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# Purification and Identification of an Antifungal Protein from an Isolated Fungus with Antagonism to *Colletotrichum gloeosporioides* MC9

**Yohana Avelia Sandy[1,2*)], Yo-Chia Chen[1)] and Liliek Sulistyowati[1,2)]**

[1)] Department of Biological Science & Technology, National Pingtung University of Science & Technology, Pingtung, Taiwan ROC
[2)] Faculty of Agriculture, Universitas Brawijaya, Malang, East Java, Indonesia

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*) Corresponding author:
E-mail: yohana_avelia@ub.ac.id

**ABSTRACT**

*Colletotrichum gloeosporioides* is the cause of anthracnose disease on mango. This disease becomes more damaging because it economically affects the harvested fruit during the postharvest season. In this research, eight isolates are isolated from the soil of a mango plantation. One of the isolates shows antifungal activity against *C. gloeosporioides* MC9. This isolate is identified as *Penicillium citrinum* isolate S1 based on the phylogenetic analysis of ribosomal rRNA sequence. From the culture of this isolate, extracellular filtrates are collected and evaluated for their antifungal activity. The mycelial growth of *C. gloeosporioides* is significantly inhibited by the culture supernatant of *P. citrinum* isolate S1. The culture filtrate is used to purify the antifungal protein using ammonium sulfate and ultrafiltration methods. Results show that the antifungal protein was estimated at around 40 kDa molecular weight when separated on a 10% Sodium dodecyl sulfate-polyacrylamide gel. After nine days of incubation, this antifungal protein's inhibition effect with a concentration of 0.94 mg/ml remained 63.6% against *C. gloeosporioides.* The LCMS result showed that the antifungal protein belongs to the L-asparaginase superfamily. Based on this result, the antifungal protein produced by P. citrinum S1 has the potential to control mango anthracnose disease caused by *C. gloeosporioides*.

## INTRODUCTION

Anthracnose disease has been widely known as one of the most important and destructive diseases in many fruits and crops, especially mango. This disease is caused by *Colletotrichum gloeosporioides* Penz and Sacc. which is the anamorph stage as the pathogenic fungus asexual stage. Anthracnose symptoms occur on all essential parts of the mango's plants, such as panicles (flower clusters), fruits, petioles, twigs, and leaves. During dry weather, lesions start as small with brown to black spots that can enlarge to form dead areas and make leaves drop out. This disease can reduce panicle yield by killing the flowers before fruits are produced. The first symptoms are small black spots that can enlarge and coalesce. The black area also appears in the twigs, stems, fruits, leaves, and flowers; another expanding lesion will be found. Anthracnose disease-causing by *C. gloeosporioides* develops sunken, prominent with dark brown to black decay spots before or after picking. After harvest, the healthy fruit can develop significant anthracnose symptoms upon ripening (Nelson, 2008). This disease becomes more damaging during the rainy season because wet, humid weather conditions favor postharvest anthracnose. And it also economically affects the harvested fruit during the postharvest season (Akem, 2006).

Currently, the control of mango anthracnose is commonly treated by specific chemicals or their combination due to their various action modes. But,

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a synthetic fungicide may have adverse effects on both the environment and consumers, such as atmosphere pollution, environmental damage, increased the harmful residues of soil, and the development of resistant isolates (Naseby, Pascual, & Lynch, 2000). Therefore, the application of synthetic fungicides must be reduced. Biological control agents are one of the most promising ways to mitigate synthetic fungicide's side effects. The most applied biocontrol agents are microbial, which have antagonistic activity against important plant pathogens, including fungi, numerous yeasts, and bacteria. Many research reports that the aggressive isolate can be used as a biocontrol agent. Newhook (1951) and Wood (1951) research show that Fusarium inhibits the growth of Botrytis cinerea spp. and *Penicillium claviforme, the* antagonistic fungi from senescent lettuce leaves. Also, control of *Botrytis cinerea* has been treated by *Trichoderma* spp. in the grape trees. Several fungi have controlled the growth of *S. sclerotiorum* on various plants (Boland & Hunter, 1988).

An alternative or supplement to reduce the use of synthetic fungicide for disease control is Biocontrol. Microbial biocontrol agents (BCAs) don't harm the environment, and it is highly impossible to develop resistance isolates because of their generally complex mode of action. The biocontrol activity against phytopathogens have different mechanisms of action: competition antibiosis, the induction of plant resistance and parasitism (Harman, Howell, Viterbo, Chet, & Lorito, 2004). Parasitism is the interaction of the antagonist against the pathogen. This interaction is called mycoparasitism which the fungi antagonist and the pathogen involve a phase of physical contact.

Among the examples of parasitic fungi are Trichoderma spp., which can attack many different pathogenic fungi such as *T. harzianum* that can inhibit pathogenic fungus *Lasiodiplodia theobromae* on rodent tuber (*Typhonium flagelliforme*) (Silfarohana et al*.,* 2022). In another case, *T. harzianum* ZC51 can inhibit *F. oxysporum* and induce the expression of *R. pseudostellariae* defense genes (Chen et al., 2021). Usually, microorganisms compete for nutrients and space (such as iron, carbon, nitrogen, and oxygen). Antagonist organism usually limits the growth of another fungi with nutrient competition. Induction of plant resistance, the induction of plant resistance is activated by inducing an agent biotic or abiotic. The process depends on the host plant's physical or chemical barriers to start the resistance. The antagonistic fungus has been well documented for mediating the induction of plant defense responses. Antibiosis is one mode of action of antagonist activity with the production of metabolites from antagonistic fungi or bacteria that can inhibit the growth of pathogenic microorganisms. It means that metabolites are essential in biocontrol activity. Antibiosis from bacteria and fungi is vital in inhibiting plant disease and is important in biocontrol activity. The process has been defined as the direct effect of the antibiotic or a compound on another microorganism (pathogenic microorganism) produced by that microorganism (antagonistic microorganism) (Lo, 1998). Antibiosis is one mode of action by microbial biocontrol agents (BCAs) that is more likely to be used as a biocontrol agent.

To date, the topic of fungal biological control agents has been reported about their efficiency (Butt & Copping, 2000). For example, antagonistic fungi *Aspergillus aculeatus* have the potential as an antagonist (biological control) against pathogenic fungi *C. gloeosporioides* (Darsini, Sudana, Suprapta, & Nyana, 2017). Moreover, much research about antifungal protein as biological control has been reported from the genus Penicillium; for example, *Penicillium adametzioides* reduce the growth of pathogenic fungi from grapes by inhibiting the mycelial by reducing the OTA production (Ahmed, Strub, Hilarie, & Schorr-Galindo, 2015). Another *Penicillium* species, *P. crysogenum* A096, isolated from Arctic sediment, inhibits the growth of *P. variotii, A. longipes, and T. viride* by producing some antifungal protein (Chen et al., 2013). The result showed that many fungi, especially from the genus of Penicillium with antagonistic activity, have the potential as a biological control agent.

In this study, an antagonistic fungi *P. citrinum* isolate S1 has been isolated from mango soil plantation and has a significant inhibition against pathogenic fungi *C. gloeosporioides* isolate MC9. *P. citrinum* isolate S1 can produce an isolated, purified, and identified antifungal protein. Then the antifungal activity of this antifungal protein is evaluated, and the result is significant in controlling *C. gloeosporioides* that cause mango anthracnose disease. Therefore, the aim of this study was to isolate an antagonistic fungi from the soil of mango plantation that can be use as Biological control of *C. Gloeosporioides*.

## MATERIALS AND METHODS

This experiment was conducted from January 2017 to August 2017 at BT211 Laboratory, Department of Biological Science & Technology, National Pingtung University of Science & Technology, Taiwan.

### Fungal Isolate

The pathogenic isolate was identified as *C. gloeosporioides* MC9. They were isolated from the infected mango fruit. Those isolates were maintained on Potato Dextrose Agar (PDA) medium and subculture every week in room temperature (RT).

### Isolation and Identification of Antagoistic Isolate

The antagonistic isolate was isolated from the soil of a mango plantation, 1 g of soil sample was diluted with 1 ml dd$H_2O$, and 100 µl was spread on RBSF selective medium. Every single isolate was purified, then the isolates were tested against the pathogen.

Morphological and molecular identification was used to identify the antagonist candidate. For microscopic analysis, the isolate was cultured in a PDA medium, grown in a sterile slide, and observed under a microscope. The molecular identification, the DNA of the candidate isolate was amplified by polymerase chain reaction (PCR) using universal prime, forward was using ITS4 (5'TCCTCCGCTTATTGATATGC-3'), and the reverse was using NS3 (5'GCAAGTCTGGTGCCAGCAGCC-3'). The PCR condition was pre-denaturation 95°C/5 minutes; denaturation 95°C/30 seconds; annealing 51.9°C/30seconds; elongation 72°C/2 minutes which repeated for 25 cycles; and last elongation 72°C/7 minutes. After PCR, the DNA was checked using electrophoresis. Then it was sent for sequenced, and the sequence was analyzed using BLAST in the database of GenBank.

### Purification of Antifungal Protein

Antagonis isolate was cultured in 250 ml Erlenmeyer flask containing 125 ml of YTM liquid medium (containing yeast extract 0.5%, mannitol 2.5%, tryptone 0.3%, glucose 1%) and incubated at 28°C for 7 days with shaking at 180 rpm. Fungal culture supernatant was centrifuge, vacuum filtration, and sterilized using a Millipore syringe filter of 0.22 µm. The sterile culture supernatant was tested for antifungal activity by antifungal assay.

Ammonium sulfate precipitation was used step by step to precipitate the protein. The amount of solid ammonium sulfate was calculated by Green & Hughes (1955). Sterile culture supernatant was prepared, and 20% solid ammonium sulfate was added slowly with rapid stirring at RT until saturated. The sample was incubated at 4°C for 90 minutes and centrifuged at 16.000 rpm for 15 minutes at 4°C to concentrate the protein. Pellet was redissolved in sterile dd$H_2O$ (1/15 v). The supernatant was used again for the next step by adding 40%, 60%, 80%, and 100% ammonium sulfate with incubated at 4°C for 90 minutes each step. Then all the crude proteins were ultrafiltration by 3 kDa Vivaspin 6 MWCO by GE Healthcare to remove the salt.

### Protein Analysis Using SDS-PAGE

The total protein concentration was determined first by Bradford Method. Approximately 1 µl PageRuler™ Prestained Protein Ladder (Fermentas) was loaded as a marker in the first line of the gel. Then, the protein sample was filled in SDS-PAGE 10% gel concentration containing stacking gel (upper) and separating gel (bottom). The gel was run in 1X TGS buffer at 80V; 60mA for 60 minutes and continued at 150V; 90 mA for additional 90 minutes. Next, the gel was washed with dd$H_2O$ three times, every 10 minutes, to remove the SDS. Then, PageBlue™ Protein Staining Solution (Fermentas #R0571) was used to visualize the protein bands by staining overnight at 4°C with shaking.

### Liquid Chromatography Mass Spectrometry (LC-MS)

The first step of the mass spectrometric identification of proteins is the sample preparation with In-gel digestion. After the in-gel digestion, the sample was run in the LC-MS. Next, the LC-MS results program for running the LC-MS was selected as usually used. Finally, the result from LC-MS was identified using a Mascot to determine the protein identity based on the NCBI database.

## RESULTS AND DISCUSSION

One of the most important fruit crops is Mango (*Magnifera indica* L.) (Nelson, 2008). These "kings of fruits" grows throughout the tropic and subtropic worldwide. Many countries are shipping a large volume of this good-quality fruit (Arauz, 2000). However, the biggest loss from this fruit is caused by anthracnose disease because this disease reduces the quality and makes the mango fruit unmarketable (Akem, 2006). Anthracnose disease of mango is

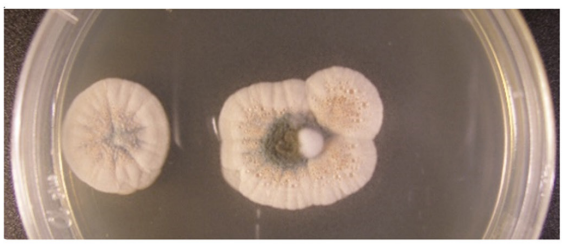
caused by pathogenic fungi *C. gloeosporioides* as the anamorph stage of *G. cingulata* (Rungjindamai, 2016).

**Identification of the Isolate and Detection of Antifungal Activity**

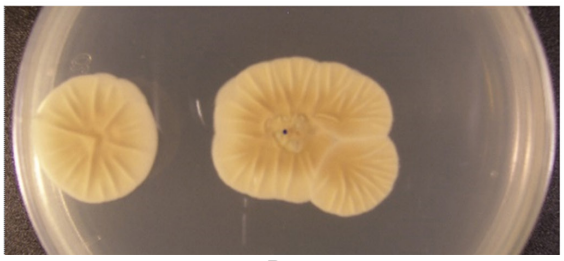
In this study, the soil sample from the mango plantation was used for screening the antagonistic fungi. Many species of fungi from soil can be used as biocontrol agents against many plant pathogens. Fungi living in the soil usually interact with other fungi, bacteria, and soil communities to colonize or plant roots by occupying plant tissue fragments (Kubicek & Harman, 1998). They are able to spread and grow in the soil because of the formation of hyphae. But there is a different interaction between these microorganisms developed with plants and the microbial community. When fungi need an association with the plant for the duration of their life cycle, they can be obligate parasites. But if they need a plant for only part of their life cycle, while they are saprophyte during the rest of their life, they are not obligate parasites. (Harman, Howell, Viterbo, Chet, & Lorito, 2004).

There were eight isolates from the result of the isolation of the antagonistic fungi. All the isolates have been purified and tested against *C. gloeosporioides* isolate MC9. One of them, known as isolate S1, showed significant aggressive activity against the pathogenic fungi *C. gloeosporioides* isolate MC9 based on the inhibition zone. The isolate S1 has an oval shape; the mycelium was white at the beginning, and after 5 days, the center of the growth mycelium became green and dark green (Fig. 1A). And the particular characteristic of this isolate was shown in the reverse side of the PDA medium, the mycelium growth with irregular shape with yellow colony colored (Fig. 1B). The microscopic identification result showed that the isolate S1 had a similar characteristic as *Penicillium* sp. Moreover, the character of hyphae of this fungi was septate with globose, smooth conidia, and smooth stipe.

According to Wen, Guo, & Chen (2014) in the Fungal Identification Manual, the mycelia of fungal *Penicillium* sp. isolate W1 on PDA plates had similar characteristics to *Penicillium* sp. First, the isolates produced many green spores and would turn to dark brown. Then the isolates produced a light yellow-green substance. Based on the morphological result, the isolated identity to the genus of *Penicillium* sp. The microscopic identification result showed that the isolate S1 had a similar characteristic as *Penicillium* sp., because it has conidium, phialide, metula, branch/ramus, branch, and stipe (Visagie et al., 2014). According to Tiwari, Jadhav, & Ashish (2011), the color of conidia of *P. citrinum* is dark green and pale yellow on the reverse side, with the character of hyphae being septate. For molecular identification, the result showed that the isolate MC9 had similarities to *Penicillium citrinum* isolate S1.



A



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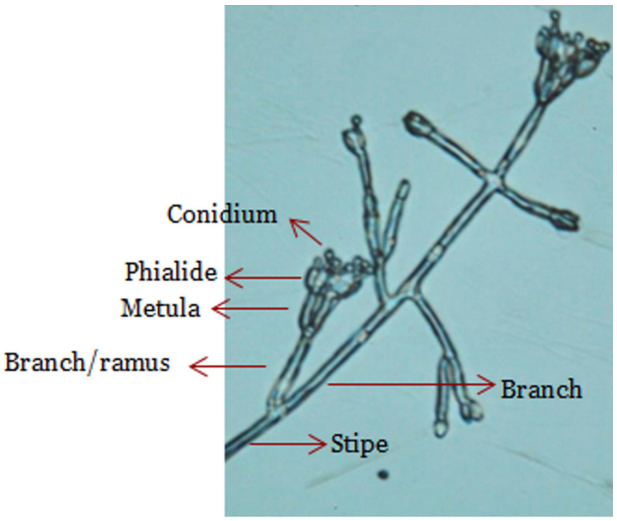



C

**Fig. 1.** Morphological identification of antagonistic isolate S1 from the front (A) and back (B) of the PDA plate and condia development (C) . Isolate was incubated on PDA plate for 7 days at room temperature

The method to identify the isolate after the sequences were obtained using a BLAST search on NCBI (http://www.ncbi.nlm.nih.gov/refseq/) (Visagie et al., 2014). This method is widely known to identify species from the sequence. Based on the molecular identification, fungal isolate S1 was determined to be *Penicillium citrinum* isolate S1, with the percentage identity of the BLAST result being 97% with E value 0.0. (Table 1). Classification of *Penicillium citrinum* is from kingdom Fungi, division Ascomycota, class Eurotiomycetes, order Eurotiales, family Trichocomaceae, genus Penicillium, species *Penicillium citrinum*. Several antagonists from *Penicillium* species have been reported to inhibit plant pathogens. The mechanisms of action are based on the production of antibiotic compounds (Nicoletti, De Stefano, De Stefano, Trincone, & Marziano, 2004), induction of resistance (Hossain, Sultana, Kubota, Koyama, & Hyakumachi, 2007), and the formation of microparasitic interactions (Sempere & Santamarina, 2008).

*Penicillium citrinum* is one of the genera *Penicillium* and is the most commonly occurring eukaryotic life form on earth. This *Penicillium* species is a filamentous fungus with a worldwide distribution (Pitt, 1979). The isolation from *Penicillium* species can be found in soil, indoor environments, (tropical) cereals, and spices (Visagie et al., 2014). PcPaf has been reported as the antifungal protein from *Penicillium citrinum* isolate W1 isolated from a Southwest Indian Ocean origin sample. This antifungal protein can inhibit the growth of a wide range of pathogenic fungi such as *Trichoderma viride, Fusarium oxysporum, Paecilomyces variotii, and Alternaria longipes* (Wen, Guo, & Chen, 2014).

Microbial antagonist is one of biological control that can be an alternative methods for plant disease control (Atanasova-Pancevska & Lovski, 2018). The naturally occurring antagonistic microorganisms surrounding the plant ecosystems will help reduce disease potetial or damage by growing vigorously (Cook, 1993). They compete for food sources with the pathogens or produce metabolites or a compound to inhibit the growth of the pathogens (Heydari & Pessarakli, 2010). The antagonistic microorganisms can produce metabolites or a compound such as protein to inhibit the growth of pathogens such as antifungal protein. Antimicrobial proteins from microorganisms have great potential for preventing and treating infection pathogens (Marx, 2004). Many antifungal proteins with different characteristics have been identified from a huge range of species of fungus. From the genus of ascomycetes, many different antifungal proteins have been derived and has a significant effect to inhibit several pathogens, such as antifungal peptide (AFP) produced by *Aspergillus giganteus*, NAF from *Penicillium nalgiovense*, PAF from *Penicillium chrysogenum*, AcAFP from *Aspergillus clavatus*, AnAFP from *Aspergillus niger*, NFAP from *Neosartorya fisheri*, and Pc-Actin from *Penicillium chrysogenum* A096 (Chen et al., 2013). AFP gene of *A.giganteus* was also identified in other species such as *Trichoderma viride* (Hao et al., 2000). On the other hand, a PAF orthologous gene is known as antifungal protein from ascomycetes and is identified in the genome of *Giberella zeae* and named as *G. zeae* antimicrobial protein A. Microorganisms especially fungi can produce secondary metabolite that have potential to inhibit the growth of the pathogens. *Saccharomyces cerevisiae* produce toxins and are named killer toxins (Tao et al., 1990). The study about killer toxin from *Ustilago maydis* has an effective killer activity against *Brettanomyces bruxellensis* (Santos, Navascues, Bravo, & Marquina, 2011). From *A. giganteus* it is called the ribotoxins α-sarcin and restrictocin, mitogillin from *A. restrictus* (Lamy & Davies, 1991). Several antagonists from *Penicillium* species have been reported to inhibit plant pathogens. For example, mechanisms of action based on the production of antibiotic compounds (Nicoletti, De Stefano, De Stefano, Trincone, & Marziano, 2004), mycoparasitic interactions formation (Sempere & Santamarina, 2008), and the induction of resistance (Hossain, Sultana, Kubota, Koyama, & Hyakumachi, 2007). One of the genera *Penicillium* is known as the most commonly occurring eukaryotic life forms on earth is *Penicillium citrinum.* This *Penicillium* species is a filamentous fungus with a worldwide distribution (Pitt, 1979). The first detection of the antifungal protein activity of *P. citrinum* isolate S1 was described in research before (Wen, Guo, & Chen, 2014).

**Table 1.** Mascot result from the antifungal protein *P. citrinum* S1

| Accession number | Score | Match case | MW (kDa) | Organism |
|---|---|---|---|---|
| gi\|510952845 | 23 | histone deacetylase RpdA | 71.49 | *P. citrinum* |
| gi\|47057722 | 18 | unnamed protein product | 40.14 | *P. citrinum* |
| gi\|23574645 | 17 | polyketide synthase | 28.23 | *P. citrinum* |
| gi\|372121070 | 15 | RNA polymerase II largest subunit, partial | 35.35 | *P. citrinum* |
| gi\|662506127 | 15 | alpha-1,2-mannosidase | 55.30 | *A. melanogenum* |

In this study, the antifungal activity has a mode of action as antibiosis based on the clear inhibition zone. Antibiosis is the inhibition of radial growth by forming an inhibition zone against the hyphae of the fungal pathogen, whereas the antibiotic metabolites inhibit the cell activity by chemical toxicity (Begum, Sariah, Zainal Abidin, Puteh, & Rahman, 2008). Antibiosis includes producing antibiotic compounds, metabolites, or proteins against plant pathogens by microorganisms. Ultrafiltration using Amicon 10 kDa Ultra centrifugal filter units (millipore) was used to separate the protein-metabolite complex, which has a high molecular weight fraction. In contrast, secondary metabolites have a low molecular weight fraction (Elkhayat & Goda, 2017). The result of this study suggested that the antibiotic is an antifungal protein because after being separated with 10 kDa, Molecular weight cut off the higher fraction has antifungal activity.

**Purification of Antifungal Protein**

The first step to identifying the antifungal protein was to induce antifungal protein production. In this study, YTM liquid medium was used. YTM liquid medium has been tested for generating the antifungal activity of fungal isolate *P. citrinum* W1, and this medium showed significant inhibitory activity against many pathogenic fungi (Wen, Guo, & Chen, 2014). Next, a good diffusion assay was used to check the antifungal potential of Culture filtrates of two different *Penicillium* species. And the result showed a high antifungal effect on mycelial growth of *Fusarium oxyporum, Fusarium solani, spergillus japonicus* var *aculeatus, Macrophomina phaseolina,* and *Cladosporium cladosporioides* with inhibition 25-68% (Khokhar, Mukhtar, & Mushtaq, 2011).

Step-by-step precipitation of Ammonium sulfate with different saturation was used to purify the antifungal protein. The culture supernatant was saturated using 20% Ammonium sulfate and incubated at 4°C for 2 hours. After that, the crude protein was collected by centrifuging the culture supernatant. The culture supernatant was used for 40%, 60%, 80%, and 100 % saturated. From Fig. 2, the concentration of the protein is different. In 20% Ammonium sulfate, the concentration was deficient at 0.199 mg/ml. Still, the concentration is an increase in 40% Ammonium sulfate became 0.653 mg/ml; the higher was in 60% Ammonium sulfate, the concentration was 1.555 mg/ml. The concentration was decrease became 1.325 mg/ml in 80% Ammonium sulfate and became 0.356 mg/ml in 100% Ammonium sulfate. The antifungal activity of the protein with different saturated Ammonium sulfate was tested. The result showed that the protein with antifungal activity was precipitated after being saturated with 60% and 80% of Ammonium sulfate. The best antifungal activity was found in the 80% Ammonium sulfate saturated, which has a concentration of 1.325 mg/ml.

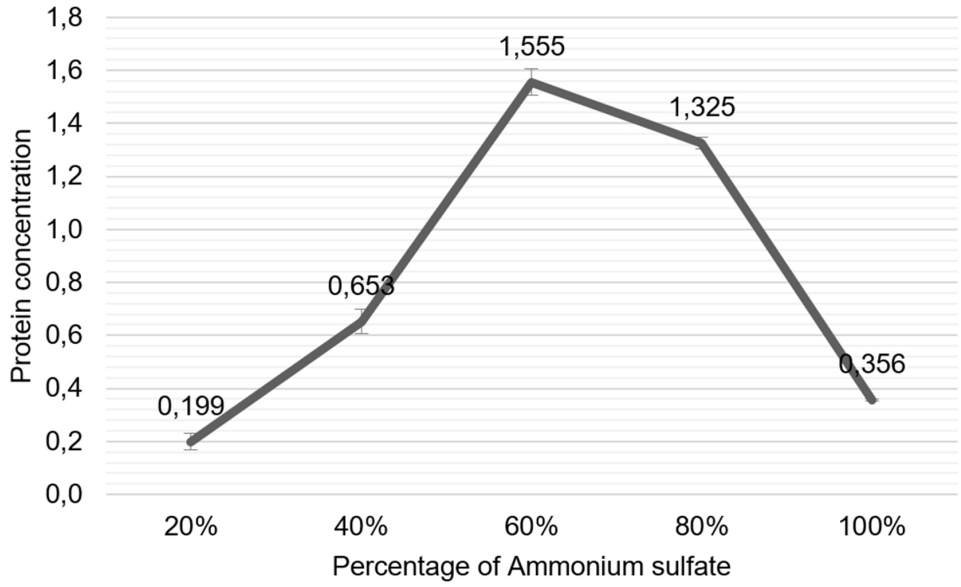


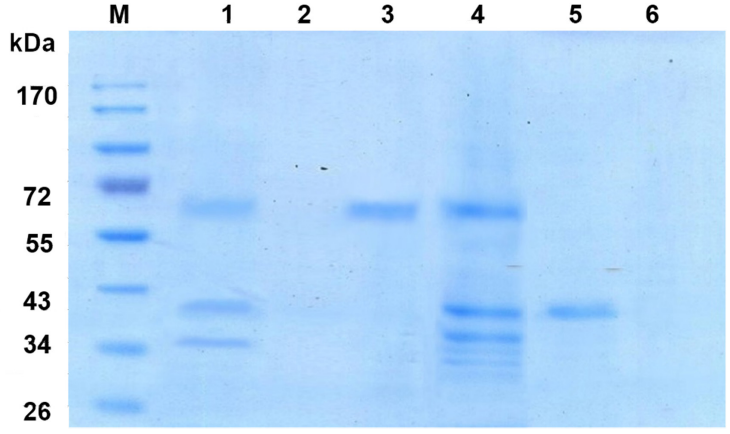

**Fig. 2.** Protein concentrations after step by step precipitated with Ammonium sulfate in different saturation

The SDS PAGE result shows that the culture supernatant has 3 bands in 70 kDa, 40 kDa, and 34 kDa (Fig. 3, line 1). After precipitating in 20% ammonium sulfate, the crude protein shows no band because the amount of the protein was meager (Fig. 3, line 2). Then the 40% crude protein has one band around 70 kDa, but when tested against the pathogen, this crude protein showed no antifungal activity (Fig. 3, line 3). The 60% crude protein has many bands around 26-70 kDa (Fig. 3, line 4). Moreover, the 80% crude protein has only one band and has antifungal activity against the pathogens (Fig. 3, line 5).

**Identification of Antifungal Protein**

The antifungal activity of the protein after precipitated using Ammonium sulfate with 80% saturation was tested and significantly inhibited the growth of *C. gloeosporioides* isolate MC-9. This protein was ultrafiltrated using a 3 kDa Molecular weight cut-off (MWCO) to remove the salt and Amicon Bioseparation Centricon YM 50 (50 kDa) to remove the large protein. After ultrafiltration, the concentration of the antifungal protein was tested and became 0.94 mg/ml. The result showed that the antifungal activity of the protein was significant. After nine days of incubation, the inhibition is 63.6% (Fig. 4). Culture supernatant was precipitated with step-by-step saturated Ammonium sulfate. Then analysis by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) for the molecular weight, and the result shows that the purified antifungal protein is around 40 kDa (Fig 5A). Then this antifungal protein is identified by LCMS. The mascot search result shows that the protein is an identity to 5 proteins (Table 1). The possible antifungal protein was the Unnamed Protein Product because it has a molecular weight of around 40.14 kDa, which was this antifungal protein is about 40 kDa as well. After BLAST Protein results of Unknown protein product, the antifungal protein belongs to the family L-asparaginase (Fig. 5B).

L-asparaginase has received increased attention in recent years because of its anti-carcinogenic potential. L-asparaginase is an enzyme that converts L-asparagine to L-aspartic acid and ammonia. This enzyme has been used as a chemotherapeutic (Raj et al., 2016). Some of the anti-tumor or antineoplastic enzymes may have potential as antifungal agents. Several investigators have studied in vitro the direct effects of standard cytotoxic and metabolic antineoplastic agents against fungi (Cardenar et al., 1999). Furthermore, in vitro susceptibility testing showed that bleomycin, carmustine, doxorubicin, daunorubicin, 5-fluorouracil and asparaginase had MICs less than 100 mg/ml. These compounds against more *C. tropicalis* than *C. glabrata* and *C. albicans* and were consistently more active. Both 5-fluorouracil and asparaginase have MICs less than 1 mg/ml for *C. tropicalis*. Against *Candida* spp, cytotoxic agents have a variable in vitro activity. The antifungal activity from both asparaginase and 5-FU were the most active against *C. tropicalis* (MIC, <1 µg/ml) (Chen, Lewis, & Kontoyiannis, 2011).




Remarks: M = Molecular weight markers

**Fig. 3.** SDS PAGE profile (B) of the YTM culture supernatant (line 1) the crude protein after precipitated wth 20% ammonium sulfate (line 2), 40% ammonium sulfate (line 3), 60% ammonium sulfate (line 4), 80% ammonium sulfate (line 5) and 100% ammonium sulfate (line 6) The predicted protein weight in kDa and position of target each protein were showed in the profile

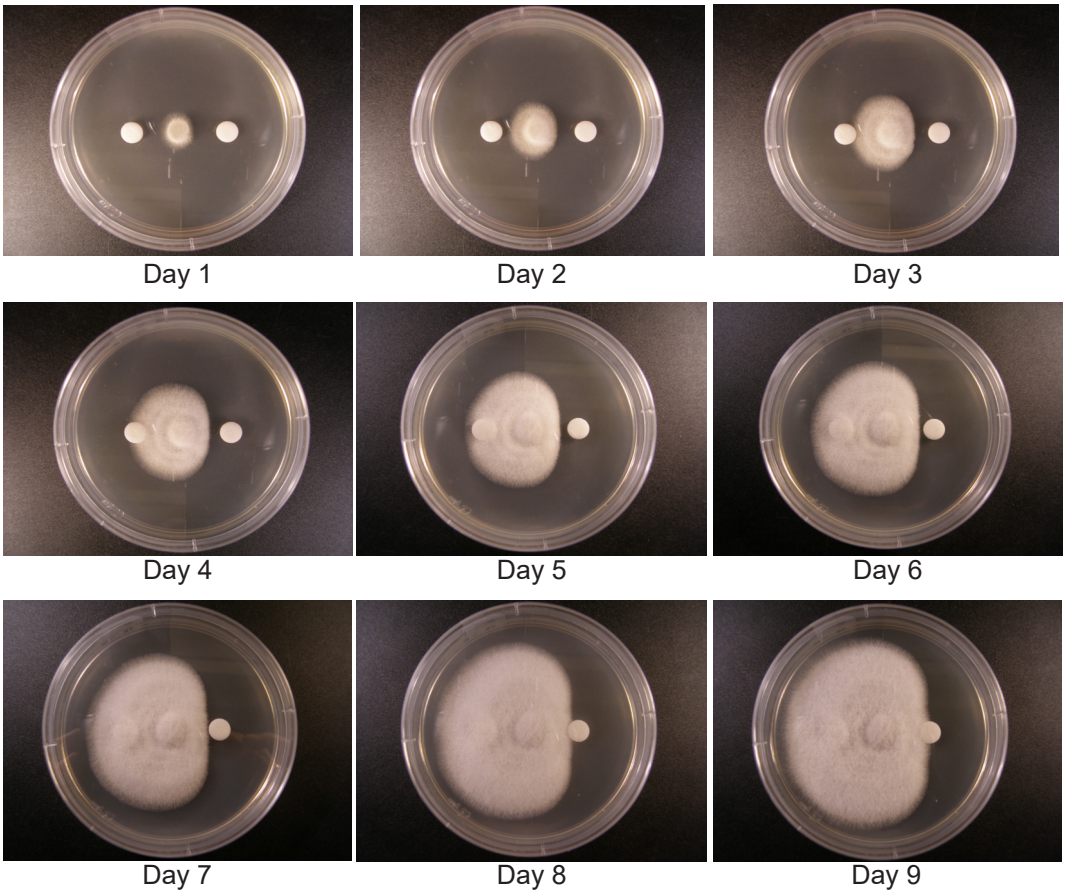



**Fig. 4.** Antifungal activity of the purified antifungal protein against *C. gloeosporioides* MC9

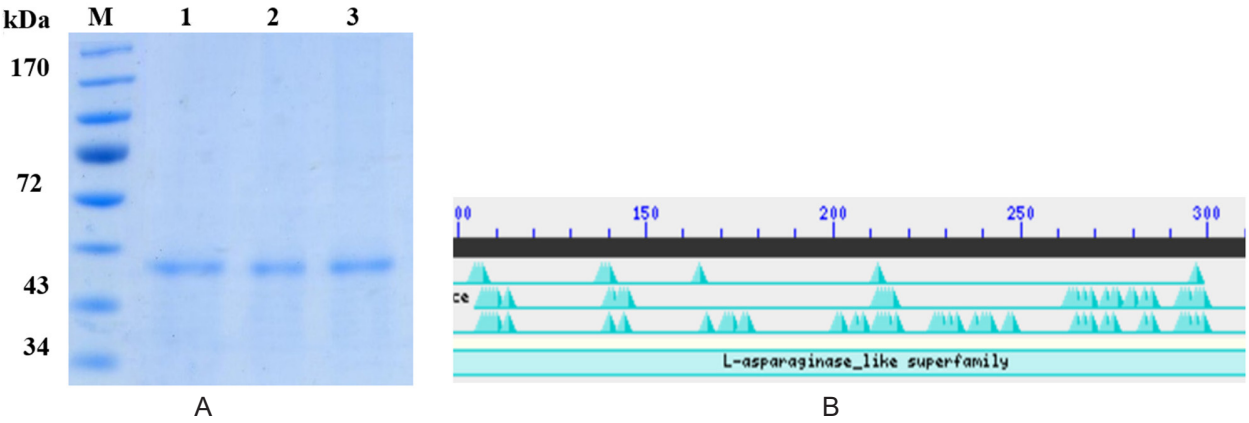



**Fig. 5.** SDS-PAGE profile of the purify antifungal protein after saturated with 80% ammonium sulfate and ultrafiltration using MWCO 50 and 3 kDa (A) and BLAST resut of the protein after LC-MS (B)

L-asparaginase and L-glutaminase-producing bacteria suggested that those bacteria can inhibit the growth of several fungal pathogens. *Aspergillus niger* and *Cryptococcus neoformans* had the highest zone of inhibition for antifungal activity from those bacteria. The lowest activity was recorded against *Mucor racemosus* and *Rhizopus arrhizus* (Raj et al., 2016)

For certain species, antibiotic production is not specific. Some species may produce the same antibiotics or secondary metabolites, but others may possess quite different antibiotics or toxic substances (Lo, 1998). Many fungal isolates have been reported well for the potential of L-asparaginase production (Patro & Gupta, 2014). Among the various fungi from the genus Aspergillus, Penicillium, and Fusarium are *Aspergillus oryzae, Penicillium nelicum, P. granulatum, P. nigricans, P. citrinum* and *F. solani* has been tested can produce this enzyme (Gulati, Saxena, & Gupta, 1997). Moreover, *P. citrinum* Thom. Isolate H can make more L-asparaginase by stimulating the enzyme production with added Fructose as a carbon source in the culture medium (Patro & Gupta, 2014).

L-asparaginase can inhibit the growth of pathogens because it can disturb the transport systems. L-asparaginase activity will convert L-asparagine to L-aspartic acid and ammonia. Asparagine and glutamine are important for fungal growth because their storage forms good nitrogen sources. It is very likely that in plant tissues, these amides are major nitrogen sources for the development of parasitic fungi. Moreover, ammonia is an effective inhibitor of the nonspecific amino acid permease in filamentous fungi and yeast. Inhibition of the mycelia of *S. botryosum* by ammonia is because of the starved of nitrogen- and carbon. Under the latter conditions, it results from the inaccessibility of the cells to ammonia transport (Breiman & Barash, 1976).

The effectiveness of this antifungal protein was tested. In this study, the minimal inhibitory concentration of this antifungal protein was 0.5 mg/ml. According to Sonderegger et al. (2016), the Minimum Inhibitory concentration of an antifungal protein PAF fsarom *P. chrysogenum* was around 0.8 mg/ml against *A. niger*. On other hand, the purified antifungal protein PcPAF from *P. citrinum* was 1.52, 6.08, 3.04, and 6.08 µg/disc against *Trichoderma viride, Paecilomyces variotii, Alternaria longipes and Fusarium oxysporum* (Wen, Guo, & Chen, 2014). Antifungal protein from *Penicillium chrysogenum* called PAF reported efectively inhibited many pre and postharvest pathogenic fungi and efects on mammalian cells and plant seedling its showed no toxic efects, PAF also can inhibit *Botrytis cinerea* on tomato plant leaves (Tóth et al., 2020a). In other hand, antifungal protein (NFAP) and its PD (γNFAPopt) from the *Neosartorya (Aspergillus) fscheri* show the antifungal efcacy and potential agricultural applicability (Tóth et al, 2020b). The study about the combination of AFPs and PDs showed the development of new biocontrol strategy against phythopathogenic fungi (Tóth et al, 2022c).

Various antifungal proteins from microorganisms like fungi may involve and disturb the synthesis, structure, and function of the fungal pathogens' cell wall. The other antifungal protein from microorganisms perturb the dtructure of the fungal membrane and make the cell lysis. Inhibition of the DNA synthesis, damage to cellular ribosomes, membrane channel and pore formation, and inhibition of the cell cycle may be the mechanisms of antifungal proteins. But many other instruments are as varied as their sources. But many proteins have an unknown mode of action and can be the subject of future study (Selitrennikoff, 2001).

## CONCLUSION AND SUGGESTION

The result of this study suggested that the antagonistic fungi *P. citrinum* isolate S1 can be used as a biological control agent against *C. gloeosporioides* MC9. *P. citrinum* isolate S1 can produce L-asparaginase as an antifungal protein. L-asparaginase has been reported as an antifungal drug with anti-carcinogenic potential. Interestingly, L-asparaginase has never been reported as an antifungal protein for biological control against plant pathogens.

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