ENDOPHYTIC ASSOCIATION OF ENTOMOPATHOGENIC FUNGI WITH RICE AND COWPEA

by

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"ENDOPHYTIC ASSOCIATION OF ENTOMOPATHOGENIC FUNGI WITH RICE AND COWPEA"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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LIST OF ABBREVIATIONS AND SYMBOLS USED

%	:	Per cent
@	:	at the rate of
µL	:	microlitre
µm	:	micro metre
CD	:	Critical difference
cm	:	Centimetre
DAI	:	Days after inoculation
DAS	:	Days after sowing
DAT	:	Days after transplanting
dATP	:	Deoxyadenosine triphosphate
dCTP	:	Deoxycytidine triphosphate
dGTP	:	Deoxyguanosine triphosphate
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
DTTP	:	Deoxythymidine triphosphate
et al.	:	Co-workers
g	:	Gram
h	:	Hour
KAU	:	Kerala Agricultural University
mg	:	Milligram
min	:	Minutes
mL^{-1}	:	millilitre
mm	:	Millimetre
°C	:	Degree Celsius
PCR	:	Polymerase chain reaction
PDA	:	Potato dextrose agar
PDB	:	Potato dextrose broth
rpm	:	Revolutions per minute
Sec	:	Second

Sp. or spp	:	Species (Singular and plural)
UV	:	Ultraviolet
viz.	:	Namely

Introduction

1. INTRODUCTION

Entomopathogenic fungi are one among the green tools in integrated pest management programs. Owing to their unique mode of action and amenability to mass production, they outnumber other microbial pesticides derived from bacteria and viruses. In the global biopesticide market, fungi occupy second position among the microbes used in plant protection (Maina *et al*., 2018).

Nevertheless, the efficacy of fungal entomopathogens is limited by abiotic factors that hinder the viability of infective propagules. Inoculating these microbes into the plants is possibly another method to encompass these adversities (Vega, 2008). Fungal endophytes are those fungi that can survive inside the plants during a part of its life cycle or during the entire life cycle, asymptomatically (Hyde and Soytong, 2008). In earlier days, endophytes were considered to be neutral organisms, neither being beneficial nor detrimental to plants. Later on, they were well-studied for their unseen roles in plants. Improved plant growth, protection against pathogens and pests were some of the roles unveiled.

Scores of naturally occurring fungal endophytes have been identified till date, most of them harbouring plants of the family, Graminae. Entomopathogenic fungi such as *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metchnikoff) Sorokin have been reported as endophytes in crop plants such as maize (Bing and Lewis, 1991), coffee (Posada and Vega, 2006), sorghum (Reddy *et al.,* 2009), beans (Akello and Sikora, 2012), cassava (Greenfield *et al.,* 2016) and wheat (Sanchez-Rodriguez *et al.,* 2017).

A very few investigations reveal their mode of action against insects, which appear to be due to antibiosis or feeding deterrence caused by the toxins produced by these organisms in plants.

Recently, some of the studies focused on establishing the endophytic association of entomopathogens, of which majority were on *B. bassiana*. The crop plants tested include maize, tomato, banana, cocoa, rice and wheat.

Standardisation of artificial inoculation methods of entomopathogens, would also pave way to successful establishment of these beneficial microbes in the plant system. Consequently, it would circumvent the adversities of being exposed to abiotic stress, which is the major hindrance in their field efficacy. Furthermore, it would reduce the quantity of inoculum and the cost of plant protection operations.

The present investigation which aimed to examine the endophytic association of entomopathogenic fungi *viz.*, *B. bassiana, M. anisopliae*, *Lecanicillium lecanii* (Zimmermann) Zare and Gams and *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno, purposefully selected two crop plants such as rice and cowpea to represent two different taxa, the monocots and dicots. It also envisaged to test different inoculation methods to establish successful association in the plants, taking into account their persistence in different plant parts and soil as well. The study would pave way to a new paradigm in the method of use of entomopathogens in biological control of crop pests.

Review of Literature

2. REVIEW OF LITERATURE

Microbial pathogens of insects such as bacteria, fungi, viruses and protozoa are intensively investigated to develop environment friendly pest management strategies in Agriculture and allied sectors. Entomopathogenic fungi like *Beauveria bassiana, Beauveria brongniartii* (Sacc.) Petch, *Metarhizium anisopliae, Lecanicillium* spp, *Hirsutella thompsonii* F.E. Fisher, *Nomuraea rileyi* (Farl.) Samson and *Isaria fumosorosea* Wize are gaining importance in pest control in the recent years. Owing to their contact mode of action and easier methods of mass production techniques, fungi are the most preferred microbial insecticides (Ramanujam *et al.*, 2014). Recently, these entomopathogenic fungi are known to colonize internally in plants asymptomatically as endophytes (Vidal and Jaber, 2015).

2.1 CLASSIFICATION OF ENDOPHYTES

Endophytic microorganisms are those that inhabit the interior of plants, especially leaves, branches and stems, showing no apparent harm to the host. They play an important role in host protection against pests and pathogens. Pest management by means of endophytic entomopathogenic microorganisms, is a good alternative to reduce the use of chemical insecticides in Agriculture (Azevedo *et al.*, 2000). Rodriguez *et al.* (2009) classified endophytic fungi based on the evolutionary relatedness, taxonomy, hosts plant and ecological functions into two major classes, Class I endophytes comprising clavicipitaceous fungi and Class II endophytes comprising non clavicipitaceous group.

Class I endophytes are known to colonize grasses. Among them, the genus *Acremonium* is well - documented in perrenial rye grass, *Lolium perenne* L. and tall fescue *Festuca arundinacea* Schreb. (Barker *et al.*, 1981). Gallagher *et al.* (1984) reported that the fungus has the potential to control many pests due to the production of certain alkaloids. In forage grasses it could control black beetle, *Heteronychus arator* F. (Ball and Prestidge, 1993), larvae of grass grub,

Costelytra zealandica White (Popay *et al.*, 1993) and argentine stem weevil, *Listronotus bonariensis* (Kuschel) (Prestidge *et al.,* 1994).

Class II endophytes are known to colonize non grasses. It includes the entomopathogenic fungi belonging to order Hypocreales. More than 700 species are reported to be entomopathogenic to different crop pests (Ali, 2014), of which *B. bassiana*, *M. anisopliae* and *Lecanicillium lecanii* are the commercially exploited species for the management of crop pests. Recent evidences proved that these fungi colonize crop plants naturally as well as by artificial inoculation.

Among class II, *B. bassiana* is a well - studied endophytic entomopathogen. Its colonization has been reported in maize (Bing and Lewis, 1991; Cherry *et al.*, 1991; Wagner and Lewis, 2000), tomato (Leckie , 2002), coffee (Posada and Vega, 2006), banana (Akello *et al.,* 2007), sorghum (Reddy *et al.,* 2009), beans (Akello and Sikora, 2012), cocoa (Posada *et al.,* 2010), rice (Jia *et al.,* 2013) and wheat (Sanchez-Rodriguez *et al.,* 2017).

M. anisopliae was reported to colonize tomato (Garcia *et al.,* 2011), beans (Akello and Sikora, 2012), soybean (Khan *et al.*,2012), mustard (Batta, 2013), sorghum (Mantozoukas *et al.*, 2015), cassava (Greenfield *et al.,* 2016), and tea (Kaushik and Dutta, 2016).

Endophytic colonisation of *Lecanicillium dimorphum* (J.D. Chen) Zare & W. Gams and *Lecanicillium psalliotae* (Treschew) Zare & W. Gams has been reported in date palm (Gomez-Vidal *et al.*, 2006). *L. lecanii* was found to colonize cotton, wheat, corn, beans, tomato and pumpkin, endophytically (Gurulingappa *et al.*, 2010).

The literature pertaining to successful endophytic association of entomopathogenic fungi in various crop plants, inoculation methods, confirmation tools for establishing endophytic association, effects on plant growth promotion, and pest incidence are reviewed below.

2.2 ENDOPHYTIC ASSOCIATION OF ENTOMOPATHOGENIC FUNGI IN CROP PLANTS

2.2.1. In Cereals

In cereals, endophytic *B. bassiana* is very well studied in corn, sorghum, wheat and rice. Bing and Lewis (1991) were the pioneers to study the endophytic colonization of *B. bassiana* in corn. *B. bassiana* inoculated in corn plants during the whorl stage, showed endophytic colonization. Further studies by Kabaluk and Ericsson (2007) revealed that *M. anisopliae* colonized the corn plants endophytically. Topical application of conidial suspension of *B. bassiana* in four weeks old sorghum seedlings lead to endophytic establishment up to 60 Days After Inoculation (DAI) (Reddy *et al.*, 2009). Gurulingappa *et al.* (2010) established endophytic association of *B. bassiana*, *L. lecanii* and *Aspergillus parasiticus* Speare in corn and wheat plants up to 21 DAI.

Jia *et al.*, (2013) found that isolates of *B. bassiana* Bb4 and Bb7 were able to colonize endophytically in the leaves of rice plants up to 15 DAI, when topically sprayed with the conidial suspension of 7.5 $x10^7$ and 7.5x 10^4 conidia mL^{-1} respectively. Colonization was absent in stem, roots and soils of paddy. *Metarhizium robertsii* J.F.Bisch., Rehner & Humber was reported to endophytically colonize the roots of *Panicum virgatum* L. and had positive effect on the root hair development (Sasan and Bidchoka, 2012).

Mantzoukas *et al.* (2015) established *B. bassiana*, *M. robertsii* and *I. fumosorosea* as endophytes in sweet sorghum. All the three fungi could be re isolated from the newly formed leaves, indicating the movement of fungi from older to new leaves. *B. bassiana* was reported to colonise wheat plants through seed soaking, root dipping and foliar spray methods (Russo *et al.*, 2015).

Renuka *et al.* (2016) tested endophytic ability of six indigenous isolates of NBAII, *B. bassiana* in maize and found that isolate Bb-23 showed 20 per cent endophytic colonization in older stem, older leaf and young stem, whereas Bb-5a

was able to colonize more in younger leaves (26.85 per cent), stating that persistence of the fungus inside the plant gradually reduced with the increase in the age of plants.

First report that showed endophytic association of *B. bassiana* in grain samples was that of Sanchez-Rodriguez *et al.* (2018). It was reported that strain, EAB 04/01-Tip endophytically colonized both bread wheat and durum wheat.

2.2.2 In Pulses

Endophytic association of *B. bassiana*, *L. lecanii* and *A. parasiticus* was reported in beans by Gurulingappa *et al.* (2010). They revealed that the fungi colonised beans up to 21 DAI. Studies by Sasan and Bidchoka (2012) revealed that *M. robertsii* endophytically colonized the roots of *Phaseolus vulgaris* L. and it had a positive effect on root hair development. Akello and Sikora (2012) tested the endophytic association of eight fungal isolates belonging to five genera in faba beans. The per cent colonization in roots was >80 in plants inoculated with *Trichoderma asperellum* Samuels, Lieckf. & Nirenberg (M2RT4) and *M. anisopliae* (S4ST7), whereas least colonization (<60 per cent) was observed in plants inoculated with *B. bassiana* (S4SU1) and *Gibberella monliformis* Wineland (E3RF20). Endophytic colonization of *B. bassiana* in beans was confirmed by Parsa *et al.* (2018).

Golo *et al.* (2014) detected the fungal toxin, destruxin (DTxs) from the cowpea plants colonized by *M. robertsii* 2575 12 DAI, but it was not detected in cucumber. This was the first report on toxin, DTxs detected inside the plants colonized by *M. robertsii*. Russo *et al.* (2015) reported that *B. bassiana* successfully colonized as an endophyte in soybean. Gathage *et al.* (2016) also confirmed that *B. bassiana* (G1LU3) and *Hypocrea lixi* Pat. (F3ST)1 endophytically colonized leaves, stem and roots of *P. vulgaris*.

Studies of Mutune *et al.* (2016) revealed that *Trichoderma atroviridae* Bissett (F5S21) and *T. asperellum* (M2RT4) showed maximum colonization in stem (86.6%) and roots (85.3%) of beans respectively. Lowest colonization of

B. bassiana (10.8%) and *H. lixi* (10.7%) was observed in leaves. *B. bassiana* ICIPE 273 and *M. anisopliae* ICIPE 273 did not colonize any plant parts.

Behie *et al.* (2015) demonstrated localised colonization of *Metarhizium* sp. in roots, whereas *B. bassiana* and *Pochonia chlamydosporia* (Goddard) Zare & W. Gams were able to colonize both below and above ground parts in haricot beans. Jaber and Enkerli (2017) observed that *B. brongniartii* strains BIPESCO2 and 2843 and *Metarhizium brunneum* Petch (BIPESCO5) colonized all plant parts of *Vicia faba* L. grown in non-sterile compost, inoculated with conidial suspension of 1×10^7 conidia mL^{-1}.

2.2.3 Vegetables

In vegetables, endophytic association of entomopathogens were well-studied in the plants belonging to the family Solanaceae and Cucurbitaceae. Endophytic colonization of *B. bassiana* has been reported in tomato by Leckie (2002), Ownley *et al.* (2008), Powell *et al.* (2009) and Sanchez-Rodriguez *et al.* (2015).

Studies of Gurulingappa *et al.* (2010) revealed that *B. basssina* and *L. lecanii* endophytically colonised both tomato and melon plants up to 21 DAI. Garcia *et al.* (2011) reported that three isolates of *M. anisopliae* (Ma 8, 10 and 20) successfully colonized leaves, stem and roots of tomato plants. Golo *et al.* (2014) proved the endophytic association of *M. robertsii* in cucumber plants up to 12 DAI. Findings of Resquin-Romero *et al.* (2016) revealed that tomato and melon plants were endophytically colonized by *B. bassiana* and *M. brunneum* in leaves, stem and roots up to 96 h after inoculation.

2.3 INOCULATION METHODS

Purview of literature disclose that methods of inoculation is a major factor that determines the endophytic association in crop plants. Colonization rate of endophytes differ with the inoculation methods used (Posada and Vega, 2006).

2.3.1 Seed Coating

Tomato seeds coated with *B. bassiana* (isolate 11-98) conidia was reported to have established as an endophyte within two weeks when grown in sterile

vermiculite medium (Leckie, 2002). For seed coating of *B. bassiana*, concentration of $1x10^7$ conidia mL^{-1} was optimum for endophytic colonization in tomato. Increase in the concentration of conidia per seed was directly proportional to the percentage recovery of the endophyte from plant (Ownley *et al.*, 2008).

Seed coating of Opium poppy was found to yield 62.5 to 100 per cent colonisation of *B. bassiana* strain, EABb 04/01-Tip throughout the crop growth period. This was the first report to show that 25 per cent *B. bassiana* was transmitted vertically to next generation (Quesada-Moraga *et al.*, 2014).

2.3.2 Seed Soaking

Akutse *et al.* (2013) studied the effect of seed soaking method in *P. vulgaris* and *V. faba* using 10 fungal isolates belonging to six genera and stated that *Beauveria*, *Hypocrea*, *Gibberella*, *Fusarium* and *Trichoderma* colonized in both the plants. *B. bassiana* isolates, G1LU3 and S4SU1 colonized only root of *V. faba* endophytically. *Metarhizium* was not found to colonise these plants.

Seed soaking lead to successful endophytic colonization of *B. bassiana* in jute (Biswas *et al.*, 2012), cotton (Lopez *et al.*, 2014), beans (Gathage *et al.*, 2016; Mutune *et al.*, 2016) and faba beans (Jaber and Enkerli, 2017).

2.3.3 Radicle Dressing

This method of inoculation was found to be less attempted to test endophytic association of entomopathogenic fungi. Posada *et al.* (2007) demonstrated that *B. bassiana* successfully colonized leaf, stem and roots of coffee seedlings up to 60 DAI, when inoculated by radicle dressing.

2.3.4 Root Dipping

According to Akello *et al.*, (2007), root dipping was the best inoculation method for endophytic colonization of *B. bassiana* in tissue cultured banana. Colonization was found to be maximum in rhizome (79%), followed by roots (68%) and pseudostem (41%). The optimal conidial concentration and treatment duration was standardised as $1.5x10^7$ conidia mL^{-1}, for two hours (Akello *et al.*, 2008).

Successful colonization of *B. bassiana* was reported by Brownbridge *et al.* (2012) in pine seedlings (both sterile and non - sterile) through root dipping and

seed coating methods. It could be recovered from one root sample in sterile condition after nine months. Though it could be recovered from soil, it was reported to be absent after nine months.

2.3.5 Soil Drenching

Lopez *et al.* (2014) reported that *B. bassiana* and *Purpureocillium lilacinum* (Thom) Luanqsa-ard, Houbraken, Hywel-Jones & Samson colonized cotton plants endophytically through soil drenching. Studies of Greenfield *et al.* (2016) revealed that in cassava, drenching conidial suspension in soil was the best method compared to immersion of stem cuttings for attaining successful colonization of the fungi, *B. bassiana* and *M. anisopliae.* Colonisation rate was higher in the proximal end of root in both the fungi. Colonisation of *B. bassiana* was 84 per cent on the seven DAI, which was reduced to 40 per cent after 47-49 DAI. Colonisation was constant in the case of *M. anisopliae* (80 %) throughout the period.

Kaushik and Dutta (2016) proved that both foliar spraying and soil drenching with *M. anisopliae* could lead to successful colonisation in the leaves and roots of tea plants, respectively. Jaber and Enkerli (2017) reported that soil drenching of 15 mL of conidial suspension of *B. bassiana* (NATURALIS) and *M. brunneum* (BIPESCO5) resulted in endophytic colonization in sweet pepper after seventh and 17th DAI. They also demonstrated that *B. bassiana* preferred leaves and stem whereas *M. brunneum* colonized more in roots and stems.

2.3.6 Foliar Spraying

Foliar spray of *B. bassiana* with conidial concentration of 10^6 spores mL^{-1} along with 0.1 % triton, 1 % molasses and 1 % titanium dioxide were found to be the best formulation strategy for the establishment of *B. bassiana* as an endophyte in *Brassica napus* L. (Lohse *et al.*, 2015). Klieber and Reineke (2016) reported that foliar spray of conidial suspension of *B. bassiana* lead to endophytic establishment in tomato leaves. Resquin - Romero *et al.* (2016) reported that three strains of *B. bassiana* (Bb04, EABb04/01-Tip, EABb 01/33-Su) and *M. brunneum* endophytically colonized tomato, alfalfa and melon plants. Colonization rate was more than 40 per cent in leaves and 30 per cent in roots. Time taken for leaf

colonization was 24 to 48 h, whereas for root colonization it was 96 h. All the strains were effective against *Spodoptera littoralis* Boisduval when topically sprayed at 10^8 conidia mL^{-1}.

Studies of Rondot and Reineke (2018) revealed that *B. bassiana* was able to colonize young grape vines up to 21 days and mature vines up to five weeks. The fungus was able to control the mealy bug, *Planocoocus ficus* Signoret in younger vines and the hopper *Empoasca vitis* Gothe in mature vines.

2.3.7 Bloom Spraying

B. bassiana colonized cocoa pods and peduncle endophytically in green house cocoa plants, when sprayed with suspension of $1x10^7$conidia mL^{-1} during full bloom stage. Colonization of fungus was found to reduce gradually which might be due to the competition with natural endophytes (Posada *et al.*, 2010).

2.4 CONFIRMATORY METHODS FOR ENDOPHYTIC ASSOCIATION

Various researchers who have focussed in the study of endophytes opined that endophytic association of entomopathogenic fungi in crop plants can be confirmed by the following methods.

2.4.1 Re isolation

Re isolation of the inoculated organism was the commonly followed method to confirm endophytic association. *B. bassiana* was re isolated from tomato (Leckie, 2002), sorghum (Reddy *et al.*, 2009), pine (Brownbridge *et al.*, 2012), rice (Jia *et al.*, 2013), rapeseed (Lohse *et al.*, 2015), cauliflower (Gautam *et al.*, 2016), maize (Renuka *et al.*, 2016) and melon (Jurado *et al.*, 2016).

Gurulingappa *et al.* (2010) re isolated *L. lecanii* from cotton, wheat, corn, beans, tomato and pumpkin. *M. anisopliae* could be re isolated from corn (Garcia *et al.*, 2011), beans (Akello and Sikora, 2012), sorghum (Mantozoukas *et al.*, 2015) and cassava (Greenfield *et al.*, 2016).

2.4.2 Use of Selective Media

Use of selective medium for detecting endophytic entomopathogens from inoculated crop plants was reported in the studies carried out by Brownbridge *et al.*

(2012). They used *Beauveria* selective media for re isolation from pine seedling and soil, which contained cycloheximide (125mg L^{-1}). Recovery rate of *B. bassiana* in dry beans inoculated by seed coating was higher in selective media, with CTAB (0.6g L^{-1}) than normal PDA medium (Ramirez-Rodriguez and Sanchez- Pena, 2016).

2.4.3 Molecular Identification

PCR amplification was reported as the fast and reliable methods when compared to conventional re isolation method. Several workers could confirm the identity of the endophytic entomopathogenic fungi at molecular level by PCR amplification (Leckie, 2002; Quesada-Moraga *et al.* 2009 and Ownley *et al.* 2008). Reddy *et al.* (2009) detected *B. bassiana* in sorghum by using PCR amplification with specific fungal primers for ß tubulin gene which yielded same 360bp, both in inoculated fungus and treated sorghum samples. Biswas *et al.* (2015) used multiple primers like SCA 14, SCA 15 and SCB 9 to detect *B. bassiana* from jute foliage and soil. The other reports using the same procedure were those of Jia *et al.*, (2013), Landa *et al.*, (2013), Lopez *et al.* (2014), Lohse *et al.*, (2015), Gautam *et al.*, (2016), Renuka *et al.*, (2016) and Jurado *et al.* (2016).

2.4.4 Use of Microscopy

It was Wagner and Lewis (2000) who studied for the first time, the colonization and mode of penetration of endophytic *B. bassiana* using light microscopy as well as transmission electron microscopy. They proved that *B. bassiana* does not require particular topographic signals for penetration. Gautam *et al.* (2016) visualized endophytic colonization of *B. bassiana* in cauliflower through scanning electron microscopic images. Other visualization method includes the use of GFP (green fluorescent protein) transformed fungal entomopathogens (Sasan and Bidochka, 2012; Landa *et al.*, 2013; Behie *et al.*, 2015; Jurado *et al.*, 2016).

2.5 EFECT OF ENDOPHYTIC ENTOMOPATHOGENIC FUNGI ON PEST INCIDENCE AND PLANT GROWTH

2.5.1 Pest Incidence

Bing and Lewis (1991) observed significant reduction in tunnelling by the European corn borer when *B bassiana* given as foliar spray. Corn plants were suspected to be colonized by the fungus endophytically at whorl stage and persisted to provide long season suppression of the pest. Endophytic *B. bassiana* in banana inoculated through root dipping, reduced the population of banana rhizome weevil 53.4 to 57.7% (Akello *et al.*, 2008) and pseudostem weevil by 50-70% (Prabhavathi *et al.*, 2013).

Reddy *et al.* (2009) reported that the stem borer, *Chilo partellus* Swinhoe did not cause any dead heart symptoms in sorghum plants which were endophytically colonized by *B. bassiana*, whereas 40 per cent of untreated plants exhibited dead heart symptom.

Gurulingappa *et al.* (2010) observed that cotton plants which were colonized by *B. bassiana*, *L. lecanii* and *A. parasiticus* had reduced the rate of feeding and reproduction of the aphid, *Aphis gossypii* Glover and in wheat the incidence of Australian plague locust, *Chortoicetes terminifera* Walker was less. Akutse *et al.* (2013) reported that endophytic *H. lixi* and *B. bassiana* (isolates ICIPE 279, S4SU1and G1LUS) were effective in causing 100 per cent mortality of leaf miner, *Liriomyza huidobrensis* Blanchard. Studies conducted by Mantzoukas *et al.* (2015) concluded that *B. bassiana*, *M. robertsii* and *I. fumosorosea* colonized sorghum plants endophytically, causing 70 to 100 per cent mortality in the larvae of *Sesamia nonagrioides* Lefebvre. Mutune *et al.* (2016) reported that endophytic *M. anisopliae* ICIPE 20 was best isolate for the control of bean stem fly maggot.

Jurado *et al.* (2016) reported that in melon plants, endophytic *B. bassiana* colonized the spaces of parenchymatous cells in aerial parts whereas *M. brunneum*, colonized roots. Secondary metabolites produced by these endophytic fungi lead to increase in the mortality of whitefly nymphs. Among them, *B. bassiana* was more

virulent causing more than 60 per cent mortality. Destruxins A (43%), produced by *M. brunneum*, was detected in dead nymphs. Sanchez-Rodriguez *et al.* (2015) reported that endophytic *B. bassiana* caused 30 to 57 per cent mortality in *S. littoralis.*

2.5.2 Plant Growth

B. bassiana was reported to have growth promoting properties in cotton, wheat, corn, beans, tomato and pumpkin when inoculated through foliar spray and soil drenching, whereas *L. lecanii* and *A. parasiticus* were not having any effects on plant growth (Gurulingappa *et al.,* 2010). Jaber and Enkerli (2017) reported that *B. brongniartii* strains BIPESCO2 and 2843 and *M. brunneum* (BIPESCO5) colonized all plant parts of *V. faba* inoculated through foliar spray and had significant positive effect on plant growth during seventh and 14th DAI. Endophytic colonization of *B. bassiana* boosted the spike production in bread and durum wheat (Sanchez-Rodriguez *et al.,* 2015).

Corn seeds treated with the conidia of *M. anisopliae* (strain F52) increased the crop stand density (78%) and fresh weight of shoots (9.6 tonne ha^{-1}) by controlling the incidence of wire worm in the crop (Kabaluk and Ericcson, 2007). Likewise, investigations of Garcia *et al.* (2011) revealed that three isolates of *M. anisopliae* (Ma 8, 10 and 20) colonized tomato plants endophytically and played an important role in plant growth promotion. Studies conducted by Sasan and Bidchoka (2012) revealed that *M. robertsii* endophytically colonized the roots of *P. virgatum* and *P. vulgaris* and had positive effect on the root hair development.

Materials and methods

3. MATERIALS AND METHODS

The study entitled "Endophytic association of entomopathogenic fungi with rice and cowpea" was carried out in Biocontrol laboratory, Department of Agricultural Entomology, College of Agriculture, Vellayani, Thiruvananthapuram during the year 2017-19.

3.1 MAINTENANCE OF FUNGAL CULTURES

Beauveria bassiana, *Metarhizium anisopliae*, *Lecanicillium lecanii* and *Lecanicillium saksenae* cultures maintained in the Biocontrol laboratory, Department of Agricultural Entomology, College of Agriculture, Vellayani were utilized for the study. *B. bassiana* isolate Bb 5, *M. anisopliae* isolate Ma 4 and *L. lecanii* isolate No. V1 8, were originally sourced from National Bureau of Agricultural Insect Resources (NBAIR), Bengaluru. *L. saksenae* (Accession no: ITCC LsVs-1-7714) is an indigenous isolate from the soils of Vellayani isolated by Rani *et al.* (2014). The cultures of *B. bassiana*, *M. anisopliae*, *L. lecanii* and *L. saksenae* were revived by passing through *Odoiporus longicollis* (Oliver), *Cosmopolites sordidus* (Germar), *Aphis craccivora* (Koch) and *Leptocorsia acuta* (Thunberg) respectively. Pure cultures were maintained in potato dextrose agar (PDA) slants and mass multiplied in potato dextrose broth (PDB).

3.1.1 Preparation of Conidial Suspensions

Conidial suspensions were prepared from 14 day old sporulating cultures of *B. bassiana* and *M. anisopliae* while for *L. lecanii* and *L. saksenae* 21 day old cultures were used. Conidial suspensions were prepared by adding 10 mL of sterile water containing 0.05% Triton X 100 into the respective culture broth in 250 mL conical flasks. Each of the mixture was blended in a mixer-grinder for 20 sec aseptically to obtain homogenous suspensions. The suspensions were filtered separately through double layered muslin cloth and collected in sterile beakers. The conidial concentration was adjusted to $1x10^8$ conidia mL^{-1} in *B. bassiana* and

M. anisopliae and $1x10^7$ conidia mL^{-1} in the case of *L. lecanii* and *L. saksenae*, by using a Neubauer haemocytometer.

3.2 *IN VITRO* STUDIES TO EXAMINE THE ASSOCIATION OF ENTOMOPATHOGENIC FUNGI WITH CROP PLANTS

Endophytic association of *B. bassiana*, *M. anisopliae*, *L. lecanii* and *L. saksenae* was tested on rice and cowpea separately. The association was tested by seven methods of inoculation, *viz.*, seed coating, seed soaking, radicle dressing, soil drenching, root dipping, foliar spraying and bloom spraying. Seeds were surface sterilized with two per cent sodium hypochlorite solution for two minutes, followed by 70 per cent ethanol for two minutes and three changes of sterile distilled water.

3.2.1 Rice

Rice variety Prathyasha (Mo 21), seeds of which procured from Coconut Research Station, Balarampuram, Thiruvananthapuram was used for the study. Surface sterilized seeds were then soaked in sterile distilled water taken in a 250 mL beaker for one day and the next day the seeds were tied in sterile muslin cloth in such a way that the muslin cloth is partially dipped in sterile water. For rice, pregerminated seeds were used for the experiment. Of the seven methods of inoculation mentioned in para 3.2, three methods *viz.,* seed coating, seed soaking and radicle dressing were adopted before planting, whereas the other methods soil drenching, root dipping, foliar spraying and bloom spraying were tested after planting.

3.2.1.1 Treatments Before Planting

Seed coating, seed soaking and radicle dressing were done before planting. The above three treatments were replicated thrice with two pots per replication and three plants per pot. Control plants were also raised in a similar manner, in which pregerminated untreated seeds were sown.

3.2.1.1.1 Seed coating

Pregerminated sterile paddy seeds (25g) dried on a sterile tissue paper were taken separately in 250 mL sterile beakers. Conidial suspensions (50 mL) of each of the fungi *B. bassiana*, *M. anisopliae*, *L. lecanii* and *L. saksenae* were added into it and kept in the dark for one hour (Plate 1A) as per the method suggested by Brownbridge *et al.* 2012). Treated seeds were taken out, dried on a sterile tissue paper and were used for sowing.

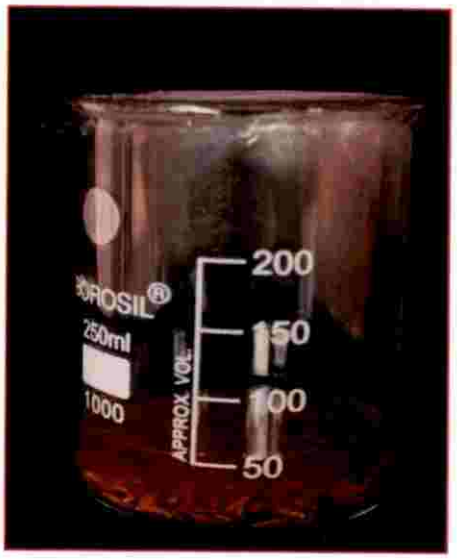
3.2.1.1.2 Seed soaking

Pregerminated seeds were treated with all the four conidial suspensions in a similar way as mentioned in para 3.2.1.1.1 and were kept soaked in darkness for 24 h, dried as per the procedure described by Akutse *et al.* (2013), before sowing.

3.2.1.1.3 Radicle dressing

Pregerminated seeds (125g) were kept in sterile petri plate lined with sterile tissue paper for five to six days for radicle emergence. Tissue paper was replaced once in two days. After radicle emergence, the seeds were transferred into five different sterile beakers, each of them containing conidial suspension of *B. bassiana*, *M. anisopliae*, *L. lecanii* and *L. saksenae* and kept immersed for two hours (Plate 1B) as suggested by Posada *et al.* (2007). After two hours, treated seeds were taken out, dried on a sterile tissue paper and were used for sowing.

The seeds treated in the above three methods were sown in plastic pots of 11 cm x 7.5 cm containing sterile potting mixture consisting of clay, soil and cow dung in the ratio of 2:1:1 and were maintained in a sterile condition by manuring with sterile dried cow dung and irrigating with sterile water. The pots were then placed in a netted cage made of aluminium frame and nylon wire mesh of 165 cm x 120 cm. It was provided with removable doors on two opposite sides that enabled to place the pots inside. The top and sides of the cage were covered with transparent polypropylene sheets to allow the entry of sunlight and also to avoid the entry of rain water.



A. Seed coating

B. Radicle dressing

C. Seed soaking

D. Root dipping

Plate 1. Inoculation methods

3.2.1.2 Treatments After Planting

Methods of inoculation such as soil drenching, root dipping, foliar spraying and bloom spraying were adopted in plants raised under sterile conditions as mentioned in 3.2.1.1.3. These treatments were replicated thrice with two pots per replication and three plants per pot.

3.2.1.2.1 Root dipping

Sterile, 25day old seedlings were uprooted and washed with sterile water. The roots were dipped in 50 mL of conidial suspensions of each of the fungus taken in 250 mL sterile beakers for two hours as per the procedure described by Akello *et al.* (2008). The treated seedlings were replanted in sterile potting mixture filled in larger pots of 23 cm x 21 cm and were maintained in sterile condition as mentioned in para 3.2.1.1.3.

3.2.1.2.2 Soil drenching

Sterile seedlings raised as mentioned in 3.2.1.1.3 were transplanted to larger pots of dimension 23 cm x 21 cm. Five days after transplanting (DAT), 10 mL of conidial suspension of each of the fungus was drenched around the root zone as specified in the methodology of Tefera and Vidal (2009). Plants were maintained in sterile condition as mentioned in para 3.2.1.1.3.

3.2.1.2.3 Foliar spraying

Sterile seedlings raised as mentioned in 3.2.1.1.3 were selected for treatments. They were transplanted to the larger pots of size 23 cm x 21 cm. Using 50 mL hand sprayer, conidial suspensions of each of the fungus (five mL per plant) were sprayed on leaves after five DAT as suggested by Gurulingappa *et al.* (2010). The plants were covered with polythene covers for 24 h to provide adequate moisture that is congenial for germination of fungi. Spraying was done during evening hours. Plants were maintained in sterile condition as mentioned in para 3.2.1.1.3.

3.2.1.2.4 Bloom spraying

Sterile seedlings raised as mentioned in 3.2.1.1.3 were maintained in netted cage up to panicle initiation stage. During this stage, the panicles were sprayed with fungal suspensions (five mL per plant) carefully, and were then covered with polyethene cover for one day as specified in the methodology of Posada *et al.* (2010). Plants were maintained in sterile condition as mentioned in para 3.2.1.1.3.

3.2.1.2.5 Untreated plants

Pregerminated sterile seeds as mentioned in the para 3.2.1 were sown in plastic pots of dimension 11cm x 7.5 cm filled with 2:1:1 sterile potting mixture comprising clay: soil: cow dung. Seeds were soaked in sterile distilled water with 0.05% Triton X-100. In each pot, three seeds were dibbled and the pots were kept in the netted cage as described in para 3.2.1.1.3.

3.2.2 Cowpea

Seeds of cowpea variety, Anaswara procured from the Department of Vegetable Science, College of Agriculture, Vellayani, Thiruvananthapuram were used for the study. Seeds were surface sterilized as mentioned in para 3.2.1 and were sown in 1:1:1 sterile potting mixture of red soil: dried cow dung: coir pith.

Treatments Before Planting

3.2.2.1.1 Seed coating

Surface sterilized seeds (75g) were coated with conidial suspensions of each of the fungi as described in para 3.2.1.1.1. Seeds were sown in plastic pots of dimension 11 cm x 7.5 cm containing sterile potting mixture as described in the para 3.2.2 and seedlings were maintained by manuring with sterile dry cow dung and irrigating with sterile water and were kept in netted cage as mentioned in para 3.2.1.1.3.

3.2.2.1.2 Seed soaking

Surface sterilized seeds (75g) were treated in a similar way as mentioned in para 3.2.1.1.2 (Plate 1C) and kept in darkness for 24 h. Pots were maintained as mentioned in para 3.2.1.1.3.

3.2.2.1.3 Radicle dressing

Surface sterilized seeds (125g) were subject to radicle dressing with each of the fungus and raised as mentioned in 3.2.1.1.3

3.2.2.2 Treatments After Planting

Methods of inoculation such as soil drenching, root dipping, foliar spraying and bloom spraying were adopted in plants raised under sterile conditions as mentioned in 3.2.2.1.1 Each treatment was replicated thrice with two pots per replication and three plants per pot.

3.2.2.2.1 Root dipping

Root dipping was done in six-day old seedlings following the method adopted in rice (para 3.2.1.2.1) (Plate 1D). Plants were maintained in sterile condition as mentioned in para 3.2.1.1.3

3.2.2.2.2 Soil drenching

Seedlings were raised and maintained as mentioned in para 3.2.2.1.1 Soil drenching was done as described in para 3.2.1.2.2. Six-day old seedlings were used for the treatment. Plants were maintained in sterile condition as mentioned in para 3.2.1.1.3.

3.2.2.2.3 Foliar spraying

Seedlings were raised and maintained as described in para 3.2.2.1.1. Spraying was done in similar way as mentioned in para 3.2.1.2.3, at three leaf stage. Plants were maintained in sterile condition as mentioned in para 3.2.1.1.3

3.2.2.2.4 Bloom spraying

Cowpea plants raised in sterile condition as mentioned in para 3.2.2.1.1 and were maintained up to flowering stage. When they attained full bloom stage, flowers were carefully sprayed with conidial suspensions of each of the fungi at the rate of five mL per plant, during evening hours. Plants were maintained in sterile condition as mentioned in para 3.2.1.1.3

3.2.2.2.5 Control plants

Surface sterilized seeds as mentioned in 3.2.1 were sown in plastic pots of dimension 11 cm x 7.5 cm filled with 1:1:1 sterile potting mixture made of red soil, dried cow dung and coir pith. Seeds were soaked in sterile distilled water with 0.05% Triton X-100 for 60 min. In each pot, three seeds were dibbled and the pots were maintained in the netted cage.

3.3 RE ISOLATION

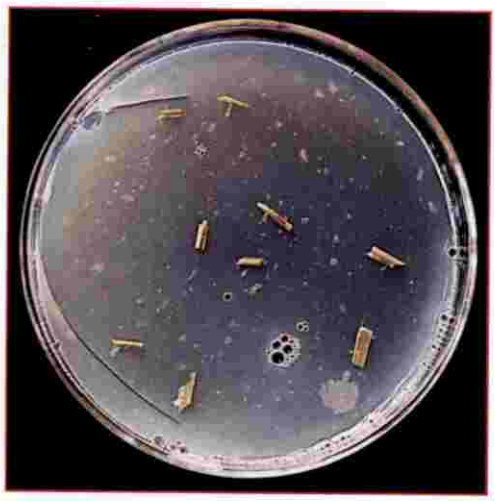
To check the colonisation of the inoculated organisms in each of the method, samples from the inoculated plants as well as soil samples taken from the root zone (in root dipping and soil drenching methods) were examined in the laboratory on 15^{th}, 30^{th} and 45^{th} DAI.

3.3.1. From Plants

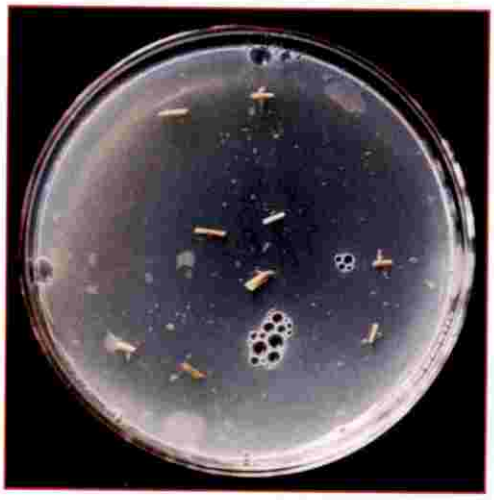
Two plants each were uprooted from one replication at random. The uprooted plant samples were washed with clean tap water and the leaves, stem, root and grains (in bloom spraying method) were separated. All the leaves, stem and roots were cut into four pieces (except grains) using a sterile blade and subjected to surface sterilization as mentioned in para 3.2. The samples were cut into small bits of size 5 mm^2 with a sharp and sterile razor blade and placed separately in Petri plates of nine cm diameter, containing sterile distilled water. The bits were dried on a sterile tissue paper and then placed aseptically on PDA plates with nine bits per plate (Plate 2). Leaves, stem, roots and grain samples were inoculated separately and incubated at room temperature for 15 days to observe the growth of the endophytes. The last rinsed sterile distilled water was smeared on PDA plates to test the efficacy of surface sterilization as specified in the methodology suggested by Schulz and Boyle (1998).

3.3.2 From Soil

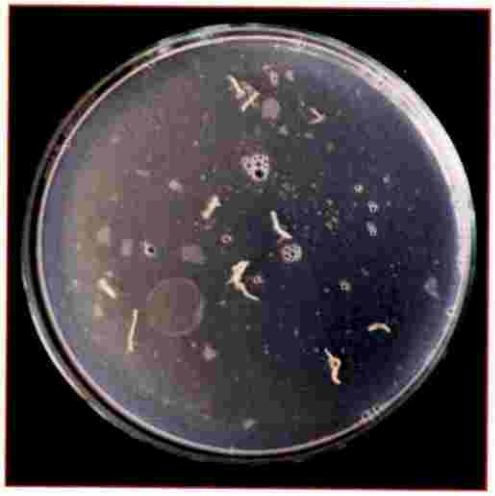
In root dipping and soil drenching methods, soil samples (50 g) were collected in polythene bags from the root zone from a depth of 10 cm. After pooling and quartering, 10 g of it was transferred into 250 mL conical flask containing 90



Leaf samples



Stem samples



Root samples

Plate 2. Surface sterilized plant samples on PDA media

mL sterile distilled water. By serial dilution, concentrations of 10^{-4} was prepared. One mL of aliquot was poured into PDA plates aseptically and incubated for 15 days at room temperature.

The inoculated plates were observed for emergence of fungal/bacterial colonies for a period of 15 days. Colonies which were suspected to be of the inoculated organism were transferred to fresh Petri plates to study their colony and conidial characters. Each of the colonies of natural endophytes that were encountered during re isolation were also transferred to separate Petri plates for further studies.

3.4 IDENTIFICATION OF ISOLATES

3.4.1 Morphological Identification

Each of the colonies observed in the isolation medium, were picked up at an earlier stage and pure cultured in separate Petri plates to study their colony characters. They were then slide cultured and examined under compound microscope with Motic cam 3.0 MP imaging software. Those organisms whose identity could not be confirmed through morphological studies were subjected to molecular characterization.

3.4.2 Molecular Characterization

Species level confirmation was done at molecular level by ITS and 16S sequencing for fungi and bacteria respectively, using the facilities available at Rajiv Gandhi Centre for Biotechnology (RGCB) for molecular identification, Thiruvananthapuram, Kerala, India.

3.4.2.1 ITS Sequencing

3.4.2.1.1 DNA isolation using NucleoSpin® plant II kit (Macherey-Nagel)

About 100 mg of the mycelium was homogenized using liquid nitrogen and the powdered mycelia was transferred to a microcentrifuge tube. 400 µL of buffer PL1 was added to powdered tissue and vortexed for one min. Then 10µL of RNase A solution was added and inverted to mix. The homogenate obtained was incubated

at 65°C for 10 min later this lysate was transferred to a nucleospin filter and centrifuged at 11000 x g for two min. The flow through liquid was collected and 450 µL of buffer PC was added. The solution was transferred to a nucleospin Plant II column, centrifuged for one min and the flow through liquid was discarded. 400 µL buffer PW1 was added to the column, centrifuged at 11000 x g for one min and flow though liquid was discarded. Then 700 µl PW2 was added, centrifuged at 11000 x g and flow through liquid was discarded. 200 µl of PW2 was added and centrifuged at 11000 x g for two min to dry the silica membrane. The column was transferred to a new 1.7 mL tube and 50 µl of buffer PE was added and incubated at 65°C for five min. The column was centrifuged at 11000 x g for one min to elute the DNA. The eluted DNA was stored at 4°C.

3.4.2.1.2 Agarose gel electrophoresis for DNA quality check

The quality of the DNA isolated was checked using agarose gel electrophoresis. One µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 μgmL^{-1} ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front has migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

3.4.2.1.3 PCR analysis

PCR amplification reactions were carried out in a 20 µl reaction volume which had 1X Phire PCR buffer (contains 1.5 mM $MgCl_2$), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 1 µl DNA, 0.2 µl Phire Hotstart II DNA polymerase enzyme, 0.1 $mgmL^{-1}$ BSA and 3% DMSO, 0.5M Betaine, 5pM of forward and reverse primers.

Primers used

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

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

PCR amplification profile for ITS

98 °C	-	30 sec	
98 °C	-	5 sec	} 40 cycles
60 °C	-	10 sec	
72 °C	-	15 sec	
72 °C	-	60 sec	
4 °C	-	∞	

3.4.2.1.4 Agarose gel electrophoresis of PCR products

The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 μgmL^{-1} ethidium bromide. One µl of 6X loading dye was mixed with five µl of PCR products and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about one-two hours, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

3.4.2.1.5 ExoSAP-IT treatment

ExoSAP-IT (GE Healthcare) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications.

Five µl of PCR product was mixed with two µl of ExoSAP-IT and incubated at 37°C for 15 min followed by enzyme inactivation at 80°C for 15 min.

3.4.2.1.6 Sequencing using big dye terminator v3.1

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol.

The PCR mix consists of following components:

PCR Product (ExoSAP treated)	-	10-20 ng
Primer	-	3.2 pM (either Forward or Reverse)
Sequencing Mix	-	0.28 µl
5x Reaction buffer	-	1.86 µl
Sterile distilled water	-	make up to 10µl

The sequencing PCR temperature profile consisted of a first cycle at 96°C for two min followed by 30 cycles at 96°C for 30 sec, 50°C for 40 sec and 60°C for 4 min for all the primers.

3.4.2.1.7 Post sequencing PCR clean up

Master mix I containing 10µl milli Q and 2 µl 125mM EDTA was added to each reaction containing 10µl of reaction contents and properly mixed. Then master mix II containing of two µl of 3M sodium acetate pH 4.6 and 50 µl of ethanol was added to each reaction and mixed by inverting. The reaction mixture was incubated at room temperature for 30 min and centrifuged at 14,000 rpm for 30 min. The supernatant was decanted and 100 µl of 70% ethanol was added and centrifuged at 14,000 rpm for 20 min. The supernatant was discarded and repeat 70% ethanol wash again supernatant was decanted and cleaned up air dried product obtained was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems).

3.4.2.1.8 Sequence analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Identity of ITS-rDNA conserved region of the fungal isolates was done by performing a similarity search in National Centre for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST) and the sequences were matched with existing database for species confirmation. The obtained results of rDNA sequences were submitted to NCBI database and accession numbers were procured.

3.4.2.2 16s Sequencing

3.4.2.2.1 Genomic DNA isolation from bacteria

Genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions.

A part of culture was taken in a microcentrifuge tube. 180µl of T1 buffer and 25µl of proteinase K was added and incubated at 56^0C in a water bath until it was completely lysed. After lysis, five µl of RNase A (100 $mgmL^{-1}$) was added and incubated at room temperature for five min. 200µl of B3 buffer was added and incubated at 70°C for 10 min. Through vortexing, 210µl of 100% ethanol was added and mixed thoroughly. The mixture was pipetted into NucleoSpin® Tissue column placed in a two mL collection tube and centrifuged at 11000 x g for one min. The NucleoSpin® Tissue column was transferred to a new two mL tube and washed with 500 µl of BW buffer. Wash step was repeated using 600 µl of B5 buffer. After washing the NucleoSpin® Tissue column was placed in a clean 1.5 mL tube and DNA was eluted out using 50 µl of BE buffer. The quality of the DNA isolated was checked using agarose gel electrophoresis as mentioned in the para 3.4.1.2.

3.4.2.2.2 PCR analysis

PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X PCR buffer (100mM Tris HCl , pH-8.3; 500mM KCl), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 2.5mM $MgCl_2$, 1 unit of AmpliTaq Gold DNA polymerase enzyme, 0.1 $mgmL^{-1}$ BSA, 4% DMSO, 5pM of forward and reverse primers and template DNA. Primers used are given below.

Target	Primer Name	Direction	Sequence (5' → 3')
16S rRNA	16S-RS-F	Forward	CAGGCCTAACACATGCAAGTC
	16S-RS-R	Reverse	GGGCGGWGTGTACAAGGC

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

PCR amplification profile

16S rRNA

95 °C	-	5.00 min	
95 °C	-	30 sec	} 35 cycles
60 °C	-	40 sec	
72 °C	-	60 sec	
72 °C	-	7.00 min	
4 °C	-	∞	

3.4.2.2.3 Agarose gel electrophoresis of PCR products

The PCR products were checked in 1.2% agarose gels as per the procedure mentioned in the para 3.4.1.4 and ExoSAP-IT Treatment was done as mentioned in the para 3.4.1.5. Sequencing using big dye terminator v3.1 was done in a PCR thermal cycler as mentioned in the para 3.4.1.6.

3.4.2.2.4 Post sequencing PCR clean up

Master mix I of 10µl milli Q and two µl 125mM EDTA per reaction and master mix II of 2 µl of 3M sodium acetate pH 4.6 and 50 µl of ethanol were prepared. 12µl of master mix I was added to each reaction containing 10µl of reaction contents and was properly mixed. Later, 52 µl of master mix II was added to each reaction. Contents were mixed by inverting and incubated at room temperature for 30 min and spun at 14,000 rpm for 30 min. After centrifuge, supernatant was decanted and 100µl of 70% ethanol was added. Again, spun at 14,000 rpm for 20

min, supernatant was decanted and repeat the 70% ethanol wash and air dried the pellet. The cleaned air-dried product was sequenced in ABI 3730 DNA Analyzer (Applied Biosystems). Sequence quality was checked and identification of obtained nucleotide sequence was done as per the procedure mentioned in the para 3.4.1.8.

3.5 *IN VIVO* STUDIES IN POTS TO ASSESS THE PERFORMANCE OF SUCCESSFUL ASSOCIATIONS

To assess the effect of endophytic entomopathogens in rice under open conditions, three successful associations were tested in pot culture experiment. Observations were recorded on the incidence of pests and diseases as well as plant growth and yield parameters.

3.5.1 Rice

As seed soaking was found to be an ideal method for getting an endophytic association of the inoculated organisms, this method was tested under open condition by raising potted rice plants. The fungi that could be re isolated from plants were selected for this experiment. Pre germinated seeds were soaked separately in the conidial suspensions of *B. bassiana* and *M. anisopliae* @ $1x10^8$ conidia mL^{-1} and *L. saksenae* @ $1x10^7$ conidia mL^{-1} for 24 h. in dark. Inoculated seeds were grown in a smaller pots of dimension 11 cm x 7.5 cm containing potting mixture as mentioned in the para 3.2.1.1.1. 25-day old seedling were transplanted to bigger pots of dimension 23 cm x 21 cm containing same potting mixture.

Observations on pest and disease incidence were taken at weekly intervals, plant height (cm) at monthly intervals and biomass and grain yield (g) at the time of harvest.

The data obtained from the experiment was subjected to analysis of variance (ANOVA) using the software, WASP 2.0.

Results

4. RESULTS

The results of the study entitled "Endophytic association of entomopathogenic fungi with rice and cowpea" carried out during 2017-19 is depicted below.

4.1 ENDOPHYTIC ASSOCIATION OF ENTOMOPATHOGENIC FUNGI WITH CROP PLANTS

Endophytic association of the entomopathogenic fungi *Beauveria bassiana*, *Metarhizium anisopliae*, *Lecanicillium lecanii* and *Lecanicillium saksenae* was tested in crop plants such as rice and cowpea separately. The association was tested by seven methods of inoculation, *viz*., seed coating, seed soaking, radicle dressing, soil drenching, root dipping, foliar spraying and bloom spraying.

4.1.1 Endophytes Associated with Rice Plants

4.1.1.1 Entomopathogenic Fungi reisolated

The details of re isolated fungi from rice plants inoculated separately with *B. bassiana*, *M. anisopliae, L. lecanii* and *L. saksenae* is presented in Table 1 and 2.

4.1.1.1.1 Seed coating

In plants inoculated by seed coating, none of the leaf, stem and root samples revealed the presence of *B. bassiana, M. anisopliae, L. lecanii* or *L. sakenae* on 15th, 30th and 45th Days After Inoculation (DAI).

4.1.1.1.2 Seed soaking

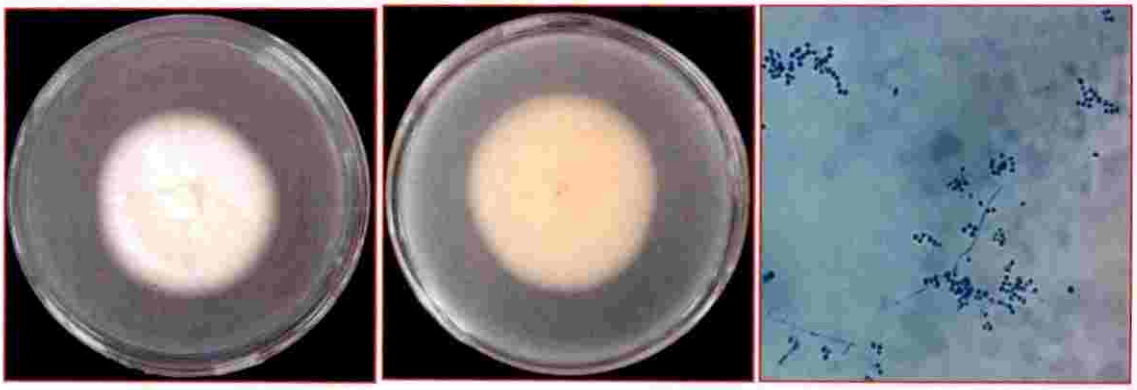
Plants inoculated with *B. bassiana* when examined in the laboratory revealed its presence in stem and root samples on 15th DAI. Re isolated colonies resembled those of the inoculated culture of *B. bassiana*. Colonies were white, regular and elevated on upper side (Plate 3Aa) and pale yellow on reverse side (Plate 3Ab). Conidia were single celled, ovoid and borne on the zig zag rachis like

Table 1. Entomopathogenic fungi re isolated from inoculated rice plants on 15th day after inoculation

Inoculation methods	Endophytes isolated						
	Leaf		Stem		Root		Grain
	Isolated species	No. of colonies	Isolated species	No. of colonies	Isolated species	No. of colonies	Isolated species
Seed coating	-	-	-	-	-	-	Nil
Seed soaking	-	-	*Beauveria bassiana*	1	*Beauveria bassiana*	1	
			Metarhizium anisopliae	1	*Lecanicillium saksenae*	2	
			Lecanicillium saksenae	2			
Radicle dressing	-	-	-	-	-	-	
Root dipping	-	-	-	-	-	-	
Soil drenching	-	-	-	-	*Lecanicillium saksenae*	2	
Foliar spray	*Lecanicillium saksenae*	3	-	-	-	-	
Bloom spray	-	-	-	-	-	-	
Control	-	-	-	-	-	-	

Table 2. Entomopathogenic fungi re isolated from inoculated rice plants on 30th and 45th day after inoculation

Inoculation methods	On 30th DAI						
	Leaf		Stem		Root		Grain
	Isolated species	No. of colonies	Isolated species	No. of colonies	Isolated species	No. of colonies	Isolated species
Seed coating	-	-	-	-	-	-	Nil
Seed soaking	-	-	*Lecanicillium saksenae*	2	*Lecanicilliium saksenae*	1	
Radicle dressing	-	-	-	-	-	-	
Root dipping	-	-	-	-	-	-	
Soil drenching	-	-	-	-	*Lecanicillium saksenae*	1	
Foliar spray	*Lecanicillium saksenae*	3	-	-	-	-	
Bloom spray	-	-	-	-	-	-	
Control	-	-	-	-	-	-	
On 45th DAI							
Seed coating	-	-	-	-	-	-	Nil
Seed soaking	-	-	*Lecanicillium saksenae*	2	*Lecanicillium saksenae*	1	
Radicle dressing	-	-	-	-	-	-	
Root dipping	-	-	-	-	-	-	
Soil drenching	-	-	-	-	*Lecanicillium saksenae*	1	
Foliar spray	*Lecanicillium saksenae*	3	-	-	-	-	
Bloom spray	-	-	-	-	-	-	
Control	-	-	-	-	-	-	

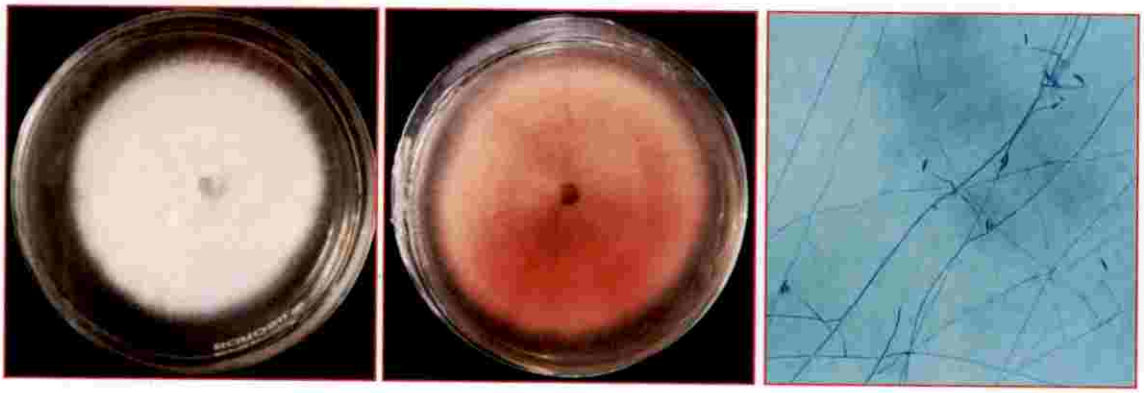


a. Upper side b. Reverse side c. Conidia
40x magnification

3A. *Beauveria bassiana*

a. Upper side b. Reverse side c. Conidia
40x magnification

3A. *Metarhizium anisopliae*



a. Upper side b. Reverse side c. Conidia
40x magnification

3C. *Lecanicillium saksenae*

Plate 3. Entomopathogenic fungi re isolated from rice plants

like a bunch of grapes (Plate 3Ac). It measured about 3.5µm x 3.4µm. It took 14 days to sporulate. Presence of the fungus was not revealed in samples examined on 30th and 45th DAI.

Plants inoculated with *M. anisopliae* through seed soaking, revealed the presence of the fungus in stem samples drawn on 15 DAI.

Re isolated colonies were white in colour, with concentric mycelial growth on the upper side, up to 10 days. After sporulation, colour of the mycelia turned into olive green (Plate 3Ba), with dark green colour on the reverse side (Plate 3Bb). It resembled the inoculated one. Conidiophores were mononematous, conidia were rod shaped (Plate 3Bc) and arranged in chains. It could not be retrieved from leaf and root samples examined.

On 30th and 45th DAI, *M. anisopliae* could not be retrieved from none of the plant samples.

On 15th, 30th and 45th DAI, *L. saksenae* could be re isolated from stem and root samples. It could not be retrieved from leaf samples.

Re isolated colonies resembled inoculated culture, typically. Colonies were pure white, regular and circular, with entire margin (Plate 3Ca). On the reverse side, vine red pigmentation was noticed (Plate 3Cb).

Conidia were anther shaped, borne singly on mononematous and verticillate branched conidiophores, and arouse directly from the mycelium. Monophialides were medium in size and solitary (Plate 3Cc). It took 21 days for sporulation. Conidia measured about 2.2µm x 4.4µm. Through colony and conidial characters, it was confirmed as *L. saksenae.*

L. lecanii could not be re isolated from plants inoculated through seed soaking on 15th, 30th or 45th DAI.

4.1.1.1.3 Radicle dressing

None of the inoculated fungi could be retrieved from plants inoculated by radicle dressing method.

4.1.1.1.4 Root dipping

Plant samples did not reveal the presence of any of the inoculated fungi when samples were drawn on 15th, 30th and 45th DAI.

L. saksenae could be retrieved from soil samples on 15th, 30th and 45th DAI (Table 3).

B. bassiana, M. anisopliae and *L. lecanii* could not be re isolated from soil samples after inoculating them through root dipping.

4.1.1.1.5 Soil drenching

Plant samples revealed the presence of *L. saksenae* in roots up to 45th DAI. Soil samples examined on 15th, 30th and 45th day revealed the presence of *L. saksenae.*

The other three fungi, *B. bassiana, M. anisopliae* and *L. lecanii* could not be detected while examining the plant and soil samples on 15th, 30th and 45th DAI.

4.1.1.1.6 Foliar spraying

L. saksenae could be retrieved from leaf samples on 15th, 30th and 45th DAI.

Plant samples did not reveal the presence of *B. bassiana, M. anisopliae* and *L. lecanii* till 45th day after treatment.

4.1.1.1.7 Bloom spraying

Inoculated entomopathogenic fungi could not be retrieved from none of the plant samples drawn on 15th, 30th and 45th DAI.

Among the four entomopathogenic fungi inoculated, *B. bassiana*, *M. anisopliae* and *L. saksenae* were found to colonise rice plants. *B. bassiana* was found to colonise in stem and roots, while *M. anisopliae* preferred stem. *L. saksenae* was found to colonise leaves when sprayed on foliage, in stem and roots when seeds were soaked in the spore suspension and in roots when the suspension was drenched into soil. *L. lecanii* was not found to colonise rice plants. Number of colonies isolated was more (21 no.) in the case of *L. saksenae* when compared to other fungi (1 no.)

Table 3. Persistence of entomopathogenic fungi in soil

Isolated on	Rice		Cowpea	
	Root dipping	Soil drenching	Root dipping	Soil drenching
15 DAI	*Lecanicillium saksenae*	*Lecanicillium saksenae*	*Metarhizium anisopliae*	*Beauveria bassiana*
				Metarhizium anisopliae
			Lecanicillium saksenae	*Lecanicillium saksenae*
30 DAI	*Lecanicillium saksenae*	*Lecanicillium saksenae*	*Metarhizium anisopliae*	*Metarhizium anisopliae*
			Lecanicillium saksenae	*Lecanicillium saksenae*
45 DAI	*Lecanicillium saksenae*	*Lecanicillium saksenae*	*Lecanicillium saksenae*	*Lecanicillium saksenae*

*DAI Days after inoculation

Seed soaking was found to be the best method for endophytic establishment of *B. bassiana*, *M. anisopliae* and for *L. saksenae* seed soaking, foliar spraying and soil drenching were found to be effective.

L. saksenae persisted in soil up to 45th days after soil drenching, while other fungi could not be retrieved from soil.

4.1.1.2 Natural Endophytes

While attempting the re isolation of inoculated organisms, 13 endophytes were encountered in rice from different plant parts (Table 4). Of these, 11 were fungal endophytes, one bacterium and another one was a fungal bacterial association. Leaf, stem and root samples harboured three fungi in common namely, *Neocomospora rubicola* L. Lombard and Crous, *Microdochium fisheri* Hern.-Restr. and Crous and *Penicillium* sp. *Pestalotiopsis* spp was found to colonize both leaves and roots, whereas *Aspergillus* spp colonized leaves and stem. Stem was found to be colonized by more endophytes (six species). A fungal bacterial association of *Sarocladium oryzae* (Sawada) W. Gams and D. Hawksw and *Kosakonia sacchari* (Zhu) Gu. was found to be predominant (seven colonies) in stem. The bacterium *Burkholderia cepacia* (Palleroni and Holmes) Yabuuchi. was found to colonize both leaves and roots. The number of bacterial endophytes isolated was less because, the experiment mainly focused to isolate fungal endophytes and the medium used for re isolation was PDA.

Morphological characters of all the natural endophytes isolated, along with the molecular characters of select endophytes that needed confirmation is presented below. NCBI Accession no. of those characterised at molecular level is given in Table 5.

Table 4. Natural endophytes isolated from different parts of rice plants

Plant parts	Fungal endophytes	No. of colonies	Bacterial endophytes	No. of colonies
Leaf	*Pestalotiopsis microspora*	4	*Burkholderia cepacia*	3
	Penicillium sp.	5		
	Neocomospora rubicola	23		
	Aspergillus sp. - 1	2		
	Pestalotiopsis sp.	4		
	Microdochium fisheri	2		
Stem	*Sarocladium oryzae* + *Kosakonia sacchari* (Endophytic fungus + endophytic bacterium association)			7
	Microdochium fisheri	5	Nil	
	Penicillium sp.	5		
	Fusarium solani	3		
	Aspergillus sp. - 1	3		
	Cladosporium sp.	2		
	Aspergillus sp. - 2	1		
	Neocomospora rubicola	15		
	Efr 11(unidentified)	4		
	Efr 14 (unidentified)	7		
Root	*Pestalotiopsis* sp.	1	*Burkholderia cepacia*	20
	Microdochium fisheri	2		
	Penicillium sp.	5		
	Neocomospora rubicola	8		
Grain	Nil		-	

Efr - Endophytic fungus from rice

Table 5. Molecular identity of natural endophytes from rice plants

Natural endophytes	NCBI accession number
Fungal endophytes	
Pestalotiopsis microspora	MN314833
Neocomospora rubicola	MN319576
Fusarium solani	MN233644
Sarocladium oryzae	MN314835
Bacterial endophytes	
Kosakonia sacchari	MN367966
Burkholderia cepacia	MN367964

4.1.1.2.1 Isolates obtained from leaves, stem and roots

4.1.1.2.1.1 Neocosmospora rubicola

Colonies were white, irregular, rhizoid in form, with filiform margin on the upper side (Plate 4Aa) and pale yellow on reverse side (Plate 4Ab). It took eight days to sporulate. Conidium measured 7.42 µm x 3.2 µm (Plate 4Ac).

DNA sequence with NCBI Accession no. MN319576 of *N. rubicola* is presented in Appendix 1.

4.1.1.2.1.2 Microdochium fisheri

The colonies exhibited pale white filamentous mycelial growth during the initial growth stages, later exhibited an alternatively arranged orange coloured conidial zone. They were elevated and flat with filamentous margins on the upper side and pale yellow on reverse. It took 12 days to sporulate.

After sporulation, the colonies appeared clumpy mottled with white and grey colours on upper side (Plate 4Ba) and black with orange concentric band on reverse side (Plate 4Bb). Conidia were pear shaped, conidiophores mononematous, arouse from the branched mycelium. Phialides were medium and solitary. Conidia were single celled, in chains or slimy masses, present in a group of six to seven, borne on thin monophialides. (Plate 4Bc). Spores measured about 2.12 µm x 4.23 µm.

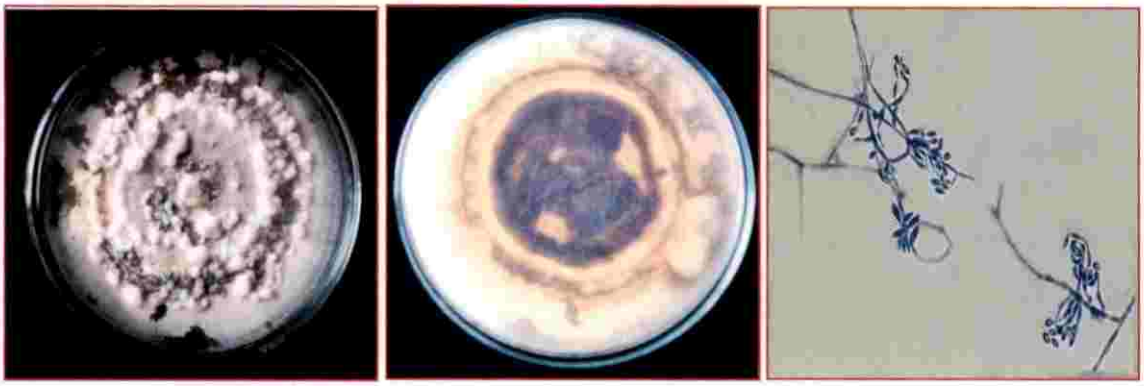
DNA sequence of *M. fisheri* is presented in Appendix 1.

4.1.1.2.1.3 Penicillium sp.

They were circular and green pigmented (Plate 4Ca), with white elevated margin on upper side and pale yellow on the reverse (Plate 4Cb). Stipes were penicillate, arising directly from the hyphae and ended in a group of phialides. Phialospores were globose, hyaline, single celled, dry, airborne and were arranged in basipetal chains (Plate 4Cc). It took five days to sporulate. Phialospores measured 3.54µm x 4.5µm.

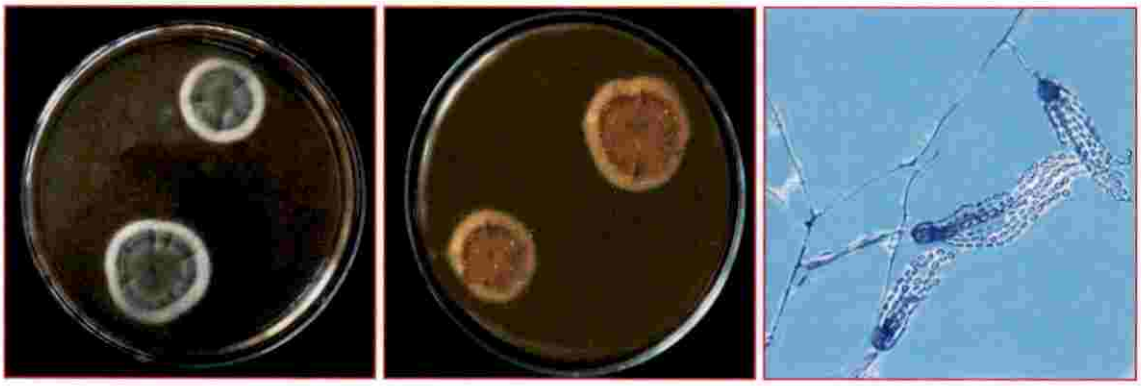
a. Upper side b. Reverse side c. Conidia
40x magnification

4A. *Neocomospora rubicola*



a. Upper side b. Reverse side c. Conidia
40x magnification

4B. *Microdichum fisheri*



a. Upper side b. Reverse side c. Conidia
40x magnification

4C. *Penicillium* sp.

Plate 4. Natural endophytes isolated from rice

Contd...

4.1.1.2.2 Isolates from leaves and stem

4.1.1.2.2.1 Aspergillus sp. - 1

Colonies were regular, mustard yellow pigmented with concentric zonation (Plate 5Aa), pale yellow on the reverse side (Plate 5Ab). The centre of the colonies was brownish and the margins were powdery upon sporulation. It took seven days for sporulation.

Stipes were simple, radiating, cylindrical and upright ending in a globose swelling, bearing phialides at the apex. Phailospores were single celled, round and arranged in a basipetal chains. They measured 2.5 µm x 3.95 µm (Plate 5Ac).

4.1.1.2.2.2 Aspergillus sp.- 2

Another species of *Aspergillus* that differed from the previously mentioned species in morphological characters, could be isolated from stem. The colonies were circular and regular, yellow pigmented with green filamentous growth in the centre on upper side (Plate 5Ba) and pale yellow on the reverse (Plate 5Bb). The margins were smooth and bordered by white concentric zone. It took ten days to sporulate.

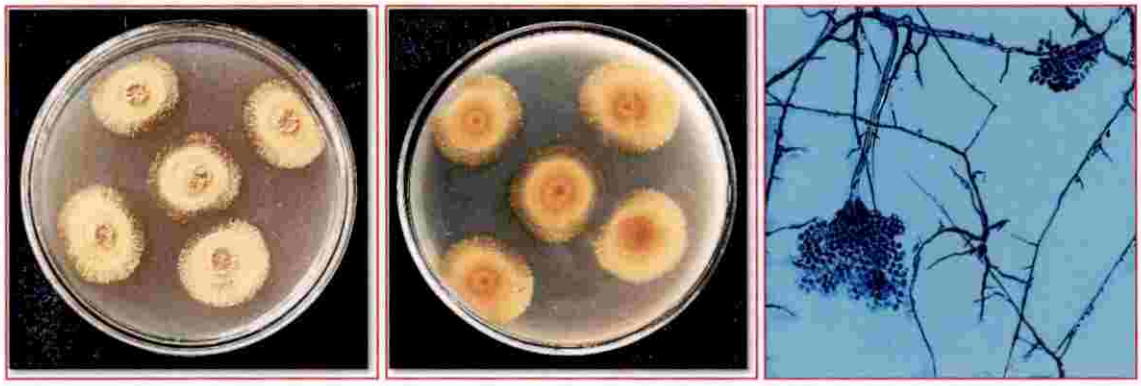
4.1.1.2.3 Isolates from leaves and roots

4.1.1.2.3.1 Pestalotiopsis microspora (Speg.) G.C. Zhao and N. Li

Colonies grown on PDA were white and fluffy on the upper side (Plate 6Aa) and pale yellow on the reverse side (Plate 6Ab). It took 10 days to sporulate.

Conidia arouse from cylindrically branched conidiophore, which were produced in black acervuli, which were profuse. Conidia were fusiform, five celled with median three cells which were dark brown in colour, bearing two spathulate appendages. They measured 4.83 µm x 3.21 µm. (Plate 6Ac).

DNA sequence with NCBI Accession no. MN314833 of *P. microspora* is presented in Appendix 1.



a. Upper side b. Reverse side c. Conidia
40x magnification

5A. *Aspergillus* sp. - 1

a. Upper side b. Reverse side

5B. *Aspergillus* sp. - 2

Plate 5. Natural endophytes isolated from rice

Contd...

a. Upper side b. Reverse side c. Conidia
40x magnification

6A. *Pestalotiopsis microspora*

a. Upper side b. Reverse side c. Conidia
40x magnification

6B. *Pestalotiopsis* sp.

6C. *Burkholderia cepacia*

Plate 6. Natural endophytes isolated from rice

Contd...

4.1.1.2.3.2 Pestalotiopsis sp.

Another species of *Pestalotiopsis* that differed from the previously mentioned species in morphological characters could be isolated from leaf and root samples.

Colonies were irregular and flat, white, rhizoid in form with fluffy growth and filamentous margin. It was pale yellow on the reverse side. Upon sporulation, black acervuli appeared on the white mycelial mat (Plate 6Ba). Colonies were pale yellow with black pigmentation on reverse side (Plate 6Bb). It sporulated profusely after 12 days (Plate 6Bc). They measured 5.43 µm x 3.13 µm.

4.1.1.2.3.3 Burkholderia cepacia

This bacterium isolated from leaf and root samples was observed to have medium sized, convex, smooth and slimy colonies with undulated margin. They were light brown pigmented on the upper side and pale yellow on reverse side (Plate 6C).

DNA sequence with NCBI Accession MN367964 of *B. cepacia* is presented in Appendix 1.

4.1.1.2.4 Isolates from stem

4.1.1.2.4.1 Fungal bacterial association

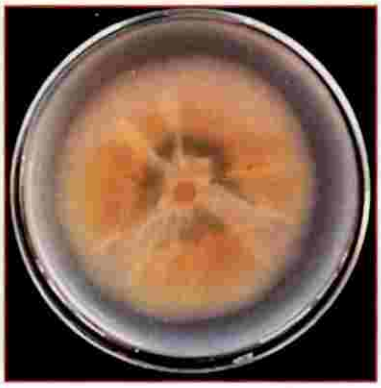
The fungus, *S. oryzae* was always found to coexist with the bacterium *K. sacchari*. Bacterial colonies always arouse from the margin of fungal colonies (Plate 7Aa).

S. oryzae colonies were pure white and filamentous (Plate 7Ab) with raised margins on upper side and pale yellow on the reverse side (Plate 7Ac). It took 15 days to sporulate. Mycelia were septate, with mononematous conidiophores arising directly from the mycelia. Conidiophores were thicker when compared to vegetative hyphae. Conidia were cylindrical, hyaline and single-celled (Plate 7Ad). It measured 3.12 µm x 3.95 µm.

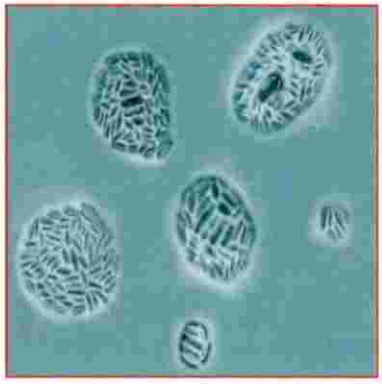
DNA sequence of *S. oryzae*, NCBI Accession no. MN314835 is presented in Appendix 1

a. Coexistence

b. Upper side

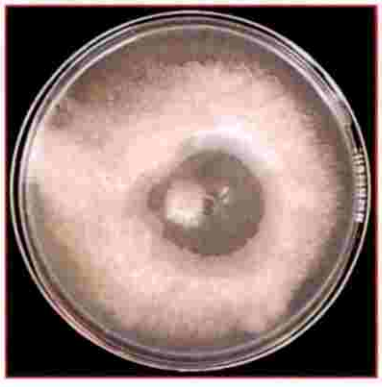


c. Reverse side

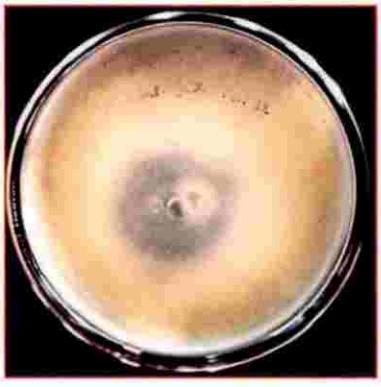


d. Conidia
40x magnification

7A. *Sarocladium oryzae* + *Kosakonia sacchari*



a. Upper side



b. Reverse side

c. Conidia
40x magnification

7B. *Fusarium solani*

Plate 7. Natural endophytes isolated from rice

Contd...

K. sacchari colonies were small and round, rough and opaque, raised and single, produced in a line. They were brown pigmented on the upper side and pale yellow on the reverse.

It was characterised at molecular level, with NCBI Accession no. MN367966 and DNA sequence is presented in Appendix 1

4.1.1.2.4.2 Fusarium solani (Mart.) Sacc.

The colonies were irregular with crateriform elevation and filiform margin (Plate 7Ba). It was a dull brownish white pigmented fungus with fluffy growth on upper side and pale yellow on reverse (Plate 7Bb). It took eight days to sporulate.

Microconidia were oval shaped, borne singly on mononematous conidiophores arising directly from mycelia. Macroconidia were sickle shaped, four to five septate (Plate 7Bc). They measured 7.42 µm x 3.2 µm.

It was characterised at molecular level, with NCBI Accession no. MN233644 and DNA sequence is presented in appendix 1

4.1.1.2.4.3 Cladosporium sp.

Colonies were greyish green pigmented on the upper side and pale yellow on the reverse. They were circular and elevated with smooth margin and bordered with white concentric zonation (Plate 8Aa). It took ten days to sporulate. Upon sporulation, they turned fluffy on upper side and dark brown on the reverse side (Plate 8Ab).

Mycelia were septate, with cylindrical conidiophores, conidia were oval to ellipsoidal in shape with rounded ends, three celled, often produced in chains (Plate 8Ac). They measured 3.2 µm x 4.3 µm

4.1.1.2.4.4 Efr 11 (Unidentified fungus)

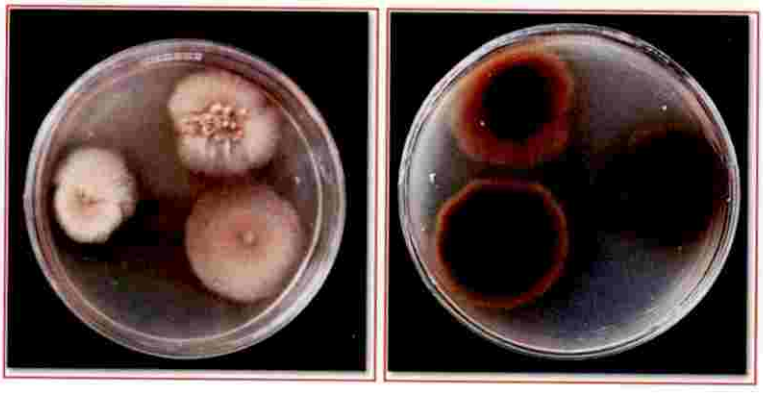
Colonies were regular, filiform margin, white on the upper side and pale yellow on reverse side. Upon sub culturing, colonies turned filamentous, bordered with orange concentric bands (Plate 8Ba and b). It took eight days to sporulate.

a. Upper side b. Reverse side c. Conidia
40x magnification

8A. *Cladosproium* sp.

a. Upper side b. Reverse side c. Conidia
40x magnification

8B. Unidentified fungus (Efr 11)



a. Upper side b. Reverse side

8C. Unidentified fungus (Efr 14)

Plate 8. Natural endophytes isolated from rice

Contd...

Mycelia were septate, branched, with mononematous conidiophores, arising directly from hyphae. Conidia were elongated, two celled, present in groups at the tip of the conidiophores (Plate 8Bc). It measured 5.1 µm x 2.9 µm.

4.1.1.2.4.5 Efr 14 (Unidentified fungus)

Colonies were round, smooth and flat with peach coloured pigmentation towards the centre and white towards the margin (Plate 8Ca). It appeared pale yellow on the lower side (Plate 8Cb). It took ten days to sporulate. Upon sporulation, brown colour gradually faded from centre to margin.

4.1.1.2.5 Grains

No endophytes could be located in grain samples taken at the time of harvest.

4.1.2 Endophytes Associated with Cowpea

4.1.2.1 Entomopathogenic Fungi reisolated

The details of re isolates obtained from cowpea plants inoculated separately with *B. bassiana*, *M. anisopliae, L. lecanii* and *L. saksenae* on 15th, 30th and 45th DAI is presented in Table 6, 7 and 8 respectively.

4.1.2.1.1 Seed coating

Plants inoculated through seed coating did not reveal the presence of inoculated fungi till 45th DAI, upon examination in the laboratory.

4.1.2.1.2 Seed soaking

Among the four fungi inoculated, only *M. anisopliae* could be retrieved from the plants inoculated through seed soaking method. The fungus was found to have colonized both in stem (Plate 9Aa and b) and roots (Plate 9Ba and b) up to 15 DAI. Its presence was not revealed in the samples examined on 30th and 45th DAI.

4.1.2.1.3 Radicle dressing

Plant samples drawn on 15th, 30th and 45th DAI, did not reveal the presence of the inoculated fungi.

Table 6. Entomopathogenic fungi re isolated from inoculated cowpea plants on 15th day after inoculation

Inoculation methods	Endophytes isolated							
	Leaf		Stem		Root		Flower	Grain
	Isolated species	No. of colonies	Isolated species	No. of colonies	Isolated species	No. of colonies	Isolated species	Isolated species
15th day after inoculation								
Seed coating	-	-	-	-	-	-	Nil	
Seed soaking	-	-	*Metarhizium anisopliae*	2	*Metarhizium anisopliae*	3		
Radicle dressing	-	-	-	-	-	-		
Root dipping	-	-	*Metarhizium anisopliae*	2	*Metarhizium anisopliae*	3		
Soil drenching	-	-	-	-	-	-		
Foliar spray	*Beauveria bassiana*	4	*Beauveria bassiana*	1	*Beauveria bassiana*	1		
	Metarhizium anisopliae	10						
	Lecanicillium lecanii	2	*Lecanicillium saksenae*	3	*Lecanicillium saksenae*	2		
	Lecanicillium saksenae	5						
Bloom spray	-	-	-	-	-	-		
Control	-	-	-	-	-	-		

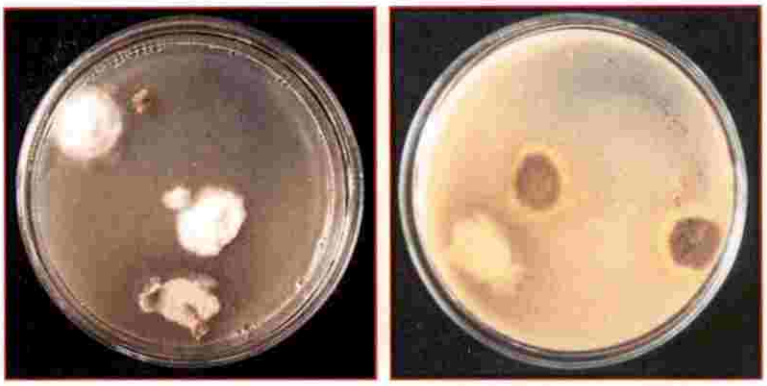
Table 7. Entomopathogenic fungi re isolated from inoculated cowpea plants on 30th day after inoculation

Inoculation methods	Endophytes isolated							
	Leaf		Stem		Root		Flower	Grain
	Isolated species	No. of colonies	Isolated species	No. of colonies	Isolated species	No. of colonies	Isolated species	Isolated species
Seed coating	-	-	-		-	-	Nil	
Seed soaking	-	-	-	-	-	-		
Radicle dressing	-	-	-	-	-	-		
Root dipping	-	-	-	-	*Metarhizium anisopliae*	5		
Soil drenching	-	-	-	-	-	-		
Foliar spray	*Beauveria bassiana*	1	*Lecanicillium saksenae*	3	*Lecanicillium saksenae*	2		
	Metarhizium anisopliae	3						
	Lecanicillium lecanii	2						
	Lecanicillium saksenae	4						
Bloom spray	-	-	-	-	-	-		
Control	-	-	-		-	-		

Table 8. Entomopathogenic fungi re isolated from inoculated cowpea plants on 45th day after inoculation

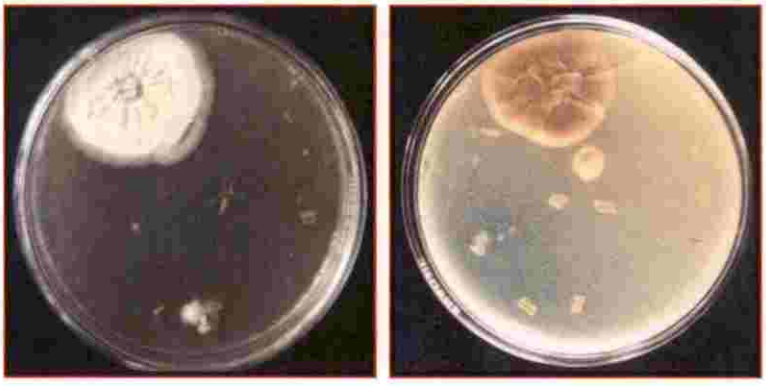
Inoculation methods	Endophytes isolated							
	Leaf		Stem		Root		Flower	Grain
	Isolated species	No. of colonies	Isolated species	No. of colonies	Isolated species	No. of colonies	Isolated species	Isolated species
Seed coating	-	-	-	-	-	-	Nil	
Seed soaking	-	-	-	-	-	-		
Radicle dressing	-	-	-	-	-	-		
Root dipping	-	-	-	-	*Metarhizium anisopliae*	4		
Soil drenching	-	-	-	-	-	-		
Foliar spray	*Metarhizium anisopliae*	2	*Lecanicillium saksenae*	3	*Lecanicillium saksenae*	2		
	Lecanicillium saksenae	4						
Bloom spray	-	-	-	-	-	-		
Control	-	-	-	-	-	-		

69



a. Upper side b. Reverse side

9A. Stem samples



a. Upper side b. Reverse side

9B. Root samples

Plate 9. Reisolated *Metarhizium anisopliae* from cowpea through seed soaking

4.1.2.1.4 Root dipping

In root dipping method, *M. anisopliae* could be retrieved from stem and root samples. On the 15th day it was retrieved from stem (Plate 10Aa and b) and root, while on the 30th day it was retrieved from root only (Plate 10Ba and b). Its association was again confirmed in root samples drawn on 45th day.

Presence of *M. anisopliae* was also noticed in soil samples drawn on 15th and 30th DAI (Plate 10Ca and b). The inoculated species *L. saksenae* was retrieved from soil on 15th, 30th and 45th DAI (Table 3).

The fungi, *B. bassiana* and *L. lecanii* could not be re isolated from plant and soil samples after inoculation.

4.1.2.1.5 Soil drenching

None of the plant samples revealed the presence of inoculated fungi when soil was drenched with their spore suspension, till 45th DAI.

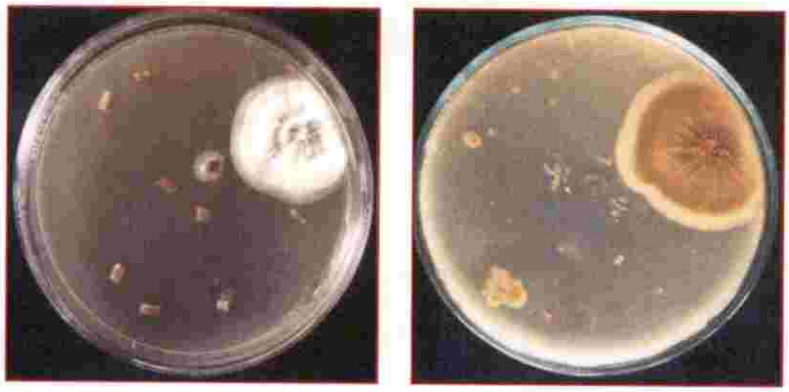
Examination of soil samples revealed the presence of *M. anisopliae* (Plate 11Aa and b) up to 30th DAI. *L. saksenae* persisted till 45th DAI. *B. bassiana* could be re isolated from soil samples on 15DAI (Plate 11Ba and b). *L. lecanii* was not found to colonise soil till the end of the experimental period.

4.1.2.1.6 Foliar spraying

Plants inoculated with *B. bassiana* through foliar spraying revealed the presence of inoculated organism in leaves, stem and roots of cowpea up to 15 DAI (Plate 12Aa, b and c). After 30DAI, it could be again retrieved from leaf samples. Its presence was not revealed in plant samples examined on 45th DAI.

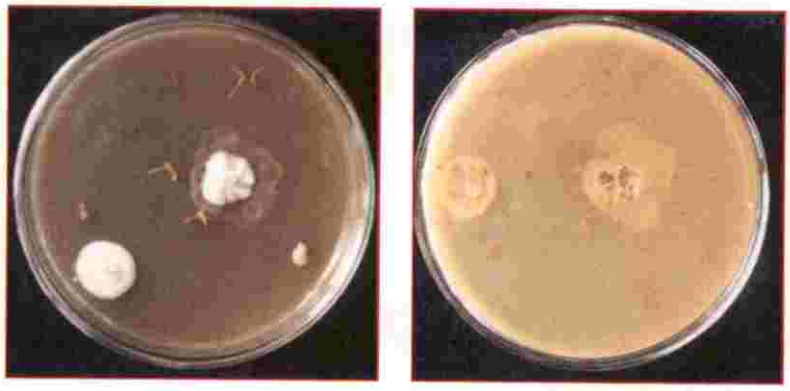
In the case of *M. anisopliae*, leaf samples revealed the presence of inoculated organism on 15th, 30th and 45th DAI (Plate 12Ba and b). Stem and root samples did not reveal the presence of *M. anisopliae.*

Plants inoculated with *L. lecanii* under examination, revealed the presence of inoculated organism from leaf samples on 15th and 30th DAI. Its presence was



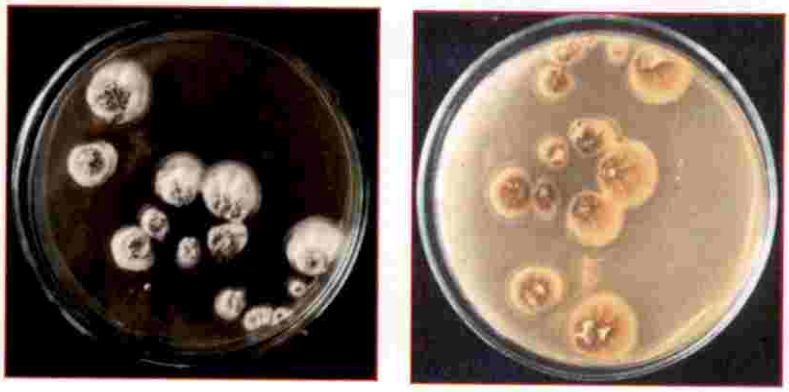
a. Upper side b. Reverse side

10A. Stem samples



a. Upper side b. Reverse side

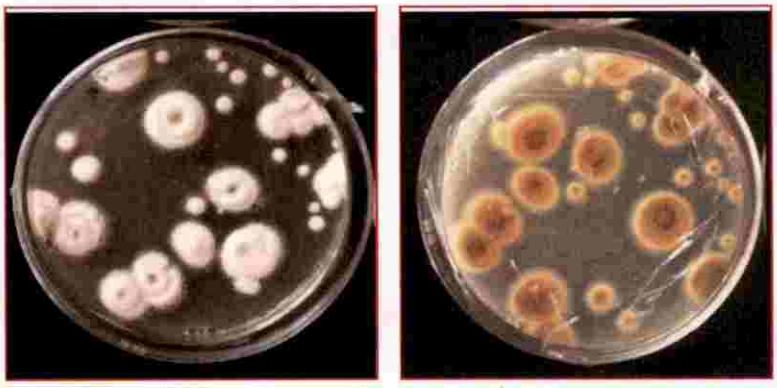
10B. Root samples



a. Upper side b. Reverse side

10C. Soil samples

Plate 10. Re isolated *Metarhizium anisopliae* from cowpea through root dipping



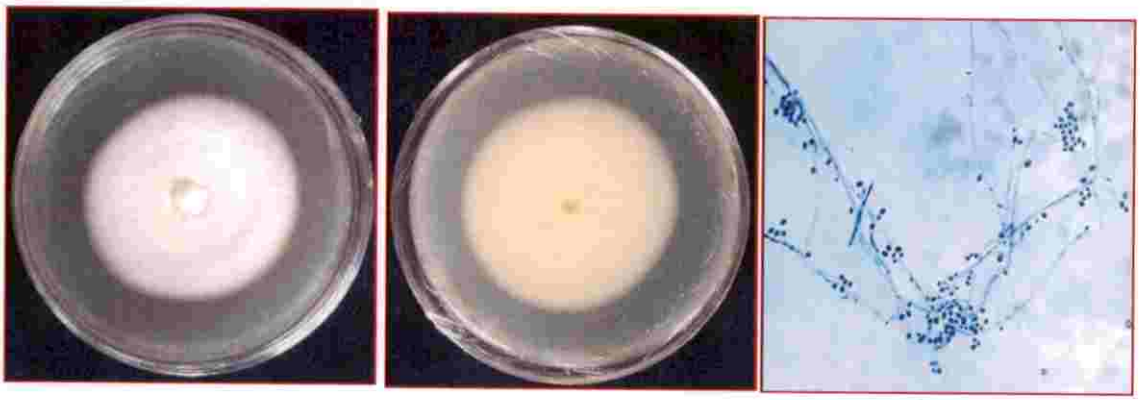
a. Upper side b. Reverse side

11A. *M. anisopliae*

a. Upper side b. Reverse side c. Conidia
40x magnification

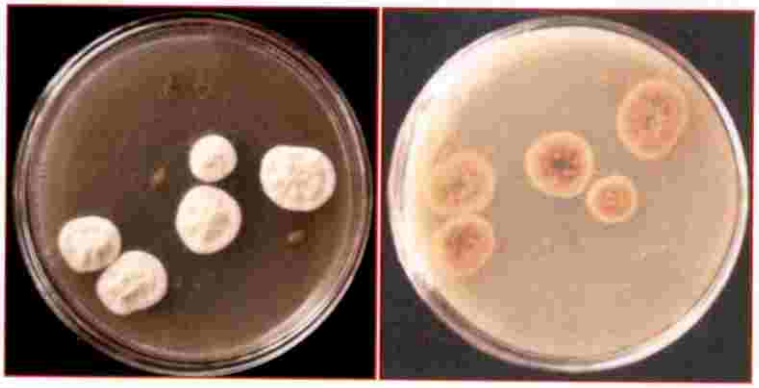
11B. *B. bassiana*

Plate 11. Entomopathogenic fungi re isolated from soils of cowpea



a. Upper side b. Reverse side c. Conidia
40x magnification

12A. *B. bassiana*



a. Upper side b. Reverse side

12B. *M. anisopliae*

a. Upper side b. Reverse side c. Conidia
40x magnification

12C. *L. lecanii*

Plate 12. Entomopathogenic fungi re isolated from cowpea through foliar spray

not revealed in samples examined on 45th DAI. Stem and root samples did not reveal the presence of *L. lecanii*.

Colony characters of re isolated organism resembled the inoculated *L. lecanii.* They were white, regular and elevated on upper side (Plate 12Ca) and pale yellow on reverse side (Plate 12Cb). Conidia typically resembled those of inoculated culture. They were globose, borne singly on verticillate branched conidiophores (Plate 12Cc), measuring 3.8µm x 3.7µm.

L. saksenae could be re isolated from leaf, stem and root samples (Plate 13) on 15th, 30th and 45th DAI, characters resembled those of inoculated culture.

4.1.2.1.7 Bloom spraying

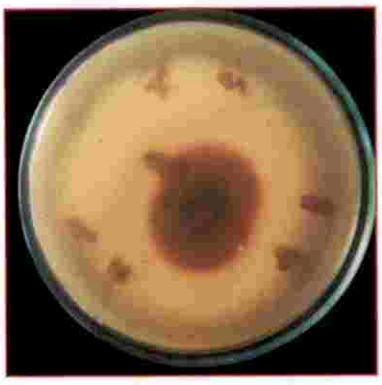
Leaves, stem, root, flower and grain samples did not reveal the presence of inoculated fungi.

B. bassiana could be re isolated from leaves, stem and roots of cowpea till 15th DAI after foliar spraying. *M. anisopliae* could be retrieved from leaf, stem and root samples by seed soaking, root dipping and foliar spraying. In seed soaking, its presence was observed from stem and roots up to 15th DAI. After root dipping, it could be re isolated from stem (up to 15th DAI) and roots (45th DAI). But through foliar spray it colonized leaves up to 45th DAI. *L. lecanii* could be re isolated from leaf samples through foliar spray up to 30th day, while *L. saksenae* could be retrieved from leaf, stem and roots samples up to 45th DAI.

4.1.2.2 Natural Endophytes

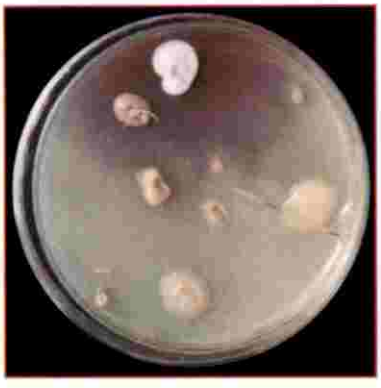
During the course of re isolation of entomopathogenic fungi from cowpea, eight natural endophytes were encountered in different plant parts (Table 9). Of these, four fungal endophytes were isolated both from leaves and stem. Among these *Cladosporium* sp. was found to be the predominant (15 colonies) one followed by *Chaetomium globosum* Kunze ex Fr. (12 colonies), *Clonostachys rosea* (Link) Schroers, Samuels, Seifert and W. Gams (10 colonies) and *Cerrena* sp. (10 colonies). The bacterial endophytes were isolated from leaves, stem as well as roots. The predominant one was *Rhizobium mayense* (10 colonies).

a. Upper side

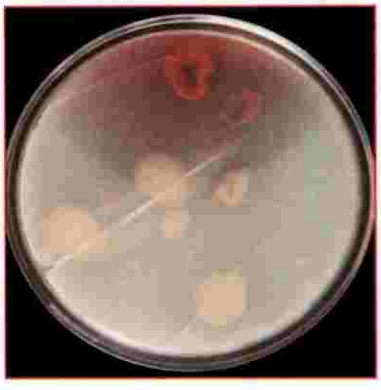


b. Reverse side

13A. Leaf samples



a. Upper side



b. Reverse side

13B. Stem samples

a. Upper side

b. Reverse side

13C. Root samples

Plate 13. Re isolated *Lecanicillium saksenae* from cowpea through foliar spray

Table 9. Natural endophytes isolated from different parts of cowpea plants

Plant parts	Fungal endophytes	No. of colonies	Bacterial endophytes	No. of colonies
Leaf	*Cladosporium* sp.	13	*Rhizobium mayense*	2
	Clonostachys rosea	3		
	Chaetomium globosum	5	*Enterobacter cloacae*	2
	Cerrena sp.	5		
Stem	*Cladosporium* sp.	2	*Rhizobium mayense*	3
	Clonostachys rosea	7		
	Chaetomium globosum	7		
	Cerrena sp.	5	*Enterobacter cloacae*	3
Root	*Fusarium* sp. + Ebc 3 (unidentified bacterium) (Endophytic fungus + endophytic bacterium association)			10
	Nil		*Rhizobium mayense*	20
			Enterobacter cloacae	10
Bloom	Nil		-	
Grain				

Ebc – Endophytic bacteria from cowpea

An association of fungus and bacteria was found to colonize predominantly in roots (10 colonies). This fungus was identified as *Fusarium* sp. and coexisting bacterium could not be identified.

Morphological characters of all the natural endophytes isolated from cowpea, with the molecular identity of select species are given below. NCBI Accession no. of those characterised at molecular level is given in Table 10.

4.1.2.2.1 Fungal isolates obtained from leaves and stem

4.1.2.2.1.1 Cladosporium sp.

This species differed from that isolated from rice stems, in its colony characters. Colonies were small and circular, regular and flat, greyish green pigmented on the upper side and pale yellow on the reverse side. Colonies were bordered with white concentric zonation during the initial days which disappeared upon sporulation. On sporulation colour changed from grey to dark green on upper side and grey pigmented on reverse side. (Plate 14Aa and b). It took ten days to sporulate.

Mycelia were septate with cylindrical conidiophores. Conidia were oval to ellipsoidal, three celled, often produced in chains (Plate 14Ac) and measured 3.2 µm x 4.3 µm in size.

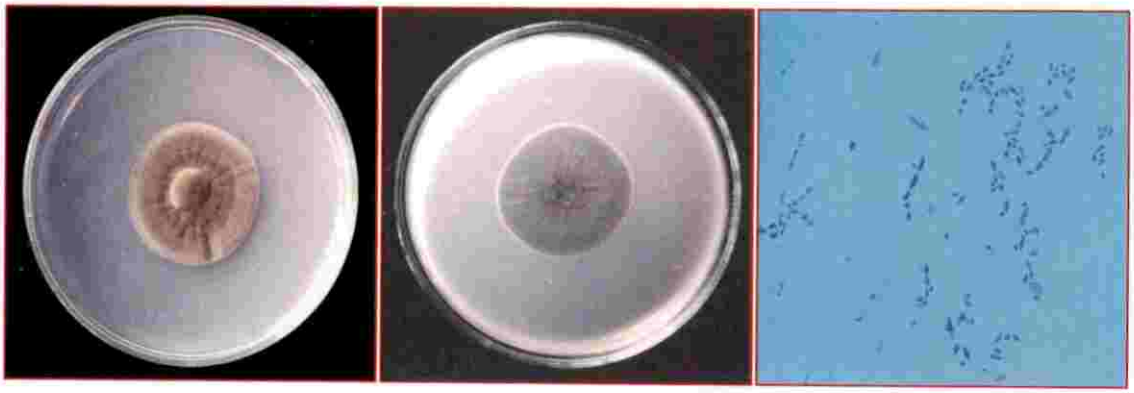
4.1.2.2.1.2 Clonostachys rosea

Colonies were regular and fluffy with filamentous margins and pure white on the upper side and reverse side (Plate 14Ba, b). Upon sporulation, the texture of the colony turned granular (Plate 14Bc) and pale yellow on reverse (Plate 14B, d). It took 12 days to sporulate.

Mycelia were septate, branched, conidiophores were mononematous, directly arising from mycelia. There were two kinds of conidiophores i.e., primary and secondary. Primary conidiophores were having opposite branches, whereas secondary conidiophores were produced at the centre of the colony which were long and penicillate (Plate 14Be). Conidia were round and smooth walled, measuring 2.7 µm x 3.5 µm.

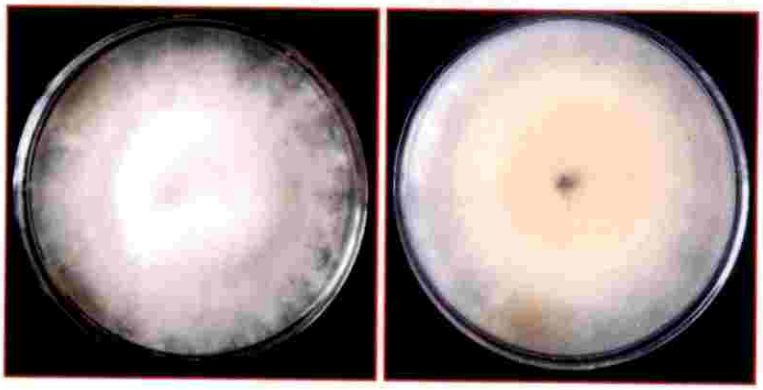
Table 10. Molecular identity of natural endophytes from cowpea plants

Natural endophytes	NCBI accession number
Fungal endophytes	
Clonostachys rosea	MN314832
Chaetomium globosum	MN314831
Cerrena sp.	MN299219
Fusarium sp.	MN398193
Bacterial endophytes	
Rhizobium mayense	MN367967
Enterobacter cloacae	MN367968



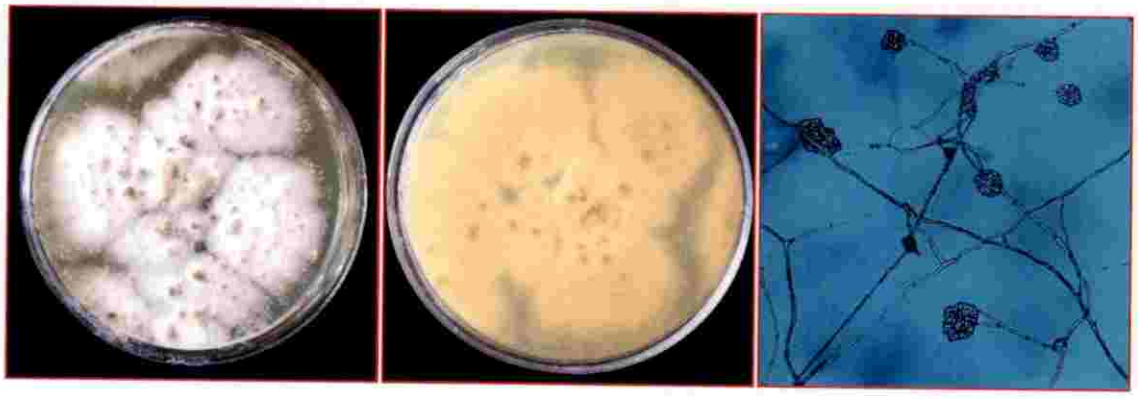
a. Upper side b. Reverse side c. Conidia
40x magnification

14A. *Cladosporium sp.*



Before sporulation

a. Upper side b. Reverse side



After sporulation

c. Upper side d. Reverse side e. Conidia
40x magnification

14B. *Clonostachys rosea*

Plate 14. Natural endophytes isolated from cowpea

Contd...

DNA sequence with NCBI Accession no. MN314832 of *C. rosea* is presented in Appendix 1.

4.1.2.2.1.3 Chaetomium globosum

Colonies were yellow and green pigmented irregularly, with white central area on upper side and brown pigmented on reverse side. Colonies were umbonate in elevation with white filamentous margin. Upon sub culturing, the colonies turned yellow with dark green coloured spores on upper side (Plate 15Aa) and pale pink on the reverse (Plate 15Ab). It took 14 days to sporulate.

Ascospores were lemon shaped (Plate 15Ac) and olive brown pigmented. Spores were comparatively larger in size and measured 8.4 µm x 7.8 µm.

DNA sequence with NCBI Accession no. MN314831 of *C. globosum* is presented in Appendix 1.

4.1.2.2.1.4 Cerrena sp.

Colonies were regular with smooth margin, pure white on upper surface (Plate 15Ba), pale yellow tinted on the reverse side (Plate 15Bb), resembling the colony of *B. bassiana*. It took 15 days to sporulate.

Mycelia coenocytic, branched, conidiophores mononematous, bearing bunch of conidia at the tip. The main distinguishing character of *Cerrena* was the bunch of conidia were not arranged on the zig zag terminal rachis of conidiophore as in *B. bassiana.* Conidia were round and single celled and measured 2µm x 2.5 µm (Plate 15c).

DNA sequence with NCBI Accession no. MN299219 of *Cerrena* sp. is presented in Appendix 1.

4.1.2.2.2 Bacterial isolates obtained from leaves, stem and roots

4.1.2.2.2.1 Rhizobium mayense Rincon-Rosales

Colonies were irregular, raised, smooth in appearance, slimy, opaque and light brown pigmented on the upper side and pale yellow on the reverse (Plate 15C).

a. Upper side b. Reverse side c. Conidia
40x magnification

15A. *Chaetomium globosum*

a. Upper side b. Reverse side c. Conidia
40x magnification

15B. *Cerrena* sp.

15C. *Rhizobium mayense*

Plate 15. Natural endophytes isolated from cowpea

Contd...

DNA sequence with NCBI Accession no. MN367967 of *R. mayense* is presented in Appendix 1.

4.1.2.2.2.2 Enterobacter cloacae Hormaeche and Edwards

Colonies were regular and raised, light brown pigmented on upper side and pale yellow on the reverse side. Colonies were glistening, translucent and sticky. Small bubble-like structure appeared on the colonies during the first seven days (Plate 16Aa). Later the bubbles gradually disappeared and the surface was shrunken (Plate 16Ab). Species level confirmation was done at molecular level.

DNA sequence with NCBI Accession no. MN367968 of *E. cloacae* is presented in Appendix 1

4.1.2.2.3 From roots

4.1.2.2.3.1 Fungal bacterial association

The fungus *Fusarium* sp. was always found to coexist with an unidentified bacterium - Ebc 3 (Plate 16Ba). Fungal colonies arouse from the top or margin of the bacterial colony. Fungal colonies were white pigmented with light pink shade, rhizoid in form with radiating margins (Plate 16Bb). It was pale yellow with pink concentric zonation on reverse side (Plate 16Bc). It took 10 days for sporulation.

Mycelia were branched, with septate hyphae. Microconidia borne on the surface of the hyphae or sometimes between the hyphae. They were rod shaped, slightly elongated present in bunches and measured 4.5 µm x 1.9 µm (Plate 16Bd).

DNA sequence of *Fusarium* sp. is presented in Appendix 1.

The bacterium, Ebc 3 had circular colonies with smooth margin, pulvinate and raised, sticky, brown pigmented and opaque on the upper surface. The reverse side appeared pale yellow.

4.1.2.2.4 From flowers and grains

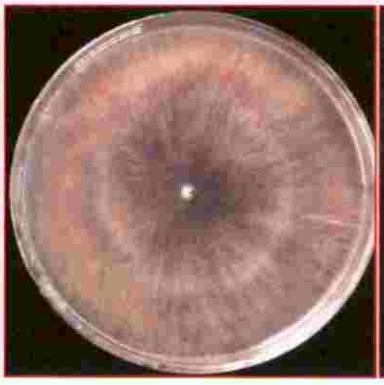
Samples examined on 15th, 30th and 45th DAI did not reveal the presence of any endophytes neither from flowers nor from grains.

a. Colonies with bubble

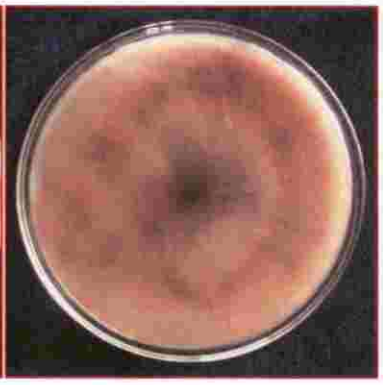
b. Bubble shrunken

16A. *Enterobacter clocae*

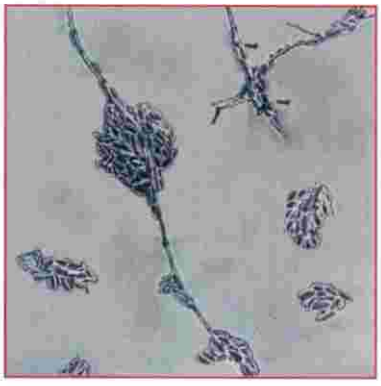
a. Coexistence



b. Upper side



c. Reverse side



d. Conidia
40x magnification

16B. *Fusarium* sp. + Ebc 3 (Unidentified bacteria)

Plate 16. Natural endophytes isolated from cowpea

In general, it was found that the natural endophytes preferred to colonize leaves followed by stem and roots. But not bloom and grains.

4.2 EFFECT OF ENDOPHYTIC ENTOMOPATHOGENS ON GROWTH, YIELD, PEST AND DISEASE INCIDENCE

Rice plants inoculated by seed soaking with the conidial suspensions of *B. bassiana*, *M. anisopliae* and *L. saksenae* when raised under open conditions, reflected significant increase in growth and yield and significant decrease in rice bug infestation (Table 11). There was no incidence of disease in treated as well as control plants.

4.2.1 Effect on Rice Bug Infestation

Percentage of chaffy grains was low in plants treated with *L. saksenae* (14.21 per cent), which significantly differed from plants treated with *B. bassiana* (20.48 per cent) and *M. anisopliae* (21.61 per cent). Whereas highest chaffy grains were observed in untreated plants (43.25 per cent).

4.2.2 Effect on Plant Height

On 30th day after sowing (DAS), mean height of plant inoculated with *B. bassiana, M. anisopliae* and *L. saksenae* was higher than that of untreated plants. It was 26.80 cm, 26.92 cm, 27.02 cm and 20.76 cm respectively. The height of inoculated plants differed in the same way on the 60th and 90th day also.

4.2.3 Effect on Plant Biomass

At the time of harvest, mean biomass of plants inoculated with *B. bassiana*, *M. anisopliae* and *L. saksenae* was significantly higher (44.98 g, 44.13 g and 45.05 g than that in untreated plants (30.56 g).

4.2.4 Effect on Grain Yield

Grain yield per plant when inoculated with *B. bassiana, M. anisopliae* and *L. saksenae* was 20.13 g, 19.98 g and 20.39 g respectively which was statistically higher than that in untreated plants (14.82 g).

Table 11. Effect of endophytes on pest incidence and growth of rice plants

Endophytes	Rice bug infestation	*Mean plant height (in cm) at monthly days interval			*Mean biomass per plant (g)	*Mean grain yield per plant (g)
	Chaffy grains per panicle (%)	First month	Second month	Third month		
B. bassiana	20.48[b]	26.80[a]	35.32[a]	73.66[a]	44.98[a]	20.13[a]
M. anisopliae	21.61[b]	26.92[a]	34.20[a]	72.76[a]	44.13[a]	19.98[a]
L. saksenae	14.21[a]	27.02[a]	35.44[a]	74.33[a]	45.05[a]	20.39[a]
Uninoculated plants	43.25[c]	20.76[b]	28.73[b]	57.39[b]	30.56[b]	14.82[c]
CD (0.05)	4.020	1.399	1.619	2.393	3.061	2.163

*Mean of nine replications. Values sharing same alphabets in superscript are statistically on par based on ANOVA

Discussion

5. DISCUSSION

Plants harbor diversity of microorganisms that may be transmitted locally or systemically. Those which do not cause any apparent symptoms are called as endophytes (Hyde and Soytong, 2008). They are omnipresent in nature, some of them act as plant growth promoters, while others impart resistance to pests and pathogens and yet another group imparts tolerance to abiotic stress as well. Growing interest on the implication of endophytes in agriculture and the possibility of exploring them for pest management has paved way to this study. The findings on endophytic association of entomopathogenic fungi in crop plants such as rice, *Oryza sativa* L. and cowpea, *Vigna unguiculata* (L.) Walp are discussed below.

ENDOPHYTIC ASSOCIATION OF ENTOMOPATHOGENIC FUNGI

In the attempt to establish endophytic association of the entomopathogenic fungi, *Beauveria bassiana*, *Metarhizium anisopliae*, *Lecanicillium lecanii* and *Lecanicillium saksenae* through different inoculation methods such as seed soaking, seed coating, radicle dressing, soil drenching, root dipping, foliar spraying and bloom spraying, it was observed that *L. saksenae* had strong endophytic association with both the crop plants, rice and cowpea whereas *M. anisopliae* had strong association with cowpea compared to rice. The rate of colonization of *B. bassiana* did not differ among rice and cowpea. *L. lecanii* was a weak colonizer in cowpea but did not colonize in rice.

B. bassiana could be re isolated from roots and stems of rice plants on 15th day after inoculation with conidial suspension of the isolate Bb5 @ 10^8 conidia mL^{-1}. It is justified with the finding of Jia *et al.* (2013) who could re isolate *B. bassiana* from stem and roots of rice when inoculated through foliar spray 15 Days After Inoculation (DAI). As observed in the study, they were also not able to re isolate it from the plants after 15 days.

In this study it was observed that *B. bassiana* when inoculated through seed soaking, colonised the roots as well as stem indicating its movement within the

plant. This finding is in accordance with that of Bing and Lewis (1991) who investigated the endophytic association of *B. bassiana.* Their study was in maize plants through stem injection and foliar spray and the fungus was recovered from stem and nodal regions. Movement of *B. bassiana* within the plants was also demonstrated in the studies carried out by Ownley *et al.* (2008), Akuste *et al.* (2013) and Quesada - Moraga *et al.* (2009).

However, this is in contrast to the findings of Akello *et al.* (2007) and Tefera and Vidal (2009). They suggested that colonisation of the *B. bassiana* is more likely to be near the area of application and less likely or not at all in the parts away from the application site. Also, studies conducted by Greenfield *et al.* (2016) revealed non systemic colonization of *B. bassiana* in roots of cassava, wherein they reported the proximal portion was the most preferred portion for colonization.

In the present investigation, *B. bassiana* could not be re isolated from the leaves of rice after inoculating them *viz.*, seed soaking, seed coating, radicle dressing, soil drenching, root dipping, foliar spraying or bloom spraying. The possible reason for this as suggested by Johnson-cicalese *et al.* (2000) is that, in grasses and other monocots most of the inoculated and natural endophytes tend to colonize between the shoot and root region and maximum endophyte concentration will be in the stem or leaf sheath. Bartlett and Lefebvre (2003) in their attempt to assess the effect of *B. bassiana* on European corn borer *Ostrinia nubilalis* (Hubner), concluded that succulent parts like pith of corn is congenial for the growth of *B. bassiana.* In rice plants, it is the stem that is pithy and hence the endophytes show a tendency to colonize away from leaves. Furthermore Posada *et al.* (2007) suggested that leaves are the poorest port of entry and colonization of *B. bassiana.*

Among the different methods of inoculation, seed soaking was found to be the best method for establishment of *B. bassiana*, as the inoculated organism could be re isolated. Seed treatment to establish fungal entomopathogens as endophytes, has been reported in the recent past, with interesting results for the management of insect pests (Akello and Sikora, 2012; Akutse *et al.*, 2013; Biswas *et al.* (2012);

Keyser *et al.* (2014); Lopez *et al.* (2014); Lopez and Sword, 2015; and Mutune *et al.* (2016).

In the experiment to establish endophytic association of *M. anisopliae* with rice plants, out of the different methods tried in this study, seed soaking was found to be the effective method as the fungus could be retrieved from sterile plant samples drawn on the 15th DAI, as with the case of *B. bassiana* inoculated rice plants. Relationship between *Metarhizium* and the endosphere of plants has been established through a number of studies. Endophytic association of *M. anisopliae* was established by Garcia *et al.* (2011) in tomato, inoculating the plants by soil drenching. They could re isolate the fungus from root and shoot samples demonstrating that the inoculated organism moves within the plant. Similarly, Mantzoukas *et al.* (2015) also opined the movement of *Metarhizium robertsii* throughout the sweet sorghum plants inoculated through foliar spray. They observed that the colonization was higher in stem when compared to leaves and roots up to 30 DAI.

On the other hand, studies of Sasan and Bidochka (2012) reported endophytic association of *M. robertsii* in the roots of switch grass *P. virgatum* up to 60 days, when inoculated through seed soaking and placing fungal plugs in soil. Their findings imply the localised colonization of *M. robertsii* in root. Similarly, Greenfield *et al.* (2016) also stated that *M. ansiopliae* colonized roots of cassava through soil drenching up to seven weeks after inoculation.

Anyhow, Leger (2008) opined that the genus *Metarhizium* is less characterised as endophytes although the principal habitat of the fungus is rhizosphere and not insect.

L. saksenae was found to be a good coloniser in rice plants as it could be retrieved from all plant parts. It could be retrieved from stem and roots through seed soaking, from root through soil drenching and from leaves through foliar spraying. Its colonization was noticed even at 45th DAI.

L. saksenae is a rare species in the genus, which was first reported by Kushwaha (1980) as a keratin degrader. Further, Sukarno *et al.* (2009) isolated the

fungus from the soil inhabiting arthropods of Kalimantan province of Indonesia. It was also reported as efficient degrader of herbicides and pesticides by Pinto *et al.*, 2012). Rani *et al.* (2014, 2015) isolated an indigenous strain of *L. saksenae* from soils of Vellayani, Kerala, India and reported it to be a promising biocontrol agent against hemipteran bugs. Jasmy (2016) reported that *L. saksenae* was pathogenic to *Aphis craccivora* Koch, *Aphis gossypii* Glover, *Bemisia tabaci* (Gennadius), *Amrasca biguttula biguttula* (Ishida), *Coccidohystrix insolita* (Green) and *Leptocorisa acuta.* Her studies revealed that conidial concentration of 1 x 10^7 conidia mL^{-1} caused mortality to the above pests mentioned, within 72 h. Entomopathogenicity of this species was further confirmed by detecting its cuticle degrading enzymes chitinase, lipase and protease and also by the toxin Dipicolinic acid. Sankar and Rani (2018) could establish its field efficacy in managing sucking pests of rice. However, it was not reported as an endophyte ever before.

L. lecanii (Vl 8) could not be retrieved from rice plants inoculated with conidial suspension @ 10^7 conidia mL^{-1}. Conversely, Gurulingappa *et al.* (2010) established *L. lecanii* as an endophyte in cotton, wheat, common beans, tomato and pumpkin after inoculating through foliar spray. They observed that its colonization was restricted to leaves up to 21 DAI, indicating that the fungus is of non-systemic nature.

Other *Lecanicillium* species such as *Lecanicillium dimorphum* and *Lecanicillium psalliotae* associated endophytically with date palm through leaf petiole injection as reported by Gomez-vidal *et al.* (2006). They found that the fungi persisted in palms up to 30 DAI. The disparity of these studies with the present finding is justified by speculating that all strains and isolates do not behave in the same way. Biswas *et al.* (2012) reported that endophytic association depend on the strain of the fungus. Another possible explanation for lack of endophytic colonization of *L. lecanii* as suggested by Posada *et al.* (2007) is that, several natural endophytes have negative impact for the endophytic association of inoculated fungus. Non colonization of *L. lecanii* might be due to its competition with other natural endophytes as put forth by Greenfield *et al.* (2016).

Another possible reason for non- retrieval of *L. lecanii* is the dominant and competitive nature of natural endophytes *Neocomospora rubicola* isolated from all plant parts and the unidentified fungus (Efr 11) from stem which inhibiting the colonisation of the slow growing *L. lecanii.* Quesada-Moraga *et al.* (2009) opined that microbiological methods like re isolation techniques are not much effective when compared to molecular based techniques because plant bits placed on PDA may contain low inoculum of the inoculated fungus.

Investigations carried out in cowpea, could establish that *B. bassiana* can associate endophytically in cowpea through foliar spraying. The inoculated organism could be re isolated from leaves, stems and roots of cowpea, 15 DAI. After 30 days the presence was detected only in leaf samples. Soil samples revealed the persistence of *B. bassiana* up to 15 DAI. This indicates the systemic nature of *B. bassiana*, moving away from the site of inoculation.

Several studies proved the systemic colonization of *B. bassiana* in other leguminous crops. Akutse *et al.* (2013) studied endophytic association of *B. bassiana* in common beans, *Phaseolus vulgaris* L. and faba beans, *Vicia faba* L. through seed soaking method and found that the inoculated fungus colonized entire plant in common beans, whereas in faba beans it colonized only stem and roots. Similarly, Mutune *et al.* (2016) and Gathage *et al.* (2016) established endophytic association of *B. bassiana* in the leaves, stem and roots of common beans. Gautam *et al.* (2016) put forth the systemic movement of *B. bassiana* in cauliflower plants inoculated through foliar spray. They stated that *B. bassiana* do not require specific location for germination of conidia. After imbibing sufficient moisture from the surrounding environment, conidia germinate on the leaf surface and penetrate the plant by rupturing the outer cell. After penetration, hyphae branches to form mycelia, grows in the intercellular spaces and colonizes throughout the plant. These findings were again supported by the findings of Jaber and Enkerli (2017) who inoculated the plants through seed soaking and stated that *B. bassiana* could be re isolated from leaves, stem and roots of faba beans up to 30 DAI.

On the contrary, non-systemic or localised colonisation was demonstrated in some other studies in leguminous crops itself. Akello and Sikora (2012) revealed that *B. bassiana* was able to colonize only roots of faba beans when inoculated by seed soaking and persisted in the plant up to 30 DAI. Their results were in accordance with investigation of Parsa *et al.* (2018), who stated that seed soaking of beans in conidial suspension of *B. bassiana* lead to root colonization only.

In the experiment to examine the endophytic association of *M. anisopliae*, it was found that the fungus could successfully associate with cowpea. It could be re isolated from plants after seed soaking, root dipping and foliar spraying. Furthermore, foliar spray was found to be superior to other methods as the frequency of occurrence in plant samples was high, compared to seed soaking and root dipping methods. It was found to persist even up to 45 DAI. In root dipping, method it was found to colonize stem and roots up to 15 and 45 DAI respectively. In seed soaking, the fungus colonized both stem and roots up to 15 DAI. However, this method resulted in low colonization. Thus, the study concluded that foliar spray of *M. anisopliae* lead to high leaf colonization and root dipping lead to high root colonization. The colonization nature was variable in *M. anisopliae* i.e., localised colonization was observed in foliar spray method and systemic colonization was observed in root dipping and seed soaking method. Soil samples also revealed the persistence of *M. anisopliae* in soil up to 30th DAI.

Localised colonization of *M. ansiopliae* was observed by Akello and Sikora (2012) and Parsa *et al.* (2018). They stated that *M. anisopliae* colonized only roots of beans through seed soaking and they were able to retrieve it from plants up to one month after inoculation.

Sasan and Bidochka (2012) were able to establish *M. robertsii* in haricot beans *P. vulgaris*, as a root endophyte in non systemic manner up to 60 DAI, inoculated through seed soaking and placing fungal plugs in soil. Through light and confocal microscopy, they observed the colonization of *M. robertsii* in intercellular spaces of roots and not in stem and leaves. However, recently, Jaber and Enkerli (2017) revealed systemic colonization of *M. brunneum* in faba beans through seed

soaking and was able to re isolate from leaves, stem and roots up to one month. These findings revealed that, systemic colonization rate was directly proportional to seed treatment duration. Increase in the seed soaking duration, increases the colonization of *M. anisopliae.*

On the contrary, the findings of Akutse *et al.* (2013); Mutune *et al.* (2016) and Gathage *et al.* (2016) stated that *M. anisopliae* failed to establish as an endophyte in bean plant, speculating the variation within strains and isolates.

L. lecanii, Vl 8 used in this study, inoculated in cowpea, could be re isolated only from the leaves on the 30th DAI. Among different methods of inoculation, foliar spraying was found to be best for *L. lecanii* association in cowpea. This finding suggests that, inoculated organism could colonise only at the site of inoculation and implies endophytic *L. lecanii* has a non systemic nature. This result is justified by the findings of Gurulingappa *et al.* (2010) which stated endophytic colonization of *L. lecanii* in the leaves of common beans, when inoculated through foliar spray up to 21 DAI. However, investigation on endophytic nature of *L. lecanii* is meagre as per the literature.

In cowpea, *L. saksenae* was successfully established as an endophyte when inoculated through foliar spraying. The fungus could be re isolated from leaf, stem and root samples when inoculated by foliar spraying till 45th day after treatment. Compared to other species *L. saksenae* was found to be a strong colonizer in plant as well as soil. The strong association is attributed to its indigenous nature. It was isolated from the soils of the region where the fungus was inoculated earlier.

INOCULATION METHODS

In the present studies, seed coating, seed soaking, radicle dressing, root dipping, soil drenching, foliar spraying and bloom spraying methods were tried to establish endophytic association of entomopathogenic fungi in rice and cowpea. Seed soaking was found to be effective for *B. bassiana* and *M. anispliae* in rice. But for *L. saksenae* seed soaking, foliar spraying and soil drenching were

successful. In cowpea, foliar spraying was found to be the best for endophytic establishment of *B. bassiana* and *L. lecanii*. But in case of *M. anisopliae* inoculated in cowpea, foliar spraying, root dipping and seed soaking were effective. This investigation clearly states that endophytic association of entomopathogenic fungi in crop plants depends on the inoculation methodology used.

Studies on the standardization of best inoculation method, dosage of inoculum used and duration of treatment in banana was conducted in detail by Akello *et al.* (2007, 2008). Their study revealed that root dipping of banana in conidial suspension of *B. bassiana* @ $1x10^7$ conidia mL^{-1} for 2 h was found to be the best inoculation method to establish endophytic association in banana. These findings clearly depict the importance of inoculation method, its dosage and duration of treatment. This implies that for every crop standard inoculation method and dosage has to be designed for successful endophytic establishment. Reddy *et al.* (2009) stated that conidial spray of *B. bassiana* in sorghum was found to be effective method rather than inoculating sporulated fungal culture on paddy straw.

In pulses, most of the studies has been conducted through seed soaking method. Akello and Sikora (2012); Akutse *et al.* (2013); Mutune *et al.* (2016); Gathage *et al.* (2016); Jaber and Enkerli (2017) and Parsa *et al.* (2018) were able to establish endophytic association of *B. bassiana* and *M. anisopliae* beans effectively through seed soaking. All these findings were in accordance to the present results of cowpea plants inoculated with *M. anisopliae*, but not for *B. bassiana* and *L. lecanii*.

However, Parsa *et al.* (2013) established successful endophytic association of *B. bassiana* in beans through foliar spray and soil drenching.

In the present study, radicle dressing and bloom spray was not effective for endophyte establishment. Conversely, Posada and Vega (2006) established *B. bassiana* as an endophyte in leaves, stem and roots of coffee seedlings through radicle inoculation. In the following investigations of Posada *et al.* (2010), they inoculated *B. bassiana* by spraying flowers of cocoa during bloom stage and later

re isolated it from pods and peduncle. These two methods were not effective in rice and cowpea, as the radicle in these crops were very small, and surface area for penetration of spores might be very less. Furthermore, during bloom stage, the natural endophytes present in crop plants might have been dominant and interrupted the colonization of artificially inoculated organisms.

Posada and Vega (2006) stated that *Fusarium* sp. and three different morphospecies of bacteria interrupted the re isolation and colonization of *B. bassiana* in coffee. Further studies need to be conducted to test these methods in different crops.

This study thus paves way to the possibility of adopting seed priming and seedling treatment with entomopathogenic fungi, which is a new vista in plant health management.

NATURAL ENDOPHYTES

While attempting re isolation of the inoculated organisms from rice plants, certain natural endophytes were also encountered. The number of fungal endophytes outnumbered the bacterial endophytes as the study concentrated on endophytic fungi and the isolation medium selected was fungus specific. It included growth promoters with nitrogen fixing properties, gibberellic acid production properties, saprophytes and plant pathogens, which are discussed below.

Pestalotiopsis microspora isolated from leaves and roots of rice plants is known for its anticancerous property due to the production of metabolite, Taxol (Strobel *et al.*, 1996). Russell *et al.* (2011) stated the importance of this fungus in bioremediation projects for biodegradation of plastics. Its endophytic association with Himalayan yew, *Taxus wallichiana* Zucc. was reported by Metz *et al.* (2000) and from *T. chinensis* (Rehder & E.H.Wilson) Rehder by Li *et al.* (2015).

N. rubicola was reported by Kim *et al.* (2017) as an endophyte in roots of *Glycyrrhiza uralensis* Fisch. ex DC. in Korea. It is a plant pathogen causing root and twig canker of citrus trees (Denis *et al.*, 2018).

Microdochium fisheri isolated from leaves, stem and roots of rice, was previously reported as an endophyte in rice stems by Restrepo *et al.* (2016), from UK. Besides, Baghela and Singh (2017) isolated this fungus from rhizosphere of paddy in India, for the first time. He reported it to be a saprophytic species.

Fusarium solani isolated from stems of rice was earlier reported as an endophyte in roots of lawn grass by Zakaria *et al.* (2010). It is a known plant pathogen of leguminous crops (Onofre *et al.*, 2013).

Cladosporium sp. isolated from rice stem is a dominant and major coloniser in rice as evidenced by the report of Clay (1988) who isolated it from leaf sheath of grasses. Fisher and Petrini (1992) isolated *Cladosporium Tenuissimum* from leaf sheath of rice plant. Hamayun *et al.* (2009) isolated *Cladosporium* sp. from the roots of soybean, *Glycine max* (L.) Merr. and stated its plant growth promoting and gibberellic acid production activities. Paul and Yu (2008) isolated two different endophytic species of *Cladosporium* sp. from needles of pine tree, *Pinus* sp. Halo *et al.* (2019) isolated *Cladosporium omanense* from the leaves of a medicinal plant, kammun, *Zygophyllum coccineum.* This genus is also known to be saprophytic as well as a plant pathogenic species.

Aspergillus sp. and *Penicillium* sp. isolated from leaves, stem and roots of rice are common saprophytic endophytes. Naik *et al.* (2009) isolated *A. flavus, A. ochraecus, P. chrysogenum* and *P. decumbens* from leaves and roots of rice.

The bacterium *Burkholderia cepacia* isolated from leaves and roots is a stable endophyte associated with rice and well adapted to the rice ecosystem (Compant *et al.*, 2005). Findings of Mendes *et al.* (2007), Mano and Morisaki (2008) and Hongrittipun *et al.* (2014) revealed that it is a growth promoting nitrogen fixing bacterium associated endophytically with rice and sugarcane.

Interestingly, a coexistence of fungus and bacterium was observed in rice stems. *Sarocladium oryzae* was found to coexist with the bacterium *Kosakonia sacchari. S. oryzae* is commonly associated with grasses. Hung Yeh and Kirschner (2014) stated that members of the genus *Sarocladium* are saprophytic

and mutualistic endophytes. It is also known to cause sheath rot of rice. *K. sacchari* is a new species in the genus *Kosakonia* (Enterobacter). It is a gram negative, non-spore forming, aerobic bacterium with motile rods. It was reported as a nitrogen fixing bacterium associated endophytically with sugarcane, having growth promoting activity (Chen *et al.*, 2014). Meng *et al.* (2015) isolated endophytic *K. oryzae* from roots of rice and reported it to be a growth promoter.

In rice, a well-studied fungal-bacterial interaction is that between a plant pathogenic fungus *Rhizopus microspores* and its endophytic bacteria, *Burkholderia rhizoxicnica* by Martinez and Hertweck (2005). *R. microspores* which is known to cause seedling blight in rice depends on the bacterium *B. rhizoxicnica* for toxin production and the bacterium in turn is benefitted for its dissemination through fungal spores thereby establishing a symbiotic life style. Therefore, the function of coexistence of *S. oryzae* and *K. sacchari* observed in this study needs further investigation.

While attempting re isolation of inoculated entomopathogenic fungi from cowpea, several natural endophytes were stumped upon in different parts of the plant. This included four fungi and two bacteria. Among these, fungal bacterial association between *Fusarium* sp. and Ebc 3 (unidentified bacterium) is remarkable.

The fungus *Clonostachys rosea* isolated from leaves and stems of cowpea in this experiment, has been earlier isolated from coffee plants by Vega *et al.* (2008). It is known as an entomopathogen of aphids and whiteflies (Anwar *et al.*, 2018) and also as a necrotrophic, mycoparasitic fungus used in the biological control of plant pathogenic fungi (Nygren *et al.*, 2018).

Chaetomium globosum isolated from leaves and stems of cowpea, has been reported earlier as an endophyte in leaves of jatropha plants (Kumar and Kaushik, 2013). *C. globosum* produces metabolites that possess antifungal activity against *Sclerotinia sclerotirum* (Lib.) de Bary (Zhao *et al.*, 2017). *Cerrena* sp. isolated from the leaves was previously isolated by Anisha and Radhakrishnan (2017) from

ginger plants. They proved it to be producer of the metabolite named N-Amino pyrrolidine, which possess antibacterial property.

Rhizobium mayense was bacterial endophyte that was found to colonise all plant parts. Zahran (1999) stated that *Rhizobium* is a diazotrophic bacteria in the root nodules of legumes. Its association in all plant parts as observed in this study was also reported by Chi *et al.* (2005) who could isolate *Rhizobium* from roots of rice, stem base, leaf sheath and leaves in rice. Saini *et al.* (2015) isolated a related species *Mesorhizobium* from the roots of chick pea which was found to be the one which helps in nodulation, nitrogen fixation and plant growth.

Enterobacter cloacae was another bacterial endophyte obtained from leaves, stem and roots of cowpea. Endophytic action of this bacterium was reported by Khalifa *et al.*, (2016) from non-nodulating roots of alfalfa, (*Medicago sativa* L.). They stated that *E. cloacae* plays a very important role in plant growth promotion.

Another coexistence of endophytes was noticed in cowpea. It was between the fungus *Fusarium* sp. and the bacterium Ebc 3 (unidentified bacteria). *Fusarium* sp. was previously isolated by Rodrigues and Menezes (2005) who identified and characterized different species of endophytic *Fusarium* at molecular level from cowpea. They stated that none of species were pathogenic, even in susceptible cowpea plants. Among different species, *F. semitectum* was most frequently seen. The interaction effects between these *Fusarium* sp. and Ebc 3 observed in this study need to be investigated in detail to know its function.

In legumes, the interaction between mycorrhizal fungus *Glomus fasiculatum* and *Azatobacter chrococcum*, was reported to have synergistic effect in growth of tomato plants (Bagyraj and Menge, 1978).

EFFECT OF ENDOPHYTES ON PEST INCIDENCE AND GROWTH OF RICE PLANTS

Rice plants inoculated with *B. bassiana, M. anisopliae* and *L. saksenae* by seed soaking method, when raised under open conditions, reflected significant increase in growth and yield. Mean plant height, biomass, and grain yield, were

superior in plants inoculated with *B. bassiana* and *M. anisopliae* when compared to those of untreated plants (Fig 1). Plants exhibited 28.79, 27.17and 29.99 per cent increase in height when inoculated with *B. bassiana*, *M. anisopliae* and *L. saksenae* respectively. So also, in these plants exhibited 47.71, 44.75 and 47.92 per cent increase in biomass and 37.73, 36.97 and 39.89 per cent increase in grain yield respectively.

This observation is substantiated by the studies conducted by Kabaluk and Ericsson (2007) in corn, where there was 26.3 per cent increase in stock and foliage weight when inoculated with *B. bassiana*. Further, Gurulingappa *et al.*, (2010) noticed 37.5 per cent increase in wet shoot weight and 44 per cent increase in wet root weight in wheat plants inoculated with that *B. bassiana* through foliar spray after 21 DAI. Studies of Sanchez-Rodriguez *et al.* (2017) reported that, endophytic *B. bassiana* inoculated through seed dressing and soil drenching significantly increased the spike production of wheat by 40 per cent.

Experiment carried out by Sasan and Bidochka (2012) revealed that switch grass treated with *M. robertsii* grew faster and promoted the root development and also helped in root hair development. They observed 33.33 per cent increase in and 120 per cent increase in root length 200 per cent increase in root hair density on the 10^{th} day after seed germination.

In this research work, rice plants inoculated with *B. bassiana, M. anisopliae* and *L. saksenae* by seed soaking method, when raised under open conditions, there was a decrease in percentage of chaffy grains. The percentage reduction was the highest (66.83) in *L. saksenae* treated plants, while in *B. bassiana* and *M. anisopliae* it was 52.43 per cent, 49.79 per cent respectively. There are many studies that support this finding, most of them being with *B. bassiana*.

In *B. bassiana* inoculated corn plants, there was 95 to 98.3 per cent reduction in tunnelling by European corn borer (Bing and Lewis, 1991, 1992); in tissue culture banana, 53.4 to 57.7 per cent reduction in infestation of rhizome weevil, Akello *et al.* (2008). In sorghum there was 100 per cent reduction in dead

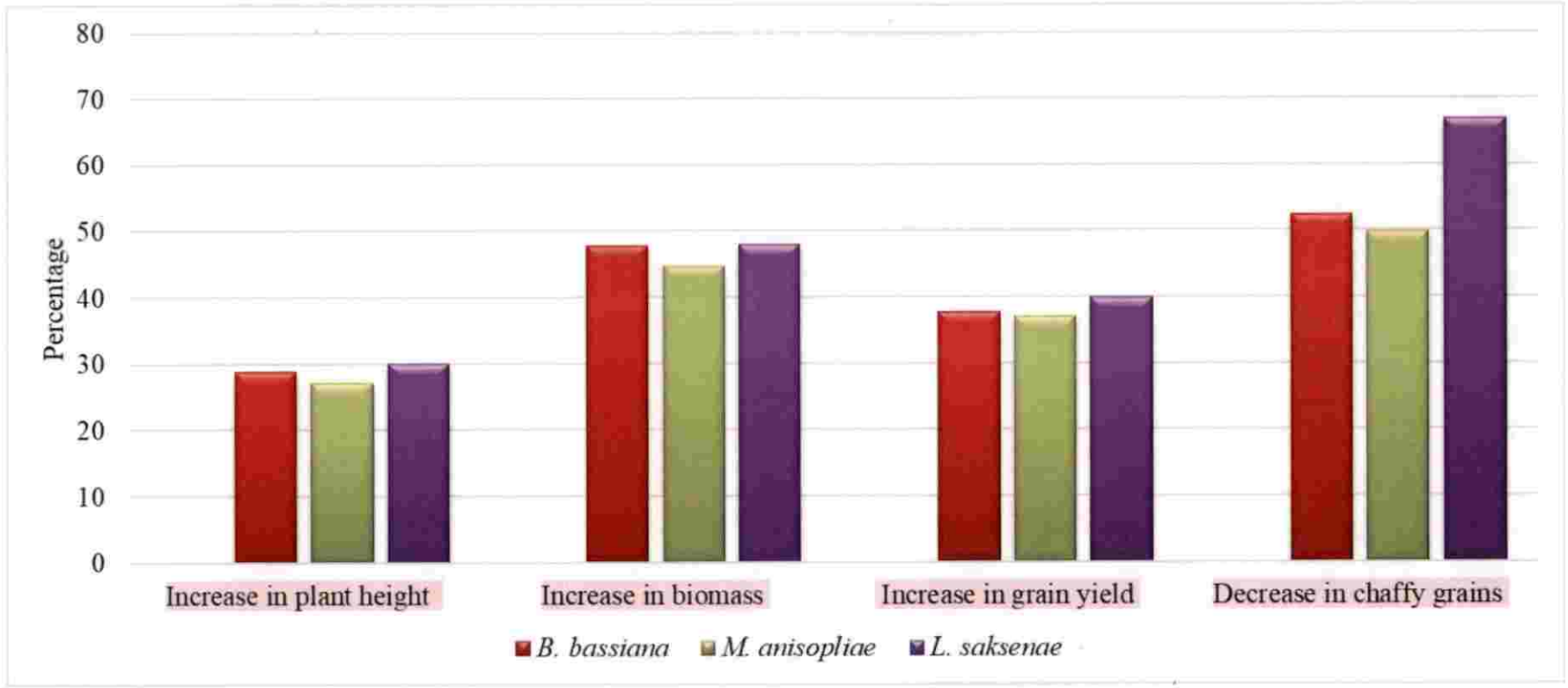


Fig. 1 Effect of endophytes on plant growth and pest incidence in rice plants

heart symptoms by *Chilo partellus* Swinhoe (Reddy *et al*., 2009). In wheat there was reduced feeding of Australian locust, *Chortoicetes terminifera* Walker (Gurulingappa *et al*. 2010). In cotton it reduced the survival of second larval instar of *Helicoverpa zeae* (Boddie) (Lopex *et al*., 2014).

Jurado *et al*. (2016) reported that in melon plants, endophytic *M. brunneum* produced secondary metabolites that caused mortality in whitefly nymphs. Destruxins A (43%), produced by *M. brunneum*, was detected in dead nymphs. Further, Kabaluk and Ericsson (2007) stated that endophytic *M. anisopliae* was able to reduce the damage of wire worm in corn significantly.

Summary

6. SUMMARY

The investigation entitled "Endophytic association of entomopathogenic fungi with rice and cowpea" aimed to examine the endophytic association of *Beauveria bassiana*, *Metarhizium anisopliae*, *Lecanicillium lecanii* and *Lecanicillium saksenae* in crop plants. Rice, *Oryza sativa* representing monocots and cowpea, *Vigna unguiculata* representing dicots were selected for the study. The association was tested by seven methods of inoculation, *viz*., seed coating, seed soaking, radicle dressing, soil drenching, root dipping, foliar spraying and bloom spraying. The fungi were tested at their effective doses by inoculating the conidial suspension.

Among the four entomopathogenic fungi inoculated, *B. bassiana*, *M. anisopliae* and *L. saksenae* were found to colonise rice plants. *B. bassiana* was found to colonise in stem and roots, while *M. anisopliae* preferred stem. Both *B. bassiana* and *M. anisopliae* colonized effectively by seed soaking method. *L. saksenae* colonised in the stem and roots in seed soaking method, and in roots in soil drenching method and in leaves in foliar spraying method.

In rice plant, *B. bassiana* and *M. anisopliae* persisted in plants up to 15th day after inoculation (DAI), while *L. saksenae* persisted till 45th DAI. In soil *L saksenae* persisted up to 45th day, while other fungi could not be retrieved from soil.

L. lecanii could not be retrieved from any of the plant parts or soil up to 45th DAI. Opposition of natural endophytes with this slow growing fungus might have obstructed its isolation in artificial medium.

Natural endophytes encountered in different parts of rice plants included 11 fungal endophytes and one bacterium, the latter being less. The number of bacterial endophytes isolated were less because, the experiment mainly focused to isolate fungal endophytes and the medium used for re isolation was PDA. The endophytes were isolated and identified with the help of colony and conidial characters. Those

fungi whose identity could not be confirmed were characterised at molecular level, through ITS sequencing and bacteria by 16S sequencing.

Natural endophytes isolated from rice, included growth promoters with nitrogen fixing properties, gibberellic acid production properties, saprophytes and plant pathogens. Most of them preferred stem followed by leaves and roots. Grains were not found to harbour any endophytes.

The endophytic fungus *Neocomospora rubicola* which is also known as plant pathogen, was found to colonise profusely in all plant parts in leaves, stem and roots of rice. The bacterium *Burkholderia cepacia* was found to colonise profusely in roots and sparsely in leaves was a nitrogen fixing bacterium. Colonisation of fungal bacterial association *Sarocladium oryzae* + *Kosakonia sacchari* was also dominant in the stem. *S. oryzae is* associated with grasses as per earlier reports with pathogenic, saprophytic and mutualistic behaviour. *K. sacchari* found in association with *S. oryzae* is a nitrogen fixing bacterium and hence a growth promoter.

The frequency of occurrence of other endophytes *viz.*, *Pestalotiopsis microspora, Cladosporium* sp., *Microdochium fisheri* and *Fusarium solani* was sparse. *P. microspora* isolated from leaves and roots of rice plants is known for its anticancerous property, bioremediation and biodegeneration of plastic. *M. fisheri* isolated from leaves, stem and roots of rice is a known endophyte in rice, while *F. solani* isolated from stems of rice is a known endophyte in lawn grass and a plant pathogen of legumes, solanaceous and bulb crops. Genus *Cladosporium*, *Aspergillus* and *Penicillium* isolated are known to be as a saprophytic as well as a plant pathogenic species.

Experiment to examine the endophytic association of entomopathogenic fungi in cowpea, proved that there was successful association of all the inoculated organisms viz. *M. anisopliae, B. bassiana, L. lecanii* and *L. saksenae* in different plant parts.

Foliar spraying was the best method in cowpea to get endophytic association of all the four fungi. While *M. anisopliae* could be retrieved through seed soaking

and root dipping methods also. *B. bassiana, M. anisopliae* and *L. saksenae* were found to colonise leaves, stem and roots, while *L. lecanii* colonised only on leaves.

M. anisopliae and *L. saksenae* were found to be more persistent in cowpea plants (up to 45th DAI), while *B. bassiana* and *L. lecanii* persisted only up to 30 DAI.

Examination of soil samples revealed the presence of *M. anisopliae* and *L. saksenae* up to 30th and 45th DAI respectively. *B. bassiana* could be re isolated from soil samples on 15th DAI. *L. lecanii* was not found to colonise soil. Grain samples in rice and bloom samples in cowpea did not reveal the presence of any endophytes.

In cowpea, several natural endophytes were found to be stumped upon in different parts, including four fungi and two bacteria. The fungal bacterial association, *Fusarium* + Ebc 3 (unidentified bacterium) was the most dominant endophyte. The fungus *Clonostachys rosea* which was isolated from leaves and stems is known to be an endophytic, necrotrophic, mycoparasitic fungus used in the biological control of plant pathogenic fungi. Another dominant species was *Cladosporium* sp. which was found to colonise profusely in leaves and sparsely in stem. *Chaetomium globosum* and *Cerrena* sp. isolated from the leaves and stem is a known endophyte in ginger with antifungal and antibacterial properties, respectively. *Rhizobium mayense* is a new species of *Rhizobium* suspected to be a nitrogen fixer. The endophyte that was found to colonise all plant parts. *Enterobacter cloacae* another bacterial endophyte obtained from leaves, stem and roots of cowpea is known for its plant growth promotion.

Rice plants inoculated by seed soaking, with the conidial suspensions of *B. bassiana*, *M. anisopliae* @ 10^8 spores mL^{-1} and *L. saksenae* @ 10^7 spores mL^{-1} reflected 27.17 to 29.99 per cent increase height, 44.75 to 47.92 per cent increase in biomass and 36.97 to 39.89 per cent increase in grain yield, without significant variation among the treatments. However, the increase was more in plants inoculated with *L. saksenae*.

Incidence of rice bug assessed in terms of percentage of chaffy grains per panicle was significantly reduced in *L. saksenae* (14.21) compared to *B. bassiana* (20.48), *M. anisopliae* (21.61) compared to control. There was no incidence of disease in treated as well as control plants.

Further confirmation of endophytic association of these entomopathogens need to be taken up by carrying out transmission and scanning electron microscopy as well as by comparison of PCR results of the inoculated and re isolated organisms. The study suggests that to take better advantage of the endophytic nature of *M. anisopliae*, *B. bassiana, L. lecanii* and *L. saksenae*, seed and seedling priming with these entomopathogens can be resorted to.

Highlights of the study

- *B. bassiana, M. anisopliae, L. saksenae* and *L. lecanii* can associate endophytically with rice and cowpea
- *L. saksenae* is a strong endophyte colonizing up to 45th day in plants. It can colonize in soil as well.
- Seed soaking is the best method for establishing endophytic association of entomopathogenic fungi in rice, while in cowpea it is foliar spraying.
- *B. bassiana*, *M. anisopliae* and *L. saksenae* has mobility in plants, moving away from the point of inoculation.
- Natural endophytes preferred stem, while in rice and in cowpea they preferred leaves.
- Plants inoculated with entomopathogenic fungi showed increase in growth and yield and decrease in incidence of rice bug. *L. saksenae* reduced the rice bug incidence significantly.

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ENDOPHYTIC ASSOCIATION OF ENTOMOPATHOGENIC FUNGI WITH RICE AND COWPEA

by

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Abstract of the thesis

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ABSTRACT

The study entitled 'Endophytic association of entomopathogenic fungi with rice and cowpea' was carried out in the Department of Agricultural Entomology, College of Agriculture, Vellayani, Thiruvananthapuram, during the year 2017-19, with the objective to examine the endophytic association of entomopathogenic fungi in rice and cowpea.

The entomopathogenic fungi, evaluated were *Beauveria bassiana*, *Metarhizium anisopliae*, *Lecanicillium lecanii* and *Lecanicillium saksenae*. They were tested through seven inoculation methods *viz*., seed coating, seed soaking, radicle dressing, root dipping, soil drenching, foliar spraying and bloom spraying by inoculating the conidial suspension at their effective doses.

Among the four entomopathogenic fungi inoculated, *B. bassiana*, *M. anisopliae* and *L. saksenae* were found to colonise rice plants. *B. bassiana* was found to colonise in stem and roots, while *M. anisopliae* preferred stem. Both *B. bassiana* and *M. anisopliae* colonized effectively by seed soaking method. *L. saksenae* colonised in the stem and roots in seed soaking, in the roots in soil drenching and in the leaves in foliar spraying method.

In plants, *B. bassiana* and *M. anisopliae* persisted up to 15th day after inoculation (DAI), while *L. saksenae* persisted till 45th DAI. In soil, *L. saksenae* persisted in soil up to 45th day, while other fungi could not be retrieved from soil. *L. lecanii* could not be retrieved from any of the plant parts or soil up to 45th DAI.

Thirteen natural endophytes were isolated from rice, which included growth promoters with nitrogen fixing properties, gibberellic acid production properties, saprophytes and plant pathogens. They were identified with the help of colony and conidial characters. Those fungal endophytes whose identity could not be confirmed were characterised at molecular level through ITS sequencing, and bacterial endophytes through 16S sequencing.

The most dominant endophyte in rice was the fungus, *Neocomospora rubicola*, a known plant pathogen followed by the nitrogen fixing bacterium *Burkholderia cepacia*.

Colonisation of fungal bacterial association *Sarocladium oryzae* + *Kosakonia sacchari* was also dominant in the stem. *S. oryzae* is a known plant pathogen while *K. Sacchari* is a nitrogen fixing bacterium. The other endophytes with less frequency of occurrence were *Pestalotiopsis microspora, Cladosporium* sp., *Microdochium fisheri*, *Fusarium solani*, *Aspergillus* and *Penicillium*.

Experiments in cowpea revealed that all the four fungi could successfully colonize different plant parts. Foliar spraying was the best method in cowpea to get endophytic association of all the four fungi, while *M. anisopliae* could also be retrieved through seed soaking and root dipping methods. *B. bassiana, M. anisopliae* and *L. saksenae* were found to colonise leaves, stem and roots, while *L. lecanii* colonised only on leaves.

In cowpea plants, *M. anisopliae* and *L. saksenae* were found to be more persistent (up to 45th DAI), while *B. bassiana* and *L. lecanii* persisted only up to 30 DAI. Soil samples revealed the presence of *B. bassiana, M. anisopliae* and *L. saksenae* up to 15th, 30th and 45th DAI respectively. *L. lecanii* was not found to colonise soil.

Grain samples in rice and bloom samples in cowpea did not reveal the presence of any endophytes.

In cowpea, several natural endophytes were found to be stumped upon in different parts, which included four fungi and two bacteria. Apart from this a fungal bacterial association, *Fusarium* + Ebc 3 (unidentified bacterium) was found to be a dominant endophyte. The bacterium *Rhizobium mayense* isolated from all plant parts was the most dominant endophyte. The other endophytes isolated were the fungi, *Cladosporium* sp., *Clonostachys rosea*, *Chaetomium globosum* and *Cerrena* sp. and the bacterium *Enterobacter cloacae*.

Rice plants inoculated by seed soaking, with the conidial suspensions of *B. bassiana* and *M. anisopliae* @ 10^8 spores ml^{-1} and *L. saksenae* @ 10^8 spores mL^{-1} reflected 28.79, 27.17 and 29.99 per cent increase height, 47.71, 44.75 and 47.92 per cent increase in biomass and 37.73, 36.97 and 39.89 per cent increase in grain yield, the highest values being those of *L. saksenae* and lowest being those of *M. anisopliae*.

Incidence of rice bug assessed in terms of percentage of chaffy grains per panicle was significantly less in *L. saksenae* (14.21), while in *B. bassiana* it was 20.48 and in *M. anisopliae,* 21.61. There was no disease incidence in treated as well as control plants.

It is concluded that *B. bassiana, M. anisopliae* and *L. saksenae* has endophytic association with rice and cowpea and *L. saksenae* is a strong endophyte.

Appendix

APPENDIX I

DNA SEQUENCE OF NATURAL ENDOPHYTES

Neocomospora rubicola

GGCATTCGAACTAACAACTCATCAACCCTGTGACATACCTATAACGTT
GCCTCGGCGGGAACAGACGGCCCCGTAACACGGGCCGCCCCCGCCAG
AGGACCCCCTAACTCTGTTTCTATAATGTTTCTTCTGAGTAAACAAGCA
AATAAATTAAAACTTTCAACAACGGATCTCTTGGCTCTGGCATCGATG
AAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGT
GAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCG
GGCATGCCTGTTCGAGCGTCATTACAACCCTCAGGCCCCCGGGCCTGG
CGTT

Microdochium fisheri

GGACCGTCGGACGTATCTAACTCCCAAACCATGTGAACTTACCACTGT
TGCCTCGGTGGAAGGCGCTGGAAACAGCGCTGGAAGCCGGTGGACAT
CTAAACTCTTGTTAATTTTTGGCATTCTGAATCATAACTAAGAAATAAG
TTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAAC
GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCAT
CGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGC
CTGTTCGAGCGTCATTTCAACCCTTAAGCCTAGCTTAGTGTTGGGAGAC
TGCCTAATACGCAGCTCCTCAAAACCAGTGGCAGAGTTTTTACGTACT
CTGAGCGCAGTAATTCTATTCTCGCTTCTGAAAACGTCTAGACGATAG
CCAGAAACCGCATGCTTCGGCAGCACTTTTTAATGGTTGACCTCGGAT
CAGGTAGGAATACCCGCTGAACTAAAAAAAAAAATTAAGCGGAGGGA
GCCTGATCATTTAAGCGGAGGAGCCTCCCAATAAGGGGGAGGAGCCT
ATGTTCCGCGGAGGAGGGGTATGGGAAAAGCGGAGGGAGACAGCGGG
AATCTGAACGATGGTAAATTTTGGGCATTCTGAAGCCGAGCTGGAAGA
GAAGTTGAAA

Pestalotiopsis microspora

CCTGTCATGGCGGTCGTATCTCTAAACCGAAGTGACTTACCATTGTTGC
CTCGGCAGAAGCTGCTCGGTGCACCTTACCTTGGAACGGCCTACCCTG
TAGCGCCTTACCCTGGAACGGCTTACCCTGCAACGGCTGCCGGTGGAC
TACCAAACTCTTGTTATTTTATGGTTATCTGAGCGTCTTATTTGAATAA
GTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAA
CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCA
TCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGC
CTGTTCGAGCGTCATTTCAACCCTTAAACCTAACTTAGTGTTGGGAGCC
TACTGCTTTTG

Burkholderia cepacia

GAATCCGGACTACGATCGGTTTTCTGGGATTAGCTCCCCCTCGCGGGTT
GGCAACCCTCTGTTCCGACCATTGTATGACGTGTGAAGCCCTACCCAT
AAGGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCA
CCGGCAGTCTCCTTAGAGTGCTCTTGCGTAGCAACTAAGGACAAGGGT
TGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACG
ACAGCCATGCAGCACCTGTGCGCCGGTTCTCTTTCGAGCACTCCCGCCT
CTCAGCAGGATTCCGACCATGTCAAGGGTAGGTAAGGTTTTTCGCGTT
GCATCGAATTAATCCACATCATCCACCGCTTGTGCGGGTCCCCGTCAA
TTCCTTTGAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGTCAACTTC
ACGCGTTAGCTACGTTACTAAGGAAATGAATCCCCAACAACTAGTTGA
CATCGTTTAGGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCC
ACGCTTTCGTGCATGAGCGTCAGTATTGGCCCAGGGGGCTGCCTTCGC
CATCGGTATTCCTCCACATCTCTACGCATTTCACTGCTACACGTGGAAT
TCTACCCCCCTCTGCCATACTCTAGCCTGCCAGTCACCAATGCAGTTCC
CAGGTTGAGCCCGGGGATTTCACATCGGTCTTAGCAAACCGCCTGCGC
ACGCTTTACGCCCAGTT

Sarocladium oryzae

GGCCCTCCGGGGACTGAAGTAGGCTCTCCACCCATTGTGACATACCTA
TCGTTCCCTCGGCGGGATCAGCGCGCGGCTGCCTCCGGGCTCCGGGCG
TCCGCCGGGGACAACCAAACTCGAATTTTATAGTGATTCTCTGAGGGG
CGAGAGCCCGAAAACAAAATAAATCAAAACTTTCAACAACGGATCTC
TTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGT
GAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGC
CCGCCGGCACTCCGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCT
CAGGAGCACCCTTCGGGGCGCACCTGGTGCTGGGGATCACGG

Kosakonia sacchari

TAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACT
ACGACGCAGTTTATGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTT
GTATGCGCCATTGTAGCACGTGTGTAGCCCTGGTCGTAAGGGCCATGA
TGACTTGACGTCATCCCCACCTTCCTCCAGTTTATCACTGGCAGTCTCC
TTTGAGTTCCCGGCCTAACCGCTGGCAACAAAGGATAAGGGTTGCGCT
CGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCC
ATGCAGCACCTGTCTCACAGTTCCCGAAGGCACCAATCCATCTCTGGA
AAGTTCTGTGGATGTCAAGACCAGGTAAGGTTCTTCGCGTTGCATCGA
ATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTG
AGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCTATTTAACGCGTTA
GCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAATAGACATCGTT
TACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTT
CGCACCTGAGCGTCAGTCTTCGTCCAGGAGGCCGCCTTCGCCACCGGT

ATTCCTCCAGATCTCTACGCATTTCACCGCTACACCTGGAATTCTACCT
CCCTCTACGAGACTCAAGCCTGCCAGTTTCGGATGCAGTTCCCA

Fusarium solani

GGCCTTCGGGGTAACAACTCATCAACCCTGTGACATACCTAAACGTTG
CTTCGGCGGGAACAGACGGCCCCGTAACACGGGCCGCCCCCGCCAGA
GGACCCCCTAACTCTGTTTCTATAATGTTTCTTCTGAGTAAAACAAGCA
AATAAATTAAAACTTTCAACAACGGATCTCTTGGCTCTGGCATCGATG
AAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGT
GAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCG
GGCATGCCTGTTCGAGCGTCATTACAACCCTCAGGCCCCCGGGCCTGG
CGTTGGGGA

Clonostachys rosea

CATACGAGAGGGAACATCATCAGTAACCCGTGTGGCATATCCTATCGT
TGCAGGCAGGCGAGGATCGCCCCGGAGCGCCTCGTGTGCCCCGGATCC
GGCGCCCGCCTAGGAACCTTAACTCTTGTTTTATTTTGGAATCTTCTGA
GTAGTTTTTACAAATAAATAAAAACTTTCAACAACGGATCTCTTGGTTC
TGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTG
CAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCA
GTATTCTGGCGGGCATGCCTGTCTGAGCGTCATTTCAACCCTCATGCCC
CTAGGGCGTGGTGTTGGGGATCGGC

Chaetomium globosum

GCGCCCCCGGGAATTGCGGCTCCCTAACCATTGTGACGTTACCTAAAC
CGTTGCTTCGGCGGGCGGCCCCGGGGTTTACCCCCCGGGCGCCCCTGG
GCCCCACCGCGGGCGCCCGCCGGAGGTCACCAAACTCTTGATAATTTA
TGGCCTCTCTGAGTCTTCTGTACTGAATAAGTCAAAACTTTCAACAACG
GATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAG
TAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACA
TTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCA
ACCATCAAGCCCCGGGCTTGTGTTGGGGACCTGCGGCTGCCGCAGGCC
CTGAAAAGCAGTGG

CGGGCTCACTGTCACACCGAGCGTAGAAACATACATCTCGCTCTGGGC
GTGCTGCGGGTTCCGGCCGTTAAACCACCTTTTA

Cerrena sp.

TGTGCGGAAAAAAGTTGGGGCGGGATTGTAGCTGGCCCCAACCGGGC
ATGTGCACATTCTGTTCATTCCATTCTCATACACCTCTGTGCACTTTAC
ATAGGTTTGGTATAGAAAAGGTCTTTATTGACTTTGGAAATACTGACC
TATGCTTTTACAAACGCTTCAGTTTTAGAATGTCATCCGCGTATAACGC
AATAAATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGA

AGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTG
AATCATCGAATCTTTGAACGCATCTTGCGCCCTTTGGTATTCCGAAGGG
CATGCCTGTTTGAGTGTCATGGTATTCTCAATACCCCAAATCTTTGCGG
ATAAGGGTGTGTTGGACTTGGAGGTTTTTGCAGGTAATGATTGTATTA
CCAGCTCCTCTTAAATGCATTAGCAGAGATAATACTGCTACTCTCCAAT
GTGATAATTGTCTACACTGTTAGTAATGCGGTATAACAAAAA

Rhizobium mayense

CGCACACTCGGCGGCCATCCTGATCCGCGATTACTAGCGATTCCAACT
TCATGGCACTCGAGTTGCAGAGTGCAATCCGAACTGAGATGGCTTTTG
GAGATTAGCTCACACTCGCGTGCTCGCTGCCCACTGTCACCACCATTGT
AGCACGTGTGTAGCCCAGCCCGTAAGGGCCATGAGGACTTGACGTCAT
CCCCACCTTCCTCTCGGCTTATCACCGGCAGTCCCCTTAGAGTGCCCAA
CTTAATGCTGGCAACTAAGGGCGAGGGTTGCGCTCGTTGCGGGACTTA
ACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTC
TCTGAGCCACCGAAGTGGAAAGTGCATCTCTGCACCGGTCCCAGGATG
TCAAGGGCTGGTAAGGTTCTGCGCGTTGCTTCGAATTAAACCACATGC
TCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTAATCTTGC
GACCGTACTCCCCAGGCGGAATGTTTAATGCGTTAGCTGCGCCACCGA
ACAGTATACTGCCCGACGGCTAACATTCATCGTTTACGGCGTGGACTA
CCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGCGTC
AGTAATGGACCAGTGAGCCGCCTTCGCCACTGGTGTTCCTCCGAATAT
CTACGAATTTCACCTCTACACTCGGAATTCCACTCACCTCTTCCATACT
CCAGATCGACAGTATCAAAGGCAGTTCCAGGGTTGAGCCCTGGGATTT
CACCCCTGACTGATCGATCCGCCTACGTGCGCTTTACGCCCAGTAATTC
CGAACAACGCTAGCCCCCTTCGTATTACCGCGGCTGCTGGGCACGAAC
TTAGCCCGGGGACTTGCTTCTCCGGAATAACCG

Enterobacter cloacae

GGACCCTTCGGTCGAACTTCTGATTCCGATTACTAGCGATTCCGACTTC
ATGGAGTCGAGTTGCAGACTGCAATCCGGACTACGACCCACTTTATGA
GGTCAGCTCCCTCTCGCGACATGGCTTCCCTTTGTATGCACCATTGTAG
CACGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGACGTCATCC
CCACCTTCCTCCGGTTTATCACTGGCAGTCTCCTTTGAGTGCCCGGCCT
AACCGGTGGGTACCTAAGAAAAAGGTTGGCCTCCTTGCGGGAATTAAC
CCCACATTTCCCAAAACCAACTGAACAACGCCCTGGCACCCCTGGCTC
ACAATTCCCCAAAGGCCCCAGGCCTCTCTGGGAAGTTCTGTGGATGGC
CAGAGCAGGTAAAGGTCTTCCCGTTGCCTCCAAATAAACCCCATGGTC
CCCCGCTTGGGCGGGCCCCCCTCCATTTCTTTGAATTTTAACCCTGGGG
GCCGAATCCCCCAGGGGGCCACTTAACGGGTTAACTTCCGAAACCAAA
CCTCCAGGGAACCACCTCCCAATTTACATCCTTTACCGGCGGGAATAA
CCAGGGATCTAATCCTGGTTGCTCCCCCCGCCTTCCCACCCGAACGTTC
GTCATCATCCAAGGGGGCCCCCTCCCCCCCCGGTAGTCCTTCCCAATAC

CTACGCTTTCAACGCCTCAACTGGAAATCCACCCCCCTCTACCAGACTC
TCAGCCTGCGCCGTTTCTGATGCCATTCCCCAGGTGAACCCGGGGATTT
TCATACCCACTTGACCGAACGCCCTGACGTGCGCCTTAACGCCAGTAA
TTTCCGAAATAACGCTGGACACCTCGGAAATAACCCCAGCTGCCTGGA
CACGAAATTAACCCGGTGCCTCATTCTCGGCTCGGTAACACGCTCAAA
CGACAGA

Fusarium sp.

AGCCATACGGAGCTACACTCCCAAACCCCTGTGACATACCAATTGTTG
CCTCGGCGGATCAGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGA
GGACCCCTAAACTCTGTTTCTATATGTAACTTCTGAGTAAAACCATAA
ATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGA
AGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTG
AATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGG
GCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCAGCTTGGTGTTG
GGACTCGCGAGTCAAATCGCGTTCCCCAAATTGATTGGCGGTCACGTC
GAGCTTCCATAGCGTAGTAGTAAAACCCTCGTTACTGGTAATCGTCGC
GGCCACGCCGTTAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGT
AAGAATACCCCAAAAAATTAAAAAAATATCAATAAGCGGAGGCGAGG
GATCATTACCGAGTTTACAACTCCCAAACCCTGTGAACATACCATTGTT
CATCAAAGAAAAACCGCTCGGCTAAAAAGGGGACGGCGCAAAGAACA
AAACTCTTTTTTTATAATGTTAAC