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EFFICACY OF NATIVE *BEAUVERIA BASSIANA* AND *B. PSEUDOBASSIANA* ISOLATES AGAINST INVASIVE BROWN MARMORATED STINK BUG, *HALYOMORPHA* HALYS (STÅL) (HEMIPTERA: PENTATOMIDAE)

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Abstract: Invasive brown marmorated stinkbug (BMSB), [*Halyomorpha halys* (Hemiptera: Pentatomidae)] are caused significant yield and quality losses in hazelnut orchards. This study evaluated the efficacy of 7 native *Beauveria bassiana* and *B. pseudobassiana* isolates against BMSB adults at 1×10^8 conidia mL^{-1} concentration under laboratory conditions. The LT_{50} and LT_{90} values for all isolates used in the study ranged between 5.37-7.74 and 9.85-18.35 days, respectively. Moreover, the mortality rates caused by all isolates were between 72 and 96%. The lowest LT_{50} value (5.37 days) was recorded for TR-SM-11, whereas the lowest LT_{90} (9.85 days) value was noted for TR-D-1 isolate. Similarly, the LT_{90} and LT_{50} values were 10.82 and 7.74 days for TR-SM-11 and TR-D-1, respectively. The LT_{90} and LT_{50} values for TR-SK-1 isolate were 6.16 and 10.25 days, respectively. These isolates (TR-D-1, TR-SK-1, TR-SM-11) caused the highest mortality rates (96, 96 and 92%, respectively) at the end of the 11th day. TR-SM-11, TR-D-1 and TR-SK-1 isolates of *B. bassiana* seemed to be one of the most promising and potential biological control agents against BMSB. However, further studies are needed to evaluate the efficacy of these isolates against BMSB under field conditions.

Keywords: Hazelnut, Invasive pest, BMSB, Entomopathogenic fungi, *Beauveria*, Biocontrol

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1. Introduction

Invasive brown marmorated stinkbug (BMSB), [*Halyomorpha halys* (Stål, 1855) (Hemiptera: Pentatomidae)] is a well-known and harmful insect. It is indigenous to China, Japan, Korea and Taiwan. It has a high infestation and spread rate throughout the world (Zhu et al., 2012). The BMSB was first recorded in North America during the mid-1990s in Pennsylvania (Hoebeke and Carter, 2003) and then spread in 44 states of the USA and 4 regions of Canada (Anonymous, 2018). In Europe, it was first detected in Switzerland in 2004 (Haye et al., 2014). Afterwards, it has been recorded in several European countries.

The BMSB is a polyphagous pest with a wide host range; therefore, capable of causing significant damage to approximately 300 plant species, including ornamental plants, agricultural crops, tree nuts and vegetables (Nielsen and Hamilton, 2009). Both adults and nymphs of the pest cause damage to plants by piercing the surface, injecting digestive enzymes and sucking plant fluids (Rice et al., 2014). Damaged products lose market value to significant extent. Moreover, significant economic losses are caused by the pest in pome and stone fruit, corn and hazelnut in the invaded areas (Bariselli et al., 2016). For this reason, excessive amounts of insecticides are applied against BMSB, which pose negative impacts to environment and human health. Moreover, excessive of insecticides disrupt integrated pest management (IPM) programs. Hence, the current studies concentrated on alternative, effective and eco-friendly management methods such as biological control (Moraglio et al., 2019).

Several natural enemies of BMSB have been identified from Asia (Leskey et al., 2012). The most promising biological control agents recorded are hymenopteran egg parasitoids, i.e., *Trissolcus japonicus* (Ashmead) and *T. mitsukurii* (Ashmead) (Hymenoptera: Scelionidae) (Yang et al., 2009). In addition, adventive populations of *T. mitsukurii* and *T. japonicus* were found out in northern and northwest northwest Italy (Peverieri et al., 2018; Moraglio et al., 2019), Switzerland (Stahl et al., 2019) and North America (Talamas et al., 2015). However, limited studies have been conducted regarding the efficacy of entomopathogenic fungi (EPF) against BMSB globally. Burjanadze (2020) reported that *B. bassiana* sensu lato (Hypocreales: Cordycipitaceae) and *Isaria cf fumosorosea* (Hypocreales: Cordycipitaceae) isolated from BMSB and caused 72-90.5% mortality of BMSB adults in Georgia. Similarly, a study conducted in the USA reported that *B. bassiana* strains were the most effective and caused 100% mortality in 12 days after the treatment (Gouli et al., 2012). In addition, Karun and Sridhar (2013) revealed that BMSB adults were naturally infected by *Ophiocordyceps nutans* (Ophiocordycipitaceae, Hypocreales) in the forests of Western Ghats of India and Japan. EPF infect insects through integument, rapidly colonize the hemocoel and then cause mortality through sporulation on the surface of the host under favorable environmental conditions (Butt et al., 2016).

Currently, the pest has established in many places along the Black Sea coast especially Artvin and Rize that continuing distrubution in Türkiye (Ak et al., 2019; Ozdemir and Tuncer, 2021). Considering the risk map prepared according to ecological requirements of BMSB in Türkiye (Kistner, 2017), hazelnut is under the major threat by BMSB in Black Sea region (Ozdemir and Tuncer, 2021). Erper et al. (2016) reported that EPF are suitable to use in the Black Sea region due to its rainy and humid environmental conditions and low annual temperature. Therefore, this study aimed to evaluate the efficacy of six native *B. bassiana* and one *B. pseudobassiana* isolates against adults of BMSB. The isolates were isolated from green shield bug [*Palomena prasina* L. (Hemiptera: Pentatomidae)], an important pest of hazelnut production areas (Ozdemir et al., 2021). The result will provide valuable insights on the possible use of EPF against BMSB. Furthermore, isolates found to be effective can contribute in the scope of IPM against BMSB in order to reduce insecticide use in hazelnut production areas of Türkiye.

2. Material and Methods

2.1. Collection of Bugs

Adults of BMSB were collected from various hazelnut, citrus and kiwi orchards situated in different districts of Rize province in eastern Black Sea Region, Türkiye. Healthy adults of BMSB were utilized from the adults brought to the laboratory (Ondokuz Mayıs University, Agriculture Faculty, Plant Protection Department, Samsun, Türkiye) in the experiments.

2.2. Preparation of Entomopathogenic Fungi

A total of 7 native EPF isolates isolated from infected *P. prasina* in hazelnuts orchards, Black Sea region of Türkiye in 2018-2019 and they were tested in this study (Table 1) (Ozdemir et al., 2021). The 7 native EPF isolates belonged to *B. bassiana* (TR-SM-10, TR-SM-11, TR-SM-2, TR-SK-1, TR-D-1, TR-D-2) and *B. pseudobassiana* (TR-SM-1). The isolates were incubated on potato dextrose agar (PDA; Merck Ltd., Darmstadt, Germany) at 25±1^{o}C for one week to obtain conidia, which were suspended in sterile distilled water, filtered through 3 layers of sterile cheesecloth and diluted to a concentration of 1×10^8 conidia mL^{-1} plus 0.02% Tween 20. The resulting spore suspension was adjusted at the concentration 1×10^8 conidia mL^{-1} using Neubauer hemocytometer under Olympus CX31 light microscope (Olympus America Inc., Lake Success, NY) (Tuncer et al., 2019).

Table 1. Species, hosts and locations of entomopathogenic fungi isolates used in the study

Species / Isolate denomination	Genbank accession numbers	Host	Location of collection
Beauveria bassiana / TR-SM-10	MT102327	*Palomena prasina*	Hazelnut orchards
Beauveria bassiana / TR-SM-11	MT102328	*Palomena prasina*	Hazelnut orchards
Beauveria bassiana / TR-SM-2	MT102329	*Palomena prasina*	Hazelnut orchards
Beauveria bassiana / TR-SK-1	MT102330	*Palomena prasina*	Hazelnut orchards
Beauveria bassiana / TR-D-1	MT102331	*Palomena prasina*	Hazelnut orchards
Beauveria bassiana / TR-D-2	MT102332	*Palomena prasina*	Hazelnut orchards
Beauveria pseudobassiana / TR-SM-1	MT102333	*Palomena prasina*	Hazelnut orchards

2.3. Application of Entomopathogenic Fungi against *Halyomorpha halys* Adults

Five adults of BMSB were released into one L plastic transparent and lid cups (disinfected by 70% ethanol) containing fresh persimmon (*Diospyros kaki* L.) fruits. Bottoms of the cups were covered by filter paper moistened with sterile-distilled water. Conidial suspension (1×10^8 conidia mL^{-1}) of each EPF isolate (TR-SM-10, TR-SM-11, TR-SM-2, TR-SK-1, TR-D-1, TR-D-2 and TR-SM-1) was applied to BMSB adults (2 mL per cup), using a Potter spray tower (Burkard, Rickmansworth, Hertz UK). The spray tower was cleaned by 70% ethanol and sterile distilled water after each application of EPF suspension. Only sterile-distilled-water containing 0.02% Tween 20 was sprayed to the control group. The cups were incubated at 25±1°C and 70±2% (RH), 16:8 h light: dark photoperiod for 11 days. Mortality rates were monitored for eleven consecutive days and the trial was repeated, using the same number of different individuals (n=25 insects/day/isolate) with 5 replications per day for each isolate to assure that each day's observations were mutually independent (Robertson et al., 2017). Mortality rates were determined for the corresponding day and the dead insects on that day were removed from the cups. The same procedure was repeated every day for the control groups (Ozdemir et al., 2021). Additionally, a procedure was used to study fungus sutructures on dead insects, and mycosis rates were calculated; for further details, see Kocacevik et al (2016).

2.4. Conidial Germination Assessment

The viability of conidia of the 7 isolates was determined. A spore suspension was adjusted to 1×10^4 conidia mL^{-1} and 0.1 mL was sprayed onto Petri dishes (9 cm diameter, containing PDA), and the dishes were incubated at 25°C for 24 h. After 24 h of incubation, percentages of germinated conidia were counted using an Olympus CX-31 compound microscope (Olympus America Inc., Lake Success, NY) at 400X magnification. Conidia were considered as germinated when they produced a germ tube at least half of the conidial length (Erper et al., 2016).

2.5. Statistical Analyses

Daily mortality rates were corrected according to the Abbott formula when mortality rate in the control exceeded 5% (Abbott, 1925). The LT_{50} and LT_{90} values were determined by probit analysis using log-probit method (POLO-PLUS ver.2.0). The slopes of regression lines were compared with each other using standard errors. The LT_{50} and LT_{90} values of the isolates were compared using confidence intervals (95%) (Ozdemir et al., 2021).

3. Results

Conidia viability of *B. bassiana* (TR-SM-2, TR- SM-10, TR-SK-1, TR-SM-11, TR-D-1, TR-D-2) and *B. pseudobassiana* (TR-SM-1) isolates used in the study before the bioassays and the conidial germination was nearly 100%. In the present study, all native fungal isolates obtained from *P. prasina* were applied against BMSB at 1×10^8 conidia mL^{-1} concentration under the laboratory conditions evaluating their efficacy (Figure 1). LT_{50} and LT_{90} values of these isolates differed ($P<0.05$) and several isolates had high virulence. Especially, TR-SM-11, TR-SK-1 and TR-D-1 isolates were more virulent than the isolates TR-SM-10, TR-D-2, TR-SM-1, TR-SM-2 and rapidly lethal to BMSB and these isolates presented LT values close to each other's (Table 2). The TR-SM-1 isolate was the least virulent among the isolates applied. In addition, approximately 100% mycosis was obtained in all treatments and at 11 days following inoculation, aggressive colonisation on BMSBs by the isolates was visible (Figure 2).

Table 2. Probit analysis data on mortality time of *Halyomorpha halys* adults after application of at 1×10^8 conidia mL^{-1} of *Beauveria bassiana* and *B. pseudobassiana* isolates

Isolates	LT_{50} (95% CI)	LT_{90} (95% CI)	Slope ± SE	Regression	X^2	*Df*	Heterogeneity
TR-SM-1	7.20(6.37-8.31)^{bc}	18.35(14.28-27.85)^{b}	3.15±0.43	y= -2.7+3.15x	36.13	53	0.68
TR-SM-2	6.73(6.12-7.42)^{bc}	13.08(11.20-16.61)^{ab}	4.44±0.54	y= -3.68+4.44x	35.37	53	0.66
TR- SM-10	6.60(6.04-7.19)^{b}	11.85(10.38-14.44)^{ab}	5.04±0.60	y= -4.13+5.04x	36.22	53	0.68
TR-SK-1	6.16(5.68-6.66)^{ab}	10.25(9.19-11-97)^{a}	5.80±0.63	y= -4.58+5.8x	42.19	53	0.79
TR-SM-11	5.37(4.82-5.92)^{a}	10.82(9.38-13.27)^{a}	4.21±0.47	y= -3.07+4.21x	49.37	53	0.93
TR-D-1	7.74(7.37-8.11)^{c}	9.85(9.27-10.74)^{a}	12.21±1.47	y= -10.86+12.21x	42.23	53	0.79
TR-D-2	6.79(6.22-7.40)^{bc}	12.19(10.65-14.96)^{ab}	5.04±0.61	y= -4.19+5.04x	48.75	53	0.92

^{a,b}Different letters in same column show statistical difference ($P<0.05$).

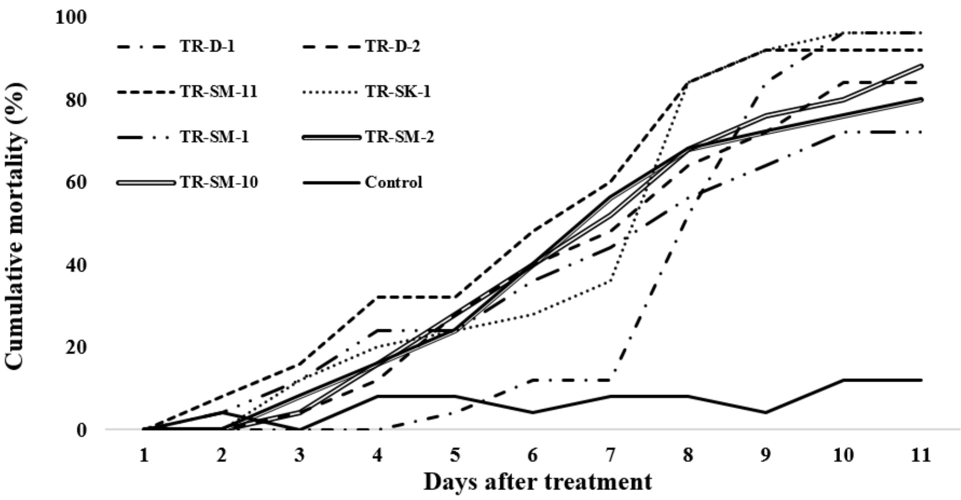

Figure 1. Daily mortality rates of adults of *Halyomorpha halys* treated with 1×10^8 conidia mL^{-1} concentration of *Beauveria bassiana* and *B. pseudobassiana* isolates.

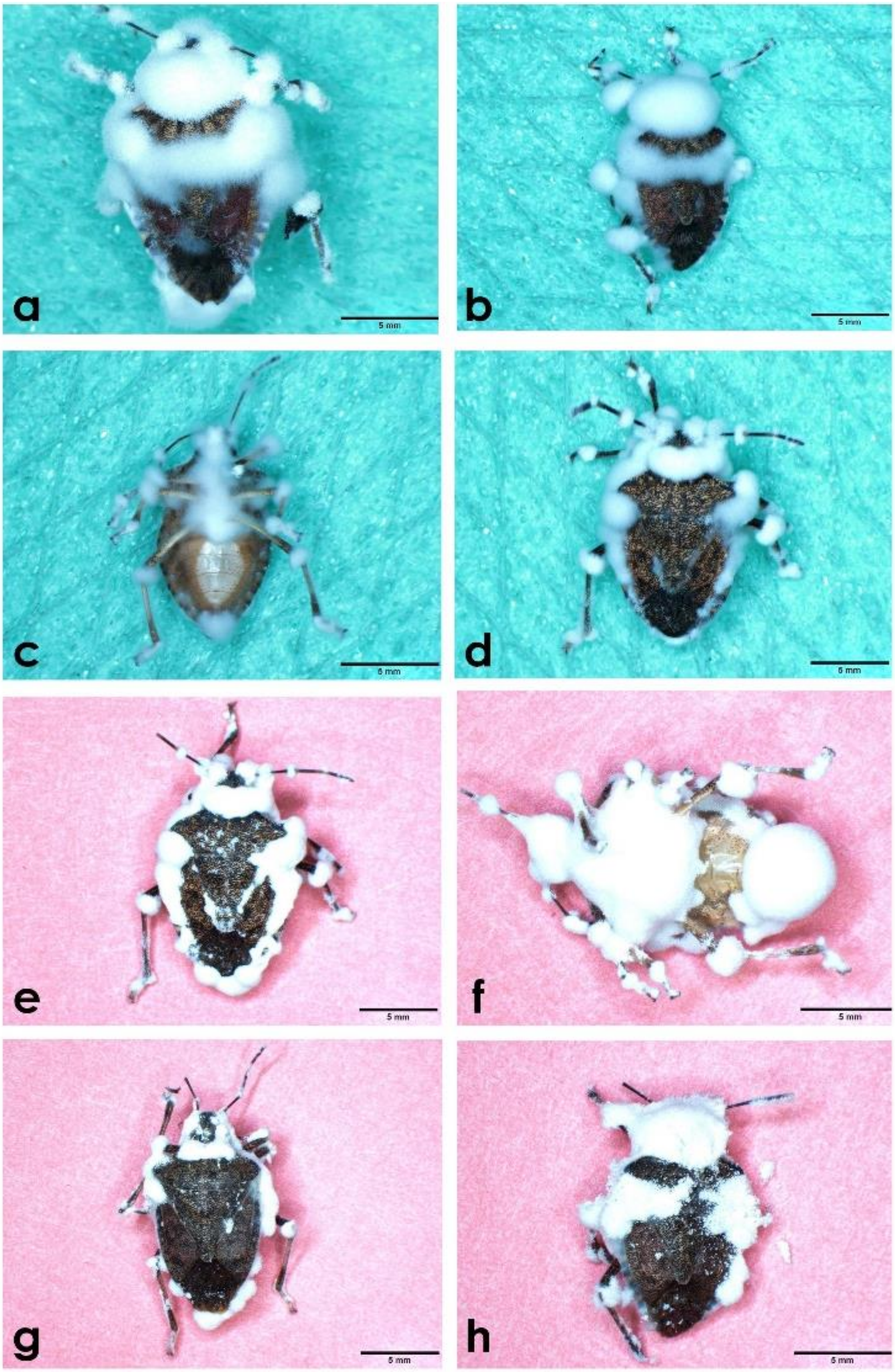

Figure 2. Mycosis of TR-SM-11 (a, b), TR-D-1 (c), TR-SK-1 (d), TR-SM-10 (e), TR-SM-1 (f), TR-SM-2 (g) and TR-D-2 (h) isolates on the body surface of *Halyomorpha halys* adults 11 days after inoculation.

4. Discussion

Some of the tested isolates were highly pathogenic against BMSB adults after 11 days of application in laboratory bioassays. In addition, in the studies conducted on BMSB by Parker et al. (2015), it was reported that adult BMSB was more resistant to the infection than nymphal stages. Therefore, possibly *Beauveria* isolates used in this study would have higher efficacy on the nymphs.

The native *B. bassiana* (TR-SM-2, TR- SM-10, TR-SK-1, TR-SM-11, TR-D-1, TR-D-2) and *B. pseudobassiana* (TR-SM-1) isolates used in the study were isolated from *P. prasina*, which had the highest population density among stink bugs and causes serious yield and quality losses to hazelnut production of Türkiye (Ozdemir et al., 2021). Evaluating the efficacy of the native *Beauveria* isolates, which are highly pathogenic against *P. prasina* is important as an alternative management method for BMSB. Bioassays in this study revealed that TR-SM-11, TR-D-1 and TR-SK-1 isolates were the most effective against BMSB. These isolates were applied at 1×10^8 conidia mL^{-1} concentration and caused 96, 96 and 92% mortality rates in BMSB adults on the end of 11th day, respectively. The mortality rates of other isolates ranged from 72 to 88% at the same concentration. It is reported that *Beauveria* isolates used in this study caused 100% mortality against *P. prasina* adults at the same concentration within 6-10 days. Moreover, TR-SM-11, TR-SK-1 and TR-D-1 isolates were the most effective isolates against native *P. prasina*, causing 100% death of the bug population 6, 7 and 8 days post application, respectively (Ozdemir et al., 2021). Similarly, in the present study, although these 3 isolates had nearly 100% efficacy against BMSB adults, however the mortality occurred longer times, i.e., 11 days after treatment. While the LT_{50} - LT_{90} values for TR-SM-11, TR-SK-1 and TR-D-1 isolates sprayed at 1×10^8 conidia mL^{-1} concentration on adult *P. prasina* were 3.65 - 6.17, 3.82 - 5.76 and 6.14 - 7.05 days, respectively (Ozdemir et al., 2021), LT_{50} - LT_{90} values of them isolates against BMSB adults were 5.37-10.82, 6.16-10.25 and 7.74-9.85 days, respectively. Considering the LT_{50} - LT_{90} values and mortality rates (%) for *P. prasina* and BMSB, the LT values for BMSB were higher, while mortality rates were lower. Sevim et al. (2010) reported that the native isolates obtained from specific regions have been ecologically compatible with the native pests. In addition, efficacy of the native isolates on non-target organism or the exotic pests such as BMSB decreased significantly. For this reason, the native isolates used in the present study showed relatively lower efficacy against exotic BMSB compared to *P. prasina*.

Gouli et al. (2012) reported that BotaniGard® commercial bio-product (*B. bassiana* strain), from 3 *B. bassiana* and 2 *Metarhizium anisopliae* isolates caused 100% mortality of BMSB adults 12 days post treatment. In a similar study conducted with same bio-product, Parker et al. (2015) reported 95-100% mortality 12 days after treatment against the 2nd nymphal stage of the bug at 1 × 107 conidia/ml concentration. Another *B. bassiana* (ET 10) isolate caused 76.19% mortality, 11 days post treatment on different nymphal stages of BMSB at 5.7×10^5 conidia/ml (Tozlu et al., 2019). Furthermore, prevalence rates of naturally-infected BMSB adults by EPF and collected from several sites, including hazelnut orchards in the Black Sea basin in Georgia had 0.9% infestation of *B. bassiana* and 0.3% of *Isaria fumosorosea*. Moreover, Bover-Ge (*B. bassiana*-024 strain) registered in Georgia as a mycoinsecticide, bioassyed at 1×10^7 and 1×10^8 conidia/ml concentration against BMSB adults under laboratory condition caused 72-90.5% mortality at the end of 12 days (Burjanadze et al., 2020). Similarly, *B. bassiana* strains of TR-D-1 and TR-SK-1 caused 96% mortality rate in this study. Thus, these isolates can be considered as a promising bio-control agent against BMSB. Additionally, *B. pseudobassiana* (TR-SM-1) had the lowest pathogenicity.

Patel et al. (2006) reported that feeding and laying egg of the pest insects infected by *B. bassiana* were decreased during infection period. However for successful field applications, ecological suitability (relative humidity) that will trigger the natural infection chain of the EPF as well as high virulence is important (Bugti et al., 2020). The native isolates that have high virulence used in this study can be used in high infestation areas, which is in hazelnut growing areas in the coastal of Black Sea region, of BMSB. Moreover, climatic suitability of the region for EPF further strengthens this inference. Chemical compounds deposited on integument of pentatomid bugs has potential to act as barrier against microbial infection (Sosa-Gómez and Moscardi, 1998). Additionally, direct contact of metathoracic gland components of some stink bugs have the detrimental effect on conidial germination of some EPFs (Lopes et al., 2015). Therefore, it is of paramount importance that the natural population of the pest can be exposed to EPF with enough amount and prolonged period, with supporting by abiotic factors of the pathogen (Bugti et al., 2020).

5. Conclusion

BMSB is a highly polyphagous invasive pest that threats many agricultural crops, particularly hazelnut in Türkiye. The Black Sea region has very suitable climatic conditions for BMSB. In this study, *Beauveria* isolates obtained from *P. prasina* in main hazelnut production areas of Türkiye were tested, the present results showed that TR-SM-11, TR-D-1, TR-SK-1 isolates belonging to *B. bassiana* seemed to be the most promising and potential biological control agents of BMSB. However, further studies are needed to evaluate their efficacy against BMSB under field conditions.

Author Contributions

I.O.O. (25%), C.T. (25%), M.U. (25%) and E.Y. (25%) designed the study, supervised the work, and wrote the manuscript with input from all authors. I.O.O. (25%), C.T. (25%), M.U. (25%) and E.Y. (25%) carried out the experiments. C.T. analyzed the data. All authors reviewed and approved final version of the manuscript.

Conflict of Interest

The authors declared that there is no conflict of interest.

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Ethical Consideration

Ethics committee approval was not required for this study due to the use of research material not included in the definition of experimental animals in the study

(Animal experiment ethics committee regulation on working procedures and principles, Article 4-d).

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