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Antifungal Potential of Cell-Free Supernatant Produced by Keratinolytic Fungi against *Ganoderma boninense*

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Abstract. Keratinolytic is a microorganism ability to degrade keratin substrates. Fungi are known to produce the enzyme keratinase. This research was carried out aiming to know the keratinolytic fungi potential in inhibiting *Ganoderma boninense* fungi. Five isolates of keratinolytic fungi coded A 31, A 18, A 12, K 18, and A 29 have antagonistic ability against *G. boninense*. A 12 and A 18 had the largest inhibitory diameter of 25.23 mm and 24 mm, respectively, after 7 days of incubation. The ability of A 12 and A 18 antagonisms was observed by testing the supernatant of keratinolytic fungi incubated in a feather meal broth medium. An 18 has the largest antagonism percentage of inhibition at 84.72% against *G. boninense*, followed by A12 (77.78%) and combination treatment of A 12 + A 18 (73.55%) after 7 days of incubation. Keratinolytic fungi can also grow on minimum salt chitin medium (MSCM) and produced a hydrolysis zone after 3 days of incubation. *G. boninense* hyphae abnormalities were observed by using a 100x magnification microscope. Moreover, hyphae lysis and dwarf, curved, rolled, and curly hyphae were also observed after giving treatment on A 12, A 18, and the combination treatment of both isolates. These results showed that both keratinolytic fungi isolates and the supernatant are powerful biocontrol agents against *G. boninense*. There is no previous research report on endophytic fungi inhibiting the growth of *Ganoderma boninense*. In the future, keratinolytic fungi can be applied in agriculture technology.

Keywords: antifungal, keratinase, *Ganoderma boninense*.

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INTRODUCTION

Poultry meat processing industry generates a large amount of feather waste production annually. In this case, the global poultry processing produced more than 4.7 million tons chicken feather waste in 2019, causing the abundance of keratin material (Li et al., 2020). Such abundant waste also produced a lot of hazardous environmental contaminant as well, whereas its bioremediation has both economic and ecological values. Uncontrolled poultry products consumption has increased environmental problems, particularly concerning the feather keratin disposal at regional and global level. This further also caused water and soil pollution, which lead to harmful effect towards surrounding people (Sharma & Kango, 2021).

Keratin is primarily found in hair, nail, horn, wool, and feather as well as being a structural component of epidermal, skeletal, and vertebrate skin (Staroń et al., 2017). Keratins have strict packing of the protein chain in either α-helices (hairs) or β-sheets (feather) frameworks, allowing them to have high biodegradation stability and resistance by non-substrate-specific protease activity i.e. papain, trypsin, and pepsin (Kalaikumari et al., 2019). In this case, 90% of feather is composed by water-insoluble macromolecules of keratinous proteins, consisting of long polypeptide chains (Kalaikumari et al., 2019). However, the results of this study showed that almost all components of keratin are proteins, so this type of waste has great potential to be applied.

Several research projects through chemical and physical experiments have been conducted to change keratinous waste into relatively small debris or protein hydrolysates that can be further applied as nutritional ingredients in the cattle feed, agricultural production, and cosmetics industrial sectors (Bhange et al., 2016). However, it could decrease the product quality and increase the environmental waste. In fact, such chemical and

physical processes can cause damage on certain amino acid essence, leading to the loss of nutritional value. In this case, the chemical material used in the feather degradation processing is in charge of the environmental pollution as the bulk effluents are released into water bodies (Jeong et al., 2012). In this context, biodegradation of feathers by keratinolytic micro-organisms is considered as an eco-friendly alternative (Dong et al., 2017; Mamangkey et al., 2019a; Mamangkey et al., 2019b; Mamangkey et al., 2020a; Mamangkey et al., 2020b; Aritonang et al., 2022).

Therefore, further study was carried out on keratinolytic micro-organisms to know the keratin waste biodegradation and changed it into more valuable products such as animal feed, nitrogen rich organic fertilizer, amino acid supplements, peptides, and ammonium ions (Fang et al., 2017; Vidmar & Vodovnik, 2018; Ghaffar et al., 2018). The discovery of new keratinolytic fungi is the first important stage in developing the most recent bioprocess and helping to minimize the quantity of keratinase waste in nature. The focus of this study was the conversion of chicken feather waste by keratinolytic fungi. Changes that were conducted on the keratin waste further produced high nitrogen material that was caused by the proteolytic activity when the fungi are active. This proteolytic activity can be further assumed as antifungal activity. It is further possible that the antifungal activity carried out by keratinolytic fungi is related to the production of secondary metabolite (Mamangkey et al., 2020c) and the secretion of hydrolytic enzymes. Specifically, the keratinolytic fungi with antagonism against *G. boninense*. Antagonistic fungi have been tested for the control of many soilborne pathogens (Daguerre et al., 2014). Coincidentally, keratinolytic fungi were obtained from chicken and goat farms around palm oil plantations. Therefore, keratinolytic fungi are antagonistic against *G. boninense*, the causal agent of Basal Stem Rot (BSR) disease in palm oil (Rajesh et al., 2014). The aim of this research was to study the screening of keratinolytic fungal antagonism against *G. boninense*, test the cell-free supernatant of keratinolytic fungi on *G. boninense*, as well as test the ability of keratinolytic fungi to produce chitinase enzymes to ensure their involvement in inhibiting the growth of *G. boninense*. Based on the literature study, presumably, this is the first report on antifungal activity of keratinolytic fungi against *G. boninense*.

METHODS

Fungi Isolation

This research employed samples in the forms of soil collected from chicken and goat farms. Fungi were further isolated from the soils collected using Skim Milk Agar (SMA) media. Furthermore, serial dilution (10^{-1}-10^{-6}) was carried out on the samples using physiological solution (NaCl 0.9%). To obtain the isolates, spread plate method was applied where inoculation was done on 0.1 mL solution of each dilution series. Incubation was further also carried out towards the colony for three days at room temperature and daily monitoring was done on the clear zone appeared around it. The fungi growth was further used in the next research.

Keratinolytic fungi antagonist test on *G. boninense*

This test was done by using a potato dextrose agar (PDA) medium. *G. boninense* studied was taken with a cork borer, then inoculated in the middle of PDA with a distance of 3.5 cm from the keratinolytic fungi and incubated under the temperature of ± 28° C for 7 days.

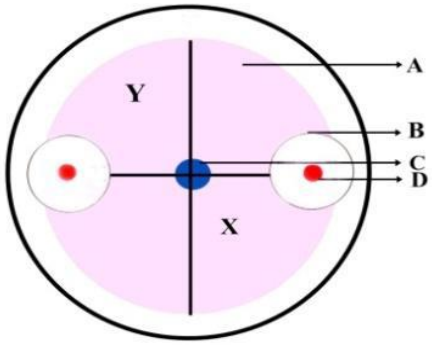


Figure 1. Method of measuring the zone of keratinolytic fungi inhibition against *G. boninense*; A. *G. boninense* colony; B. Inhibition zone against *G. boninense*; C. Placement point of *G. boninense*; D. Keratinolytic Fungi Colony; Y. Normal diameter of fungal colony; X. Inhibition zone diameter (Cavello et al., 2015)

Enzyme keratinase production

Keratinolytic fungi isolates were rejuvenated on PDA and incubated for 5 days. The fungal spores was put into Feather Meal Broth (FMB) media and incubated for 12 days at an orbital shaker speed of 120 rpm, ± 28°C. After that, the supernatant enzyme extract was measured every three days, with the centrifugation at 10,000 rpm for 15 minutes. The measurement was done according to Mamangkey et al. (2020b) method. In addition, the protein content was measured using Bradford method.

Measurement of keratinase activity

Keratinase enzyme activity measurement was determined according to the modified Gousterova et al. (2012). Keratinase enzyme as much as 0.5 ml was dissolved with 0.5 ml of potassium phosphate buffer solution (pH 7) then as much as 0.5 ml of 0.5% keratin solution was added. After that, it was incubated for 15 minutes at 31° C. Furthermore, 1 ml of trichloroacetate (TCA) was added after the incubation for 15 min and was centrifuged at 10,000 rpm for 10 min. The filtrate was measured at a wavelength of 280 nm. Control was done by adding 1 ml of TCA solution before adding 0.5% keratin. One unit of keratinase activity (U/ml) was defined as an increase in absorption of 0.01.

Keratinolytic fungi supernatant test on *G. boninense*

Antifungal ability test of supernatant keratinolytic fungi to inhibit the growth of *G. boninense* was done in two stages. First, 15 mL PDA was mixed with 5 mL supernatant (Isolate A 12 or A 18) poured on a sterile petridish. Second, 15 mL PDA was mixed with 5 mL supernatant (2.5 mL isolate A 12 + 2.5 mL isolate A 18) poured on a sterile petridish. Pathogenic *G. boninense* fungus (with the diameter of 9 mm) was placed in the center of the petridish. The observation was carried out by measuring the growth of *G. boninense* fungal colonies and comparing the growth of no-treatment control by keratinolytic fungi supernatants. Moreover, the inhibitory zone diameters were observed and counted on the fourth and seventh days.

Observation of *G. boninense* hyphae abnormalities

The microscopic observation was made by cutting off the tip of the *G. boninense* hyphae on a block square PDA, then placed on a glass object and observed under a microscope (100x magnification). Lactophenol blue stain was used to get a clear look at the intact hyphae.

Screening chitinase-producing fungi

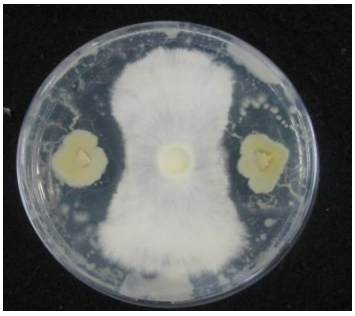
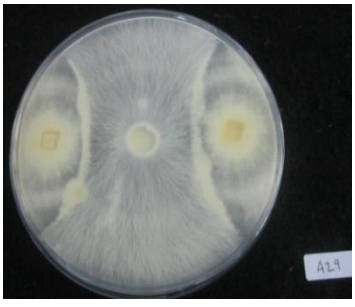
The admixture containing 72.7 mL of 12.5% colloidal chitin in salt medium (0.7 g dipotassium phosphate; 0.3 g monopotassium phosphate; 0.5 g magnesium sulfate heptahydrate; 0.01 g ferrous sulfate heptahydrate; 0.001 g zinc sulfate; 0.001 g manganese(II) chloride; 20 g agar bacteriological (oxoid); pH 6.8) with 1000 mL of sterile aquadest was used. The fungal spore was placed in the center of the petridish and was incubated at ± 28° C for three days. The metabolite ability of keratinolytic fungi supernatant to produce chitinase enzymes was indicated by a clear zone around the colony resulted in chitinase activity (Soesanto et al., 2021).

RESULTS AND DISCUSSION

Antagonistic properties of keratinolytic fungi

Five fungi isolated with keratinolytic activity were selected from chicken and goat farms. The fungal isolates coded A 31, A 18, A 12, and A 29 were got from a chicken farm. Meanwhile, isolate K 18 was from a goat farm. A 12 and A 18 have the best inhibition zone of *Ganoderma bonin* in sequence 25.23 mm and 24 mm (Table 1), respectively, at ± 28°C after the 7 days of incubation.

Table 1. Interaction Result of Dual Culture Assays between the *G. boninense* Pathogen and Keratinolytic Fungi Isolates on potato dextrose agar

Antagonism assay	Figure		Inhibition zone (mm) of 7 days
	Top View	Bottom View	
A 31			14.85
A 18			24
A 12			25.23
K 18			23.5
A 29			23.25

The observation of the inhibition zone on each keratinolytic fungus that appeared was made on the fourth day of incubation. *Ganoderma boninense* mycelium growth was inhibited on PDA after seven days. The antagonism of each keratinolytic fungi isolate did not show a significant difference. A 12 and A 18 isolates showed the best activity, both isolates were from chicken farms and were believed to have biological activities that can affect the growth

of *G. boninense*. This is supported by the process of keratinolytic fungi adaptation in soil with natural competition against other fungi, especially *G. boninense*. The sample was taken from a chicken farm adjacent to the location of the palm oil plantation made the *G. boninense* spores spread around the soil with the help of wind, water, and insects.

The proteolysis process as a part of chicken feather keratin waste degradation mechanism is very possible to be an important factor in the presence of protease activity to inhibit *G. boninense*. Furthermore, other keratinolytic fungi activities have biological multiactivity such as hydrolytic enzymes and bioactive compounds. Interestingly, there has been no report about keratinolytic fungi that inhibit the growth of *G. boninense*. Hence, the researcher hoped that this research would open new insight into the antagonistic nature of keratinolytic fungi from chicken and goat farms against *G. boninense*.

Fungal keratinase activity

A 12 and A 18 (Figure 2) were selected based on the best inhibition against *G. boninense*. Before testing the keratinase fungal supernatant, the highest keratinase activity after the incubation period was discovered. The incubation was carried out for twelve days at three days intervals. Six days of incubation were the best time for fungi keratinase production. A 12 and A 18 activities were 51.8 U/mL and 11.8 U/mL, respectively, after six days of incubation. The keratinase activity of the two fungal isolates decreased after 9 days of incubation. Keratinase production will decrease at the end of the growth stationary phase (Dalee et al., 2018), indicating the end of the stationary phase of A 12 and A 18 isolates after 9 incubation days.

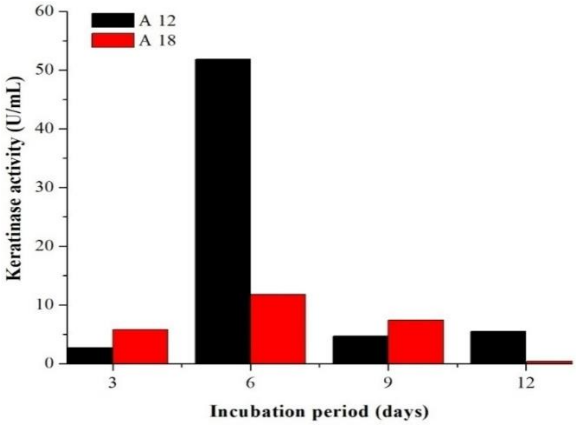


Figure 3. Profile of Fungal Keratinase Activity

The observation of the microscopic morphology of isolates A 12 and A 18 clearly showed that they have conidia and conidiophores (Figure 4). Isolate A 12 was different for it produced violet pigment, while the mycelium morphology of isolate A 18 was white to greyish-orange, with conidial masses greyish-turquoise. Based on the microscope visualization, the fungal A 18 isolate bear similarities to Penicillium.

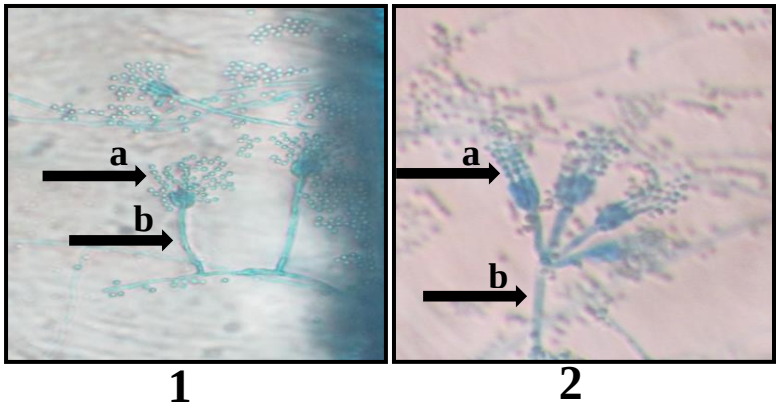


Figure 4. Microscopic Morphology of A 12 (1) and A 18 isolate (2). Parts observed: (a) Conidia and (b) Conidiophores

Antagonism properties of culture supernatant

Culture supernatant antifungal activity against selected *G. boninense* was estimated through a growth inhibition assay. A 12 and A 18 were cultured in a liquid feather meal broth medium, then the supernatant antagonisms were tested using *G. boninense* (Figure 5). The supernatant harvesting was done after six days of incubation and was based on its highest keratinase activity. In Figure 1, the growth rate of each function in the absence and presence of the supernatant culture as well as the inhibition ratio (%) is shown. *G. boninense* was controlled by cell-free supernatants with inhibition ratios higher than 50%. Every single combination treatments showed slightly different results.

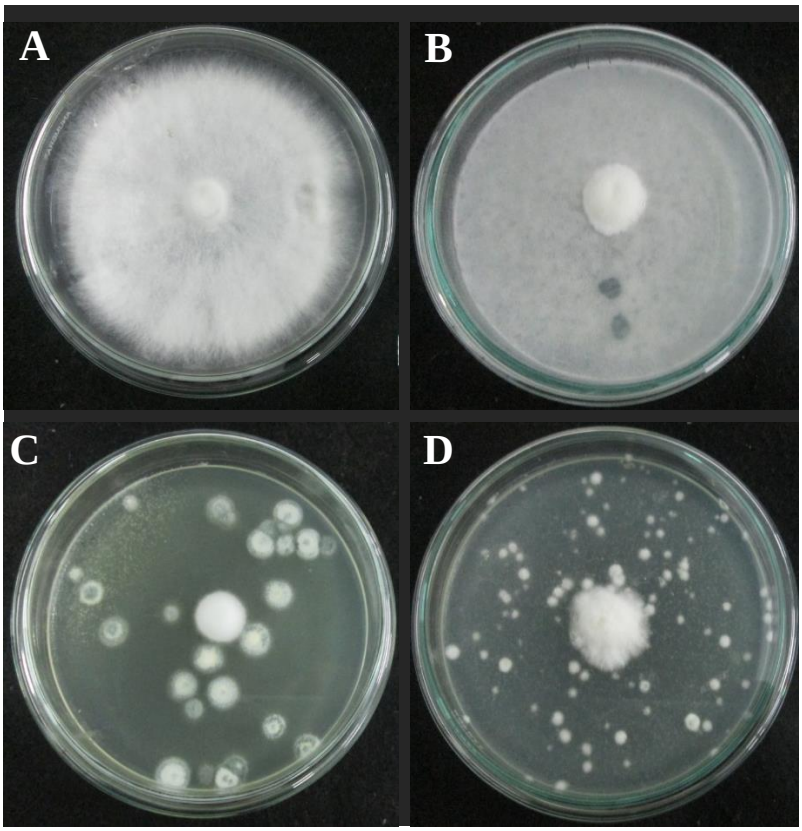


Figure 5. Supernatant antifungal activity assay between *G. boninense* pathogen and keratinolytic fungi isolates on PDA. In each set of three plates, a single culture of *G. boninense* is shown on A (control), the dual culture in the middle and the keratinolytic fungi isolate mix on the PDA medium. In cell-free antifungal supernatant, isolates (B) A 12, (C) A 18, and (D) A 12 + A 18 inhibited *G. boninense* after 7 days of incubation

Cell-free supernatant inhibitory activity of keratinolytic fungi was apparent after four days of incubation and increased after seven days of incubation (Figure 6). The A 18 antagonism towards *G. boninense* has the largest percentage of inhibition, which was 84.72% of incubation for seven days. When compared with the combination treatment of A 12 + A 18 (73.55%), treatment A 12 (77.78%) and A 18 were still better. This likely happens due to the nutrition competition in growth media and adaptation of abiotic factors such as pH and temperature that greatly affected the biological activity of each fungal isolate. Furthermore, the two keratinolytic fungi isolates also had different secondary metabolites due to inhibitory compounds interaction between A 12 and A 18 that further affected the biological activities of both isolates, mutually supported or inhibited as well as led to the regeneration process of pathogenic fungi that grow around it.

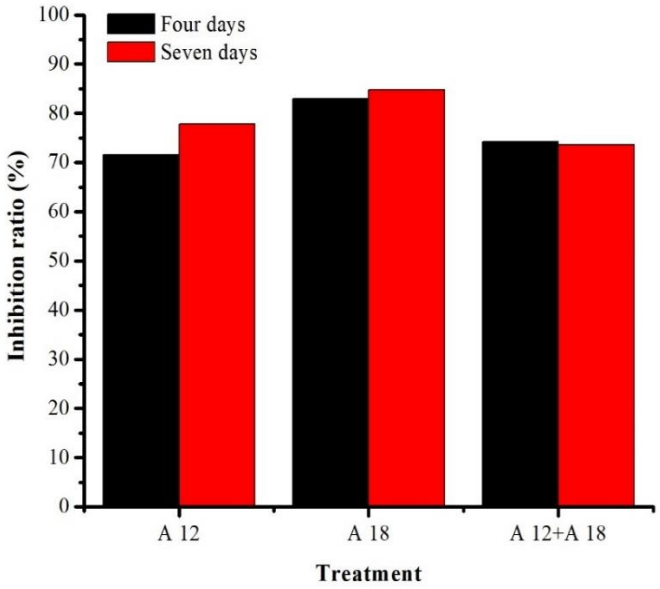


Figure 6. Antagonistic Effect of Supernatant Keratinolytic Fungi on Percentage of Mycelial Growth Inhibition of *G. boninense*.

The phenomenon of antagonism toward fungi can be explained through several mechanisms, including antibiosis and parasitism. Cavello et al. (2015) reported that *P. lilacinum* keratinolytic fungi supernatant had inhibition percentages of *A. alternata* (57%), *A. niger* (27%), *F. culmorum* (34%), and *F. graminearum* (27%). This means that keratinolytic fungi can be used as the focus of the study to assist the process of plant growth through inhibition to eradicate pathogenic fungi. In some cases, hydrolytic enzymes such as chitinases, glucanases, or proteases can act against the fungal cell wall (Sayyed et al., 2013).

Gousterova et al. (2012) successfully utilized feather protein-rich hydrolyzate waste using a mixture of thermophilic actinomycete strains culture which the antifungal activity was tested against several plant pathogenic fungi such as *F. solani*, *F. oxysporum*, *Mucor* sp., and *A. niger*. Keratinolytic fungi isolates can be used as a new alternative source for the production of protease groups that can be used as fungicides against *G. boninense*. The supernatant A 12 and A 18 used chicken feather waste medium which had antifungal activity as a sign of the presence of several hydrolytic enzymes as well as ammonia in the supernatant. The role of ammonia in controlling the growth of phytopathogenic fungi was explained by Weise et al. (2013) that ammonia is the only gas in sufficient concentration in soil to inhibit the growth of soil fungi.

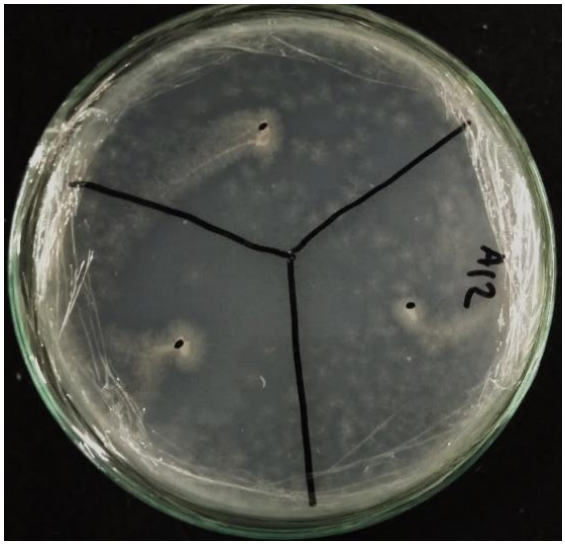


Figure 7. Chitinase Activity of Keratinolytic Fungi on Minimal Medium Containing Colloidal Chitin

In addition, the initial screening for chitinase activity was also carried out. After 3 days of incubation, A 12 and A 18 showed chitinase activity. The indicator was the visualization of the hydrolysis zone around the chitin medium (Figure 7). Chitinase which is owned by keratinolytic fungi can attack the chitin cell wall of *G. boninense*. Chitin fungi cell walls are a linear-chain polymer composed of 1,4-linked 2-acet-amido-2-deoxy-β-D-glucopyranose units and classified as γ-chitin (Fernando et al., 2021). For the Ganoderma genus, a study carried out by Chen et al. (2012) successfully identified 40 genes assignable to the CAZy family GH18 that acted as chitin decomposition in *the Ganoderma lucidum* cell wall. The study on A 12 and A 18 and other keratinolytic fungi isolates can be continued toward biotechnological application products that can replace chemicals to make them more environmentally friendly for biopesticides.

G. boninense hyphae abnormalities

G. boninense hyphae abnormalities were observed on the seventh day. The observation was made after learning about the antagonistic interaction on PDA. All single treatments with A 12, A 18 and combination of A 12 with A 18 showed inhibition of the growth of *G. boninense* hyphae (Figure 8).

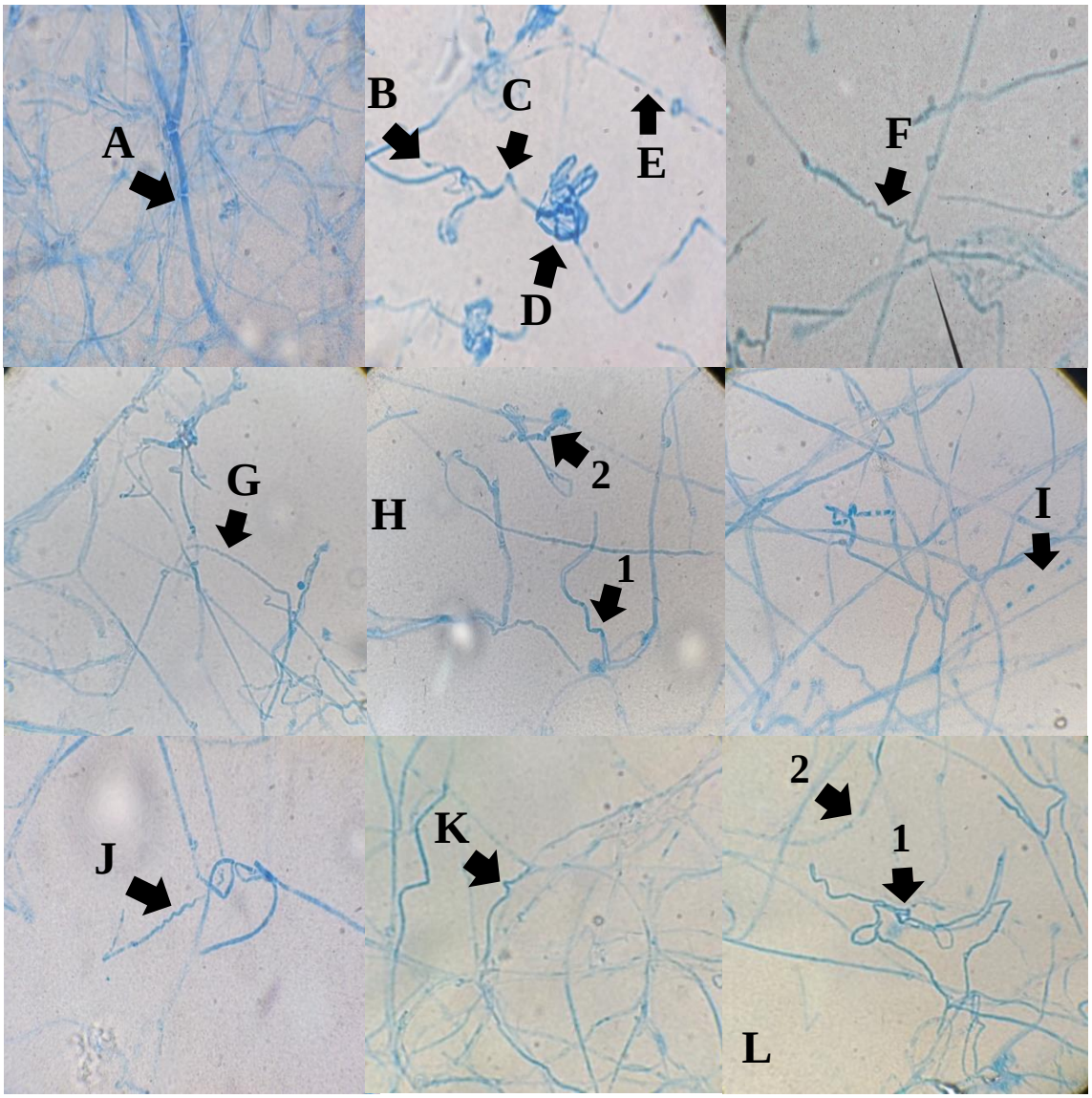


Figure 8. *G. boninense* Abnormal Hyphae: A. Hypha without keratinolytic fungi treatment, B. (dwarf hyphae), C. (curved hyphae), D. (rolled hyphae), E. (hyphae lysis), F (curly hyphae) on *G. boninense* after treatment of isolate A 12; G. (curly hyphae), H1. (curved hyphae), H2. (curved hyphae), I. (hyphae lysis) on *G. boninense* after treatment of isolate A 18; J. (curly hyphae), K. (curved hyphae), L1. (rolled hyphae), L2. (hyphae lysis) after the combination treatment of A 12 and A 18. The incubation was done under the temperature of ± 28° C for 7 days

Hyphae abnormalities can be caused by keratinolytic fungi biological activity such as hydrolytic enzyme reactions, proteases, and chitinases. The biological activity of keratinolytic fungi A 12 and A 18 caused changes in the structure of *G. boninense* hyphae: dwarf hyphae, curved hyphae, rolled hyphae, curly hyphae, and hyphae lysis. The growth of *G. boninense* mycelium was inhibited due to the depletion that leads to cell wall damage. The stunted growth of *G. boninense* after being observed with a microscope at 100X magnification was started on the fourth day of incubation, this result is in line with the visualized inhibition reaction in PDA media. Optimum keratinolytic fungi A 12 and A 18 required seven days to damage until the *G. boninense* hyphae lysis which made it unable to reach the nutrients needed. Therefore, mycelium growth is abnormal. Jadhav & Sayyed (2016) stated that hydrolytic enzymes (One of them is keratinase) produced by fungi can degrade fungi cells and cause lysis on fungi pathogenic cells.

CONCLUSION

Keratinolytic fungi A 12 and A 18 showed the best antagonistic activity against *G. boninense* which were 25.23 mm and 24 mm, respectively. The cell free-supernatant A 18 inhibition activity against *G. boninense* showed the largest percentage of inhibition at 84.72%, followed by A 12 (77.78%), and the combination of A 12 + A 18 (73.55%) after seven days of incubation. A 12 and A 18 were confirmed to have chitinase activity after 3 days of incubation. *G. boninense* hyphae abnormalities found were dwarf, curved, rolled, curly hyphae, and hyphae lysis after the treatment done on A 12 isolate. The curly, curved, rolled hyphae, as well as hyphae lysis, was found after the treatment was done on A 18 isolate. Moreover, curly, curved, and rolled hyphae, as well as hyphae lysis were found after the combination treatment of A12 and A18 isolates which were incubated under a temperature of ± 28° C for 7 days. In the future, keratinase from keratinolytic fungi can be developed as a biotechnology application to inhibit the growth and even removed *G. boninense* that infects living plant tissue.

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