Biological Forum – An International Journal **13(2): 358-361(2021)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Substrate Evaluation for Mass Multiplication of Entomopathogenic Fungal Isolates for Management of Walnut Weevil

Mushtaq Ahmad Guroo[1*], Rakesh Kumar Gupta[1], Hafeez Ahmad[1], Mudasir Gani[2], Kamlesh Bali[1], Mohd. Ayoub Mantoo[2], Amit K. Singh[1], Ajay Gupta[3] and Muneeshwar Sharma[3]

[1]Division of Entomology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Chatha, Jammu-180009 (J & K), India.

[2]Division of Entomology, Faculty of Agriculture, Sher-e-Kashmir University of Agricultural Sciences and Technology, Kashmir-193 201 (J & K), India.

[3]Krshi Vigyan Kendra, Poonch, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Chatha, Jammu-185101 (J & K), India.

(Corresponding author: Mushtaq Ahmad Guroo)*

(Received 06 April 2021, Accepted 14 June, 2021)

(Published by Research Trend, Website: www.researchtrend.net)

ABSTRACT: The selection of suitable media with respect to growth and sporulation of entomopathogenic fungi is an obligatory precondition for their large scale mass production and field application henceforth. In tandem with this, laboratory experiments were conducted to demonstrate the efficacy of using different liquid and solid media for the mass multiplication and estimation of conidial count of entomopathogenic fungal isolates of *B. bassiana* and *M. anisopliae* for the management of walnut weevil, *Alcidodes porrectirostris* Marshall. The results of mass culturing on liquid media tested, indicated that highest conidial count per unit area after inoculation was obtained for Bb-BH2 isolate (32.02×10^4 conidia/cm^2) followed by Bb-BH1 (31.08×10^4 conidia/cm^2) on Potato-dextrose agar (PDA) medium compared to Sabouraud dextrose agar (SDA)and Oat-meal agar (OMA) media. In case of solid media evaluated, maximum sporulation of Bb-BH2 was recorded on rice grains (6.62×10^6spore/g) while as in Ma-PO1 isolate, maximum sporulation was recorded on wheat grains (4.36×10^6 spore/g). These results propose rationale basis for sound conidia production for laboratory and commercial use for the management of walnut weevil, *Alcidodes porrectirostris*.

Keywords: Media, conidia, isolate, rice, control, experiment, multiplication

INTRODUCTION

The walnut weevil, *Alcidodes porrectirostris* Marshall has become a major pest of walnut, *Juglans regia* over the years especially in the Jammu region of Jammu and Kashmir, where it has stimulated a considerable yield loss (Guroo *et al*., 2021). The major portion of damage to the fruit (kernel) is caused by the cryptic stage (larvae) of this pest which renders the use of insecticides quite unproductive. The use of suitable entomopathogenic fungi under such circumstances offers an effective alternative to utilize them for the control of such pests as they have the ability to manage the concealed pests and perpetuate on their own. The use of insect pathogenic fungi is quite safe, environmentally pure and non-accumulating (Edde 2012). These entomopathogenic fungi infect the host insect both by direct treatment and by transmission of inoculum from treated insects or cadavers to untreated insects through the production of new spores (Quesada-Moraga *et al*., 2004). Several valuable findings on the use of entomopathogenic fungi as an effective control strategy against various insect pests have been documented by various scientists with special reference to coleopteran insect pests (Francardi *et al*., 2012; Kavallieratos *et al*., 2006; Sun *et al*., 2016; Ozdemir *et al*., 2020). Among these entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* offer the promising potential as bioncontrol agents against several weevils (Batta *et al*., 2004). These fungi grow naturally in soils throughout the world and, as parasitoids, are capable of causing disease in various insects. Among the microbial biopesticides, entomopathogenic fungi are the second-highest selling, accounting for around 9% of all microbial biopesticides sold globally (Glare *et al*., 2012). Over the years these fungi have been mass produced and made marketable by growing them on various types of solid and liquid media. However, optimum conidia production depends not only on the type of isolate used but also on selection of appropriate media (Kamp and Bidochka 2002) for mass multiplication of these entomopathogenic fungi which determines their measurable pathogenicity ((McCoy *et al*., 1975). This is because the type of growth medium used affects conidial/spore production of these entomopathogenic fungi that forms the basis of their field application. There is no general factor that stimulates sporulation in these fungi (Marchant 1984). However, the nutritional constituents coupled with the amount of accessible nutrients can have a reflective effect on culture growth, spolulation and morphology in entomopathogenic fungi. Keeping the above facts in view, the present study was conducted in this direction

to assess different economical and easily accessible substrates viz. Potato-dextrose agar (PDA), Sabouraud dextrose agar (SDA) and Oat-meal agar (OMA) as liquid media and rice, wheat and oats as non-synthetic solid media for mass multiplication of *B. bassiana* and *M. anisopliae* isolates for the management of walnut weevil, *Alcidodes porrectirostris* Marshall.

MATERIAL AND METHODS

The present study was conducted in the Biological control laboratory, Division of Entomology, SKUAST-Jammu. The isolates of *Beauveria bassiana* and *Metarhizium anisopliae* used were isolated from naturally infected larvae of walnut weevil, *Alcidodes porrectirostris* and soil.

A. Liquid media mass production of fungal isolates

Three different culture media with their respective compositions *viz*., Potato-dextrose agar (PDA) medium (peeled potato 200 g + dextrose 20 g + agar-agar 20 g), Sabouraud dextrose agar (SDA) medium (dextrose 40 g + peptone 10 g + agar-agar 20 g), and Oat-meal agar (OMA) medium (rolled oats 30g + agar-agar 20) were prepared as per standard procedure. The entire media were autoclaved for 20 minutes at 15 lbs pressure and poured in Petri-plates separately and a disc of 5mm of different isolates of *Beauveria bassiana* and *Metarhizium anisopliae* from the stock culture were aseptically transferred into the different media. The inoculated plates were incubated at 25±1°C, 85–90% (R.H.) and photophase of 12 hours for 12 days.

*B. Conidia production and radial growth of B. bassiana and M. anisopliae***.**

Observations were recorded on the number of days required for the sporulation of the isolates on different media. The radial growth in cm was recorded on 3rd, 6th, and 9th days after inoculation (DAI).The surface colonies were measured by using two diameters (cm) of fungus for calculating radial growth (cm/day). A 1-cm diameter plug was taken exactly halfway between the centre and edge of the colony of each isolate and then suspended in 0.3 % Tween 80 solution in sterile distilled water to ensure maximum conidial harvesting. The suspensions were vortexed for 3 minutes to produce a homogenous suspension. The suspensions were filtered through several layers of cheese cloth to remove mycelia and debris. Subsequently, the spore concentrations were determined using a haemocytometer, and the spore amount per unit area was calculated (Kamp and Bidochka 2002).

C. Solid media mass production of fungal isolates

Non-synthetic solid media *viz*. rice, wheat and oats were used for estimating the sporulation of *B. bassiana* and *M. anisopliae* at 25±1°C. Purified 500 grams of each grain type were washed and soaked in water overnight except rice which were soaked for 3-4h prior to starting of experiments. The excess water was drained by pouring and shade drying for half an hour to further remove the surplus moisture. The grains were packed separately in 500 ml conical flask, with cotton plug and auto calved at 15 psi for 20 minutes. After cooling, 1 ml of the spore suspension of isolates of *B. bassiana* and *M. anisopliae* were inoculated into each conical flask under laminar flow chamber followed by incubation in BOD incubator at 25±1°C for 20 days. Four replications were maintained for each grain type. In order to avoid stomping, after 5 days of inoculation, the flasks were shaken robustly to separate the grain and to break the mycelial mat. After 20 days of incubation, 10 g homogenous grain sample drawn from each replicate of consistently sporulating flasks was transferred to 100 ml sterilized distilled water containing 0.3 % Tween 80 solution in 250 ml conical flasks. The flasks were shaken in mechanical shaker for 10 minutes. The suspensions were filtered through double layered cheese cloth to remove mycelia and debris. Counting of spores was made using a haemocytometer and a binocular microscope at 400x magnification and expressed as conidia per gram (g).

D. Statistical analysis

Data was statistically analysed by using the SPSS version 16.0 and further subjected to post hoc tests for comparison of means.

RESULTS AND DISCUSSION

Differential conidial count of selected isolates of *B. bassiana* and *M. anisopliae* using potato-dextrose agar (PDA), Sabouraud dextrose agar (SDA), and Oat-meal agar (OMA) media is presented in Table 1. Among the different substrates evaluated, highest conidial count after inoculation (32.02×10^4 conidia/cm^2) was obtained for Bb-BH2 isolate, followed by Bb-BH1 (31.08×10^4 conidia/cm^2), Ma-PO1 (28.52×10^4 conidia/cm^2) and Ma-PO2 isolate (20.31×10^4 conidia/cm^2) on PDA medium, respectively. Significant differences were observed in the conidial count on different media (PDA, F = 34.321, df=3, P=0.000; SDA, F = 29.432, df=3, P=0.000; OMA, F = 26.523, df=3, P=0.000).The variation in sporulation and conidia production of entomopathogenic fungi may be due to the fungal isolate (Kamp and Bidochka 2002) and nutritional composition of the media and carbon: nitrogen ratio (Shah *et al*., 2005). For example, a high conidia production and radial growth rate was reported when *B. bassiana* B14841 (37.10 mm/ day) was cultured on Czapeck Dox agar (CDA) which may be due to sodium nitrate and minerals which are needed for the growth of this fungi (Altomare *et al*., 1999). Similarly highest radial growth of 50.50 mm/day was reported when *B. bassiana* was grown on a basal medium mixed with sodium nitrate (Sabbour *et al*., 2011). To single out a particular medium for best conidial production of entomopathogenic fungi is a matter of long debate.

Table 1: Spore counts of *Beauveria bassiana* and *Metarrhizium anisopliae* fungi using different liquid media.

Media	Bb-BH1 ($x10^4$ spores/cm^2)	Bb-BH2 ($x10^4$ spores/cm^2)	Ma-PO1 ($x10^4$ spores/cm^2)	Ma-PO2 ($x10^4$ spores/cm^2)
PDA	31.08±3.73c	32.02±0.02c	28.52±0.52c	20.31±0.22b
SDA	28.31±1.28b	30.11±2.23c	24.11±1.23b	17.8± 0.32a
Oat meal	21.18±0.43a	23.32±0.22b	17.52±0.12a	15.31±2.22a

Mean ± SE followed by different letters within the same column and same isolates are significantly different at $p < 0.05$ level.

Some researchers believe that PDA is the best medium for culture of fungi (Zhang *et al*., 2001) While as others have reported Sabouraud dextrose agar (SDA) as a best medium for *M. anisopliae* and *B. bassiana* (Hallsworth *et al*., 1996) multiplication. The spore dimensions measured using a phase contrast research microscope revealed that aerial conidia size of Bb-BH1 and Bb-BH2 isolates showed considerable variability with spore mean sizes ranging from 2.49 to 2.51 µm and from 2.38 to 2.40 µm for Ma-PO2 and Ma-PO1 isolates (Table 2) respectively. No relation could be demonstrated between the morphological traits (spore size and radial growth) and pathogenicity of *B. bassiana* and *M. anisopliae* isolates to *A. porrectirostris*. These results are in agreement with the findings of Talaei-Hassanloui *et al*., (2006) who did not find any correlation between morphological traits (growth rate, spore size and germination rate) and pathogenicity of *B. bassiana* against *Leptinotarsa decemilneata* and *Plutella xylostella*.

Methods for commercial production of conidia are usually done on solid substrates that consist of cereal grains such as rice or other starch-based substrate. All the solid media substrates used in the present study ensured good growth of different isolates used (Table 3). Maximum sporulation was recorded on rice grains in case of Bb-BH2 (6.62×10^6spore/g) while as in Ma-PO1, maximum sporulation was recorded on wheat grains (4.36×10^6spore/g). Significant differences were observed in the conidial count on different media (Rice, $F = 21.234$, df = 3, $P=0.000$; Wheat, $F = 19.512$, df=3, $P=0.000$; Oats, $F = 17.320$, df = 3, $P=0.000$). On the whole, rice and wheat grains provided significantly more sporulation in case of Bb-BH2 and Ma-PO1respectively, compared to oats. The ability of *B. bassiana* and *M. anisopliae* isolates to grow and sporulate on artificial media is one of the main advantages in the commercial development of these fungi. Methods of mass production of these entomopathogenic fungi using different cereal grains such as rice or other starch based substrates have been employed globally (Goettel and Roberts 1992). Our results are in agreement with findings of Sharma *et al*. (2002) who reported maximum sporulation of *B. bassiana* isolate Bb-1 and Bb-2 (9.7×10^7 and 7.56×10^7 conidia/ g) on rice grains followed by sorghum and maize.

Table 2: Growth rate and sporulation of different entomopathogenic fungal isolates.

Strain	Colony diameter (cm)			Sporulation time(days)	Spore size (µm)	Spore shape
	3 days	6 days	9 days			
Bb-BH1	2.0a	3.6b	6.6c	3a	2.49 ±0.02a	Round
Bb-BH2	2.5b	4.8b	7.2c	3a	2.51±0.01a	Oval
Ma-PO1	2.0a	3.6bc	5.8b	4b	2.40± 0.05b	Ellipsoidal
Ma-PO2	2.2b	3.7bc	5.5b	4b	2.38 ±0.01b	Ellipsoidal

Mean ± SE followed by different letters within the same column and same isolates are significantly different at $p < 0.05$ level.

Table 3: Sporulation of different isolates of *Beauveria bassiana* and *Metarhizium anisopliae* on locally available solid media.

Media	Bb-BH1 ($x10^6$spores/g)	Bb-BH2 ($x10^6$spores/g)	Ma-PO1 ($x10^6$spores/g)	Ma-PO2 ($x10^6$spores/g)
Rice	6.02 ±0.45a	6.62 ±0.25a	3.22 ±0.15a	2.35 ±0.25a
Wheat	4.75±0.20b	5.20±0.50b	4.36 ±0.17b	4.05 ±0.17b
Oats	4.12±0.20c	4.27±0.70c	3.96 ±0.25b	2.96 ±0.25a

Mean ± SE followed by different letters within the same column and same isolates are significantly different at $p < 0.05$ level.

CONCLUSIONS

There were two findings in this study. First, growth and sporulation of *B. bassiana* and *M. anisopliae* isolates is affected by different liquid and solid substrate culture conditions. Second, among the liquid media tested, PDA medium ensured the maximum growth and sporulation while as among the solid media, maximum hyphal growth and conidial count of *B. bassiana* occurred on rice grains and that of *M. anisopliae* isolates, it occurred on wheat grains. These media should therefore be used for the mass multiplication of these isolates for managing *A. porrectirostris* when used under field conditions.

ACKNOWLEDGEMENTS

The authors are highly thankful to the Division of Plant Pathology, SKUAST-Jammu for their cooperation and help during the experimentation. We sincerely thank the anonymous reviewers for their valuable comments and suggestions which improved the paper.

Conflict of Interest: The authors assert that they have no conflict of interest.

REFERENCES

Altomare, C., Norvell, W.A., Bjorkman, T., & Harman, G.E. (1999). Solubilization of phosphates and micronutrients by the plantgrowth-promoting and biocontrol fungus *Trichodenna tianianum*. *Applications of Environmental Microbiology*, *65*: 2926-33.

Batta, Y.A. (2004). Control of rice weevil (*Sitophilus oryzae* L., Coleoptera: Curculionidae**)** with various formulations of *Metarhizium anisopliae*. *Crop Protection*, *23*: 103-108**.**

Edde, P.A. (2012). A review of the biology and control of *Rhyzopertha dominica* (F.) the lesser grain borer. *Journal of Stored Products Research*, *48*: 1–18.

Francardi, V., Benvenuti, C., Roversi, P.F., Rumine, P., & Barzanti, G. (2012). Entomopathogenicity of *Beauveria bassiana* (Bals.) Vuill. and *Metarhizium anisopliae* (Metsch.) Sorokin isolated from different sources in the control of *Rhynchophorus ferrugineus* (Olivier) (Coleoptera Curculionidae). *Redia*, *95*: 49–55.

Glare, T., Caradus, J., Gelernter, W., Jackson, T., Keyhani, N., Kohl, J., Marrone, P., Morin, L. & Stewart, A. (2012). Have biopesticides come of age? *Trends Biotechnology*, *30*: 250–258.

Goettel, M.S., & Roberts, D.W. (1992). Mass production, formulation and field application of entomopathogenic fungi. In: Biological Control of Locusts and Grasshoppers (ed.) Lomer, C.J. and Prior, C. pp. 230–238. Wallingford, UK: CAB International.

Guroo, M.A., Ahmad, H., Gupta, R.K., Gani, M., Bali, K., Mir, M., & Jamwal, S. S. (2021). Estimation of Yield Loss Caused by *Alcidodes porrectirostris* in Walnut Growing Areas of Jammu Division (J &K). *Biological Forum-An International Journal*, *13* (2): 133-137.

Hallsworth, J.E., & Magan N. (1996). Culture age, temperature, and pH affect the polyol and trehalose contents of fungal propagules. *Applied and Environmental Microbiology*. *62*: 2435-42.

Kamp, A.M., & Bidochka, M.J. (2002).Conidium production by insect pathogenic fungi on commercially available agars. *Letters in Applied Microbiology*, *35*: 74–77.

Kavallaieratos, N.G., Athanassiaou, C.G., & Mchalaki, M.P. (2006). Effect of combined use of *Metarhizium anisopliae* (Metschinkoff) Sorokin and diatomaceous earth for the control of three stored beetle species. *Crop protection***,** *25*: 1087-1094**.**

Marchant, R. (1984). The ultrastructure and physiology of sporulation in Fusarium. In: The Applied Mycology of Fusarium British Mycological Society Symposium Series (7), pp. 15–37. Queen Mary College, London: Cambridge University Press.

McCoy, C.W., Hill. A.J., & Kanavel, R.F. (1975). Large scale production of the fungal pathogens *Hirsutela thomsonii* in submerged culture and its formulation for application in the field. *Entomophaga*, *20*: 229-240.

Ozdemir, I.O., Tuncer, C., Erper, I. & Kushiyev, R. (2020). Efficacy of the entomopathogenic fungi: *Beauveria bassiana* and *Metarhizium anisopliae* against the cowpea weevil, *Callosobruchus maculatus* F. (Coleoptera: Chrysomelidae: Bruchinae). *Egyptian Journal of Biological Pest Control*, *30*: 24-29.

Quesada-Moraga, E., Santos-Quiros, R., & Valverde-Garcia, P. (2004). Virulence horizontal transmission and sub-lethal reproductive effects of *Metarhizium anisopliae* (anamorphic fungi) on the German cockroach (Blattodea: Blattellidae). *Journal of invertebrate pathology*, *87*: 51-58.

Sabbour, M. M., Ragei, M., & Abd-El Rahman, A. (2011). Effect of some ecological factors on the growth of *Beauveria bassiana* and *Paecilomyces fumosoroseus* against Com Borers. *Australian Journal of Basic and Applied Sciences*, *5*: 228-35.

Shah, F.A., Wang, C.S., & Butt, T.M. (2005). Nutrition influences growth and virulence of the insect-pathogenic fungus *Metarhizium anisopliae*. *FEMS Microbiol Letters*, *251*: 259-66.

Sharma, S., Gupta, R.B.L., & Yadav, C.P.S. (2002). Selection of suitable medium for mass multiplication of entomofungal pathogens. *Indian Journal of Entomology*, *64*: 254-261.

Sun, X. Y., Qin, W., Zhang, W., Niu, J., Ma, G. & Li, F. (2016). Screening of tropical isolates of *Metarhizium anisopliae* for virulence to the red palm weevil *Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae). *Springer Plus*, *5*: 1100. DOI 10.1186/s40064-016-2780-6.

Talaei-Hassanloui, R., Kharazi-Pakdel, A., Goettel, M., & Mozaffari, A. (2006). Variation in virulence of *Beauveria bassiana* isolates and its relatedness to some morphological characteristics. *Biocontrol Science and Technology*, *16*: 525-534.

Zhang, W., Sulz, M., & Bailey, K.L. (2001). Growth and spore production of *Plectosporium tabacinum*. *Canadian Journal of Botany*, *79*: 1297-306.

How to cite this article: Guroo, M. A., Gupta, R. K., Ahmad, H., Gani, M., Bali, K., Mantoo, M. A., Singh, A. K., Gupta, A. and Sharma, M. (2021). Substrate Evaluation for Mass Multiplication of Entomopathogenic Fungal Isolates for Management of Walnut Weevil. *Biological Forum – An International Journal, 13*(2): 358-361.