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Examining Some Cereals for Mass Production of *Beauveria bassiana* (Balsamo) Vuillemin Conidia by Solid State Fermentation

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ABSTRACT
This study was conducted to test five substrates (whole rice, broken rice, dövme, wheat and bulgur) for the mass production of *Beauveria bassiana* isolate 5-4, known to be affective against stored-cereal pests. A solid-state fermentation technique was used, and amount of product, number of conidia, germination, hydrophobicity and thermotolerance of conidia, and their virulence on *Rhyzopertha dominica* adults were used to evaluate the success of substrates. For production, 100 g of a substrate was used in a polypropylene bag. After sterilization and inoculation, the bags were sealed and incubated for 14 days at 25ºC. Hydrophobicity was assessed using the aqueous-solvent partitioning method with PM buffer, and liquid paraffin as two phases. Thermotolerance tests were conducted by exposing conidia to 35, 40 and 45°C for 15, 30 and 60 minutes. In virulence test, adults were released into 40 gr wheat mixed with conidia and kept at 25ºC and 65% relative humidity in darkness for 14 days. The highest amount of product was obtained by using whole rice (4.10g/bag), followed by broken rice, wheat, dövme and bulgur. The highest number of conidia was achieved using broken rice ($10.24x10^{10}$conidia/g) and whole rice ($9.62x10^{10}$conidia/g). The germination rates of conidia did not vary significantly. Significantly higher hydrophobicity rate was obtained using wheat (88.73%). The conidia from bulgur and wheat showed higher thermotolerance than those from other substrates. The conidia obtained from whole rice and broken rice caused 80.3% and 72.0% mortalities, respectively, significantly higher than the others. Rice was found to be a better choice for this isolate as the ultimate purpose is to suppress pest populations, with awareness of its shortcomings. It was found crucial to test all important characteristics of produced conidia together for assessment of substrates.

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Beauveria bassiana Konidilerinin Katı Faz Fermantasyonu ile Kitlesel Üretimi için Bazı Tahılların İncelenmesi

ÖZET
Bu çalışma, depolanmış tahıl zararlılarına karşı etkili olduğu bilinen *Beauveria bassiana* izolatı 5-4'ün kitlesel üretimi için beş substratı (tam pirinç, kırık pirinç, dövme, buğday ve bulgur) test etmek için yürütülmüştür. Katı faz fermantasyon tekniği kullanılmış ve substratların başarısını değerlendirmek için ürün miktarı, konidi sayısı, çimlenme, hidrofobisite ve konidi termotoleransı ve elde edilen konidilerin *Rhyzopertha dominica* erginleri üzerindeki virülenslikleri kullanılmıştır. Üretim için, polipropilen bir torba içinde 100 g substrat sterilize edilmiş ve inokulasyondan sonra torbalar kapatılıp 25°C sıcaklıkta 14 gün inkübe edilmiştir. Hidrofobisite, iki faz olarak likid paraffin ve PM tamponu ile ayrıştırma yöntemi kullanılarak değerlendirilmiştir. Termotolerans testleri, konidilerin 35, 40 ve 45°C sıcaklığa 15, 30 ve 60 dakika süreyle maruz bırakılmasıyla gerçekleştirilmiştir. Virülenslik testinde erginler, konidi ile muamele

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edilmiş olan 40 gr buğdayın içine bırakılmış ve 25°C sıcaklık ve %65 nispi nemde karanlık ortamda 14 gün bekletilmiştir. En yüksek ürün miktarı tam pirinç (4.10 gr/torba) kullanılarak elde edilirken, bunu kırık pirinç, buğday, dövme ve bulgur izlemiştir. En yüksek konidi sayısı, kırık pirinç ($10.24x10^{10}$konidi/g) ve tam pirinç ($9.62x10^{10}$konidi/g) kullanılarak elde edilmiştir. Konidilerin çimlenme oranları önemli ölçüde değişmemiştir. Buğday kullanılarak önemli ölçüde daha yüksek hidrofobisite oranı elde edilmiştir (%88.73). Bulgur ve buğdaydan elde edilen konidiler, diğer substratlardan elde edilen konidilere kıyasla daha yüksek termotolerans göstermiştir. Tam pirinç ve kırık pirinçten elde edilen konidi, sırasıyla %80.3 ve %72.0 ile diğerlerinden önemli ölçüde daha yüksek ölüme neden olmuştur. Zayıf özellikleri bulunmakla birlikte, nihai amaç zararlı popülasyonunu baskılamak olduğundan bu izolat için princin daha iyi bir seçim olduğu belirlenmiştir. Substratların değerlendirilmesinde üretilen konidilerin tüm kritik özelliklerinin birlikte değerlendirilmesinin çok önemli olduğu bulunmuştur.

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INTRODUCTION

Avoiding qualitative and quantitative loss due to insect pests during storage periods is quite important and challenging for agricultural commodities (Moino et al., 1998; Padin et al., 2002). The most commonly practiced method to suppress the populations of these pests is the use of synthetic chemical insecticides (Athanassiou and Palyvos, 2006). However, because of the adverse effects associated with such chemicals on non-target organisms, environment and humans there has been an increasing demand for alternative and more eco-friendly options (Arthur, 1996; Michalaki et al., 2007). The use of entomopathogenic fungi is an acceptable alternative to synthetic insecticides to control a variety of pests (Wraight and Carruters, 1999; Lomer et al., 1999; Wraight et al., 2001). Entomopathogenic fungi have also been investigated as alternatives for the management of stored grain pests. A number of studies demonstrated this potential with a range of success depending on both fungus and pest species (Rice ve Cogburn, 1999; Cherry et al., 2005; Shams et al., 2011; Sewify et al., 2014). In these studies, researchers have focused on *Beauveria bassiana* being a potential species that can be developed as a biological control agent in grain storages. In order to demonstrate its potential in actual storage conditions, its application in larger quantities of grains needs to be fulfilled. That in turn requires mass production of *B. bassiana* spores with an appropriate procedure, as the procedure itself can have effects on the ultimate success of the fungus to control grain pests.

Mass production of entomopathogenic fungi for the management of pest insects has been investigated for producing three types of fungal propagules. One of them is fungal vegetative cells called blastoconidia, which are produced in liquid culture media as submerged. The second propagule type is mycelium, which are grown in liquid fermentation. The most resistant and more persistent stage of the fungal life cycle, conidia, is the third propagule type. These conidia can be produced by surface culturing on a solid medium. The medium is usually inoculated with blastoconidia obtained from culturing in liquid fermentation. Conidia produced using solid media have a hydrophobic property, which is important for their adherence to targeted insect surface. Besides, the shelf life of conidia produced in solid media can have a longer shelf life (Moore and Higgins, 1997).

Furthermore, in mass production procedures, additive substances have various effects on the properties of entomopathogenic fungi in the final product (Humphreys et al.,1989; Ying and Feng, 2004; Kassa et al., 2008; Gangwar, 2013; Patil et al., 2014). It is also known that properties of yielded conidia can be altered depending on ambient conditions like temperature, humidity and pH together with the water activity of the product (Borisade and Magan, 2014; Lizzy et al., 2015). This study was conducted to test various mass production substrates for developing a mass production procedure of the conidia of a *B. bassiana* isolate (5-4), known to be affective against stored-cereal pests. A solid-state fermentation technique was chosen as the production technique as the final product will need to be a long lasting powder with a hydrophobic property, so that the product can be mixed into cereal kernels, can stay for required period of time for sufficient efficacy, and can adhere easily to the insect surface.

MATERIAL and METHOD

Insect culture

Rhyzopertha dominica (F.) (Coleoptera: Bostrichidae) adults were obtained from a laboratory culture

originally established from individuals collected in Kahramanmaraş. The cultures were maintained under controlled conditions at 30±5 ºC and 65±5% R.H in darkness and kept on wheat (*Triticium aestivum*). One week old adults were used for pathogenicity tests.

Fungus culture and inoculum preparation

B. bassiana isolate used in tests (5-4) is a single spore culture of *B. bassiana* 151138, which was originally isolated from a *R. dominica* adult. This isolate was taken from the Department of Plant Protection, University of Kahramanmaraş Sütçü İmam, Turkey.

In order to prepare inoculum for mass production, the fungus was grown on PDA (potato dextrose agar) in sealed Petri plates for 14 days at 25±2º C in dark and the conidia in the cultures were harvested in 10 ml sterile distilled water with 0.01% Tween80. Spore count of the suspension was determined by using a hemocytometer and the suspension concentration was adjusted to $2x10^7$conidia/ml.

Production of conidia

Whole rice, broken rice, dövme, wheat and bulgur were used as substrates for mass production of *B. bassiana* conidia. 100 g of each substrate was placed individually in an autoclavable polypropylene bag (25cmx38cm). Tap water was added into the bags and they were kept overnight. Thereafter, excess water was removed and 1.5g $CaSO_4$ and 1.5g $CaCO_3$ were added and mixed thoroughly to get a uniform coating over the substrate (Nirmala et al., 2005). Then, the prepared bags were sterilized by autoclaving at 121 ºC for 20 minutes. After cooling, each bag was inoculated with 10 ml of inoculum (conidial suspension at $2x10^7$conidia/ml concentration). The bags were sealed and incubated for 14 days at 25±2ºC and 12:12h light:dark photoperiod Once the fungi in bags sporulated, the bags were opened and the contents were allowed to dry at 25º C until the moisture content was low enough for sieving to separate the conidia from the substrate. The obtained product was stored at +4° C until it is used. The whole process was carried out in 5 replications for each substrate.

Obtained conidia from each replication was weighed by using a precision scale and recorded. In order to determine count of conidia in the products, one milligram conidia was mixed within 10 ml of 1% Tween 80 solution and vortexed. The concentration of the conidial suspension was determined by counting on a hemocytometer under a light microscope at 40x magnification. This process was repeated five times for each sample. Number of conidia per gram of product was then calculated.

Germination tests

After determining the concentration, each conidial suspension was adjusted to the concentration of $1x10^6$ conidia/ml before spreading 100µl on PDA. They were incubated 24 hours at 25±2° C in darkness. Thereafter, the conidia were examined under a light microscope at 40x magnification and those with germ tubes equal or longer than the conidia were considered germinated. The germination ratios were calculated after examining a minimum of 300 conidia from each of the five replicate plates.

Determination of hydrophobicity

Conidial hydrophobicity was tested according to the aqueous-solvent partitioning method described by Shah et al. (2007). Obtained conidia in the products were suspended in PM buffer containing 0.02% (v/v) Tween 80, and the concentration of the suspension was adjusted to $2x10^7$ conidia/ml. As the organic phase, 40 µl liquid paraffin was added to each 4 ml of the suspension. The mixture was vortexed for 3 minutes. After the two phases (organic phase and aqueous phase) are separated from each other, the number of conidia in the aqueous phase was found by counting on a Neubauer hemocytometer under a microscope. The test was repeated five times for conidia obtained from each substrate. The hydrophobicity rate (Hr) was calculated as $Hr = (1-C/C_0) \times 100$, where C_0 is concentration of conidia in the suspension before addition of liquid paraffin, and C is the concentration of conidia in the aqueous phase after the separation of two phases.

Determination of thermotolerance

The conidia from the products using different substrates were subjected to thermotolerance tests at 35, 40 and 45° C. For each replication of each product, 1 mg of conidia was added into 10 ml of 0.1% Tween 80. The suspension was mixed using vortex and then 1 ml of suspension was transferred into an Eppendorf tube. The tubes were heated in a water bath at above mentioned temperatures for 15, 30 and 60 minutes. The tests were conducted with five replications and the viability of treated and non-treated control conidia was determined by the germination tests.

Virulence of conidia

For each treatment, 40 gr durum wheat homogenously mixed with the required amount of conidia to provide the concentrations of $2x10^7$ conidia/g wheat in a 50 ml centrifuge tube. Twenty *R. dominica* adults were placed inside each tube. For control, clean wheat without fungal conidia was used. All the experimental tubes were placed in a humidity chamber, a sealed plastic container (45x31x18.5 cm) with saturated $NaNO_2$ solution. The experiment had four replications for each product, and was conducted at 25±2° C and 65±5% relative humidity in darkness. Adult mortalities were recorded 14 days after treatment.

Statistical Analysis

The data (hydrophobicity, thermotolerance, germination rates, conidia amounts and mortality rates) were subjected to one-way ANOVA followed by Tukey multiple comparison test ($P \leq 0.05$) and were arcsine transformed prior to the statistical analysis. All statistical analyses were conducted by using SPSS statistics program.

RESULTS

Amount of conidia, their germination and hydrophobicity

Beauveria bassiana conidia were mass produced on different substrates and obtained amount of products, number of conidia in the products, germination rates and hydrophobicity of the conidia were presented in Table 1. Substrate was significantly important in terms of amount of product, number of conidia and hydrophobicity, while germination of conidia in all cases were similarly high. The highest amount of product was obtained by using whole rice as substrate (4.10 g/bag), followed by broken rice (3.20 g/bag), wheat (2.01 g/bag), dövme (1.61 g/bag) and bulgur (1.23 g/bag). The highest number of conidia was achieved using broken rice (10.24×10^{10} conidia/g) and whole rice (9.62×10^{10} conidia/g) while the other substrates resulted in significantly lower number of conidia. The germination rates of conidia obtained from different substrates did not show any significant differences ($F_{4,20}= 0.61$; $P=0.658$) and all had a germination rate of 95% or higher. The hydrophobicity rates of conidia varied between 81.60% and 88.73%. The highest hydrophobicity rate was obtained using wheat as substrate (88.73%). This was significantly higher than the rest, while the differences among the rest were insignificant.

Table 1. Effects of substrate in mass production of *Beauveria bassiana* conidia in terms of amount, germination and hydrophobicity (±s.e.)

Çizelge 1. *Beauveria bassiana konidilerinin kitlesel üretiminde substratların hidrofobisite, çimlenme ve miktar açısından etkisi(±s.h.)*

Substrates	Amount of product per bag (g)	Number of conidia in products ($x10^{10}$conidia/g)	Germination (%)	Hydrophobicity rate (%)
Whole rice	4.10±0.21 a	9.62±0.15 a	95.72±1.18 a	83.04±0.77 b
Broken rice	3.25±0.20 b	10.24±0.09 a	94.93±1.30 a	82.10±0.97 b
Dövme	1.61±0.12 cd	3.06±0.63 b	96.20±0.62 a	82.13±0.36 b
Wheat	2.10±0.17 c	0.87±0.15 c	97.13±0.50 a	88.73±0.64 a
Bulgur	1.23±0.05 d	0.48±0.10 c	96.33±0.64 a	81.60±0.73 b
ANOVA results	$F_{4,20}$=35.16 P<0.0001	$F_{4,20}$=241.9 P<0.0001	$F_{4,20}$=0.61 P=0.658	$F_{4,20}$= 16.71 P<0.0001

* Different letters within each column indicate significant differences according to Tukey multiple comparison test (P<0.05).

Thermotolerance of conidia

Germination rates of *B. bassiana* conidia exposed to high temperatures for 15, 30 and 60 mins. were presented in Tables 2, 3 and 4, respectively. The germination rates of untreated conidia were >95% for all substrates at 24 hours. Examining all the tables shows that temperature, exposing time and substrate are important factors for the thermotolerance of *B. bassiana* conidia. When exposed to 35°C for 15 mins, tolerance of conidia did not differ except for those obtained using bulgur, which tolerated significantly better than the rest. Bulgur was better than the rest except for wheat as substrate for conidial tolerance at 40°C for 15 mins (Table 2). Substrate did not significantly change the tolerance of conidia at 35° C for 30 mins. After exposing to 40° C for 30 mins, conidia from bulgur, wheat and dövme germinated better than those obtained using whole and broken rice (Table 3). Treatment of the conidia for 60 mins at 35° C did not cause any significant difference while at 40° C conidia grown on whole and broken rice were more susceptible than the others (Table 4). At all exposure times, *B. bassiana* conidial germination was impeded severely at 45° C. Overall the conidia from bulgur and wheat showed higher germination rates than the others.

Virulence of conidia

Rhyzopertha dominica adult mortalities caused by application of *B. bassiana* conidia produced using different substrates are presented in Figure 1. There were significant differences among obtained *R. dominica* adult mortalities ($F_{4,20}$=35.16; P<0.0001). The most virulent conidia were produced on rice while the least virulent conidia were produced on wheat and bulgur. At 14 days post-treatment, the conidia obtained from whole rice and broken rice caused 80.3% and 72.0% mortality, respectively. However, the conidia obtained from dövme, wheat, bulgur caused significantly lower mortalities, 56.7%, 43.5%, 39.3%, respectively.

DISCUSSION

Five different substrates were evaluated for developing a mass production procedure of the conidia of a *B. bassiana* isolate (5-4), efficiency of which has previously been demonstrated to be high against

stored-cereal pests. The results show that both quantity (amount of product and number of conidia) and quality (germination rate, hydrophobicity rate and thermotolerance) of conidia were influenced by substrate used for production. The only characteristic that did not show significant change depending on substrate was the germination rate of *B. bassiana* conidia in this study. It seems that once conidia were produced by the fungus, almost all are capable of germinating, at least soon after production.

The tested substrates significantly affected the quantity of conidia and the highest yield of conidia was obtained using rice, which is used predominantly for mass production of entomopathogenic fungi (Mar and Lumyong, 2012; Ye et al., 2006; Cherry et al., 1999; Nirmala et al., 2005; Patil et al., 2011). There are numerous studies demonstrating the significance of substrate or nutrient content of the medium where entomopathogenic fungi are produced on the virulence of obtained conidia (Feng et al., 2000;Shah et al., 2005;

Table 2. Effect of exposing *Beauveria bassiana* conidia to high temperatures for 15 minutes on their germinations (%) (±s.e.)

Çizelge 2. *Beauveria bassiana konidilerinin yüksek sıcaklıklara 15 dakika maruz bırakılmasının çimlenmeleri üzerine etkisi (%) (±s.h.)*

Temperature / Substrates	35°C	40°C	45°C	ANOVA results
Whole rice	91.66 ± 1.26 bA	83.00 ± 2.41 cdB	26.00 ± 1.70 bC	$F_{2,12}$=148.135 P<0.0001
Broken rice	93.00 ± 1.28 bA	78.00 ± 2.02 dB	46.66 ± 1.42 aC	$F_{2,12}$=98.651 P<0.0001
Dövme	89.66 ± 0.51 bA	86.66 ± 0.96 bcA	14.00 ± 1.12 cB	$F_{2,12}$=947.471 P<0.0001
Wheat	92.66 ± 1.00 bA	91.66 ± 0.54 abA	3.00 ± 0.70 dB	$F_{2,12}$=2212.345 P<0.0001
Bulgur	97.00 ± 0.51 aA	96.66 ± 0.85 aA	22.66 ± 0.99 bB	$F_{2,12}$=1357.107 P<0.0001
ANOVA results	$F_{4,20}$=14.195 P<0.0001	$F_{4,20}$=19.953 P<0.0001	$F_{4,20}$=91.827 P<0.0001	

* Different capital letters in a row and different small letters in a column indicate significant differences according to Tukey multiple comparison tests (P<0.05).

Table 3. Effect of exposing *Beauveria bassiana* conidia to high temperatures for 30 minutes on their germinations (%) (±s.e.)

Çizelge 3. *Beauveria bassiana konidilerinin yüksek sıcaklıklara 30 dakika maruz bırakılmasının çimlenmeleri üzerine etkisi (%) (±s.h.)*

Temperature / Substrates	35°C	40°C	45°C	ANOVA results
Whole rice	88.33 ± 1.13 aA	60.66 ± 3.23 bB	10.00 ± 0.77 bcC	$F_{2,12}$=164.468 P<0.0001
Broken rice	88.00 ± 1.29 aA	59.66 ± 4.13 bB	19.66 ± 1.53 aC	$F_{2,12}$=66.889 P<0.0001
Dövme	84.00 ± 0.88 aA	80.33 ± 1.04 aA	6.66 ± 0.90 cB	$F_{2,12}$=949.921 P<0.0001
Wheat	87.00 ± 0.85 aA	86.33 ± 0.57 aA	1.00 ± 0.57 dB	$F_{2,12}$=2939.383 P<0.0001
Bulgur	88.66 ± 0.70 aA	89.00 ± 0.55 aA	13.66 ± 0.69 bB	$F_{2,12}$=1902.601 P<0.0001
ANOVA results	$F_{4,20}$=2.765 P=0.056	$F_{4,20}$=16.064 P<0.0001	$F_{4,20}$=69.430 P<0.0001	

* Different capital letters in a row and different small letters in a column indicate significant differences according to Tukey multiple comparison tests (P<0.05).

Table 4. Effect of exposing *Beauveria bassiana* conidia to high temperatures for 60 minutes on their germinations (%) (±s.e.)

Çizelge 4. *Beauveria bassiana konidilerinin yüksek sıcaklıklara 60 dakika maruz bırakılmasının çimlenmeleri üzerine etkisi (%) (±s.h.)*

Temperature / Substrates	35°C	40°C	45°C	ANOVA results
Whole rice	74.00 ± 2.07 bA	27.00 ± 3.89 bB	3.66 ± 0.99 bcC	$F_{2,12}$=86.787 P<0.0001
Broken rice	78.00 ± 1.30 abA	28.33 ± 4.00 bB	5.00 ± 1.29 bC	$F_{2,12}$=94.934 P<0.0001
Dövme	76.00 ± 0.83 bA	59.66 ± 0.41 aB	2.33 ± 0.95 cC	$F_{2,12}$=1428.977 P<0.0001
Wheat	84.33 ± 0.38 aA	76.33 ± 1.05 aB	0.00 dC	$F_{2,12}$=1366.243 P<0.0001
Bulgur	84.66 ± 0.38 aA	84.00 ± 0.38 aA	10.33 ± 0.52 aB	$F_{2,12}$=4114.225 P<0.0001
ANOVA results	$F_{4,20}$=6.881 P<0.001	$F_{4,20}$=42.315 P<0.0001	$F_{4,20}$=61.119 P<0.0001	

* Different capital letters in a row and different small letters in a column indicate significant differences according to Tukey multiple comparison tests (P<0.05).

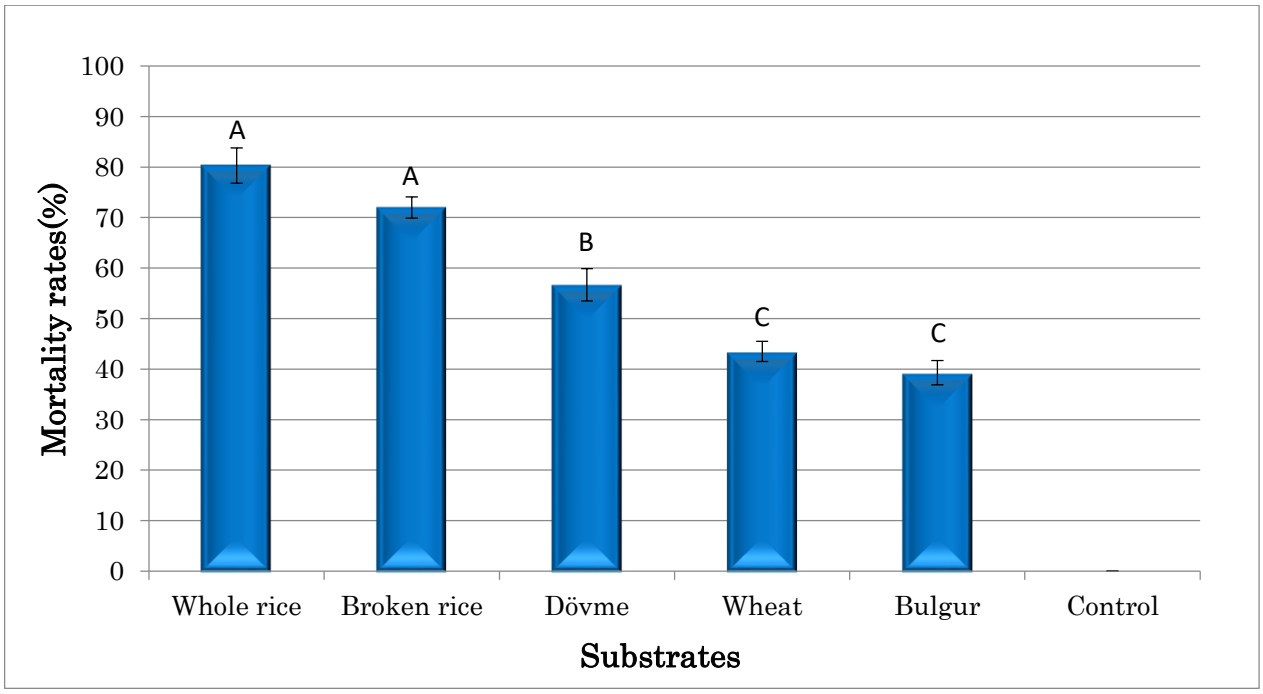

Fig. 1. Virulence of *Beauveria bassiana* conidia produced using different substrates against*Rhyzopertha dominica* adults at the concentration of 2x10^{7}conidia/g wheat (Bars represent standard errors, n=4)

Şekil 1. *Farklı substratlar kullanılarak üretilen Beauveria bassiana konidilerinin 2x10^{7}konidi/g buğday konsantrasyonda Rhyzopertha dominica erginlerine karşı virülenslikleri (Barlar standart hatayı belirtmektedir, n=4)*

Shah et al., 2007; Latifian et al., 2013). In the present study conidia with the highest virulence against *R. dominica* adults were obtained when whole or broken rice was used for mass production of *B. bassiana* conidia. This is probably another reason for rice to be commonly preferred in mass production of entomopathogenic fungi (Mar and Lumyong, 2012; Ye et al., 2006; Cherry et al., 1999; Nirmala et al., 2005; Patil et al., 2011). Although these results indicated that rice would be the best choice for production of *B. bassiana* conidia, some properties of the conidia were found to be better when other substrates were used. The conidial thermotolerance of entomopathogenic fungi has been shown to be significantly influenced by ambient conditions where fungi are grown. It was found that during mycelial growth, especially the substrate used can enhance conidial thermotolerance (Ying and Feng, 2006; Kim et al., 2011). The present study supports these results. The thermotolerance of conidia significantly varied depending on the substrate and the most tolerant conidia was obtained by using wheat and bulgur. Kim et al. (2011) and Ying and Feng (2004) found a positive relationship between thermotolerance and hydrophobicity of conidia. Similarly, according to the results of the present study, the conidia produced by using wheat showed both high thermotolerance and high hydrophobicity amongst tested substrates.

It is once more demonstrated that choice of substrate is a very important step in developing a mass production procedure for entomopathogenic fungi. Although the results may apply to most closely related entomopathogenic fungi, at least for mass production of the tested *B. bassiana* isolate (5-4), as substrate, rice should be preferred to achieve highly virulent more conidia while wheat should be the choice to reach more hydrophobic and thermotolerant conidia. In this study, all the tested characteristics were evaluated together for the first time for an entomopathogenic fungus. These results demonstrated that in the decision making stage for mass production of entomopathogenic fungi, all important characteristics should be assessed together. In this study, As the main purpose of evaluating substrates for the mass production of the tested *B. bassiana* isolate (5-4) is to obtain conidia for the control of stored cereal pests, rice can be selected as substrate to achieve high mortality and thus better suppression of the pest, while ways to improve their lower features are to be sought in feature researches.

Statement of Conflict of Interest

Authors have declared no conflict of interest.

Author's Contributions

The contribution of the authors is equal.

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