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Antagonism Mechanism of Fungal Contamination Animal Feed using Phylloplane Yeasts Isolated from the Bintaro Plant (*Cerbera manghas*) Bekasi in Java, Indonesia

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A B S T R A C T

Cerbera manghas has many qualities, the leaves have bioactive constituents of insecticides, cosmetics, pesticides, medicinal plants, and antifungal activities. We investigated the diversity yeasts species associated with leaves of *C. manghans*, which is prerequisite to understand the phylloplane yeasts and their antagonistic abilities against molds from chicken feed. Based on the on similarity of colony morphology, three representative molds isolates were selected and identified. Two representative molds are capable of damage of chicken feed, are genus *Aspergillus*, and *Penicillium*. A total of 28 yeasts isolates were obtained. The yeasts were able to inhibit growth and sporulation of molds (21 isolate yeasts inhibit *Aspergillus*; 10 isolate yeasts inhibit *Penicillium*). Two antagonistic yeasts were able to inhibit growth and sporulation of molds with high zone were code T1.3.5 and T4.5.3.R.The results showed yeasts from phylloplane Bintaro can be used as agents of antagonism in the ability to inhibit mold contaminant origin animal feeds.

Introduction

*Cerbera manghans*is plants can grow in the tropical Indo-Pacific region from Seychelles to French Polynesia including Indonesia. Natural habitat is coastal and mangrove forests (mangroves). *Cerbera manghans*is known by several vernacular names, such as Bintaro, Blind Rhino, Mangga Sea, Wood Octopus, Kanyeri White (Bali), Bilutasi (NTT), Wabo (Ambon), Goro - Goro Guwae (Ternate), Madangkapo (Minangkabau), Bintan (Malay), Lambuto (Makassar), Goro - Goro (Manado) (Utami, 2010).

Cerbera manghans utilization for the people of Indonesia is used as an ornamental plant, urban greening, raw materials handicraft, botanical pesticides, and medicinal plants. (Utami, 2010). In traditionally *C. manghans* leaves used as laxatives, emetics, anti - rheumatism, sedative, anti – nociceptive, toxic activity in the central nervous system (Ahmed *et al*., 2008).

Numerous studies have highlighted about potential aspects of *C. manghans* in particular field of health and the safety of

food. Sa'diyah *et al*. (1995) reported *C. manghans* contains several compounds are secondary metabolites, such as saponins, polyphenols, terpenoids, and alkaloids. Compound *C. manghans* are polar because it contains nitrogen and compounds the phenol-soluble polar solvents and semipolar. The seeds of the Bintaro contain cerberin compounds that have the potential to Carcinogenicity (Cheenpracha *et al*., 2004; Liu *et al*., 2008).

Research of*C. manghas* only regarding the utilization of plant extracts. Kariba, 2001 reported of methanol extract of leaves of *Schizozygia coffaeoides* (*Apocynaceae*) has been investigated as a fungistatic against *Trichophyton mentagrophytes*, *Microsporum gypseum, Cladosporium cucumerinum*, and *Candida albican*s. Murniana (2011) reported the chemical compounds from the seeds of *Cerbera odollam* which is one genus with *C. manghas* for antifungal activity against test the fungus *C. albicans*. However, there no report on isolation of yeasts from *C.manghas* and there is no information on the bioactive compounds and potential antagonism of this microorganism.

The surface of the leaf (phylloplane) is habitat for yeast. The existence of phylloplane yeasts on can get carried away by soil particles and other particles from the air (Watkinson, 1995), and are carried by insects from one plant to another plant (Spencer &Spencer, 1997).

Some reports about the epiphyte diversity of yeasts derived from leaves, among others, the following: Sukmawati *et al*. (2015) reported the phylloplane yeasts isolated from the leaves of *Broussonetia papyrifera* from Bandung, Garut, Trowulan and produced 17 genera and 32 species of yeasts is composed of 11 genera (18 species) of the phylum *Ascomycota* and 6 genera (14 species) of the phylum *Basidiomycota*. Sjamsuridzal (2007) to isolate and identify the phylloplane yeasts of plants in Cikurutug, Cikaniki and mount Halimun National Park of Mount Kendeng Ciujung, West Java. There has been no report of research on the phylloplane yeasts from *C.manghas.*

Mold is one of the microorganisms that can cause contamination (Widhiastuti, 2006). Contamination can occur in a variety of organic substrates. One of the contaminants that occur are on cattle feed. Handayani & Sulistyo (2000) reported the fungus *Aspergillus* spp., *Cladosporium* spp., *Absidia* spp., *Montha* spp., *Mucor* spp., *Moniliella* spp., and *Rhizopus* spp.a well-known contaminate feed on farm poultryand is the main organism responsible for the production of mycotoxin.Mycotoxins are known to be produced by toxigenic fungi, especially under suitable culture conditions.

The effect of contamination pathogen fungi from livestock feed is mycotoxin. Mycotoxin can show a variety of biological effects on chicken such as a decrease in food intake (Merril *et al*., 2001 *in* Ahmad, 2009), decrease in immune response (Maruanovic *et al*., 1991), diminution lymphoid organs, especially the thymus (Tabbu, 2002), a decrease in the efficiency of breeding and neurotoxic (Pestka, 2007 *in* Ahmad, 2009) as well as cause the disruption of productivity (D'Mello &Macdonald, 1998). It can cause harm to the chicken food. Recently breeders used use antibiotics for reduce contamination of pathogen molds. However excessive use of antibiotics can lead to declining health in humans.

Therefore, it is necessary alternative mold prevention more effective, does not induce resistance, and safe for human health and the environment. One of the ways that can be

done is by utilizing yeasts which have the ability of antagonistic (Druvefors, 2004). The ability of yeast was produce a barrier to the growth of other microorganisms (Madigan *et al*., 2012). In this study, we isolated yeast indigenus Indonesia from Bintaro (*C.manghas*) which has potential as an agent that can inhibit the growth of mold (*Aspergillus* sp. and *Penicillium* sp.) from chicken feed.

Materials and Methods

Sample and Sampling Location

Samples of fresh leaves of *C.manghas* were retrieved from old plants in Bekasi West Java. A total sample of leaves used amounted to 16 leaves. Leaf samples were obtained from four plants of each plant in Bintaro, take 4 strands of leaves.

Isolation of Yeast Isolates

Isolation of yeasts from the fresh leaves of *C. manghas* using the method of washing method based on Sjamsuridzal *et al*. (2013). The leaves are weighed (1 g), cut into pieces and became part of the minor. Foliage washed with sterile 25 ml akuades (in the conical tube 50 ml) and vortex for 10 minutes.

Washed pieces of leaves were placed directly onto isolation colloidal chitin medium (1%) containing 1% colloidal chitin, 4.7 1% MgSO, 0.05% H_2O, 0.1%KH_2PO_4, 0.05%yeast extract, and 2% agar, in 1 liter akuades and added with 0.05 %tetracycline after sterilization. After that 0.1 ml suspension leaf leaching results *C. manghas* was inoculated directly onto 1%colloidal chitin agar medium in three replicates. Plates were incubated at room temperature (27-28°C), and after three days all single colonies were picked up using sterile toothpicks and placed into new plates to create colony libraries. The representative colonies of each morphological type were purified at least two times on yeast malt extract agar (YMA), maintained on potato dextrose agar (PDA) slants, and stored at –20°C. The cultures from this study were deposited in the Jakarta State University Culture Collection (UNJCC), Department of biology, Faculty of mathematics and natural sciences, State University of Jakarta.

Isolation of Molds Isolates from Feed Chicken

Feed chicken samples taken at a chicken farm villages of Bekasi, West Java. Sampling technique based on Shareef (2010) by the method of purposive sampling. Samples taken from two of the plot i.e. the feed shed and chicken coop. On each plot consists of 5 subplots.

Isolation of molds isolate from feed chicken samples using the method of *dilution plating* by Labuda *et al.*, 2006). The feed chicken samples are weighed (1 g), put into sterile water (9 ml) then make dilution at 10^{-4}, 10^{-5} and 10^{-6}.After that 0.1 ml suspension was inoculated directly onto PDA medium. And after seven days all single colonies were picked up using sterile toothpicks and placed into new plates to create colony libraries at a temperature of 25^{o}C-30^{o}C.

Antagonism Test Method

Antagonism test method that is carried out for early screening method based on point, by Csutak, *et al*. (1995). This antagonism test using yeast isolate from the fresh leaves of *C. manghans* and molds isolate (*Aspergillus* sp. and *Penicillium* sp.) from animal feed. Antagonism test using PDA medium. One medium PDA is is divided into four quadrants. After that yeast isolate (48 h incubation) scratch on the medium. On the central part of medium was inoculation with mold (5 d incubation). The

ability of yeasts in inhibiting the growth of mold is determined by reduced growth of mycelium on the mold. Incubation is done during 5 days at a temperature of 26-28 °C.

Results and Discussion

Isolation of Yeasts from Fresh Leaves of *C. manghans*

Thirty six yeast and mold isolates (20 yeast and 16 mold isolates) have been obtained (Table 1). Yeast isolates obtained have a difference in colors and textures. The colors and textures of yeast colonies, and their percentages are as follows: mucoid orange (25%), butyrous white (30%), butyrous blackish (20%), butyrous cream (20%), and butyrous orange (1%)on YMA after 3 days incubation at room temperature (27-28°C) (Table 2).

In this study we found variety of texture and colors of yeast isolates. It indicates that the surface of the leaves is a proper habitat for the growth of yeast. Yeast can grow on the surface of the leaves have a characteristic morphology dominated with texture butyrous (75%) and with variety pigment. Fonseca and Inacio (2006) reported the yeast isolates from phylloplane that have been dominated by the pigments such as beige, orange, blackish, cream and pink.Dufosse (2006) reported the yeasts can produce pigments, among others: pink (astaxanthin), black (melanin), orange (torularhodin) and red (β carotene). The pigment on the yeasts serve to protect yeasts of unfavorable environmental conditions such as light intensity and ultraviolet radiation (Luciana *et al*., 1998).

Isolation of Molds from Animal Feed Chicken

Macroscopic and Microscopic Observation of Mold Isolates

In this study we were obtained two genera of mold from cattle feed in Bekasi, West Java. Genera were *Penicillium* and *Aspergillus*. Observations of macroscopic and microscopic morphology have been done in the medium Malt Extract Agar (MEA) and incubated at a temperature of 27-28 °C.

Observations colony of the genus *Aspergillus* sp. at the age of five days in medium MEA show cedar green with the edge of the ivory, the texture, the granules have exudate drops, growing zone, zoning and radial furrow, the color behind the colony of brown. On microscopic observations, the genus *Aspergillus* sp. This genus contained conidiophore, vesicles, and head of conidia.

Colony morphology of the genus *Aspergillus* sp. in accordance with the description by Klich (2002), namely, molds of the genus *Aspergillus* at 7 days in medium Malt Extract Agar (MEA) has a granular texture, mycelium is white, head of the conidia of the old Brown to black, and greyish-yellow colonies rather than too colorless. Based on observations colonies of *Aspergillus*sp. microscopically: the hypha has septa, conidiophore on the ends rounded form vesicles, metula and phialid (Fig.1).

Observations colony of the genus *Penicillium* sp. shows the colored mold colony night green with colored edges white, texture granule, has a growing zone, exudate drop and zone, has no radial furrow, the color behind the colony brunt ochre. On microscopic observations, a colony of *Penicillium* sp. has a characteristic that is conidiophore on the ends of branches, has the metula and phialid as a place of conidia which vary from round, semi rounded and ovoid (Fig. 2).

Colony morphology in the genus *Penicillium* sp. in accordance with the description according to Gams, *et al*. (1987)

a colony of *Penicillium* sp. is usually white, sometimes green, most have conidiophore. Single Conidiophore (mononematus) or compound (synematous), consisting of a single trunk dividing some of the phialid (simple/monoverticillata). Phialid is the structure that sustains conidia, cylindrical drier basal narrowing in neck, or lanceolate (the most basal part embedded at the end of the tip). Conidia long chain-shaped, elliptical, globular, transparent, or greenish, with smooth or wavy walls (Gandjar *et al*. 1984).

Screening Antagonism of Original Phylloplane Yeast Isolates *C. manghas* with *Aspergillus* sp. and *Penicillium* sp

Screening antagonism of original phylloplane yeast isolates *C. manghas* with *Aspergillus* sp. and *Penicillium* sp. using PDA medium incubation 3 days at a temperature of 27-28 ° C. The results showed of the antagonism of 20 original yeast isolates of *C. manghas* against mold, *Aspergillus* sp. and *Penicillium* sp. showed positive results. A total 100% isolate yeasts capable of inhibiting the mold *Aspergillus* sp. with average inhibition (0.85--6.31) mm and 40% isolate yeasts are able to inhibit mold *Penicillium* spwith average inhibition (0.79--6.91) (Fig 3-4).The distance of the largest shown by the inhibition of yeast isolates code T 1.3.5 with a diameter inhibition of 6.31 mm on the mold *Aspergillus* sp. and yeast isolates code T 4.5.3. R too *Penicillium* sp. mold with diameter inhibition 6.91 mm (Table 3-4).

We found that the phylloplane yeast isolates of *C. manghas* were be potential antagonism mechanism inhibited mold contamination especially *Aspergillus* sp. and *Penicillium* sp. Interaction antagonism between yeast isolates against *Aspergillus* sp. and *Penicillium* sp.thought to be competition of nutrient and living space. We suggest too this antagonism mechanism involves chitinase enzyme. This yeast isolates were produced chitinase enzyme due to we used colloidal medium (1%) for isolation. The yeast isolates were can growed on colloidal chitin medium supposedly had the ability to produce the chitinase enzyme. Chitinase enzyme on yeast extracellular enzymes is to acquire nutrition and parasitism (Patil *et al*. 2000). Yeast has the ability of antagonistic towards pathogenic mold growth through the mechanism of competition against space and nutrients on their substrates. The inherent yeasts hypha mold then secrete enzymes degradation cell wall mold, i.e. chitinase enzyme (Ge *et al*., 2010). Yeast species can produce chitinase, i.e. *Saccharomyces cerevisiae* (Ahmad, 2007); *Metschnikowia fruticola* (Banani *et al*. 2015); *Pichia guilliermondii* K14 and *Bulleromyces albus* K7 (Preechasuth *et al*. 2015).

The results showed yeast isolate from phylloplane of *C. manghans* have activity antagonism. Mechanisms antagonism with mold mycelium growth reduction after grown along with yeasts. Interaction antagonism between yeast origin teak leaves with the mold *Aspergillus* sp., and *Penicillium* sp. and thought to be the mechanism of nutrient competition and living space. These result supported with Spadaro (2003) reported testing antagonism between yeast *Metschnikowia pulcherrima* with different cell number (10^6until 10^8 CFU/ml) mold against *Bacillus cinerea.*

Table.1 Mold and Yeast Isolates Obtained from Leaves of *C. manghas using* Colloidal Chitin Medium Isolation Agar (1%) for 3 Days Incubation Temperature 30 ^{0C}

Tree	Code of isolates	Fungi	Observation on the morphology
1	T1.1.1	Mold	Filament, greenish white
	T1.3.1	Mold	Filament, white
	T1.1.2	Yeast	Butyrous, white
	T1.3.1.1	Yeast	Mucoid, orange
	T1.3.2	Yeast	Butyrous, cream
	T1.3.3	Yeast	Butyrous, cream
	T1.3.5	Yeast	Mucoid, orange
	T1.2.1	Yeast	Mucoid, orange
	T1.3.4	Yeast	Mucoid, orange
2	T2.1.1	Mold	Filament, white
	T2.4.1	Mold	Filament, white
	T2.4.2	Mold	Filament, white
	T2.2.1R	Yeast	Butyrous, white
	T2.2.1	Yeast	Butyrous, blackish white
	T2.2.3	Yeast	Butyrous, white
	T.2.2.2	Yeast	Butyrous, cream
	T.2.3.1R	Yeast	Butyrous, cream
	T2.5.1	Yeast	Butyrous, white
3	T3.3.1	Mold	Filament, white
	T3.3.2	Mold	Filament, white
	T3.5.1	Mold	Filament, white
	T3.1.1	Yeast	Butyrous, blackish
	T3.1.2	Yeast	Mucoid, orange
	T3.1.3	Yeast	Butyrous, blackish
	T3.1.4	Yeast	Butyrous, white
4	T4.1.1	Mold	Filament, white
	T4.1.2	Mold	Filament, white
	T4.2.1	Mold	Filament, white
	T4.2.2	Mold	Filament, white
	T4.4.1	Mold	Filament, white
	T4.4.2	Mold	Filament, white
	T4.5.1	Mold	Filament, white
	T4.5.2	Mold	Filament, white
	T4.5.3R	Yeast	Butyrous, white
	T4.5.1R	Yeast	Butyrous, orange
	T4.2.2R	Yeast	Butyrous,blackish

Table.2 Morphology Colony of Yeast Isolates Obtained from Leaves of *C. manghas using* YMA Medium for 3 Days Incubation Temperature 30 ^{0C}

Tree	Total number of isolates	Mucoid, orange	Butyrous, white	Butyrous, blackish	Butyrous, cream	Butyrous, orange
1	7	4	1		2	
2	6		3	1	2	
3	4	1	1	2		
4	3		1	1		1
Total	20	5 (25%)	6 (30%)	4 (20%)	4 (20%)	1 (5%)

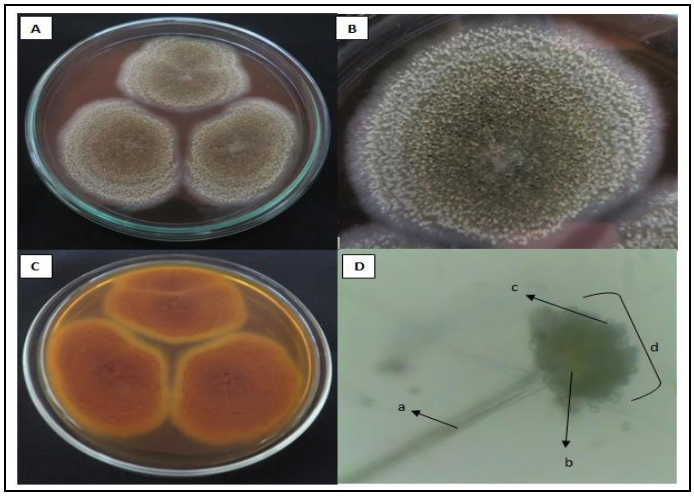
Table.3 Inhibitation Yeasts Isolates from *C. manghans* with the Mold *Aspergillus* sp.on PDA Medium Incubation3 Day at Temperature 27-28 ° C

Yeast isolates	Inhibitation of yeast isolates (1)	Inhibitation of yeast isolates (2)	Average of zone inhibitation	Antagonism Yeast Vs *Aspergillus*
T1.1.2	3.73	3.71	3.72	+ antagonism
T1.2.1	5.21	5.23	5.22	+ antagonism
T1.3.1.1	6.02	6.01	6.02	+ antagonism
T1.3.2	2.55	2.57	2.56	+ antagonism
T1.3.3	2.83	2.82	2.83	+ antagonism
T1.3.4	0.84	0.85	0.85	+ antagonism
T1.3.5	**6.28**	**6.33**	**6.31**	**+ antagonism**
T2.2.1	2.04	2.14	2.09	+ antagonism
T2.2.1.R	1.57	1.55	1.56	+ antagonism
T2.2.2	2.83	2.82	2.83	+ antagonism
T2.2.3	4.84	4.82	4.83	+ antagonism
T2.3.1R	5.11	5.12	5.12	+ antagonism
T2.5.1	4.29	4.28	4.29	+ antagonism
T3.1.1	1.22	1.23	1.23	+ antagonism
T3.1.2	1.62	1.63	1.63	+ antagonism
T3.1.3	1.83	1.9	1.87	+ antagonism
T3.1.4	5.88	5.89	5.89	+ antagonism
T4.2.2.R	1.41	1.4	1.41	+ antagonism
T4.5.1.R	3.32	3.31	3.32	+ antagonism
T4.5.3.R	3.83	3.82	3.83	+ antagonism

Table.4 Inhibitation Yeasts Isolates from *C. manghans* with the Mold *Penicillium* sp.on PDA Medium Incubation3 Day at Temperature 27-28 ° C

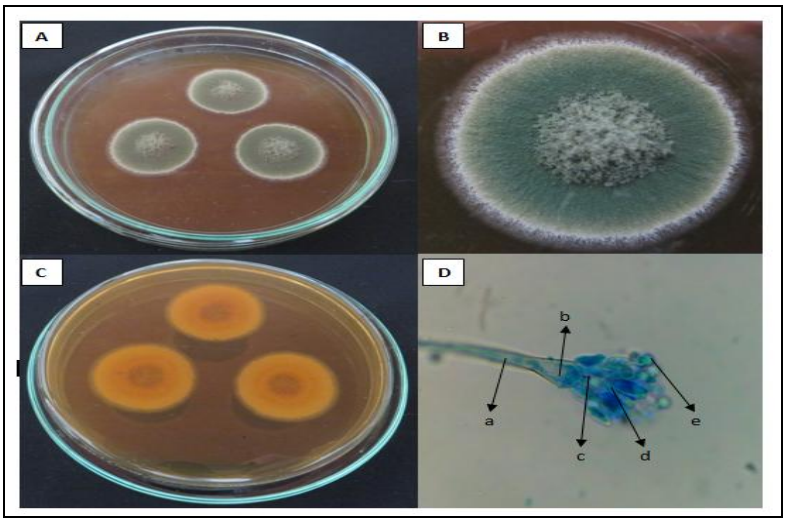
Yeast isolates	Inhibitation of yeast isolates (1)	Inhibitation of yeast isolates (2)	Average of zone inhibitation	Antagonism Yeast Vs *Penicillium* sp.
T1.1.2	4.22	4.32	4.27	+ antagonism
T1.2.1	-	-	-	-
T1.3.1.1	-	-	-	-
T1.3.2	3.23	3.33	3.28	+ antagonism
T1.3.3	-	-	-	-
T1.3.4	-	-	-	-
T1.3.5	1.15	1.1	1.13	+ antagonism
T2.2.1	1.07	1.08	1.08	+ antagonism
T2.2.1.R	0.78	0.79	0.79	+ antagonism
T2.2.2	-	-	-	-
T2.2.3	1.4	1.42	1.41	+ antagonism
T2.3.1R	-	-	-	-
T2.5.1	-	-	-	-
T3.1.1	-	-	-	-
T3.1.2	-	-	-	-
T3.1.3	4.92	4.93	4.93	+ antagonism
T3.1.4	-	-	-	-
T4.2.2.R	-	-	-	-
T4.5.1.R	-	-	-	-
T4.5.3.R	**6.92**	**6.89**	**6.91**	**+ antagonism**

Fig.1 Observations Colony of Mold *Aspergillus sp*. in medium MEA was 5 Days at a Temperature of 27-28°C



(A). Colonies of mold (B). Macroscopic and microscopic molds, (C). Mold colonies, (D) the microscopic mold magnification 1000 x (a conidiophore, b conidia, c vesicles, d head conidia)

Fig.2 Colonies of Mold Observations *Penicillium* sp. in Medium MEA was 5 Days at a Temperature of 27-28°C



(A). Colonies of mold (B). Macroscopic and microscopic molds, (C). Mold colonies, (D) the microscopic mold magnification 1000 x (a conidiophore, b vesicle, c metula, d phialid, and econidia)

Fig.3 Testing of Antagonism Phylloplane Yeasts Isolates from *C. manghans* with the Mold *Aspergillus* sp. on PDA Medium Incubation3 Day at Temperature27-28 ˚C

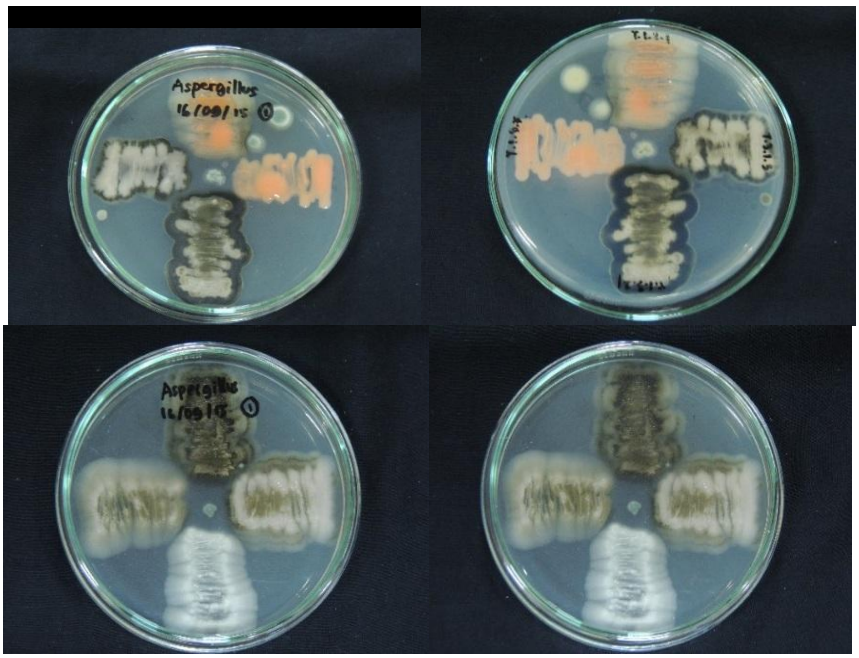
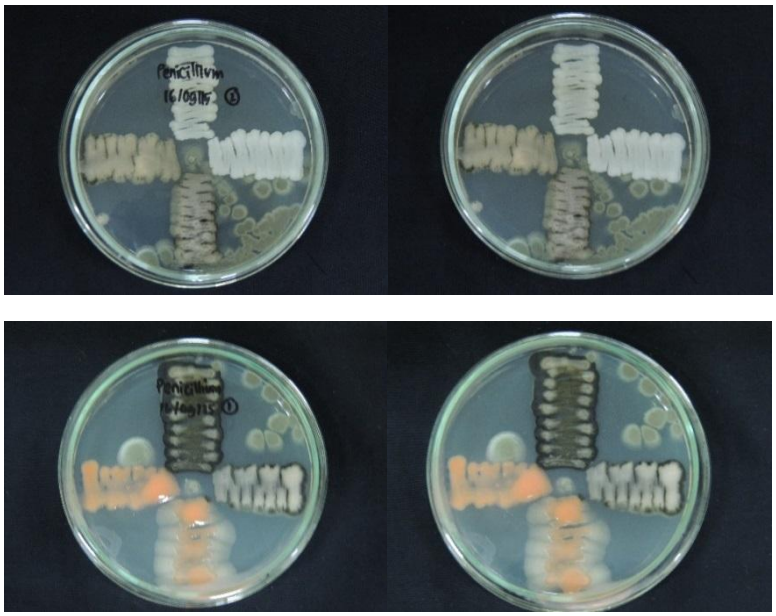


Fig.4 Testing of Antagonism Phylloplane Yeasts Isolates from *C. manghans* with the Mold *Penicillium* sp. on PDA Medium Incubation3 Day at Temperature 27-28°C



Metschnikowia pulcherrima with cell number 10^8 CFU/ml was capable inhibiting the growth of *B. cinerea* mold better than *M. pulcherrima* with cell number 10^6 CFU/ml and 10^7 CFU/ml. *Metschnikowia pulcherrima* is assumed to use nutrients more than mold. The increase in the population of yeast can cause nutrient deficiencies. The deficiencies nutrient can result mold germination spore become obstructed.

In conclusion, two antagonistic yeasts were able to inhibit growth and sporulation of moulds, i.e. *Aspergillus* sp., and *Penicillium* sp. These yeast isolate with code T4.5.3.R and T1.3.5 have potential as agent antagonisms which was in reducing mycelium growth with mechanism of nutrient competition and living space.

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