Investigation of the effects of ambient humidity on the combined application of Beauveria bassiana (Balsamo) Vuillemin and diatomaceous earth against the lesser grain borer, Rhyzopertha dominica (F.) (Coleoptera: Bostrichidae)

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Ortam neminin, Beauveria bassiana (Balsamo) Vuillemin ve diatomlu toprağun Ekin kambur biti Rhyzopertha dominica (F.) (Coleoptera: Bostrichidae)'ya karşı kombine uygulamaları üzerine etkinliğinin araştırılması

Öz: Entomopatojen fungus (EF) ve diytatom toprağın (DE) depolanmış tahıl zararlıklarına karşı tek başlarına veya birlikte etkili olduğu bilinmektedir. Bu çalışmada, bir Türk DE (ACN) ile birleştirilmiş iki yerel Beauveria bassiana (Balsamo) Vuillemin izolatı (5-4, 1-1) seçilen ortam koşullarının birleşik uygulamalarının etkinliği üzerindeki etkisini ortaya çıkarmak için üç nispi nemde (%45, %55, %65) ve iki sıcaklık değerinde (25ºC, 30ºC) Ekin kambur biti Rhyzopertha dominica (F.) (Coleoptera: Bostrichidae) erginlerine karşı test edilmiştir. Denemeler için 40 g buğday alan 50 ml kapasiteli santrifüj tüpleri kullanılmıştır. Kontrol için sadece buğday kullanılmıştır. EF ve DE ayrı ayrı 300 ppm (w/w) konsantrasyonunda ve birlikte 300ppm+300ppm konsantrasyonunda kullanılmıştır. Böcek ölümleri 7-14 gün sonra kaydedilmiştir. Elde edilen sonuçlara göre, B. bassiana'nın DE ile birlikte uygulanması, test edilen nispi nem seviyelerinde % R. dominica'ya karşı aynı derecede etkilidir ve bu yüzden etkinlik kaybı olmadan bir dizi ortam koşulunda kullanılabilir.

Anahtar sözcükler: Beauveria bassiana, diatom toprağı, nispi nem, Rhyzopertha dominica

Abstract: Entomopathogenic fungi (EF) and diatomaceous earth (DE) are effective alone or in combination against stored-grain insects. In this study, two Turkish isolates of Beauveria bassiana (Balsamo) Vuillemin and a Turkish DE were tested separately and in combination at three relative humidities (45%, 55%, 65%) and two temperatures (25ºC, 30ºC) to determine their efficacy against adults of the lesser grain borer Rhyzopertha dominica (F.) (Coleoptera: Bostrichidae). For the bioassays, 50 mL centrifuge tubes containing 40 g of wheat were used. For the controls, wheat alone was used. The EFs and DE were used at 300 ppm (w/w) individually and 300 ppm+300 ppm in combination. Insect mortality was recorded after 7 and 14 days. The application of B. bassiana with DE was equally effective against R. dominica at the three levels of RH used. Therefore, the further testing of combinations of entomopathogenic fungi and diatomaceous earths in grain storages under a range of ambient condition is recommended.

Key words: Beauveria bassiana, diatomaceous earth, relative humidity, Rhyzopertha dominica

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Alınış (Recieved): 30.09.2019 Kabul ediliş (Accepted): 05.12.2019
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**Introduction**

Cereals comprise an important part of the agricultural production sector in Turkey. These products are subject to damage by stored-product pests which affect the quantity, nutritional value and other commercial properties of the products. The control of insect pests of stored products mostly depends upon chemical insecticides (Karaca and Ayyıldız, 2018). Although they can kill the pests in a short time, these chemicals are hazardous to the environment and human health (Altukat et al, 2009). In addition, the wide use of insecticides also causes pests to gain resistance to these chemicals (Çakır and Yamanel, 2005). Due to all such problems, alternative methods are continually under investigation.

The use of entomopathogenic fungi (EF) is an alternative control technique against stored-product pests. The hyphae of pathogenic fungi develop in the insect’s epidermis and continue to proliferate in the body and hemolymph, causing the death of the insect (Deacon, 1983).

In many studies, promising results have been obtained with the use of EF in laboratory tests against stored-product pests (Michalaki et al, 2006; Mahdneshin et al, 2009; Wakil et al, 2010; Wakil et al, 2011; Şahin and Er, 2014; Sewify et al, 2014; Er et al, 2015). There are commercially available EF products (Um et al., 2017). Diatomaceous earth (DE) is another alternative to insecticides that are used against stored-product pests. DE is non-toxic to mammals and does not leave residues in commodities and is in the GRAS (generally recognized as safe) category, according to the Environment Protection Agency of the USA, and thus it is possible to use DE as a food additive (FDA, 1995). Diatomaceous earth causes insects to dehydrate and die by absorbing the oils and fats from the cuticle of the insect's exoskeleton. Its sharp edges are abrasive, which speeds up the process. It remains effective as long as it is kept dry and preserved (Bunch, et al, 2013). Turkish DE that could be used commercially were reported by Doğanay et al (2014). Studies have shown that commercially available DEs are highly effective against stored product pests (Athanassiou et al., 2003; Athanassiou et al, 2005; Beriş et al, 2010). Furthermore, it has been shown that the combined use of EFs and DEs has a high efficacy against stored product pests at low concentrations (Vassilakos et al, 2006; Athanassiou et al., 2007a; Athanassiou et al, 2008; Riasat et al, 2011; Sabbour et al, 2012; Shafighi et al, 2014).

In this laboratory study, the efficacy of the combined use of the EF, *Beauveria bassiana* (Balsamo) Vuillemin, and a DE, against *Rhyzopertha dominica* (F.), was investigated under three different relative humidities (RHs).

**Materials and Methods**

**Fungal culture**

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The EF (B. bassiana) cultures were started with spores isolated from R. dominica collected in Adana Province in Turkey. Specifically, spores of the two Turkish isolates of B. bassiana (5-4, 1-1) used in testing were mass produced (Barış, 2016). After adding 1.5 g CaCO3 and CaSO4 per bag for getting rice as uniform to polyethylene bags containing rice, the bags were sterilized in an autoclave at 121°C for 20 minutes. When the completion of the autoclaving process was done, the polyethylene bags were allowed for 2 hours until they reached room temperature. Ten mL of spore suspension at 2x10^7 spores/mL was added to each polyethylene bag and mixed with the rice for inoculation purposes. The polyethylene bags were then sealed with a 30 cm wide bag sealer. Thereafter, the bags were kept for 14 days at 25±2°C and 12:12 hour photoperiod for fungal growth and sporulation. Following that procedure, the polyethylene bags were opened and kept at 25±2°C until the contents were dry. After that, the fungi spores were separated from the rice grains by sieving through a 500 μm sieve. The obtained spores were kept at +4°C until used for testing.

**Insect culture**

The R. dominica culture was obtained from samples collected from grain storages in Kahramanmaraş, Turkey. Wheat was used for the culturing of R. dominica. For getting plenty of R. dominica, many adults were placed in 1 L glass jars with 250 g of wheat, and for oviposition purposes were kept for three days at 30±5°C and 65±5% RH in continuous darkness. The eggs were then separated from the wheat and added to new jars which containing clean wheat. In this way, the continuity of the culture was ensured during the study period. One week old females and males from this culture were used for testing.

**Diatomaceous earth**

Diatomaceous earth (coded CAN) was collected from DE reserves located in central Anatolia, Turkey.

Table 1. Description of the diatomaceous earth used with Beauveria bassiana against Rhyzopertha dominica

| DE Code | SiO₂ (%) | Median particle size* (µm) |
|---------|----------|---------------------------|
| ACN     | 73.80    | 14.297                    |

*The analyses were carried out in the Analysis Laboratories of the General Directorate of Mineral Research and Exploration, Turkey

**Experimental procedure**

The germination rate of the B. bassiana spores was evaluated before each experiment. A dilute suspension of conidia in sterile, distilled water including 0.01% Tween 80, was spread on PDA and incubated for 24 h at 25±2°C in darkness. One hundred spores were checked then under a light microscope (Olympus BX51) and those with a germination tube with a length at least equal to the diameter of the spore
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Centrifuge tubes of 50 mL capacity were used for the bioassays and each tube had 40 g of wheat added to it. Three hundred ppm EF+300ppm DE were added to each tube and shaking for 5 minutes by hand. Finally, 20 insects were placed in each tube. The same procedure was used to test the EF and DE individually. For the control units, wheat alone was used. Tests were performed at 25°C and 30°C at 45%, 55% and 65% RH. Potassium nitrite (KNO$_3$), calcium nitrate (Ca(NO$_3$)$_2$) and sodium nitrite (NaNO$_2$) saturated salt solutions were used to achieve the required RH levels (Greenspan, 1977). Dead insects were taken into moisture circle which is in petri dishes, fungal growth on the insects was monitored and mortalities were evaluated according to fungal growth on adults. Insect mortalities were recorded after 14 days for the statistical analyses.

**Statistical analysis**

After the mortality rates were corrected by using Abbott's formula (Abbott, 1925), the statistical analyses were performed. After arcsine transformation was used to fit the data, the 7th and 14th-day mortality rates were subjected to variance analysis (one-way ANOVA) by using the SPSS 24 statistics program (SPSS Inc., 2015). Differences between treatments were determined by using the Duncan multiple comparison test at the 5% significance level. In the tests with two treatments, the t-test was applied to determine the differences between mean mortalities.

**Results and Discussion**

The 7-day mortality rates for *R. dominica* adults obtained at three different relative humidities at 25°C are given in Table 2. According to the one-way ANOVA results, there was a significant difference between the effects of the applied agents on the death rates of adult *R. dominica* at 25°C ($F_{2,36}=50.766$, $P<0.001$); moreover, it was determined that ambient humidity affects the mortality rate ($F_{2,36}=4.572$, $P=0.017$) but the interaction between the two factors was not significant ($F_{4,36}=2.034$, $P=0.110$). While there was no significant difference for RH for the *B. bassiana* 5-4 isolate and ACN combination, the activity of *B. bassiana* 1-1 was statistically lower at 65% RH. The 14-day mortality rates of *R. dominica* adults obtained with the three different RH treatments at 25°C are given in Table 3. According to the one-way ANOVA results, there was a significant difference between the effects of the applied agents on the death rates of adult *R. dominica* ($F_{2,36}=30.664$, $P<0.001$), ambient humidity did not significantly affect the mortality rate ($F_{2,36}=2.378$, $P=0.107$), and the interaction between the two factors was significant ($F_{4,36}=0.281$, $P=0.030$). In addition, isolate 5-4 caused a significantly higher mortality level than ACN in all three RH environments.
Table 2. Corrected mortality at 7 days for the application of a Turkish diatomaceous earth (ACN) and two isolates of Beauveria bassiana (5-4 and 1-1) to Rhyzopertha dominica adults at 25°C at three ambient relative humidities

| Treatments     | Corrected mortality (%)* ± SEM | F and P values |
|----------------|---------------------------------|---------------|
|                | 45%                             | 55%           | 65%           |                  |
| 300 ppm 5-4 EP | 59.55±3.57                      | 63.15±6.14    | 64.54±4.12    | F$_{2,12}$=0.296 |
|                | Aa                              | Aa            | Aa            | P=0.749          |
| 300 ppm 1-1 EP | 56.09±4.74                      | 66.81±3.57    | 44.78±3.27    | F$_{2,12}$=7.936 |
|                | Ab                              | Aa            | Bb            | P=0.006          |
| 300 ppm ACN    | 13.73±4.71                      | 31.68±6.42    | 21.59±4.81    | F$_{2,12}$=2.664 |
|                | Ab                              | Ab            | Ac            | P=0.111          |
| Control        | 13.00±2.55                      | 9.00±4.30     | 4.00±1.87     |                |
| F and P values | F$_{2,12}$=21.883                | F$_{2,12}$=11.298 | F$_{2,12}$=22.462 |             |

Differences between the means were determined according to the Duncan test at the 5% significance level. Different uppercase letters in the same row and different lowercase letters in the same column indicate that the means are significantly different from each other (n=5).

Table 3. Corrected mortality at 14 days after the application of a Turkish diatomaceous earth (ACN) and two isolates of Beauveria bassiana (5-4 and 1-1) against Rhyzopertha dominica adults at 25°C at three ambient relative humidities

| Treatments     | Corrected mortality (%)* ± SEM | F and P values |
|----------------|---------------------------------|---------------|
|                | 45%                             | 55%           | 65%           |                  |
| 300 ppm 5-4 EP | 89.65±3.89                      | 93.89±3.66    | 88.56±4.38    | F$_{2,12}$=2.285 |
|                | Aa                              | Aa            | Aa            | P=0.98           |
| 300 ppm 1-1 EP | 78.43±7.54                      | 83.45±4.28    | 65.61±3.74    | F$_{2,12}$=0.524 |
|                | Aab                             | Aa            | Ab            | P=0.98           |
| 300 ppm ACN    | 52.68±10.52                     | 53.59±9.17    | 43.40±6.16    | F$_{2,12}$=2.285 |
|                | Ab                              | Ab            | Ac            | P=0.605          |
| Control        | 14.00±2.44                      | 9.00±4.30     | 6.00±2.91     |                |
| F and P values | F$_{2,12}$=6.008                 | F$_{2,12}$=12.218 | F$_{2,12}$=16.724 |             |

Differences between the means were determined according to the Duncan test at the 5% significance level. Different uppercase letters in the same row and different lowercase letters in the same column indicate that the means are significantly different from each other (n=5).

The 7 and 14 day mortality rates obtained with the application of the combinations of ACN and B. bassiana isolates against R. dominica adults at 25°C and three different relative humidities are given in Tables 4 and 5. There were no differences in the 7th day mortalities obtained from the applications in the different RH treatments. However, at day 14, the application of the combination of 1-1+ACN caused a significantly lower level of mortality at 65% RH. Although the effect of the combinations was generally not significantly different on days 7 and 14, a significant difference was found between mortalities on day 7 at 65% RH (Table 4).
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Table 4. Corrected mortality rates 7 days after the application of combinations of Turkish diatomaceous earth (ACN) and isolates of *Beauveria bassiana* (5-4 and 1-1) against *Rhyzopertha dominica* adults at 25°C and three ambient relative humidities

| Treatments               | Corrected mortality (%) ± SEM | F and P values |
|--------------------------|-------------------------------|----------------|
|                          | 45%                           | 55% | 65%        |
| 300 ppm 5-4 EP+300 ppm ACN | 72.16±4.84 82.23±3.67 74.81±5.99 | F2,12=1.234 P=0.331 |
| 300 ppm 1-1 EP+300 ppm ACN | 68.35±5.43 64.91±6.69 51.83±4.99 | F2,12=2.241 P=0.149 |
| Control                  | 13.0±2.54 9.0±4.30 4.0±1.87  |                |
| T and P values           | T0=0.505 T1=2.231 T2=2.988 | P=0.627 P=0.56 P=0.017 |

*Differences between the means were determined with the Duncan test at the 5% significance level. Different uppercase letters in the same row and different lowercase letters in the same column indicate that the means are significantly different from each other (n=5).*

Table 5. Corrected mortality rates 14 days after the application of combinations of Turkish diatomaceous earth (ACN) and two isolates of *Beauveria bassiana* against *Rhyzopertha dominica* adults at 25°C and three ambient relative humidities

| Treatments               | Corrected mortality (%) ± SEM | F and P values |
|--------------------------|-------------------------------|----------------|
|                          | 45%                           | 55% | 65%        |
| 300 ppm 5-4 EP+300 ppm ACN | 92.86±2.87 97.61±1.47 91.62±4.86 | F2,12=0.891 P=0.436 |
| 300 ppm 1-1 EP+300 ppm ACN | 96.47±1.44 92.12±4.09 79.99±3.26 | F2,12=5.551 P=0.020 |
| Control                  | 14.0±2.44 9.0±2.44 6.0±2.91  |                |
| T and P values           | T0=0.505 T1=0.505 T2=0.505 | P=0.627 P=0.627 P=0.627 |

*Differences between the means were determined with the Duncan test at the 5% significance level. Different uppercase letters in the same row and different lowercase letters in the same column indicate that the means are significantly different from each other (n=5).*

The 7-day mortality rates of adult *R. dominica* obtained from the treatments at 30°C under three different RH conditions are given in Table 6. According to the one-way ANOVA results, there was a significant difference between the effects of the applications on the mortality rate of adult *R. dominica* (F2,36=72.124, P<0.001). On the other hand, RH did not affect mortality (F2,36=0.693, P=0.507). Also, the interaction between the two applications was not significant (F4,36=0.764, P=0.556). In addition, in all three RH environments, there was no significant difference between the effects of the two fungal isolates. Separately, the mortalities caused by both isolates were significantly higher than for ACN on day 7.
The day 14 mortality rates at 30°C are presented in Table 7. One-way ANOVA demonstrated that the two *B. bassiana* applications and ambient humidity did not have a significant effect on mortality ($F_{2,36}=43.590, P<0.001; F_{2,36}=3.499, P=0.041$, respectively) and the interaction between them was not significant ($F_{4,36}=1.224, P=0.318$).

Table 6. Corrected mortality of *Rhyzopertha dominica* adults 7 days after the application of a Turkish diatomaceous earth (ACN) and two isolates of *Beauveria bassiana* at three ambient relative humidities at 30°C

| Treatments   | Corrected mortality (% ) ± SEM | F and P values |
|--------------|--------------------------------|---------------|
|              | 45%                            | 55%           | 65%           |
| 300 ppm 5-4  | 49.13±4.74                     | 57.63±8.15    | 54.0±5.09     |
| EP           | Aa                             | Aa            | Aa            |
| 300 ppm 1-1  | 57.54±5.12                     | 53.52±4.68    | 45.0±3.53     |
| EP           | Aa                             | Aa            | Aa            |
| 300 ppm ACN  | 7.0±4.89                       | 10.31±3.33    | 8.0±2.54      |
| Control      | 7.0±3.74                       | 3.0±1.22      | 0±0           |

Table 7. Corrected mortality of *Rhyzopertha dominica* adults at 14 days after the application of a Turkish diatomaceous earth (ACN) and two isolates of *Beauveria bassiana* at three ambient relative humidities at 30°C

| Treatments   | Corrected mortality rates (%)* ± SEM | F and P values |
|--------------|--------------------------------------|---------------|
|              | 45%                                  | 55%           | 65%           |
| 300 ppm 5-4  | 70.99±5.02                          | 83.15±5.61    | 69.80±4.57    |
| EP           | Aa                                   | Aa            | Aa            |
| 300 ppm 1-1  | 83.85±1.19                          | 81.15±5.46    | 83.40±4.30    |
| EP           | Aa                                   | Aa            | Aa            |
| 300 ppm ACN  | 26.63±8.63                          | 49.21±9.94    | 25.64±6.59    |
| Control      | 8.0±3.39                            | 4.0±1.0       | 4.0±1.87      |

*R. dominica* adult mortalities after the applications of different combinations of a Turkish DE and *B. bassiana* isolates at three different relative humidities and 30°C are given in Table 8 (day 7 mortalities) and Table 9 (day 14 mortalities). There was no significant difference in the effects of both combinations in all RH environments
The effects of *Beauveria bassiana* and diatomaceous earth against the lesser grain borer on both day 7 and day 14. Corrected mortality rates 7 days after the application of combinations of local diatomaceous earth ACN and isolates of *Beauveria bassiana* (5-4 and 1-1) against *R. dominica* adults at 30°C and at three ambient relative humidities.

Table 8. Corrected mortality of *Rhyzopertha dominica* adults 7 days after the application of a Turkish diatomaceous earth (ACN) and two isolates of *Beauveria bassiana* at three ambient relative humidities at 30°C

| Treatments                        | Corrected mortality (% ) ± SEM | F and P values |
|-----------------------------------|--------------------------------|----------------|
|                                   | 45%                           | 55%            | 65%            |
| 300 ppm 5-4 EP+300 ppm ACN       | 76.7±4.83                     | Aa             | 77.26±4.96     | Aa             | 68.0±4.89     | Aa             |
| 300 ppm 1-1 EP+300 ppm ACN       | 79.8±2.08                     | Aa             | 74.10±5.27     | Aa             | 72.0±3.0     | Aa             |
| Control                           | 7.0±3.74                      | 3.0±1.22       | 0±0            |                |               |                |
| T and P values                    |                               |                | T₃=0.501      | P=0.630        | T₃=0.219      | P=0.832        |

*Differences between the means were determined with the Duncan test at the 5% significance level. Different uppercase letters in the same row and different lowercase letters in the same column indicate that the means are significantly different from each other (n=5).*

Table 9. Corrected mortality rates 14 days after the application of combinations of a Turkish diatomaceous earth ACN and isolates of *Beauveria bassiana* (5-4 and 1-1) against *Rhyzopertha dominica* adults at 30°C and at three ambient relative humidities

| Treatments                        | Corrected mortality (% ) ± SEM | F and P values |
|-----------------------------------|--------------------------------|----------------|
|                                   | 45%                           | 55%            | 65%            |
| 300 ppm 5-4 EP+300 ppm ACN       | 92.73±3.91                    | Aa             | 91.57±4.27     | Aa             | 86.57±3.72   | Aa             |
| 300 ppm 1-1 EP+300 ppm ACN       | 93.42±1.94                    | Aa             | 88.52±6.74     | Aa             | 88.51±1.09   | Aa             |
| Control                           | 8.0±3.39                      | 4.0±1.0        | 4.0±1.87       |                |               |                |
| T and P values                    |                               |                | T₃=0.219      | P=0.832        | T₃=0.238      | P=0.818        | T₃=0.278      | P=0.788        |

*Differences between the means were determined with the Duncan test at the 5% significance level. Different uppercase letters in the same row and different lowercase letters in the same column indicate that the means are significantly different from each other (n=5).*

When the effect of RH on the efficacies of the treatments against *R. dominica* was determined, lower effects of the *B. bassiana* isolate1-1 on 7th day and 1-1+ACN on the 14th day were observed at 65% RH and 25°C. In general, the efficacies of treatments at both temperatures (25°C and 30°C) were not different in the RH range tested.
The relative humidity is one of the crucial factors in the determination of the efficacy of both fungus and DE (Moore et al., 2000). It has been reported that DEs are not very effective against stored product insects at high RH values (Fields and Korunic, 2000; Vayias and Athanassiou, 2004). In the previous studies, it is understood that the decrease in the effectiveness of the combination in high humidity, environments were due in particular to the effect of RH on the ACN in the combination. Stathers et al. (2004) reported that RH changes the effect of DE on insects and thus our findings support theirs. Athanassiou et al. (2014) also reported that RH affects the efficacy of DE. In their application of 200 ppm DE, 99.4% mortality was achieved at 55% humidity after 14 days, while it was 86.1% at 75% humidity. The results of both studies indicate that high RH decreases the effectiveness of DE.

In conclusion, ambient humidity in the tested range did not change the level of effectiveness of combined applications of EF and DE on the pest insect. Our results demonstrate that B. bassiana can be used successfully in conjunction with DE against R. dominica under laboratory conditions. It is important to test these two factors in combination in a range of environments to better understand their interaction, with the goal of achieving greater efficacy of treatment. Separately, the search for a fungal strain that is equally or more effective against R. dominica at low RH levels should be prioritised.

Acknowledgments
This study, which was accepted as an abstract for oral presentation at the 7th International Entomopathogens and Microbial Control Congress held from 11 to 13 September 2019 in Kayseri, Turkey is a partial summary of the first author’s master thesis and was supported by Kahramanmaras Sutcu Imam University Scientific Research Projects Unit (2016/3-38 YLS).

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