under field condition

Treatments		Number of leaves		
		40 DAS	60 DAS	80 DAS
T_1	*Beauveria bassiana* (NBAIR strain) as seed inoculation	68.73[a]	88.55	114.45
T_2	*Beauveria bassiana* (NBAIR strain) as soil application	57.00[b]	90.00	124.80
T_3	*Beauveria bassiana* (NBAIR strain) as foliar application	57.47[b]	93.40	111.47
T_4	*Purpureocillium lilacinum* (EEF 4) as seed inoculation	60.53[ab]	107.27	121.80
T_5	*Purpureocillium lilacinum* (EEF 4) as soil application	52.33[b]	105.40	118.93
T_6	*Purpureocillium lilacinum* (EEF 4) as foliar application	58.67[b]	98.40	101.13
T_7	Control 1 (Seed inoculation)	52.73[b]	93.62	94.10
T_8	Control 2 (Soil application)	54.87[b]	104.40	105.40
T_9	Control 3 (Foliar application)	52.60[b]	85.60	98.40
	CD (P=0.05)	8.677	NS	NS

*DAS – days after sowing

In a column, mean followed by a common letter are not significantly different by DMRT (P=0.05)

Table 24. Effect of fungal colonisation on height of cowpea under field condition

Treatments		Height (cm)		
		40DAS	60 DAS	80 DAS
T_1	*Beauveria bassiana* (NBAIR strain) as seed inoculation	47.47[a]	67.87	90.00[d]
T_2	*Beauveria bassiana* (NBAIR strain) as soil application	48.40[a]	73.60	91.00[cd]
T_3	*Beauveria bassiana* (NBAIR strain) as foliar application	39.67[bc]	58.20	93.00[cd]
T_4	*Purpureocillium lilacinum* (EEF 4) as seed inoculation	44.73[ab]	67.33	102.40[bc]
T_5	*Purpureocillium lilacinum* (EEF 4) as soil application	46.27[ab]	86.80	120.67[a]
T_6	*Purpureocillium lilacinum* (EEF 4) as foliar application	45.80[ab]	80.87	113.33[ab]
T_7	Control 1 (Seed inoculation)	39.13[bc]	79.80	85.93[d]
T_8	Control 2 (Soil application)	40.40[bc]	71.53	88.13[d]
T_9	Control 3 (Foliar application)	34.73[c]	55.07	80.60[d]
	CD (P=0.05)	7.221	NS	12.595

*DAS – days after sowing

In a column, mean followed by a common letter are not significantly different by DMRT (P=0.05)

Table 25. Effect of fungal colonisation on total and marketable pod yield of cowpea

Treatments		Pod yield (g plant^{-1})	Marketable pod yield (g plant^{-1})
T_1	*Beauveria bassiana* (NBAIR strain) as seed inoculation	170.13^{ab}	147.00^{a}
T_2	*Beauveria bassiana* (NBAIR strain) as soil application	132.87^{c}	109.67^{bc}
T_3	*Beauveria bassiana* (NBAIR strain) as foliar application	173.00^{a}	152.83^{a}
T_4	*Purpureocillium lilacinum* (EEF 4) as seed inoculation	163.33^{ab}	137.67^{ab}
T_5	*Purpureocillium lilacinum* (EEF 4) as soil application	169.33^{ab}	144.67^{a}
T_6	*Purpureocillium lilacinum* (EEF 4) as foliar application	173.67^{a}	149.33^{a}
T_7	Control 1 (Seed inoculation)	121.00^{c}	96.33^{c}
T_8	Control 2 (Soil application)	140.20^{bc}	113.17^{bc}
T_9	Control 3 (Foliar application)	140.67^{bc}	109.67^{bc}
	CD (P=0.05)	30.144	28.666

In a column, mean followed by a common letter are not significantly different by DMRT (P=0.05)

Table 26. Effect of fungal colonisation on infestation by spotted pod borer

Treatments		Infestation on flowers (%)	Infestation on pods (%)
T_1	*Beauveria bassiana* (NBAIR strain) as seed inoculation	9.71	14.94^{cd}
T_2	*Beauveria bassiana* (NBAIR strain) as soil application	10.17	20.71^{abc}
T_3	*Beauveria bassiana* (NBAIR strain) as foliar application	7.72	12.53^{d}
T_4	*Purpureocillium lilacinum* (EEF 4) as seed inoculation	9.80	15.91^{bcd}
T_5	*Purpureocillium lilacinum* (EEF 4) as soil application	7.63	16.39^{bcd}
T_6	*Purpureocillium lilacinum* (EEF 4) as foliar application	8.32	14.66^{cd}
T_7	Control 1 (Seed inoculation)	10.61	22.07^{ab}
T_8	Control 2 (Soil application)	10.03	20.38^{abc}
T_9	Control 3 (Foliar application)	10.25	25.44^{a}
	CD (P=0.05)	NS	6.752

In a column, mean followed by a common letter are not significantly different by DMRT (P=0.05)

4.8.1 Effect of endophytic entomopathogenic fungi on infestation by spotted pod borer

Effect of endophytic entomopathogenic fungi on infestation by spotted pod borer on flowers and pods was compared with the chemical check flubendiamide (Table 27).

4.8.1.1 Infestation on flowers

The treatments varied significantly with respect to infestation on flowers. The insecticide flubendiamide was significantly superior over other treatments with a mean value of 7.09 per cent. Plants treated with *B. bassiana* had 20.78 per cent of flowers infested on an average, and was on par with untreated control with 26.40 per cent incidence.

4.8.1.2 Infestation on pods

Significant difference was also recorded among these tratements with respect to infestation on the basis of mean number of pods. Application of flubendiamide resulted in the lowest mean pod infestation of 8.41 per cent and was followed by *B. bassiana* as foliar application with 15.05 per cent. Control had the highest mean infestation of 21.28 per cent.

4.8.2 Effect of endophytic entomopathogenic fungi on yield of cowpea

Effect of endophytic entomopathogenic fungi on total and marketable yield of cowpea is illustrated in Table 28.

4.8.2.1 Yield per plant

All the three treatments were on par with each other with respect to total yield per plant although application of *B. bassiana* resulted in higher yield of 181.57g as against flubendiamide and untreated control with 176.43 and 171.57g respectively.

4.8.2.2 Marketable pod yield per plant

Flubendiamide was found to be statistically superior to other treatments with a mean marketable yield of 166.14g. *B. bassiana* yielded 155.14g of marketable pods. Both the above treatments were significantly superior to control with a value of 139.29g.

Table 27. Effect of different treatments on infestation by *Maruca vitrata* on cowpea

Treatments		Infestation on flowers (%)	Infestation on pods (%)
T_1	*Beauveria bassiana* as foliar application	20.78^{ab}	15.05^{b}
T_2	Flubendiamide	7.09^{c}	8.41^{c}
T_3	Control	26.40^{a}	21.28^{a}
	CD (P=0.05)	5.682	4.402

In a column, mean followed by a common letter are not significantly different by DMRT (P=0.05)

Table 28. Effect of different treatments on total and marketable pod yield of cowpea

Treatments		Yield (g plant^{-1})	Marketable pod yield (g plant^{-1})
T_1	*Beauveria bassiana* as foliar application	181.57	155.14^{b}
T_2	Flubendiamide	176.43	166.14^{a}
T_3	Control	171.57	139.29^{c}
	CD (P=0.05)	NS	10.078

In a column, mean followed by a common letter are not significantly different by DMRT (P=0.05)

Discussion

5. DISCUSSION

The spotted pod borer, *Maruca vitrata* Fab. (Lepidoptera: Crambidae) is one of the most important insect pests of cowpea. Management strategies against the pest are predominantly insecticide centric. However, the cryptic feeding nature of the larvae renders it difficult to manage the pest through chemical control. The use of the pods as fresh vegetable causes concern about the dependence on insecticide-based tactics. The need for an alternate and safe management has led to the exploration of biocontrol options, including the use of endophytic entomopathogens. Endophytic colonisation of fungi considered to be more advantageous than conventional methods because of its potential for cost effective and long term protection from insect pests.

In the above backdrop, the present study was undertaken to identify potential endophytic entomopathogenic fungi against cowpea pod borer, standardize the method of inoculation of endophytic fungi in cowpea plant and evaluate the effect of inoculated EEF against spotted pod borer. The results obtained in the different experiments are discussed in this chapter.

5.1 ISOLATION OF ENDOPHYTIC FUNGI

An attempt was made to identify potential fungal endophytes from cowpea plants in selected districts of Kerala. Isolation of endophytic fungi from accessions with varying degrees of resistance was also attempted.

5.1.1 Survey and isolation of endophytes from different geographical areas of Kerala

Survey was conducted in the major cowpea growing areas of Kozhikode, Thrissur, Kottayam and Thiruvananthapuram districts of Kerala. Plant samples were collected from ten organically grown plots or homesteads in each district at reproductive stage for isolation. A total of 235 endophytic fungi were isolated from different plant parts of cowpea.

Duing the survey, highest number of isolates was obtained from roots of cowpea (Figs 11 to 15). Results of fungal entophytes from plant samples of cowpea obtained during survey indicated that more than forty per cent of the endophtic fungi colonized the roots across all locations, followed by stem colonisation with 26.81 per cent (Fig. 16). According to Arnold *et al*. (2000) spatial heterogenity for fungal endophytes may be difficult to discern because stratum, substrate and host preferences confound spatial patterns.

In the present investigation, consistent site specific differences were observed in endophytic abundance. Roots and stems harbored more fungal endophytes than leaves and flowers. According to Yahr *et al.* (2006) fungal endophytes show geographic variation and endophytic fungal segregation was impacted by environmental differences.

Spatial variability in the distribution of endophytes has been documented by Arnold *et al*. (2001). Such spatial heterogeneity has been attributed to differences in abiotic factors such as temperature, humidity, rainfall as well as soil conditions (Photita *et al*., 2001).

Pimentel *et al*. (2006) studied the soybean microbiota in both field and green house grown plants and found that more isolates were obtained from plant parts that were more proximal to the ground in case of field grown plants than from parts farther from the soil. This difference was ascribed to the fact that infection in the field occurs mainly through the roots.

Zakaria *et al.* (2010), however, reported that they could obtain only seven isolates from roots of rice out of a total of 110 fungal isolates. In comparison, as much as 40 isolates were obtained from seeds, followed by leaf blade and leaf sheath with 32 and 21 isolates respectively.

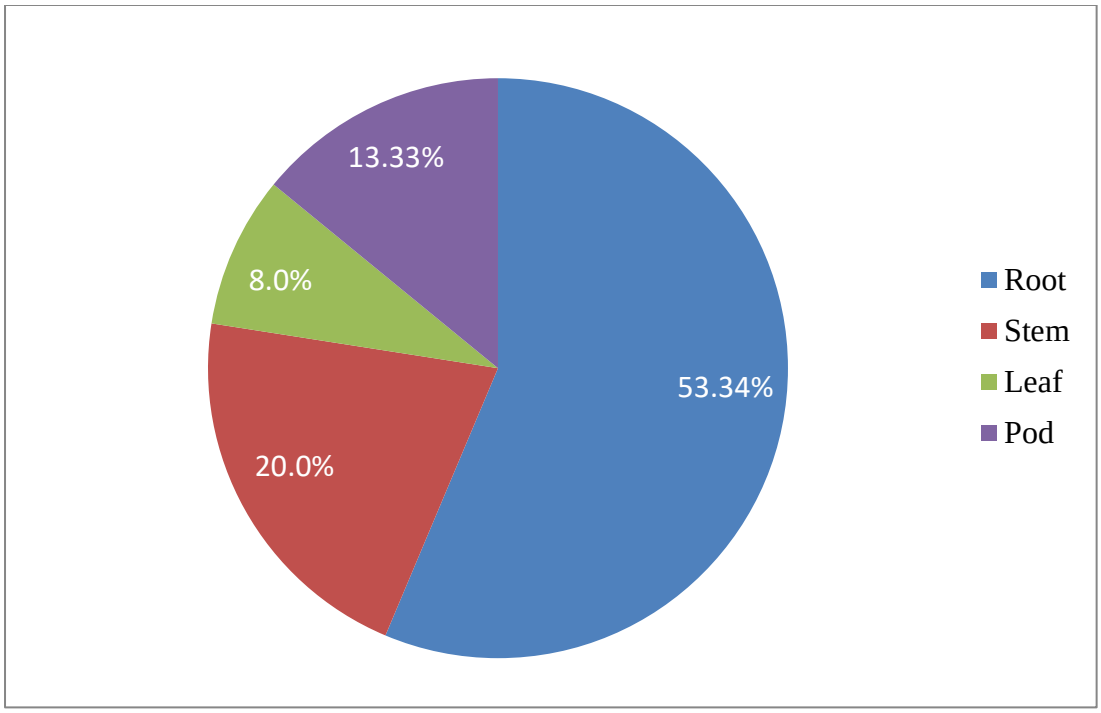


Fig. 11. Proportion of fungal endophytes obtained from Kozhikode district

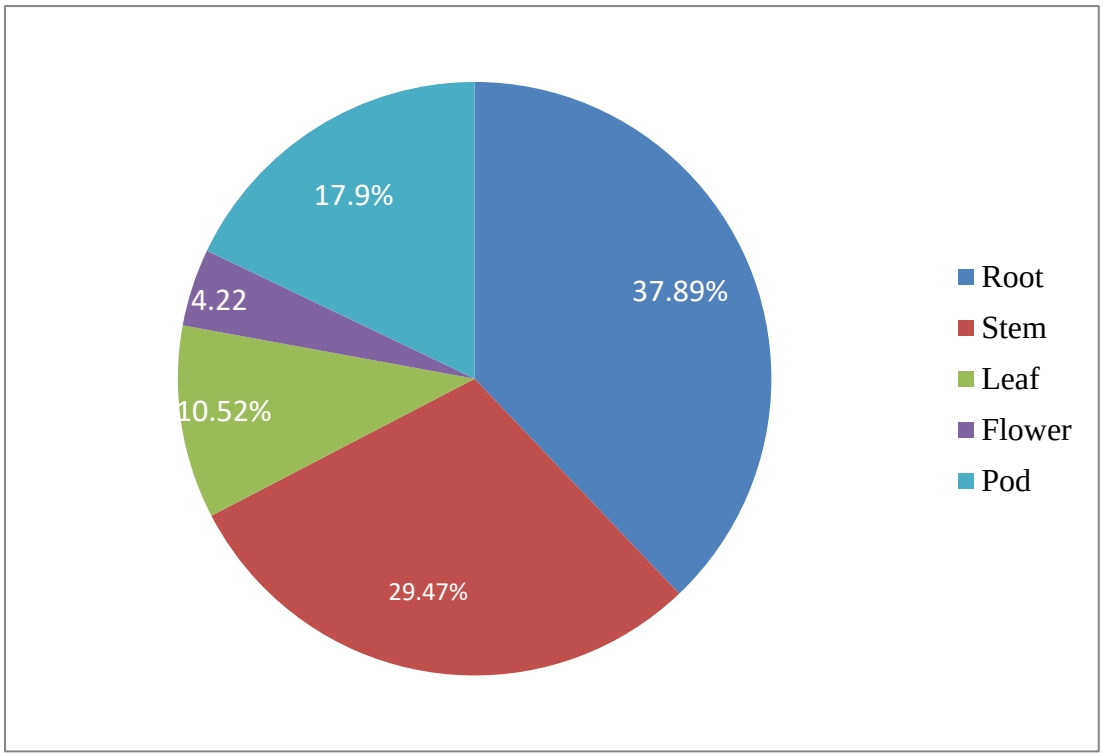


Fig. 12. Proportion of fungal endophytes obtained from Thrissur district

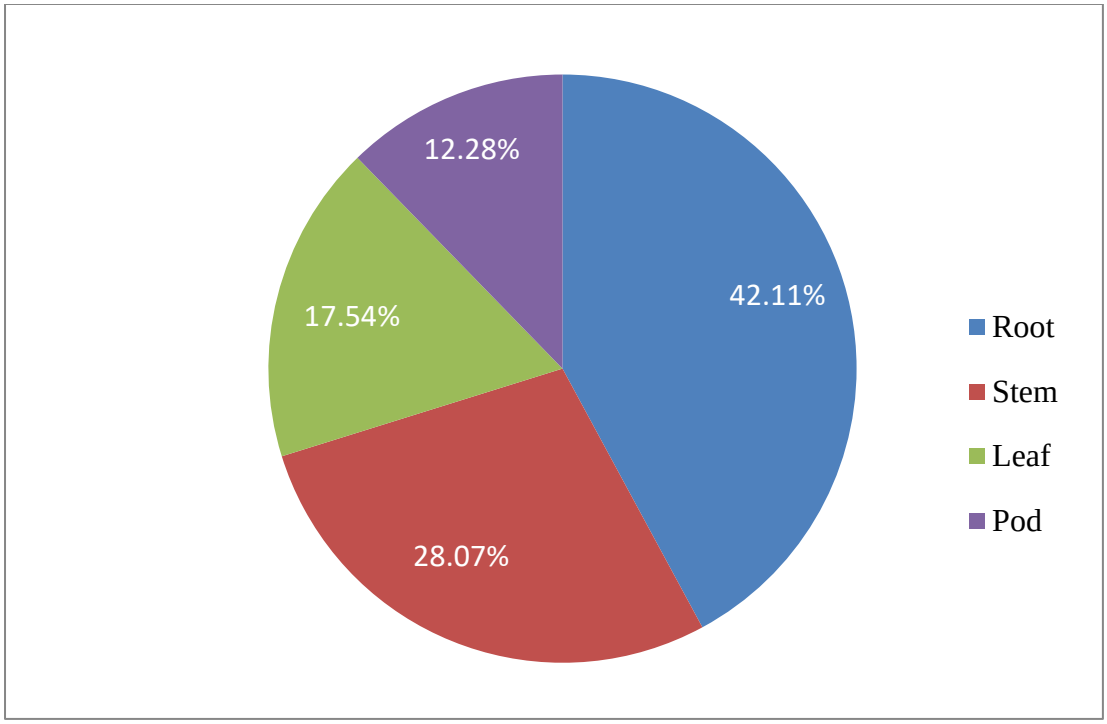


Fig. 13. Proportion of fungal endophytes obtained from Kottayam district

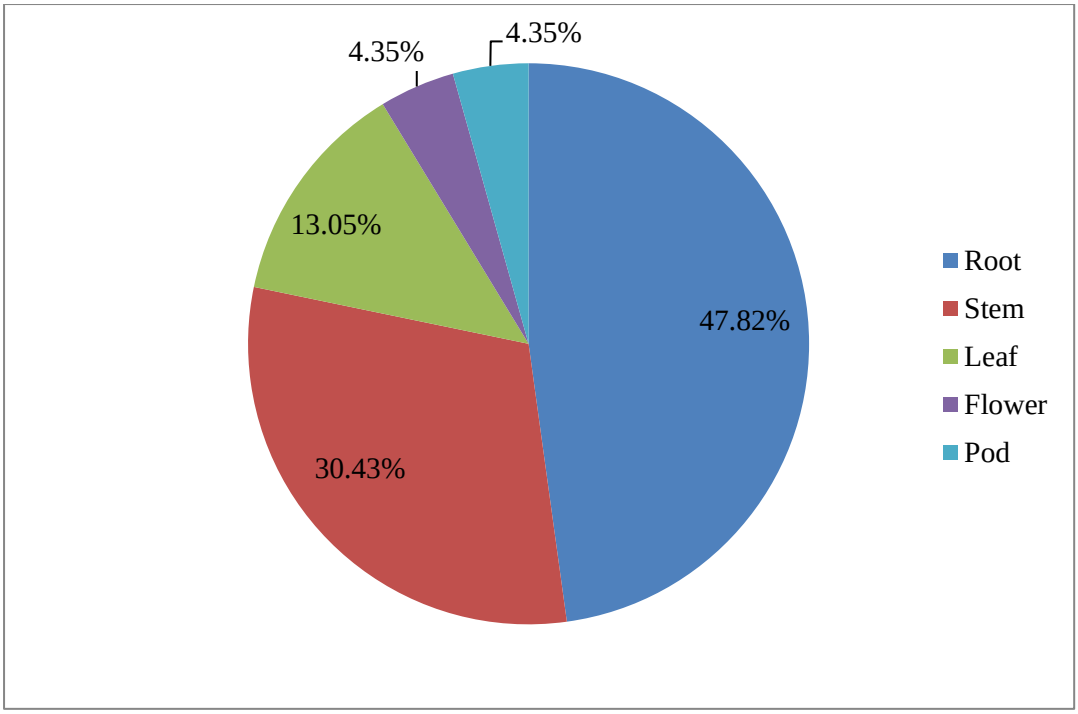


Fig. 14. Proportion of fungal endophytes obtained from Thiruvananthapuram district

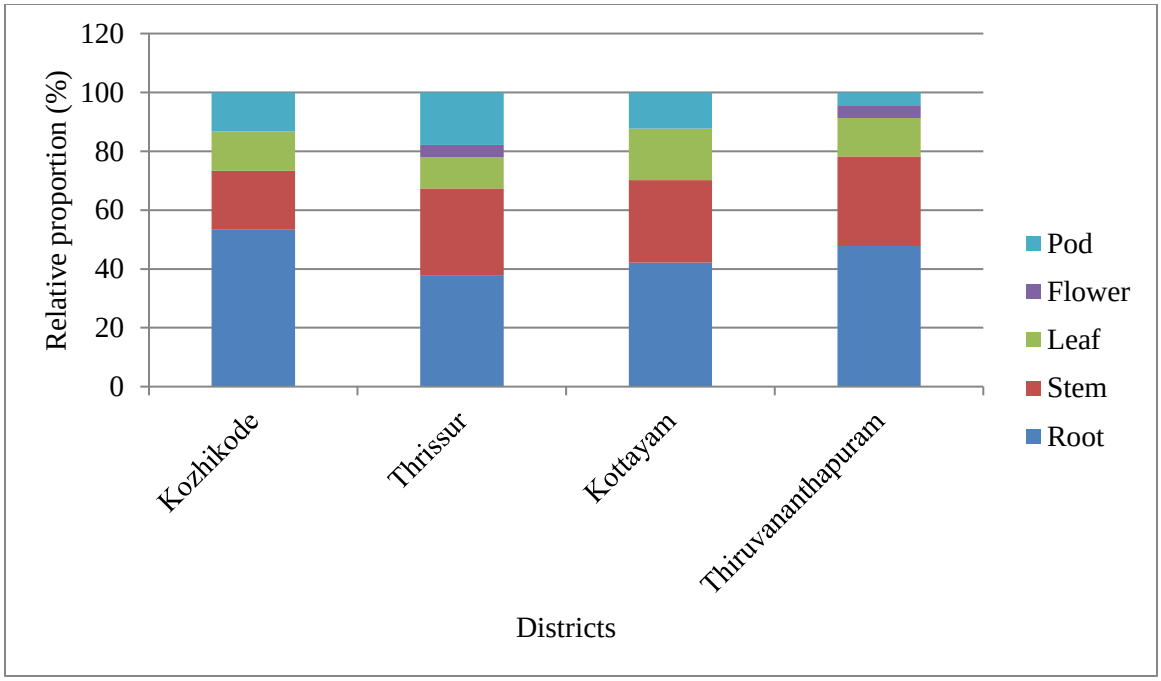


Fig. 15. Extent of colonization of plant parts by fungal isolates obtained during the survey

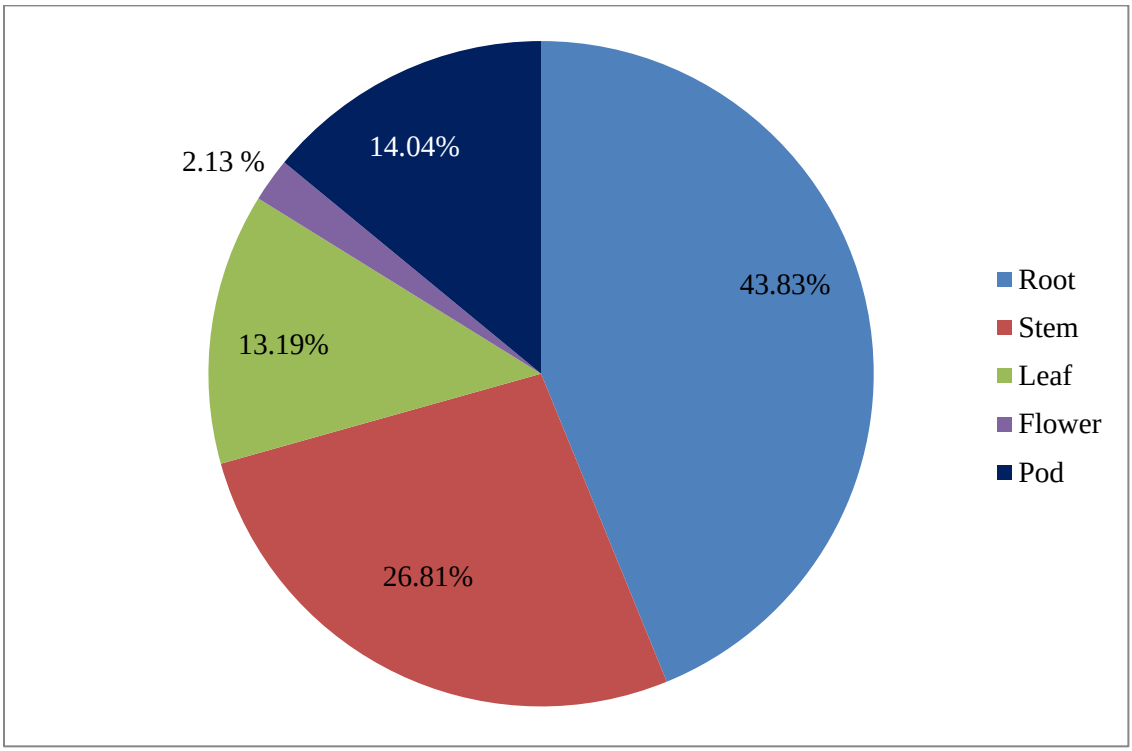


Fig. 16. Proportion of fungal isolates from different parts of cowpea plant obtained during the survey

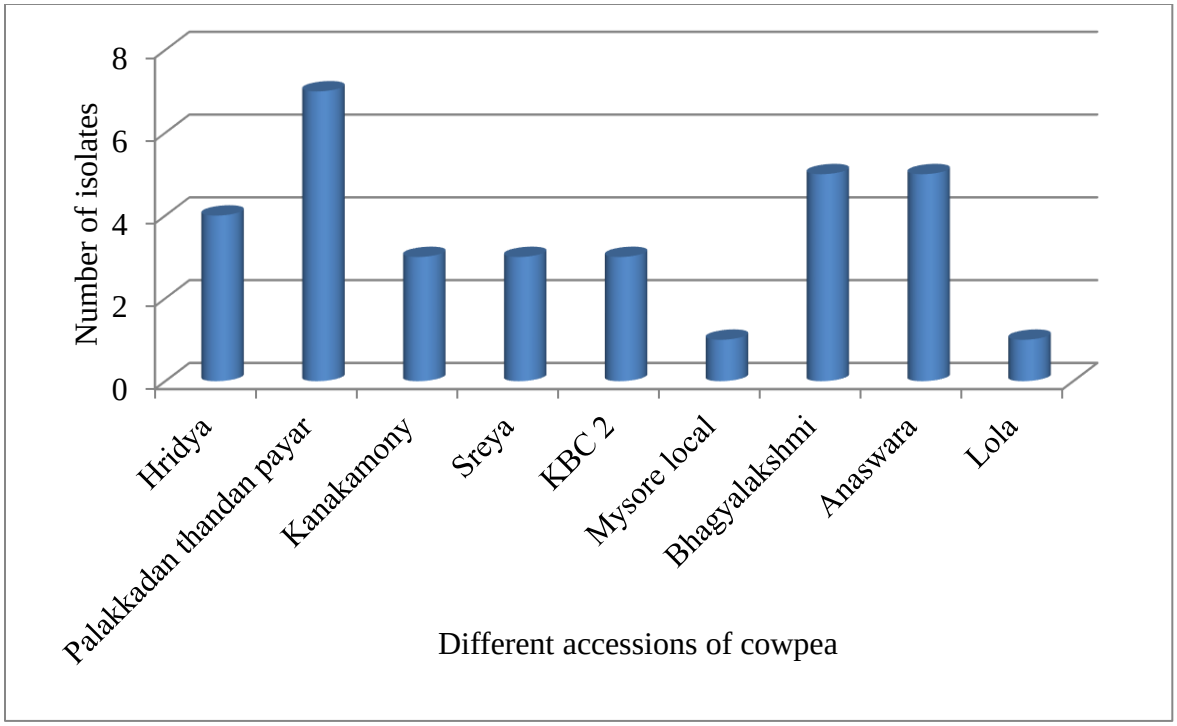


Fig. 17. Fungal isolates obtained from different accessions of cowpea

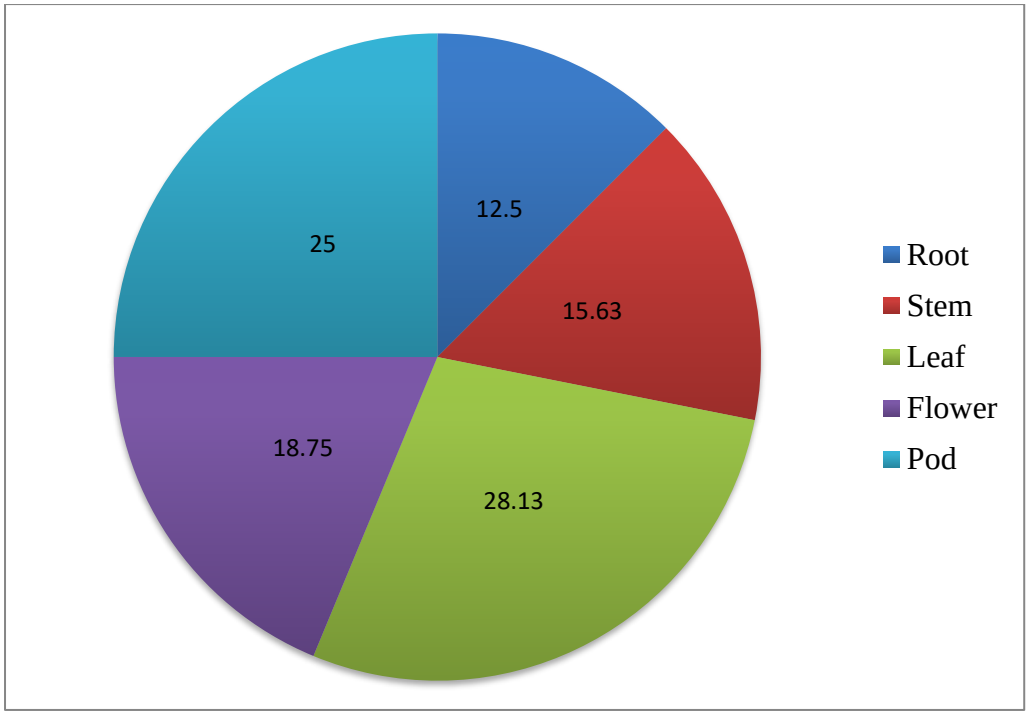


Fig. 18. Proportion of fungal endophytes from different parts of cowpea accessions

5.1.2 Isolation of endophytic fungi from different accessions of cowpea

All the nine accessions were found to harbor endophytic fungi to varying extents (Fig. 17 and 18). Among the nine accessions, Palakkadan thandan payar, a highly resistant variety yielded the highest number of seven endophytes followed by Bhagyalakshmi and Anaswara with five each.

The distribution of endophytic fungi within the plants of different accessions also showed variation. Unlike in case of field collected samples, leaves followed by pods on an average harbored more fungi than other plant parts. It might be due to the spatial homogenity. This finding was in conformity with Huang *et al.* (2001) who reported more number of endophytic fungi in leaf and stem samples (549 and 568) of medicinal plants *viz*., *Taxus mairei*, *Cephalotaxus fortunei* and *Torreya grandis*. Less number of isolates was obtained from other plant parts such as flowers (28) and roots (11).

5.2 EVALUATION OF BIOEFFICACY OF ENOPHYTIC FUNGAL ISOLATES

All the endophytic fungi isolated during the survey were tested for pathogenicity against third instar larvae of *Galleria mellonella*. The three (EEF 1, EEF 4 and EEF 64) out of 267 isolates were found to be pathogenic to the test insect. Fungal isolates were reisolated from the infected cadavers and compared with that of original isolates.

After preliminary bioassay, the above three isolates were evaluated for pathogenicity to target pest, *Maruca vitrata*. Second instar larvae of *M. vitrata* were inoculated with these three isolates and were reisolated from infected cadavers and proved the Koch's postulate. Identity of these isolates was confirmed by both morphological and molecular characterization.

5.3 CHARACTERIZATION OF ENDOPHYTIC ENTOMOPATHOGENIC FUNGAL ISOLATES

The above three fungal isolates which were shown to be pathogenic to pod borer were subjected to morphological and molecular characterization.

5.3.1 Morphological and cultural characterization

The identity of the three isolates was confirmed as *Fusarium oxysporum* (EEF 1) and *Purpureocillium lilacinum* (EEF 4 and EEF 64) with the aid of standard descriptors.

5.3.1.1 *Fusarium oxysporum*

The colony of *F. oxysporum* (EEF 1) was off white in colour during initial stages. But later changed to peach with a purple tinge. Growth of the fungus was fast in PDA and had floccose outgrowth.

The fungus produced both micro and macro conidia. Average size of macroconidia was 25.81 x 3.37 µm and microconidia were 5.48 x 2.99 µm. Hyphae were branched to unbranched and smooth walled. Phialides were simple to polyphialidic and variable in shape and size. These observations are in conformity with the characters of fungus described by Booth (1971) and Nelson *et al.* (1983).

5.3.1.2 *Purpureocillium lilacinum*

Colonies of EEF 4 and EEF 64 had lilac shade and showed moderate growth on PDA. Spores were formed from the ends of phialides in long chains. Spores were ellipsoidal to fusiform and smooth walled to slightly roughened. Size of conidia ranged from 2.5–3 × 2.0-2.2 µm with an average of 2.76 x 2.11 µm. Chlamydopsores were absent. Morphological characters of EEF 64 were similar to that of EEF 4 with slight variation. Average size of conidia of EEF 64 was 2.79 x 2.20 µm. The colony and spore

characteristics observed in the current study matched the description of the above genus by Luangsa-Ard *et al.* (2011).

The fungal isolates were further subjected to molecular characterization to confirm the identity.

5.3.2 Molecular characterization and phylogenetic analysis

The identity of above three isolates was confirmed through molecular characterization using Internal Transcribed Spacer (ITS) sequencing and phylogenetic analysis. Ghaffar *et al*. (2016) concluded that analysis of ITS regions give taxonomical evidence regarding the related organisms.

ITS sequencing of EEF 1 yielded 553 base pair (bp) sequences and Basic Local Alignment Search Tool (BLAST) at National Center for Biotechnology Information (NCBI) identified the same as *Fusarium* sp. Similarly the isolates, EEF 4 and EEF 64 with 454 and 550 base pair (bp) sequences were identified as *P. lilacinum*.

Zarrin *et al.* (2016) reported that ITS region augmentation with primers ITS 1 and ITS 4 produces nearly 550 bp band in all 50 isolates of *Fusarium*. Phylogenetic analysis based on ITS sequences helped to reveal the evolutionary relationship. In the present study the phylogenetic analysis confirms the identity of *F. oxysporum* and *P. lilacinum* and explicitly confirms the previous phylogenetic investigation (Luangsa-Ard *et al*., 2011; Nirmaladevi *et al*., 2016).

Based on the morphological, cultural, molecular characterization and phylogenetic analysis, the three fungal isolates were confirmed as *F. oxysporum* (EEF 1) and *P. lilacinum* (EEF 4 and 64).

5.3.3 Entomopathogenicity of endophytic fungal isolates

5.3.3.1 *Fusarium oxysporum* (EEF 1)

In the present study, the entomopathogenic isolate of *F. oxysporum* (EEF 1) was isolated from the pod of cowpea (Table 12). *Fusarium* has been reported to be one of the most common endophytic fungi recovered from plants and have been reported from rice, soybean and cowpea seeds (Pacin *et al.*, 2002; Rodrigues and Menezes, 2005). The entomopathogenic potential of different species of *Fusarium* has been well documented by Sharma and Marquez (2018).

Ali-Shtayeh *et al.* (2003) had reported the pathogenicity of *F. oxysporum* against larvae of *G. mellonella.* The pathogenicity of *Fusarium* has been attributed to insecticidal compounds like Beauvericin produced by the fungus. Several workers have isolated Beuvericin from *Fusarium* spp.

Gupta *et al*. (1991) isolated insect toxin, Beuvericin from *Fusarium semitectum* and *Fusarium moniliforme* var. *subglutinans.* Logrieco *et al.* (1998) also reported the production of insect toxin, Beuavericin by *Fusarium* spp. Jastoi (2008) revealed that various *Fusarium* spp. produced insect pathogenic toxins like enniatins, belonging to the enniatin antibiotic family. Waweru *et al.* (2014) reported that the endophytic colonisation by the fungus *F. oxysporum* can result in increased host resistance to pests and diseases as well as greater biomass production.

5.3.3.2 Isolates of *Purpureocillium lilacinum* (EEF 4 and EEF 64)

In this study, the entomopathogenic isolates of *P. lilacinum* (EEF 4 and EEF 64) were isolated from stem and root of cowpea (Table 12). Schulz *et al.* (1998) had reported the presence of the genera *Paecilomyces* and *Cladosporium* in barley and other plants. The fungus *P. lilacinum* (=*P. lilacinus*) has been mainly considered a nematophagous, egg-parasitizing fungus. However, several scientists have reported of it being pathogenic

to insects including *Aphis gossypii, Ceratitis capitata* and *Triatoma infestans* (Marti *et al*., 2006; Imoulan, 2011; Wakil *et al*., 2012). Ek-Ramos *et al.* (2013) carried out a survey to isolate endophytic fungi from upland cotton (*Gossypium hirsutum*) to find out potential biocontrol agents and isolated *P. lilacinum* among different species of biocontrol agents. Lopez (2015) reported the pathogenic potential of *P. lilacinum* to cotton aphid (*Aphis gossypii*) and boll worm (*Heliothis zea*) infesting cotton.

5.4 PLANT PATHOGENICITY TEST

The above fungal isolates were tested for plant pathogenic effect against cowpea following standard procedures. None of the isolates, including *F. oxysporum* were found to be pathogenic to cowpea. This finding is in agreement with the findings of Kuruvilla and Jacob (1978) who reported the non-infectivity of *F. oxysporum* to tomato, cotton and rice. Hareendranath *et al.* (1987) reported the non pathogenic effect of entomopathogen, *F. pallidoroseum* on okra, rice, tomato and chillies. The absence of any adverse effects by the endophytes on the host plant is an encouraging indication for assessing their beneficial role as endophytes. However, *F. oxysporum* was not used in further studies as it may cause disease in favourable condition.

5.5 EVALUATING THE BIOEFFICACY OF ENTOMOPATHOGENIC ENDOPHYTIC FUNGAL ISOLATES

Bioefficacy of the native endophytic entomopathogenic fungal isolates *viz*., EEF 4 and EEF 64 of *P. lilacinum* along with *Beauveria bassiana* (NBAIR strain) to the second instar larvae of *M. vitrata* was assessed through contact toxicity bioassay at different concentrations *viz*., 10^5, 10^6, 10^7, 10^8 and 10^9 spores ml^{-1} for further studies.

The pathogenic nature of the three entomopathogenic fungi to *M. vitrata* was confirmed through the bioefficacy studies. *B. bassiana* consistently recorded higher mortality as compared to the *P. lilacinum* isolates throughout the study period. The mortality also showed a positive linear relationship with spore concentration, with highest

mortality occurring consistently at the highest concentration of 10^9 spores ml^{-1} throughout the study period.

Mortality also increased with time of exposure to the EEF. Thus, *B. bassiana* applied at the rate of 10^9 spores ml^{-1} recorded highest mean mortality of 20.00 per cent among all treatments two days after treatment, which increased to 50.00, 66.67, 67.14 and 70.71 per cent at 4^{th}, 6^{th}, 8^{th} and 10^{th} day respectively. A similar trend was discernable in case of the two *P. lilacinum* isolates as well. The highest mortality recorded by EEF 4 for the corresponding period was 10.00, 26.67, 50.00, 50.33 and 57.23 per cent respectively, all resulting at a spore concentration of 10^9 spores ml^{-1}. In comparison, mortality at the highest concentration of 10^9 spores ml^{-1} ranged from 3.33 to 50.50 per cent in case of EEF 64 (Fig. 19).

Several authors have reported the entomopathogenic potential of *B. bassiana* against lepidopteran insect pests. Sivasanakran *et al*. (1990) reported mortality of third instar larvae of *Chilo infuscatellus* ranging from 68.53 to 75.93 per cent on exposure to *B. bassiana* at $1x10^7$ spores ml^{-1}.

Jayanthi and Padmavathiamma (2001) had evaluated the bioefficacy of *B. bassiana* against *Spodoptera litura* and recorded mortality of 96.67, 80.67, 80.00, 70.67 and 61.33 per cent respectively for first, second, third, fourth and fifth instar larvae at a concentration of $1x10^9$ spores ml^{-1}.

Purpureocillium lilacinum though considered as a nematophagous, egg parasitizing fungus (Lopez *et al*., 2014), it has also been shown to be pathogenic to insects. Johny *et al.* (2012) isolated and characterized *P. lilacinum* associated with the emerald ash borer, *Agrilus planipennis*. The fungus caused 50.00 per cent mortality of adult beetles in the laboratory when applied at a concentration of $2x10^7$ spores ml^{-1}.

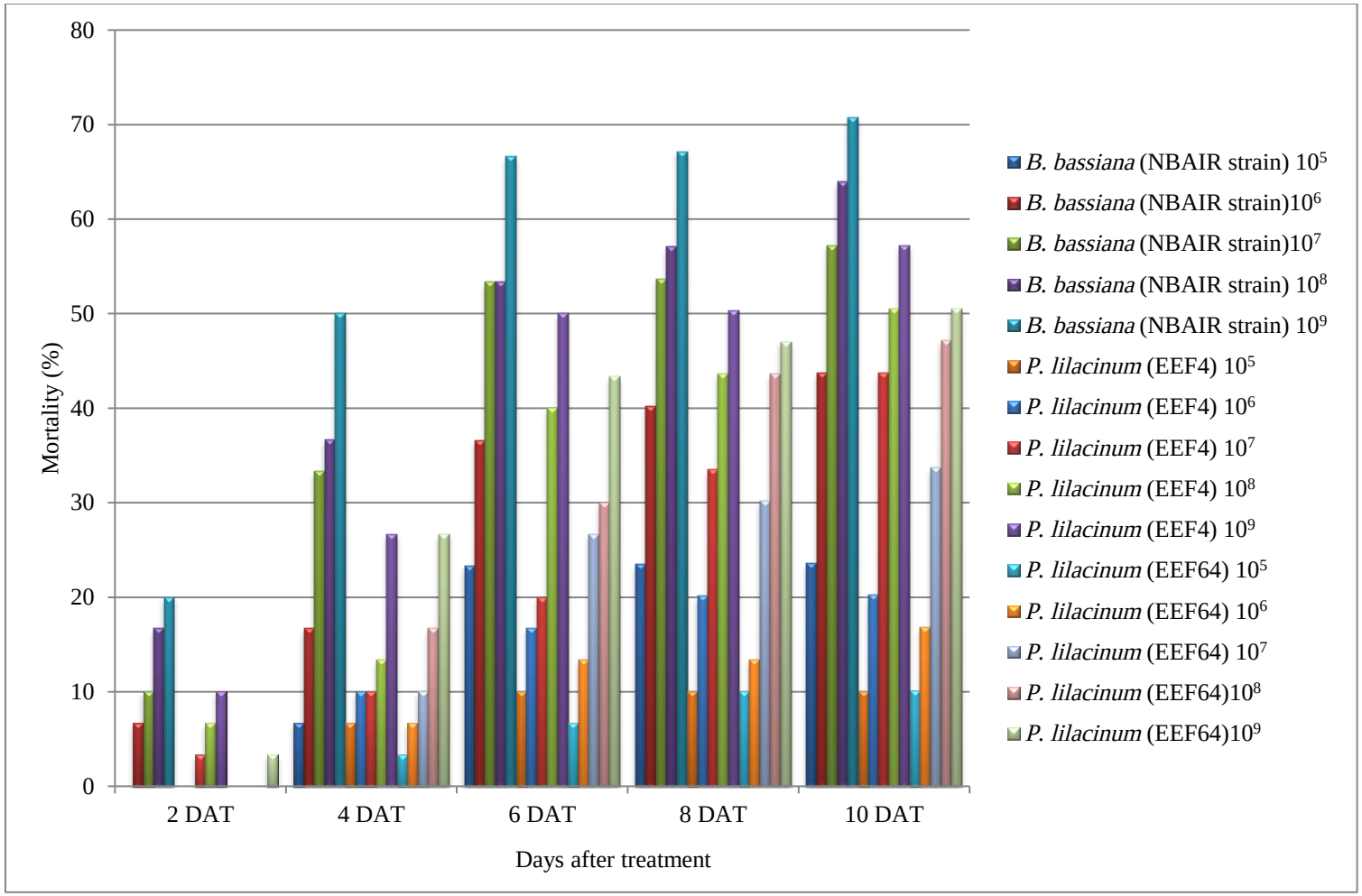


Fig. 19. Bioefficacy of fungal isolates against second instar larvae of *Maruca vitrata*

5.6 STANDARDIZATION OF THE INOCULATION TECHNIQUE FOR ENDOPHYTIC COLONISATION

The best two organisms identified in the bioefficacy test *viz*., *B. bassiana* (NBAIR strain) and *P. lilacinum* (EEF 4) were used for endophytic colonisation in cowpea through seed, soil and foliar inoculation methods. Colonisation by the fungal isolates was confirmed by re-isolation and molecular methods.

5.6.1 Re-isolation

Colonisation study demonstrated that *B. bassiana* and *P. lilacinum* were capable of endophytic colonisation in cowpea plants to varying degrees depending on the method of colonisation employed. Variability was also observed with regard to the nature of plant parts that the fungi chose to colonize.

5.6.1.1 Colonisation of fungal endophytes in stem tissues of cowpea

Both *B. bassiana* (NBAIR strain) and *P. lilacinum* (EEF 4) could colonize different parts of cowpea plant irrespective of method of inoculation except *B. bassiana* as soil application in young stem tissues (Fig. 20 and 21).

Beauveria bassiana showed highest extent of 36.11 per cent colonisation of young tissues 30 days after foliar inoculation. The mean colonisation was significantly lower with value of 8.33 per cent when inoculated through seeds, and the fungus failed to colonize younger stem tissues through soil inoculation. In older stem tissues, the colonisation of *B. bassiana* was highest at 16.67 per cent through foliar inoculation 55 days after sowing, followed by 11.11 per cent through seed inoculation. The mean colonization was high in foliar inoculated plants with a value of 9.26 per cent. On the whole, *B. bassiana* appeared to prefer younger stem tissues for colonisation irrespective of the inoculation method.

Purpureocillium lilacinum, showed relatively greater degree of colonisation of younger stem tissues of cowpea, irrespective of method of inoculation. As much as 75.0 per cent of samples were colonized 55 days after sowing when inoculated through leaves, though colonisation was significantly lower through both seed and soil inoculation. The mean colonization was also high in foliar inoculated plants with a value of 62.04 per cent.

Purpureocillium lilacinum also showed greater colonisation of older stem tissues as well, when compared to *B. bassiana*, with a value of 55.56 per cent at 55 days after sowing through both seed and foliar inoculation. *P. lilacinum* as foliar application registered higher value for mean colonisation (40.74%).

5.6.1.2 Colonisation of fungal endophytes in leaf tissues of cowpea

Colonisation of young leaves by *B. bassiana* varied from 7.41 to 24.08 per cent, foliar inoculation once again recording the highest value. No colonisation was observed in plants subjected to soil inoculation with *B. bassiana* (Fig. 22). Mean colonisation of young leaves by *P. lilacinum* was higher compared to *B. bassiana*, with 15.74 to 24.08 per cent of young leaves harbouring the endophyte.

Colonisation of old leaves by *B. bassiana* ranged fom 1.85 to 10.50 per cent, foliar inoculation once again recording the highest value. No colonisation in old leaves was observed when plants were subjected to soil application with *P. lilacinum*. Mean colonisation values were ranged from 1.82 to 2.59 per cent (Fig. 23).

5.6.1.3 Colonisation of fungal endophytes in root tissues of cowpea

Colonisation by *B. bassiana* in roots ranged from 3.40 to 9.26 per cent with soil inoculation resulting in highest colonization (Fig. 24).

Purpureocillium lilacinum proved to be significantly superior as a root endophyte, having colonized 20.37 to 31.48 per cent of samples analysed. Greater degree of colonisation was observed through foliar and soil inoculation compared to seed treatment.

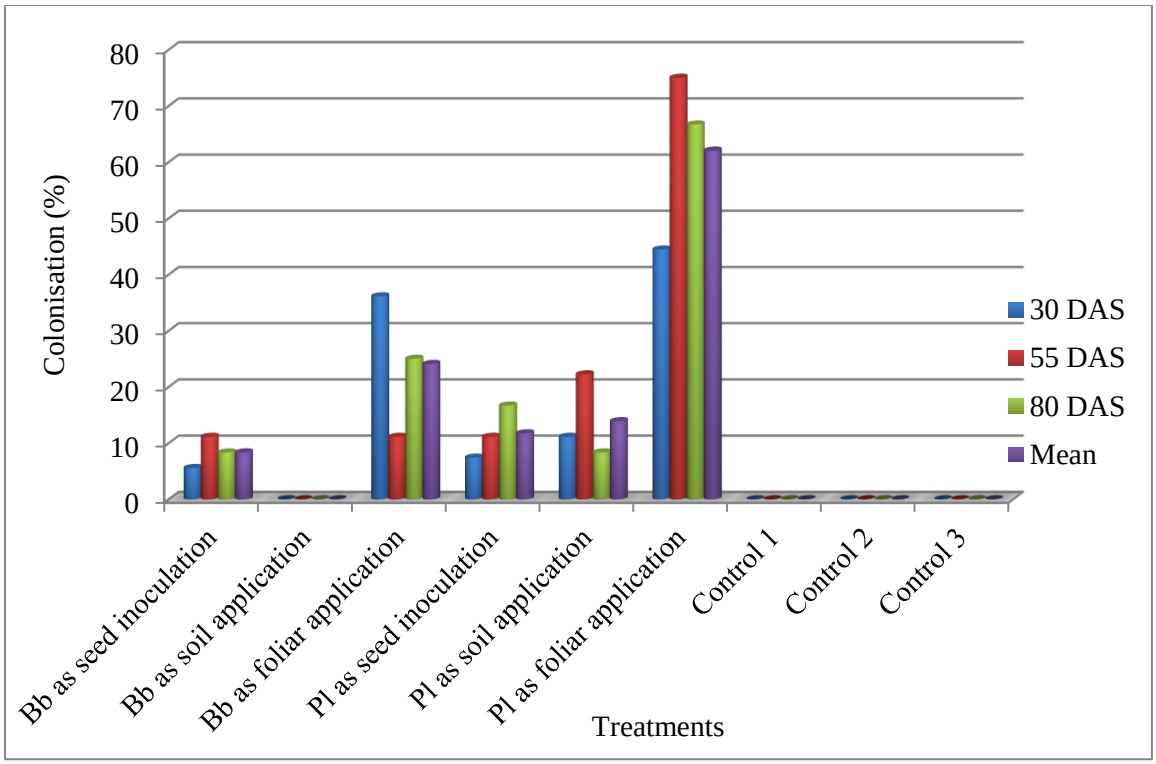


Fig. 20. Colonisation of fungal endophytes in young stem tissues of cowpea

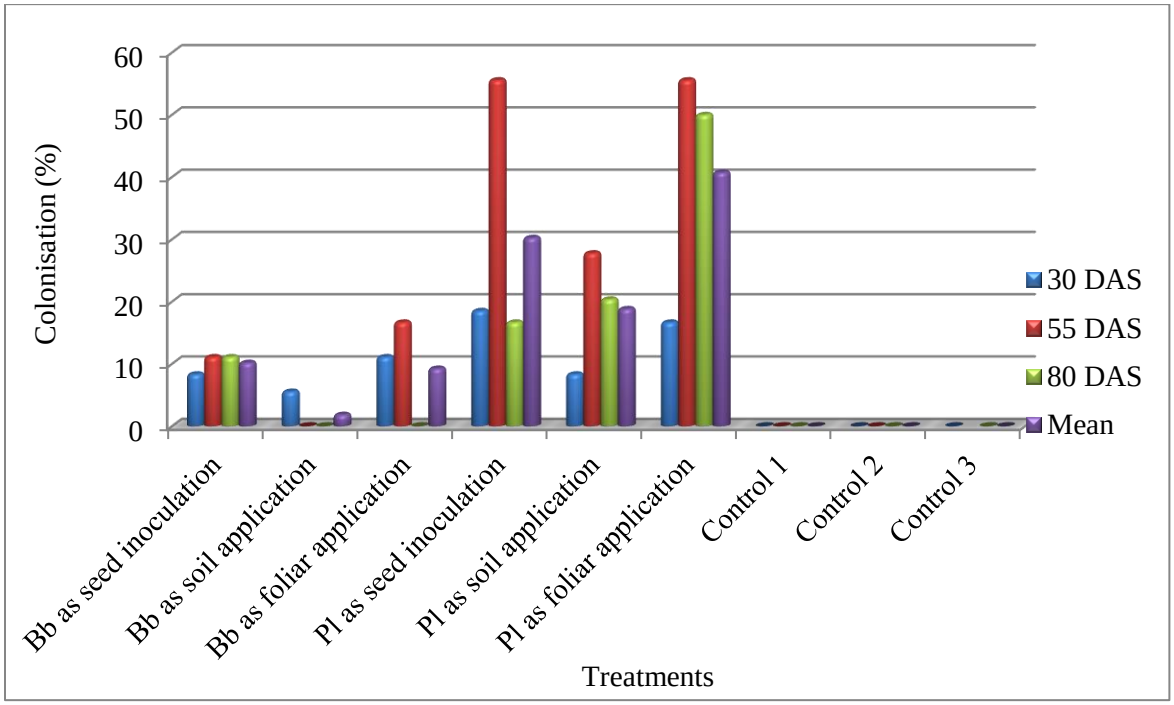


Fig. 21. Colonisation of fungal endophytes in old stem tissues of cowpea

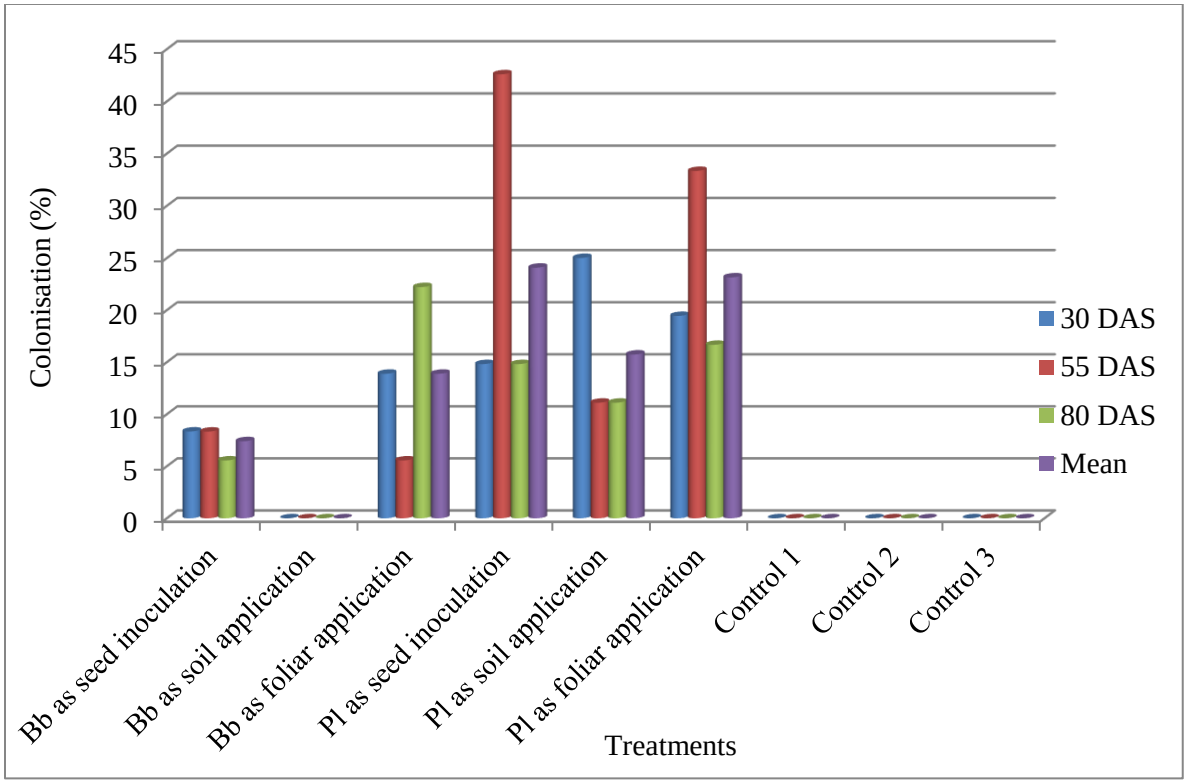


Fig. 22. Colonisation of fungal endophytes in young leaf tissues of cowpea

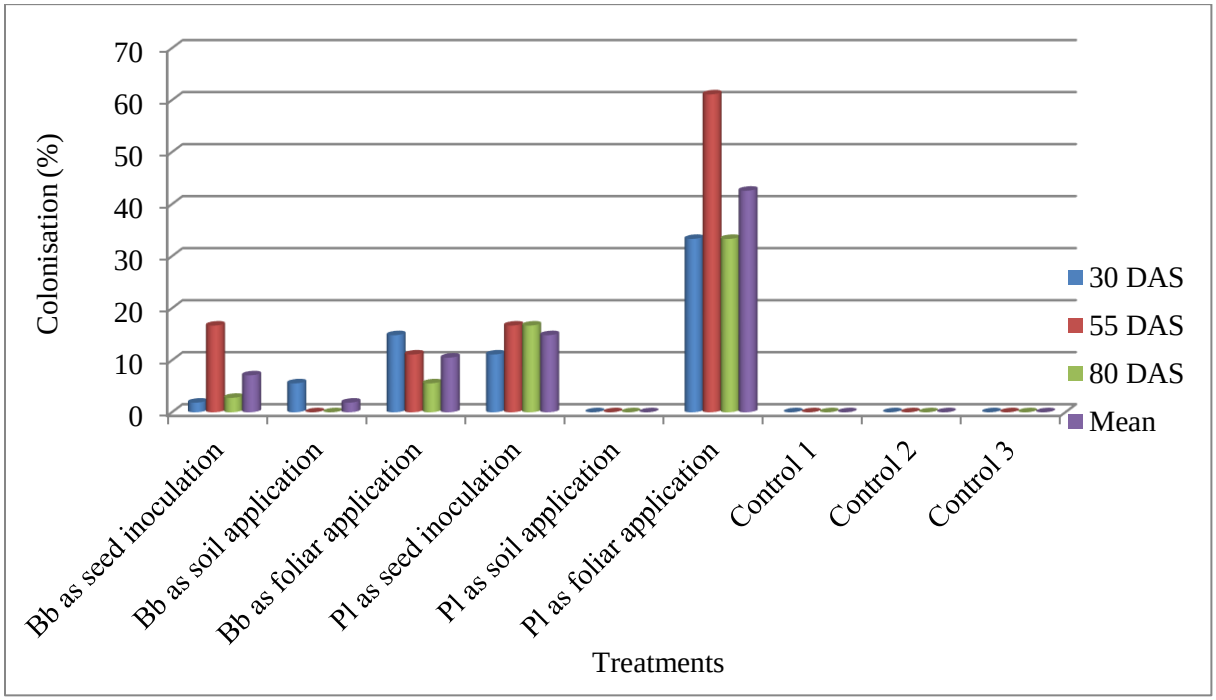


Fig. 23. Colonisation of fungal endophytes in old leaf tissues of cowpea

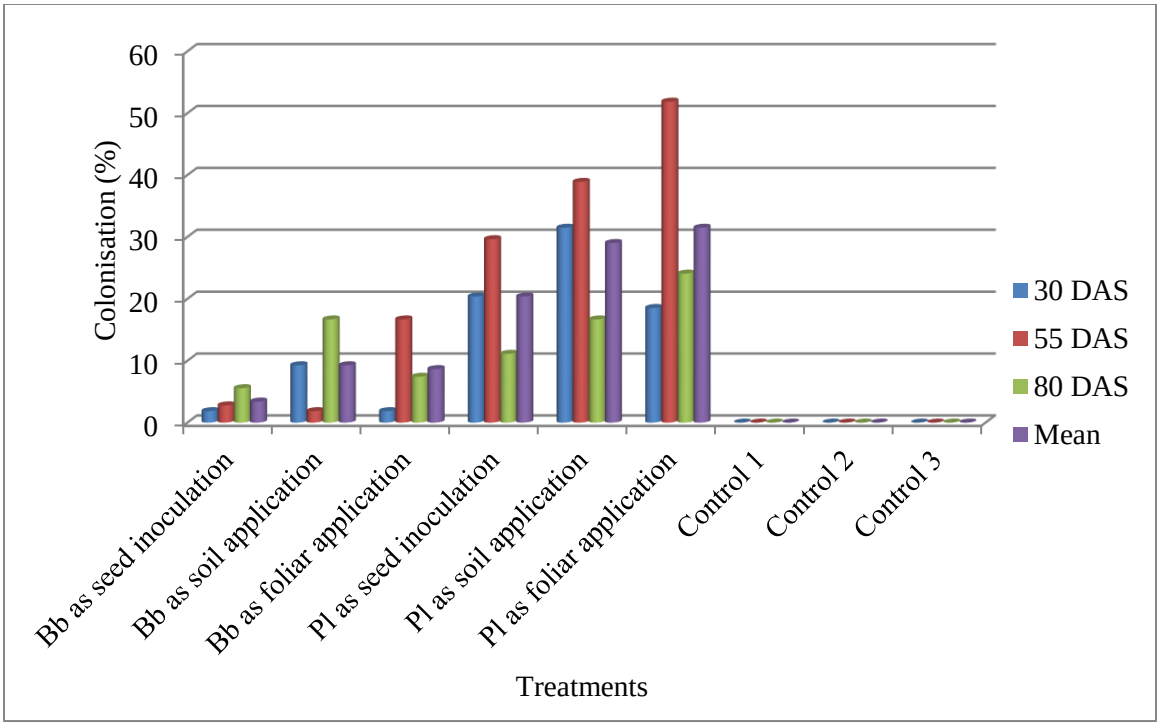


Fig. 24. Colonisation of fungal endophytes in root tissues of cowpea

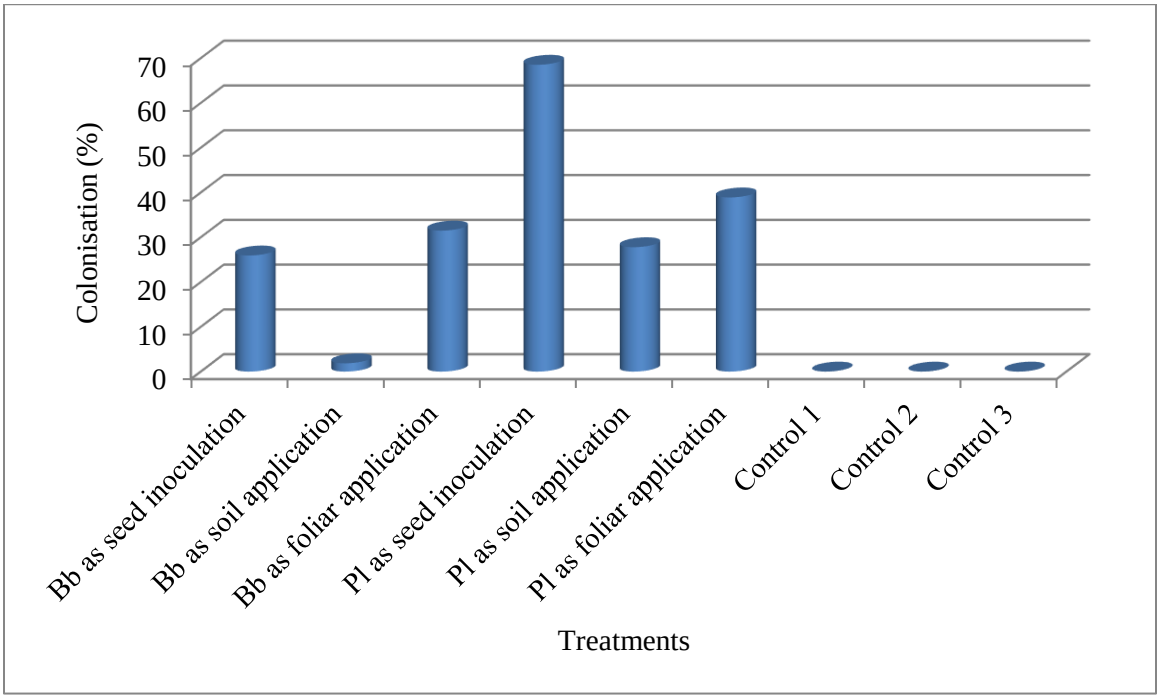


Fig. 25. Colonisation of fungal endophytes in pod tissues of cowpea

In both *B. bassiana* and *P. lilacinum* treated plants, soil and foliar application recorded higher colonisation in root tissues than seed treatment. The reason for the low endophytic colonisation in root tissues with seed inoculation is not clear and requires further investigation.

5.6.1.4 Colonisation of fungal endophytes in pod tissues

Colonisation by *B. bassiana* in pods, when inoculated through soil and leaves averaged 25.93 and 31.48 per cent respectively (Fig. 25), though soil inoculation resulted in low level of endophytic establishment in pods (1.85%). Mean colonisation by *P. lilacinum* varied from 27.78 to 68.52 per cent. Foliar inoculation resulted in highest colonisation and was followed by seed inoculation (38.89%).

The present study confirmed the ability of the two selected entomopathogenic fungi for endophytic colonisation and also helped to standardize the best method of inoculation through inoculation by three different methods followed by reisolation at different phases of plant growth. Throughout the experiment, foliar inoculation of fungal isolates was found to colonise different parts of plant to a greater extent.

5.6.2 Molecular technique

PCR products of re-isolated fungi were compared with that of original isolates. The PCR technique confirmed the colonisaton of respective fungal isolates in different parts of cowpea plant (Plates 31 to 36).

Reisolation and PCR technique confirmed the colonization of both fungal isolates in cowpea plants. Among the two microbes, *B. bassiana* has been reported as an endophyte in an array of crops such as maize (Cherry *et al*., 2004; Renuka *et al*., 2016), tomato (Leckie, 2002), cotton (Ownley *et al*., 2004), banana (Akello *et al*., 2008) and coffee (Vega *et al*., 2008). Studies on endophytic ability of *P. lilacinum* however, have

been limited, though Lopez (2015) reported the successful colonisation of a native isolate of *P. lilacinum* in cotton plants.

Foliar inoculation consistently resulted in higher colonization of different plant parts by both *B. bassiana* and *P. lilacinum* except in case of colonisation of pods by *P. lilacinum*, where seed inoculation proved to be superior. Several studies support the above results of our study. Our results are entirely in agreement with the finding of Tefera and Vidal (2009), who reported foliar application to be the best method for inoculating *B. bassiana* in sorghum plants. Resquín-Romero *et al*. (2016) sprayed leaves of melon, alfalfa and tomato plants with suspensions of different strains of *B. bassiana* and *M. brunneum* and all the fungal strains were able to successfully colonize the leaves, stems, and roots of the three host plants to the extent of around 40.0 per cent.

Soil inoculation of *B. bassiana* and *P. lilacinum* caused low colonisation in stem, leaf, root and pod tissues of cowpea. Kessler *et al.* (2003) reported the influence of abiotic and biotic soil factors on the occurrence of the entomopathogenic fungus *B. brongniartii* after application.

The two fungi varied considerably in the level of colonisation and persistence in different plant tissues. *B. bassiana* appeared to prefer younger tissues of stem and leaves with plant colonisation, in general, being highest up to 55 DAS. *P. lilacinum* proved to be far more proficient as an endophyte, with 40.0 to 50.0 per cent colonisation in different tissues. Age and tissue based variations in colonisation by endophytes have been reported by several authors such as, Posada *et al*. (2007), Vega *et al*. (2008) and Gurulingappa *et al*. (2010). The observation by Akutse *et al*. (2013) that endophytic colonisation of *B. bassiana* depends upon the inoculation methods, fungal isolates and plant species is supportive of the findings in the present study. Similarly, Liang-Dong *et al*. (2008) reported that endophytic fungi showed tissue specificity because they are adapted to particular conditions present in the given plant part.

The recovery from plant tissues also suggested that the endophytes were capable of movement within the plant though the extent and nature of such movement was not studied. Re-isolation of a systemic fungus from plant parts distant from the site of inoculation indicates the systemic colonization. Wagner and Lewis (2000) reported the passive movement of *B. bassiana* in the plant through xylem vessels. The present results are in agreement with Akello *et al.* (2007) who reported the endophytic potential of different strains of *B. bassiana* in tissue cultured banana plants and colonization in different parts after plant dip method.

The presence of fungal endophytes in young stem and leaf tissues 80 days after sowing is encouraging from a pest management perspective. Equally noteworthy is the fact that fungal endophyte colonized pods to the highest extent which is the most critical concern for pod borer management.

5.6.3 Effect of fungal colonisation on growth and yield parameters under polyhouse condition

5.6.3.1 Growth parameters

The effect of fungal colonisation on plant growth was determined by measuring the number of leaves per plant and height of plant (Fig. 26 and 27).

Significant difference was observed among different treatments at 40 and 60 days after sowing with regard to number of leaves. Seed inoculation with *B. bassiana* consistently recorded the highest number of 39.93 and 54.93 leaves at 40 and 60 days after sowing respectively. It was, however, on par with foliar application of *B. bassiana*. Eighty days after sowing, no significant difference was observed between the treatments in terms of number of leaves. The present study confirmed that there was no negative influence on the number of leaves of cowpea plants due to treatment with endophytes.

An earlier study by Lopez and Sword (2015), had reported a positive influence of seed treatment of *B. bassiana* and *P. lilacinum* on cotton dry biomass and number of nodes.

Inoculation with endophytes had little impact on the height of plants with all the treatments being at par. Plants inoculated through soil application of *P. lilacinum* had registered the highest plant height with higher values of 121.33 cm, 184.87 cm and 221.33 cm, respectively among all the treatments at 40, 60 and 80 days after sowing.

Results of the current study suggest that that endophytic colonisation in cowpea has little adverse impact on plant growth and could even be promoting it. Reports of growth promotion by endophytic fungal entomopathogens have been previously documented by several authors such as Gurulingappa *et al*. (2010), Sasan and Bidochka (2012), and Lopez and Sword (2015).

Garcia *et al*. (2011) reported that the soil inoculation with Ma-8 and Ma-10 strains of the entomopathogenic fungus, *M. anisopliae* recorded an average increase of 48.0 and 33.2 per cent in height and shoot dry weight of tomato plants respectively, when compared with untreated control plants.

Jaber and Araj (2017) evaluated the effect of colonisation by *B. bassiana* and *Metarhizium brunneum* and recorded a positive influence of endophytic colonisation by both the fungi. Treatment with *B. bassiana* (NATURALIS$^{@}$) led to an increase in plant growth parameters of sweet pepper, *Capsicum annum* at seven days post inoculation. *B. bassiana* recorded an increase in shoot height, leaf number, root length, fresh shoot weight and fresh root weight by 20.00, 5.56, 13.85, 12.5 and 33.00 per cent respectively than the untreated plants. While *M. brunneum* (BIPESCO5$^{@}$) had also recorded an increase by 23.00, 2.86, 13.85, 12.50 and 20.00 per cent respectively for the corresponding parameters, when compared to the control plants.

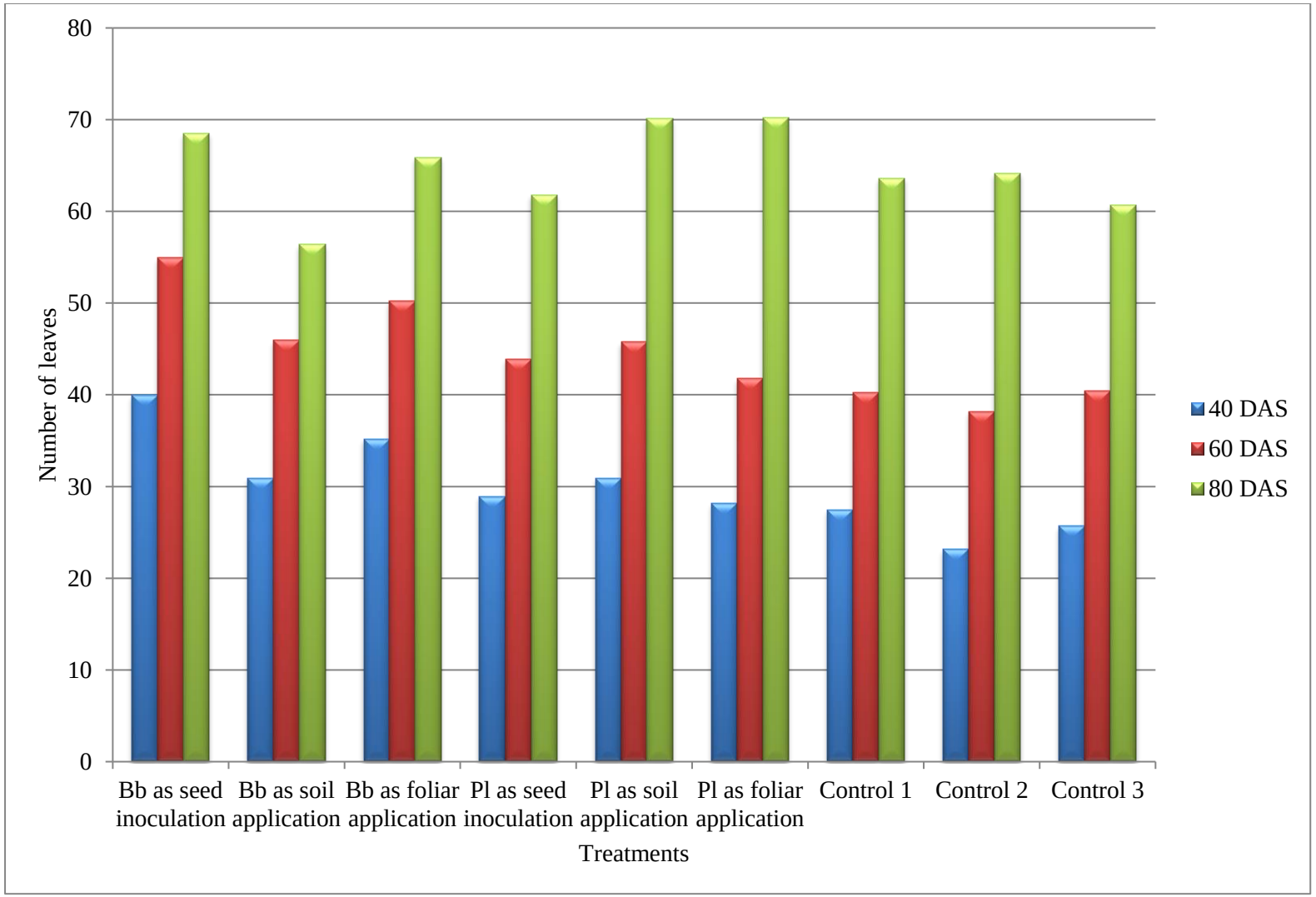


Fig. 26. Effect of fungal colonization on number of leaves of cowpea under polyhouse condition

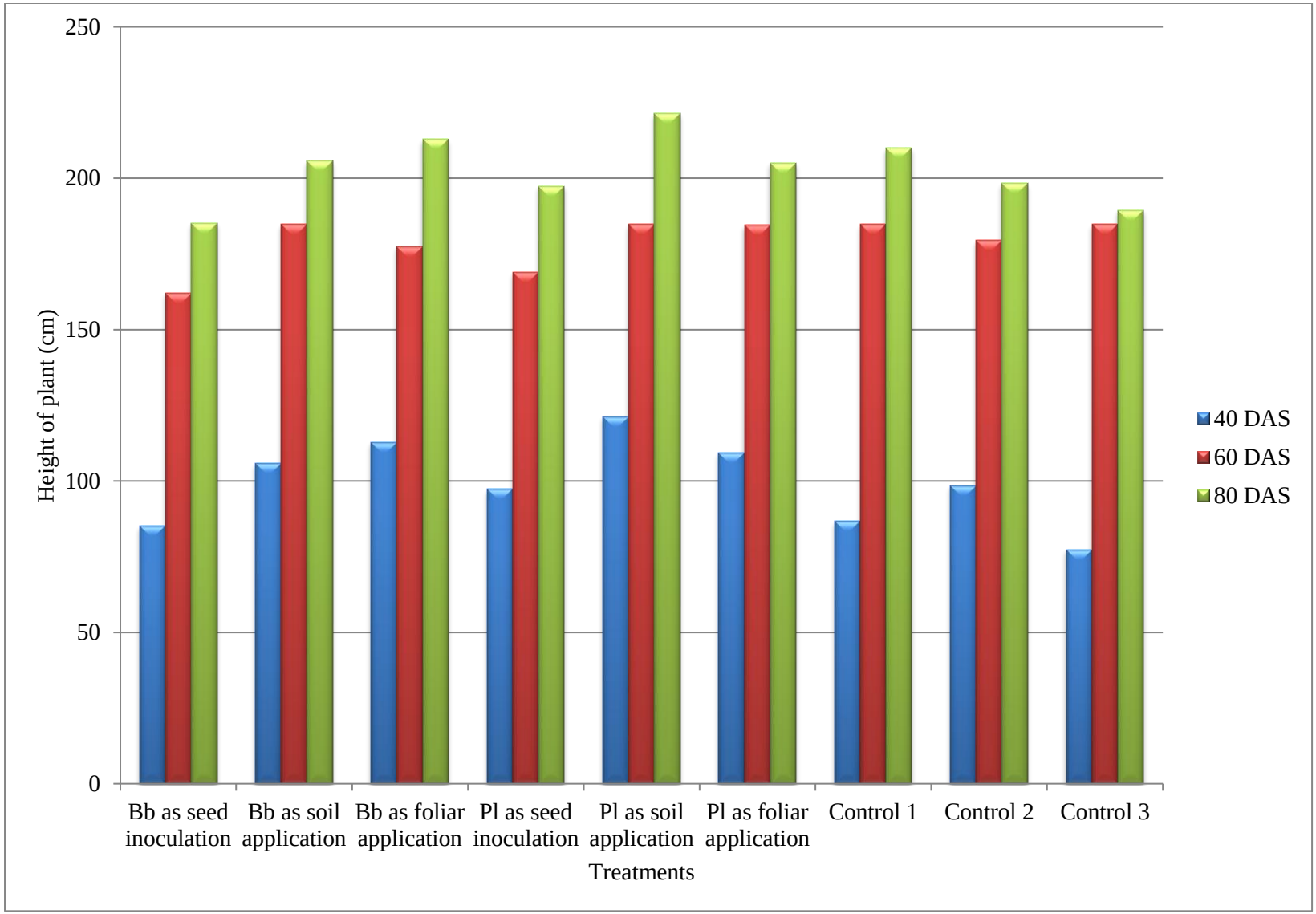


Fig. 27. Effect of fungal colonization on height of cowpea under polyhouse condition

5.6.3.2 Pod yield per plant

In polyhouse study, plants inoculated with *B. bassiana* through seed recorded the highest pod yield of 171.20g plant^{-1}, and was on par with leaf as well as soil inoculation registering 157.70 and 150.00g pods plant^{-1} respectively (Fig. 28).

Treatments involving *P. lilacinum*, with yields ranging from 134.13 to 142.40g plant^{-1} which again were on par with each other and also with the control treatments.

The results on pod yield underlined the earlier observation that inoculation with endophytic entomopathogenic fungi had little adverse effect on the growth and yield of cowpea.

This result confirms the research findings of Sanchez-Rodriguez *et al.* (2017), who applied *B. bassiana* on wheat plants using three inoculation methods *ie.,* soil treatment, seed inoculation and leaf spraying and obtained increased grain yield by about 40.0 per cent in addition to negative effect on cotton leafworm (*Spodoptera littoralis*).

5.7 EFFECT OF COLONISATION ON GROWTH, YIELD AND INFESTATION OF SPOTTED POD BORER IN COWPEA UNDER FIELD CONDITION

After standardizing the inoculation methods through polyhouse studies, the effect of fungal colonisation on infestation of spotted pod borer in cowpea was assessed through pot culture experiment under field condition. The effect on growth and yield parameters were also studied.

5.7.1 Effect of fungal colonisation on growth parameters of cowpea under field condition

The effect of fungal colonisation on plant growth was evaluated by measuring height and number of leaves per plant (Fig. 29 and 30).

Significant difference in the total number of leaves was observed in vegetative stage of crop growth, when cowpea plants seed inoculated with *B. bassiana* had significantly higher number of 68.73 leaves. This was followed by seed inoculation of *P. lilacinum* with an average of 60.53 leaves. All other treatments were on par with each other. This result indicates the influence of seed inoculation on number of leaves in initial stages of crop growth irrespective of isolates. No significant difference among the treatments were observed during the later stages, suggesting that the endophytes did not have any adverse effect on the leaf production by treated plants irrespective of the method of inoculation.

The effect of seed inoculation of *B. bassiana* on number of leaves of cowpea plant is in agreement with the results of Lopez and Sword (2015), who reported the positive influence of seed treatment of *B. bassiana* and *P. lilacinum* on cotton dry biomass and number of nodes.

Both soil and seed inoculation with endophytes had a positive influence on the mean height of the treated plants during the earlier stages of crop growth. While the difference between the treatments was not significant at 60 days after sowing, *P. lilacinum* continued to exert a significant effect on the mean height of the plants, irrespective of the method of inoculation. At 80 days after sowing, endophyte colonisation showed a positive influence on height of plant. These findings were consistent with those of the pot culture studies as well and support the concept that endophytic colonisation in cowpea promotes plant growth.

Bing and Lewis (1991) reported that colonisation of *B. bassiana* did not have any negative impact on plant growth parameters. Reports of growth promotion by endophytic fungal entomopathogens have been previously documented (Gurulingappa *et al*., 2010; Sasan and Bidochka, 2012; Lopez and Sword, 2015).

Soil inoculation of wheat plants with *B. bassiana* had significantly increased dry and wet shoot and root weights at 20 days post inoculation as compared to control plants

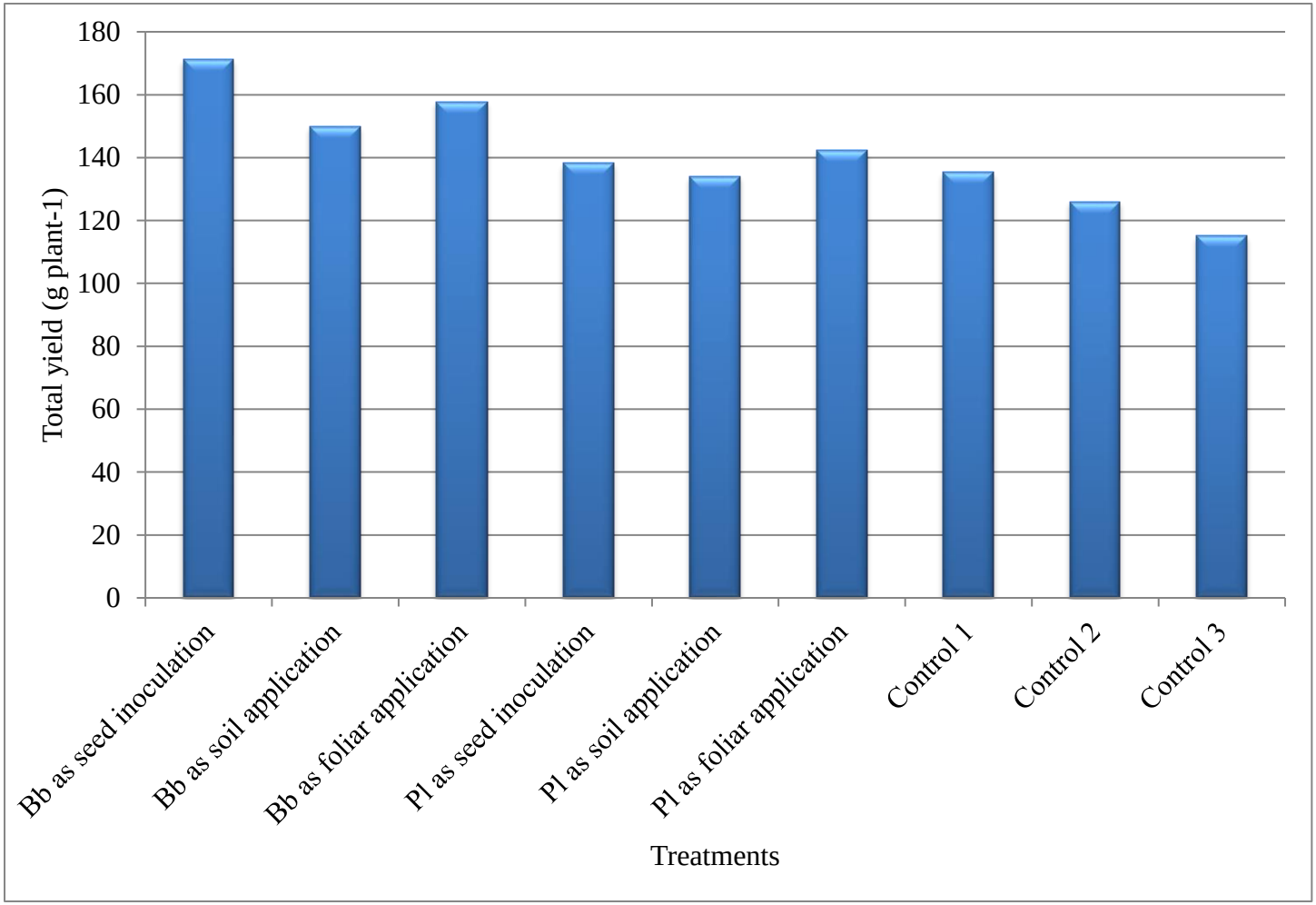


Fig. 28. Effect of fungal colonization on yield of cowpea under polyhouse condition

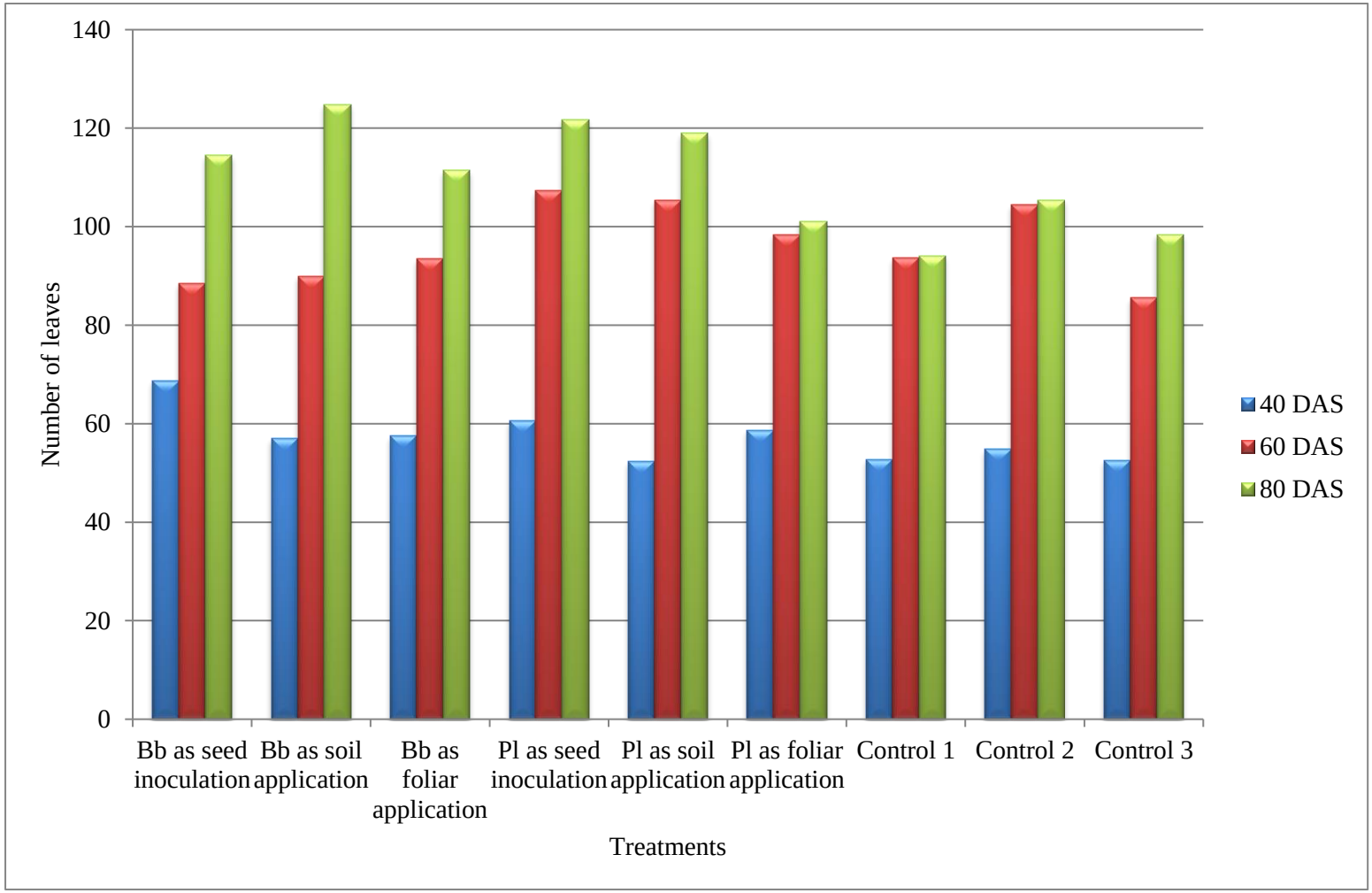


Fig. 29. Effect of fungal colonization on number of leaves of cowpea under field condition

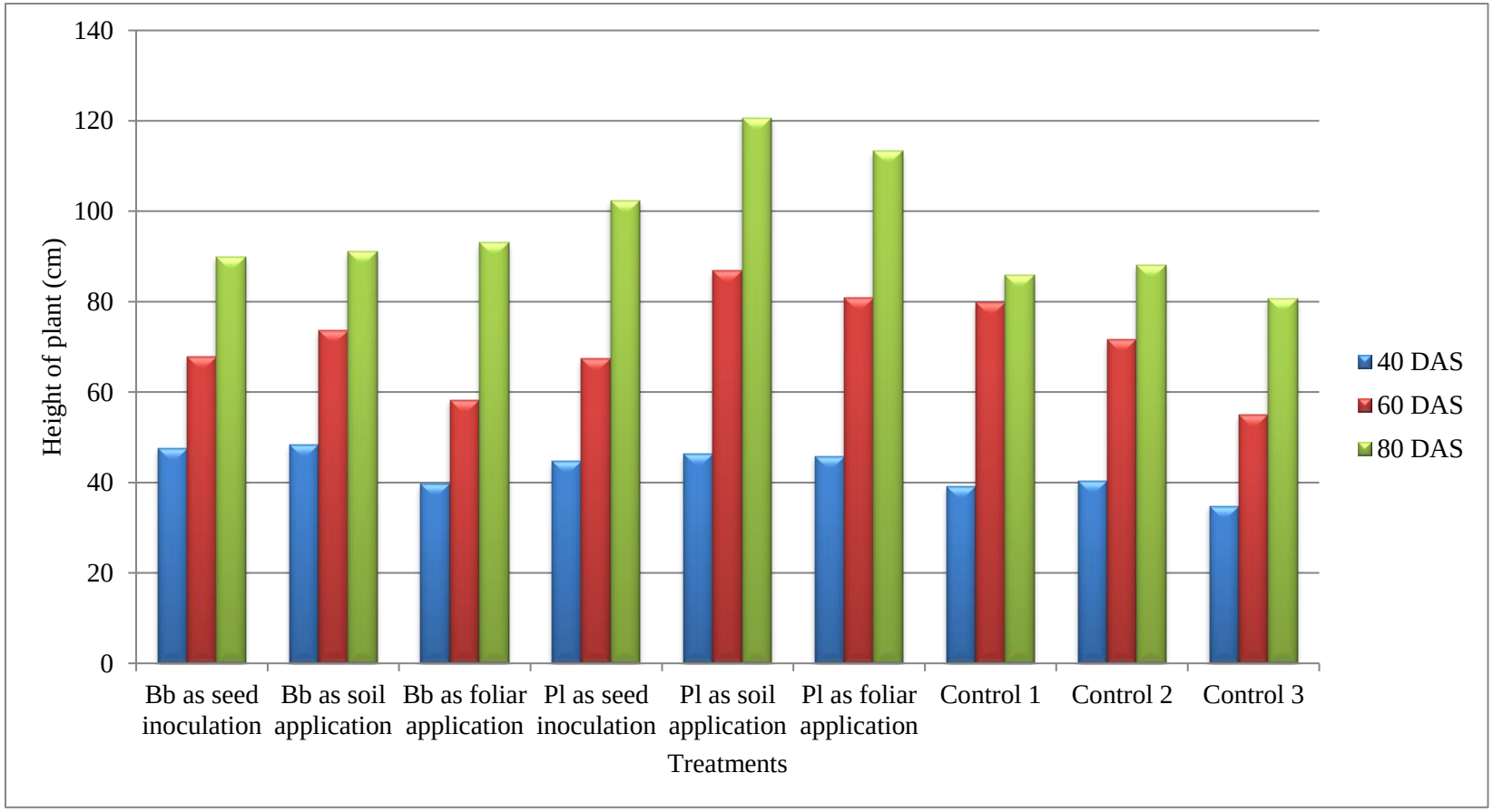


Fig. 30. Effect of fungal colonization on height of cowpea under field condition

(Gurulingappa *et al*., 2010). Garcia *et al*. (2011) also had reported that soil inoculation with entomopathogen, *M. anisopliae* caused a significant increase in root length, height, shoot and root weight of tomato plants when compared with untreated control plants.

5.7.2 Effect of fungal colonisation on yield parameters under field condition

Cowpea plants inoculated with endophytic fungi recorded overall pod yield of 163.33 to 173.67g plant^{-1} and marketable yield of 137.67 to 152.83g plant^{-1}. The above values were on par with each other except soil application of *B. bassiana* and control plants. The present result confirms the influence of endophyte colonisation on yield parameters (Fig. 31). In this study, chemical fertilizers are not applied to the plants. It might have reflected on total and marketable pod yield per plant.

The positive influence of fungal colonization on yield parameters in the present study is in conformity with the findings of Schardl *et al.* (2004) who reported that endophytic fungi can promote host nutrient uptake in colonised plants with the production of various alkaloids (Schardl *et al*., 2004). Das and Varma (2009) also reported that production of bioactive compounds by the endophytes themselves or through induction of the host plant to produce secondary metabolites promote plant growth and yield.

5.7.3 Effect of fungal colonisation on infestation by spotted pod borer

Identical to the values obtained in the previous studies (Beegum, 2015), overall infestation was found to be low. Overall infestation by spotted pod borer on pods was low in the experimental plots with untreated plants recording 20.38 to 25.44 per cent damage. Both of the inoculated endophytes recorded comparable damage values ranging from 12.53 to 16.39 per cent in terms of number of pods. They were on par with each other except soil application of *B. bassiana*. Foliar inoculation with *B. bassiana* registered the lowest mean damage of 12.53 per cent (Fig. 32).

There was no significant difference among different treatments with respect to pod borer infestation on flowers (Fig. 33). However, lowest value of 7.72 per cent was recorded in case of foliar inoculation with *B. bassiana*, followed by soil application of *P. lilacinum* and foliar application *P. lilacinum* with values of 7.63 and 8.32 per cent respectively.

Evaluation of the efficacy of the two endophytes on pod borer infestation indicated that inoculation with the endophytes resulted in a reduction in infestation by the borer. While the differences between the two fungi as well as among the treatments were not often significant, a comparison with respective control reveals that foliar inoculation with *B. bassiana* was significantly superior to the control treatment. The above treatment also caused the highest reduction in infestation over control. The results are supported by the infestation levels on flowers as well, though the differences were not significant.

The present results showed that the inoculated plants negatively affected the spotted pod borer. Some researchers have found that the adverse effect of fungal endophytes on insect pests may be due to the production of superoxides and secondary metabolites, due to their indirect systemic defense responses or changes in the phytosterol profile of the plants (Hartley and Gange, 2009; White and Torres, 2010). In contrast to this study, in some investigations, mycosis of *Helicovepa zea* was observed in tomato plants colonised by *B. bassiana* (Powel *et al*., 2009).

5.8 EFFECT OF ENDOPHYTIC ENTOMOPATHOGENIC FUNGI ON INFESTATION BY SPOTTED POD BORER

Foliar application of *B. bassiana*, which was identified as the most effective treatment against pod borer in the previous study, was selected for comparative evaluation with the diamide insecticide flubendiamide against *M. vitrata* through a pot culture experiment using the same variety, Anaswara.

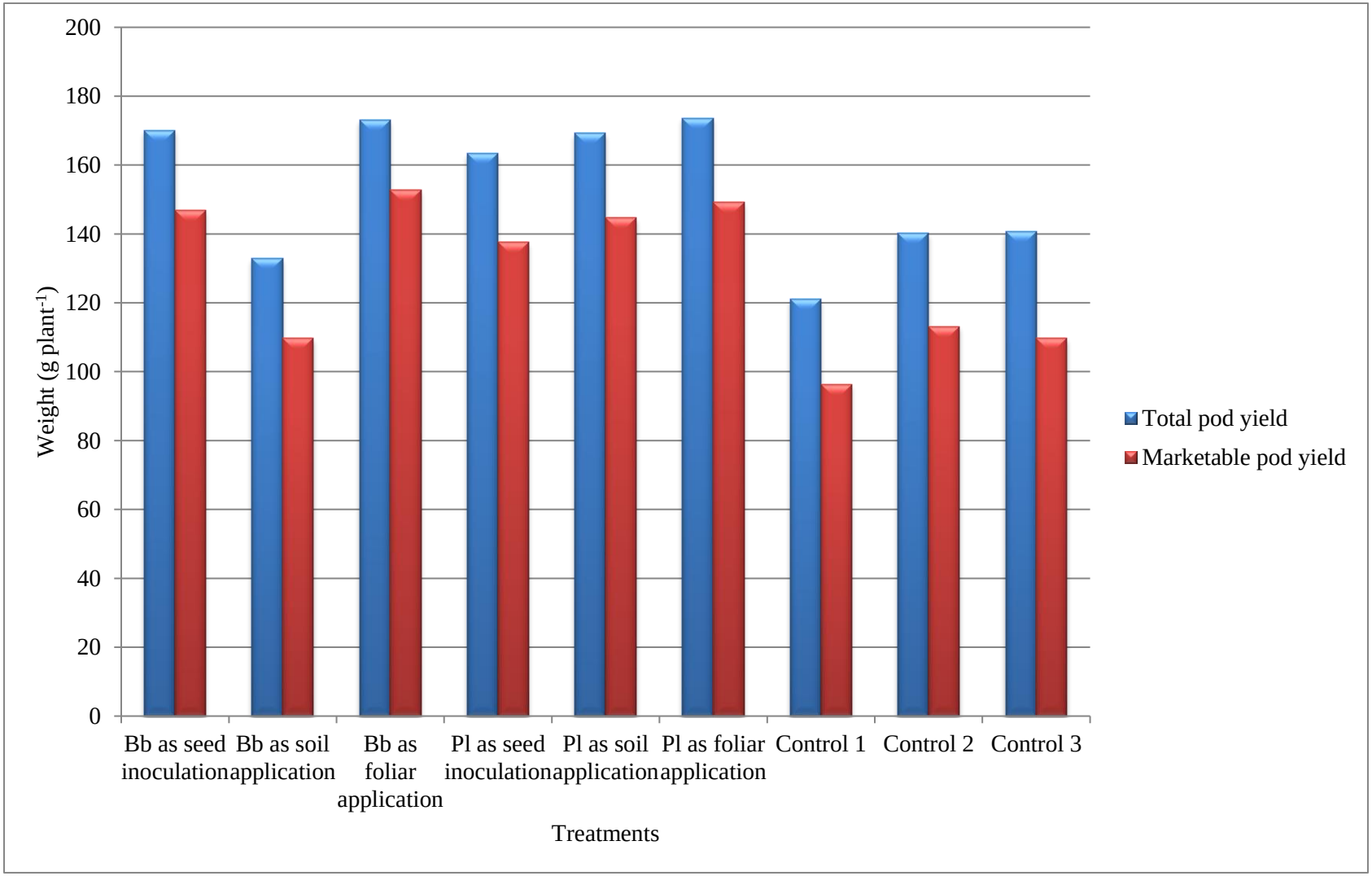


Fig. 31. Effect of fungal colonization on total and marketable pod yield of cowpea under field condition

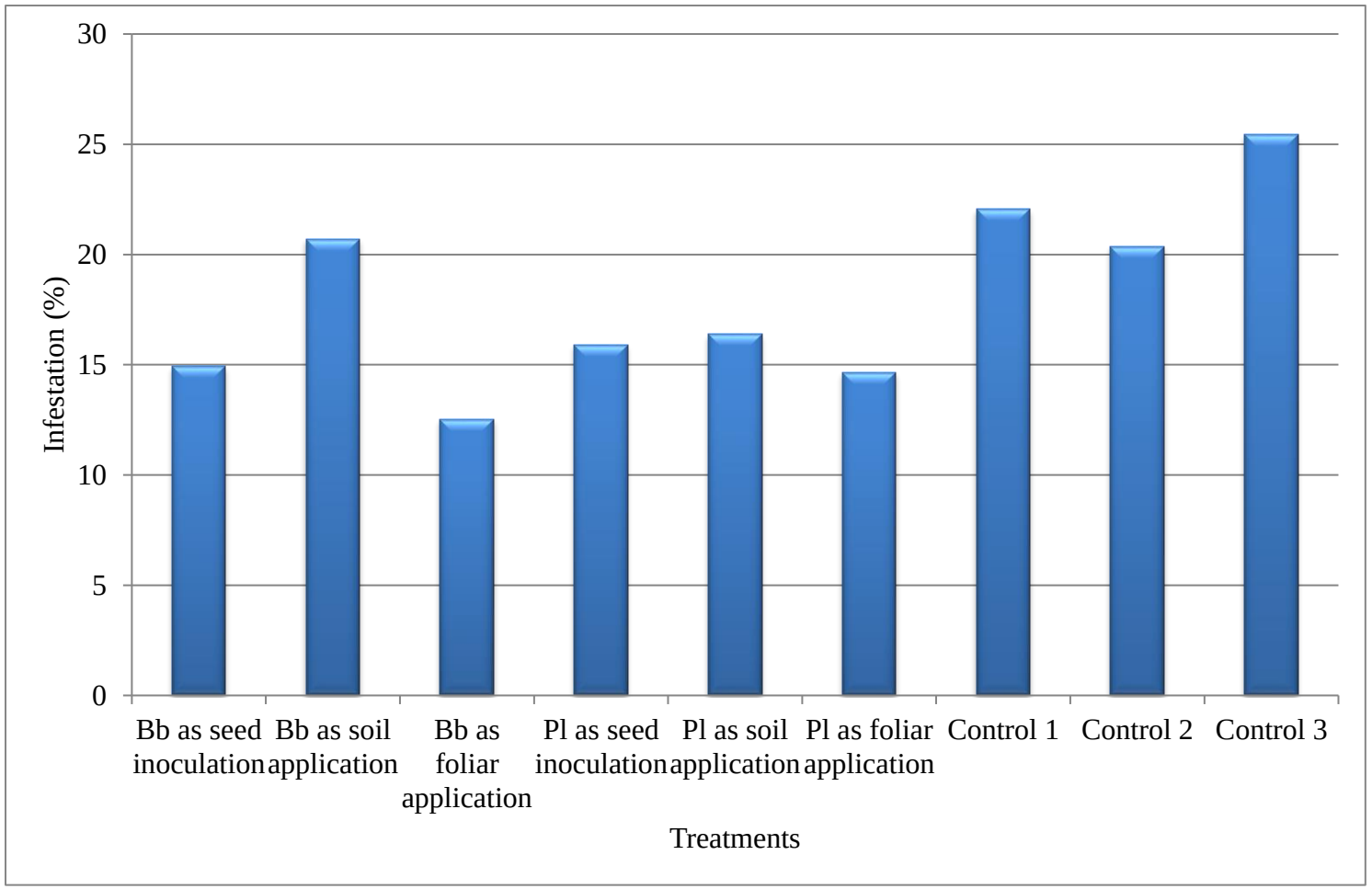


Fig. 32. Effect of fungal colonization on infestation of *Maruca vitrata* on pods of cowpea under field condition

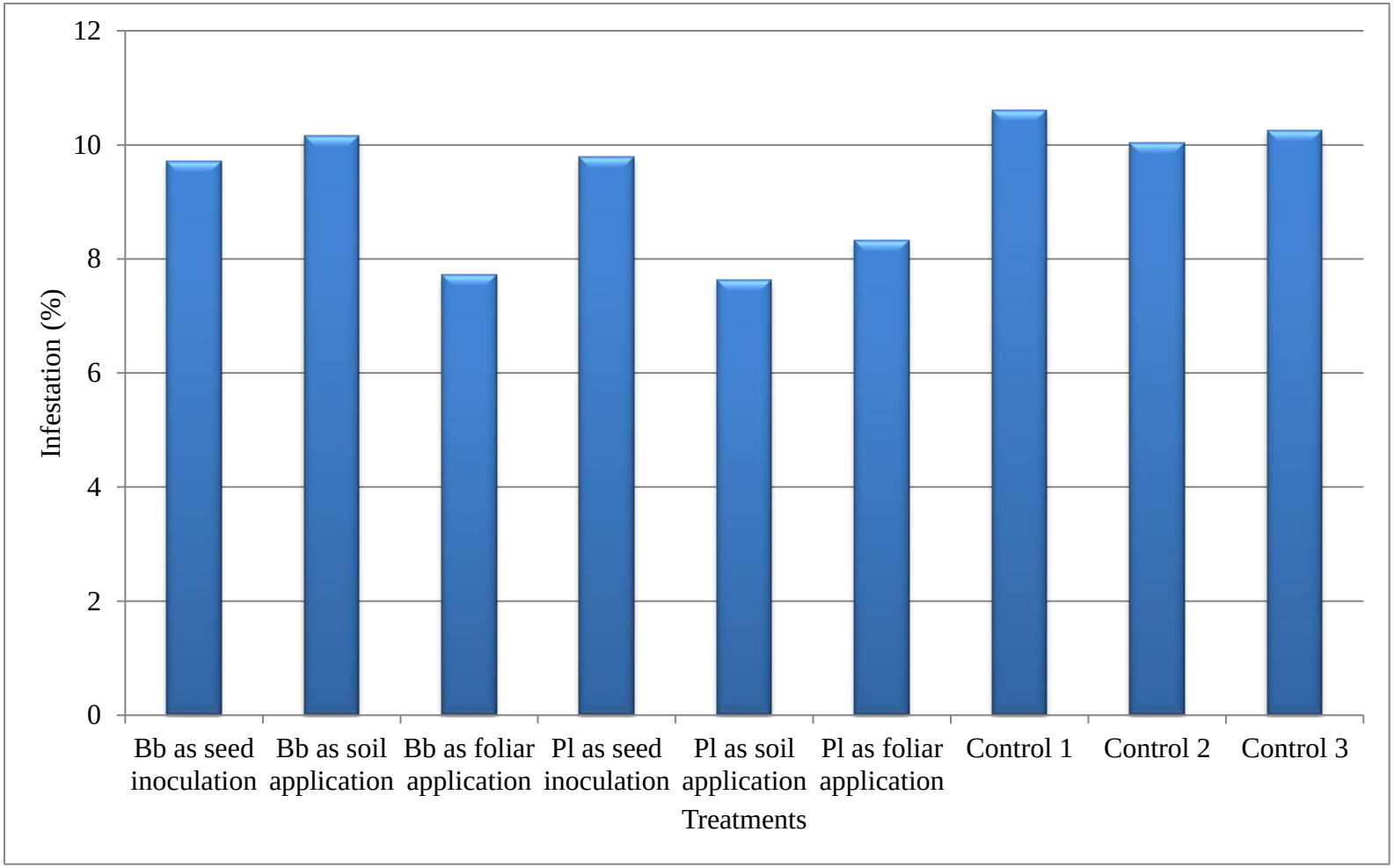


Fig. 33. Effect of fungal colonization on infestation of *Maruca vitrata* on flowers of cowpea under field condition

5.8.1 Effect of endophytic entomopathogenic fungi on infestation by spotted pod borer

Effect of endophytic entomopathogenic fungi on infestation by spotted pod borer on flowers and pods was compared with the chemical check, flubendiamide (Fig. 34).

Flubendiamide proved to be the best treatment and was significantly superior to other two treatments with a mean infestation of 7.09 per cent. Control plants recorded the highest infestation on flowers (26.40%) and were on par with foliar application of *B. bassiana* with a mean value of 20.78 per cent. All the three treatments varied significantly with respect to infestation on pods. Cowpea plants treated with flubendiamide and *B. bassiana* had significantly less number of infested pods than control, with 8.41 and 15.05 per cent infestation respectively, and insecticide proving to be the best treatment among the treatments.

5.8.2 Effect of endophytic entomopathogenic fungi on yield of cowpea

The treatments varied significantly in terms of marketable yield, with flubendiamide recording the highest mean marketable yield of 166.14g, followed by foliar application of *B. bassiana*. Both these treatments were yielded significantly higher pod yield compared to control plants (Fig. 35). Chemical fertilizers are not applied to the plants. It might have reflected on the total and marketable pod yield.

The field studies thus conclusively demonstrated that foliar inoculation with *B. bassiana* leads to significant reduction in infestation by spotted pod borer, *M. vitrata*. This study confirmed the positive influence of endophyte colonisation on yield parameters also.

The above results are in agreement with the findings of Ramanujam *et al*. (2017) who reported successful colonisation by *B. bassiana* in maize through foliar inoculation with marked reduction in infestation by the stem borer, *Chilo partellus*. Foliar inoculation

of three isolates of *B. bassiana* (Bb 5a, Bb 23 and Bb 45) and one isolate of *M. anisopliae* (Ma 35) under field conditions led to successful colonisation of maize plants by all the isolates. Mean number of dead hearts ranged from 9.00 to 18.50 per cent in inoculated plots as against untreated control which recorded a significantly higher 19.42 per cent.

The endophytic activity of *B. bassiana* leading to effective management of insect pests have also been reported across a range of insect herbivores like *Ostrinia nubilais* in corn (Bing and Lewis, 1991), *Sesamia calamistis* in corn (Cherry *et al*., 2004) and *Cosmopolites sordidus* in banana (Akello *et al*., 2008). The findings of the present study in cowpea are in agreement with the above reports.

The exact mechanism through which endophytic colonisation leads to reduction in infestation is far from clear. While *B. bassiana* have been known to produce metabolites leading to antibiosis, antixenotic effects through feeding deterrence have also been postulated (Cherry *et al*., 2004; Vega *et al*., 2008). Bing and Lewis (1991) suggested that the reduced tunneling of *Ostrinia nubilalis* in maize following endophytic colonisation by *B. bassiana*, could be due to the presence of fungal metabolites that cause antibiosis or feeding deterrence. Studies by Cherry *et al*. (2004) on *Sesamia calamistis* also support the feeding deterrence or antibiosis hypothesis since larvae feeding on corn plants injected with *B. bassiana* were smaller than those in the control plants.

Richardson (2000) reported that fungal endophytes increase apoplastic carbohydrate concentration, altering C:N ratio of tissues and making them a less efficient source of proteins. This effect was compounded when the fungual endophytes also uses plant nitrogen to form N- based secondary metabolites.

Gange and Nice (1997) reported that reduction in availability of nitrogen in fungal endophyte colonized plants affected the performance of thistle gall fly (*Urophora cardui* L.).

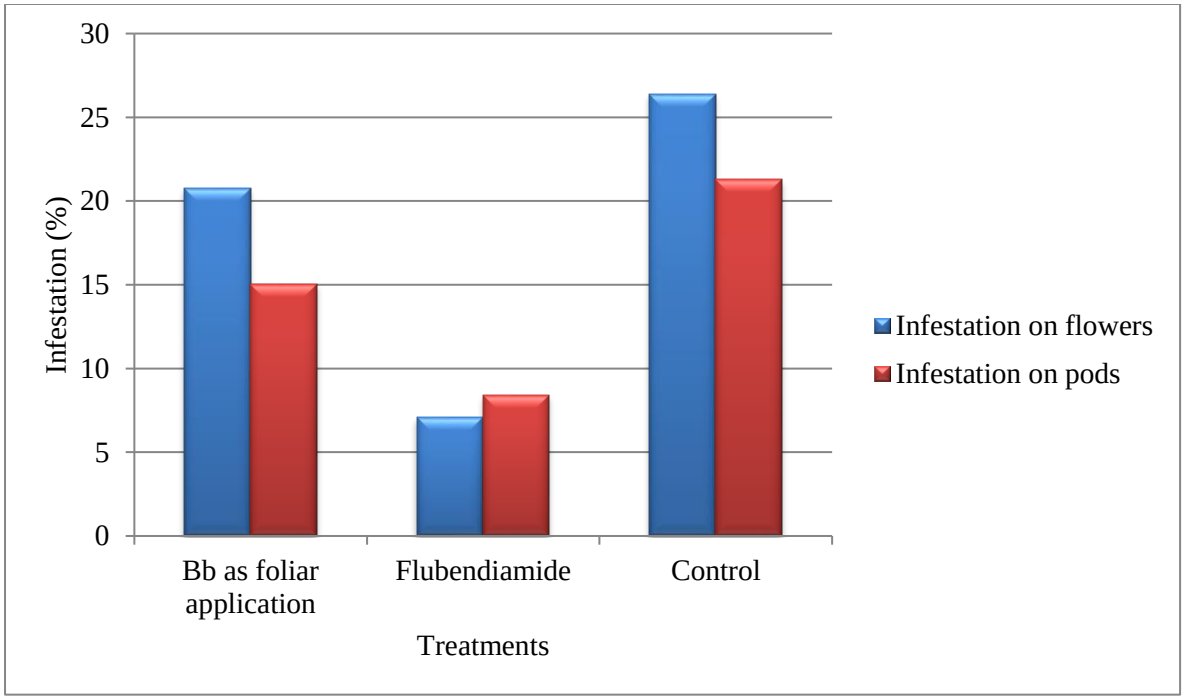


Fig. 34. Effect of different treatments on infestation of *Maruca vitrata* on flowers and pods of cowpea

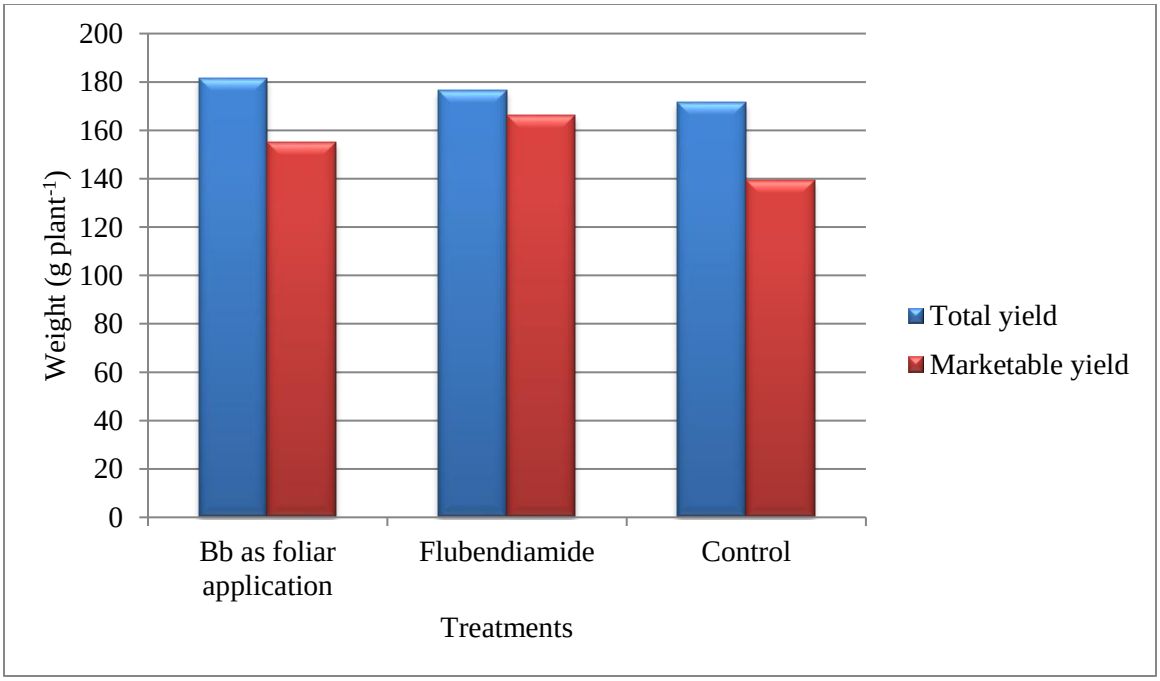


Fig. 35. Effect of different treatments on total and marketable yield of cowpea

Several studies have reported the production of primary and secondary metabolites due to colonisation of fungal endophytes and their effect on insect herbivores. Bhagyashree (2013) carried out biochemical analysis of endophyte colonized maize and reported correlation between colonisation of *B. bassiana* and changes in levels of the primary and secondary metabolites in plants. She reported higher levels of C:N ratio, primary metabolites and lower levels of secondary metabolites in control plants. Meanwhile, *B. bassiana* colonized plants had lower levels of primary metabolites (proteins and sugars) and higher levels of secondary metabolites such as total phenols.

Yan *et al.* (2015) suggested that systemic colonisation of fungal endophytes is not necessary for pest control due to the secondary metabolites produced by the fungus that confer antagonistic protection to the host plant.

In conclusion, this study has revealed the significant interaction between endophytic entomopathogenic fungi and plants. The study successfully isolated and characterized endophytic entomoapthogenic fungi from cowpea. The method of inoculation of entomopathogenic fungi in cowpea was standardized and evaluated against spotted pod borer of cowpea. Foliar inoculation of *B. bassiana* fourteen days after sowing was shown to be effective in managing population of spotted pod borer in cowpea.

But, there is a need for further research to understand the mechanisms through which the endophytes protect plants from herbivores and promote plant growth and yield.

Summary

6. SUMMARY

Cowpea, [*Vigna unguiculata* (L.) Walp.], commonly known in India as *lobia* is one of the important *kharif* pulse crops grown in the country. It is a multipurpose crop, used as grain legume, vegetable, forage crop and green manure. Cowpea is rich in proteins, vitamins and essential micronutrients and hence is often known as vegetable meat.

Infestation by the spotted pod borer, *Maruca vitrata* Fab. (Lepidoptera: Crambidae) is a major constraint in the production of legumes because of its wide host range, distribution and destructiveness. The larvae initially damage flower buds, flowers, tender pods and tender leaf axils by webbing them together. This feeding habit protects the larvae from natural enemies, adverse environmental conditions and chemical sprays.

Management of spotted pod borer is by and large based on frequent application of pesticides, though application of pesticides can hardly be recommended in cowpea as the pods are harvested at alternate days.

Biological control of *M. vitrata* using entomopathogenic microorganisms has received considerable attention in recent times. Many of these entomopathogens enjoy endosymbiotic relationships with plants which have opened up the possibility of engineering such associations for crop protection against insects. Endophytic entomopathogenic fungi colonize different plant parts and infect insects attacking those parts. In addition, they also act as plant disease antagonists, plant growth promoters and rhizosphere colonizers. Utilization of these endophytic entomopathogenic fungi confers several advantages such as season long protection, cost effectiveness and environment neutrality as compared to conventional approaches.

Hence, the present study entitled “Endophytic fungi for the management of spotted pod borer, *Maruca vitrata* Fab. (Lepidoptera: Crambidae) in cowpea” was carried out in the Department of Agricultural Entomology, College of Agriculture, Vellanikkara,

Thrissur, Kerala during 2016-2019. The salient findings of the study are summarized below.

Purposive sampling surveys were carried out in the major cowpea growing areas of Kozhikode, Thrissur, Kottayam and Thiruvananthapuram districts of Kerala. A total of 235 endophytic fungal isolates were obtained from the plant samples collected from 40 locations surveyed. This comprised of 103 isolates from roots, 63 from stems, 31 from leaves, 33 from pods and five from flowers. The highest occurrence of fungal endophytes was in roots (43.83%) and was followed by stems (26.81%). Isolation of endophytic fungi from different accessions with varying levels of resistance was also attempted. All the nine accessions were found to harbor endophytic fungi. Out of 32 isolates from cowpea accessions, Palakkadan thandan payar, a highly resistant variety yielded the highest number of seven endophytic fungi. Lola and Mysore local had the lowest number of one isolate each. In contrast to the results of survey, the leaves of cowpea plant harbored more endophytic fungi than other plant parts.

Out of the total of 267 isolates obtained from survey samples and cowpea accessions, three were found to be pathogenic to *M. vitrata*. They were identified as *Fusarium oxysporum* (EEF 1) and two isolates of *Purpureocillium lilacinum* (EEF 4 and EEF 64) through morphological and molecular characterization.

Fungal isolates were subjected to plant pathogenicity test on cowpea variety, Anaswara. None of the plants showed any symptoms of fungal infection even up to three months after inoculation. However, *F. oxysporum* was not selected for further studies as the fungus is widely regarded as a facultative plant pathogen.

The two endophytic isolates of *P. lilacinum* obtained in the previous experiment were evaluated along with *Beauveria bassiana* (NBAIR strain) for their bioefficacy against second instar larvae of *M. vitrata* through contact toxicity bioassay at five different concentrations *viz*., 10^5, 10^6, 10^7, 10^8 and 10^9 spores ml^{-1}. *B. bassiana* applied at the rate of 10^9 spores ml^{-1} induced the highest mortality of 66.67 per cent six days after

treatment. In comparison, *P. lilacinum* (EEF 4) applied at the rate of 10^9 spores ml^{-1} induced a significantly lower mortality of 50.00 per cent six days after treatment, while *P. lilacinum* (EEF 64) recorded mortality values of 43.33 per cent at 10^9 spores ml^{-1} for the corresponding period.

The best two organisms in the bioefficacy study *viz*., *B. bassiana* (NBAIR strain) and *P. lilacinum* (EEF 4) were used for standardizing the inoculation technique for endophytic colonization in cowpea plant. Foliar inoculation of *B. bassiana* recorded the highest extent of 24.08 per cent mean colonization in younger stem tissues. No colonization in younger stem tissues resulted through soil application of *B. bassiana*. *P. lilacinum* showed relatively greater colonization of younger stems irrespective of the method of inoculation. Mean colonization values were 11.73, 13.89 and 62.04 per cent when the isolate was applied through seed, soil and foliar application respectively.

Colonization by *B. bassiana* averaged from 1.85 to 10.19 per cent in older stem tissues, with the highest value being recorded in seed inoculation. The mean colonization by *P. lilacinum* in old stem tissues ranged from 18.83 to 40.74 per cent with foliar application proving to be the most effective.

No colonization in young leaves resulted through soil application of *B. bassiana*. Among *B. bassiana* treatments, highest extent of colonization was recorded in foliar application with mean value of 13.89 per cent. Mean colonization values for *P. lilacinum* were 24.08, 15.74 and 23.15 per cent for seed, soil and foliar inoculation respectively. Mean colonization by *B. bassiana* isolate in older leaf tissues was between 1.85 in soil and 10.50 per cent in case of foliar inoculation, the treatments being at par. *P. lilacinum* failed to colonize older leaves when applied through soil. Foliar inoculation of *P. lilacinum* led to the highest colonization of 42.59 per cent, which was significantly superior to other treatments.

Mean colonization by *B. bassiana* in roots ranged from 3.40 to 9.26 per cent with soil inoculation resulting in the highest colonization (9.26%), followed by foliar

inoculation (8.03%). *P. lilacinum* colonized 20.37, 29.01 and 31.48 per cent of roots through seed, soil and foliar inoculation respectively, the latter two treatments being at par.

Colonization by *B. bassiana* in pods, when inoculated through soil and leaves averaged 25.93 and 31.48 per cent respectively, the values being on par with each other. Soil inoculation did not result in endophytic establishment in pods. Mean colonization by *P. lilacinum*, at 27.78 per cent was the lowest in case of soil inoculation. Seed and foliar inoculation resulted in colonization of 38.89 and 68.52 per cent respectively.

The colonized endophytes were reisolated and subjected to PCR technique to confirm the colonization by the inoculated fungi.

Seed and foliar application of *B. bassiana* resulted in significantly higher number of leaves in cowpea plants. The influence of different treatments was found to be non-significant with respect to height of the plant at different stages of growth. Inoculation of *B. bassiana* recorded significantly higher pod yield as well. Foliar inoculation with *B. bassiana* yielded 171.20g plant^{-1} and was on par with leaf and soil inoculation of *B. bassiana* with mean yields of 157.70 and 150.00g plant^{-1} respectively.

Following standardization of inoculation methods, the effect of fungal colonization on infestation of spotted pod borer on cowpea was assessed through pot culture experiment under field conditions. In addition to infestation, effect on growth parameters was also recorded. At 40 days after sowing, seed inoculation of *B. bassiana* recorded significantly higher number of leaves as in case of polyhouse studies. Forty days after sowing, all the treatments resulted in comparable increase in mean plant height with values ranging from 34.73 to 48.40 cm. *B. bassiana* as soil inoculation recorded the highest value for mean height (48.40 cm).

Beauveria bassiana inoculated through leaves resulted in the highest marketable pod yield of 152.83g, followed by *P. lilacinum* as foliar application (149.33g). Foliar inoculation with *B. bassiana* registered the lowest mean pod damage of 12.53 per cent.

Foliar application of *B. bassiana*, which was identified as the most effective treatment against pod borer in the previous studies, was selected for comparative evaluation with the diamide insecticide flubendiamide against *M. vitrata.* Cowpea plants treated with both flubendiamide and *B. bassiana* had significantly less number of infested pods than control at 8.41 and 15.05 per cent respectively. Control plants recorded the highest infestation of 21.28 per cent. Mean marketable pod yield showed significant difference between flubendiamide (166.14g plant^{-1}) and foliar inoculation of *B. bassiana* (155.14g plant^{-1}). Both these treatments also had significantly higher pod yield compared to control plants (139.29g plant^{-1}).

The study thus successfully isolated and characterized endophytic entomoapthogenic fungi from cowpea. The method of inoculation of endophytic entomopathogenic fungi in cowpea was standardized and evaluated against spotted pod borer of cowpea. Foliar inoculation of *B. bassiana* fourteen days after sowing was shown to be effective in managing population of spotted pod borer in cowpea.

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Appendices

Appendix I

Details of endophytic fungal isolates from Kozhikode district					
Sl. No./ Location code	Isolate No.	Plant part	Sl. No./ Location code	Isolate No.	Plant part
KZD1			30	EF -220	Root
1	EF - 176	Root	31	EF -257	Root
2	EF - 222	Stem	32	EF -264	Root
KZD2			33	EF -270	Root
3	EF - 64	Root	34	EF -209	Leaf
4	EF - 177	Leaf	35	EF -210	Leaf
5	EF - 181	Stem	36	EF -206	Pod
KZD3			37	EF -217	Pod
6	EF -107	Root	38	EF -214	Pod
7	EF -119	Root	**KZD7**		
8	EF -184	Root	39	EF -265	Root
9	EF -121	Pod	40	EF -266	Root
10	EF -122	Stem	41	EF -219	Pod
KZD4			**KZD8**		
11	EF -105	Leaf	42	EF -194	Root
12	EF -118	Root	43	EF -225	Root
13	EF -172	Stem	44	EF -226	Root
14	EF -182	Stem	45	EF -228	Root
KZD5			46	EF -230	Root
15	EF -99	Stem	47	EF -231	Root
16	EF -205	Stem	48	EF -236	Root
17	EF -221	Stem	49	EF -237	Root
18	EF -159	Root	50	EF -216	Stem
19	EF -141	Root	51	EF -223	Stem
20	EF -204	Pod	52	EF -224	Stem
21	EF -160	Pod	53	EF -234	Leaf
22	EF -267	Pod	54	EF -235	Leaf
KZD6			**KZD9**		
23	EF -192	Root	55	EF -227	Leaf
24	EF -200	Root	**KZD10**		
25	EF -201	Root	56	EF -195	Leaf
26	EF -207	Root	57	EF -212	Root
27	EF -208	Root	58	EF -213	Root
28	EF -211	Root	59	EF -233	Root
29	EF -218	Root	60	EF -232	Stem

Appendix II

Details of endophytic fungal isolates from Thrissur district (Contd..)					
Sl. No./ Location code	Isolate No.	Plant part	Sl. No./ Location code	Isolate No.	Plant part
TSR1			**TSR6**		
1	EF -14	Leaf	33	EF -111	Root
2	EF -35	Stem	34	EF -115	Root
3	EF -41	Root	35	EF -170	Leaf
4	EF -47	Root	36	EF -175	Stem
5	EF -5	Pod	37	EF -178	Stem
6	EF -40	Root	38	EF -183	Stem
TSR2			**TSR7**		
7	EF -63	Root	39	EF -75	Leaf
8	EF -48	Root	40	EF -116	Leaf
9	EF -60	Pod	41	EF -112	Leaf
10	EF -27	Root	42	EF -103	Root
TSR3			43	EF -110	Root
11	EF -31	Flower	44	EF -114	Pod
12	EF -32	Flower	45	EF -78	Pod
13	EF -46	Root	46	EF -174	Stem
14	EF -58	Root	**TSR8**		
TSR4			47	EF -90	Leaf
15	EF -39	Pod	48	EF -92	Leaf
16	EF -37	Pod	49	EF -91	Stem
17	EF -38	Pod	50	EF -154	Stem
18	EF -55	Leaf	51	EF -155	Stem
TSR5			52	EF -125	Root
19	EF -77	Root	53	EF -142	Root
20	EF -79	Root	54	EF -143	Root
21	EF -80	Root	55	EF -149	Root
22	EF -81	Root	56	EF -157	Root
23	EF -113	Root	57	EF -158	Root
24	EF -97	Stem	58	EF -203	Root
25	EF -106	Stem	59	EF -215	Root
26	EF -126	Stem	60	EF -101	Pod
27	EF -76	Pod	**TSR9**		
28	EF -124	Pod	61	EF -71	Root
29	EF -153	Pod	62	EF -74	Root
30	EF -171	Pod	63	EF -102	Root
31	EF -179	Pod	64	EF -104	Root
32	EF -180	Pod	65	EF -272	Root

Appendix II

(Contd..) Details of endophytic fungal isolates from Thrissur district					
Sl. No./ Location code	Isolate No.	Plant part	Sl. No./ Location code	Isolate No.	Plant part
66	EF -73	Stem	81	EF -252	Stem
67	EF -93	Stem	82	EF -254	Stem
68	EF -94	Stem	83	EF -278	Stem
69	EF -95	Stem	84	EF -193	Root
70	EF -96	Stem	85	EF -250	Root
71	EF -98	Stem	86	EF -256	Root
72	EF -156	Stem	87	EF -261	Root
73	EF -173	Leaf	88	EF -268	Root
TSR10			89	EF -275	Root
74	EF -185	Stem	90	EF -255	Pod
75	EF -186	Stem	91	EF -273	Pod
76	EF -196	Stem	92	EF -276	Pod
77	EF -197	Stem	93	EF -260	Flower
78	EF -198	Stem	94	EF -269	Flower
79	EF -202	Stem	95	EF -259	Leaf
80	EF -251	Stem			

Appendix III

Details of endophytic fungal isolates from Kottayam district					
Sl. No./ Location code	Isolate No.	Plant part	Sl. No./ Location code	Isolate No.	Plant part
KTM1			29	EF -280	Pod
1	EF -2	Stem	30	EF -281	Leaf
2	EF -56	Root	**KTM7**		
3	EF -282	Leaf	31	EF -1	Pod
4	EF -18	Leaf	32	EF -277	Leaf
5	EF -34	Leaf	33	EF -262	Pod
6	EF -11	Root	**KTM8**		
KTM2			34	EF -187	Leaf
7	EF -25	Root	35	EF -199	Leaf
8	EF -19	Pod	36	EF -247	Leaf
KTM3			37	EF -189	Stem
9	EF -43	Root	38	EF -239	Stem
10	EF -29	Leaf	39	EF -240	Stem
KTM4			40	EF -241	Stem
11	EF -57	Pod	41	EF -242	Stem
12	EF -42	Root	42	EF -253	Stem
KTM5			43	EF -258	Stem
13	EF -26	Stem	44	EF -190	Root
14	EF -59	Pod	45	EF -229	Root
KTM6			46	EF -244	Root
15	EF -127	Stem	47	EF -245	Root
16	EF -129	Stem	**KTM9**		
17	EF -146	Stem	48	EF -130	Root
18	EF -147	Stem	49	EF -131	Root
19	EF -188	Stem	50	EF -243	Root
20	EF - 4	Stem	51	EF -248	Root
21	EF -145	Root	52	EF -152	Leaf
22	EF -150	Root	53	EF -246	Stem
23	EF -271	Root	**KTM10**		
24	EF -274	Root	54	EF -100	Root
25	EF -279	Root	55	EF -128	Root
26	EF -283	Root	56	EF -148	Root
27	EF -284	Root	57	EF -151	Stem
28	EF -263	Pod			

Appendix IV

Details of endophytic fungal isolates from Thiruvananthapuram district					
Sl. No./ Location code	Isolate No.	Plant part	Sl. No./ Location code	Isolate No.	Plant part
TVM1			**TVM5**		
1	EF -87	Stem	13	EF -120	Stem
2	EF -88	Root	14	EF -304	Stem
3	EF -89	Leaf	**TVM6**		
TVM2			15	EF -308	Root
4	EF -305	Stem	16	EF -309	Root
5	EF -306	Root	**TVM7**		
TVM3			17	EF -310	Stem
6	EF -307	Leaf	18	EF -311	Leaf
7	EF -117	Stem	**TVM8**		
8	EF -302	Root	19	EF -312	Root
TVM4			20	EF -313	Stem
9	EF -191	Root	**TVM9**		
10	EF -301	Root	21	EF -314	Root
11	EF -123	Flower	22	EF -315	Pod
12	EF -303	Root	**TVM10**		
			23	EF -316	Root

Appendix V

COMPOSITION OF DIFFERENT MEDIA

Composition of PDA (1L)

- Potato – 200g
- Dextrose – 20g
- Agar – 20g
- Distilled water – 1 L

Composition of SDAY for re-isolation of *B. bassiana* (1L)

- Dextrose – 40g
- Mycological peptone – 10g
- Yeast extract – 5g
- Agar – 20g
- Distilled water – 1 L

Composition of oatmeal agar with CTAB (1L)

- Rolled oatmeal – 20 g
- Agar - 20g
- CTAB – 0.6 g
- Chloramphenicol – 0.5 g
- Distilled water – 1 L

ENDOPHYTIC FUNGI FOR THE MANAGEMENT OF SPOTTED POD BORER, *Maruca vitrata* Fab. (LEPIDOPTERA: CRAMBIDAE) IN COWPEA

By

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(2016-21-012)

ABSTRACT OF THE THESIS

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Endophytic fungi for the management of spotted pod borer, *Maruca vitrata* Fab. (Lepidoptera: Crambidae) in cowpea

Abstract

The spotted pod borer, *Maruca vitrata* Fab. (Lepidoptera: Crambidae) is a major constraint in the production of legumes because of its wide host range, distribution and destructiveness. In cowpea, the loss due to pod borer infestation varies from 20 to 60 per cent, often reaching upto 80 per cent in severe cases. Application of pesticides can hardly be recommended in cowpea as the pods are harvested at alternate days.

Biological control of *M. vitrata* using entomopathogenic microorganisms has received considerable attention in recent times. Previous studies have demonstrated the potential of entomopathogenic organisms to colonise an array of plants and confer protection from insect pests. In this context, a study entitled "Endophytic fungi for the management of spotted pod borer, *Maruca vitrata* Fab. (Lepidoptera: Crambidae) in cowpea" was undertaken with an objective of isolation and characterization of endophytic entomopathogenic fungi in cowpea, standardization of method of inoculation of entomopathogenic fungi in cowpea, and evaluation of selected endophytic fungi for management of the spotted pod borer, *M. vitrata*.

Purposive sampling surveys were conducted in the major cowpea growing areas of Kozhikode, Thrissur, Kottayam and Thiruvananthapuram districts of Kerala. A total of 235 endophytic fungal isolates were obtained from the cowpea plant samples collected from 40 locations. This comprised of 103 isolates from roots, 63 from stems, 31 from leaves, 33 from pods and five from flowers. Nine accessions of cowpea with different levels of resistance were also screened for the isolation of fungal endophytes. All the accessions were found to harbor endophytic fungi and yielded 32 isolates. Among these accessions, *Palakkadan thandan payar* yielded the maximum number of seven endophytic fungi. Lola and Mysore local had the lowest number of isolates. In contrast to the results of survey, the leaves of cowpea plant harbored more endophytic fungi than other plant parts. A total of 267 isolates were obtained from survey samples and cowpea accessions. Three isolates were found to be pathogenic to *M. vitrata*. They were identified as *Fusarium oxysporum*

(EEF 1) and two isolates of *Purpureocillium lilacinum* (EEF 4 and EEF 64) through morphological and molecular characterization. These isolates were evaluated along with *Beauveria bassiana* (NBAIR strain) for their bioefficacy against *M. vitrata*.

The best two organisms in the bioefficacy studies, *viz*., *B. bassiana* (NBAIR strain) and *P. lilacinum* (EEF 4) were used for standardizing the inoculation technique for endophytic colonization in cowpea plant. Three different methods of inoculation *viz*., seed, soil and foliar inoculation were evaluated for identifying the best method for colonization of entomopathogenic fungi in cowpea plants. The effect of fungal colonization on infestation of spotted pod borer in cowpea was further assessed under field condition.

Foliar inoculation with *B. bassiana* registered the lowest mean pod damage of 12.53 per cent. *B. bassiana* applied as foliar application resulted in the highest marketable pod yield of 152.83g plant^{-1}, followed by *P. lilacinum* as foliar application (149.33g plant^{-1}). Based on the polyhouse and field studies, it was inferred that foliar application of *B. bassiana* was found to be the best treatment against the target pest, *M. vitrata*. In addition, the results of the current study suggested that that endophytic colonization in cowpea had little adverse impact on plant growth and yield.

Foliar application of *B. bassiana*, which was identified as the most effective treatment against pod borer in the previous studies, was selected for comparative evaluation with the diamide insecticide, flubendiamide against *M. vitrata.* Cowpea plants treated with both flubendiamide and *B. bassiana* had significantly less number of infested pods than control, with 8.41 and 15.05 per cent infestation respectively. Control plants recorded the highest infestation of 21.28 per cent. Mean marketable pod yield showed significant difference between flubendiamide (166.14g plant^{-1}) and foliar inoculation of *B. bassiana* (155.14g plant^{-1}). Both these treatments also had significantly higher marketable pod yield compared to control plants (139.29g plant^{-1}).

In conclusion, the present study revealed that use of *B. bassiana* as an endophyte could be a useful tool in integrated pest management of pod borer in cowpea. However, further studies are needed to understand the mechanisms through which the endophytes protect plants from herbivores and promote plant growth and yield.