Antagonistic activity of wild growing mushrooms against various fungal rice pathogen.

Nurkhalida Mohd Baseri
Universiti Putra Malaysia
Muhammad Salahudin Kheirel Anuar
Universiti Putra Malaysia
Nur Alia Syamimi Shamsuhazli
Universiti Putra Malaysia
Mohd Amirul Faiz Zulkifli
Universiti Putra Malaysia
Helmi Wasoh
Universiti Putra Malaysia
Mohd Termizi Yusof (mohdtermizi@upm.edu.my)
Universiti Putra Malaysia https://orcid.org/0000-0002-5241-3443

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Abstract

Paddy is an important crop in Malaysia. There are various pathogens able to infect paddy causing a loss in yield's production. In this study, dual culture method, volatile organic compounds (VOCs) analysis and non-volatile compound analysis were used to assess the ability of mushroom to control fungal rice pathogens including *Curvularia lunata*, *Bipolaris panici-miliacei* and *Nigrospora* sp. Four mushroom isolates were further analysed for their antagonistic activity against rice pathogen. The highest percentage inhibition of radial growth (PIRG) was recorded between 45.55–73.68% observed in isolate 42b. The 4 isolates with the highest PIRG based on the dual culture analysis were then tested for their production of VOCs and non-volatile compound. Internal transcribed spacer (ITS) region analysis of the 4 mushroom isolates revealed their identity as *Coprinellus disseminates* (isolate 12b), *Marasmiellus palmivorus* (isolate 42b), *Trametes maxima* (isolate 56e), and *Lentinus sajor-caju* (isolate 60a). This study showed that mushroom isolates have the potential of antagonistic effect on various fungal rice pathogens tested by the production of secondary metabolites and mycoparasitic interaction.

Introduction

In Malaysia, paddy is one of the most important commercial crops planted after oil palm and rubber (Zakaria et al. 2010). According to Crop Statistics (Food Crops Sub-Sector) 2017 released by the Department of Agriculture Peninsular Malaysia, in 2016, the nation produced a total of 2.7 million metric ton of paddy. Farmers lose an estimated average of 37% of their crop to pest and diseases each year (Savary et al. 2000). Diseases related to paddy are caused by various species of fungi including *Curvularia lunata*, *Bipolaris panici-miliacei*, and *Nigrospora* sp. (Kusai et al. 2015; Mohana et al. 2011). In order to control or prevent diseases, farmers rely on the usage of fungicides. However, the use of chemical pesticides has been known to cause various environmental and health problems (Sharma and Singhvi 2017). Therefore, one of the alternative ways to replace the use of fungicide is by using microbial antagonists as a biological control agent. Biological control agent is the exploitation of naturally occurring living organisms and their metabolites to control the pathogen or pest (Sodhi and Kapoor 2014). Using living organisms as an agent is considered as natural and environmentally acceptable compared to the use of chemicals. The use of biocontrol agent to control plant pathogen has been studied extensively and applied in some agricultural practices (Han et al. 2019; Jagtap and Suryawanshi 2015; Anand and Jayarama 2009; Rahman et al. 2009). Mushrooms are abundantly found in various habitats throughout Malaysia. Studies related to mushroom are somehow limited to their nutritional benefit as a food source. There is little information available on the antimicrobial properties of locally grown mushroom (Wong et al. 2009) especially against plant pathogen. Mushrooms, like other plants, secrete antimicrobial compounds in order to survive and flourish in the environment (Reis et al. 2017).

Materials And Methods

Isolation of mushroom samples

Samples were collected from various locations in Selangor, Malaysia. The collected samples were kept in a paper bag and stored at 4ºC prior to analysis. The mushroom fruiting body was surface sterilize using 10% sodium hypochlorite, sterile distilled water, and 70% ethanol for 5 to 10 sec respectively. The fungal tissue was then blotted dry and cut into smaller pieces. Next, each piece of the samples was transferred into Potato Dextrose Agar (PDA) medium and incubated at room temperature.

Dual Culture Analysis

A 6 mm agar disc of the 5-day-old mushroom isolate was placed 2 cm away from the periphery of the petri dish and the same sized agar disc of the test fungus was placed similarly on the opposite side of the mushroom isolate. As a control, the test fungus was placed in a similar manner on a fresh PDA plate without any mushroom isolate. All pairings were carried out in triplicate and incubated at room temperature. Mycoparasitic interaction was recorded 5 days after incubation by measuring the radius of the test fungus (R_1) on the control plate and the radius of the test fungus in the direction to the mushroom isolate (R_2) in a dual culture plate. The percentage inhibition of radial growth (PIRG) was calculated as described by Sundram (2013).

Volatile Organic Compounds (Vocs) Analysis

Analysis was performed as described by Siddiquee et al. (2009). A 6 mm agar disc of test fungus was placed at the centre of PDA plate and the same size agar disc of 5-days old mushroom isolate was placed on another PDA plate similarly at the centre. The lids of both plates were removed, and the test fungus plate was immediately placed on top of the mushroom isolate plate. Both plates were held together with parafilm. For the control plate, only test fungus was cultured on the PDA. All plates were incubated at room temperature for 5 days. The percentage inhibition of mycelium growth (PIMG) was calculated by measuring the diameter of test fungus mycelium (R_1) on the control plate and diameter of test fungus mycelium (R_2) on the test plate.

Non-volatile Organic Compounds Analysis

Analysis was performed as described by Siddiquee et al. (2009). Each PDA plate was overlaid with a sterilized cellophane membrane. A 6 mm agar disc of the mushroom isolate was placed at the centre of the cellophane membrane and incubate at room temperature for 5 days. After 5 days, the cellophane membrane adhering with the mushroom isolate mycelium was removed. After that, a 6 mm agar disc of test fungus was placed at the centre of the plate. For the control plate, the same size of test fungus was placed at the centre of the PDA plate. All plates were then incubated at room temperature for 5 days and the diameter of test fungus was recorded.

Molecular Identification

Analysis was done by extracting DNA from fresh mycelia grown on the PDA laid with cellophane. PCR was performed by amplifying internal transcribed spacer (ITS) region using ITS1 and ITS4 primers. The total volume of the reaction was 50 µL consisting of 25 µL Taq PCR Master mix, 19 µL ultrapure water, 2 µL of ITS1 and of ITS4 primer each and 50 µg of genomic DNA template. The PCR mixture was amplified for 30 cycles in the following manner: initial denaturation at 94°C for 2 min, denaturation at 94°C for 15 sec, annealing at 49°C for 30 sec, extension at 72°C for 1 min and the final extension at 72°C for 7 min. Amplicons were purified and sequenced using ABI PRISM 3730 × 1 Genetic Analyzer (Applied Biosystem, USA). Sequences were finally edited in Chromas software and MEGA 5.0 and were compared in NCBI GenBank sequences using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). Alignments were performed using ClustalW. A phylogenetic tree was created using the neighbor-joining (NJ) method and a bootstrap analysis of 1000 replicas implemented in Phylogeny.fr (Dereeper et al., 2008). The sequences of *Polyporus* tubiformis and *Mycetinis scorodonius* are used as the outgroup.

Results

Dual culture analysis

Twenty mushroom isolates were successfully isolated and subjected to dual culture assay. Only 4 isolates show positive inhibition with PIRG value between 20–80%. Mushroom isolate 42b gave the highest PIRG value against all three test fungi with 45.55% growth suppression on *C. lunata*, 68.68% growth suppression on *B. panici-miliacei*, and 73.68% growth suppression on *Nigrospora* sp. (Fig. 1). *Curvularia lunata* was the least affected by this interaction compared to the other test fungi with PIRG value of all isolates ranging from 28.88–45.55%.

Volatile Organic Compounds (Vocs) Analysis

The mushroom isolates varied in their abilities to produce volatile compounds that can inhibit the growth of the test fungi. VOCs analysis of the mushroom isolates showed PIMG value ranging from 4–40% (Fig. 2). Isolate 12b shows the highest PIMG value with 33.11% against *B. panici-miliacei* and 36.06% against *Nigrospora* sp. While isolate 56e shows the highest PIMG value against *C. lunata* with 18.65%. However, all mushroom isolates exhibit only minimum growth inhibition against the tested fungi.

Non-volatile Compounds Analysis

All mushroom isolates released non-volatile compounds that diffused into the PDA medium resulting growth inhibition of the test fungi. PIMG value recorded ranging from 5–90% growth inhibition for the test fungi. Isolate 60a gives the highest PIMG value with 38.35%, 83.77%, and 59.56% against *C. lunata*, *B. panici-miliacei*, and *Nigrospora* sp. respectively. The growth *B. panici-miliacei*, was greatly inhibited by all mushroom isolates with PIMG value ranging from 69.72–83.77% while *C. lunata* showed the least growth inhibition, between 5.25–38.35% only (Fig. 3).

Identification Of Fungal Isolated

Sequence analysis of PCR products were compared with the NCBI database sequences using the BLAST program. Isolate 12b has 99% sequence homology to *Coprinellus disseminates*, isolate 42b has 98% sequence homology to *Marasmiellus palmivorus*, isolate 56e has 99% sequence homology to *Trametes maxima*, and isolate 60a had 97% sequence homology to *Lentinus sajor-caju* (Table 1). To further confirm the identity of the mushroom isolates, phylogenetic trees were constructed and compared to several reference species (Fig. 4).

Table 1
The BLAST result of sequence analysis by NCBI for the identification of mushroom isolates.

Isolate	Class	Order	Family	Genus	Species	Identical (%)	Accession
12b	Agaricomycetes	Agaricales	Psathyrellaceae	*Coprinellus*	*disseminatus*	99	KX017207.1
42b	Agaricomycetes	Agaricales	Marasmiaceae	*Marasmiellus*	*palmivorus*	98	JQ653427.1
56e	Agaricomycetes	Polyporales	Polyporaceae	*Trametes*	*maxima*	98	JN164918.1
60a	Agaricomycetes	Polyporales	Polyporaceae	*Lentinus*	*sajor-caju*	96	KP283493.1

Discussion

Mycoparasitic interaction is a basic indicator to assess the antagonistic properties of microorganisms (Rahman et al. 2009). There are several methods that can be used to assess this interaction and the most common one is the dual culture method. In this study, four mushroom isolates which have been identified as *Cop. disseminates* (12b), *M. palmivorus* (42b), *T. maxima* (56e) and *L. sajor-caju* (60a) show a various degree of inhibition toward test fungi based on the dual culture analysis. *M. palmivorus* gave the highest PIRG value compared to the other isolates. A similar observation was reported by Lallawmsanga et al. (2016), *M. palmivorus, L. sajor-caju*, and *T. hirsute* show antifungal activity towards *Fusarium oxysporum, Candida albicans*, and *Fusarium proliferatum*. Abdullah et al. (2005) also showed Malaysian tropical mushrooms such as *Marasmiellus* sp. and *L. squarrosulus* have the ability to produce secondary metabolites that have an antibacterial and antifungal effect.

One of the possible inhibition mechanisms exert by this mycoparasitic interaction are mycelium interaction. Different mycelium density and inhibition pattern on the agar plates were observed. Boddy (2016) reported mycelium interaction could be observed by naked eye including the growth rate of the mycelium, changes in mycelium morphology, pigmentation and the presence of lysis zones. Other mechanisms are through the ability of mushroom isolates to grow faster than the test fungi.

These mushroom isolates also suppress the growth of the test fungi through the production of various secondary metabolites including volatile and non-volatile metabolites. Based on the *in vitro* analysis, we able to observe the growth inhibition of test fungi when exposed to the volatile compounds produced by the mushroom isolates. However, none of the mushroom isolates had done above 45% PIMG value. This shows that their volatile compound does not give a strong inhibitory effect. Some volatile compounds were capable to suppress other fungal or microorganisms generally. Gary et al. (2001) identified five classes of volatile compounds such as esters, lipids, alcohols, acids and ketones. A volatile compound such as junipal from *Daedalea juniperina* has been reported to possessed fungistatic and fungicidal effects (Fries 1973). Furthermore, Osaki-Oka et al. (2019) identified Isovelleral, a volatile compound from fruiting bodies of *Russula* spp., able to inhibit mycelial growth and conidial germination of a phytopathogenic fungus, *Alternaria brassicicola.*

Mushroom isolates are also capable of producing a diffusible toxic substance which non-volatile. In general, microorganisms will generate waste products which also known as metabolites during the growing phase. Some of these metabolites have properties that can inhibit other microorganisms. In this study, *L. sajor-caju* shows highest PIMG value indicating that their non-volatile compound has inhibitory properties and *B. panici-miliacei* was effectively inhibited compared to another test fungus. A similar study was done on basidiomycete *Earliella scabrosa* that capable to produce active metabolites such as 2(3H)-furanone, 5-heptyldihydro-, 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- and triacetin to inhibit the growth of fungi on rubberwood (Peng and Mat Don, 2013). This finding also proves that basidiomycete has the ability to produce usable metabolites. Study on *Trichoderma viride* shows positive inhibition through the production of diffusible metabolites on *Pythium* sp. (Patil et al. 2012). Similar study on *Trichoderma* spp shows positive inhibition through production of antifungal non-volatile metabolites on soil borne diseases of chickpea (Nagamani et al. 2017). According to Naher et al. (2014), secondary metabolites can exist in the form of the volatile and non-volatile compound. 6-pentyl-alpha-pyrone (6PAP) is an example of a well-known volatile antifungal compound which has been characterised as a secondary metabolite (Siddiquee et al. 2009). Some of these volatile compounds are common to many fungi, and some are unique to certain species.

From all the tests performed, metabolites produced by mushroom isolates were effective inhibitors for the growth of *B. panici-miliacei* and *Nigrospora* sp. meanwhile, the opposite result was showed for *C. lunata*. This is because *C. lunata* is well adapted to the environmental changes and able to produce their own defence compound. A study by Jagtap and Suryawanshi (2015) used *C. lunata* as biocontrol agent candidate in controlling *Fusarium oxysporum* which causes basal rot of onion in their studies. In another study of Avinash et al. (2015) the crude extract of *C. lunata* showed antimicrobial activity towards bacteria and fungi.

Conclusions

In conclusion, each mushroom isolate has their own antagonistic potential toward the tested rice pathogen. Characterization and identification of these metabolites will improve our understanding of mechanisms involved in suppressing the pathogen growth as well as promoting plant growth. This information will further give the idea of how to design the application of the fungal biocontrol to the field in order to protect the crops from diseases. The implementation of the biocontrol may improve the production of the crops, environmentally friendly, and reduce the use of chemicals that affect human health.

Declarations

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Authors' contributions

All the authors contributed equally and substantially to the work.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

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Figures

Figure 1

(a) Dual culture analysis of indigenous mushroom isolates against various rice pathogens. (b) Percentage inhibition of radial growth (%) of various rice pathogens against mushroom isolates based on dual culture analysis. Data represent means ± SEM, n=3.

Figure 2

(a) The effect of volatile organic compounds produced by mushroom isolates against various rice pathogens. (b) Percentage inhibition of mycelium growth analysis of various rice pathogen in the presence of volatile compound produced by mushroom isolates. Data represent means ± SEM, n=3.

Figure 3

(a) The effect of non-volatile organic compounds produced by indigenous mushroom isolates against various rice pathogens. (b) Percentage inhibition of mycelium growth analysis of various rice pathogen in the presence of non-volatile compound produced by mushroom isolates. Data represent means ± SEM, n=3.

Figure 4

A phylogram (neighbor-joining method) showing genetic relationship between (a) isolate 12b, (b) isolate 42b, (c) isolate 56e and (d) isolate 60a with other related reference fungi based on the ITS region sequence analysis. Species name are followed by the accession numbers of their ITS region sequences. The numbers at branching points or nodes refer to bootstrap values, based on 1,000 re-samplings. *Polyporus* tubiformis and *Mycetinis scorodonius* are used as the outgroup.