"COLLECTION, ISOLATION AND SCREENING OF ENTOMOPATHOGENIC FUNGI, *Metarhizium anisopliae* (Metchnikoff) Sorokin"

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"COLLECTION, ISOLATION AND SCREENING OF ENTOMOPATHOGENIC FUNGI, *Metarhizium anisopliae* (Metchnikoff) Sorokin"

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By

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C E R T I F I C A T E

This is to certify that the thesis entitled **"COLLECTION, ISOLATION AND SCREENING OF ENTOMOPATHOGENIC FUNGI, *Metarhizium anisopliae* (Metchnikoff) Sorokin"** submitted by **G. SOWMYA ID No. UHS14PGM417** for the degree of **MASTER OF SCIENCE (HORTICULTURE)** in **ENTOMOLOGY** to the University of Horticultural Sciences, Bagalkot is a record of research work carried out by her during the period of her study in this university under my guidance and supervision, the thesis has not previously formed the basis of the award of any degree, diploma, associateship, fellowship or other similar titles.

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Heartly Dedicated To

My Grandfather

Late Sri Mahalingayya

and

Ever loving Parents

Smt. M. Annapurna

Sri G. Vishwamurthy

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1. INTRODUCTION

Insect pest management in ancient time was by using non-chemical components like chalk powder, talc and ash to control the storage insect pests. Apart from these, the botanicals like neem oil, neem leaves and other plant originated materials were used for seed treatment by Chinese people. Indians during Harappa civilization came to know about the use of neem against the storage insect pests and later tobacco extract was used to spray the plants for management of sucking insect pests in field conditions. The discovery of insecticidal property of DDT during 1939 followed by HCH, Cyclodiens, Organophosphates, Carbamates, Synthetic pyrethroids and novel group of insecticides has lead to era of pesticides. During this period, the maximum utilization of synthetic chemicals is a plant protection tool and still it is the major component of pest management programmes against various insect pests of agricultural and horticultural crops among the farming community. Quick knock down is the only reason which made the farmers to depend on the chemicals for insect pest management.

The direct effect due to indiscriminate use of synthetic organic chemicals has lead to the development of pest resurgence, pest resistance and secondary outbreaks of pest. On the other hand, indirect ill effects are like health disorders in human beings, environmental pollution and its effect on nature, decline in the population of natural enemies (predators and parasitoids), insect pollinators, effect on aquatic ecosystem, pollution of natural resources such as water, soil, air *etc.* Understanding the direct and indirect effects of chemical insecticides on the environment and other non target organism including human beings, the researchers and environmentalists were forced to search an alternative for the chemical insect pest control. Therefore, the concept of Integrated Pest Management (IPM) came into existence during 1975. At the same period Rachel Carson, the first naturalist to raise the issues on impact of synthetic chemicals on environment (1962) who wrote a book "*Silent Spring*" highlighting the effects of synthetic chemicals on environment specifically on death of bird species due to pesticide bioaccumulation.

The ill effects of insecticides as well as increased cost of other agricultural inputs in general and plant protection inputs in particular year by year have made the inclination of farmers towards synthetic chemicals and showing interest in organic

farming and drive the serious interest in the biological control rather than chemical control of insect pests. Biological control consists of the introduction of beneficial predatory or parasitic species into cultivation systems where they were previously absent or present only at low population level (Gliessman, 2001). Biological agents based bio pesticides often have considerable scope as plant protection agents against several insect pests (Noris *et al*., 2002) compared to other bio agents like predators and parasitoids since, they require live host for mass production which is also a cumbersome process.

Recently the use of entomopathogens *viz*., bacteria, nematodes, virus and fungi for insect pest management in cultivated crops is popularising. Among these, Entomopathogenic Fungi (EPF) specially *Metarhizium anisopliae* (Metchnikoff) Sorokin is gaining more importance because of its wider host range infecting almost all soil inhabiting insects like root grubs, termites, scarabids *etc*. The EPF can kill the host just by coming in contact with host (cuticle) whereas bacteria, nematodes and virus require natural openings like mouth, anus and spiracles or by ingestion of food. The fungal-host relationship occurs through the adhesion and germination of conidia on the surface of the insect, followed by hyphae penetration through the cuticle. The process of host colonization initiates after penetration, with the penetrating hyphae becoming thicker and ramify within the integument and the hemocoel of the insect forming blastospores. The hyphae continue to grow and invade various internal organs after the death of the host and will subsequently emerge from the insect body producing conidia that disseminate and infect other individuals (Alves, 1998).

It is thought that there are about 750 species of EPF that cause infections in insects or mites (Shahid *et al.,* 2012). Successful programmes of microbial control using EPF to combat arthropod pests in soils and aquatic environments have been developed, principally utilizing the genera *Metarhizium, Beauveria*, *Sporothrix, Lecanicillium*, *Nomuraea*, *Hirsutella*, *Aschersonia*, *Isaria*, *Paecilomyces*, and *Enthomophthora* (Alves and Lopes, 2008). Several workers have reported that many of the EPF are very effective against wide range of insect pests (soil insects, leaf eating caterpillar, sucking insects *etc*.) in agricultural, horticultural and forest ecosystems (Kaur and Padmaja, 2008; Ripoll *et al*., 2008; Malarvannan *et al*., 2010; Prasad and Syed, 2010; Wraighta *et al.,* 2010; Manisegaran *et al*., 2011; Prabhu *et al*., 2011; Sahayaraj and Namachivayam,

2011). The genus *Metarhizium* has a world-wide distribution and has been described as having yellowish green, olivaceous, dark-herbage green, pink or vinaceous buff (Brady, 1979).

Many of the EPF isolates with wide host range and adaptability to different environmental conditions have been discovered against various insect pests across the world. These exhibit high level of variation among the isolates in relation to their pathogenicity, optimal temperature and viability at different climatic conditions. Therefore, the regional specific isolates for Belagavi district of Karnataka state need to be identified. In this connection, the present investigation was undertaken with the following objectives, *viz.*

1. To collect and isolate the EPF, *Metarhizium anisopliae* fungi from soil and insect samples.

2. To standardize the protocol for the isolation and selective medium for EPF, *Metarhizium anisopliae.*

3. To screen the EPF, *Metarhizium anisopliae* isolates against *Spodoptera litura*.

2. REVIEW OF LITERATURE

The reviews related to collection, isolation and screening of *Metarhizium anisopliae* are envisaged under below as objective wise of the present study.

In recent years, the study on regulation of insect pest in horticulture ecosystem has usually ignored the contribution of entomopathogens *viz.*, bacteria, viruses, fungi, nematodes, actinomycetes *etc*. Entomopathogenic fungi like *Beauveria bassiana* (Balsamo) Vuillemin and *M. anisopliae, Lecanicillium lecanii* (Zimmermann) and *Nomourea relyi* (Farl.) Samson *etc.*, are playing an important role in maintaining the insect pest population below economic threshold level in undisturbed natural ecosystems. These are having wide host range of insects and are cosmopolitan distribution in nature (Roberts and St. Leger, 2004; Rehner, 2005). Unlike other entomopathogens, EPF cause infection by direct penetration through cuticle without requirement for ingestion. Among the entomopathogenic fungi, *M. anisopliae* (Green Muscardine Fungus) is taking upper hand and popularizing among the farming community due to effective control of soil insect pest and some of the caterpillars. It has been reported that *M. anisopliae* is effective in controlling more than 200 species of insect pests (Pu and Li, 1996). It plays a significant role in the regulation of insect populations in nature and may potentially be explored for their commercial uses in biological control of insect pests. Therefore, literature pertaining to the objectives of study were collected and presented here under in different headings.

2.1 Distribution and abundance of *Metarhizium anisopliae* in soil

The occurrence and abundance of EPF varied from different agro climatic conditions. The pathogenecity, virulence and sustainability of EPF conidial spores are affected with wide range of environmental conditions like temperature, humidity, rain fall, soil type *etc.*

Vanninen (1996) reported the occurrence of *M. anisopliae, B. bassiana, Paecilomyces farinosus* and *P. fumosoroseus* in natural and agricultural soils of Finland. Among 590 soil samples tested, *B. bassiana* was isolated from 19.8 per cent, *M. anisopliae* from 15.6 per cent, *P. farinosus* from 9.2 per cent and *P. fumosoroseus* from one per cent of the samples. Vanninen also reported that, geographical location was the strongest factor determining the occurrence of *M. anisopliae*. *B. bassiana* and *P.*

farinosus were adversely affected by cultivation. Finally it was found that soil type did not affect the occurrence of any of the species. Chandler *et al*. (1997) studied on occurrence and sampling of EPF in UK soils in both cultivated and uncultivated habitat over three years (two pilot studies in 1991 and 1992 and main study in 1993) and revealed that overall 15.9 per cent of EPF occurrence was found, of which *B. bassiana* share 6 per cent, *P. farinosus* share 5 per cent, *P. fumosoroseus* 2.5 per cent, *M. anisopliae* 1.3 per cent, *V. lecanii* 0.8 per cent and *Aspergillus parasiticus* 0.3 per cent.

Ali-Shtayeh *et al*. (2002) studied on distribution and occurrence of EPF in irrigated vegetable fields and citrus orchard soils over a period of nine months (April-December). Entomopathogenic fungi were found in 33.6 per cent of soil samples studied, with positive samples yielding 70 fungal isolates, belonging to 20 species from 13 genera.

Asensio *et al.* (2003) carried out a survey in different areas of agriculture and forest in the province of Alicante (South East Spain). The bait trap method was used to know the frequency of insect pathogens. The most abundant EPF were *B. bassiana* (32.8%), *M. anisopliae* and (6.4%) *L. lecanii* (4.8%). Among the soil of different areas analyzed, the forest soil displayed the highest number of EPF (62.5%) as compared to agriculture soil (50%), natural vegetation (30%) and garden soil (14%). Similarly Tkaczuk *et al.,* (2014) studied on the occurrence of EPF in a conventional and organically cultivated fields in different locations of north-eastern region of Lubelskie Voivodeship using selective media and bait trap technique as two isolation methods and reported that, a total of four species of fungi were isolated *viz., B. bassiana, M. anisopliae, Isaria fumosorosea and Lecanicillium sp.* The number of EPF were more abundant in organically cultivated fields as compared to conventionally cultivated fields. Among these *M. anisopliae* being the most abundant species.

A survey was conducted by Keller *et al*. (2003) to know the diversity of EPF in agricultural fields of Switzerland. A total of 82 samples were collected out of which 96 per cent samples had *M. anisopliae.* Further, they also reported other EPF like *B. bassiana, P. fumosoroseus* and *Conidiobolus* sp. However, the per cent occurrence of these EPF was very less (18%).

The study on distribution of *M. anisopliae* was conducted in Tamil Nadu by Sahayaraj and Borgio (2006) with a collection of 40 soil samples from agricultural and horticultural fields. Among the samples collected, the presence of *M. anisopliae* was noticed in 50 per cent of the samples.

Diversity of EPF in Spain was observed by Moraga *et al.* (2007). Out of 244 soil samples, 175 soil samples possessed the EPF. Of the 175 soil samples, 104 yielded *B. bassiana* (42.6 %), 18 yielded *M. anisopliae* (7.3 %), and 53 samples (21.7 %) harbored both fungi. Investigation for the distribution of soil borne EPF in crop fields was conducted in Iran. Among 150 soil samples collected, EPF occurred at 78 per cent of soil samples from which 40 per cent of *B. bassiana*, 21 per cent *M. anisopliae* and 17 per cent had both species. They found that occurrence and distribution of EPF was not significantly affected by pH and texture of soil (Derakhshan, 2008).

A study on occurrence and species diversity of insect-associated fungi from forest habitats was conducted in China. Among 425 soil samples, 55.5 per cent had EPF. A total of 377 fungi belonging to 46 species and 27 genera were isolated and identified which included *P. farinosus* (19.6%), *B. bassiana* (14.1%) and *M. anisopliae* var. *anisopliae* (10.6%) as predominant one (Sun and Liu, 2008).

A study was carried in Mauritius by collecting 224 soil samples from 19 locations representing three climatic zones and covering vegetable cultivated, sugarcane plantation and natural sites. Results revealed that entomophagous fungi were isolated from 77 soil samples out of 224 (38.6%). *Metarhizium anisopliae* was isolated from 42 (18.8%) samples and *B. bassiana* from 24 (10.7%) samples. They also observed that *M. anisopliae* was isolated more frequently from soils under vegetables as compared to soils under sugarcane or habitat with natural vegetation. Further, they noticed that *M. anisopliae* isolates were recovered more frequently at higher temperatures (25-28^{0}C). Later they also tested for pathogenicity of all the isolates of *M. anisopliae* against fruit fly in cucurbits and recorded 2-94 per cent mortality at five days of post-treatment (Sookar *et al*., 2008).

The distribution of EPF in central Brazilian soils was studied by Rocha *et al*. (2012). Results revealed that a total of 107 *Metarhizium* isolates were obtained from

soils collected and gene sequences from 63 of these were obtained for comparison. Among these, one was confirmed to be *M. anisopliae* and 53 were very closely allied to the same species.

Investigation on the occurrence and distribution of EPF was done in Iraq province agro-ecosystem during two seasons (fall and spring) for two years (2012-2013) using bait trap technique (Khudhair *et al.,* 2014). The results revealed that out of 94 isolates, 75.5 per cent had *B. bassiana* and 18.1 per cent had *M. anisopliae* followed by *L. lecanii* with 4.3 per cent.

Occurrence of insecticides fungi in soils from monoculture crops of *Festuca pratensis* (Huds.) was studied by Kolczarek and Jankowski (2014) in Krzyzewo and Uhnin (Poland) where three species of EPF *viz., B. bassiana, I. fumosorosea* and *M. anisopliae* were isolated and identified. The soil samples were collected from forest plantations that are a natural habitat of the large pine weevil, *Hylobius abietis* L. during spring and autumn 2010 and 2011 in forest districts of northern, central and southern Poland. About five species of EPF were isolated *viz., B. bassiana, I. farinosa, I. fumosorosea, M. anisopliae* and *V. lecanii*, of which, *I. fumosorosea* and *M. anisopliae* were most abundant (Popowska-Nowak *et al.*, 2016).

2.2 Standardization of protocol for the isolation of fungus and selective medium for *Metarhizium anisopliae*

2.2.1 Standardization of selective medium for *Metarhizium anisopliae*

A wide range of fungi inhabiting soil can grow on any artificial media provided. Number of methods for isolating the EPF from soil samples was developed by earlier workers (Chandler *et al.,* 1997; Asensio *et al.,* 2003; Meyling and Eilenberg, 2007; Jackson *et al.,* 2005; Khudhair *et al.,* 2014; Tkaczuk *et al.,* 2014). However, they varied with pH conditions of the samples and hence, the selective media for specific locations is needed. Previously developed protocols for isolation of EPF were tested for selective media for isolation of EPF from soil samples testing conidial germination, colony growth *etc.,* are here under.

A study was carried out for testing the conidial production of EPF on various depths and types of commercially available agars like Corn Meal Agar (CMA), Nutrient Agar (NA), Malt Extract Agar (MEA), Sabouraud Dextrose Agar (SDA), Yeast Peptone Dextrose Agar (YPDA) and Potato Dextrose Agar (PDA) and it was found that PDA at a depth of 2 mm showed greatest conidium production for *M. anisopliae* and *B. bassiana* isolate and as Yeast Extract-Peptone-Dextrose Agar (YPDA) for *V. lecanii* irrespective of depth (Kamp and Bidochka, 2002).

Investigation was undertaken on different culture media like 1% Agar-water (AW), Minimal medium (MM), PDA, potato-dextrose-1% yeast extract agar (PDAY), Sabouraud-dextrose-1% yeast extract agar (SDAY) and Complete medium (CM) (Edimara *et al.*, 2006; Pontecorvo *et al.*, 1953; Azevedo & Costa, 1973; Alves *et al.*, 1998) to test the conidia germination of *L. lecanii*, *B. bassiana* and *P. fumosoroseus* isolates. The results showed that for *L. lecanii* CM and PDA recorded the highest germination percentages, while the lowest percentages were obtained with SDAY and AW medium, for *B. bassiana* highest germination of conidia was observed in PDA, PDAY, SDAY, and CM while MM and AW medium showed lower germination and the highest percentages for conidial germination of *P. fumosoroseus* isolates were obtained on CM and PDA and the lowest percentages on SDAY. Higher germination percent was observed for the nutrient-rich media (PDA, PDAY, SDAY and CM).

Study on various selective media for EPF isolation prepared with the modification of previously reported D0C2 medium (Shimazu and Sato, 1996) and Veens semi selective medium (Veen and Ferron , 1996) like D0C2- 10, D0C2- 4, D0C2- 50%, D0C2-PDA and SMA medium revealed that SM (10~100 µg/ml Dodine, 100 µg/ml Chloramphenicol, 50 µg/ml Streptomycin, 32.5g SDA in 500ml distilled water) was proved to be best one, because the more EPF and inhibition of non-entomopathogenic fungi can be grown on Dodine contained than on $CuCl_2$ contained medium (Shin *et al.*, 2010).

Liu *et al.* (2012) evaluated the different selective media (PDA with 1% (w/v) peptone agar medium and oatmeal agar medium) for isolation of *M. anisopliae.* Results showed that potato dextrose with 1% (w/v) peptone agar medium was the optimal culture medium for colony growth and conidial yield after three to seven days of

incubation. Samir *et al*. (2015) prepared different types of isolation media by again with modification of previously reported D0C2 medium (Shimazu and Sato, 1996) using Cetyl trimethyl ammonium bromide (CTAB) and oatmeal agar (OT) as a basal medium as introduced recently by Posados *et al.* (2012) for the isolation of fungi from soil *viz.*, D0C2, D0C2+ Oatmeal agar, D0C2 +CTAB and OTA + CTAB. Among these D0C2 +CTAB (0.6g CTAB, 0.2 g $Cucl_2$, 2 mg Crystal violet, 15 g agar, 0.5g Chloramphenicol, one liter distilled water) and OT + CTAB (0.6 g CTAB, 15 g agar, 0.5g Chloramphenicol, 50 g oat, 1litre distilled water) media were able recover two EPF true entomopathogenic species *L. lecanii* and *M. anisopliae* successfully whereas, D0C2 alone and D0C2 + OT medium failed to recover the two species. This result indicated that addition of CTAB to media was vital factor for the recovery of the two EPF.

2.2.2 Standardization of protocol for the isolation of *Metarhizium anisopliae*

The EPF can be isolated both from soil and infected insect sample. There are various methods (Warcup, 1950; Asensio *et al.*, 2003) for the isolation of entomopathogens, which differ in their efficacy of isolating maximum number of positive isolates. Hence, there is a need for standardizing the best isolation method for getting maximum number of isolates.

Azaz (2003) isolated and identified soil borne fungi in fields irrigated in Harran plain used two isolation methods *i.e.*, soil dilution plate method and soil wash technique. The results revealed that, out of 109 samples isolated 62 were isolated through soil dilution plate method, seven were through soil wash technique and about 40 were through both methods.

Four different isolation methods *viz.,* insect bait method, serial dilution plate method, soil plating (Warcup method) method and from insect cadaver with surface sterilization were followed by Rani *et al.* (2015) from both soil and infected soil samples in their study on isolation and screening of indigenous EPF against sucking pests of vegetables. The results found that total of three isolates were isolated, one each from the insect bait method, serial dilution plate method and other from insect cadaver with surface sterilization.

2.3 Screening of *Metarhizium anisopliae* against insect pests

The strains isolated from soil or insect vary in their infectivity against the insect pests. Many of them will be either host specific or region specific. Infectivity and sporulation also varies with climatic conditions like fluctuating temperature and humidity. So there is a need to identify the region specific strains for effective pest management and mass production for commercial use by farmers. Following reviews may help in screening and indentifying the virulent strains.

Different formulations (crude, wettable powder and oil) of *M. anisopliae*, *B. bassiana* and *V. lecanii* were tested against different sucking pests like aphids, thrips, whitefly and mite under field condition during *kharif* season and the results indicated that the oil based formulation of *V. lecanii* was best in controlling the aphids and whitefly population. The plot treated with *M. anisopliae* and *B. bassiana* noticed least population of leaf hoppers and thrips, respectively. The oil based formulation treated plot gave the maximum yield as compared to other formulations (Harischandra and Shekharappa, 2009).

A laboratory study on pathogenicity, ovicidal effect and median lethal concentration (LC_{50}) of *B. bassiana*, *M. anisopliae*, *L. lecanii* and *P. fumosoroseus* against exotic spiraling whitefly was conducted by Boopathi *et al*. (2013). The results revealed that, *P. fumosoroseus* showed high pathogenicity with 100 per cent mortality and lowest LC_{50} whereas *M. anisopliae* showing more ovicidal effect (37.3%) and *L. lecanii* with minimum egg hatchability (23.2%) at $2x10^9$ conidia/ml.

A field experiment was conducted for two seasons (2011-2012 and 2012-2013) to evaluate the efficacy of EPFs *viz.*, *B. bassiana* and *M. anisopliae*, *L. lecanii* & *I. fumosorosea* in managing the exotic spiraling whitefly, *Aleurodicus dispersus* (Hemiptera: Aleyrodidae) on cassava (*Manihot esculenta* Cranrz.) and the results revealed that *I. fumosorosea* and *L. lecanii* were promising in controlling the spiraling whitefly (> 70 % mortality) compared to *B. bassiana* and *M. anisopliae* (Boopathi *et al*., 2015). Similarly, field experiment was conducted by Janghel *et al*. (2015) during *kharif* 2013 and summer 2013-14 to evaluate different bioagents and biopesticides like *B. bassiana*, *M. anisopliae*, *L. lecanii* and *Chrysoperla zastrowi* sillemi along with

neemazal and acetamiprid against okra aphid and reported that the *C. zastrowi* sillemii @ 1 lakh/ha with 80.21 per cent and *L. lecanii* @ $1x10^8$ cfu/g with 79.49 per cent reduction in the aphid population indicating *C. zastrowi* and *L. lecanii* to be the best biopesticides .

Murad *et al*. (2006) screened 10 isolates of *M. anisopliae* var. *anisopliae* against the cowpea weevil in Brazil. Results revealed that, the pathogenicity of *M. anisopliae* towards the mentioned pest. Bioassay indicated that the three isolates (CG34, CG292 and CG100) showed the considerable mortalities (10-26.7%) compared to other isolates. When LC_{50} was performed, isolate CG34 showed increased lethality.

In 2013, Norhelina *et al.,* studied the infectivity of 5 strains of *M. anisopliae* against the 2^{nd} and 4^{th}instars of brinjal whitefly. Among these, strain GJ4 had the lowest LT_{50} value indicating its high virulence. Using this strain, dosage response assays on nymphal mortality rates were conducted with four different concentrations (10^7, 10^5, 10^3, 10). Results revealed that 2^{nd} instar was more sensitive than 4^{th} instar nymphs.

The effect of two isolates of *M. anisopliae* on cabbage root fly under glass house and field conditions was studied in UK by Chandler and Davidson (2005). Under glasshouse condition, the isolate 389.93 gave 90 per cent reduction when compared to others. It also increased the mean plant weight. In field experiment, it gave only 30 per cent mortality. Iprodione and tebuconazole were compatible with EPF when tested.

Ravindran *et al.* (2015) screened 4 isolates of *M. anisopliae* for their pathogenicity against subterranean termites among which Tk-4 and Md-1 were highly virulent producing mortality per cent of approximately 86.6±1.7% and 83.3±1.3% respectively on 4^{th} day of post inoculation. Days required for sporulation was also less (5-7 days) compared to other two strains.

An on farm trial in Kerala for management of Rhinoceros beetle grubs in coconut with common salt, Naphthalene balls+ sand+ *Clerodendron infortunatum* and *M. anisopliae* (300 ml/l of water/m^2) revealed that incidence of grubs in 1 m^2 area was low (27.93%), pest attack was low (10.28 %), pest reduction was high (70%), nut yield was high (80 nuts/palm/year) and B: C ratio was also high (2.06) by using *M. anisopliae* when compared to other methods (Yamini Verma, 2013).

In Belgium, the virulence of 34 EPF isolates against white grub was tested by Ansari *et al.* (2004). Among these, two isolates of *M. anisopliae* (CLO 53 and CLO 54) caused the highest mortality (90%) 10 weeks post inoculation when compared to other isolates. Screening bioassays revealed that CLO 53 was highly virulent with increased mortality and LT_{50}values decreased with increase in temperature and conidial concentrations.

Virulence of two fungal isolates, *M. anisopliae* FT83 and *P. fumosoroseus* FG340 against the second instar larva of beet army worm was investigated in Korea. Results revealed that mortality increased and the LT_{50} values decreased with increased conidial concentration. Mortality caused by both the isolates was temperature dependent and increased from 20-30^{o} C but decreased at 35^{o} C. The mortality of larvae treated with *M. anisopliae* FT83 at various RH was higher with increased RH but, *P. fumosoroseus* FG340 showed high control effects in whole ranges of RHs (Han *et al.*, 2014).

Susceptibility of Red palm weevil (RPW) to 7 isolates of *M. anisopliae* and 3 isolates of *B. bassiana* was studied by Gindin *et al.* (2006) in Israel. The Ru and MA isolates of *M. anisopliae* were considered to be most virulent (100%) 7 days post inoculation. The averaged mortalities of treated eggs and hatched larvae for Ru isolate were more compared to MA but there was no significant difference. When Ru isolate was further tested on adults with two methods of application *viz.*, Spraying of spore suspension and application of dry spores, quick mortality was observed in second method. They also reported reduced the oviposition time and weight of the larvae however the results were not significant.

Loc and Chi (2007) studied the bio control potential of *M. anisopliae*, *B. bassiana*, Crymax and Atabron against diamond back moth (DBM), in Vietnam. Among these, *M. anisopliae* [*M. a* (OM_3 STO)] gave the highest mortality 7 days after treatment at 10^7 conidia/ ml in the field which indicated its highest pathogenicity. Population density of DBM larvae in greenhouse experimental cauliflower field was also the lowest (19.2 /m^2) when compared to other treatments but there was no significant difference between them.

Virulence of three isolates of *M. anisopliae* and four isolates of *B. bassiana* was reported in New York. Results showed that *M. anisopliae* strain 346 caused 100 per cent mortality in only 3 hours of exposure with least LT_{50} (<0.13) when compared to other isolates indicating highest pathogenicity. Topical application of conidial suspensions was performed where, *B. bassiana* strain 787 had the lowest LT_{50} indicating its high virulence at low concentrations (Jones *et al.*, 1996).

Godonou *et al.* (2009) screened the isolates of *M. anisopliae* and *B. bassiana* in Benin for their virulence to third instar larvae of DBM. Results revealed that larval mortality caused by both Bba5653 and Ma182 was higher when compared to other isolates. However, the mortality caused by Bba5653 was significantly higher (94%) than that of Ma182.

Fakoorziba *et al.* (2014) screened 4 strains of *M. anisopliae* and 6 strains of *B. bassiana* against early larval instars mosquito in Iran. All the 4 strains of *M. anisopliae* and Bb429C, Bb796C strains of *B. bassiana* gave 100 per cent mortality 7 days after exposure. Ma1018c was considered to be the most virulent strain of *M. anisopliae*, since highest mortality was seen 3 days after exposure. Mortality rates in early instars were different at different concentrations. LC_{50} and LT_{50} values were not determined since mortality rates showed no significant difference.

The pathogenicity of five isolates of *M. anisopliae* to adult groundnut beetle was investigated in Nigeria. All the isolates were virulent to the beetle but their pathogenecity varied. Isolate CPD 4 was superior in terms of mortality of the beetle (100 %), low pod damage (2.3%), reduction in the progeny production (0.00%) and highest repellency to the beetle (78.5 %) at the concentration of 1.0 g conidia/50g of pods. Same results were obtained when the pods were treated with pirimiphos-methyl at 10 ppm (Ekesi *et al*., 2000).

Gutierrez *et al*. (2000) reported the virulence of 20 isolates of the *M. anisopliae* against the last instar and adult emergence of Mexican fruitfly in Mexico. Among these Ma2, Ma8 and Ma16 were most virulent at the concentration of 10^8 CFU/ml showing highest mortality (98.750%) and lowest LC_{50} values (02.0-04.0). Adult emergence in the field-cage condition was also reduced in the soils treated with Ma2 isolate.

Pathogenicity of 17 isolates of *M. anisopliae* and 2 isolates of *B. bassiana* against the tobacco spider mite was studied in Kenya. Results confirmed that all fungal isolates were pathogenic but mortality varied among them. *B. metete*, ICIPE 78, MA/GPK isolates of *M. anisopliae* and Bb/GPK isolate of *B. bassiana* were most virulent showing highest per cent mortality (77.9- 82.6) and lowest LC_{50}values (2.3-1.1X10^7 conidia/ml). LT_{50} was also low (4.8-4.6 days) indicating its high virulence (Wekesa *et al*., 2005).

In Mauritius, Sooker *et al*. (2008) studied the pathogenicity of seven isolates of *M. anisopliae*, five isolates of *B. bassiana* and two isolates of *P. fumosoroseus* towards the adults of *Bactrocera zonata* and *B. cucurbita* by topical application of conidial suspension of 1×10^6 conidia/ml. All the isolates tested were pathogenic to the two fruit fly species. Mortality of *B. zonata* varied between 12.0 and 98.0 per cent and between 2.0 and 94.0 per cent in *B. cucurbitae* at 5 days post-treatment.

Dimbi *et al.* (2003) in Kenya investigated the pathogenicity of two isolates of *B. bassiana* and 12 isolates of *M. anisopliae* against fruit fly species and reported that among all these, ICIPE 40 isolate of *M. anisopliae* gave the highest mortality (100%) with lowest LT_{90} values (3.27 at 4 days post treatment) against *Ceratitis capitata* and *C. rosa* var. *fasciventris* except for *C. cosyra*. Most pathogenic isolate to both of these isolates were tested against *C. cosyra* but no significant differences were observed.

Biocontrol potential of *M. anisopliae* on red cotton bug was studied in Tamil Nadu. The lowest LC_{50} value (2.25×10^5) was recorded for the isolate CPRC16 indicating highest pathogenicity among all the 21 isolates (Sahayaraj and Borgio, 2006).

Ekesi *et al.* (2000) evaluated the persistence and infectivity of aqueous, oil/aqueous (50:50) and granular formulations of *M. anisopliae* against pupariating larvae of three species of fruit flies in the field cage experiments. All formulations of the fungus and the chemical insecticide (diazinon) significantly reduced emergence of fruit flies from treated soil.

Barra *et al*. (2013) evaluated the EPF against the three stored pests of maize *viz.*, *Tribolium confusum*, *Sitophilus zeamais* and *Rhyzopertha dominica* in Argentina**.** Results revealed that isolate JQ926223 showed the lowest LT_{50} for *T. confusum* (4, 66 days) and *R. dominica* (9, 38 days) but the isolate JQ926212 demonstrated similar LT_{50} for the three insects. However, the maximum mortality rate was observed in JQ926223 isolate.

3. MATERIAL AND METHODS

Investigations on the collection and isolation of *Metarhizium anisopliae* isolate from soil and insect samples, standardization of protocol for the isolation of *M. anisopliae* from soil samples, selective medium for *M. anisopliae* and screening for pathogenicity and sub lethal effect of *M. anisopliae* isolates on laboratory reared insect *Spodoptera litura* were carried out during 2015-16 at Biological Control laboratory, Department of Entomology, Kittur Rani Channamma College of Horticulture Arabhavi, University of Horticultural Sciences, Bagalkot. The materials used during the investigations and methodologies followed are described here under.

3.1 Collection and isolation of *Metarhizium anisopliae* from soil samples

3.1.1 Roving survey

A roving survey was conducted at different villages of Belagavi district covering major area of vegetable crops. During the survey, soil samples from vegetable fields and naturally infected insect specimens were collected and brought to the laboratory and isolated the *M. anisopliae* isolates. A total of 30 places which included six taluks of Belagavi district and in each taluk five villages and one field for each village were selected for soil sampling and collection of infected insect with *M. anisopliae* (Plate 1 and 2). The taluks of Belagavi district selected for collection of soil samples and infected insects are presented in Table 1.

3.2 Standardization of protocol and selective medium for isolation of *Metarhizium anisopliae* from soil and insect samples

To determine the optimal selective medium for EPF, various media were prepared by the modifying previously reported D0C2 medium (Shimazu and Sato, 1996) and Veens semi selective medium (Veen and Ferron, 1996).

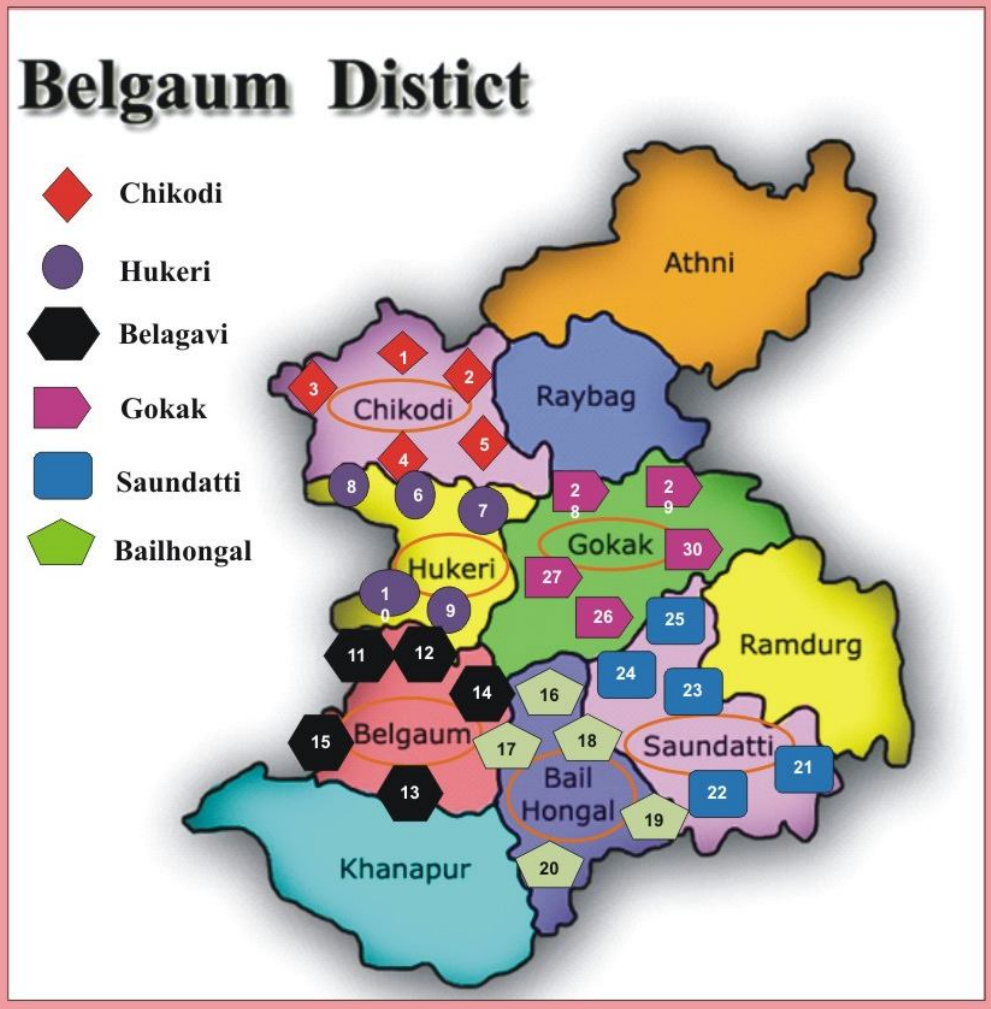


Plate 1: Map showing location of different villages in Belagavi District

a. Collection of soil sample from cabbage field of Arabhavi village

b. Collection of soil sample from tomato field of Benakatti village

c. Collection of soil samples in polythene bag

d. Labeling the collected soil samples

e. Root grub sample collected to isolate entomopathogenic fungi.

f. Diamond back moth sample collected to isolate entomopathogenic fungi.

Plate 2: Collection of soil and insect samples from fields

Table 1: Roving survey to collect the soil and insect samples for the study

Sl. No.	Taluks	Name of the villages
1	Gokak	i. Arabhavi
		ii. Kallolli
		iii. Pamaladhinne
		iv. Ghataprabha
		v. Duradundi
2	Chikodi	i. Examba
		ii. Nagaral
		iii. Hirekodi
		iv. Belakud
		v. Donwad
3	Hukeri	i. Bellada Bagewadi
		ii. Ammanagi
		iii. Borgall
		iv. Amminabhavi
		v. Handigood
4	Belagavi	i. Devagiri
		ii.Kanagav
		iii. Hudali
		iv. Honaga
		v. Bastavad
5	Saundatti	i. Yaragatti
		ii. Munavalli
		iii. Shindogi
		iv. Yaraganavi
		v. Benakatti
6	Bailhongal	i. Naganur
		ii. Sanikoppa
		iii.Sampagaon
		iv. Belavadi
		v.Bagewadi

Table 2: The selective media used for the isolation of *Metarhizium anisopliae* from soil samples

Selective medium	Components
D0C2-10	3 g Bactopeptone, 0.2 g $CuCl_2$, 2 mg crystal violet, 15 g agar, 1,000 ml distilled water, pH 10.0 with Na_2CO_3
D0C2-4	3 g Bactopeptone, 0.2 g $CuCl_2$, 2 mg crystal violet, 15 g agar, 1,000 ml distilled water, pH 4.0 with HCl
D0C2-50%	3 g Bactopeptone, 0.1 g $CuCl_2$, 2 mg crystal violet, 15 g agar, 1,000 ml distilled water, pH 4.0 with HCl
D0C2-PDA	0.2 g $CuCl_2$, 2 mg crystal violet, 39 g PDA (potato dextrose agar), 1,000 ml distilled water, pH 4.0 with HCl
SDA medium	65 g SDA (Sabouraud dextrose agar), 1,000 ml distilled water

3.2.1 Isolation of *Metarhizium anisopliae* from soil and insect samples

3.2.1.1 Serial dilution method with selective media

A 0.2 g of soil sample was placed in a 1.5 ml micro tube with 1.3 ml of 0.02% Tween-80 solution and vortexed for 15 min. The resulting suspension was serially diluted (10^{-9}) and plated on selective medium. After incubation for 6 days at 25^{o}C, the putative EPF were selected by morphological characteristics [(aspects of the colonies, such as color, diameter and mycelia texture) (Shin *et al.*, 2010)] (Plate 3).

3.2.1.2 Soil Washing Technique

A 20 g of fresh soil was placed in a funnel lined with muslin cloth (pore size 0.5 mm) for isolation using the soil washing technique. The soil samples were first washed with 2 litres of tap water and the outflow was collected in a funnel. The procedure was then repeated using 2 litres of sterile water. After this treatment, the muslin cloth and its contents were transferred into a sterile petri dish with the same water containing streptomycin (a pinch). Organic particles floating on the surface of the water and the washed soil particles were picked up with a loop and forceps and transferred onto plates

of Peptone Dextrose Agar with Rose Bengal. The plates were incubated at 25°C for 10 days (Azaz, 2003) (Plate 4).

3.2.1.3 Soil direct Plate Method

A small amount of soil sample (0.005- 0.015g) was taken from the main sample by means of sterile spatula with flattened tip and it was dropped into a sterile plate. Agar medium was poured and particles were distributed throughout the medium by swirling the plate. After solidification, plates were incubated at room temperature for one week and observed for growth of the fungi (Warcup, 1950) (Plate 5).

3.2.1.4 Plating of insect with surface sterilized

The symptomatic insect specimens for mycosis were brought to the laboratory from various vegetable growing fields and kept for one or two days. Upon death or mycosis, the specimens were surface sterilized using one per cent sodium hypochlorite followed by 70 per cent alcohol and three repeated changes of sterile distilled water. They were then inoculated on selective media and kept under incubation for one week under room temperature. On development of fungal mycelia they were transferred to fresh media for further growth. The pure cultures thus developed were stored under refrigeration for identification (Rani *et al*., 2015) (Plate 6).

3.2.1.5 Direct plating of insects samples

The defoliating caterpillar samples infected with EPF were collected from the different vegetable fields and brought to laboratory for isolation of *M. anisopliae*. The samples were directly plated on the media and allowed for drying. After drying plates were wrapped with Para film wax paper and kept for incubation under room temperature for one week (Plate 7).

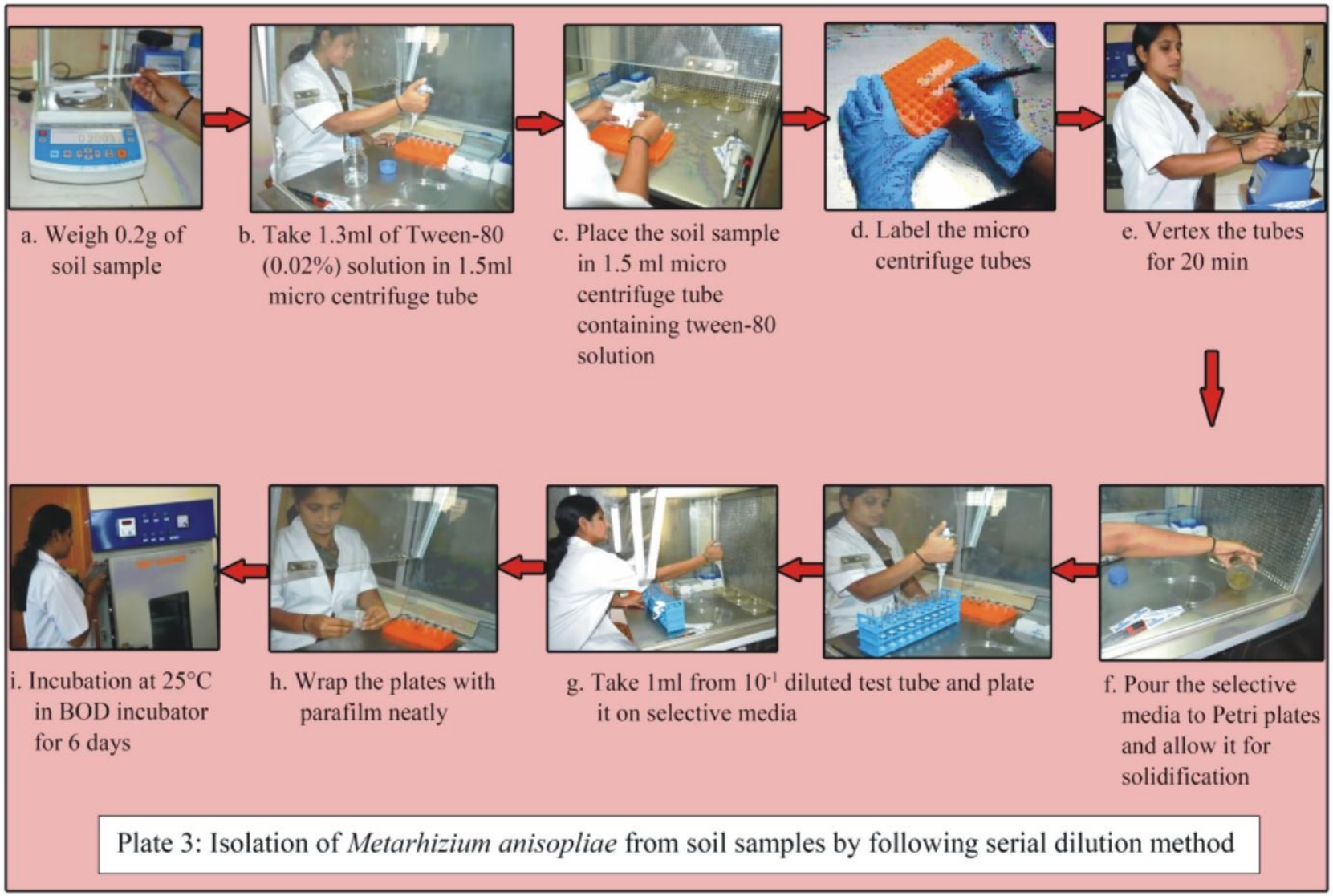


Plate 3: Isolation of *Metarhizium anisopliae* from soil samples by following serial dilution method

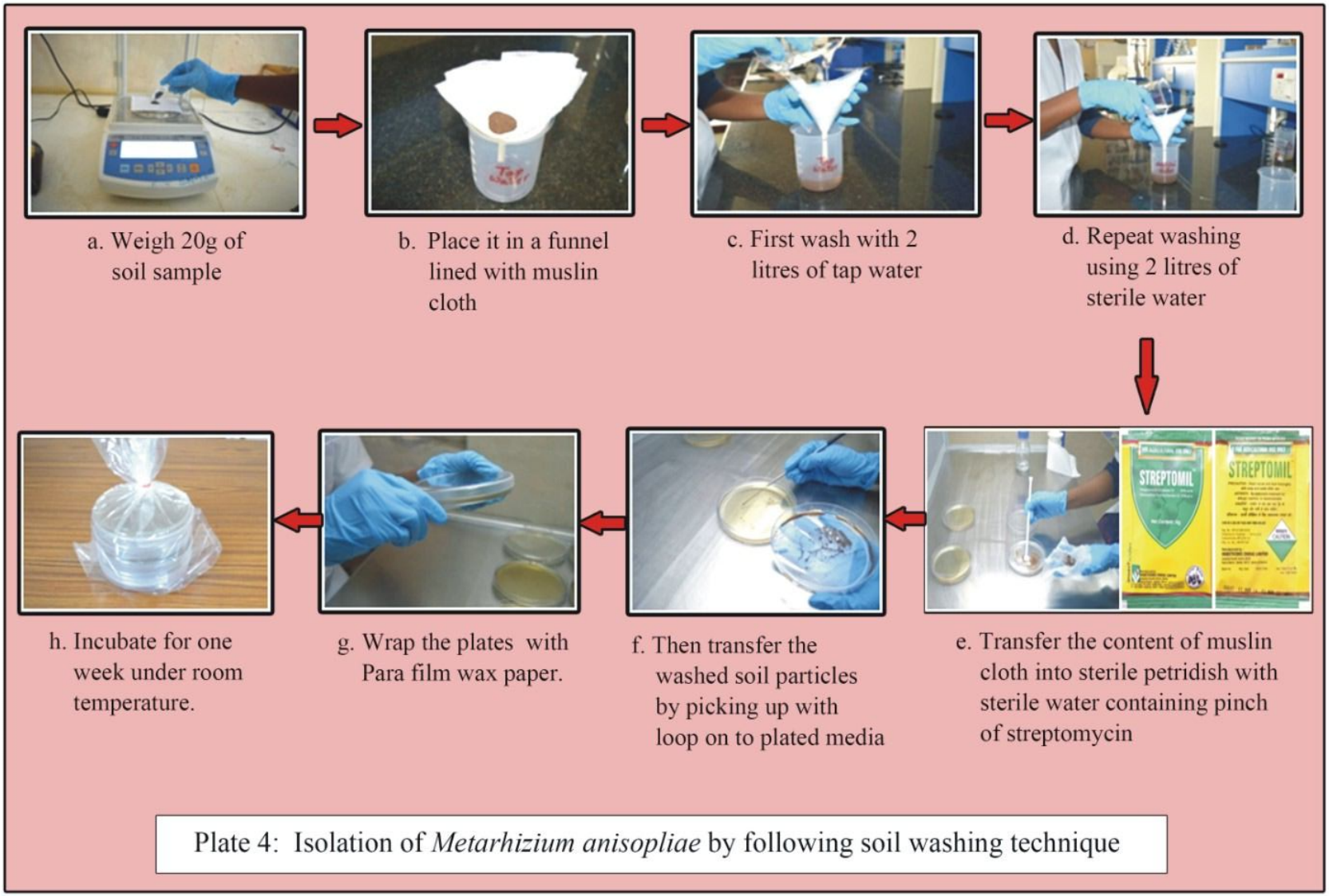
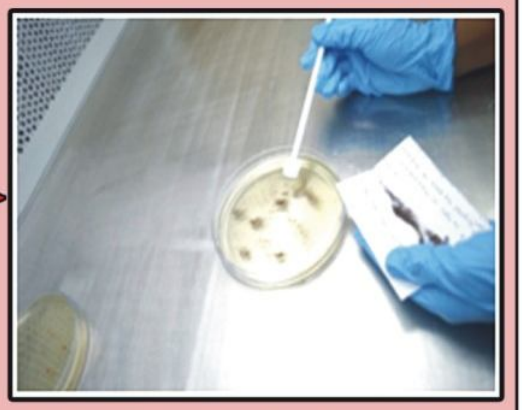


Plate 4: Isolation of *Metarhizium anisopliae* by following soil washing technique

a. Weigh a small amount of soil sample (0.005- 0.015g) from the collected soil sample



b. Drop the weighed soil into a sterile plate containing selective media

c. After drying wrap the plates with para film wax paper

d. Incubate for one week under room temperature

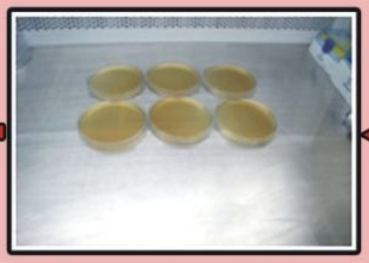
Plate 5: Isolation of *Metarhizium anisopliae* by following soil direct plate method

a. Collect insect specimens from field suspected to be infected by mycosis

b. Observe for mycosis infection on insect under stereo binocular microscope and separately collect in petri plates

c. Surface sterilize the infected insects by dipping them for one min. in a solution of one per cent sodium hypochlorite, followed by 70 per cent alcohol and three repeated changes of sterile distilled water

d. Plating of sterilized insect on selective media



e. Allow it for solidification aseptically

f. Wrap the plates with Para film wax paper

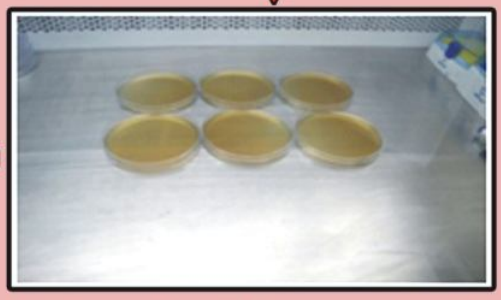
g. Incubate for one week under room temperature

Plate 6: Isolation of *Metarhizium anisopliae* by plating of surface sterilized insect

a. Collect insect specimens from field suspected to be infected by mycosis

b. Observe for mycosis infection on insect under stereo binocular microscope and separately collect

c. Place insect directly on selective media



d. Allow it for solidification aseptically

e. Wrap the plates with Para film wax paper

f. Incubate under room temperature for growth of entomopathogenic fungi

Plate 7: Isolation of *Metarhizium anisopliae* by direct plating of insect samples

3.3 Screening for pathogenicity of *Metarhizium anisopliae* isolate against *Spodoptera litura*

3.3.1 The insect

Wild population of the test insect, *S. litura* was collected from vegetable fields of KRCCH, Arabhavi and light sources of nearby hostels. The culture of the test insect was maintained on castor, *Ricinus communis* leaves under laboratory conditions in plastic tubs. The larvae of second instar were taken from the culture, as and when required. All the experiments were conducted in the Bio control laboratory, Department of Entomology, KRC College of Horticulture Arabhavi (Plate 8).

3.3.2 Isolates of *Metarhizium anisopliae*

After isolating the different strains from field collected soil samples and infected insects, these were used for the screening purpose to identify the best one.

3.3.3 Mortality study of *Metarhizium anisopliae* isolates against *Spodopetra litura*

Two ml of spore suspension with different formulations (3×10^3, 3×10^4, 3×10^5, 3×10^6, 3×10^7 and 3×10^8 CFU/1000ml) were prepared. Leaf discs of 5cm diameter were dipped in spore suspension for two minutes and air dried. Ten 2nd instar larvae of *S. litura* were released in a petri dish (10cm diameter) and allowed to feed on the treated leaf disc. Larvae were maintained at room temperature 27.0 ± 1.0 ºC and the relative humidity of 70.0 ± 5.0% RH. Another group, 10 larvae were allowed to feed the leaf disc treated with distilled water and reared under the above mentioned conditions, served as control. The isolates which showed pathogenicity (mortality) were further investigated for their effect on growth and developmental parameters of *S. litura*. A chronic feeding experiment was conducted wherein freshly treated castor leaves were fed for two days to the second instar larvae of *S. litura* followed by untreated leaves until the initiation of pupation. The observations were recorded as pupation (%), adult emergence (%) and fecundity (%) (Plate 9).

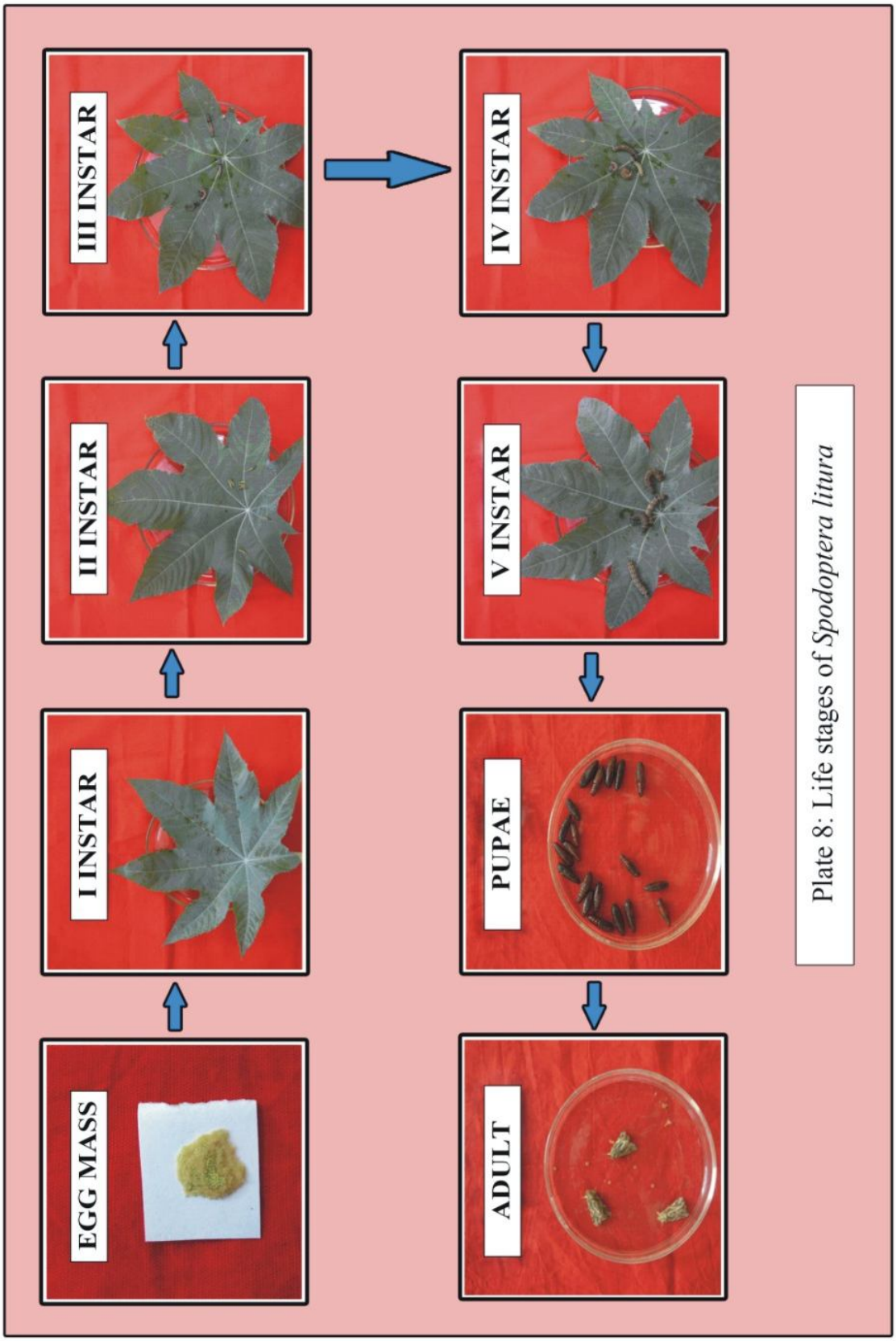


Plate 8: Life stages of *Spodoptera litura*

a. Prepare the required concentration of spore solution using Heamocytometer

b. Castor leaf disc treated with *Metarhizium anisopliae* spore suspension.

c. Release ten 2nd instar larvae of *S. litura* into petri dish and allow them to feed the treated leaf

d. Cover the petri dish with muslin cloth and keep in a stand (free from ant damage)

Plate 9: Bioassay study of *Metarhizium anisopliae* isolates against *Spodoptera litura*

Formulae

Pupation (%) = (No. of pupae/Total no. of larvae) ×100

Adult emergence (%) = (No. of adults emerged from pupae/ Total no. of pupae) ×100

Fecundity (%) = (No. of eggs laid from the emerged adults/Total no. of adults) ×100

(Bhoopathi *et al.*, 2013)

Statistical Analysis

The data of the experiment on mortality and sub lethal effects were analyzed by ANOVA, Completely Randomized Design (CRD) and means were separated by DMRT (p=0.01).

4. EXPERIMENTAL RESULTS

The results obtained during the present investigations on survey for collection of soil and insect samples for isolation of EPF, screening for virulent strains conducted at Biological Control laboratory, Department of Entomology, Kittur Rani Channamma College of Horticulture, Arabhavi during 2015-16 are presented hereunder.

4.1 Collection and isolation of *Metarhizium anisopliae*

A roving survey was conducted in Belagavi district of Karnataka state covering a total of 30 places for collection of soil and insect samples from August 2015 to February 2016. Thirty soil samples and thirty insect samples were collected from six taluks and five villages in each taluk. The soil samples were serially diluted in sterile water, whereas insects were surface sterilized and plated on selective media for growth of fungi. Eighteen isolates (60%) of 30 soil samples and six isolates (20%) of 30 insect samples were identified as entomopathogenic fungi based on their morphological and colony characteristics *viz.,* green conidia with white margin with diameter of 3.2 cm at room temperature and later were confirmed by microscopic examination with the help of mycologist of our college (Table 3 and 4).

4.1.1 Selection of entomopathogenic isolates through pathogenicity test against *Spodoptera litura*

The isolated fungi were screened for their pathogenicity against tobacco caterpillar, *S. litura*. Out of 24 isolates collected from the soil and insect samples, only three isolates (12.5%) from the soil samples yielded positive results for their infectivity against *S. litura*. However, none of the isolates obtained from the insect samples yielded positive results of pathogenicity.

The infective *M. anisopliae* isolates were obtained from the soil samples of Devagiri and Bastavad villages of Belagavi taluk and Benakatti village of Savadatti taluk which were coded as SBvD, SBvB and SSB, respectively. The isolate SBvD was found to be cause mortality of *S. litura* at two days after infection. While, the rest of two isolates *viz.,* SBvB and SSB were brought the mortality of *S. litura* after three days of infection (Table 5). However, the growth of mycelia on cadaver was very poor in all the

Table 3: The occurrence of *Metarhizium anisopliae* isolates in soil samples and their pathogenicity against *Spodoptera litura*

Sl. No.	Taluks	Name of the villages	Isolates grown on the selective medium	Isolates shown positive results for infectivity
1	Gokak	i. Arabhavi	✓	-
		ii. Kallolli	-	-
		iii. Pamaladhinne	✓	-
		iv. Ghataprabha	✓	-
		v. Duradundi	✓	-
2	Chikodi	i. Examba	-	-
		ii. Nagral	✓	-
		iii. Hirekodi	✓	-
		iv. Belakood	✓	-
		v. Donvaad	-	-
3	Hukeri	i. Bellada Bagewadi	✓	-
		ii. Ammanagi	✓	-
		iii. Borgall	-	-
		iv. Ammanabhavi	✓	-
		v. Handigood	-	-
4	Belagavi	i. Devagiri	✓	+
		ii.Kanagav	-	-
		iii. Hudali	✓	-
		iv. Honaga	-	-
		v. Bastwad	✓	+
5	Saundatti	i. Yaragatti	-	-
		ii. Munavalli	✓	-
		iii. Shindogi	-	-
		iv. Yaraganavi	-	-
		v. Benakatti	✓	+
6	Bailhongal	i. Nagnur	-	-
		ii. Sanikoppa	✓	-
		iii.Sampgavi	✓	-
		iv. Belavadi	-	-
		v.Baagewadi	✓	-

✓ **:** Isolates grown on selective media

\- **:** No growth on selective media and no infectivity

\- \+ **:** Infective against *Spodoptera litura*

Table 4: The occurrence of *Metarhizium anisopliae* isolates in insect samples and their pathogenecity against *Spodoptera litura*

Sl. No.	Taluk	Name of the villages	Specimens	Isolates grown on selective medium	Isolates shown positive results for infectivity
1	Gokak	i. Arabahvi	Diamond back moth	-	-
		ii. Kallolli	Brinjal shoot and fruit borer	✓	-
		iii. Pamaladhinne	Castor hairy caterpillar, Diamond back moth	-	-
		iv. Ghataprabha	Cabbage looper	✓	-
		v. Duradundi	Raddish caterpillar, chilli fruit borer	-	-
2	Chikodi	i. Examba	Bhendi fruit borer	✓	-
		ii. Nagral	Ridgegourd fruitfly	-	-
		iii. Hirekodi	Dolichus bean caterpillar	-	-
		iv. Belakood	Cabbage semilooper	-	-
		v. Donvaad	-	-	-
3	Hukeri	i. Bellada Bagewadi	Brinjal shoot and fruit borer	-	-
		ii. Ammanagi	Dolichus bean caterpillar	-	-
		iii. Borgall	Cow pea pod borer	-	-
		iv. Ammanabhavi	Cucumber and ridge gourd fruit fly	-	-
		v. Handigood	Diamond back moth	✓	-

4	Belagavi	i. Devagiri	Chilli fruit borer	-	-
		ii.Kanagav	Brinjal Ash weevil	✓	-
		iii. Hudali	Ridgegourd fruitfly	-	-
		iv. Honaga	-	-	-
		v. Bastwad	Brinjal shoot and fruit borer	-	-
5	Saundatti	i. Yaragatti	Tomato leaf miner	✓	-
		ii. Munavalli	Castor hairy caterpillar	-	-
		iii. Shindogi	Diamond back moth	-	-
		iv. Yaraganavi	-	-	-
		v. Benakatti	Cucumber fruit fly	-	-
6	Bailhongal	i. Nagnur	-	-	-
		ii. Sanikoppa	-	-	-
		iii.Sampgavi	-	-	-
		iv. Belavadi	-	-	-
		v.Baagewadi	Cow pea pod borer	-	-

Table 5: Pathogenicity test of *Metarhizium anisopliae* against *Spodoptera litura* under laboratory conditions

Sl. No.	Isolates	Infectivity	Time taken to kill the test insect (days)
1.	SGD	-Ve	-
2.	SGP	-Ve	-
3.	SGA	-Ve	-
4.	SGG	-Ve	-
5.	SCN	-Ve	-
6.	SCH	-Ve	-
7.	SCB	-Ve	-
8.	SHAm	-Ve	-
9.	SHB	-Ve	-
10.	SHAb	-Ve	-
11.	**SBvD**	**+Ve**	**2**
12.	SBvH	-Ve	-
13.	**SBvB**	**+Ve**	**3**
14.	SSM	-Ve	-
15.	**SSB**	**+Ve**	**3**
16.	SBhSp	-Ve	-
17.	SBhSm	-Ve	-
18.	SBhB	-Ve	-
19.	GKBSFB	-Ve	-
20.	GGCL	-Ve	-
21.	CEBFB	-Ve	-
22.	HHDBM	-Ve	-
23.	BKBAW	-Ve	-
24.	SYTLM	-Ve	-

three isolates. Therefore, the cadavers were placed on agar medium to observe for the growth of *M. anisopliae*. Later, all the isolates of *M. anisopliae* were got identified from the National Fungal Culture Collection of India, Mycology and Plant Pathology Group, Agharkar Research Institute, GG Agharkar Road, Pune, India.

4.2 Standardization of protocol for isolation and selective medium for isolation of *Metarhizium anisopliae* from soil and insect samples

4.2.1 Standardization and selection of appropriate protocol for isolation of *Metarhizium anisopliae*

Different methods were employed with appropriate modification for the isolation of *M. anisopliae* from the soil and insect samples of different locations of Belagavi district. The results of the different techniques are presented here under.

4.2.1.1 Soil washing technique

The results of the experiment revealed that the soil washing technique did not have of any fruitful growth of *M. anisopliae* colonies in the potato dextrose agar (PDA) medium. However, other plant pathogenic fungal colonies were grown in the plates. Hence, the next technique was approached rejecting the present one (Plate 10).

4.2.1.2 Soil direct plate method

The direct plate method followed for isolation of *M. anisopliae* resulted in overlapping growth of fungal colonies and was difficult to isolate the pure culture colonies required for culturing. Therefore, this method was also discarded (Plate 11).

4.2.1.3 Serial dilution method

The result of serial dilution method was found to be positive for the growth of *M. anisopliae*. Out of nine dilutions (10^{-9}, 10^{-8}, 10^{-7} and 10^{-6}) tried, the dilution of 10^{-9} was found to be positive for the growth and identification of pure, individual and non over lapping of *M. anisopliae* colonies in the plate. Therefore, this method was better

for isolation of *M. anisopliae* from soil samples compared to the soil wash technique and soil direct plate method (Plate 12).

4.2.1.4 Isolation of *M. anisopliae* from insects by direct plating

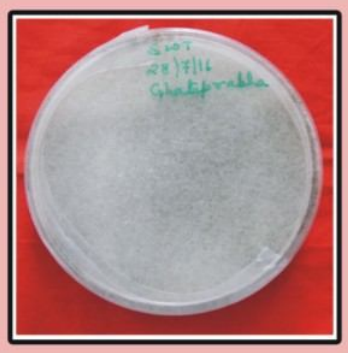
The insect samples were directly placed on the selective media for the growth of *M. anisopliae*. The result of the experiment was found to be positive for the growth of many fungal colonies including *M. anisopliae* with overlapping growth. It was difficult to differentiate the individual colony of *M. anisopliae* for subsequent culturing to obtaine the pure culture of *M. anisopliae* (Plate 13).

4.2.1.5 Isolation of *M. anisopliae* from the insect samples with surface sterilization

The insects were surface sterilized with one per cent sodium hypochlorite followed by 70 per cent alcohol to remove external micro-biota from the insect body surface and to reduce the overlapping growth of unwanted micro-flora in the plate. The results of this method showed the growth of few *M. anisopliae* colonies in the media. This EPF was within the body of surface sterilized insect and that could ease the isolation of *M. anisopliae* for further sub- culturing to obtain pure *M. anisopliae* (Plate 14).

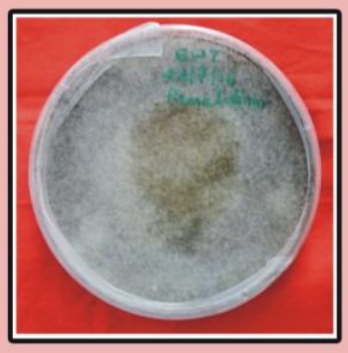
4.2.2 Standardization of selective medium for *M. anisopliae*

Five selective media *viz.,* D0C2-10, D0C2-4, D0C2-50%, D0C2-PDA and SMA media with appropriate modification (Shimazu and Sato, 1996) and Veens semi selective medium (Veen and Ferron, 1996) were evaluated in order to determine the optimal selective medium for isolation of EPF, *M. anisopliae*. Following the results of Shin *et al.* (2010), the two media *viz.,* D0C2-PDA and SMA media were selected and used for the isolation of *M. anisopliae* from soil and insect samples. The results showed that all the isolates of *M. anisopliae* were found to be grown on above said media. Among the five media, SMA and D0C2-PDA yielded more number of colonies compared to D0C2-10 and D0C2-4 which were yielded moderate number of colonies. The medium D0C2-50% showed very poor colonies of *M. anisopliae* (Table 6; Plate 15). Among the D0C2- PDA and SMA media, the SMA showed better sporulation of *M. anisopliae* (Table 7). The sporulation was quantified by Neubauer Haemocytometer (Silver counting, Rohan instruments Pvt. Ltd., Satpur, Nasik, India).



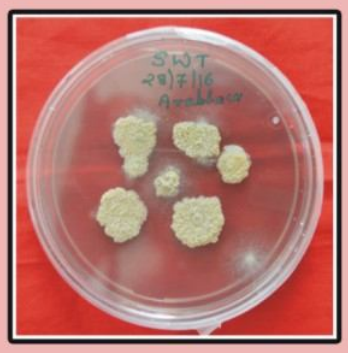
SGG Isolate

SGK Isolate



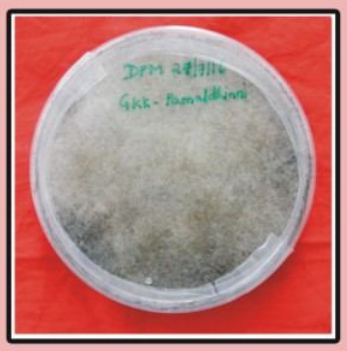
SGP Isolate

SGD Isolate

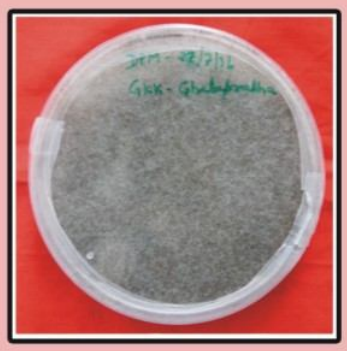


SGA Isolate

Plate 10: Growth of fungi on PDA medium by following soil washing technique

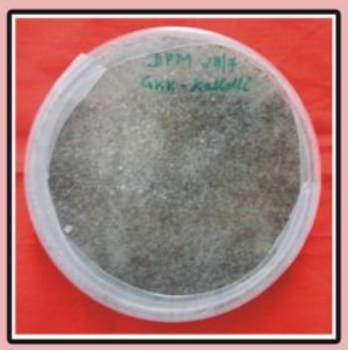


SGP Isolate

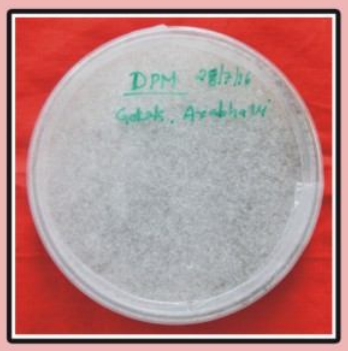


SGG Isolate

SGD Isolate

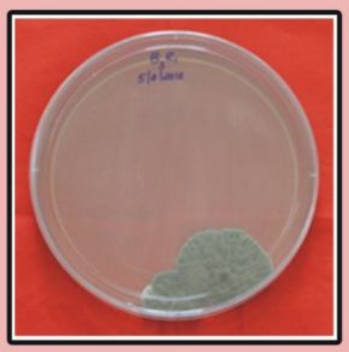


SGK Isolate

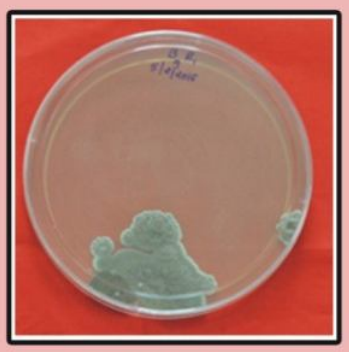


SGA Isolate

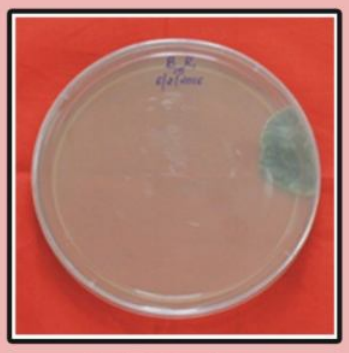
Plate 11: Growth of fungi on PDA medium by following direct plate method



SGA Isolate

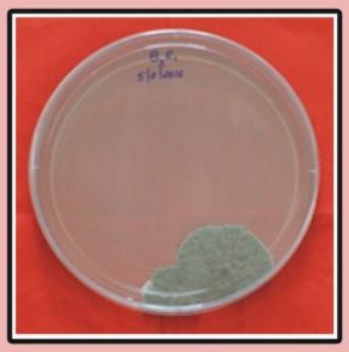


SGP Isolate



SGG Isolate

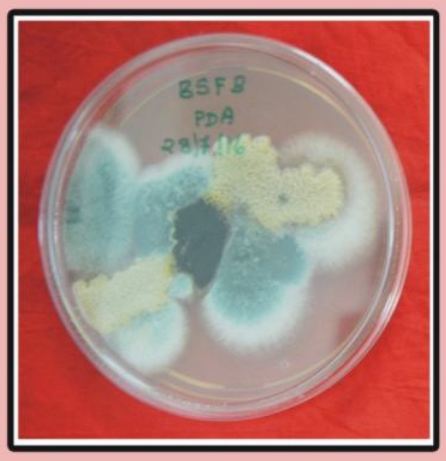
SGD Isolate



SGN Isolate

Plate 12: The growth of fungi on PDA medium by following serial dilution method

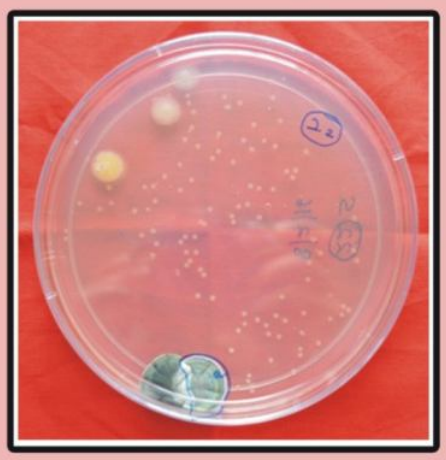
GGCL Isolate
(Cabbage looper)



GKBSFB Isolate
(Brinjal fruit and shoot borer)

Plate 13: Fungal growth on PDA medium following direct plating of insect method

Pure colonies of
GGCL Isolate



Pure colonies of
GKBSFB Isolate

Plate 14: Fungal growth on PDA medium by plating of surface sterilized insect

Table 6: Influence of selective media on growth of different isolates of *Metarhizium anisopliae*

Isolates	Selective media				
	D0C2-50%	**D0C2-10**	**D0C2-4**	**D0C2-PDA**	**SMA Medium**
SSB	+	++	++	+++	+++
SBvD	+	++	++	+++	+++
SBvB	+	++	++	+++	+++

(+): Least growth of colonies; (++): Moderate growth of colonies; (+++): Good growth of colonies

Table 7: Influence of selective media on Colony forming Unit (CFU) of Different isolates of *Metarhizium anisopliae*

Isolates	Selective media (CFU/ml)				
	D0C2-50%	**D0C2-10**	**D0C2-4**	**D0C2-PDA**	**SMA Medium**
SSB	1.2×10^{2}	2.3×10^{3}	3.2×10^{3}	4.13×10^{5}	5.12×10^{5}
SBvD	1.0×10	2.0×10^{2}	2×10^{3}	3.5×10^{4}	4.03×10^{5}
SBvB	1.1×10^{2}	2.2×10^{2}	3×10^{3}	4.8×10^{2}	5.06×10^{3}

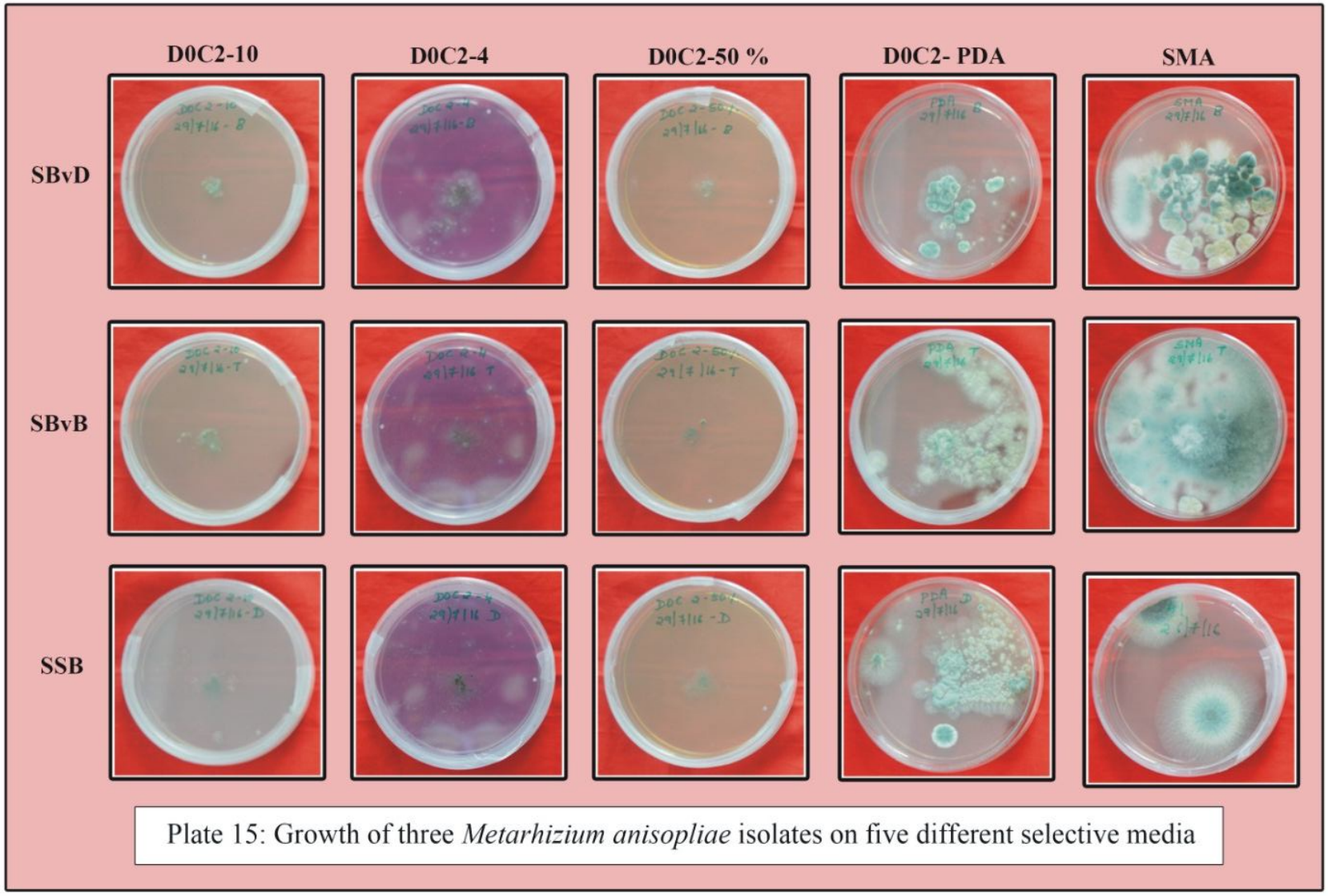


Plate 15: Growth of three *Metarhizium anisopliae* isolates on five different selective media

4.3 Screening the isolates of *Metarhizium anisopliae* against *Spodoptera litura*

Three isolates of *M. anisopliae* obtained by soil samples (SSB, SBvB and SBvD) were screened for their bio-efficacy against laboratory reared, *S. litura* following leaf dip method. The results of the experiment were found to be varied with different concentrations at different days after treatment.

4.3.1 SSB isolate

The pathogenicity of the isolate SSB revealed that the mortality varied with different concentrations at different days of treatment. The mortality of *S. litura* was increased significantly from three to six days after treatment. The significantly highest mortality of *S. litura* was recorded at the concentration of 3×10^8 cfu per1000 ml which caused 56.67 and 76.67 per cent at three and six days after treatment, respectively. While, it was lowest in the concentration of 3×10^3 cfu per 1000 ml and recorded 10.0 and 16.67 per cent at three and six days after treatment, respectively. No significant difference was observed with respect to mortality between the concentrations of 3×10^6, 3×10^5 and 3×10^4 cfu per 1000 ml at both three and six days after treatment. The mortality of *S. litura* was also noticed in the control treatment at six days after treatment (3.33 per cent). However, it was significantly lowest compared to its lower concentration (3×10^3 cfu per 1000 ml) of *M. anisopliae* (Table 8; Plate 16).

The isolates of *M. anisopliae*, which showed the varied per cent mortality at different days after treatment, were further investigated for their effect on growth and development of *S. litura* under laboratory conditions. The observations on the per cent pupation revealed that, all the concentrations of SSB isolate caused a reduction in per cent pupation compared to control. The lowest (23.33 %) pupation was recorded in the concentration of 3×10^8 cfu per 1000 ml and it was highest at 3×10^3 cfu per 1000 ml recorded 83.33 per cent. The control treatment recorded the highest pupation (96.67 %) compared to all other concentrations of SSB isolate.

The observations related to adult emergence of *S. litura* revealed that all the concentrations of SSB isolate caused a reduction in adult emergence in comparison to control (73.33 %). The lowest (6.67 %) adult emergence was noticed in the

concentration of 3×10^8 cfu per 1000 ml and it was highest (60.00%) at 3×10^3 and followed by 3×10^4, 3×10^5, 3×10^6 and 3×10^7cfu per 1000 ml which were recorded 60.00 and 46.67, 43.33, 33.33 and 16.67 per cent, respectively. The control treatment recorded the highest (73.33) adult emergence and it was *on par* with its lower concentration of 3×10^3 cfu per 1000 ml.

No fecundity was observed in the adults of *S. litura* emergence from the treatment (T_1), 3×10^8 cfu per 1000 ml. However, the fecundity in the adults emerged from lowest concentration was higher compared to adults emerged from other treatments. The control treatment recorded significantly highest (91.00 %) fecundity compared to all the concentrations of *M. anisopliae*. No significant difference was observed with respect to fecundity between the concentrations 3×10^4 and 3×10^5 cfu per 1000 ml and between 3×10^5 and 3×10^6 cfu per 1000 ml (Table 9).

4.3.2 SBvB isolate

The similar results were obtained by the SBvB isolate against the mortality of *S. litura*. Significantly highest (56.67 %) mortality of *S. litura* was recorded in the concentration of 3×10^8 cfu per 1000 ml compared to all lower concentrations at three days after treatment while no significant difference with the concentration of 3×10^7 cfu per 1000 ml at six days after treatment. Least mortality of 3.33 and 13.33 per cent was recorded at the concentration of 3×10^3 cfu per 1000 ml at three and six days after treatment, respectively. No significant difference was observed with respect to mortality between 3×10^6, 3×10^5 and 3×10^4 cfu per 1000 ml concentrations both at three and six days after treatment. The mortality of *S. litura* in the control treatment was same (3.33 per cent) as that of experiment conducted for SSB isolate and it was also significantly lowest compared to all the concentrations of *M. anisopliae* (Table 10).

All the concentrations of SBvB isolate caused a reduction in per cent pupation compared to control. However, lower concentrations (3×10^3, 3×10^4, 3×10^5 cfu per 1000 ml) of *M. anisopliae* were *on par* with the control treatment and recorded 86.67, 76.67 and 76.67 per cent, respectively. The lowest (36.67 %) pupation was recorded in the highest concentration of 3×10^8 cfu per 1000 ml. Similarly, adult emergence of *S. litura* revealed that all the concentrations of SSB isolate caused a reduction in per cent adult emergence in comparison to control. The lowest (3.33 %) adult emergence was noticed

in the concentration of 3×10^8 cfu per 1000 ml. The control treatment recorded the highest adult emergence (73.33 %) and it is *on par* with the concentrations of 3×10^3, 3×10^4, $3\times10^5$and 3×10^6 cfu per 1000 ml.

The fecundity of *S. litura* was absent in the concentration of 3×10^8 cfu per 1000 ml, while it was maximum (73.33 %) at lowest concentration of *M. anisopliae* (3×10^3 cfu per 1000 ml). The control treatment recorded significantly highest (87.67 %) fecundity compared to all the concentrations. No significant difference was observed with respect to fecundity between the concentrations 3×10^3 and 3×10^4 cfu per 1000 ml and between 3×10^4, 3×10^5 and 3×10^6 cfu per 1000 ml. Similarly there is no significant difference between the concentrations 3×10^5, 3×10^6 and 3×10^7 (Table 11).

4.3.3 SBvD isolate

Similarly, the bio efficacy of the isolate SBvD was done against *S. litura* and mortality was found to be varied across different concentrations at three and six days of treatment was recorded. The mortality of *S. litura* was increased from three to six days after treatment. The highest mortality of 43.33 and 50.00 per cent was recorded at the concentration of 3×10^8 cfu per 1000 ml at three and six days after treatment, respectively and it was *on par* with the next lower concentration of 3×10^7 cfu per 1000 ml at both days after treatment. No significant difference was observed with respect to mortality of *S. litura* in other lower concentrations of 3×10^6, 3×10^5 and 3×10^4 cfu per 1000 ml both at three and six days after treatment (Table 12).

The observations on the per cent pupation varied across the different treatment. The lowest (50.00 %) pupation was recorded in the concentration of 3×10^8 cfu per 1000 ml and it was highest at the concentrations of 3×10^3 followed by 3×10^4, 3×10^5, 3×10^6 and 3×10^7cfu per 1000 ml recorded 93.33 and 86.67, 83.33, 73.33 and 60.00 per cent, respectively. The control treatment recorded the highest pupation (96.67 %) and it is *on par* with lower concentration of 3×10^3 cfu per 1000 ml.

The observations on the per cent adult emergence of *S. litura* revealed that all the concentrations of SSB isolate caused a reduction in per cent adult emergence in comparison to control. The lowest (23.33 %) adult emergence was noticed in the concentration of 3×10^8 cfu per 1000 ml. The control treatment recorded the highest

adult emergence (76.67 %) and it is on par with the concentration 3×10^3 cfu per 1000 ml.

The fecundity of *S. litura* was lowest (23.00 %) in the concentration of 3×10^8 cfu per 1000 ml while it was maximum (87.33 %) at lowest concentration of *M. anisopliae* (3×10^3 cfu per 1000 ml). The highest per cent fecundity (88.67) was recorded in control and it was on par with the concentrations 3×10^3, 3×10^4, 3×10^5 and 3×10^6 (Table 13).

Table 8: Bio-efficacy of SSB isolate at different concentrations against *Spodoptera litura*

Treatments	Concentrations (cfu/1000ml)	Mortality (%)	
		3DAT	6 DAT
T_1	3×10^8	56.67 (48.93)[a]	76.67 (61.22)[a]
T_2	3×10^7	46.67 (43.08)[ab]	66.67 (54.78)[a]
T_3	3×10^6	30.00 (33.00)[bc]	40.00 (39.15)[b]
T_4	3×10^5	26.67 (31.00)[bc]	30.00 (33.21)[b]
T_5	3×10^4	20.00 (26.07)[cd]	26.67 (31.00)[bc]
T_6	3×10^3	10.00 (15.00)[d]	16.67 (23.86)[c]
T_7	Control (Distilled water)	0.00 (0.00)[e]	3.33 (6.14)[d]
$F_{6,12}$		*	*
S. Em±		4.22	2.76
CD		13.00	8.52
CV		14.84	7.70

Figures in parenthesis are angular transformed values/ Arc sin transformed values

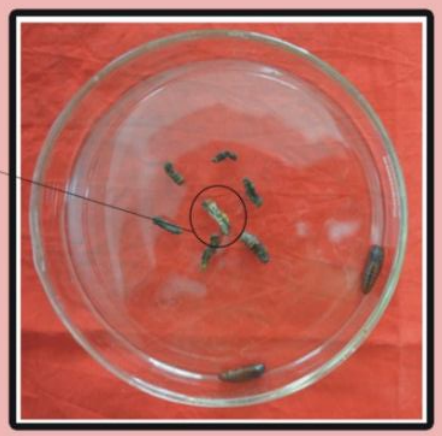
* F- test is significant at 5% probability, ** F- test is significant at 1% probability

The values in the column following same alphabet letters are not significantly different from each other

DAT- Days After Treatment

Cadaver of *Spodoptera litura* caused by *Metarhizium anisopliae*

Growth of *Metarhizium anisopliae* from cadaver when plated on agar medium



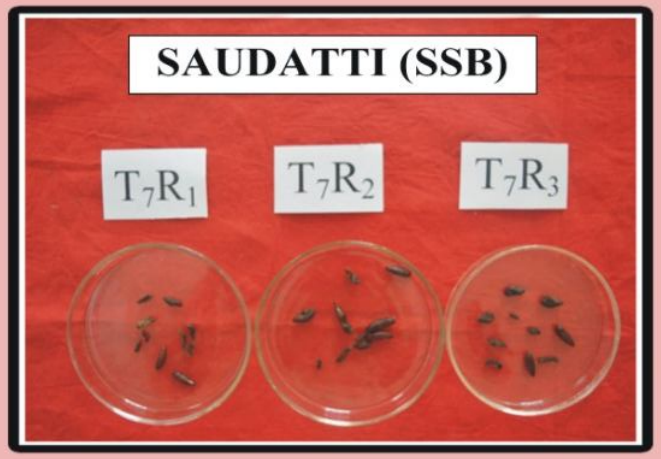


Plate 16: Mortality of *Spodoptera litura* caused by Saundatti isolate, SSB

Table 9: Effect of SSB isolate on growth and development of *Spodoptera litura*

Treatments	Concentrations (cfu/1000ml)	Pupation (%)	Adult emergence (%)	Fecundity (%)
T_1	3×10^8	23.33 (28.78)^{d}	6.67 (12.29)^{e}	0.00 (0.00)^{e}
T_2	3×10^7	33.33 (35.22)^{d}	16.67 (23.86)^{d}	16.33 (4.94)^{e}
T_3	3×10^6	60.00 (50.85)^{c}	33.33 (35.22)^{c}	44.33 (43.90)^{d}
T_4	3×10^5	70.00 (56.79)^{bc}	43.33 (41.15)^{bc}	57.67 (49.93)^{cd}
T_5	3×10^4	73.33 (59.00)^{bc}	46.67 (43.08)^{bc}	65.67 (54.68)^{c}
T_6	3×10^3	83.33 (66.14)^{b}	60.00 (50.77)^{ab}	78.33 (64.13)^{b}
T_7	Control (Distilled water)	96.67 (83.86)^{a}	73.33 (59.00)^{a}	91.00 (73.27)^{a}
$F_{6,12}$		*	*	*
S. Em±		2.76	3.13	2.32
CD		8.52	9.66	7.18
CV		5.03	8.23	5.54

Figures in parenthesis are angular transformed values/ Arc sin transformed values

* F- test is significant at 5% probability, ** F- test is significant at 1% probability

The values in the column following same alphabet letters are not significantly different from each other

Table 10: Bio-efficacy of SBvB isolate at different concentrations against *Spodoptera litura*

Treatments	Concentrations (cfu/1000ml)	Mortality (%)	
		3DAT	6 DAT
T_1	3×10^8	56.67 (48.85)[a]	63.33 (52.78)[a]
T_2	3×10^7	33.33 (35.22)[b]	46.67 (43.08)[ab]
T_3	3×10^6	20.00 (26.07)[bc]	33.33 (35.01)[bc]
T_4	3×10^5	16.67 (23.86)[c]	23.33 (28.29)[cd]
T_5	3×10^4	10.00 (18.43)[c]	23.33 (28.78)[cd]
T_6	3×10^3	3.33 (6.14)[d]	13.33 (21.14)[d]
T_7	Control (Distilled water)	0.00 (0.00)[d]	3.33 (6.14)[e]
$F_{6,12}$		*	*
S. Em±		3.06	3.80
CD		9.43	11.70
CV		13.37	12.23

Figures in parenthesis are angular transformed values/ Arc sin transformed values

* F- test is significant at 5% probability, ** F- test is significant at 1% probability

The values in the column following same alphabet letters are not significantly different from each other

DAT- Days After Treatment

Table 11: Effect of SBvB isolate on growth and development of *Spodoptera litura*

Treatments	Concentrations (cfu/1000ml)	Pupation (%)	Adult emergence (%)	Fecundity (%)
T_1	3×10^8	36.67 $(37.22)^{d}$	3.33 $(6.14)^{c}$	0.00 $(0.00)^{e}$
T_2	3×10^7	53.33 $(46.92)^{cd}$	26.67 $(31.00)^{b}$	42.33 $(40.56)^{d}$
T_3	3×10^6	66.67 $(54.99)^{bc}$	53.33 $(47.01)^{a}$	49.67 $(44.85)^{cd}$
T_4	3×10^5	76.67 $(61.72)^{abc}$	53.33 $(46.92)^{a}$	54.33 $(47.49)^{cd}$
T_5	3×10^4	76.67 $(61.22)^{abc}$	53.33 $(46.92)^{a}$	64.33 $(53.37)^{bc}$
T_6	3×10^3	86.67 $(66.14)^{ab}$	73.33 $(59.21)^{a}$	73.33 $(59.04)^{b}$
T_7	Control (Distilled water)	96.67 $(75.00)^{a}$	73.33 $(59.00)^{a}$	87.67 $(69.83)^{a}$
$F_{6,12}$		*	*	*
S. Em±		4.45	3.99	2.57
CD		13.74	12.30	7.93
CV		7.67	9.33	5.65

Figures in parenthesis are angular transformed values/ Arc sin transformed values

* F- test is significant at 5% probability, ** F- test is significant at 1% probability

The values in the column following same alphabet letters are not significantly different from each other

Table 12: Bio-efficacy of SBvD isolate at different concentration against *Spodoptera litura*

Treatments	Concentrations (cfu/1000ml)	Mortality (%)	
		3DAT	6 DAT
T_1	3×10^8	43.33 (41.07)^a	50.00 (45.00)^a
T_2	3×10^7	23.33 (28.78)^{ab}	40.00 (39.23)^{ab}
T_3	3×10^6	13.33 (21.14)^{bc}	26.67 (31.00)^{bc}
T_4	3×10^5	10.00 (15.00)^{bcd}	16.67 (23.86)^c
T_5	3×10^4	6.67 (12.29)^{cd}	13.33 (21.14)^{cd}
T_6	3×10^3	3.33 (6.14)^{cd}	6.67 (12.29)^{de}
T_7	Control (Distilled water)	0.00 (0.00)^d	3.33 (6.14)^e
$F_{6,12}$		*	*
S. Em±		4.78	3.53
CD		14.71	10.90
CV		26.60	13.71

Figures in parenthesis are angular transformed values/ Arc sin transformed values

* F- test is significant at 5% probability, ** F- test is significant at 1% probability

The values in the column following same alphabet letters are not significantly different from each other

DAT- Days After Treatment

Table 13: Effect of SBvD isolate on growth and development of *Spodoptera litura*

Treatments	Concentrations (spores/1000ml)	Pupation (%)	Adult emergence (%)	Fecundity (%)
T_1	3×10^8	50.00 $(45.00)^{e}$	23.33 $(28.78)^{e}$	23.00 $(23.98)^{c}$
T_2	3×10^7	60.00 $(50.77)^{de}$	40.00 $(39.15)^{d}$	47.33 $(43.47)^{bc}$
T_3	3×10^6	73.33 $(59.00)^{cd}$	46.67 $(43.08)^{cd}$	65.67 $(54.24)^{ab}$
T_4	3×10^5	83.33 $(66.14)^{bc}$	63.33 $(52.78)^{b}$	74.00 $(59.59)^{ab}$
T_5	3×10^4	86.67 $(68.86)^{bc}$	60.00 $(50.77)^{bc}$	70.33 $(57.37)^{ab}$
T_6	3×10^3	93.33 $(77.71)^{ab}$	66.67 $(54.78)^{ab}$	87.33 $(71.27)^{a}$
T_7	Control (Distilled water)	96.67 $(83.86)^{a}$	76.67 $(61.22)^{a}$	88.67 $(70.70)^{a}$
$F_{6,12}$		*	*	*
S. Em±		3.53	2.32	6.06
CD		10.89	7.17	18.68
CV		5.42	4.88	11.04

Figures in parenthesis are angular transformed values/ Arc sin transformed values

* F- test is significant at 5% probability, ** F- test is significant at 1% probability

The values in the column following same alphabet letters are not significantly different from each other

5. DISCUSSION

Among various other alternatives to the use of chemical insecticides is the use of entomopathogens like fungi. These possesses greater potential under selective environmental conditions. Though these pathogens have narrow spectrum of activity than chemicals, they are very much safe to the environment. Epizootics due to fungi occur naturally and there is no need for ingestion, mere contact with insect body is sufficient for entry and serves as natural regulators of pest populations (Tanada and Fuxa, 1987). Further, they are associated with insects in diverse habitats and thus they have wider scope in biological control programmes in different cropping ecosystem. Therefore, entomopathogenic fungi are emerging as potential bio-agents. Currently there is considerable interest in identifying strategies for biological control that may enable a progressive reduction in the use of chemical agents with a heavy environmental impact. One among the entomopathogenic fungi is *M. anisopliae*. It is considered as an important biological control agent against the insect pests of Coleoptera, Lepidoptera and Orthoptera and Isopteran orders (Stephan *et al.*, 1996).

However, these fungi are region specific which exhibit high level of variation among the isolates of a particular region with respect to pathogenicity, virulence, viability and environmental conditions. Hence, a study was undertaken to isolate the virulent strains *M. anisopliae* and standardization of isolation method and selective media for *M. anisopliae*. The results of the present findings are discussed and correlated with available literature in this chapter under the respective objectives.

5.1 Collection and isolation of *Metarhizium anisopliae*

Among the 60 samples collected (includes 30 soil and insect samples each) during the survey only 24 isolates of *M. anisopliae* were obtained based on their morphological study. Out of these, 18 were isolated from soil and six were from insect samples contributing 40 per cent occurrence of *M. anisopliae* collected samples. Further, pathogenicity study of all the isolates of *M. anisopliae* revealed that only three isolates (SSB, SBvB and SBvD) were caused the mortality of test insect, *S. litura* under laboratory conditions. Entomopathogenic fungi as well as in general fungi were required high relative humidity and moisture content in the environment for their

multiplication. Since, *M. anisopliae* infesting to the soil inhabiting insect pests and therefore the occurrence of *M. anisopliae* was more in the soil samples as compared to insect samples collected from vegetative part of the plant.

The present study was in agreement with the other workers reported across the world. Sahayaraj and Borgio (2006) reported about 50 per cent occurrence of *M. anisopliae* in total collected soil samples from agricultural and horticultural fields. In another study, Derakhshan (2008) collected a total of 150 soil samples from crop fields of Iran and reported that 21 per cent of samples has *M. anisopliae* isolates. Similarly, Sookar *et al*. (2008) collected about 224 soil samples from vegetable cultivation, sugarcane plantation and natural site and obtained about 18.8 per cent isolates of *M. anisopliae*. Further, they also reported that the occurrence of *M. anisopliae* was more frequent from soils under vegetables as compared to soils under sugarcane or habitat with natural vegetation. Popowska-Nowak *et al.* (2016) reported the occurrence of five entomopathogenic fungi among them *M. anisopliae* was the most dominant species. Similarly, about 55.5 per cent occurrence of entomopathogenic fungi were reported by Sun and Liu (2008) of which *M. anisopliae, P. farinosus,* and *B. bassiana* were identified as predominant one.

5.2 Standardization of protocol for the isolation and selective medium for *Metarhizium anisopliae*

5.2.1 Standardization of protocol for the isolation of *Metarhizium anisopliae*

Five different methods were evaluated for the isolation of *M. anisopliae viz.*, soil wash technique, direct plating of soil, serial dilution method, direct plating of insects and plating of insects with surface sterilized. Among the different methods, serial dilution and plating of insect with surface sterilize methods yielded positive results for isolation of *M. anisopliae.* Whereas soil washing technique, soil direct plate method and direct plating of insects did not yield any positive results. In soil wash technique, since the washing of 20 g soil with two liters of tap and distilled water respectively removed maximum spore load which sometimes remained with no spores and hence no colonies were obtained in the plate. Even though, the number of colonies was more in both direct plating of soil and insect methods, however the non availability of pure *M. anisopliae*

isolate in these methods were very poor. It was mainly due to heavy spore load of both entomopathogenic and non entomopathogenic fungi and because of overlapping of colonies it was difficult to distinguish the *M. anisopliae* isolate with other pathogenic fungi grown in the plate. Hence, these two methods were not that accurate in getting pure *M. anisopliae* isolate from direct plating of soil and insect.

A total of 24 isolates were obtained out of which 18 were through the serial dilution method and six were from insect with surface sterilization method. In serial dilution method, the dilution of soil samples reduced the spore load and could get few colonies in a plate which was easy for clear distinguishing of EPF colonies with other pathogenic fungi. These were further cultured for getting pure isolate of *M. anisopliae*. The direct plating of surface sterilized insect method was also good in isolating the EPF. This method helped in removal of spore load adhering to the surface of the insects including EPF and other plant pathogenic fungi. However, the growth of colonies of only *M. anisopliae* was possible through the further development of existing mycelium present inside the body cavity of the insect. The similar studies were undertaken by earlier researchers for isolating the EPF from soil and insects.

The literature on isolation protocol for *M. anisopliae* was meager. However, the available work so far showed that serial dilution plate method and insect surface sterilization methods were better in isolating pure and individual colonies of *M. anisopliae* compared to all other methods reported. Azaz (2003) reported soil dilution plate method was better compared to soil wash techniques and isolated 62 and seven entomopathogenic fungi respectively from 109 soil samples. Similarly Rani *et al.* (2015) also reported that serial dilution plate method and from insect cadaver with surface sterilization showed positive results for isolating *M. anisopliae* compared to all other methods tested.

5.2.2 Standardization of selective medium for *Metarhizium anisopliae*

The selective media *viz.,* D0C2-10, D0C2-4, D0C2-50%, D0C2-PDA and SMA were evaluated for the isolation of *M. anisopliae***.** Among the five, the media SMA and D0C2-PDA yielded more number of colonies when compared to D0C2-10 and D0C2-4 which yielded moderate number of colonies. D0C2-50% showed poor growth of *M.*

anisopliae. The findings of the present the study are in contrary with opinion of other scientists.

The findings of Shin *et al.* (2010) reported contrary to our present findings that, medium D0C2-PDA showed poor for culturing entomopathogenic fungi. While during present investigation, the medium D0C2-PDA performed better for isolation of *M. anisopliae*. Further, they also used various selective media *viz.,* D0C2-10, D0C2-4, D0C2-50% and SMA for isolation of entomopathogenic fungi with the modification of previously reported D0C2 medium (Shimazu and Sato, 1996) and Veens semi selective medium (Veen and Ferron, 1996) and found SM medium which contains of 10~100 µg/ml Chloramphenicol, 50 µg/ml Streptomycin, 32.5 g SDA (Sabouraud dextrose agar) in 500 ml distilled water was better for isolation of entomopathogenic fungi. Similarly, Samir *et al.* (2015) also found that media D0C2+CTAB and OTA+CTAB were the best for cent per cent recovery of true entomopathogenic species including *M. anisopliae* and *L. lecanii* successfully.

In another study conducted by Kamp and Bidochka (2002) reported that Potato Dextrose Agar (PDA) showed greatest conidia production for *M. anisopliae* irrespective of depth of the medium. Liu *et al.* (2012) opined that potato dextrose with 1% (w/v) peptone agar medium was the optimal culture medium for colony growth and conidial yield after three to seven days of incubation. Various authors reported PDA and SMA media with different combinations of yeast extracts, agar, fungicides and different sugars with varied pH condition of media for isolation of *M. anisopliae* across the world showed better recovery of entomopathogenic fungi, *M. anisopliae.*

5.3 Screening for pathogenicity of *Metarhizium anisopliae* isolate against *Spodoptera litura*

Generally the EPF exhibit high level of variation among the isolates of a particular region with respect to pathogenicity, virulence and viability. Therefore, the identification of region specific isolates is an utmost important. In this connection, the present study was under taken to screen the collected *M. anisopliae* isolates against *S. litura* under laboratory conditions.

During the present study a total of 24 isolates were obtained from the collected soil and insect samples of Belagavi district. All the obtained isolates were screened for their pathogenicity against laboratory reared *S. litura*. Among these, only three isolates (SSB, SBvB and SBvD) were found pathogenic to *S. litura*. Within the three, the isolate SSB was found to be most virulent and recorded highest mortality of 76.67 per cent at six days after treatment. While, the isolates SBvB (63.33%) and SBvD (50.00%) were less virulent against *S. litura* compared to SSB isolate.

The work on pathogenicity, virulence and sub lethal effects of *M. anisopliae* against the chewing insect pests was studied by earlier workers. Ravindran *et al.* (2015) reported that the isolates, Tk-4 and Md-1 were highly virulent to termite and caused mortality of 86.6±1.7 and 83.3±1.3 per cent, respectively on 4^{th} day of post inoculation. Ansari *et al.* (2004) reported the *M. anisopliae* isolate; CLO 53 and CLO 54 caused the highest mortality of 90 per cent after 10 weeks post inoculation when compared to other isolates collected by them. The ICIPE 78 and MA/GPK isolates of *M. anisopliae* were most virulent and showed highest (77.9% - 82.6%, respectively) mortality compared to others (Wekesa *et al*., 2005). Virulence of *M. anisopliae* isolate (FT83) against the second instar larva of beet army worm was temperature dependent and increased from 20-30^{o} C but decreased at 35^{o} C (Han *et al.,* 2014). The mortality of second instar nymph of brinjal white fly was more compared to fourth instar nymphs by the isolate (GJ4) of *M. anisopliae* (Norhelina *et al.,* 2013).

The effect of these isolates (SSB, SSvB, SSvD) on normal growth and development of *S. litura* was varied with respect to pupation, adult emergence and fecundity. In all the isolates of *M. anisopliae,* it was observed that per cent pupation, adult emergence and fecundity were very poor as compared to control. The order of sub lethal effect of these isolates were SSB>SSvB>SSvD. The isolate SBvD recorded the highest per cent pupation, adult emergence and fecundity indicating the least virulent against *S. litura*. The present findings were in line with the report of Gutierrez *et al*. (2000) that Ma2, Ma8 and Ma16 isolates of *M. anisopliae* were most virulent at the concentration of 10^{8} CFU/ml and showed the reduced adult emergence of Mexican fruit fly in the field-cage condition. Similarly, Boopathi *et al*. (2013) reported that *M. anisopliae* recorded more ovicidal effect (37.3%) at $2x10^{9}$conidia/ml against exotic spiraling whitefly.

6. SUMMARY AND CONCLUSION

The results of present investigations on collection, isolation, standardization of protocol and selective media for *M. anisopliae* and screening for their pathogenicity and sub lethal effect of *M. anisopliae* isolates against *S. litura* were carried out at Biological Control Laboratory, Department of Entomology, Kittur Rani Channamma College of Horticulture, Arabhavi, University of Horticultural Sciences Bagalkot. The results obtained from above said studies have been summarized and concluded hereunder.

The growing demand for reducing chemical inputs in agriculture combined with increased resistance to insecticides have provided great impetus to the development of alternative forms of insect pest management. A striking substitute for the use of chemical pesticides is use of bio-agents like entomopathogenic fungi which are naturally occurring organisms that are perceived as less damaging to the environment. Their mode of action appears little complex which makes it highly unlikely that resistance could be developed to a bio-pesticide. Past research has shown some promise of the use of fungi as a selective pesticide. Among the EPF, *M. anisopliae* proved effective against the defoliating and soil dwelling insect pests.

Roving survey was conducted during August 2015-2016 in Belagavi district of Karnataka state, covering six taluks with five villages in each taluk and collected about 60 samples of including 30 samples of soil and 30 samples of insect. A total of 24 isolates (40%) were obtained out of which 18 were from soil samples and six were from insect samples.

In another study, on standardization of protocol and selective media for isolation of *M. anisopliae* following different isolation methods *viz.,* soil wash technique, direct plating of soil, serial dilution method, direct plating of insects and plating of insects with surface sterilized were assessed. Among these methods, serial dilution and plating of insect with surface sterilized methods yielded positive results for isolating *M. anisopliae*. Whereas, soil wash technique, soil direct plate method and direct plating of insects showed negative results and were not suitable for isolation of *M. anisopliae.*

Similarly, selective media (D0C-10, D0C2-4, D0C2-50%, D0C2-PDA and SMA) were evaluated for isolation of the *M. anisopliae*. Among the different media

used for isolation, the media SMA and D0C2-PDA yielded more number of colonies when compared to D0C2-10 and D0C2-4 which were yielded moderate number of colonies. The medium D0C2-50% showed very poor colonies of *M. anisopliae*. Hence, the media D0C2-PDA and SMA were proved better for isolation of *M. anisopliae*.

The obtained isolates of *M. anisopliae* were screened for their pathogenicity against *S. litura*. Among the 24 isolates obtained, only three isolates (SSB, SBvD and SBvB) were found to be pathogenic. Out of these three, the isolate SSB was found to be the most virulent with highest mortality (76.67%) of *S. litura* at six days after treatment. While poor virulence was noticed for the isolate SBvB and SBvD with the mortality of 63.33 per cent and 50.00 per cent at six days of treatment respectively. These three isolates were further investigated for their sub lethal effect on growth and development of *S. litura* under laboratory conditions. The isolate SSB recorded lowest per cent pupation (23.33) and SBvB recorded lowest per cent adult emergence (6.67) at the concentration of 3×10^8 cfu per 1000 ml when compared to the isolate SBvD. The fecundity of *S. litura* was absent at the concentration of 3×10^8 cfu per 1000 ml in the isolates SSB and SBvB while the highest was recorded in the isolate SBvD at 3×10^3 cfu per 1000 ml.

The diversity and distribution of entomopathogenic fungi varied from one geographical area to another. They require higher relative humidity and moisture content in the soil and environment for the persistence throughout the year. Therefore, the abundance of these EPF differs from season to season, crop area to forest area *etc*. Since the *M. anisopliae* infesting soil dwelling insect pests hence, the occurrence of this was more in soil samples compared to insect samples.

Our results confirmed that serial dilution plate method and insect surface sterilization methods were better in isolating pure and individual colonies of *M. anisopliae* compared to all other methods reported. These results were also in accordance with other earlier findings. Hence, it is better to go for serial dilution plate method and insect sterilization methods for isolating pure and individual colonies of *M. anisopliae* from soil and insect samples, respectively.

Similarly, earlier workers reported that PDA and SMA media with different combinations of yeast extracts, agar, fungicides and different sugars with varied pH

condition of media for isolation of *M. anisopliae* across the world showed better recovery of EPF, *M. anisopliae.* In the present investigation, the media D0C2-PDA and SMA were proved better for isolating *M. anisopliae*. Therefore, it is better to go for isolating *M. anisopliae* without combination of above mentioned components to save the cost of isolation of *M. anisopliae*.

The reports on the effectiveness of *M. anisopliae* on various defoliating pests indicate that it is one of the best biological control agents without affecting the non-target organisms. The results of the present findings also showed that all the three isolates were able to cause disease and mortality at different intervals of time. However, these isolates of *M. anisopliae* performed better under laboratory conditions and required evaluation under field conditions against various chewing pests of horticulture crops.

Future line of work

- The study on diversity and abundance of entomopathogenic fungi, *M. anisopliae* across the different agro-ecosystems of Belagavi district needs to be undertaken.
- Development of various formulations of *M. anisopliae* needs to be carried out.
- Storability and efficacy studies of different formulations need to be undertaken.
- Farm level mass production technology for *M. ansolpliae* to be developed.
- Field level evaluation of the *M. anisopliae* in vegetable crops needs to be undertaken.
- Molecular characterization for more virulent strains of *M. ansolpliae* is required.
- Effect of *M. anisopliae* on soil inhabiting microorganisms.

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Appendix-1: Weather data of the experimental period for the year 2015-16

Month	Temperature ^{o}C				RH %	Actual		Normal (15 Yrs)		Evaporation (mm)
	Min.	Min. Nor	Max.	Max. Nor.		Rainfall (mm)	No. of rainy days	Rainfall (mm)	No. of rainy days	
August-15	19.9	19.1	30.9	28.5	88.3	33.7	04	51.5	5	7.4
September-15	18.9	18.3	31.9	29.2	89.4	53.7	03	66.9	6	4.6
October-15	18.3	14.7	34.6	30.0	89.9	53.3	04	99.0	6	3.9
November-15	14.8	16.5	30.9	29.6	87.7	3.2	01	24.3	2	4.2
December -15	14.0	13.2	32.6	29.2	91.3	0.0	00	5.4	0	3.8
January -16	10.4	9.3	31.5	29.4	88.9	0.0	00	0.3	00	3.6
February -16	14.5	10.2	35.9	30.9	91.7	0.0	00	1.1	00	4.1

COLLECTION, ISOLATION AND SCREENING OF ENTOMOPATHOGENIC FUNGI, *Metarhizium anisopliae* (Metchnikoff) Sorokin"

2016

G. SOWMYA **Dr. JAYAPPA. J**
(Major advisor)

ABSTRACT

A roving survey was conducted randomly in Belagavi district of Karnataka state for collection of soil and insect samples in vegetable fields from August 2015 to February 2016. A total of 30 soil and insect samples each were collected from six taluks covering five villages in each taluk. About eighteen isolates (60%) from soil and six isolates (20%) from insect samples were identified as entomopathogenic fungi.

Five protocols (soil washing technique, soil direct plate method, serial dilution method, direct plating of insects and plating of surface sterilized insect) were tested for isolation of *Metarhizium anisopliae* from soil and insects samples. Among them, soil serial dilution method and plating of surface sterilized insect proved to be best isolation techniques. Five selective media *viz.,* D0C2-10, D0C2-4, D0C2-50%, D0C2-PDA and SMA were used for isolation. The media SMA and D0C2-PDA yielded more number of colonies compared to other media.

All the eighteen isolates of *M. anisopliae* from soil and six isolates from insect samples were screened for their pathogenicity against the laboratory reared tobacco caterpillar, *Spodoptera litura.* The isolates SSB, SBvB and SBvD from the soil samples yielded positive results for their infectivity. Further they were screened for their bio-efficacy against the test insect following leaf dip method. The isolate SSB was found to be the most virulent with highest mortality (76.67%) of *S. litura* at the concentration of 3×10^8 cfu per 1000 ml at six days after treatment when compared to other two isolates. The sub lethal effects were also investigated wherein, the isolate SSB recorded lowest per cent pupation (23.33), SBvB recorded lowest per cent adult emergence (6.67) at the concentration of 3×10^8 cfu per 1000 ml when compared to SBvD. The isolates SSB and SBvB recorded zero per cent oviposition at the concentration of 3×10^8 cfu per 1000 ml.

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