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Compatibility Between *Beauveria bassiana* (Bals.) and Neem Extract against Brown Plant-Hopper (*Nilaparvata lugens* Stal.)

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ABSTRACT
Beauveria bassiana fungi and neem extract as botanical insecticide can be combined to get the synergizing effect to control brown planthopper (BPH). This study was conducted to determine the best combination between the spore density of B. *bassiana* and concentration of neem extract to control BPH effectively. The research was arranged in Completely Randomized Design consisting of two factors with three replications within each treatment combination. The treatments consisted of control and combination between *B. bassiana* concentration (106 Colony Forming Unit (CFU) and 107 CFU) and neem extract concentration (5%, 10%, 15%). The data were analyzed using ANOVA and LSD at 5%. The test was conducted in vitro and bio assay of testing insects, BPH, was conducted using dipping method. B. *bassiana* was compatible with neem extract with T value ranged from 78.58- 90.38. The mortality of BPH occurred on day 5 after application. The highest mortality (91.67%) occurred in the treatment of *B. bassiana* 107 CFU + neem extract 10 %. The shortest LT50 was observed in the treatment of *B. bassiana* 10^6 CFU + neem extract 15% (2.74 days) but not significantly different from treatment of *B. bassiana* 10^7 CFU + neem extract 15% (2.76 days). *B. bassiana* spores combined with neem extract are assumed to speed up the mortality of BPH by increasing the concentration.

Keywords: Brown planthopper, fungi, botanical insecticide.

INTRODUCTION

Awareness of the negative impacts of synthetic pesticides has led us to find the alternative control measures which are environmental friendly. Among alternative control measures, entomopathogenic fungi and botanical insecticide are commonly used. One of the entomopathogenic fungi used is *B. bassiana*, a microorganism able to cause disease on insects and serve as biological agent. This fungus infects herbivorous insects selectively and is not dangerous for predators and parasitoids, thus it suits the principle of sustainable agriculture (Yazdi and Eilenberg, 2007).

One of botanical insecticides used is neem (*Azadirachta indica* A. Juss). Neem has several uses such as functioning as anti-bacteria and insecticide that can disturb neurosecretory cell which has adverse effects on protein stimulation and metamorphosis regulation which in turn cause the mortality of larva or slight disruption which slow down the insect growth (Mordue and Blackwell, 1993 *cit.* Sunarto and Nurindah, 2009). The botanical insecticide is sometimes combined with biological insecticide in their usage. The leaf extract of Mimosa pudica is compatible with *B. bassiana*, but when the composition of concentrations and doses is not compatible, it can disrupt the growth of *B. bassiana* mycelia (Astoni *et al*., 2015; Astoni *et al*., Ummidi and Vadlamani (2014) said that *B. bassiana* was compatible to be combined with almond oil and other entomopathogenic fungi and Metharizium anisopliae was compatible with

sesame oil. Both combinations can be used to control Spodoptera litura (Ummidi and Vadlamani, 2014). Other study found that *B. bassiana* was compatible with neem oil and effective to control cocoa bugs, *Helopeltis antonii*. But it was not compatible with citronella and clove oils (Rohimatun *et al*., 2015; Rohimatun *et al*., 2014). These findings prove that the synergy between botanical and biological insecticides depends on the type of insecticides and the target pests. For these reasons, this study aimed to determine the best combination between the spore density of *B. bassiana* and concentration of neem extract to control BPH effectively.

MATERIALS AND METHODS

Research design

The study was conducted in Laboratory of Agronomy and Plant Protection, University of Muria Kudus (17 m dpl) from February to July 2018. Factorial treatments consisting of control , concentration of *B. bassiana* (106 Colony Forming Unit (CFU) and 107 CFU) and concentration of neem extract (5%, 10%, 15%) were tested in this research. The treatments were arranged in Completely Randomized Design with three replications.

Rearing of brown planthopper (BPH) *Nilaparvata lugens*, Stahl.

BPH used was Kudus isolate which had been reared since 2017. They were reared in Faculty of Agriculture, University of Muria. The rearing was conducted using modified glass containers (diameter of 30 cm) filled with one-week rice seedlings as feed in room with the temperature was 24ºC and humidity 75%. After the eggs hatched, the nymphs were moved to separate containers to produce F2 and the following generations (Heong *et al*., 2011.).

Propagation and suspension preparation of Beauveria bassiana

Propagation of *B. bassiana* fungi (isolate from Universitas Gadjah Mada, February 2018) was conducted in LAF (Laminar Air Flow) which was first cleaned using alcohol then sterilized with Ultra Violet light for 15 minutes. *Beauveria bassiana* fungus was taken using inoculating loop and inoculated to media of PDA (Potato Dextrose Agar) in petri dishes and then covered with aluminum foil. The petri dishes were placed in rack until the fungi covered the media (usually within 21 days). Propagation of these fungi was also done using corn media used as suspension for dipping treatment.

Suspension solution of *B. bassiana* was made from 1 g of rice propagated inoculum which was fined and diluted in 100 mL distilled water, then was shaken using shaker (Optima type OS-762) for 24 hours, 150 rpm (revolution per minutes). The density obtained was 107 CFU and was diluted ten times to obtain the density 10^6 CFU.

Extraction of neem leaves

The leaves of neem were cleaned and weighed 125 g and then chopped and dried for 2 hours. It was then placed into an erlenmeyer and added with 1 L alcohol 70% and covered tightly. Erlenmeyer containing the substance was then covered with black fabric and kept for 4 days. After 4 days, the substance was screened using filter paper at a glass funnel while squeezing the leaves.

Purification of the extract was continued by evaporating it at water bath with regulator temperature at scale 6. Temperature was stabilized for 15 minutes before the substance was placed inside. Evaporation was conducted until the substance was left a half volume. This resulted in concentrated extract.

Mortality and LT_{50} of BPH

Testing was done using dipping method. Fourteen days-old rice seedlings were dipped for 5 minutes in a single or mixed (neem extract and *B. bassiana* suspension) solution, then were dried at room temperature for 10 seconds. Twenty nymphs of 3rd instar of BPH were placed in a modified cup (diameter 15 cm). Three plastic cups were needed for one treatment where cup 1 was filled with water, cup 2 was cut a half and given 3 holes for 6 rice seedlings, and cup 3 was given fine holes using needle and was used as a cover. Mortality of BPH was observed until 6 days after treatment. Percentage of BPH mortality was calculated by the following formula:

$$\mathbf{P = \frac{a}{a+b} x\ 100\ \%} \quad \text{.....(1)}$$

Remarks:
P = Percentage of mortality; a = Number of dead BPH; b = Number of alive BPH

LT_{50} (Lethal $Time_{50}$) was obtained from linier regression with the equation: y= ax + b where "y" was mortality and "x" was time taken before death.

Colony growth of *Beauveria bassiana*

Fungi were grown PDA media and incubated to four days at 25ºC. After 4 days 1 mL neem extract (based on treatment) was dropped in to PDA micelia of *B. bassiana*. The diameter of fungi colony was measured on the 15th using ruler. The percentage

of colony growth decrease was calculated with the following formula:

$$Nr = \frac{N1-N2}{N1} x\ 100\% \quad \text{.....(2)}$$

Remarks:
Nr: percentage of colony growth decrease; N1: the growth of fungi colony on media without neem extract; N2: the growth of fungi colony on media with neem extract.

Sporulation capacity

One gram of spore was taken from PDA media that had been incubated for 15 days at temperature of 25°C and given neem extract 4 day after inoculation based on treatment. One gram incubated spore then was dissolved in 100 ml of distilled water at the erlenmeyer and added with 2 drops methyl blue for staining then shaken using 'shaker' (Optima type OS-762) for 3 hours with the speed of 150 rpm. 0.1 ml suspension of *B. bassiana* was taken to count the number of spores. The spores were counted using hemocytometer with formula of Gabriel and Riyatno (1989) in (Herlinda *et al*., 2006). The formula was as following:

$$C = \frac{t}{n\ x\ 0.25} x\ 10^6 \quad \text{.....(3)}$$

Remarks:
C: spore density per ml solution or CFU ; t: total number of spores in a sample box observed; N: number of sample boxes (5 big boxes × 16 small boxes) 0.25: correction factor for using small sample box at hemocytometer.

The percentage of sporulation decrease was counted using the following formula:

$$Sr = \frac{S1-S2}{S1} x\ 100\% \quad \text{.....(4)}$$

Calculation of compatibility value

The effect of neem extract on *B. bassiana* fungi (compatibility value) was determined using the following formula of T from Alves *et al*. (1998) *cit.* Depieri *et al*., (2005):

$$T = \frac{\{20(PK)+80(SP)\}}{100} \quad \text{.....(5)}$$

Remarks:
T = Compatibility value; PK = Relative value of colony growth on treatment compared to control (%); SP = relative value of sporulation on treatment compared to control (%).

Criteria of T value were: 0–30 very toxic; 31–45 toxic; 46–60 less toxic; and > 60 not toxic or compatible. Data were analyzed using analysis of variance and continued with the Least Significant Difference (LSD) test at α=5% using Microsoft Excel 2016.

RESULTS AND DISCUSSION

The data obtained from the spore density of *B. bassiana* showed that the addition of neem extract on the fungi colony decreased the spore density from 6% to 17% compared to control. The spore density decreased significantly at the neem extract 15% (2.71×10^7 CFU) (Table 1) but the spore in all treatments was still on the density of 10^7 CFU and was still considered in good category. The growth of *B. bassiana* colony in PDA media with the addition of neem extract also decreased from 19% to 23% compared to control but the colony growth was not significant proof to show that the *B. bassiana* was compatible with the neem extract (Tabel 1). Compatibility analysis between neem extract and *B. bassiana* using Alves formula resulted in the value of T ranging from 78.58 to 90.38 (Table 2). When the T value is higher than 60, it means that the neem extract is not toxic to the spores of *B. bassiana* or they are compatible when applied in combination (Depieri *et al*., 2005). The activity of *B. bassiana* was not affected significantly by the insecticidal activity of neem extract. This result was supported by Rohimatun *et al*. (2014) who found that neem oil was compatible with *B. bassiana*. Further, Sahayaraj *et al*. (2011)

Table 1. Spore cpacity and growth of *B. bassiana* after contact with neem extract

Treatment	Spore Dencity 15th DAI* (x 10^7 CFU)	Sporulation decrease (%)	Colony Growth 15th DAI* (cm)	Growth Decrease (%)
Control	3.28 a	--	8.10 a	--
5% Neem Extr.	3.08 a	6%	6.60 a	19%
10% Neem Extr.	2.84 a	13%	6.20 a	23%
15% Neem Extr	2.71 b	17%	5.00 ab	38%

Note: Numbers followed by the same letters at the column was not significantly different based on LSD 5%.
*DAI: Day After Inoculation

Table 2. Compatibility of *B. Bassiana* and Neem Extract

Treatment	T	Criteria
Bvr + 5% neem extract	90.38	Compatible
Bvr +10% neem extract	85.51	Compatible
Bvr +15% neem extract	78.58	Compatible

Table 3. The mortality of brown planthoppers at day 3, 4, and 5 after given different treatments of *B. bassiana* and neem extract

Treatments	Percentage of Mortality on day		
	3	4	5
Control	3.67 e	4.00 e	5.67 c
Bvr 10^6	20.0 d	61.67 cd	83.33 ab
Bvr 10^7	40.00 c	68.33 bcd	83.33 ab
Bvr 10^6 + neem extract 5%	50.00 c	61.67 cd	81.67 ab
Bvr 10^6 + neem extract 10%	66.67 ab	75.00 abc	83.33 ab
Bvr 10^6 + neem extract 15%	76.67 a	83.33 a	90.00 a
Bvr 10^7 + neem extract 5%	50.00 c	60.00 d	78.33 b
Bvr 10^7 + neem extract 10%	65.00 b	73.33 abcd	91.67 a
Bvr 10^7 + neem extract 15%	73.33 ab	78.33 ab	90.00 a

Note: Numbers followed by the same letters at the column was not significantly different based on LSD 5%

Table 4. LT_{50} and linear regression of brown plant hoppers after given different treatments of *B. bassiana* and neem extract

Treatments	LT50 (Days)	Linear Regression
Bvr 10^6	3.69	y = 21.83x – 30.5
Bvr 10^7	3.39	y = 22x – 24.67
Bvr 10^6 + neem extract 5%	3.31	y = 20x – 16.33
Bvr 10^6 + neem extract 10%	3.04	y = 22.17x – 17.5
Bvr 10^6 + neem extract 15%	2.74	y = 23.33x – 14
Bvr 10^7 + neem extract 5%	3.22	y = 19.17x – 11.83
Bvr 10^7 + neem extract 10%	2.91	y = 22.67x – 16
Bvr 10^7 + neem extract 15%	2.76	y = 22.33x – 11.67

reported that entomopathogenic fungi were more tolerant to leaf extracts.

The highest mortality of BPH on the 3rd day (Table 3) was showed in combination of *B. bassiana* 10^6 CFU + neem extract 15% reaching 76.67% but was not significantly different from that intreatment combination of *B. bassiana* 10^6 CFU + neem extract 10% (73.33%) and 10^7 CFU + neem extract 15% (78.33%). The mortality of BPH condition on the 4th day (Table 3) was still similar to previous day. But on the 5th day, the highest mortality was found in combination of *B. bassiana* 10^7 CFU + neem extract 10% (91.67%), which was significantly different from that in other treatments, except control. The mortality in all treatments was significantly higher than control. It implied that *B. bassiana* and neem extract combinations were effective to control BPH. Data in Table 3 clarify that both neem extract and *B. bassiana* synergize in suppressing the population of BPH. *B. bassiana* combined with neem oil synergized because both could decrease the synthesis of cuticle, digestive tract and respiration (Rohimatun *et al*., 2015). In this case, using neem and *B. bassina* after one and another could be considered without being worried about the negative impacts.

The result of LT50 from linear regression, y= ax + b, showed that the increase of neem concentration and spore density of *B. bassina* could shorten LT50 (Table 3). It implied that the death of BPH became quicker when combination of neem extract with *B.*

bassiana was applied. The shortest LT50 occurred in all treatments with neem extract 15% + *B. bassiana* 10^6 CFU (LT50= 2.74 days), which was not significantly different from that in neem extract 15% + 10^7 CFU (LT50= 2.76 days) (Tabel 4). The treatments of neem extract and *B. bassiana* separately, indeed, showed significant effect on the population of BPH. A study conducted by Wisuda (2015) indicated that 96 hours after application of neem extract to BPH, the LC50 was found to be at low concentration (6.77%) and caused mortality (71.88%) at concentration of 10% neem extract.

Other study using Spodoptera litura proved that *B. bassiana* was compatible with almond oil with LT50 4.95 days and mortality 95.5%. The dose and concentration of both neem extract and *B. bassiana* could be reduced (Ummidi and Vadlamani, 2014). In this case, the spore density of fungi could be reduced and the concentration of botanical insecticide could be increased. Neem extract and *B. bassiana* had good tolerance in all range combination. In another case, the botanical insecticide Mimosa pudica leaf extract at concentration 1% dan 2% were compatible with *B. bassiana* 10^5 CFU. However, the *M. pudica* leaf extract with concentration of 0.4%; 1%; 2% were not compatible with *B. bassiana* 10^3, 10^5 and 10^7 CFU (Astoni *et al*., 2015). This indicates that not all botanical insecticides are compatible with entomopathogenic fungi but it depends on the species and concentration. Neem has good insecticidal effects. According to Aziz *et al*. (2014), the leaf extract and sap of neem showed repellent effect (97.98%) on Lipaphis erysimi and were safe for its natural enemy, Syrphid fly. In other case, *M. anisopliae* and *B. bassiana* were effective in suppressing the population of BPH and were safe for its natural enemy, group of spiders (Reddy *et al*., 2013),

CONCLUSIONS

Neem extract was compatible with *B. bassiana* with T value's ranging from 76.6 to 89.4. Both of them also showed synergistic effects so that they could be used at the same time or one after another. The highest mortality of BPH (91.67%) occured on day 5 with the treatment of *B. bassiana* 107 CFU + neem extract 10%. The shortest LT50 was found on treatment of *B. basiana* 106 CFU + neem extract 15% (2.74 days) and B. basiana 107 CFU + neem extract 15% (2.74 days). Neem is assumed to speed up the mortality of BPH by increasing its concentration.

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