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Efficacy of *Metarhizium anisopliae* combined with diatomaceous earth against *Sitophilus oryzae* (Coleoptera: Curculionidae) under laboratory conditions

Eficacia de *Metarhizium anisopliae* combinado con tierra de diatomeas contra *Sitophilus oryzae* (Coleoptera: Curculionidae) en condiciones de laboratorio

WAQAS WAKIL[1], M. USMAN GHAZANFAR[2], MUHAMMAD YASIN[1] and YONG JUNG KWON[3]

Abstract: The objective of the present study was to assess the efficacy of an indigenous isolate of *Metarhizium anisopliae* alone and with diatomaceous earth (DE) formulation against the rice weevil, *Sitophilus oryzae* (Coleoptera: Curculionidae). Rice grains were admixed with three doses of *M. anisopliae*, i.e., 2.78×10^6, 2.78×10^7 and 2.78×10^8 conidia kg-1 alone and in combination with DE Protect-It™ applied at the rate 100 and 200 ppm, respectively. The weevil's mortality was determined after 7, 14 and 21d of exposure intervals at 25 ± 2 ºC and 55% R. H. After each mortality count, the dead adult insects were removed and after the last exposure interval, the treated material was kept for the following 62d to check the progeny production. Rice grains treated with the highest dose rate of *M. anisopliae* combined with 200 ppm of Protect-It™ provided the maximum mortality and reduced progeny production of *S. oryzae*. These findings suggest that the combined application of entomopathogenic fungi and DE is more effective than when applied singly against *S. oryzae*. Moreover, it was also observed that the low doses of *M. anisopliae* showed the highest rate of mycosis and sporulation in the dead adults of *S. oryzae*.

Key words: Insect pathogenic fungi. Weevil. Rice grains. Mycosis. Sporulation.

Resumen: El objetivo de esta investigación fue evaluar la eficacia de un aislamiento nativo de *Metarhizium anisopliae* (Ascomycota: Hypocreales), sólo y en conjunto con tierra de diatomeas (TD) en contra de los adultos del gorgojo del arroz, *Sitophilus oryzae* (Coleoptera: Curculionidae). Los granos de arroz fueron tratados con tres dosis diferentes, i.e., $2{,}78 \times 10^6$, 10^7 y $2{,}78 \times 2{,}78 \times 10^8$ conidias kg-1 por sí solos y en combinación con TD Protect-It® a 100 y 200 ppm, respectivamente. La mortalidad de los gorgojos adultos se determinó a los 7, 14 y 21 d después de exposición a 25 ± 2 ºC y 55% H. R. Posterior a cada recuento de mortalidad, los cadáveres de adultos fueron retirados y se incubaron durante los 62d siguientes para estimar la producción de la progenie. Los resultados revelan que los granos de arroz tratados con la más alta dosis de *M. anisopliae* y en combinación con 200 ppm de Protect-It® producen la máxima mortalidad y reducen la progenie de *S. oryzae*. Estos resultados mostraron que los hongos entomopatógenos cuando se combinan con TD son más eficaces en comparación con las aplicaciones simples contra *S. oryzae*. Además, las dosis bajas de *M. anisopliae* desplegaron la máxima esporulación en cadáveres de *S. oryzae*.

Palabras clave: Hongos entomopatógenos. Gorgojo. Granos de arroz. Micosis. Esporulación.

Introduction

Rice weevil, *Sitophilus oryzae* (Linnaeus, 1763) (Coleoptera: Curculionidae), is considered among the most devastating species of primary stored product insects (Pinto *et al*. 1997). As an internal feeder it affects directly protein, vitamins and carbohydrate contents of the grains and reduces germination rate (Dal-Bello *et al.* 2001). The use of synthetic chemicals and fumigants either as grain protectants or structural treatments is very common and traditional method used against *S. oryzae* and various other stored grain insect pests (Collins and Cook 2006). However, the continuous use of these synthetic chemicals has induced tolerance or resistance to these substances. This problem diverted the focus of researchers towards the use of innovative and safe insect control measures like the application of entomopathogenic bacteria, viruses, protozoa, fungi (Moore *et al.* 2000) and nematodes (Laznik *et al.* 2010). Several valuable findings on the use of entomopathogenic fungi as an effective control strategy have been documented by various scientists particularly with special reference to Coleopteran insect pests (Kavallieratos *et al.* 2006; Bourassa *et al*. 2001). After the introduction of different Hypocreales fungi in the field of insect pest management more research is needed to identify the new species of entomopathogenic fungi with high virulence, and to evaluate the pathogenicity of different native and exotic isolates of already available fungal species (Adane *et al.* 1996; Ekesi *et al.* 1998; Moore *et al.* 2000; Batta 2005).

It is well established that the fungal efficacy can be enhanced by the simultaneous presence of other substances with different mode of action (Lemanceau and Alabouvette 1993; Wakil *et al*. 2011; 2012). In this regards, desiccant dusts have proved the most compatible synergist with various formulations of Deuteromycotina fungi (Vassilakos *et al.* 2006; Michalaki *et al*. 2007).

The most important category of desiccant dusts for use in stored-product protection is diatomaceous earths (DEs) (Korunic 1998). DEs are generally very effective against a broad range of stored grain insect pests (Subramanyam and

[1] Department of Entomology, University of Agriculture, Faisalabad, Pakistan. *arid1972@yahoo.com*. Corresponding author. [2] Department of Plant Pathology, University College of Agriculture, University of Sargodha, Pakistan. [3] College of Agriculture and Life Sciences, Kyungpook National University, Daegu, Korea.

Roesli 2000; Stathers *et al.* 2004; Wakil *et al.* 2010). Most of the work, this regards, to investigate insect response to any local or exotic fungal isolate individually or in combined treatment with DE primarily using *Beauveria bassiana* (Wakil and Ghazanfar 2010; Riasat *et al.* 2013) and secondarily with *Metarhizium anisopliae* (Adane *et al.* 1996; Athanassiou *et al.* 2008; Meikle *et al.* 2001). As far as *M. anisopliae* is concerned, indigenous and exotic isolates have been tested not only for stored product insect management (Wakefield *et al.* 2005; Michalaki *et al*. 2006) but also against field crop pests (Ekesi *et al.* 1998). In the present study, the potential efficacy of an indigenous isolate of *M. anisopliae* was assessed alone as well as in combination with DE. In addition to the mortality effect, the production of progeny and the influence of fungal dose rates on mycosis and sporulation in the cadavers of *S. oryzae* were also measured.

Materials and methods

Insects, DE and fungus. *Sitophilus oryzae* was mass cultured on wheat, at 25 ± 2 ºC and 70 ± 5% R. H., and maintained under dark conditions in IPM Laboratory (Insect Pathology) of Department of Entomology, University of Agriculture, Faisalabad, Pakistan. The enhanced DE formulation used in the tests was Protect-It™ (Hedley Technologies Limited, Mississauga ON, Canada), a mix of 90% SiO_2 and 10% silica gel. The fungal strain of *M. anisopliae* was isolated from *Coccinella septempunctata* L. (Coccinellidae: Coleoptera) using the single spore method (Choi *et al.* 1999). Samples were collected from different geographical locations of Faisalabad (31º30'N 73º6'W; Altitude: 184 m), Punjab, Pakistan. The infested insects, with visible externally developed fungal growth, were plated on 0.5% SDAY (Sabouraud Dextrose Agar + Yeast) for mass production of conidia. The harvested conidia were suspended in sterile 0.02% Tween-80 solution to measure its concentration under improved Neubauer hemocytometer.

Commodity. Infestation-free clean rice kernels, with moisture content ranging between 11.4 - 12.2% were used in the bioassays. The rice moisture content was determined by Dickey-John grain moisture meter (Dickey-John Co., USA).

Bioassays. To test the insecticidal toxicity of both the substances, 100g rice kernels were taken in plastic jars (4 inches high and 2.5 inches in diameter) which served as an experimental unit and were admixed with corresponding dosages of DE (100 and 200 ppm) and fungi (2.78×10^6, 2.78×10^7, 2.78×10^8 conidia kg^{-1} of rice kernels). In this way eleven different treatments (two for DE, three for fungi, three for fungi with 100 ppm of DE and three for fungi with 200 ppm of DE) were made and each treatment repeated for three times. An additional set of jars served as control as the commodity in these jars was left untreated. These treated as well as untreated units were further infested by the release of 50 less than 2 old mixed sex *S. oryzae* adults in each unit. The conidial viability in the form of percent germination was checked periodically by inoculating the fungal conidia on SDAY plates under optimum conditions. All jars were incubated (Sony Corp.) at 55% R. H. and 25 ± 2 ºC; the required humidity level was maintained by using saturated salt solution of magnesium nitrate reported by Greenspan (1977). The adult mortality was scored after 7, 14 and 21d of exposure by removing the contents of each jar on plastic trays (16 × 10.5 cm) made for the purpose. The dead adults were removed subsequently after each exposure period returning the material back into jars with live adults. After the last mortality count at 21d of exposure all dead and live adults removed and the substrate, held under same conditions for further 62d in order to determine the F1 progeny production. The dead cadavers were collected for mycosis data from each replication and each individual was dipped in 0.05% sodium hypochlorite solution for 2-3 minutes for the surface sterilization, followed by three time washings with the sterilized distilled water, and then finally were placed on SDAY plates. The plates were then incubated at 25 ± 2 ºC, 75% R. H. for 7 days and the cadavers showing external fungal growth were determined under the stereomicroscope. Sporulation production was determined by mixing 20 individuals in 20 ml distilled water with the drop of Tween-80 in beaker reported by Riasat *et al.* (2011) and Tefera and Pringle (2003), after thorough stirring the solution the number of conidia was counted using the hemocytometer.

Statistical analysis. The adult mortality was corrected by Abbott's formula (1925); however, the control mortality was low enough and ranged from 1 - 2% during entire experimental period. The whole data was subjected to analysis of variance (ANOVA) under GLM procedure, whereas, for means comparison the Tukey-Kramer (HSD) test was used at 5% level of significance (Sokal and Rohlf 1995).

Results

Mortality, mycosis and sporulation of *S. oryzae*. Both main effects were significant at $P < 0.01$ level (treatment $F = 53.36$; interval $F = 130.07$; $df = 10.66$ and 2.66, respectively), however their associated interaction i.e. treatment × interval was non significant at $P = 0.44$; $F = 1.03$; $df = 20.66$.

After 7d of exposure, significantly less number of adult weevils (12%) were dead on rice treated with fungus alone specifically where it was applied at its lowest dose rate i.e. 2.78×10^6 conidia kg^{-1} of rice (Fig. 1). The resultant mortality level was increased up to 32% with the increase of exposure interval and application rate of the fungus (Fig. 2). On the other hand, DE alone was significantly more effective than fungus alone but less effective than its combined treatment

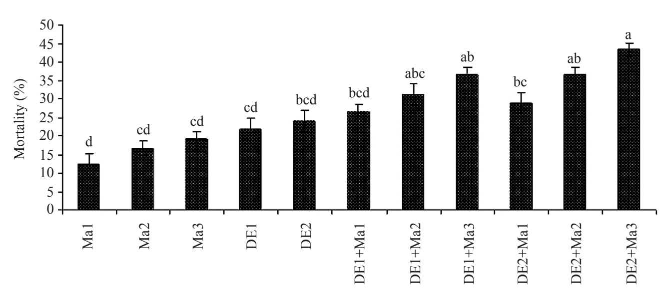

Figure 1. Mean mortality (± SE) of *S. oryzae* adults exposed for 7 days on rice kernels treated with *M. anisopliae* alone and combined with diatomaceous earth (DE1: 100 ppm; DE2: 200 ppm; Ma1: 2.78×10^6; Ma2: 2.78×10^7 and Ma3: 2.78×10^8 conidia kg-1 of rice), the means with the same letters are not significantly different; Tukey-Kramer test at P = 0.05.

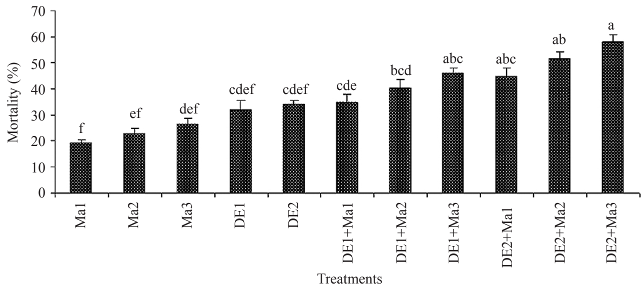

Figure 2. Mean mortality (±SE) of *S. oryzae* adults exposed for 14 days on rice kernels treated with *M. anisopliae* alone and combined with diatomaceous earth (DE1: 100 ppm; DE2: 200 ppm; Ma1: 2.78 x 10^6; Ma2: 2.78 x 10^7 and Ma3: 2.78 x 10^8 conidia kg-1 of rice), the means with the same letters are not significantly different; Tukey-Kramer test at P = 0.05.

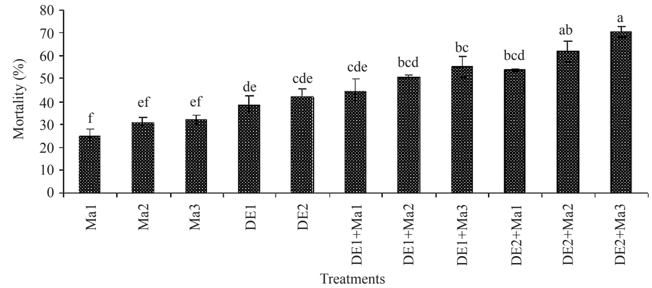

Figure 3. Mean mortality (±SE) of *S. oryzae* adults exposed for 21 days on rice kernels treated with *M. anisopliae* alone and combined with diatomaceous earth (DE1: 100 ppm; DE2: 200 ppm; Ma1: 2.78 x 10^6; Ma2: 2.78 x 10^7 and Ma3: 2.78 x 10^8 conidia kg-1 of rice), the means with the same letters are not significantly different; Tukey-Kramer test at P = 0.05.

with fungus, as it caused 42% mortality. This mortality was increased with the increase of exposure and the DE dose rate.

In the combined treatment, the highest fungal dose with 200 ppm of DE was significantly more effective in comparison with the application of each substance alone, after 21 days of exposure (Fig. 3). Additionally, for all combinations in which either 100 or 200 ppm of DE was mixed with each fungal dose, mortality was generally higher in comparison with the application of fungus or DE alone, however, despite significant differences in doses, mortality did not exceed 71%.

The highest mycosis (86.47%) and sporulation (153.22 conidia ml^{-1}) was observed in treatments which received the individual lowest concentration of *M. anisopliae* 2.78×10^6 conidia kg^{-1} (Fig. 5 and 6), however, low rate of mycosis and sporulation was observed in the treatments where high concentration of DE was applied with *M. anisopliae*.

Progeny production. For the progeny production of *S. oryzae*, the main treatment effect was significant at $P < 0.01$ ($F = 890.19$; $df = 11.24$). The individuals of F1 generation were found to be 46, 40, 32 and 20 number of live insects, respectively treated with highest fungal (2.78×10^6), highest DE (200 ppm), 100 ppm of DE + 2.78×10^7 of fungus and 200 ppm of DE + 2.78×10^8 of fungus. The highly suppressed progeny production was seen (Fig. 4) where 200 ppm of DE was mixed with 2.78×10^8 fungal conidia kg^{-1} of rice. In contrast, significantly more F1 individuals were recorded in jars containing commodity treated with the lowest fungal rate (2.78×10^6 conidia kg^{-1} of rice).

Discussion

The efficacy of various DE formulations and pathogenicity of different fungal species has been evaluated against *S. oryzae* in several studies (Sheeba *et al*. 2001; Arthur 2002; Athanassiou *et al.* 2008). In the same scenario, the results of current study demonstrated that the efficacy level of *M. anisopliae* and DE product Protect-It™ against the rice weevil is in agreement with previous works (Kavallieratos *et al.* 2006; Athanassiou *et al.* 2008). Several studies have shown that the efficacy of DE product varies according to the host commodity (Kavallieratos *et al.* 2007), its application rates (Subramanyam and Roesli 2000), similarly, the degree of the attachment of DE particles (Athanassiou and Kavallieratos 2005), and the physical and morphological characteristics of various DE formulations (Fields and Korunic 2000). The results from our bioassays exhibited similar trends, but with lower mortality levels, probably due to the fact that Protect-It™ was used at 200 ppm and not at its recommended rate of 400 ppm. Athanassiou *et al*. (2005) used the same DE formulation against *S. oryzae* and found 100% mortality, but at higher dose rates. On the other hand, against the

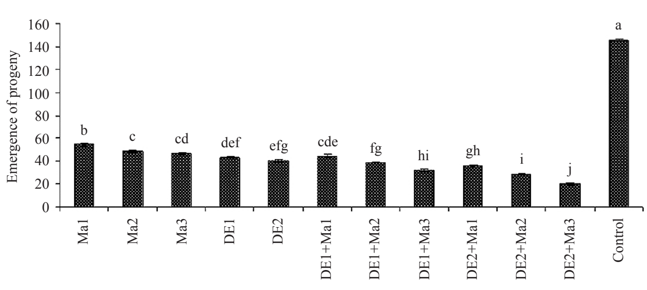

Figure 4. Production of progeny (number of alive adults per jar) (±SE) of *S. oryzae* on rice kernels treated with *M. anisopliae* alone and combined with diatomaceous earth (DE1: 100 ppm; DE2: 200 ppm; Ma1: 2.78 x 10^6; Ma2: 2.78 x 10^7 and Ma3: 2.78 x 10^8 conidia kg-1 of rice), the means with the same letters are not significantly different; Tukey-Kramer test at P = 0.05.

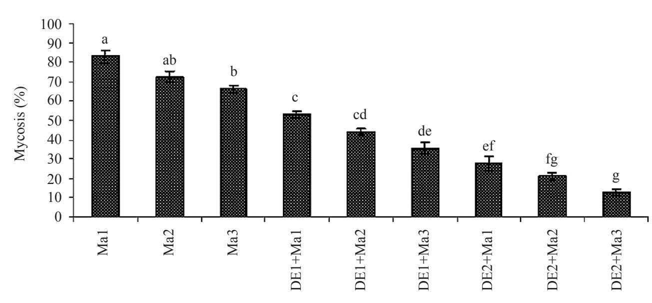

Figure 5. Mycosis (%) in cadavers of *S. oryzae* treated with *M. anisopliae* alone and combined with diatomaceous earth (DE1: 100 ppm; DE2: 200 ppm; Ma1: 2.78 x 10^6; Ma2: 2.78 x 10^7 and Ma3: 2.78 x 10^8 conidia kg-1 of rice), the means with the same letters are not significantly different; Tukey-Kramer test at P = 0.05.

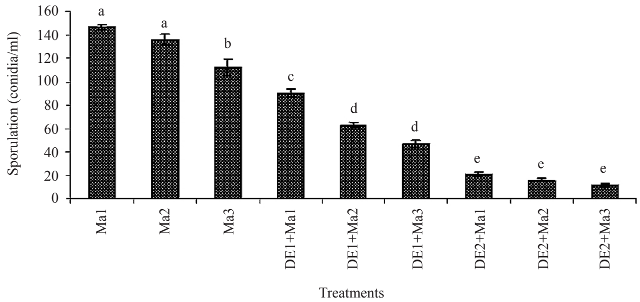

Figure 6. Sporulation (conidia/ml) on cadavers of *S. oryzae* treated with *M. anisopliae* alone and combined with diatomaceous earth (DE1: 100 ppm; DE2: 200 ppm; Ma1: 2.78 x 10^6; Ma2: 2.78 x 10^7 and Ma3: 2.78 x 10^8 conidia kg-1 of rice), the means with the same letters are not significantly different; Tukey-Kramer test at P = 0.05.

lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae), Chanbang *et al.* (2007) found that Protect-It™ caused mortality that did not exceed 70% even at 400 ppm on rough rice.

The relatively reduced efficacy of entomopathogenic fungi could be attributed to the capacity of the target species to moderate the effect of the virulence of the test isolate (Moino *et al.* 1998). Moreover, rice kernels may be less suitable than other grain commodities for the optimum insecticidal effect of DE (Kavallieratos *et al.* 2005) as generally, DE particle attachment and retention varies among grains (Athanassiou and Kavallieratos 2005).

Mortality was higher where *M. anisopliae* was applied in conjunction with DE. Similar trends were also noted from the same fungus species by Michalaki *et al.* (2006), however, Dal-Bello *et al.* (2001) reported reduced effectiveness of *M. anisopliae* against adults of *S. oryzae*, when it was applied in combination with *B. bassiana*. The combined use of entomopathogenic fungi with diatomaceous earth is advocated not only to enhance the relative effectiveness of both substances (Lord 2001) but the dust formulation of the fungus ensures easy handling of the conidia, prolonged or unaffected conidial viability (Akbar *et al*. 2004).

The increased weevil mortality with the extended exposure period has been observed and described by numerous previous studies. Vassilakos *et al.* (2006) examined that when DE formulation SilicoSec and *B. bassiana* was applied either alone or in combination, the adult mortality rates of *R. dominica* and *S. oryzae* were higher after 14d than any other exposure interval. Similarly the mortality of three stored product insects *R. dominica*, *T. confusum* and *S. oryzae* was increased with exposure period and highest percentages of dead adults were recorded after 14d than after 7d of exposure (Kavallieratos *et al.* 2006). The same has also been noted in our experiment where highest mortalities were observed after 21d of exposure.

The high level of adult mortality at the highest combination rate were in accordance with the results of Athanassiou *et al.* (2008) who integrated *M. anisopliae* with 250 ppm of DE against *S. oryzae* and *R. dominica*, using maize and wheat as test commodities. As an important parameter of insecticidal efficacy assessment, progeny production was also highly affected by the simultaneous use of DE and fungi as compared to their individual application. Previous studies on rice indicated that in comparison with *R. dominica*, progeny production was higher in the case of *S. oryzae* (Kavallieratos *et al.* 2006; Athanassiou *et al.* 2008). Further studies are needed to explicate the potential of *M. anisopliae* against other insect pests of stored-grain commodities not only under variable environmental conditions but also as an admixture with certain other natural control measures.

The percent mycosis and sporulation was higher in the cadavers previously treated with the lower dose of *M. anisopliae* alone compared to other treatments. These findings are in confirmation with Tefera and Pringle (2003), as they also found the maximum mycosis and sporulation percent in the dead adults *Chilo partellus* (Lepidoptera: Pyralidae) treated with the lower dose rates of *B. bassiana* in comparison with the highest concentration, this may be due to the self inhibition mechanism at highest fungal concentrations (Garraway and Evans 1984). Similarly, the sporulation on *S. zeamais* (Coleoptera: Curculionidae) by different fungal isolates with maximum by isolate 190-520 of *B. bassiana* has also been reported (Adane *et al.* 1996). The results from this study indicated that the enhanced efficacy of *M. anisopliae* in the presence of diatomaceous earth can be used effectively in the integrated management programs of *S. oryzae* in stored grains.

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