Evaluation of biodegradable agricultural substrates for mass production of entomopathogenic fungi

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The indiscriminate use of chemical pesticides is resulting in pest resurgence, pesticide resistance and health hazards as well as negatively affecting natural beneficial insects and the crop ecosystems in recent years. Thus the biological control becomes a safer alternative to chemical pesticides. Biopesticides based on bacteria, viruses, entomopathogenic fungi and nematodes often have considerable scope as insect-pest management agents (Noris *et al*., 2002). Entomopathogenic fungi cause lethal infections, perpetuate in target insects and regulate insects and mite population in nature by epizootics (Carruthers and Soper, 1987; Ferron, 1987; McCoy *et al*., 1988). Their characteristics, namely, safety to non-target organisms, ease and economic feasibility for *in vitro* mass multiplication, make them one of the preferred options for pest management. Among the fungal biopesticides, *Beauveria bassiana*, *Metarhizium anisopliae*, *Nomuraea rileyi* and *Lecanicillium lecanii* (Zimm.) Zare and Gams have been reported to be promising under Indian conditions. Various strains and formulations of *M. anisopliae* have been shown to infect 100 foliar and other soil dwelling insect pest species belonging to different genera (Butt *et al*., 2001; Kim *et al*., 2001; Horaczek and Viernstein, 2004; Bruck *et al*., 2005; Fisher and Bruck, 2008; Jin *et al*., 2009; Ansari *et al*., 2009 and 2010).

L. lecanii (formerly *Verticillium lecanii* (Zimm.) Viegas), known as a "white-halo" fungus, belongs to the class Sordariomycetes and reproduces asexually (anamorphic stage) by conidia without fruiting bodies (Hajek, 1997; Zare and Gams, 2001). The market for biocontrol agents (BCAs) has been growing continuously over the last few decades due to improved awareness on the adverse environmental impacts of chemical pesticides. Production of adequate quantities of a good quality inoculum of any biocontrol organism is a prerequisite for receiving excellent results of a biocontrol programme. Production of entomopathogenic fungi has been successfully accomplished in many synthetic and semisynthetic media. Carbon and nitrogen are the most vital nutrients required for growth and sporulation (Campbell *et al*., 1983). Natural media, which are invariably rich in carbon and nitrogen, were proved to support the growth and sporulation of fungi. Broken sorghum grain is reported to produce lesser conidia of *L. lecanii* than cooked rice and bran per gram substrate (Feng *et al*., 2000; Lakshmi *et al*., 2001). Malt extract peptone medium yielded more conidia /cm^2 of *L. lecanii*, compared to dextrose peptone yeast extract agar medium (Kamp and Bidochka, 2002). Similarly, the biphasic state production by using molasses yeast broth and rice grains produced the spore count of 1.70g /100g (Derakhshan *et al*., 2008) and in potato dextrose broth and rice after 10 days, it ranges from 0.23 to 1.75 × 10^8 spores /ml (Nirmala *et al*., 2006). Though different methods of mass production of entomopathogenic fungi are available,

The development of cheaper method of mass production using locally available materials will be highly useful. Thus, the present study was undertaken to evaluate agriculture products like grains of rice, wheat, ragi, sorghum, pearl millet and maize alone and in combination with carbon and nitrogen sources *i.e*., 2% jaggery and 0.5% yeast, to find out economically viable substrate for the mass production of *Metarhizium anisopliae* and *L. lecanii* with good number of spores.

The present study was carried out at Center for Biological Control (CBC), National Institute of Plant Health Management (NIPHM) during 2012 and 2013. Laboratory experiments were conducted to test the effect of different agricultural products in solid and enriched solid agricultural substrates on mass production of entomopathogenic fungi (EPFs). The cadavers of the insects or the small hoppers that appeared to be infected by fungi were collected in screw cap vials (18 × 4 mm) during survey and brought to the laboratory and entomopathogens were isolated on specific media. Mycosed insects collected from the fields were surface sterilized with 5 per cent sodium hypochlorite for few seconds and then thoroughly rinsed with sterile distilled water several times. The excess water was removed by keeping the diseased insects on Whatman filter paper

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No. 1. In a sterile Petri dish, the diseased-insect specimens were cut into small pieces with the help of sterile blade and the bits were aseptically transferred to a culture plate containing selective medium [sabourand maltose agar enriched with 1% yeast extract (SMYA)] with the help of sterile inoculation needle. The inoculated Petri plates were kept in BOD at 25±1 °C under constant observation for the growth and development of microorganisms. Diseased larvae were also kept on moist filter paper in Petri dish for mycelial growth and sporulation. The diseased larvae either showed whitish mycelial surface growth as characteristic of *L. lecanii* or slight darker green colour growth on the rice plant hoppers as characteristic of *M. anisopliae*. After 5 days of incubation, the microorganisms were sub-cultured for purification. Preliminary identification of fungi was made based on the morphological character with the help of the Atlas of entomopathogenic fungi (MaCoy *et al.*,1988; Humber, 1997). The identified fungi *M. anisopliae* Ma-11 (Accession No. NFCT 5805.13) and *Lecanicillium lecanii* (Accession No. NFCT 5806.13) are available at CBC-NIPHM. All the cultures were maintained on SMYA and potato dextrose agar (PDA) slants. A loopfull of inoculum from sub cultured plates of *M. anisopliae* and *L. lecanii* was transferred to PDA slants and maintained as pure culture. For laboratory studies, both fungi were cultured on PDA medium, separately. After sporulation, conidia from the medium were harvested by washing them thoroughly with sterilized water containing Tween-80 (0.2%) for immediate use. Otherwise, spores were harvested with the help of a small sterile metal spatula. Harvested conidia were air dried under laminar air flow and stored in small air tight screw cap vials (10 cm with 2.5 cm diameter) in refrigerator at 4 °C for further studies.

Whole grains and their by-products of rice, wheat, sorghum, pearl millet, finger millet, maize, barley, black gram seed bran, rice bran were collected from the local market and used for mass multiplication and estimating the sporulation of *M. anisopliae* and *L. lecanii* at 28 °C. Each sample of 100 g grains was taken, washed well and equal amount of distilled water (w/v) (pH 7.0) was added and packed separately in individual autoclavable polypropylene bags [Hi Media 7" (B) x 11" (H) size]. The bags were plugged using non-absorbent cotton with the support of a piece of 1.5 inch PVC pipe at the opening of the bag and sterilized by autoclaving at 121 °C and 15 lbs pressure for 20 minutes (Mazumdar *et al.*, 1995). Each bag was considered as a replicate and three bags were used for each treatment. In addition to the set of plain grain media, another set of all grain media amended with 0.5% yeast and 2% Sugarcane "Jaggery " (65-85% sucrose) separately for nutrient enrichment was taken. For enrichment of these grain media, 5 g yeast and 20 g sugarcane "jaggery" were mixed in 1000 ml distilled water. Hundred gram grains of each crop were cleaned, washed well transferred separately in 7" (B) × 11" (H) autoclavable bags (Hi Media) and 100 ml of yeast and sugarcane "jaggery" solution was added to each bag separately and sterilized as mentioned earlier. Three replications were maintained for each grain medium. After, autoclaving the bags were allowed to cool down upto the lukewarm temperature and 1 ml of freshly prepared conidial suspension of *M. anisopliae* and *L. lecanii* (10-days old) was inoculated in each bags, separately under aseptic conditions. The inoculated bags were incubated in BOD incubator at 25 ± 1°C separately for 10 days. To avoid clumping, after every three-days of inoculation, the bags were shaken vigorously to separate the grain and to break the mycelial mat. Ten days after incubation, grain along with fungal biomass of each sample was transferred in sterilized porcelain trays separately and dried. The biomass was dried to constant weight in hot air oven at 60-65 °C for 12 hours. The determination of dry mycelial weight done separately by weighing the biomass along with fungal mycelium and conidia (Cochrane, 1958).

After 10 days of incubation, 1g homogenous dried grain media sample drawn from each replicate of uniformly sporulating bags was transferred to 10 ml sterilized distilled water containing Tween 80 (0.2%) solution in test tubes. The test tubes were shaken in mechanical shaker for 10 min. The suspension was filtered through double layered muslin cloth and then filtered through Whatman filter paper No.1. The filtered suspension was subjected to the serial dilution up to 12 times. A drop of conidial suspension of *M. anisopliae* and *L. lecanii* was placed on separate hemocytometer. The number of conidia of entomopathogenic fungi was counted under phase contrast microscope at higher resolution in the middle square (V) of Neubauer hemocytometer. The conidial count was determined to find out the number of conidia in 1 g of the dry biomass obtained from each grain media separately using the formula: Conidia per ml = the average count per square (1 mm^2) × the dilution factor × 10^4. Colony forming units (cfu) were estimated by plating technique. From the filtered stock solution, further dilutions were made to upto 10^{-8} dilution. The study was carried out in a randomized complete block design (RCBD) with three replications. The biomass and conidial production of both the fungi from different substrates were subjected to analysis of variance (ANOVA). The means were separated using least significant difference (LSD) and differences between treatments were considered significant at probability level P = 0.05.

The results presented in Table 1 revealed that among the grain media tested, the dry weight of *M. anisopliae* per 100 g media was significantly higher in pearl millet (47.7 g) followed by finger millet (45.5 g) and maize (44.2 g) than other grain media at 10 days after inoculation as compared to other grain media. However, there was no significant difference (P=0.05) among these three grain media in terms of dry biomass weight of *M. anisopliae*. The significantly highest dry biomass of *L. lecanii* in sorghum (62.6 g) followed by wheat and maize grain media, 60.0 and 58.6 g, respectively than others. There was no significant difference between wheat and maize in terms of dry biomass weight. Compared to the other grain media the spore production of *M. anisopliae* was significantly higher on wheat (11.6×10^{10}) and rice (11.5×10^{10}) followed by sorghum (10.4×10^{10}) than others. The spore count of *L. lecanii* was highest on sorghum and pearl millet enriched with yeast and "jaggery" media (11.4×10^{10} and 11.2×10^{10}) should not be superscript, respectively, but not significantly different from each other.

In case of enrichment of these grain media with 0.5% yeast and 2% sugarcane "jaggery" (65-85% sucrose), there was a significant boost in dry biomass production as well as spore count. In case of *M. anisopliae*, enriched pearl millet jaggery and finger millet jaggery media recorded the highest dry weight per 100 g (54.6 and 53.1, respectively) but both were at par with finger millet and pearl millet was statistically insignificant. In case of *L. lecanii*, enriched sorghum jaggery medium resulted in the highest dry biomass yield (66.6 g per 100 g). This was followed by sorghum alone, enriched maize and wheat media (62.6, 61.4 and 61.1, respectively). There was no statistically significant difference among maize, wheat, and sorghum enriched media and jaggery and sorghum alone. The spore count of *M. anisopliae* was recorded highest on enriched rice jaggery and wheat jaggery media (12.8×10^{10} and 12.7×10^{10} per gram of dry biomass, respectively). But the statistical comparison between the rice and wheat media was insignificant. However, the spore count of

Table 1. Effect of various grain media alone and or enriched with jaggery plus yeast (JY) on biomass and spore production of *Metarhizium anisopliae and Lecanicillium lecanii*

	Spore count × 10^{10}/substrate alone		Fresh weight (g)		Dry weight (g)	
Media	*M. anisopliae*	*L. lecanii*	*M. anisopliae*	*L. lecanii*	*M. anisopliae*	*L. lecanii*
Rice	11.5	10.2	81.5	82.0	34.9	44.9
Wheat	11.6	9.4	64.1	86.8	33.1	60.0
Sorghum	10.4	11.4	62.1	89.3	42.5	62.6
Pearl millet	9.8	11.2	91.5	90.6	47.7	42.0
Finger millet	10.2	10.4	89.3	88.2	45.5	41.2
Maize	9.5	10.3	94.8	93.8	44.2	58.6
Barley	10.2	10.5	75.9	85.1	27.4	41.0
Black gram seed bran	10.2	9.5	59.6	65.3	41.1	43.1
Rice bran	7.2	6.5	60.1	63.9	42.4	45.2
Rice +JY	12.8	12.7	83.3	83.6	35.5	49.7
Wheat +JY	12.7	11.2	83.5	88.5	36.2	61.1
Sorghum+ JY	11.6	12.6	63.0	91.4	45.6	66.6
Pearl millet + JY	11.1	11.8	92.8	91.0	54.6	45.8
Finger millet +JY	10.6	11.2	91.8	87.9	53.1	44.3
Maize + JY	10.7	11.4	95.7	95.6	46.8	61.4
Barley +JY	11.4	11.8	78.4	86.3	31.3	43.6
Black gram seed bran +JY	11.3	10.9	64.3	68.2	42.5	44.9
Rice bran +JY	8.4	7.8	65.8	67.9	43.9	46.7
SEm ±	0.995	1.033	3.233	3.778	2.271	2.657
CD (P = 0.05)	2.022	2.099	6.569	6.864	4.615	5.403

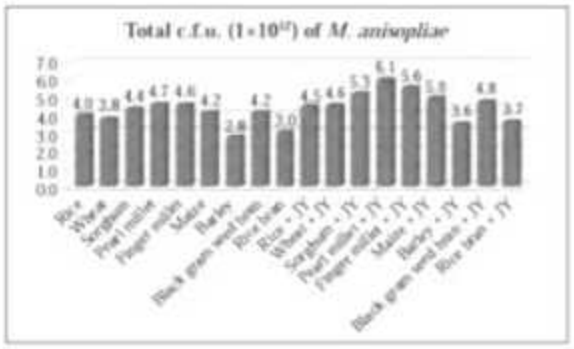

Figure 1. Total c.f.u. (1 × 1012/100 g) of *Metarhizium anisopliae* recorded on different media alone and enriched with jaggery plus yeast (JY)

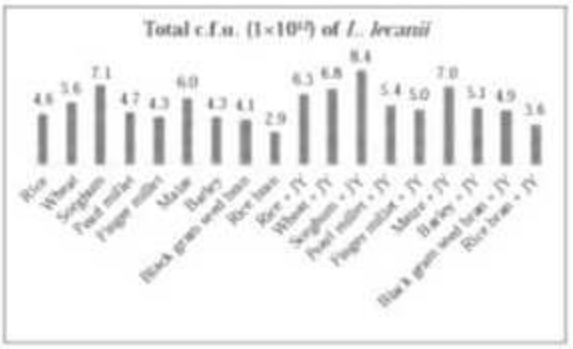

Figure 2. Total c.f.u. (1 × 10^{12}/100 g) *Lecanicillium lecanii* recorded on different media alone and enriched with jaggery plus yeast (JY)

L. lecanii was recorded highest but at par on rice and sorghum medium enriched with yeast plus jaggery (12.7 × 10^{10} and 12.6 × 10^{10}, respectively). Overall, selection of medium was done based on total spore production in terms of total c.f.u. from the harvested biomass. The highest number of c.f.u of *M. anisopliae* was recorded in pearl millet medium enriched with yeast plus jaggery (5.6 × 10^{12} per 100 g); however, the highest c.f.u. (8.4 × 10^{12}/100 g) of *L. lecanii* was recorded in sorghum medium enriched with yeast plus jaggery. Similarly, sorghum was reported to be ideal for the mass production of *L. lecanii* (11.31×10^{10} spores/100 g) while, pearl millet was also found as suitable medium for the spore production (10.17×10^{10} spores/100g) (Sahayaraj *et al.*, 2008) and the spore production can further be maximized by incorporation of molasses at 4% concentration (Derakhshan *et al.*, 2008). Broken sorghum grains are reported to produce 15 × 10^{8} conidia /g (Lakshmi *et al.*, 2001), which is in confirmation with the findings of the current study. While Feng *et al.* (2000) found cooked rice and bran substrate to produce higher spores of 1.5 and 1.4 × 10^{9} spores /g for *L. lecanii*. Grains are cheap, easily available and act as best nutritive media for the mass multiplication of many micro and macro organisms. According to Ibrahim and Low (1993) and Sharma *et al.* (2002), rice was found to be the suitable medium for the mass culture of deuteromycete fungi. The above findings are closely in agreement with Bharati *et al.* (2007), who recorded considerably low spore yields in agro wastes as compared to food grains and Sahayaraj *et al.* (2008) who have observed that sorghum recorded maximum spore production of *Paecilomyces fumosoroseus* and *L. lecanii*. Dangar *et al.* (1991) also observed similar findings in *M. anisopliae*. In Brazil, *M. anisopliae* has been found to produce conidial yields of 5-15 times higher using rice bran/rice husk substrate mixtures than yields usually obtained from rice grains, with viabilities of higher than 85% (Dorta *et al.*, 1990). Sahayaraj *et al.* (2008) found that among other grain media sorghum recorded maximum spore production of *P. fumosoroseus* and *L. lecanii*. Puzari *et al.* (1997) further reported that rice husk supplemented with 2% dextrose solution recorded more sporulation of *M. anisopliae*.

In conclusion, the tested fungi were able to grow on a wide variety of agriculture products and by products of solid state and this can be useful to farmers to culture these fungi easily. Moreover, the grain media enriched with low cost carbon and nitrogen sources could further enhance the fungal biomass production. The pearl millet medium enriched with yeast plus jaggery showed significantly high growth rate of *M. anisopliae* in terms of total c.f.u production (8.4 × 10^{12} per 100 gram) as compared to other media. However, comparatively sorghum grain media enriched with jaggery plus yeast showed significantly enhanced growth rate of *L. lecanii* in terms of total c.f.u production (8.4 × 10^{12}/100 g). For each new strain and species of entomopathogenic fungi, the optimal conditions and the source of nutrition required for large scale production may differ from those found previously and need to be tested to produce a high level of infective inoculum at minimal cost. The grain media can be a very good low cost and a suitable alternative for small scale industry and farm level mass multiplication of these beneficial microbial fungi. This method can ultimately serve as an economic way of making quality biopesticides easily available to the farmers.

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