Detection of fungi from rice black bug <i>Paraeucosmetus pallicornis</i> Dallas (Hemiptera: Lygaeidae) and inhibition with crude extract of <i>Calatropis gigantea</i> (Asclepiadaceae)

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Abstract. Rice black bug (<i>P. pallicornis</i>) is one of the pests that attack the rice plants in the generative phase that causes the rice easily destroyed when milled and tasted bitter after cooking hence reduces the quality and quantity of rice. The bitter taste in rice may be due to the fungus associated with rice black bug. The aimed of this research was to detect the associated fungi with rice black bug <i>P. pallicornis</i> using some sterilization methods and inhibition with of leaf crude extract of <i>C. gigatae</i>. Detection of fungi from <i>P. pallicornis</i> was conducted using three sterilization methods and control (without sterilization) namely: (1) sterilization with aquades + alcohol 70% (5, 10, 15 and 20 times dipping) + aquades; (2) aquades + alcohol 70% (10 and 20 times dipping); (3) Aquades + alcohol 90 % + NaCl 0.5 % + alcohol 90 % + aquades . Inhibition of fungi from <i>P. pallicornis</i> with crude extract of <i>C. gigantea</i> obtained by maceration method and then made some concentration to see the effect of its inhibition on the fungi associated with the <i>P. pallicornis</i>. The results showed that without sterilization, four microbe were obtained: <i>Gliocladium</i> sp., <i>Aspergillus</i> sp., black hyphae fungus and white hyphae fungus, sterilization method of Aquades + alcohol 70% with 5 times dipping in alcohol obtained <i>Gliocladium</i> sp., 10 and 20 times dipping found <i>Aspergillus</i> sp. and <i>Gliocladium</i> sp and 15 times dipping found <i>Aspergillus</i> sp. Sterilization with 10 and 20 times dipping in alcohol 70% then washing 2 times with aquades found <i>Gliogladium</i> sp. and <i>Aspergillus</i> sp. Sterilization with Aquades + alcohol 90 % + NaCl 0.5% + alcohol 90% + aquades found <i>Gliocladium</i> sp. Crude extract of <i>C. gigantea</i> had the potential to inhibit fungi <i>Aspergillus</i> sp. and <i>Gliocladium</i> sp. from rice black bug.

1. Introduction
Rice black bug <i>P. pallicornis</i> (Dallas) (Hemiptera: Lygaeidae) is a new pest in rice plants belonging to quarantine pest with A2 categories and its currently a major problem for farmers and has spread in several rice production centers in South Sulawesi and West Sulawesi with the intensity moderate to severe thereby decreasing production. In the vegetative phase the pest attacks the rice by sucking the stem liquid with symptoms of leaves of yellowish-colored rice and generally occur in young leaves and the generative phase the pest attacks by sucking the base of the stem and the rice grain and causing bitter taste in rice due to the association with the fungus [1] this affecting the quality of the rice. The results of the laboratory studies show that on the grain and rice that has been attacked by <i>Aspergillus</i> sp. And <i>Gliocladium</i> sp. The fungus contained in rice plants attacked by <i>P. pallicornis</i> is pathogenic because it
causes death in young rice leaves [2]. The previous results also showed that by sterilization method on the surface of the body of P. pallicornis was found many fungi [3]. Therefore, to reassure the assumption of an association between fungus and P. pallicornis, it is necessary to detect this by using different sterilization method from previous sterilization. Alleged existence of this association which says that the fungal origin of P. pallicornis can be pathogenic to rice crops then the necessity is also to a control method which can only control P. pallicornis but also can inhibit the development of the fungus associated with P. pallicornis by using plant extract C. gigantea. The antifungal activity of C. gigantea was also reported in some studies and it provides an important option for the biological control a plant pathogenic fungus [4] and can be used for the remedy of infectious diseases caused by pathogenic bacteria and fungi as well as for the control of insect [5]. Extract of Calatropis are harmless and nonphytotoxic, inhibitory effects on germination and on the viability of fungal spores as well and moderate to good activity against A. niger as it is a saprophyte in soil causing black mould of onion, garlic and shallot, stem rot of Dracaena, root stalk rot of Sansevieria, and boll rot of cotton; spoilage of cashew kernels, dates, figs, vanilla pods and dried prune [6]

2. Material and methods

2.1. Isolation and Identification of Fungi Emerged from P. pallicornis
Samples of P. pallicornis were obtained from Gowa Regencies at South Sulawesi. The adults were kept in a cage containing rice seedlings during the trip to the laboratory. P. pallicornis was randomly selected and stored in an airtight glass container which placed in a freezer with a temperature of ± -5°C for 1 hour before the fungus isolation process. Isolation of fungi associated with the separated insects done in three successive stages as follows: detection of fungi from P. pallicornis with three method sterilization was modified based on [7] and without sterilization. The process detection of fungi from P. pallicornis: (1). Adults were washed with aquades and Sterilized with alcohol 70% (5, 10, 15 and 20 times dipping) and washed again with aquades, (2). The adults were washed with Aquades and sterilized with alcohol 70% (10 and 20 times dipping), (3). The adults were washed with Aquades and sterilized with alcohol 90% and subsequently with NaCl 0.5% + 90% alcohol and the finally washed with aquades. The sterilized insects were put in petri dish containing of 13 ml PDA medium. The dishes were checked for fungal growth after 7 days. The emerged fungi were picked-up and transfered to new PDA medium and identified by characterizing the morphology were described according to their macroscopic features such as colour, shape and growth of cultured colonies, as well as microscopic characteristics like structure of hyphae, conidia and conidiophores. Obtained data then compared with the descriptions of fungi species present in the literature [8].

2.2. Preparation of Calotropis gigantea Extract
Fresh leaves of C. gigantea were collected from areas in and around Takalar Regency of South Sulawesi, Indonesia and shade-dried for 2–3 days, macerated with methanol solvent for 7 days and filtered through filter paper. The solvent was removed by rotary-evaporator for 6–8 h to obtain crude extract in the form of paste. It was then placed in a reagent bottle, and stored in the refrigerator as a stock solution prior to its use. The extract were prepared 3 concentration that is 1%, 1.5%, and 3% by diluting the stock solution with acetone and then suspended with 1% ml of tween-80 for each concentration.

2.3. Preparation Liquid Media for Inhibition Extract to Fungi from P. pallicornis
The extract potato containing sugar 20 grams and 1 Chloromphenicol grain were homogenized in the erlenmeyer. Furthermore, the erlenmeyer was wrapped with aluminum foil and plastic wrapping and then sterilized in an autoclave for 2 hours at 121°C.

2.4. Method of Treatment
The liquid media was prepared as much as 100 ml and add the extract in accordance with concentration and then grow fungi from Rice black bug (Aspergillus sp. and Gliocladium sp.). Observations were
made 2 times each at the age of fungi 7 HST and 14 HST were to calculate the weight of dried mycelium of fungi after being treated with C. gigantea leaf extract by filtering the fungus until no water dripped from the sieve. Then the dried mycelium is weighed using a digital scale.

3. Results and Discussion

The assessment of fungal detection in P. pallicornis revealed that the presence of Aspergillus sp. dan Gliocladium sp. was the most frequent with the process where adults were washed with aquades and sterilized with alcohol 70% with 5, 10, 15 and 20 times dipping and washed again with aquades. In NaCl treatment only presence Gliocladium. Aspergillus sp., Gliocladium sp., white fungi and black fungi was detected in not sterilized as control. Macroscopic and microscopic observations of Gliocladium sp and Aspergillus are shown in table 1 and table 2.

These results show that Aspergillus sp. and Gliocladium sp. were consistently found in various sterilization methods. Interpretation of these data that the fungi are associated with P. pallicornis. The results also show that the presence of Aspergillus is not only found in adult insects but also on the nymph of P. pallicornis from rearing in the laboratory which is F1. From the results of previous tests showed that fungus was found in the left side body of the P. pallicornis. This may be because these fungi accumulate in the left gastrointestinal tract. The similarity between Aspergillus sp. isolated from insect and from rice grain, suggested that Aspergillus sp. from rice grain may come from P. pallicornis insect and that the fungi probably reside in the digestive tract including the mouth parts. From these sites, the fungi could be transferred to the rice grain through their stylets when they feed [1].

Association between Aspergillus sp. and insect attacking plant have been reported that A. flavus to be associated grew from spiracle, recta and mouth parts on surface insects, internal organs of Lygus and stink bug and colonies of A. flavus developed from intestinal tissue [9].

| Table 1. Characterization of fungi in adults P. pallicornis |
|-----------------|-----------------|-----------------|
| No. | Genera | Macroscois | Microscois |
| 1 | Aspergillus sp. | Colonies on agar culture are spread with pale dark brown colour. Growth with irregular shape. | Conidiophores, simple, bearing conidial head. |
| 2 | Gliocladium sp. | Colonies on agar culture are brownish green colony color. Growth with circular thick shape and entire margin. | Conidiophores hyaline, erect, simple or branched oppositely or verticillately especially in metula bearing spore masses at phialides on the apical branches. |
| 3 | White hyphae fungi | Colonies on agar culture are white. Growth like cotton with circular thick shape and entire margin. | Have no conidiophore and unbranched. |
| 4 | Black hyphae fungi | Colonies on agar culture are dark cotton with white edge. Growth with circular thick shape and entire margin. | Have no conidiophore and branched. |

Aspergillus flavus has been widely accepted occurs in the field were damaging preharvest maize. Insect pest M. nigrenvenella collected feeding on maize ears was found to be significantly correlated to A. flavus infection and aflatoxin contamination causes damage before and after harvest [10]. The presence of aflatoxin content may cause damage to plants in the vegetative phase causing yellow color while in the rice grain cause bitter taste. P. pallicornis can carry a fungus that produces a toxic substance and Aspergillus sp. isolated from rice grains was similar to that isolated from the adults P. pallicornis and from rice seed was just isolated Aspergillus sp., while from adult insect Isolated Aspergillus sp. and Gliocladium sp [1]. Further, this research confirmed the occurrence of an association between Aspergillus fungus and insect pest of P. Pallicornis in causing toxicity symptoms in rice.
Gliocladium sp is an antagonist fungus used to control some plant diseases so it is likely to cause damage to very small rice. Gliocladium sp. isolated from P. pallicornis does not give effect to the damage of seeds and rice plants [11]. The existence of Gliocladium sp may be derived from planting because of the habit of P. pallicornis who likes to hide in the fractures of the soil and most species of Gliocladium are saprophytes in various environments and can be associated with healthy plant roots to protect them from pathogenic [12] and Gliocladium can be carried by adults P. pallicornis. Sometimes many saprophytic fungi in the soil can be found in the area of the digestive system of insects [13]. The ability of various species of the genus Gliocladium is to produce hydrolytic enzymes and antifungal, and antibacterial compounds [7]. In addition, some species of the genus Gliocladium have the ability to encapsulate foreign substances. This is what causes the application of various insecticides against P. pallicornis have no real effect.

| Num. | Genera          | Macroscopic | Microskopic |
|------|----------------|-------------|-------------|
| 1    | Aspergillus sp | ![Image](image1) | ![Image](image2) |
| 2    | Gliocladium sp | ![Image](image3) | ![Image](image4) |
| 3    | White fungi    | ![Image](image5) | ![Image](image6) |
| 4    | Black fungi    | ![Image](image7) | ![Image](image8) |

**Table 2.** Macrosopic and microscopic identification of fungi in P. pallicornis

**Figure 1.** Percentage of inhibition fungi from P. pallicornis by C. gigantea leaf extract
Inhibition of fungi *Aspergillus* and *Gliocladium* from *P. pallicornis* with crude extract *C. gigantea* indicated that all concentrations of *C. gigantea* crude extract (1%, 1.5%, and 3%) were able to inhibit the development of *Aspergillus* sp. and *Gliocladium* sp. (figure 1). It can be seen that the weight of *Aspergillus* sp. and *Gliocladium* sp. after application of concentration was lower than control. This clearly shows that 3% concentrations inhibit than concentrations of 1.5% and 1%. *Calotropis* (Asclepiadaceae) commonly known as is a useful medicinal plant. The two species i.e. *C. gigantea* and *C. procera* are to a great extent having a very similar chemical properties like of tannins, saponin, flavonoids, terpenoids and cardiac glycosides [14, 15] cardenolide, glucosides, a non-protein, amino acid, flavonoids and steroids [16] and aqueous extracts of leaves have demonstrated strong inhibitory effect microorganisms with the mechanism of action could be by inhibition of fungal cell wall, protein and amino acid, sphingolipid biosynthesis and electron transport chain [17].

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**References**

[1] Rosmana A, Sylvia S, Dian E S, Uvan N, Akbar A H, Ala A and Nasruddin G 2014 Fungi associated with *P. pallicornis* causing apparent symptoms of toxicity in rice grains and rice seedlings *Int. J. Curr. Microbiol. App. Sci.* **3** (2) 407-414

[2] Rosmana A, Sylvia S, Dian E S, Uvan N, Ala A and Nasruddin 2012 Identification of fungi associated with *P. pallicornis* (Dallas) (Hemiptera: Lygaeidae) and study of its impact on incidence of rice seedlings with toxicity symptoms.

[3] Amin N, Daha L, Agus N, Rosmana A and Fadhlan M 2015 Diversity of some endophytic fungi associated with rice black bug *P. pallicornis* on rice plant. *J. of Chemic. and Pharma. Res.* **7** (4) 1246-1253

[4] Gaurav K, Loganathan K and Kokati V B R 2010 Antibacterial activity of aqueous extract *C. gigantea* leaves- an in vitro study *Int. J. of Pharmaceut. Sci. Rev. and Res.* **4** (2)

[5] Shumaia P, Kader Md A, Aktar U C, Shuaib Md A R and Ekramul Md H 2013 Antibacterial, antifungal and insecticidal activities of the n-hexane and ethyl-acetate fractions of methanolic extract of the leaves of *C. gigantea* Linn. *J. of Pharmacog. and Phytochem.* **2** (5) 47-51

[6] Varahalarao V, Mohan B, Kaladhar D S V G K, Kumar S S V N, Seshagiri B and. John P M 2012 Antimicrobial profile of crude extracts *Calotropis proceria* and *Centella asiatica* against some important pathogens. *Indian J. of Sci and Tech.* **5** (8) ISSN: 0974- 6846

[7] Hallmann J, Berg G and Schulz B 2006 Isolation Procedures for Endophytic Microorganisms (Microbial Root Endophytes (Soil biology)) ed. Schulz B J E et al. (Heidelberg: Springer) pp 300–319

[8] Barnett H L and Hunter B B 1972 *Illustrated Genera of Imperfect Fungi* 3rd edition (USA: Burgess Publishing Co.)

[9] Stephenson L W and Russel T E 1974 *The Association of A. flavius with Hemipterous and other Insects Infesting Cotton Bract and Foliage* (Publication No .2298) (USA: Agriculture experiment station University of Arizona)

[10] Sétamou M, Cardwell K F, Schulthess F and Hell K 1997 *Aspergillus flavus* infection and aflatoxin contamination of preharvest maize in the Republic of Benin *Plant Disease* **81**323-1327

[11] Sari D E 2012 Pengaruh Cendawan yang Diisolasi dari Kepik Hitam *Paraeucosmetus pallicornis* Dallas (Hemiptera : Lygaeidae) Terhadap Kerusakan Bibit Tanaman Padi (*Oryza sativa*)
(Makassar: Program Studi Ilmu Hama dan Penyakit Tumbuhan, Fakultas Pertanian, Universitas Hasanuddin)

[12] Heyler 2014 *Biological Control In Plant Protection: A Colous Handbook* Second Edition (London: CRC Press)

[13] Tanada Y and Kaya H K 1993 *Insect Pathology* (New York: Academic Press Inc.)

[14] Verma V N 2014 The chemical study of Calotropis *Int. Letters of Chem. Phy. and Astronomy* 20 74-90

[15] Singh S, Sanchita Singh, Mishra R M and Shrivastava M P 2014 Preliminary phytochemical screening of *C. gigantea* leaf *Int. J. of Sci. and Res. 4* (2) 1-3

[16] Kori P and Alawa P 2014 Antimicrobial activity and phytochemical analysis *C. gigantea* root latex extract *IOSR J. of Pharmacy 4* (6) 7-11

[17] Hassan S W, Bilbis F L, Ladan M J, Umar R A, Dangoggo S M, Saidu Y, *et al* 2006 Evaluation of antifungal activity and phytochemical analysis of leaves, roots, and stem barks extracts of *C. procera* (Asclepiadaceae) *Pak. J. Biol. Sci 9* (14) 2624-9