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# *In Vitro* Management of *Ganoderma* Basal Stem Rot Disease on Oil Palm Using Wild Basidiomycetes Fruiting on the Ground after Rainfall

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***Abstract*:** *Basal stem rot (BSR) has been reported becoming a major treat to oil palm industry in Malaysia and Indonesia. Many control methods have been adopted for management of BSR but still not able to control the disease. As alternative, the use of biocontrol agents such as wild basidiomycete fungi (macrofungi) are ideal options, apart from chemical and cultural methods to reduce Ganoderma problem in oil palm plantations due to the production of some potent secondary metabolites which have been recognized with anti-phytofungal, anti-phytobacterial, anti-phytoviral, phyto-nematicidal and mosquito larvicidal activities. Thus, the ultimate aims of this study were to survey and identify wild basidiomycetes that have in vitro effect on the radial growth of Ganoderma isolate that caused basal stem rot on oil palm. Subsequently, wild basidiomycetes can be used as the potential targets for seeking unknown and useful natural products that could lead for the development new biopesticides or provide potentially new biocontrol agents in sustainable pest and disease managements. In this study, diverse macrofungi belong to phylum basidiomycota have been collected in the Jasin district of Melaka during rainy season excepted for two isolates which were found at Pedas Linggi Laybay, Plus Highway (south bound), Negeri Sembilan and at the roadside of Jalan Gapam, Melaka, respectively. All macrofungi isolates were isolated, grow and maintained on potato dextrose agar (PDA) from tissue culturing of the mushroom fruiting body. In vitro antagonistic activity of wild basidiomycetes on Ganoderma sp. was determined in a plate assay by using dual inoculation method. During survey study, 56 wild unknown basidiomycete isolates have been discovered and initially identified based on their fruiting body morphological characteristics. Many of them were either reluctant to grow or show low growth rate (yielding only small fungal colonies in size) on PDA after 7 days incubation at 28-30$^0$C. Further identification of all those wild basidiomycetes have been done based on their cultural characteristics; morphological characteristics of spores, hyphae and asexual reproductive structures but were still not very helpful or inadequate findings for fungal recognition. A total of seven wild basidiomycetes have been known and exhibited different level of antagonism to suppress the mycelial growth of Ganoderma isolate on PDA medium. But only three isolates (mushroom isolate no. 10, 17 and 56) exhibited strongest antagonism and the rest (four isolates) displayed moderate or slight antagonistic activity against Ganoderma sp. based on their antagonistic rating scales. The results suggest that wild basidiomycetes fruiting on the ground during rainy season may be potentially useful sources of new biocontrol agents which could inhibit the growth of Ganoderma sp. and therefore reduce the Ganoderma basal stem rot disease infection on oil palm.*

***Keywords:*** *Basal stem rot, Basidiomycetes, Ganoderma sp., biocontrol agents, in vitro antagonistic activity.*

# 1. Introduction

Basal stem rot (BSR) has been considered as the most destructive disease on oil palm in South East Asia. In Malaysia, four different species of *Ganoderma* have been identified and associated with BSR which *Ganoderma boninense* is the most aggressive species to cause the disease on oil palm [10], [14]. Many control methods have been adopted for management of BSR such as mechanical and chemical treatments, plus cultural practices but still have *not* proved satisfactory to control the disease due to the fact that *G. boninense* had various resting stages (melanised mycelium, basidiospores and pseudosclerotia) [33]. Thus, reference [33] and [26] suggested that alternative control approaches to overcome the *Ganoderma* problem were focused on the use of biocontrol agents and planting resistant materials.

Several promising antagonistic fungi and bacteria have been reported as biocontrol agents against *G. boninense* on oil palm [33], [23] which mostly concentrated to either on seedlings level or in small scale experiment or under glass house condition. Among all microbes, *Trichoderma harzianum* and *Gliocladium viride* were reported be superior to *Bacillus* sp. in large scale trial which the disease incidence became lower in a field treated with those fungi rather than in untreated fields [33]. Although *Trichoderma* spp. have been identified as the most effective biocontrol agents for managing BSR on oil palm, but they only protect the plants at the very early stages of the disease and were not able to cure highly infected palms [12], [28], [33]. Currently, there is no single approach proven effectively control the disease in the field, thus finding other options or alternatives are extremely important to us.

Bolhassan *et al*. (2012) been identified 60 species of macrofungi from five families in Peninsular Malaysia. In this study, biocontrol properties of basidiomycetes (mushrooms) were evaluated against *Ganoderma* isolate by *in vitro* studies. According to Sivanandhan *et al*. (2017), basidiomycetes were flourished with secondary metabolites which possessed antimicrobial, antitumor and antioxidant properties. Consequently, searching for safe and more potent alternate products for controlling plant pathogens and pests can be performed with basidiomycete extracts wherein ideal for sustainable agricultural production with minimum damage to the environment. Some previous studies have shown that basidiomycete culture filtrates, methanol and water extracts consisted of antifungal agents against several plant fungal pathogens [13], [34]

In order to exert antagonistic effect of fungal isolates, the colony interactions were studied by inoculating the various basidiomycetes with *Ganoderma* sp. in dual culture method. Antagonistic interaction and mycoparasitism can be used to determine potential biocontrol agents of plant diseases caused by fungi. By some modifications, the mode of interaction was identified according to [9] and [36]. Parameters used for the measurement of inhibition were the width of inhibition zone and demarcation line or observations for the mycelium of the aggressor advances on a front over the mycelium of the victim (antagonistic interaction through direct hyphae contact).

Although biocontrol agents have not provided a complete solution for BSR control but the technique has become a notable approach compared to others because did not cause any damage to the plant and leaving any toxic elements which are harmful to beneficial microbes and soil environment. Thus, the objectives of the study pursued to survey, identify fleshy basidiomycete isolates grown on the ground during rainy season in Jasin District, Melaka which have antagonistic activity for *in vitro* management of basal stem rot disease on oil palm.

# 2. Materials and Methods

## 2.1. Sample Collection of Ganoderma Basidiocarps and Raising of a Pure Culture

The *Ganoderma* isolates were isolated from basidiocarps of *Ganoderma*-infected oil palm trees growing in Kampung Seri Mendapat, Merlimau, Melaka with severe basal stem damages. Field samples were brought to laboratory and potato dextrose agar (PDA) used to obtain the isolates and culture maintenance. Isolation of fungal pathogen have been done in accordance to method suggested by [22], with some modifications. Small pieces (approximately 5 mm x 5 mm x 5 mm) from inside or the central core of freshly *Ganoderma* basidiocarp

tissues were removed or cut by using flame-sterilized scalpel. They are then sterilized by dipping in 95% ethanol and sterilized again by exposure to a Bunsen burner flame for a few seconds. The sterilizing process was repeated in triplicate to kill all potential contaminating organisms. After transferring the sterilized infected tissues into PDA medium, the fungal culture was incubated in incubator at 28-30$^0$C to induce quick growth of fungal pathogen. The identification of fungal pathogen was confirmed based on spore morphology and cultural characteristics. According to Narayanan (2011), the morphological characteristics of spores, hyphae, asexual and sexual reproductive structures can be used as the basis identification up to genus or species of fungi.

## 2.2. Survey and Isolation of Wild Basidiomycetes from Fresh Fruiting Bodies

In this study, fungi were isolated from fresh fruiting bodies of 56 wild mushroom samples fruiting on the ground during raining season roughly undertaken from November 2016 till May 2017 in Jasin district of Melaka excepted for two isolates which were found at Pedas Linggi Laybay, Plus Highway (south bound), Negeri Sembilan and at the roadside of Jalan Gapam, Melaka, respectively. As proposed by [34], each fresh fruiting body was cut with a sterile flame-sterilized scalpel. Small fragments of fungal tissues (approximately 5 mm x 5 mm x 5 mm) from each fruiting body were cut out and surface-sterilized in 80% ethanol for 2 min and 1% sodium hypochlorite for 3 min before triplicate rinsed in sterile distilled water for 2 min. The fungal tissue samples were gently blotted *dry* with sterile filter paper to remove adhering solution before cultured on PDA at 28-30$^o$C for 7 days. Fungal isolates that emerged after the inoculation were transferred to new PDA medium and maintained on PDA slants until use.

## 2.3. Identification of Fungi

Besides cultural-based morphological approaches, microscopic examination was conducted to observe the structure and characteristic of fungi for fungal identification. A sterile scalpel was used to pick a small portion of hyphae reproducing spores and placed onto a sterile glass slide before stained with lactophenol cotton blue. According to [16] the lactophenol cotton blue (LPCB) wet mount preparation was the most widely used and simple method to observe micromorphological characterization of fungi under the microscope. The morphological and cultural characteristics observed for *Ganoderma* isolates were compared with structures in the identification guides for *Ganoderma* species by [10] and [11] For Basidiomycete isolate identification, a study by Lee *et al.* (2012) was referred to identify all those fungi besides other reliable resources or information searched in internet.

## 2.4. In Vitro Effect of Wild Basidiomycetes to Ganoderma isolate

*In vitro* antagonistic activity was observed between *Ganoderma* isolate and each wild basidiomycete isolate in a plate assay. Around 5 mm x 5 mm square block from the edge of the seven days old of *Ganoderma* isolate was placed 2 cm at the edge on PDA plates and allowed to grow for 3 days. In similar square shape and size, each wild basidiomycete isolate was transferred approximately 4 cm apart at the opposite side of PDA plates containing *Ganoderma* sp. Inoculated plates were incubated at *28-30$^o$C* for 7 days. The mode of interaction was identified according to [9] and [36] but with some modifications. Examples no visible sign of inhibition of pathogenic fungus which its mycelium overgrown the potential antagonistic fungus (antagonistic rating scale = 0), both organisms stopped growing on contact at the centre or close to the centre of the petri dish (1), pathogen with inhibition zone less than 1 cm in width (2), pathogen with inhibition zone more than 1 cm width (3), test antagonistic fungus had grown across the centre line of the petri dish (4) and pathogen by overgrowth or displacement of pathogen by the antagonistic fungus (5).

Fig. 1a *Ganoderma* fruiting bodies found on the trunk of infected oil palm.

Fig. 1b Pure culture of *Ganoderma* isolate on PDA medium.

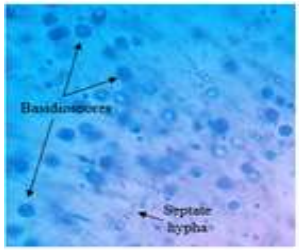



Fig. 1c Basidiospores and hyphae of *Ganoderma* isolate observed under light microscope at 400x magnification.

Fig.1: *Ganoderma* brackets formed at the base of a BSR infected oil palm and its *in vitro* micromorphological characteristics under light microscope

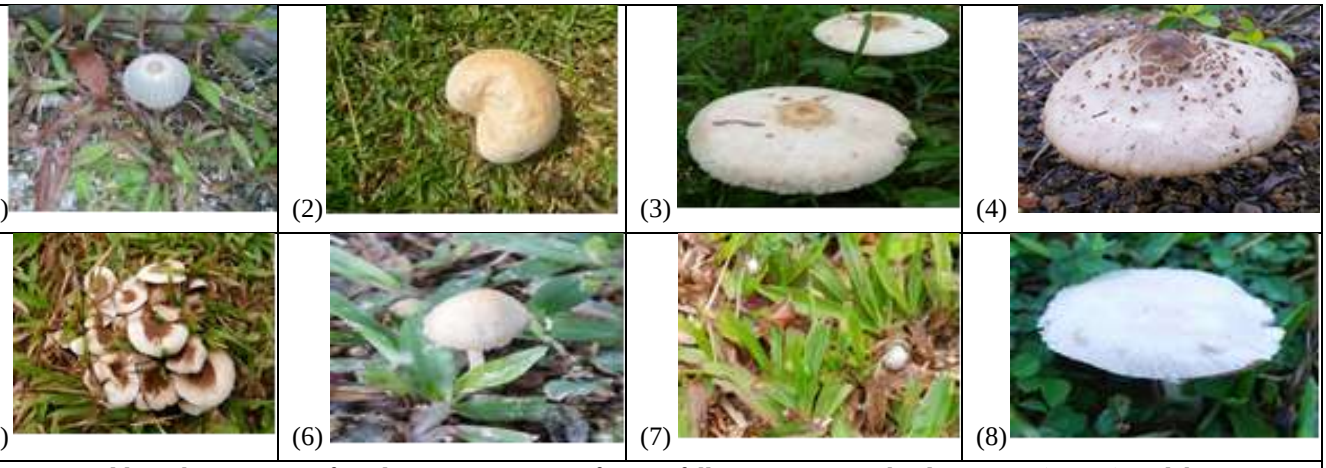



Fig. 2 Wild mushrooms were found growing in grass after rainfall at Universiti Teknologi Mara (UiTM) Melaka, Jasin Campus, Merlimau, Melaka

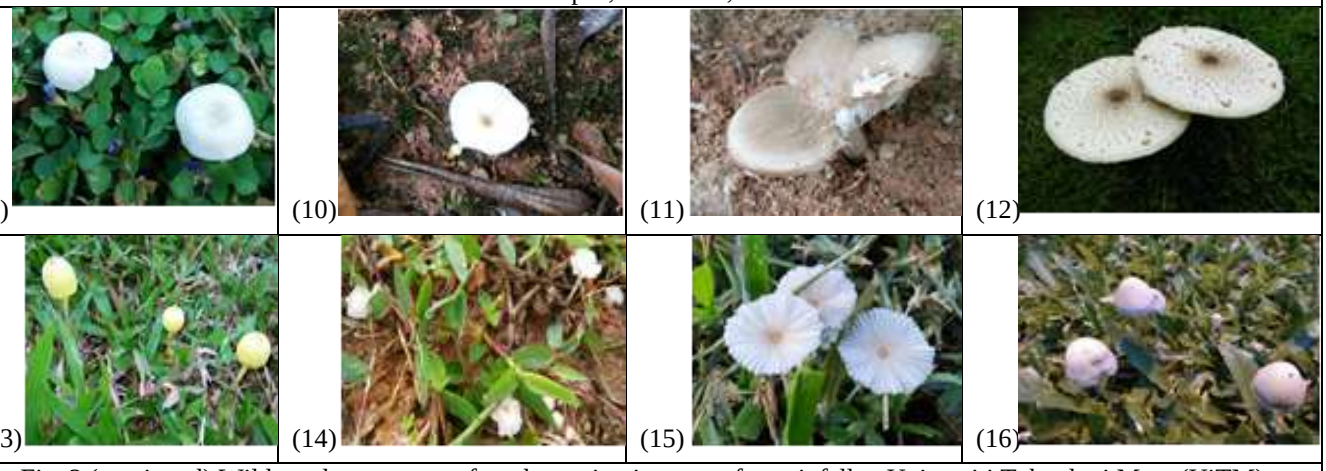
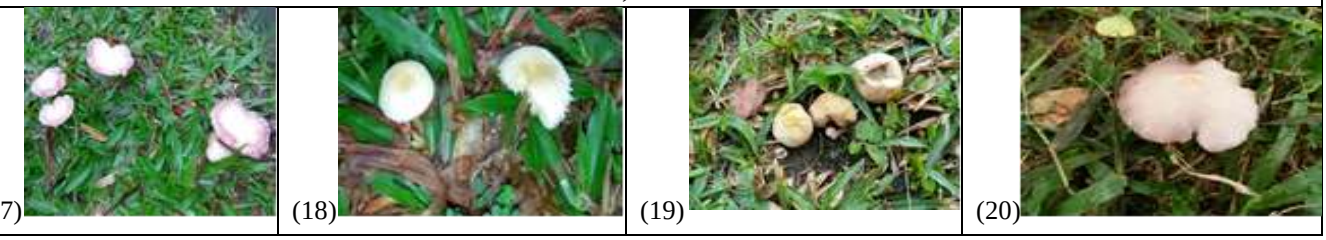
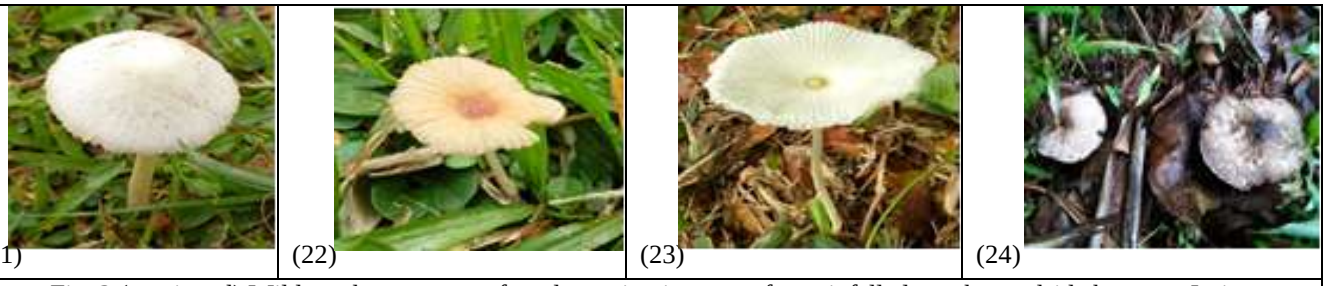


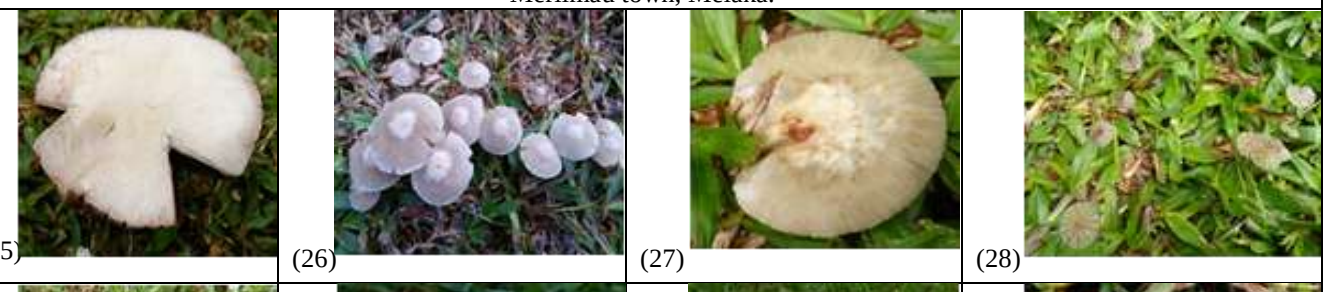

Fig. 2 (continued) Wild mushrooms were found growing in grass after rainfall at Universiti Teknologi Mara (UiTM) Melaka, Jasin Campus, Merlimau, Melaka (no. 9-12). The rest (no. 13-16) were discovered at Taman Lipat Kajang Perdana, Jasin, Melaka



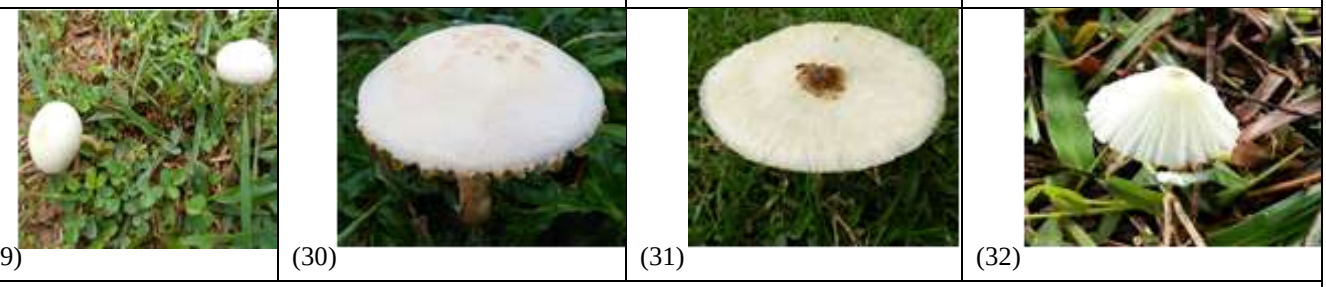


(21) (22) (23) (24)

Fig. 2 (continued**)** Wild mushrooms were found growing in grass after rainfall along the roadside between Jasin to Merlimau town, Melaka.

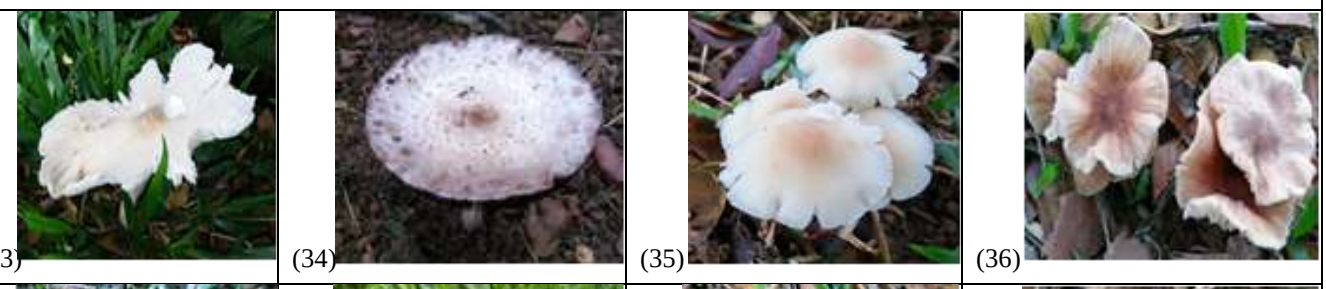


(25) (26) (27) (28)

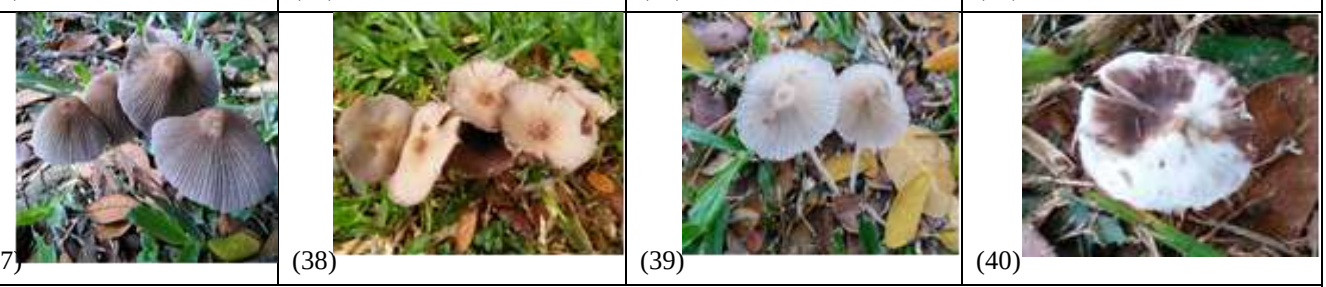


(29) (30) (31) (32)

Fig 2 (continued) Wild mushrooms were found growing in grass after rainfall along the roadside between Jasin to Merlimau town, Melaka (no. 25-29). Fungal fruiting body no. 30 was discovered at Taman Simpang Kerayong, Jasin, Melaka and both wild mushrooms (no. 31 & 32) were originated from wood industrial area, Merlimau, Melaka.



(33) (34) (35) (36)



(37) (38) (39) (40)

Fig. 2(continued**)** Wild mushrooms were found growing in grass after rainfall along a main route to the North-South Expressway Southern Route via the Jasin Interchange area.

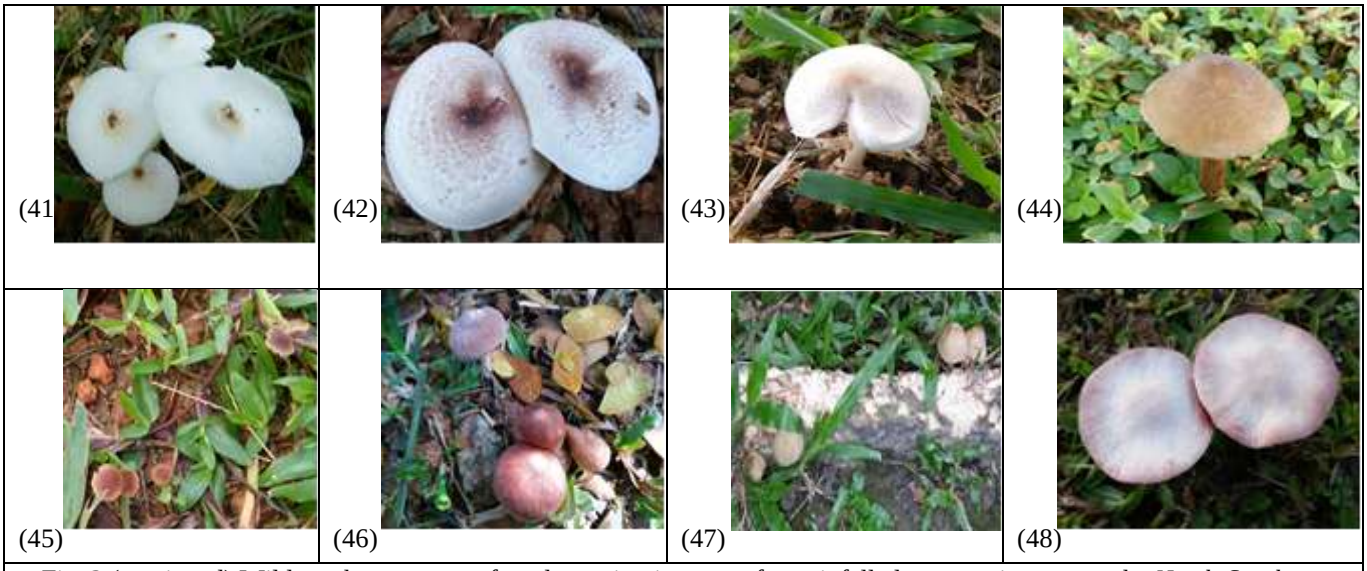



Fig .2 (continued) Wild mushrooms were found growing in grass after rainfall along a main route to the *North-South Expressway Southern Route via* the *Jasin Interchange area (no. 41 & 42)*. Fungal isolates no. 43-46 was emerged at roadside in Kg. Bukit Kepok, Merlimau, Melaka. Fruiting bodies of unknown fungus no. 47 were found at Pedas Linggi Laybay, Plus Highway (south bound), Negeri Sembilan and no. 48 observed at the roadside of Kg. Felcra Seri Mendapat, Merlimau, Melaka.


Fig. 2 (continued) Wild mushrooms were found growing in grass after rainfall in different locations in Melaka. No. 49 & 50 - were found in Jasin Town; No. 51 & 52 – At the roadside in Kg. Terentang, Jasin; No. 53 – at the roadside near to Chinchin, Jasin; No. 54 – At the roadside of Jalan Gapam, Air Keroh; No. 55 – At the roadside of Alor Gajah-Melaka-Jasin (*AMJ*) Highway and No. 56 - At the roadside in Bemban Town.

## 3. Result and Discussion

### 3.1. Morphological Features of Ganoderma isolates

During survey study, *Ganoderma* isolates were characterized by their woody basidiocarps (Fig 1a). They were sessile, flat and bracket-shaped on the trunks of infected palms. The dorsal surface was laccate character, blackish-brown colour with concentric markings. The edge was white and the under surface was also white in colour. Based on the *in vitro* study, typical characteristics of the fungal mycelium were shown in Fig 1b. Cultural characteristics of *Ganoderma* isolate had an undulating surface appearance and white in colour. Similar results have been reported by [11] for the mycelial growth characteristics of *Ganoderma boninense* on PDA medium. Moreover, the morphological characteristics of their basidiospores were ovoid in shape, double-walls and with septate hyphae (Fig 1c) as similar observations have been reported by [11] and [20] for *in vitro* growth

of *Ganoderma* isolates viewed under light microscope. [7] described Ganodermataceae have a unique double-*walled* basidiospore which called ganodermatoid.

### 3.2. Fruiting Body Collection

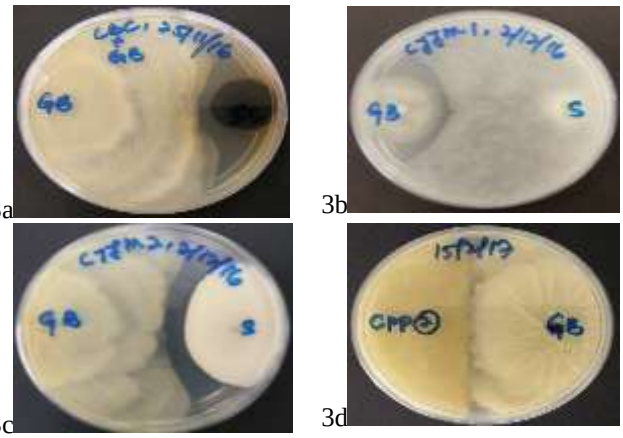
A total of 56 species of mushrooms with an amazing amount of morphological diversity were recovered from the survey study and photographed in their habitats (Fig. 2). They displayed a noticeable variation in their shape, size, colour, texture, smell and locality found in Jasin District, Melaka. Most of them were found fruiting in grass excepted for five mushroom isolates which grown on the ground (Fungal isolate no. 4, 10, 11, 34 and 54). Among the fungal isolates, two pear-shaped puffball mushrooms have been discovered (Fungal isolate no. 2 and 19). A few of them were reproduced and growth in cluster such as for isolate no. 5 and 51 and the rest in singly form.

### 3.3. Fungal isolation and identification

In this study, all mushrooms were classified belong to members of the phylum basidiomycota, commonly known as basidiomycetes. According to [21] basidiomycetes were well known for the production of large spore-bearing structures (mushrooms, brackets, puffballs, false-truffles, cup fungi, etc.) that formed above ground and can be visible to naked eye based on their morphological structures (Fig 2). Pure cultures of wild mushrooms were then obtained on PDA medium but majority of them did not grow on it at all. Some were successful isolated and grown on PDA medium but with poor mycelia growth rate (produced only small fungal colonies with small size) after incubated at 28-30$^{o}$C for 7 days. Morphological, cultural and microscopic characteristics of basidiomycete isolates also have been done but produced insufficient information to provide full identification of mushroom-forming species. Only seven *fast-growing* fungal isolates (fungal growth begins from 72 hours to 10 days after the provision of growing conditions) and easily propagated on PDA medium were used to determine its significant attention for antagonistic activity against *Ganoderma* isolate in dual culture assessment.

### 3.4. *In vitro* antagonistic activity for selected fungal species against *Ganoderma* sp.

On the basis of their growth rate, seven fast-growing basidiomycetes were subjected to determine their antagonistic activity against *Ganoderma* isolate *in vitro dual culture* assays. In Fig 3, all fungal isolates show its antagonistic activity which were suspected due to differences in nutrient resources utilization capacity between both target and test fungi, growth rate; or by the mycoparasitism process or production of some secondary metabolites (antibiosis or mutual inhibition at a distance) for lytic activity. Table I shows colony interactions and mycoparasitism between seven basidiomycete isolates with *Ganoderma* sp. which have been observed in dual culture assays. It was appeared that three basidiomycete isolates overgrown the colony of *Ganoderma* isolate through direct hyphae contact (mycoparasitism or antagonism), whereas the presence of inhibition zones in dual culture plates inoculated with basidiomycete isolate CBC and CJJM2, respectively indicate the production of extracellular metabolites (such as antibiotics and lytic enzymes) by the antagonistic fungi against the pathogen (*Ganoderma* isolate).



3a 3b

3c 3d

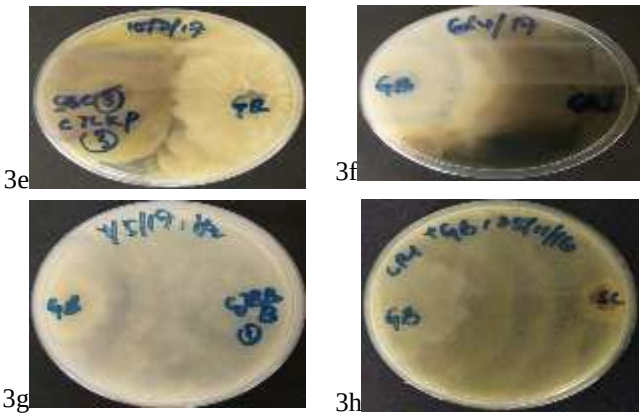


Fig. 3 Antagonistic assays of wild basidiomycetes against *Ganoderma* sp. Abbreviations: GB: *Ganoderma* isolate; CBC or SC (3a, wild mushroom no. 5), CJJM1 or S (3b, wild mushroom no. 17), CJJM2 or S (3c, wild mushroom no. 18), CPP2 (3d, wild mushroom no.6), CTLKP3 (3e, wild mushroom no. 47), CS1 (3f, wild mushroom no. 10) and SJBB1 (3g, wild mushroom no. 56) referred to all basidiomycetes tested. 3h shows an example of negative growth suppression behaviour of unidentified basidiomycete isolate (labelled as CR1 or SC) against *Ganoderma* isolate.

TABLE I: Antagonistic Interaction Features Among Different Species of Wild Basidiomycete Isolates Against *Ganoderma* Sp.

| No. | Antagonists | Antagonistic rating | Mode of interactions |
|---|---|---|---|
| 1. | CBC or SC (3a, wild mushroom no.5) | 2 | Antibiosis/mutual inhibition at a distance |
| 2. | CJJM1 or S (3b, wild mushroom no.17) | 4 | Mycoparasitism/ antagonism |
| 3. | CJJM2 or S (3c, wild mushroom no.18) | 3 | Antibiosis/mutual inhibition at a distance |
| 4. | CPP2 (3d, wild mushroom no.6) | 1 | Mycoparasitism/mutual slight inhibition |
| 5. | CTLKP3 (3e, wild mushroom no.47) | 1 | Mycoparasitism /mutual slight inhibition |
| 6. | CS1 (3f, wild mushroom no.10) | 4 | Mycoparasitism/antagonism |
| 7. | SJBB1 (3g, wild mushroom no.56) | 4 | Mycoparasitism/antagonism |

In the present study, collected mushrooms samples which have been sprouted abundantly and had species richness (different types of species) after rainfall were the fruiting bodies of the fungi found growing on the ground and in the grass, predominantly during the rainy season in Jasin District, Melaka. They were the fruiting bodies arising from a soil-inhabiting fungi. The fungi fed as a saprophyte by decomposing organic matters such as thatch at the top or surface soil which organic materials, nutrients and biological activity at the highest. The right combination between moisture (wet weather), shade or cloudy weather and organic materials in the soil has yielded a bumper crop of wild fleshy mushrooms in the survey areas. According to Mahajan *et al*. (2008), mushrooms grew well at relative humidity levels around 95-100% and substrate moisture levels at 50 to 75%. Majority of them were expected as non-edible basidiomycetous mushroom or poisonous or edibility is not known. A similar finding has been reported by [3] that majority of macrofungi fruiting on ground or ground litter in six Aeta tribal communities in Central Luzon, Philippines which found during the rainy season were identified not edible or poisonous mushrooms.

The collected mushroom samples with distinguish characteristics have been identified based on their macroscopic or phenotypic characters (shape, size, texture, colour and odour of the fruiting bodies) and microscopic (spore and hyphal morphologies) features but not really helpful, tedious, difficult to be done and seek help from an expert in fungal taxonomy. Moreover, details morphometric data on each of the specimens must be collected such as the different features of the cap, gills and stalk of the mushrooms. A spore print should also be prepared in identification process of fleshy mushrooms before can be compared their morphologies with published literature such as [18] and [15]

In future, *Ganoderma* isolate and field-*collected* basidiomycete isolates will be identified by using molecular approaches for species identification. According to [24], DNA-based molecular markers with particular PCR-based methods were quick and reliable approaches to reveal identities of wild mushrooms. Many studies proven that internal transcribed spacer (ITS) region can be used as a potential DNA barcode marker for fungal phylogenetic analyses and species identification [30], [4], [29], [24], [27]. Meanwhile [35] suggested that the taxonomical classification of mushroom species can be done in combination between their morphological and molecular descriptions which then could give more accurate knowledge and information about their identity and diversity.

During isolation process, most of the mushroom-forming basidiomycete isolates either cannot be grown on PDA medium or emerged, grown, proliferated and sporulated with small colony sizes after incubation at 28-30$^{o}$C for seven days. Although PDA is the common and widely used medium in fungal isolation and culturing, but still cannot stimulate and favour rapid mycelial growth of most fungal isolates that have been collected in this study. Due to the lack of sufficient variability of media composition, replication of the exact environmental conditions in the laboratory, suspected to cause majority of them were unculturable or exhibited very low growth rate [1]. According to [37], the medium had a significant effect on the growth rates of the fungi as well as the production of, and response to, volatile and non-volatile antibiotic compounds and hyphal interactions. Responses to the media appeared to be related both to inherent properties of the fungi and to their natural ecological behaviour. Besides, [31] also stated that nutrition could influence growth, sporulation and virulence of the insect pathogenic fungus, *Metarhizium anisopliae*.

Here we report the first evidences of the wild basidiomycetes which could exhibit antagonistic activity against *Ganoderma* sp. that caused BSR on oil palm via dual culture technique. A total of seven basidiomycete isolates have been recognized which have the potential to inhibit *Ganoderma* sp. growth on PDA medium with different mode of antagonistic interactions and antagonistic rating rates. Antibiosis/mutual inhibition at a distance, mycoparasitism/mutual slight inhibition and Mycoparasitism/ antagonism were observed for colony interactions between basidiomycete isolates when paired with *Ganoderma* isolate on PDA medium (Table 1). A total of two pairs displayed mutual inhibition at a distance (mushroom isolate no. 5 & 18), another two pairs recorded mycoparasitism/mutual slight inhibition (mushroom isolate no. 6 & 47) and three pairs exhibited mycoparasitism/antagonism (mushroom isolate no. 10, 17 & 56). According to [5], *in vitro* inhibition of fungi can be happened due to some factors such as antibiotic production and pH changes in the growth medium. In addition, [36] reported that when two opposite species produce inhibitory metabolites, mutual inhibition can be occurred. The existence of an inhibition zone in dual culture without hyphae interaction revealed the secretion of diffusible non-volatile inhibitory substance of fungal organisms. For mutual slight inhibition, both fungi mutually intermingled each other until almost in contact and there was a narrow demarcation line of 0.1- 2 mm in range, while mutual inhibition at a distance had a visible distance of more than 2 mm between two competitive fungi [8]. Whereas, a result of antagonism due to parasitism shown the aggressive forms of fungal behaviour which the hyphae of one competitor spread into the mycelium of the other and destroy it by overgrowth via hyphal interactions to take possession of the available resource in the growth medium. Reference [6] reported that hyphal interference can be happened through coiling, folding and secretion of cell wall enzymes which could limit the fungal growth because of the fungus act as a nutrient source for the other. Furthermore, distinct interaction patterns between competing fungal species on the grain surface of barley were identified and determined by rate of hyphal extension and branching which were (a) faster growth of one species causing progressive inhibition of the slower-growing species, (b) faster growth initially of one species which is then inhibited by the slower-growing species, (c) one species grew faster than the other but with no adverse effects, (d) one species grew faster than the other initially, but growth rates of both declined later during interaction, (e) both species grew at similar rates initially but growth rate of one declined during competition and (f) both species grew at similar rates initially but later reduced the growth of each other [25]

## 4. Conclusion

The study has discovered abundance of the wild mushrooms fruiting on the ground during the rainy season and harvested in Jasin District, Melaka. In summary, 56 isolates of wild mushroom have been identified which seven species could show *in vitro* antagonistic activities against *Ganoderma* sp. that caused BSR disease to oil palms. Mycoparasitism and antibiosis were identified as the mechanism of action of the basidomycete isolates *in vitro* studies. In future, an intensive study should be done for colony interactions and mycoparasitism behaviour between wild basidiomycetes and *Ganoderma* sp. due to have great potential uses in *Ganoderma* basal stem rot disease biocontrol strategy. Lastly, this is the first preliminary report of macrofungi produced fruiting bodies above ground after heavily rainfall in Jasin District, Melaka.

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