Application of *Beauveria bassiana* (Bals.) Vuil. (Hypocreales: Cordycipitaceae) in rice seed and its effect on mortality of green leaf hopper, *Nephotettix virescens* (Distant) (Homoptera: Cicadellidae)

T Abdullah, T Kuswinanti, A Nurariaty, I D Daud, A Nasruddin, R Risal, S Utami and M Tuwo

1Plant Pest and Disease Department, Faculty of Agriculture, Universitas Hasanuddin, Makassar, 90245, Indonesia.
2Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, 90245, Indonesia.

Abstract. The use of synthetic chemical insecticides in controlling rice pest insects has various negative impacts. Chemical insecticides can stimulate pest resistance, the emergence of secondary pests, killing of useful organisms and natural enemies, and contamination of residues in food, soil and water. Another potential effective control alternative is the use of entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuil. (Hypocreales: Cordycipitaceae). The effectiveness of *B. bassiana* application by spraying on plants or pests directly, can decrease due to the influence of high temperatures, low humidity, and exposure to ultra violet light. The study of the application of *B. bassiana* by treatment of rice seeds was carried out to determine the cumulative mortality of green leaf hopper, which was carried out in two-time periods at the Bioecology Laboratory for Natural Pests and Enemies, Department of Plant Pests and Diseases, Faculty of Agriculture, Hasanuddin University. The first series of studies used *B. bassiana* collection culture of Bioecology Laboratory, and the second series used the commercially available *B. bassiana* formulation. Completely Randomized Design (CRD), with each of the 4 treatments and 5 replications was used in this study. The results showed that, coating or soaking rice seeds with entomopathogenic fungi *B. bassiana* was able to cause mortality to green leafhoppers. Although the mortality is lower than spraying on rice plants or penetrating the body of green leafhoppers, however, with the covering or soaking rice seeds, the mortality of green leafhoppers is significantly higher than control without the application of *B. bassiana*. This indicates basic potential that requires a study for development of rice seed coating and immersion techniques to increase its effectiveness in controlling green leafhoppers.

1. Introduction

Rice is an important commodity and staple food for more than half of the world's population. Rice contains nutrients for main energy sources for human needs [1, 2]. Indonesia is one of rice producer countries with a large population facing challenges to meet the food needs of the population [3, 4]. To increase the productivity of lowland rice plants can be done by extensification, intensification, and agricultural diversification. But to strive to increase production, many obstacles must be faced. One obstacle to increasing the productivity of lowland rice is the presence of plant pests. Plant-disturbing organisms are any organism that can interfere with the growth and development of plants, so that plants become damaged, stunted growth, and or death [5].
One of the important pests in rice plants, especially in several countries located in South and Southeast Asia is Green Wereng (Nephotettix sp.) [6]. Planthopper pest is the main pest that has attacked rice plants which are quite extensive with heavy attack intensity. In Indonesia, planthopper populations are often found in high numbers, resulting in dry rice or called hopper burn. Various control efforts have been carried out, including by planting resistant varieties, the use of insecticides [7] but the use of insecticides causes environmental damage because only 20% of pesticides hit the target while 80% fell to the ground [8]. To examine this problem, it is necessary to develop an alternative control method that does not cause environmental, human and plant pollution such as the use of the entomopathogenic fungus Beauveria bassiana [9].

Entomopathogenic fungi are able to suppress the green leafhopper imago population with a double action that can directly kill and indirectly suppress green leafhopper [10]. In addition to green leafhoppers, B. bassiana is also able to suppress Cylas formicarius (Acarina: Tertachidae) sweet potato plant pests by dipping the cuttings into a conidia suspension of the fungus for 30 minutes. The working system of B. bassiana fungus spores enters the body of host insect through the skin, digestive tract, spiracles and other holes. In addition, the fungus inoculum attached to body of the host insect can germinate and develop to form a sprout tube, then enter cuticle of insect's body. Penetration is done mechanically or chemically by releasing an enzyme or toxin called beauvericin, this antibiotic can cause interference with the insect's hemolymph function, resulting in swelling accompanied by hardening that causes damage to the insect's body tissue and within days the insect will die. After that, fungus mycelia will grow to all parts of insect's body [11, 12].

The ability of endophytic entomopathogenic B. bassiana as an insect pathogen and cause death in several types of insect pests were reported in several previous studies. B. bassiana can also live as an endophyte in cocoa fruit and is able to kill larvae of Tenebrio molitor (Coleoptera: Tenebrionidae) of cereals plant pests up to 100% and more found in leaves than those isolated from branches and insects fruit [13]. Beauveria bassiana as an endophytic fungus of rice plants is able to colonize each part of the plant, especially in root and stem tissue [14]. The application method can be carried out by spraying on plant parts, sprinkled on the surface of soil and mixed with soil/compost, soaking seeds and injection on plant stems [15]. The mechanism of endophytic fungi in protecting plants against insects or pathogens includes direct inhibition of pathogen growth, indirect inhibition through induction of plant resistance in the formation of secondary metabolites, stimulation of plant growth, colonization of plant tissue so that pathogens are difficult to penetrate, and hyperparasite [16]. This research was conducted to determine the effect of Beauveria bassiana entomopathogenic fungus application on the mortality of green leafhopper Nephotettix virescens (Distant). in rice plants (Oryza sativa L).

2. Materials and methods

2.1. Preparation of B. bassiana and experimental treatment

The first series of experiments using isolates of B. bassiana laboratory collections. The spore concentration used was 10^8, the result of scouring that was rejuvenated on PDA media. Spore density was calculated using a hemocytometer with dilution. The experimental treatment consisted of: (a) the application of the contact body of the test insect by dripping B. bassiana suspension; (b) rice seeds soaked for two days in suspension B. bassiana 10^8; (c) rice seeds covered with B. bassiana suspension from propagation by corn media; and (d) without the application of B. bassiana as a control. Soaking rice seeds with water only (without B. bassiana) for two days, carried out on treatments a, c, and d. For treatment c, seeds that have been cultivated are then covered with B. bassiana and left in the room for seven hours. Seed sheath material is a paste from corn flour, sweet potato flour and starch mixed with a ratio of 6: 3: 1. Then the seeds are germinated in a plastic pot with a height of 12 cm, a diameter of 10 cm. The lid of the jar is glued by a used plastic water container with a height of 29 cm, a diameter of 8.5 cm as a cage of rice seedlings and green leafhopper nymphs tested. Rice seedlings from the treatment of seeds above, then used as feed (host) for test insects to determine the effect of B. bassiana application on rice seeds on mortality of green leafhopper nymphs. The rice seeds used were Mekongga rice varieties, which were never treated with fungicide-application. To avoid contamination, the seeds are washed with distilled water.
In this second series of experiments, commercial ready-to-use formulations of *B. bassiana* are in powder form. For this test, 0.8 gram was used which was dissolved into 100 ml distilled water for each test treatment. The second series experiment also used four treatments, namely: (a) *B. bassiana* solution was sprayed directly on rice seedlings and planthopper nymphs in the test container; (b) rice seeds soaked in *B. bassiana* solution for two days, then ripened for one day; (c) *B. bassiana* solution is splashed to the soil in a rice plant growing media pot; and (d) without application of *B. bassiana*, as a control. Seeds for treatments a, c, and d, soaked with distilled water for 2 days, then ripened for a day. Inpari 30 rice seed varieties, never received seed fungicide treatment were used for seed treatment with *B. bassiana*, to test their effect on mortality of green leafhoppers. Growing media for planting rice seeds treatment is soil that has been cleaned of small rocks, sterilized using an autoclave at a pressure of 1.5 bar for 1 hour 30 minutes.

2.2. Propagation of green leafhoppers

The rice seedlings used for the propagation of green leafhoppers are TN-1 variety seeds. The seeds were obtained from the Tungro Research Center in Sidrap District, South Sulawesi, where the variety is susceptible to green leafhoppers, making it suitable for the maintenance and propagation of green leafhoppers. The seeds are soaked for 2 × 24 hours, then cured for 1 × 24 hours, then spread on gutters containing soil that has been sterilized before. The gutters containing 14-day-old rice seeds then put into a cage as food for the propagation of green leafhoppers. Maintenance of green leafhoppers using cages with a length of 100 cm, width 55 cm, height 100, and 70 cm feet. Then covered using a tile cloth (or gauze). Green leafhopper are collected from the field by catching them using sweep nets in the paddy field location of the Tungro Research Center in Sidrap, South Sulawesi. Green leafhopper cultivation is carried out to produce a new generation. For testing, nymphs were transferred into the test container, two days after the second skin change occurred, meaning the green leafhopper used as a test insect was a two-day-old instar 3 nymph.

2.3. Cultivation of green leafhopper

Cultivation of green leafhoppers using cages with a length of 100 cm, width 55 cm, height 100, and 70 cm feet. Then covered using a yellow tile cloth. Green leafhoppers obtained in the field are put in a cage, then maintained until an instar 1 nymph appears. The green leaflet from egg to instar 1 nymph takes about 14 days. Instar changes are characterized by skin changes in nymphs that occur 5 times within a period of 15 days to reach imago again. The day after the formation of instar 3 nymph, it was only infested in the test plant.

2.4. Testing, observation and data analysis

For each unit of the experiment (one treatment for each test), 10 instar-3 leafhopper green nymphs were used. Observations were made every day, starting from day-1 to day-10 after the test nymphs were placed in the treatment. The parameters observed were daily mortality and cumulative mortality of green leafhoppers. Data on mortality of green leafhopper nymphs *Nephotettix virescens* (Distant) were analyzed using analysis of variance.

3. Results and discussion

3.1. Death of green leafhopper after application of *B. bassiana* isolate

Death of green leafhopper nymphs began to occur in treatments dripped with *B. bassiana* suspension on the 4th day. Early death of green leafhopper nymphs on rice whose seeds have been soaked or covered with *B. bassiana*, occurred on the 5th day of testing (table 1). The effect of *B. bassiana* application was not significantly different in the three treatments that appeared on the 6th day, but all three began to show significant differences with controls (which were not applied with *B. bassiana*).
Table 1. Results of observations of the average cumulative mortality of green leafhopper nymphs on days 1-10

| Treatment with B. bassiana | Mean cumulative mortality of nymphs (%) / day |
|----------------------------|---------------------------------------------|
|                            | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  |
| Dripping on the body of green leafhopper | 0   | 0   | 0   | 8a  | 20a | 30a | 52a | 72a | 82a | 74a |
| Soaking the rice seeds     | 0   | 0   | 0   | 0b  | 10ab| 18a | 20b | 30b | 46b | 48ab|
| Coverage of rice seeds     | 0   | 0   | 0   | 0b  | 4bc | 18a | 26b | 34b | 44b | 38ab|
| Without application of B. bassiana | 0   | 0   | 0   | 0b  | 0c  | 0b  | 0c  | 0c  | 0c  | 0c  |

*Mortality rates followed by the same letter, in the same column, were not significantly different from the Tukey test at α 0.05

The pattern of influence of B. bassiana on mortality of green leafhopper nymphs appeared to be the same on 7th, 8th, and 9th days in the test period. B. bassiana test on the body of green leafhopper gives the highest mortality and is significantly different from the others. Immersion and coverage of seeds have an effect on mortality that is not significantly different between both. Furthermore, this first series of experiments showed the final results on 10th day that immersion, or coverage of seeds with B. bassiana, their effects on mortality of green leafhoppers were more or less the same, lower compared to direct contact or drops on the body of nymph, but mortality was markedly higher compared without the application B. bassiana or control.

3.2. Death of green leafhopper after application of B. bassiana

Based on observations of the mortality test (tables 1 & 2), entomopathogenic B. bassiana fungi caused mortality of the green leafhopper nymph Nephotettix virescens (Distant) significantly higher than control. Data shows that B. bassiana in the drops treatment effectively suppressed the death of green leafhopper nymphs Nephotettix virescens (Distant) by more than 80% at 10^8 spore densities. The B. bassiana fungus after 7 days after application (DAA) and 10 DAA the faster the entomopathogenic fungus kills the insect vector, the smaller the possibility of spread of tungro virus. In direct inoculation of nymphs and food apart from conidia which are faster to attach and germinate in the body of nymph, conidia can also enter directly into the larval body through digestive system of nymph, due to infections that occur from outside and in the body simultaneously will further accelerate mortality nymph. The more the number of conidia attached to the body of nymph, the faster the mortality will be, what else is supported by conditions of temperature and humidity in accordance with desired entomopathogenic fungi. The large number of entomopathogenic fungi conidia is related to the level of concentration used, because the higher the concentration, the higher the number of conidia, and the mortality will also be higher [17, 18].

Table 2. Results of observations of the average cumulative mortality of green leafhopper nymphs applied to B. bassiana in the second series of experiments

| Treatment with B. bassiana | Average cumulative mortality in nymphs (%) on day… |
|----------------------------|---------------------------------------------|
|                            | 3   | 4   | 5   | 6   | 7   | 8   |
| Spraying planthopper        | 24.00b | 50.00c | 66.00c | 92.00c | 98.00c | 100.00c |
| Soaking the rice seeds      | 4.00a  | 16.00b | 36.00b | 52.00b | 64.00b | 76.00b |
| Sprinkling the rice media   | 4.00a  | 18.00b | 38.00b | 52.00b | 68.00b | 88.00b |
| Without application B. bassiana | 2.00a | 2.00a | 4.00a | 8.00a | 8.00a | 8.00a |

*Mortality rates followed by the same letter, in the same column, were not significantly different from the Tukey test at α 0.05
The green leafhopper nymph *Nephotettix virescens* (Distant) which died from *B. bassiana* infection is characterized by the presence of white mycelia or conidia on the surface of the insect's body, white mycelia begin to penetrate the cuticle out of insect's body, then develop continuously and eventually cover the entire insect's body. In general, this pathogen enters the body of host insect through intersegmental membrane, spreading to all layers of body's walls with the help of enzymes proteinase, lipase, and chitinase. Dead insects are not always accompanied by symptoms of spore growth. If conditions are less favorable, the development of fungus only takes place in the body of insect without coming out through integument. Sometimes in infected insects, fungal mycelia are only found at the ends of body and are not clearly visible. This can occur due to conditions of temperature and humidity of room is not suitable so that the fungus can not grow well on the surface of insect's body. Death of leafhoppers nymphs *Nephotettix virescens* (Distant) occurs due to growth and development of fungi in the body.

![Figure 1. Nymph N. virescens (Distant) is infected with entomopathogenic fungus B. bassiana](image)

Based on the results of mortality test (tables 1 and 2), entomopathogenic *B. bassiana* fungus with a spore concentration of $10^8$ and selected to test the time of insect death showed that mortality occurred on the fourth day after application with an average of 8% (table 1). The entomopathogenic fungus *B. bassiana* and *Metarhizium* sp were effective in killing green leafhopper nymphs [19] and brown plant hopper in the fastest time of about 4 days [20]. Then the highest mortality occurred on the seventh day with an average of 22% (Table 1) on treatment of drops, then the highest mortality occurred on ninth day with an average of 82% (table 2). This shows that entomopathogenic fungus *B. bassiana* is effective when used as a biological control agent for green leafhopper nymphs. Prayogo Y and Tengkano [21] also found that *B. bassiana* has a higher virulence compared to *Verticillium lecanii* in killing *Spodoptera litura*.

The length of time pest death due to entomopathogenic fungal infections caused by fungi requires the process and stages to infect and kill larvae, namely inoculation of contacts between fungus propagules and body of insects, attachment and germination, penetration, destruction and colonization in hemolymph, infecting food tract and system new respiration then the insect will die this process generally takes 1-2 days in suitable environmental conditions. According to [22] nymphs of *N. virescens* are rarely reported to reach population levels that can cause damage directly. However, as a *N. virescens* vector insect has a high dispersal ability and this results in a higher chance of spreading the tungro virus. With application of *B. bassiana* it is expected that there will be a decrease in distribution of *N. virescens* as a vector of tungro virus so that it will reduce the spread of tungro virus to a wider area.

From the data (table 1) shows that *B. bassiana* in spray treatment effectively suppressed the death of green leafhopper compared to other treatments. This is because the spray treatment is a direct contact treatment on green leafhoppers and rice plants, where *B. bassiana* is sprayed attached to the body of host insect to germinate and develop to form a sprout tube, then enter the cuticle of insect's body. By removing an enzyme or toxin called beauvericin, this can cause disruption in function of insect haemolymph, resulting in swelling accompanied by hardening that makes insect's body tissue damage and within days the insect will die [11]. In addition to sticking to the body of insects, *B. bassiana* also attaches itself to plants, so that when plant hopper consumes the rice plant, *B. bassiana*
enters directly into stomach of insect and development of fungus occurs more quickly. The mechanism of entry of *B. bassiana* in seeds by immersion treatment, according to [23] states that the seeds are soaked in *B. bassiana* suspension, the seeds absorb water. Diffusion of water into the seeds results in physical changes of the seed coat to become soft so that the conidia are attached to the seed tissue. Once in the seed, the fungus can develop quickly because the nutrients needed by the fungus are starch and carbohydrates.

As for watering treatment, *B. bassiana* can be attached to the root surface, germinate and spread in plant tissue. This is consistent with what was stated by Agrios [23] that some fungi in soil have the ability to penetrate roots and spread in plant tissue. The spread begins at root and then goes to stems and leaves, although the incubation period required for *B. bassiana* to spread into plant tissue is not known with certainty and is clear. It is assumed that roots play an important role in spread of fungi to other parts of plant where the fungus joins with water and other elements present in the soil. Imago *N. virescens* that died from *B. bassiana* infection is characterized by presence of white mycelia or conidia on surface of insect's body, white mycelia begin to penetrate the cuticle out of insect's body, then develop and eventually cover the entire insect's body. But in this study, imago which was infected by *B. bassiana* did not show symptoms of mummification. Based on the results of mortality test (table 1) shows that mortality occurred on the third day after application with an average of 26% (table 1). On observation of the first day to the third day after application of nymph and imago deaths have not been found this is due to fungus *B. bassiana* still need time to penetrate the integument to cause infection and death in nymph. Then the highest mortality occurred on the eighth day with an average of 90% (table 1) in spray treatment. The results of [24] who tried three concentrations of *B. bassiana* conidia were $1.10 \times 10^8; 3.36 \times 10^7$; and $1.68 \times 10^7$ which was applied directly to the body of *H. antonii* imago in the laboratory, showing a mortality rate of 94-98 percent six days after application. This shows that the entomopathogenic fungus *B. bassiana* is effectively used as a biological control agent for green leafhopper pests.

Application of *B. bassiana* in egg stages aged 0 to 4 days can thwart hatching eggs up to 100% and can thwart hatching eggs between 26-56% at the age of eggs 5-7 days [25]. The application of *B. bassiana* to larval stage influences the mortality rate, ie the smaller the instar treated with *B. bassiana*, the higher the mortality rate. Application on instar 1 can cause death 100%, on instar 2 is 96.67%, instar 3 is 90%, instar 4 is 70%, and instar 5 is 63.33%. While applications in the imago stage can cause death between 80-97% with an average of 84.50% in laboratory testing [26].

4. Conclusion

Application of entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuil. can provide different effects on mortality of green leafhopper nymphs *Nephotettix virescens* (Distant) in rice plants (*Oryza sativa* L). Application of *B. bassiana* isolates with drops treatment can give the highest mortality from the first day to the tenth day after application. The application of *B. bassiana* formulation with spray treatment is more effective to influence the mortality of green leafhopper nymphs *Nephotettix virescens.*

References

[1] Heriqbaldi U, Purwono R, Haryanto T and Primanthi M R 2017 An Analysis of Technical Efficiency of Rice Production in Indonesia *Asian Soc. Sci.* **11**

[2] Manurung E M, Tobing M C, Lubis L and Priwiratama H 2012 Efficacy of several *Metarhizium anisopliae* formulations against *Oryctes rhinoceros* L. larvae. (Coleoptera: scarabaeidae) di insektarium *J. Online Agroekoteknologi* **1** 47-63

[3] Anggraini F, Suryanto A and Aini N 2013 Sistem tanam dan umur bibit pada tanaman padi sawah (*Oryza sativa* L.) varietas inpari 13 *Jurnal Produksi Tanaman* **1** 92 52-60.

[4] Rahmah M 2017 The Protection of Agricultural Products under Geographical Indication : An Alternative Tool for Agricultural Development in Indonesia *J. Intellect. Prop. Rights* **22** 90–103

[5] Sembel D T 2012 *Fundamentals of Plant Protection* Andi Yogyakarta

[6] Padmavathi G, Siddiq E A and Kole C 2001 Inheritance of protein markers detecting polymorphism among rice genotypes with contrasting host respose to green leafhopper *Current Science* **80** 1111-1112
[7] Widiarta I N, Matsumura M, Suzuki Y and Nakasuji F 2001 Effects of sublethal doses of imidacloprid on the fecundity of green leafhoppers, *Nephotettix* spp. (Hemiptera: Cicadellidae) and their natural enemies *Applied Entomology and Zoology* 36 501-507

[8] Hernayanti 2015 *Dangers of Pesticides on the Environment* Faculty of Biology Place (Purwokerto: Universitas Jenderal Sudirman)

[9] Hasnah, Susanna and Sably H 2012 Effectiveness of fungus *Beauveria bassiana* Vuill on mortality of *Nezara viridula* L. on stadia nymph and imago *J. Floratok*. 7 13-24

[10] Widiarta I N, Kusdianman D and Hasanuddin A 1999 Dinamika populasi *Nephotettix virescens* pada dua pola tanam padi sawah *Jurnal Perlindungan Tanaman Indonesia* 5 42-49

[11] Thomas M B and Read A F 2007 Infection by fungal entomopathogens *Microbiol.* 5 377-383

[12] Deciyanio S and Indrayani I G A 2008 *Beauveria bassiana* entomopathogenic fungus: Its potential and prospects in mite pest control *Perspektif* 8 65-73

[13] Trizelia and Winarto 2016 Diversity of endophytic entomopathogenic fungus from *Theobroma cacao* *Proceedings of National Seminar on Indonesian Biodiversity Society* 2 277-281

[14] Wardhani P 2017 Potential of Entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) as endophytic fungus of upland rice plants and their effects on leaf bacterial leaves disease *Thesis* (Malang: Universitas Brawijaya)

[15] Tefera T and Vidal S 2009 Effect inoculation method and plant growth medium on endophytic colonization of sorghum by entomopathogenic fungus *Beauveria bassiana* *BioControl* 54 663-669

[16] Yulianti T 2017 Potential of entomopathogenic fungi *Verticillium lecanii* and *Beauveria bassiana* in controlling green leafhopper and suppressing the intensity of tungro disease *Food Crop Agriculture Research* 30 2 114-120

[17] Fatahuddin, Amin N, Daud I D and Chandra Y 2003 Ability Test of *Beauveria bassiana* Vuillemin (Hypomycetes: Moniliales) as endophytes in cabbage plants and their effects on *Plutella xylostella* larvae (Lepidoptera: Yponomeutidae) *Indonesian Journal of Phytomedicine Fitomedika* 5 16-19

[18] Atmadja W R, Wahyono T E, Savitri T H and Karmawati E 2000 The effectiveness of *beauveria bassiana* against *Helopeltis antonii* Sign. p 176-186 *cit Sukartana P, Prasadja I, Arifin M, Wikardi E A, Kaomini and Soesilawati (eds) Proceedings of National Seminar III Wise Insect Management Towards Production Optimization* i Bogor, 6 November 2001

[19] Pangestu B D 2011 Efficacy of three entomopathogenic isolate cendawan *Beauveria bassiana* (Vuill.) Balsm in controlling *Cylas formicarius* (F.) Boleng Pest (Coleoptera: Formicidae) in sweet potatoes *Undergraduate Thesis* (Malang: Biology Department Faculty of Mathematics and Natural Sciences Universitas Negeri Malang)