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Growth response of *Metarhizium anisopliae* in indigenous media and efficacy to control rice black bug under greenhouse condition

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Abstract Results revealed that the highest conidial count among media incubated at room temperature was in T7 (rice bran) with 13.1 x 10^9 conidia/ml which was significantly higher than other treatments. The same result was obtained in cultures under air conditioned environment wherein T7 (rice bran) had 15.5 x 10^9 conidial/ml. On substrates incubated at room temperature, the highest conidial count was observed in sorghum seeds (12.8 x 10^9) but not significantly different from rice bran (12.4 x 10^9), ground corn (11.6 x 10^9) and chipped camote tubers (11.1 x 10^9). Under air conditioned environment, the highest conidial count was observed in sorghum seeds (14.1 x 10^9) but not significantly different from rice bran (13.5 x 10^9). The highest mortality of Rice Black Bug (RBB) under greenhouse condition was observed in rice sprayed with *M. anisopliae* cultured in rice bran (70.00%) but not significantly different from palay seeds (65.00%), corn cob (58.33%) and ground corn (56.67%).

Keywords: *Metarhizium anisopliae*, Rice Black Bug, Indigenous media and substrate

Introduction

Rice Black Bug (RBB) has become one of the most serious insect pests in the Philippines today, as it attacks almost all the stages of the rice crop, particularly from maximum tillering to ripening (Reissig *et al*., 1986; PhilRice, 2000). Simbajon (1992) said that they are destructive because the nymphs can feed at the basal part of the rice crop for up to 42 days. RBBs prefer stem nodes as feeding sites because of the large sap reservoirs (Reissig *et al*., 1986).

Moreover, feeding by large number of bugs can cause plants to be stunted (IRRI, 1983; Lim, 1975). Historically, in the Philippines, the insect was only known during the infestation in 1979 at Bataraza, Palawan Island. It was followed by major outbreak in 1982 covering 4,500 hectares of rice fields. In

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1992, RBB was observed in Zamboanga City damaging about 2,070 hectares. Moreover, in 1995, the pest invaded the whole of Region 9 including the Autonomous Region of Muslim Mindanao (ARMM) and other parts of the Philippines specifically in the Visayas Region (Cuaterno, 2011).

M. anisopliae has the ability to kill insects due to its capability to produce cyclopeptide toxins such as destruxin A, B, C, D, E and desmethyl destruxin (Widiyanti and Muyadihardja, 2004). Moreover, according to Aw and Hue (2017), destruxin A and E are more insecticidal because they are synthesized to suppress the body's immune response of insects. The toxin damages the tissues and the fungus absorbs the fluid from the larva's body thus, causing the larvae to dry up and die (Widiyanti and Muyadihardja, 2004).

The search for alternative media for the culture of microorganisms is becoming a trend nowadays. This is due mainly to the high cost of synthetic or commercial media available in the market. Moreover, there is a need to cater to the needs of the farmers where these laboratory grade media and substrates are not readily available. Indigenous media for the culture of fungal species were evaluated and were proven to be beneficial to the end users such as sugarcane bagasse for the culture of *Aspergillus niger*, *Fusarium* sp., F1, F2, F3, F5, *Candida albicans,* and *Saccharomyces cerevisiae.* (Sidana and Farooq, 2014). In addition, Omodara and Adebolu (2014) found that sweet potatoes dextrose agar (SPDA), and cassava dextrose agar (CDA) can be used as culture media for the growth of *Fusarium moniliforme, F. oxysporium, Aspergillus niger, A flavus, Penincilliun notatum, Rhizopus stolonifer, Mucor mucedo* and *Aspergillus fumigatus.*

The objectives were to determine the growth response of *M. anisopliae* in various indigenous culture media and substrates, and the efficacy of *M. anisopliae* grown using the different substrates.

Materials and methods

Locale and time

The study was conducted at the Central Luzon Integrated Agricultural Research Center for Upland Development in Magalang, Pampanga, Philippines from May to November 2021.

Culture media

The different raw materials used in the different treatments were oatmeal (control), rice wash, potato, sweet potato, corn, mungbean, rice bran, soya, white corn, cassava, peanut and white sorghum.

Thirty grams of each sample was cooked separately in 500 ml distilled water was used. All the media were added with 20 gram gulaman. Each resulting concoction was boiled to dissolve the gulaman then dispensed into flat bottles. Each bottle contained 30ml. The flat bottles were plugged with cotton and covered with aluminum foil. The bottles were sterilized through autoclaving at 15 psi for 30 minutes. The bottles were cooled in slanting position to increase the surface area of the medium. The bottles were inoculated with pure culture of *M. anisopliae* obtained from Regional Crop Protection Center (RCPC), Maligaya, Science City Of Munoz, Nueva Ecija, Philippines. One set of inoculated bottles (15) were incubated at room temperature (non-AC) while another 15 bottles were incubated under air conditioned (AC) environment. Conidial counting was done on the 14th day of incubation.

Substrates

The different substrates used in this study were ground corn, rice straw, sawdust, rice bran, soil, sorghum seeds, palay seeds, sugarcane bagasse, rice hull, corn cob, ground coconut dried meat, chipped camote tubers, tobacco midribs, dried water lily, dried tiger grass, white corn and peanut rind. The dry raw materials were soaked overnight, chopped into 1-inch length and air-dried. When the moisture content was approximately 60-70%, they were individually placed into a 8x16 inches polypropylene plastic bags. Each bag weighed 400 grams. The bags were sterilized by autoclaving at 15 psi for 30 minutes. Upon cooling, they were individually inoculated with pure culture of *M. anisopliae*. One set of bags (15) were incubated at room temperature while another 15 bags were incubated under air conditioned environment. Conidial counting was done on the 14th day of incubation.

Conidia counting

Ten grams from each sample of substrate grown with *M. anisopliae* was mixed with 10 ml distilled water and added with 1% tween 20 surfactant. The mixture was stirred using sterile stirring rod to dislodge the conidia. The solid particles were separated and the liquid portion containing the conidia was stained with 1% or 0.01 uL methylene blue prior to microscopic examination. The stain was applied to distinguish dead from live spores. After staining, two-fold dilution was done where 1 mL of the suspension was dispensed in a haemacytometer slide and observed under a microscope. Conidia were calculated using the equation (Avin, 2019):

$$conidia/ml = (n) \times 10^{2} \times 10^{6}$$

where: (n)= the average cell count; 10^2= the dilution factor made; 10^6 the six corner squares. Triplicate reading was done per treatment.

Efficacy test using rice black bug under greenhouse condition

In the greenhouse, a total of twenty seven pots grown with rice were setup representing nine treatments with growth of *M. anisopliae* and replicated three times were tested for efficacy.

Twenty adult live rice black bugs reared and obtained from the Department of Agriculture Regional Crop Protection Center were introduced into 14 days rice seedlings in pots at the vegetative stage.

After 7 days of rice black bug introduction, a total of 50 grams of substrates with conidia of *M. anisopliae* was mixed in 1 liter tap water, added with 1% tween 20 surfactant to disperse the conidia. From that mixture, approximately 50 ml was sprayed per treatment. Spraying was done early in the morning (6 a.m – 8 a.m) and late in the afternoon (4 p.m – 6 p.m), every three days for three weeks. Three replications per treatment were made. Data on rice black bug mortality were monitored and recorded after 45 days.

Experimental design and statistical analysis

All experiments were laid out in Complete Randomized Design. Statistical significance was determined using one-way Analysis of Variance at 5% level of significance. Moreover, if variation was significant, means were compared using Duncan’s Multiple Range Test (DMRT) at 5% level. A t-test at 5% level of significance was also done to compare conidial counts in two different incubation conditions

Results

Culture media

The conidial count in each culture medium transferred with *M. anisopliae* and incubated under room (non-AC) and air conditioned (AC) environment are presented in Table 1. The range at room temperature was 33.9-35.3^{o}C and the mean was 34.60^{o}C while in air conditioned environment was 23.9-33.2^{o}C with a mean of 28.55^{o}C.

Table 1. Conidial count of *M. anisopliae* in different culture media incubated at room and air conditioned environment

Treatments	Conidia (x10^9) non AC	Conidia (x10^9) AC
T1 Oatmeal	10.7c	**7.8d**
T2 Rice wash	5.3d	**10.4c**
T3 Potato	2.9e	**4.9e**
T4 Sweet	3.0e	**3.4f**
T5 Corn	2.7e	**4.9e**
T6 Mungbean	12.9b	**12.1b**
T7 Rice bran	13.1a	**15.5a**
T8 Soya	0.36f	**1.5g**
T9 White Corn	1.06f	**3.4f**
T10 Cassava	0.83f	**1.0g**
T11 Peanut	0.36f	**1.0g**
T12 White Sorghum	**0.46f**	**1.1g**

- Means followed by the same letter do not vary significantly at 5% level of significance using DMRT

Results revealed that the highest conidial count among media incubated at room temperature was in T7 (rice bran) with 13.1 x 10^9 conidia/ml which was significantly higher than all other treatments. The same result was obtained in cultures under air conditioned environment wherein T7 (rice bran) had 15.5 x 10^9 conidia/ml. The standard oatmeal being used for the culture of *M. anisopliae* ranked third and was surpassed by mungbean at room temperature condition which mungbean and rice washing under air conditioned environment. No significant difference using t-test was observed on the effect of incubation condition on the conidial count.

Substrates

The conidial count in each substrate was transferred with *M. anisopliae* which incubated under room and air conditioned environment as presented in Table 2. The room temperature was 33.9-35.3^oC and the mean was 34.60^oC while in air conditioned environment was 23.9-33.2^oC with a mean of 28.55^oC. The substrates incubated at room temperature showed the highest conidial count in sorghum seeds (12.8 x 10^9) but not significantly different from rice bran (12.4 x 10^9), ground corn (11.6 x 10^9) and chipped camote tubers (11.1 x 10^9). Under air conditioned environment, showed the highest conidial count in sorghum seeds (14.1 x 109) but not significantly different from rice bran (13.5 x 10^9). No significant difference was observed between incubation conditions (non-AC vs, AC) using t-test at 5% level of significance. No conidia was observed in rice straw, sawdust, soil, sugarcane bagasse, rice hull, tobacco midribs, water lily, tiger grass and peanut rind.

Table 2. Conidial count of *M. anisopliae* in different substrate incubated at room temperature and air conditioned environment

Treatments	**Conidia (x10^9) non AC**	**Conidia (x10^9) AC**
T1 Ground corn	11.6 a	**11.7 bc**
T2 Rice straw	-	**-**
T3 Sawdust	-	**-**
T4 Rice bran	12.4 a	**13.5 ab**
T5 Soil	-	**-**
T6 Sorghum seeds	12.8 a	**14.1 a**
T7 Palay seeds	3.3 c	**9.7 cd**
T8 Sugarcane bagasse	-	**-**
T9 Rice Hull	-	**-**
T10 Corn cob	4.5 c	**3.6 fg**
T11 Ground coconut dried meat	4.8 c	**5.5 e**
T12 Chipped camote tubers	11.1 ab	**7.7 de**
T13 Tobacco midribs	8.4 b	**10. 9 c**
T14 Dried water lily	-	**-**
T15 Dried tiger grass	-	**-**
T16 White corn	2.3 c	**2.0 g**
T17 Peanut rind	**-**	**-**

- No growth observed
- Means followed by the same letter do not vary significantly at 5% level of significance using DMRT

Efficacy test using rice black bug under greenhouse condition

Metarhizium anisopliae cultured and incubated at room temperature (non-AC) were used for efficacy test. The mortality was observed every 3 days. After 45 days the total mortality was added in each treatment.

The Percentage Mortality of Rice Black Bug after 45 days is presented in Table 3. The highest mortality was observed in rice sprayed with *M. anisopliae* cultured in rice bran (70.00%) but not significantly different from palay seeds (65.00%), corn cob (58.33%) and ground corn (56.67%). This result implies that the above mentioned indigenous substrates could be used as alternative substrates for the production of *M. anisopliae* inoculum for the control of RBB.

Table 3. Percentage Mortality of Rice Black Bug treated with *M. anisopliae* after 45 days

	Mortality (%)
T1 Ground corn	**56.67 abc**
T2 Rice straw	**-**
T3 Sawdust	**-**
T4 Rice bran	**70.00 a**
T5 Soil	**-**
T6 Sorghum seeds	**33.33 de**
T7 Palay seeds	**65.00 ab**
T8 Sugarcane bagasse	**-**
T9 Rice Hull	**-**
T10 Corn cob	**58.33 abc**
T11 Ground coconut dried meat	**45.00 bcd**
T12 Chipped camote tubers	**25.00 de**
T13 Tobacco midribs	**13.33 e**
T14 Dried water lily	**-**
T15 Dried tiger grass	**-**
T16 White corn	**43.33 cd**
T17 Peanut rind	**-**

- Not done since no growth in the result of previous experiment (Table 4)
- Means followed by the same letter do not vary significantly at 5% level of significance using DMRT

Discussion

Results of the current study revealed that *M. anisopliae* grown in rice bran produced the most number of conidia both at room and air conditioned environment. Studies showed that nitrogen compounds that supported growth were less favourable for conidia germination and, since different amino acids stimulated particular stages of growth and sporulation, a complex nitrogen source was required to optimise these processes (Li and Holdom, 1995). Rice bran contains energy, amino acids such as Leucine, Isoleucine, Lysine, Cysteine, Phenylalanine, Tryptophan as well as vitamins and minerals such as Thiamine, Niacine, Calcium, Zinc, Riboflavin, Iron and Phosporus (Chakraborty and Budhwar, 2018). Nutritional elements play an important role in conidia and blastospore production (Orquidea, P.G. *et al.,* 2021). Cereal grains are generally rich from carbohydrates and other substrates for conidia production, (Bena-Molaei *et al*., 2011). Furthermore, cereals and industrial organic by-products such as rice bran, crushed corn, wheat and barley are the most important source of hydrocarbons and other nutrients for *M. anisopliae* and other entomopathogenic fungi (EL Damir, 2006). Sorghum is the most commonly used substrate for the culture fungi such as *Pleurotus* species. It contains protein (7.84-9.23%), fat (0.5-0.9%), Ash (0.7-0.9%), fibre (1.7-2.0%) and carbohydrate (80-90%) according to Okoye and Obi (2017). In the cultivation of *Volvariella* spp., the use of wheat with rice bran resulted in significantly faster mycelial growth and highest production (fructification) as compared to other substrates (Tripathy, 2010). Conidial yield of *M. anisopliae* did not appear to be linked with radial growth as an increased in radial growth that did not resulted in simultaneous increased in conidial yield, but it is dependent upon the fungal strain and nutrition (Shah *et al.,* 2005).

The observed mean temperature during incubation in this study was 34.60^{o}C (non-AC) and 28.55^{o}C (AC) for both culture media and substrate. The optimal growth for most *M. anisopliae* isolates occur in the temperature range of 25 and 30 ℃ (Bugeme *et al.,* 2009) with 25 ℃ was most frequently recorded (Ekesi *et al.,* 1999). Maximum growth at 30 ℃ was observed for isolate M. anisopliae V90 which showed the fastest growth (≈1.7 mm per day) at 30°C, and was surprisingly able to grow at 40 ℃ (Hallsworth and Magan, 1999). The growth rates of some isolates of *M. anisopliae* were evaluated at temperatures between 28 and 40 ℃ and it was found that some French and Brazilian isolates grew at 37.5 ℃ but no growth above that temperature (Brooks *et al.,* (2004). Moreover, in the study of Humpherson-Jones and Phelps (1989) on climatic factors influenced conidia production in *Alternaria brassicae* and *Alternaria brassicicola,* it was found that the optimum temperatures for sporulation were

18–24 ℃ for *A. brassicae* and 20–30 ℃ for *A. brassicicola* at which temperatures both fungi produced conidia. There were no conidia in rice straw, sawdust, soil, sugarcane bagasse, rice hull, tobacco midribs, water lily, tiger grass and peanut rind which could be due to low nutrient content of this substrates which was not enough to support conidia formation of *M. anisopliae*.

The high mortality rate of RBB sprayed with *M. anisopliae* grown in rice bran could be attributed to the high density of conidia (12.4 x 10^9). Siswanto and Trisawa (2017) stated that the high conidial density of the entomopathogenic fungus~~,~~ leading to the higher the infection against the insect. Moreover, Wicaksono *et al.* (2015) added that a high conidia density resulted to faster penetration, development and infection by the fungus than low concentrations. The direct application of *M. anisopliae* to control *O. rhinoceros* pests has been reported by Parinduri *et al.* (2017) who stated that the concentration of 30 g per liter water was able to cause 62.5% mortality. Moreover, Sihombing *et al.* (2014) stated that the concentration of 75 g.l-1 water was able to cause mortality in larvae at 89.6%. Gabarty *et al.* (2013) stated that when the conidia attach to the cuticle of the insect, it will germinate and penetrate the insect's skin and then it will produce toxin and damage the insect's immune system. *Metarhizium anisopliae* has also expressed larvicidal activity because of cyclopeptide toxin, destruxin A, B, C, D, E and desmethyldestruxin B and has been considered as an insecticide for a new generation. The destruxin effects the target cells organells (mitochondria, endoplasmic reticulum and nucleus membrane) which causes cells paralysis and abnormalities in the function of the middle stomach, tubules malphigi, hemocytes and muscle tissue (Widiyanti and Muyadihardja, 2004).

It is interesting to note that high conidial concentration does not always lead to high mortality. Gopal *et al.* (2005) stated that the time needed to kill the tested insects depends not only on the conidia concentration but also depends on the weather that supports the activity of the fungus *M. anisopliae.* In like manner, the factors that affect the effectiveness of a fungus in causing the death of target insects, including conidia density, quality of growing media, type of pest to be controlled, age of pest status, time and frequency of application as well as the environment (Fauzana *et al*., 2020) Moreover, Wicaksono *et al.*, (2015) stated that the time of death of test insects was faster in the application method of entomopathagus fungi sprayed on test insects than sprayed on compost, and the virulence of entomopathogenic fungi needed time to infect to kill the insect, infection began from attaching conidia, germination and penetration.

It is concluded that the conidia number was higher by culturing in rice bran and mungbean than oatmeal. The substrates for multiplication growth of *M.*

anisopliae are shown in sorghum, rice bran, ground corn and chipped camote tubers which shown to be the best alternative subtrates. Overall, rice bran was found to be excellent substrate for the culture of *M. anisopliae* which the fungus grown in rice bran proved to cause high mortality to insect.

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