International Journal on Advanced Science Engineering Information Technology

Vol.7 (2017) No. 1
ISSN: 2088-5334

Exploring Antagonistic Candidate Fungi for Controling Pathogenic Fungi (*Colletotricum gloeosporioides*) Causing Anthracnose Disease in Kintamani Siam Orange Plants (*Citrus Nobillis Lour Var. Hass*)

Ni Nyoman Darsini[#], I Made Sudana[*], I Dewa Ngurah Suprapta[*], Dewa Nyoman Nyana[*]

[#] *Plant Disease Laboratory, Faculty of Agriculture, Udayana University, Denpasar-Bali, Indonesia*
E-mail: darsinikoming@yahoo.com

** Postgraduate Agricultural Science Program, Udayana University, Denpasar-Bali, Indonesia*

Abstract*— Orange plantation centre in Bali are located in Bangli Regency, Kintamani District. Kintamani orange plantations cultivated three types of oranges: tangerine, selayar, and mandarin oranges. The famous orange in Bali today is Kintamani orange, tangerine type. The typical flavour and aroma of Kintamani tangerine make it is favoured by consumers from various regions. Based on the information from Bangli District Agriculture Office and the results of field surveys in the last two years (in 2013 and 2014), cultivation of orange in Kintamani has been infected with anthracnose disease. The disease is characterized by symptoms whereby brown twigs spread to the leaves and fruit, and the fruits which are about to be harvested rot simultaneously and eventually fall due to decay. This condition causes farmers to suffer significant losses. The cause of anthracnose on Kintamani orange is Colletotrichum gloeosporioides . The Control of these diseases has been carried out by farmers with various synthetic fungicides but the disease is still widespread. It is feared that the uncontrolled use of synthetic pesticides can harm the environment, cause resistance to C. gloeosporioides fungi, and kill non-target beneficial micro-organism. It is necessary to conduct research that aims to control anthracnose biologically to maintain the ecological balance and environmental safety. Based on the results of this research by exploring the fungi on healthy plants around orange trees infected with anthracnose diseases, nine isolates of antagonist candidate fungus were obtained based on colony colour of fungal hyphae (IS1, IS2, IS3, IS4, IS5. IS6, IS7, IS8, IS9). Based on the test results of the in vitro dual culture, two candidates of antagonistic fungal isolates were selected, the isolates IS4 and IS7. It was because on day 7 after the second dual culture, these two isolates had the highest percentage of inhibition, 89.22% and 85.11 % respectively. Based on the conventional and molecular identifications, it is known that the two candidate isolates of antagonistic fungus (IS4 and IS7) belong to one species of the *Aspergillus aculeatus*.

Keywords*— orange; antagonists fungi; kintamani tangerine; anthracnose; *Cilletotricum gloeosporioides; Aspergillus aculeatus.

I. INTRODUCTION

Orange is one type of horticultural crops that become the important commodity in Bali. Orange plant centre in Bali are located in Bangli Regency, Kintamani District. Currently, there are three types of oranges cultivated at the heart of Kintamani orange plantations: tangerine, selayar, and mandarin oranges. Tangerine type is the famous most widely grown by farmers because of this type has a sweeter taste, thinner rind, delicate fruit pulp, and prices in the market better than selayar oranges and mandarin. Kintamani orange fruit (Figure 1) has typical taste and aroma that make it is favoured by consumers from various regions.

The production of Kintamani orange fruit is approximately 500 tons per day in its peak season. In fact, today, almost every well-known supermarket and even in traditional markets the imported orange are still widely sold. This indicates that the production of orange plants still needs to be improved both in quality and quantity to reduce imports. One reason is the cultivation of oranges in Kintamani has been threatened by anthracnose (Figure 2). The disease is characterized by symptoms whereby brown twigs spread to the leaves and fruit, orange fruit which is about to be harvested simultaneously rot, and eventually, fall for the foul [1].

This condition causes the farmers to suffer significant losses, and if it is continue it will cause the cultivation and development of tangerine in Kintamani to be unsustainable. Anthracnose on Kintamani orange plants needs to be handled properly so that orange fruit remains available to meet the consumer needs and farmers can get optimal revenue. Counter measures of anthracnose in orange have

been carried out by farmers using synthetic fungicides, but the attack of the disease has been getting worse.

Fig. 1 Kintamani Tangerine

Fig. 2 Orange fruit harvest fall due to being infected with anthracnose

The orange fruits to be harvested decrease drastically due to anthracnose disease. The uncontrolled use of fungicides is feared to be harmful to the environment. Synthetic pesticides are also very dangerous for human health especially the farmers. It will also cause pathogenic resistance when it is used without any control by farmers. Sometimes it causes respiratory diseases and other diseases for farmers. Lately many negative effects of excessive application of synthetic pesticides have been reported.

The use of synthetic fungicides intensively was reported to cause some pathogens to become resistant to benomyl, quintozene, and blasticidin as well as the presence of residues in agricultural products. Biological control of anthracnose for ecosystem security is important to be done [2].

Ref. [3] conducted a study on the use *Debaryomyces* sp. and *Schizosacccaromyces* sp. as microorganisms antagonistic to fungal pathogens (*Colletotricum* sp.) which cause anthracnose on mango (*Mangifera* sp.). Based on the research findings, it was reported that the two microorganisms categorized as yeast are able to suppress the development of anthracnose on mango shown with a diameter blotch substantially smaller. [4] conducted research on fungi antagonistic towards *Gloesporium piperatum* which causes anthracnose on large chilli. Based on his research, it is known that there are three fungal antagonists that can be used to control *G. piperatum* namely: *Aspergillus niger*, *A. flavus* and *Mucor* sp. The percentage inhibition of *A. niger* amounted to 50.66%; *A. flavus* 49.33% and *Mucor* sp. 40.67%. The mechanism of inhibition of *A. niger* and *Mucor* sp against *G. piperatum* is antibiosis and *A. flavus* is competition and parasitism. Based on the above description, it is very important to do research on the identification of fungi that cause anthracnose and utilization of antagonistic fungi as natural enemies for the biological control of anthracnose on Kintamani orange crops (*Citrus* sp.) in Bangli Regency Bali Province.

II. Materials and Methods

A. Field survey

The research was conducted in two stages, consisting of field surveys and research in the laboratory. A field survey conducted was the observation of plants infected with anthracnose and healthy plants that are not infected with the disease.

B. Fungus isolation antagonists (in vitro)

Isolation of fungal antagonists was done by taking part of the healthy orange organ (fruit, twigs, and leaves) located around orange planting area infected with anthracnose disease. These organs are cut and put in a new plastic bag and labelled form of the date and location and time. The samples were stored in a place protected from direct sun exposure and taken to the laboratory. Isolation antagonist fungi were conducted at the Laboratory of Plant Diseases, Faculty of Agriculture, University of Udayana. The samples were cut to a certain size and then grown on PDA that has been provided beforehand. Isolation of endophytic fungi antagonist was done by splitting the stems and twigs that have been sterilized on the tissue surface using alcohol 95% for 5 minutes, then the inside of the tissue was taken and cut into pieces of a certain size. The pieces of tissue are then inoculated on PDA. Fungus growing colonies were isolated based on the colour and shapes of different colonies. The preparation was then incubated for five days to obtain some fungal antagonist candidates which were assumed that one or more of the fungi were beneficial as fungi antagonistic to pathogenic fungi.

C. Dual Culture process / antagonistic Test

Fungal isolates that had been obtained from the isolation of antagonistic fungi were antagonistically tested in vitro against pathogenic fungi that cause anthracnose disease in orange. The method used was the dual culture method [5], isolates of fungal antagonist candidate were placed on the opposite side of the pathogenic fungi with a certain range of pathogenic fungi mycelia.

The most accurate method for calculating the inhibition of fungal antagonist is by calculating the inhibition by using mm block paper. The growth areas of the pathogenic and antagonist fungi were respectively calculated with mm block paper, with the help of tracing paper and carbon paper. The formula used was as follows:

$$I = \frac{K - P}{K} X\,100\% \qquad (1)$$

Description:
I = Percentage inhibition of antagonist fungi (%)
K = Growth area of the control pathogenic fungi (without antagonist)
P = Growth area of pathogenic fungi after being antagonistically treated

The preparation was incubated at room temperature (26-28^{o}C) for 7 days. The data obtained were analysed using CoStat to determine whether there was a significant difference between treatment and control. Based on the results of calculation of the percentage inhibition of fungal antagonist candidates, antagonistic fungal candidates that have highest inhibiting activities could be determined.

D. Identification of Antagonist Fungal Candidate Species

Identification of antagonist fungal candidates species was done by observing the shape, the colour of the vegetative organs matched with the identification guide of fungal species in the CMI (Commonwealth Mycology Institute) book, conducted at the laboratory of Plant Pathology Faculty of Agriculture, University of Udayana. Molecular identification was carried out in LIPI (Indonesian Institute of Sciences), Cibinong Bogor-Indonesia. Molecular identification was done by using PCR (Polymerase Chain Reaction) using ITS eukaryote-specific primary to amplify the ITS region of the fungus and the amplification product in sequence.

E. Statistic analysis

The design of experiments was conducted using a completely randomized design (CRD) with 4 replications and treatment of 9 antagonist fungal candidate isolates and added with 1 control (total of at least 10 treatments). Control treatment as a comparison was carried out by isolating the pathogen on PDA medium untreated with fungal antagonist candidates. The preparation was incubated at room temperature (26-28^{o}C) for 7 days. The data obtained were analysed using CoStat to determine whether there was a significant difference between treatment and control.

III. Results and Discussion

A. Field Survey Results

Exploration of antagonist fungi begun on June 17, 2015, the antagonist fungal candidates were isolated from samples of healthy orange plants (branches, fruit and leaves) that were around plants affected with anthracnose. It was highly likely caused by healthy orange plants that are in locations of orange plants infected with anthracnose there were fungi which were able to suppress the growth of fungi that cause anthracnose.

B. Isolated Results of Antagonist Fungus Candidates

Samples were taken to the lab later, washed with running water, drained, and then dipped in 95% alcohol. On June 18, 2015, antagonist fungus candidate isolation was conducted in a Plant Pathology Laboratory, Faculty of Agriculture, University of Udayana. Materials were cut according to the size.

On the second day after isolation colonies of white fungi began to be found; on the fourth day, there grew a colony of light gray fungi other than white fungal colonies; on day 5, there were fungi with mycelia which had green, brown, light gray, gray, dark brown, and black colour. On day 9, fungi with white mycelia fungus can be distinguished from the appearance of colonies, namely white as cotton (white 1), a thin white (white 2), and the snow white (white 3). So, nine (9) antagonist fungal candidates were found, pathogenic fungi causing anthracnose disease on Kintamani orange crops. On day 10, after the isolation process of a fungal candidate was carried out, separation of antagonist fungal candidates was made based on colour and its colonies until it was completely pure on PDA. Based on the results of this separation, nine isolates of antagonistic fungi had been found.

C. Dual Culture Process Results

On October 15, 2015 dual culture process was conducted simultaneously between pathogenic fungi and 9 antagonist fungal candidates with 4replications. Dual culture process was carried out to determine the candidate fungi that have the highest percentage of inhibition of the growth of pathogenic fungi. Measurement of the power resistor candidate fungi antagonistic to pathogenic fungi began one day after the dual culture was done. It can see on Table 1.

TABLE 1.
PERCENTAGE OF INHIBITION ACTIVITIES BY ANTAGONISTS FUNGUS CANDIDATE THAT GROW ON PATHOGENS FROM DAY 1 TO DAY 7 AFTER DUAL CULTURE PROCESS

Days	Treatment								
	IS 1	**IS 2**	**IS3**	**IS4**	**IS 5**	**IS6**	**IS7**	**IS8**	**IS9**
1	26.27	13.43	21.23	0	0	0	22.35	1.92	0
2	0	0	0	7.14	0	0	21.75	0	0
3	2.28	31.13	20.5	61.14	12.84	44.67	52.35	7.96	42.65
4	28.87	43.16	22.49	79.24	32.6	51.81	64.61	26.06	50.57
5	44.82	54.45	43.42	79.76	52.76	67.45	77.1	42.4	66.72
6	59.6	72.73	53.61	87.35	62.51	75.6	83.62	51.11	74.8
7	69.38	63.54	52.18	89.22	66.35	79.23	85.11	52.28	75.81

Based on Table 1 above, it can be explained that on day 1 and day 2 after the dual culture was implemented, the inhibiting activities of all the antagonist candidate fungi were still all under 40%. Antagonist candidate fungi IS4, IS5, IS6, and IS9 percentage of the inhibiting activities against pathogens growth was 0%, but on day 2 for fungi IS4 antagonist candidates had started to rise.

Day 3 percentage of the inhibiting activity of dark chocolate fungal candidates (IS6) was 44.67%, green candidate fungi (IS9) amounted to 42.65%, fungi light gray candidate fungi (IS7) amounted to 52.35%, while the inhibiting activities of other antagonist candidate fungi were still below 40%.

Day 4 white candidate fungi 1 (IS5) inhibiting power amounted to 43.16%, green candidate fungi (IS9) amounted to 50.57%, dark chocolate candidate fungi (IS6) amounted to 51.81%, light gray candidate fungi (IS7) amounted to 64.61%, the brown candidate fungi (IS4) amounted to 79.24%, while other fungi inhibiting activities was still below 40%.

Day 5 all the inhibiting activities of the antagonist fungi was above 40%, i.e. each candidate fungi white 3 (IS8) amounted to 42.40%, black candidate fungi (IS3) 43.42%, gray candidate fungi (IS1) was 44, 82%, candidate fungi white 2 (IS5) amounted to 53.76%, the candidate fungi white 1 (IS2) amounted to 67.45%, light gray candidate fungi (IS7) amounted to 77.10%, the brown candidate fungi (IS4) amounted to 79.76%.

Day 6 the inhibiting activities of all kinds of fungal antagonist were already above 50%, the snow white candidate (IS8) amounted to 51.11%, black (IS3) amounted to 53.61%, gray (IS1) amounted to 59.60%, white 2 (IS5) amounted to 62.91%, white 1 (IS2) amounted to 72.73%, green (IS9) amounted to 74.80%, dark chocolate (IS6) amounted to 75.60%, light gray at 83, 62%, and chocolate candidate 87.35%.

Day 7 the inhibiting activities of black candidate fungi(IS3) amounted to 52.18%, candidate white 3 (IS8) amounted to 52.28%, white candidate 1 (IS2) amounted to 63.54%, candidate white 2 (IS5) 66.35%, gray candidates (IS1) 69.38%, candidates white 3 (IS8) amounted to 75.81%, dark chocolate candidate (IS6) 79.23%, the light gray candidate (IS7) amounted to 85.11% and the candidate chocolate (IS4) amounted to 89.22%. Observations on day 7, all petri dish were already filled with pathogenic fungal hyphae and fungal hyphae candidate antagonist.

Observation on day 7 was the last observation because petri dish had been filled with the candidate antagonist fungal hyphae and fungal pathogens. Fungal antagonist candidate which had the inhibiting activities above 80% was prepared for testing in vivo in the field. Based on the results of statistical analysis it was explained that on day 1 to day 4 inhibition of 9 fungal isolates antagonist candidate were not significantly different from the control (the fungal pathogen without fungal isolates antagonist candidates).

On day 5 to day 7 after the dual culture, statistical test results showed significant difference about the inhibiting activities of 9 isolates of fungal antagonist against controls. This means that the nine isolates of antagonist candidate fungus had the ability to inhibit the growth of antagonist fungi. The inhibiting activities of the isolates of antagonist candidate fungi having the most significant difference from control were isolates of candidate fungi with brown hyphae (IS4). The growth of fungal isolates IS4 was very quick from day 2 to 7. Fungal growth media was quickly controlled by antagonistic fungal candidate so that the growth of the fungal pathogen was very limited. In can be see on Figure 3.

Fig. 3 Dual Culture Process on day 7 Antagonist candidate Fungi IS4 (left) Vs Pathogenic fungi (right).

D. Molecular identification of antagonistic fungi IS4 and IS7 Pathogenic Fungi Causing Anthracnose disease

Molecular identification of the 2 selected antagonist candidate fungi (IS4 and IS7) was held in LIPI Cibinong, Bogor. Identification of fungal species was conventionally done by observing the shape of fungal vegetative and reproductive organs and matched with CMI Books; and [6] conducted at the Laboratory of Plant Pathology, Faculty of Agriculture, Udayana University while the molecular identification was done at Mycology Laboratory, Indonesian Institute of Sciences (LIPI) Cibinong, Bogor, using the PCR using specific primers eukaryotic ITS, to amplify the area of ITS fungi, and after amplification product was sequenced then the sequencing results were compared with the data sequences in NCBI gene Bank through BLAST-N program, in order to obtain images to determine the phylogenic proximity of tree species that were already registered with Gene Bank.

Extraction was done by extracting genomic DNA of *Colletotricum* spp (pathogen) by taking the hyphae of fungal colonies which were then put in a centrifuge tube and mixed with 100 mL of Prep Man ultra reagent (Prep Man Ultra Protocol, applied Biosystems, USA). Furthermore sample was spined using vortex for 30 seconds was later put on the heat block temperature 95^{o}C-100^{o}C for 10 minutes and then placed at room temperature for 2 minutes and the pellet was taken as a DNA extract to be used for further processing [7].

The next process is the DNA amplification by PCR, 18S rRNA gene was amplified by PCR using a primer Internal Transcript Spacer (ITS) 1 (5-TCCGTAGGTGAACCTGCGG-3) and ITS 4 (5-TCCTCCGCTTATTGATATGC-3). The reaction took place by using Takara PCR thermal cycler Personal (Takara Bio, Otsu, Japan) with Ex Tag (Takara Bio, Otsu, Japan) with conditions: pre-denaturation 940 C for 4 minutes, followed by 35 cycles of denaturation 940C for 35 seconds and then annealing 520C for 55 seconds, elongation 72°C for 2 minutes and post elongation 72°C for 10 minutes [8].

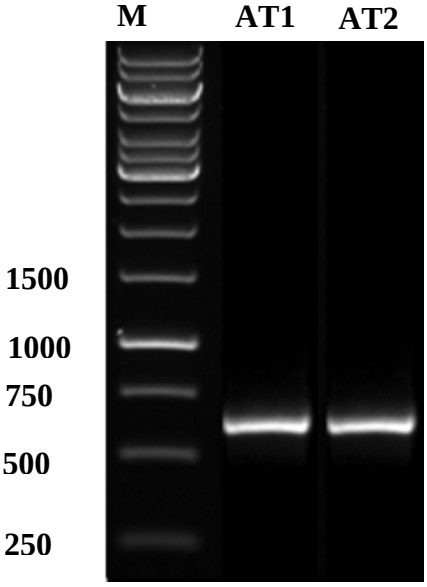

Fig. 4 Result of PCR amplification process to ITS gene\ with ITS_5F and ITS_4R primary; M = marker 1 Kb ladder ; production of PCR Antagonistic sample 1 and antagonistic sample 2 (AT1, AT2.)

The next process was the ITS region sequencing and DNA sequence computer analysis. Nucleotide sequence was determined by using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Apple Biosystems, Foster City, CA, USA) according to the guidelines of the tool and the PE Applied Biosystems Automated DNA Sequencer (model 313xl, Applied Biosystems). DNA sequences double

helix assembled and analysed using Genetyx version 11.0 and Genetyx-ATSQ (version 4.0) software (Genetyx, Tokyo, Japan), consecutively and compared to the same DNA sequences taken from DDBJ / EMBL / Gen Bank through NCBI BLAST program [9]. The results process of molecular identification can see on figure 4.

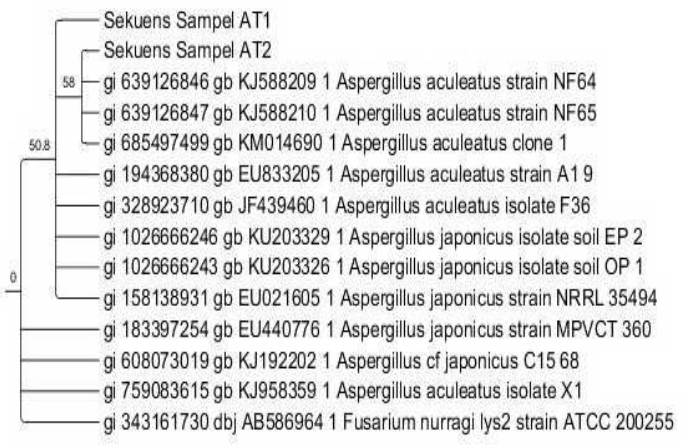

Fig. 5 Phylogeny tree built from ITS Sequence of Library Gen Bank of 2 Aspergillus spp antagonist candidate for pathogenic causing anthracnose disease on tangerine variety of Kintamani orange

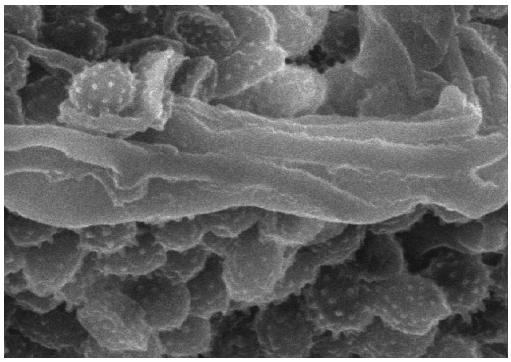

Fig. 6 Spore of Apergillus aculeatus documented by SEM method

Based on the results of molecular identification, it was known that the two candidates fungal antagonist (IS4 and IS7) belong to 100% the same species i.e.*Aspergillus aculeatu* swhich has 100% similarities to *Aspergillusa culeatus* strain A1. 9 (Assesion number Gene Bank EU833205.1), *Aspergillus japonicus* strain.chai.1 (Assesion number KF613717.1). Having 99% similarity to *Aspergillus japonicas* strain chai.1 (Assesion number Gene Bank KF613717.1) and *Aspergillus japonicus* strain SB1 VIT (Assesion Number Gen Bank KC128815.1). Having a 97% similarity to *Aspergillus* sp 2011.9 (Assesion number Gene Bank KP668958.1). Having a 96% similarity to *Aspergillus uvarum* strain A 3297 (Assesion number Gene Bank JQ316520.1), *Aspergillus niger* strains GZ 4 (Assesion Number Gen Bank KT726919.1), and *Aspergillus indologenus* isolates CBS 114 80 (Assesion Number Gen Bank AJ280005.1). Having a 94% similarity to the strain *Aspergillus violaceofuscus* CCF 4079 (Assesion Number Gen Bank FR733805.1) and *Aspergillus aculeatus* isolate P72 30 (Assesion Number Gen Bank KJ439163.1). Having a 35% similarity to nurragilys2 Fusarium strain ATCC 200 255 (Assesion Number Gen Bank AB586964.1).

Biopesticides are appropriate alternatives to be chosen as a natural pesticide that can be used to control plan pests. Particularly; this study had found one natural enemy organism or antagonist fungus as biological biopesticides.

The fungal organism is *Aspergillus aculeatus*. The fungus is a natural enemy or controlling bioavailable (biopesticides) for fungal pathogens *Colletotricum gloeosporioides* causing anthracnose on Kintamani orange crops. Based on the UHPLC-DAD-HRMS, it can be seen that the fungus Aspergillus aculeatus can produce new compounds namely dioxomorpholine, okaramine, aflavinine, aculenes A-C. calbistrins, ocaramines and secalonic acid. These compounds are able to kill fungal pathogens in this case *Colletotricum gloeosporioides* fungus. Antibiosis mechanism occurs because of secondary metabolites produced by microbes in the form of antibiotics and mycotoxins. Mycotoxins are secondary metabolites resulting from fungal metabolism and cytotoxic in nature, damaging cell structures such as membranes, as well as the process of forming cells, such as proteins. Naturally a number of fungi produce mycotoxins during the process of metabolism [10]. Control of plant diseases that are environmentally friendly and support sustainable agriculture can be achieved by using biological agents. Biological control aims to reduce the use of synthetic pesticides that have an impact on the environment and food.

Utilization of microbes or microbial products to control plant diseases and to improve plant production is an integral part of sustainable agriculture [11]. The benefit of biological pesticides include: cheap and easy to make and do not cause toxicity in plants and do not cause resistance in pest, relatively safe for the environment, compatible when combined with other control methods, yields of healthy agricultural and residue-free pesticide, under going degradation / decomposition quickly by sunlight, having an effect / influence quickly, stopping insects' appetite even though they rarely cause death, toxicity is generally low for animals and relatively safe in humans, having a broad control spectrum and selective, reliable to cope with pests that are resistant to synthetic pesticides. Low phytotoxicity that does not poison and damage the crop, the raw material is abundant and available in nature. Because it is selective, it is relatively safe to organisms that act as predators or natural predators. It is easy to make and it can even be processed by a layman, It may also serve as a liquid organic fertilizer.

Aspergillus spp grows quickly and is antagonistic to other fungi to produce antafumicin antibiotics that kill the opponent fungi. The fungal antagonist mechanism occurs are competition, mycoparasitic, and antibiosis. *A. flavus, A. fumigatus, A. niger* hamper by way of competition, lysis of the cell walls of pathogens, and produce an antibiotic substance called antibiosis mechanism, which opens plant roots against pathogen attack. If there are antagonist microorganisms in this area, the pathogen will be dealing with the antagonistic microorganisms during the spread and infect the roots. This condition is called natural obstacles, antagonistic microbes is very potential to be developed as a biological control agent. Microorganisms that can live in the rhizosphere area are very suitable for use as a biological control agent, given that rhizosphere is the main area where

the plant roots are open to be attacked by pathogens. If there are antagonist microorganisms in this area, the pathogen will be dealing with the antagonistic microorganisms during the spread and infect the roots. This circumstance is called natural barriers [12]. Research conducted by Ref. [13] about Test antagonists *Pyriculariagrisea Sacc.* Blast Cause in Rice used Fungus rhizosphere Local Isolates of the fungus *A. flavus, A. fumigatus, A. niger* hamper by way of competition, lysis of the cell walls of pathogens, and produce an antibiotic substance called antibiosis mechanism. Research by [14]; [15] found that *Aspergillus* sp. was potential as microbial antagonists against *Fusarium oxysporum* in banana plants. Food competition occurs in terms of utilizing the growing medium as a source of food. It can be seen from the macroscopic observation antagonist test showing that the edges *Colletotricum gloeosporioides* pathogenic fungal colonies are tangent to isolate the *Aspergillus aculeatuas* antagonists; growth continues to decrease due to competition for space is growing. Antagonist fungal and pathogen equally need nutrients from food to grow. PDA (Potato Dextrose Agar) Media used in the antagonist test is an element of main nutrients such as carbohydrates, amino acids, proteins, minerals, and trace nutrients such as phosphorus (P), magnesium (Mg), potassium (K) also vitamin C and vitamin B required by both types of fungi [16].

Isolates of *Aspergillus aculeatus* have been used to produce a number of important industrial enzymes (cellulases, hemicellulase, and protease) which are used commercially in the food industry. *Aspergillus aculeatus* as a general member of the community of microbes are found in the soil, with the production of a variety of hydrolytic and oxidative enzymes involved in the breakdown of lingo cellulosic crops. Aspergillus species are an important source of enzymes and play an important role in the global carbon cycle [17].

Based on the research by [18] on the selection and identification of antagonistic fungi as a biocontrol agent of white root fungus (*Rigidoporus mikroporus*) selected several fungal isolate antagonists include *Trighoderma* and *aspergillus*. Both of these isolates are a potential antagonist for controlling fungal diseases on rubber JAP. [19] also reported the use bio-fungicide namely *Aspergillus* sp. which can inhibit the growth of pathogenic *R. microporus* more than 50%. Besides being resistant to drought and the condition of the copy, fungus *Aspegillus* survival in drought areas also can live in soil with high salinity, but the fungus *Aspergillus* also have the ability to decipher the cellulose compound into simple carbon compounds needed by soil microbes as a source of carbon (C). This fungus can also dissolve rock phosphate into organic compounds and capable of producing IAA hormone that can enhance plant growth. The addition of biological fertilizers containing *Aspergillus* fungus can promote the growth of rice plants of Ciherang variety planted on land with a salinity of 0.5%. Increasing growth occurs on plant height, number of tiller (tiller), total dry weight biomass (straw) [20].

Aspergillus spp. (*A. niger, A. fumigatus* and *A. repens*) has been experimented *in vitro*, it can suppress the growth of fungi P. *Palmivora* causing fruit rot disease of cocoa. *Aspergillus niger* has the highest inhibiting activities compared to the other two fungi (*A. fumigatus* and *A. repens*), which amounted to 54% [21]. *Aspergillus niger* can improve biological control of bacterial inoculants (P. *fluorescens*) against the disease root knot nematodes, fungal antagonist can be used to protect the sun flower seeds from attack of *Fusarium* spp. [22]; [23] *Aspergillus* spp. (*A. niger, A. fumigatus* and *A. repens*) have been experimented *in vitro*, they can suppress the growth of fungi *P. palmivora* causing fruit rot disease of cocoa. *Aspergillus niger* has the highest inhibiting activities compared to the other two fungi (*A. fumigatus* and *A. repens*), which amounted to 54% [21].

Aspergillus terreus can inhibit the growth of pathogenic fungi *Botrytis cinerea* by releasing volatile compounds such as α-phellandrene, acetic acid pentyl ester and 2-acetyl-5-methyl furan [24]. *Aspergillus nidulans* can be antagonistic to *Colletotrichum gloeosporioides* causes diseases in plants antraknose vanilla. Results of research by [25] found that *A. niger, A. flAvus, A. terreus* and *A. versicolor* can inhibit the growth of *Fusarium* spp. by forming a barrier zone in vitro. [26] also found that Aspergillus spp. can inhibit the growth of pathogenic fungi *Sclerotium rolfsii* with inhibition of 73.12 to 88.35%. The process of inhibition caused by *Aspergillus* spp. produces the enzyme chitinase and β-1, 3 glucanase Laminarinase) that has the ability to break down the cell wall components of fungal pathogens such as chitin and β-1, 3 glucan.

Based on the discussion above, it can be concluded that the fungus genus of *Aspergillus* in general has been proven by many researchers as an antagonistic fungi against various plants diseases. In this study it was found that the fungus *Aspergillus aculeatus* as antagonistic fungi of pathogenic fungi *Colletotroticum gloesporioides* causing anthracnose disease on Kintamani orange crops to produce antibiotics that kill fungal pathogens. Antagonistic mechanisms done by fungus *A. aculeatus*, besides antibiosis, it is also capable of colonizing pathogenic fungi *C. gloeosporioides*. *A. aculeatus* also produces the chitanase enzyme and β-1, 3 glucanase Laminarinase that has the ability to break down the cell wall components of pathogenic fungi such as chitin and β-1, 3 glucan.

IV. CONCLUSIONS

Based on the research and discussion above, it can be concluded that *in vitro* fungi that can control growth of *Colletotricum gloeosporioides* is *Aspergillus aculeatus* fungus. This study had found one natural enemy organism or antagonist fungus as biological bio-pesticides. Bio-pesticides are appropriate choice as a natural pesticide that can be used to control anthracnose disease of Kintamani Siam Orange Plant (*Citrus Nobillis Lour* Var. Hass).

REFERENCES

[1] BPTP Bali. 2013. Organic Fertilizer Increase Productivity of Orange from Kintamani. BPTP Bali. Unpublished report.

[2] Brimer, FY, and G.J. Boland. 2003. A Review of the non-target effects of fungi used to biologically control plant diseases. Agriculture Ecosystems and Environment 100: 3-16.

[3] Indratmi, 2009. Use of Debaryomyces sp. and Schizosaccaromyces sp. as microorganisms antagonistic to fungal pathogens (Colletotricum sp) causes anthracnose on mango (Manggiferaindica). Faculty of Agriculture and Animal Husbandry Department of Agronomy, University of Muhammadiyah Malang.

[4] Rumayomi T.R.O. 2010. Identification of Fungi Filoplan Characteristically Antagonists Against Gloesporium piperatum Cause

Antraknose At Large Chilli. Study Program Plant Pests and Diseases, Faculty of Agriculture, University of Papua. Manokwari.
[5] Khamma; S.A. Yukota; J.F. Peberdy and S. Lumyong. 2009. Antifungal Activity of Streptomyces spp. Isolated from Rhizosphere of Thai Medicinal Plant. International Journal of Integrative Biology. 6: 143-147.
[6] Pit, J.I. and A. D. Hocking. 1997. Fungi and food Spoilage. Blackie Academic and Professional. Second Edition. London-Weinhein-New York-Tokyo-Melbourne-Madras.
[7] Cano, J., guarro, J. and Gene, J. 2004.moleculer and morphological identification of Colletotricum species of clinical interest (American Society for Microbiology). Journal of Clinical Microbiology 42: 2450-2454.
[8] Nishizawa, T .; M. Zhaorigetu; Y. Komatsuzaki; N. Sato, Kaneko and H. Ohta. 2010. Molecular Characterization of Fungal Communities in Not-tilled, Cover-Cropped Upland Rice Field Soils. Microbes and environment 25 (3): 204-2010. [Cited on 4 Nov. 2011] Available from: http.//wwwsoc.nii.ac.jp/jsme2/doi:10.1264/jsme2. ME10108.
[9] Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F, and HIggis, D. G. 1997. The Clustal_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25 (24): 4876-4884.
[10] Samson, R.A., E. S. Hoekstra, and A. N. V. Oorschot. 1981. Introduction to Food-Borne Fungi. Central bureau-Voor Schimmel cultures. Institute of the Royal Netherlands. Academic Of Art and Science.
[11] Haggag, W.M. and H. A.L. A. Muhamed, 2007. Biotechnological Aspects of Microorganisms Used in Plant Biological Control. American-Eurasian Journal of Sustainable Agriculture, 1 (1): 7-12.
[12] Budiarti, L and Nurhayati. 2014. The abundance of fungi antagonists on Long Bean (Vignasinensis (L.) Savi ax Hassk. Prosiding National Seminar on Land Suboptimal. Sriwijaya University in Palembang.
[13] Meinawati; S. Khotimah, Mukarlina. 2014. Uji Antagonis Pyrycularia grisea Sacc. Penyebab Penyakit Blas Pada Tanaman Padi Menggunakan Jamur Risosfir Lokal. Protobiont. Vol.3(1) : 17-24
[14] Sudarma, I Made 2011. Potenensi Antagonist Fungus Derived from Plants Habitat Pisang dengan and without symptoms of Fusarium wilt to Control Fusariumoxy sporumf.sp. cubense In vitro. Agroecotechnology Faculty of Agriculture, University of Udayana.
[15] Suprapta, D.N and Sudarma I Made, 2016. Potential antagonist fungus Derived from Plants Habitat Bananas with and without symptoms of Fusarium wilt to control Fusarium oxysporium cubense In vitro. Department of Agrotechnology Faculty of Agriculture, University of Udayana.Denpasar. Bali
[16] Djafaruddin, 2004. Fundamentals Pengendalia Plant Disease, Publisher Earth Literacy
[17] Petersen, L.M; Casper A; Jens C; Charlote H; Gotfredsen; Thomas O. L. 2014. Direplication Guide Discovery of Secondary metabolites of Mixed
[18] Amaria, W; Efi, T; Rita H. 2013.Selection and Identification of Fungi antagonists as White Root Fungus Biological Agents (Rigidoporus microporus) at the Rubber Plant. Research Institute of Plant Industry.Sukabumi, Indonesia.
[19] Kaewchai, S. and K. Soytong. 2010. Application of biofungicides against Rigidoporus microporus causing white root disease of rubber trees. Journal of Agricultural Technology 6 (2): 349-363
[20] Subowo YB. 2009. Isolation and selection of fungi Ascomycetes pengur some extreme environment in West Kalimantan. Proceeding National Seminar on Economic Sector Empowerment and Culture-Based Environmental and initiation of the Establishment of the Association of Indonesian Environment, University of March Surakarta, August 15 to 16, 2009.
[21] Adebola, M.O. and J.E. Amadi, 2010. Screening three Aspergillus species for antagonistic activities against the cocoa black pod organism (Phytophthora palmivora). Agriculture and Biology Journal of North America. 1(3): 362-365.
[22] Siddiqui, I.A., S.S. Shaukat and A. Khan. 2004. Differential impact of some Aspergillus species on Meloidogyne javanica biocontrol by Pseudomonas fluorescens CHA0 strain. Applied Microbiology. 39: 74-83.
[23] Dawar, S., S. Hayat, M. Anis and M.J. Zaki. 2008. Effect of Seed Coating Material In The Efficacy of Microbail A antagonists For The Control of Root Fungi On Okra And Sunflower. Sir. J. Bot., 40 (3): 1269-1278.
[24] Ting, A.S.Y., S.W. Mah and C.S. Tee. 2010. Identification of Volatile metabolites from Fungal Endophytes with Potential Biocontrol towards Fusarium oxysporum F. sp. cubense Race4. American Journal of Agricultural and Biological Sciences, 5 (2): 177-182.
[25] Fakhsannisa, M.A and A. Ghafar. 2006. In Vitro Interaction of Fusarium spp. With other Fungi. Pak. J. Bot, 38(4) : 1317 - 1322
[26] Bosah,O; C.A. Igeleke and V.I. Omorusi. 2010. In Vitro Microbial Control of Pathogenic Sclerotium rolfsii. Int. J. Agric. 12 : 474-476