# INTERACTIONS OF *PHLEBOPUS SPONGIOSUS* WITH SEVERAL SOIL FUNGI AND ANTIBACTERIAL ACTIVITY OF ITS CULTURE BROTH

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(Received: April 10, 2019; Revised: May 13, 2019; Accepted: May 21, 2019)

**ABSTRACT**

An edible ectomycorrhizal fungus *Phlebopus spongiosus* have been found in pomelo orchards (*Citrus maxima*). The culture broth of *Ph. spongiosus* became darker after ca. 3 weeks of inoculation. The dry production of culture broth extract (culture extract) was 0.30 ± 0.09 g per culture (20 ml broth in a 50 ml flask). Both 5% and 10% solution of the culture extract shows the antibacterial activities on growth of all tested Gram positive bacteria *Bacillus subtilis*, *Bacillus thuringiensis* and a Gram negative bacterium *Gluconobacter oxydans* but not on the other Gram negative bacteria *Escherichia coli* and *Asaia bogorensis*. On PDA plates, *Ph. spongiosus* showed the inhibition on the growth of soil fungi *Penicillium citrinum* and *Aspergillus niger*, whereas it was invaded by that of a mycoparasite *Trichoderma viride*. Further studies on physiological and ecological characteristics and principal components for the activities of culture exudates in laboratory is necessary to find the applicable profits from this fungus.

**Keywords:** Bioactivity; Culture broth; Ectomycorrhizal mushroom; *In vitro* interaction; Mycelium.

## 1. Introduction

Fungi, especially saprotrophs, also work as a decomposer for recycling remainder of organisms and supplying nutrient again to other living organisms (Stamets, 2005). They also share the same habitat inside the mycobiota in soil and interact with each other, other microbes, small insects and plants. From the ecological viewpoint, any biological interaction in nature should be place into several radiate interaction with other organisms which sharing the same habitat. Largely, interaction physiology has been studies using plate culture in which the two species, usually, or exacting substances of a species and another species are opposed for assessing outcome (Cooke and Whipps, 1993). The interaction between a fungus – a fungus, with or without of other organisms, on both *in situ* and *in vitro* has been paid the attention by mycologists. By the sequencing publishes of Frankland et al (1982), Cooke and Rayner (1984), and Woodland and Boddy (2008), several patterns and definitions of fungus – fungus interaction were established.

Besides, fungi look like "the amazing

chemical factories" (Wainwright 2010) which is used to produce a range of commercial products from clothes dyes to life-saving drugs (antibiotic). In a long time from ancient, mushrooms have been considered to have medicinal value. Fungi were assumed to use firstly as "a magic drugs" or a hallucinogen, not as food, from 7000-9000 years ago in Sahara Desert areas (Rutter, 2010). Then in Asia, some fungi were used as a superior healthy supplementary by ancient Chinese in ca. 2000 years ago such as *Ganoderma lucidum* (Chang and Miles, 2004). About 700 species of fungi were known medicinal properties and about 1800 species of mushrooms were reckoned to potential medicinal attributes (Chang and Miles, 2004).

The fungal values usually derive from their exudates or their inside chemical substances. Most are the intra-basidiomata compounds such as cordycepin in *Cordyceps sinnesis*, garnoderic acid in *Ganoderma lucidum* or a recent compound ergothioneine in many edible mushrooms (Ohshima, 2011). However, some are the mycelial compounds as a case of krestin (PSK) in *Tramates versicolor*; mycelial culture broth as schizophyllan in *Schizophyllum commune* and some from all part of the fungus as case of *Ganoderma lucidum*. The example about pharmaceutical values from different origins of some common mushrooms was shown in Table 1 following Chang and Miles (2004).

**Table 1**

The example about pharmaceutical values from different origins of some common mushrooms (Chang and Miles 2004)

| Species | Cultivated fruiting body | Cultivated mycelium | Culture broth |
|---|---|---|---|
| *Agaricus blazei* | ++ | + | + |
| *Flammulina velutipes* | ++ | + | — |
| *Ganoderma lucidum* | ++ | + | + |
| *Grifola frondosa* | + | — | — |
| *Hericium erinaceus* | ++ | + | — |
| *Lentinula edodes* | ++ | + | + |
| *Schizophyllum commune* | — | — | ++ |
| *Tramates versicolor* | + + | + | — |
| *Volvariella volvacea* | + | — | — |

*Note:*++ High bioactive effects; + moderate bioactive effects; - not available

*Phlebopus spongiosus* is a terrestrial, edible, ectomycorrhizal fungus and most of its basidiomata have been found in pomelo orchards (*Citrus maxima*), appearing around the bases of the plants (Pham et al 2012a, b). In our study, this fungus was investigated several physiological and ecological characteristics in laboratory as the fundamental data. Some of applicable profits from these characteristics would be also recorded from these data.

During cultivation of *Ph. spongiosus*, its colonies altered several media, both broth and agar, to darker by the mycelial exudate. The culture broth is seemed to be a suitable candidate applicable substance(s) from this edible fungus. The extract of culture broth of

*Ph. spongiosus* in Ohta medium was collected and applied to test antibacterial activity. For investigating the relationship between this fungus and other soil fungi, the *in vitro* interactions of colonies of ectomycorrhizal fungus *Ph. spongiosus* with some common soil fungi *Aspergillus niger*, *Penicillium citrinum* and *Trichoderma viride* were studies from view of ecology.

## 2. Materials and Methods

### *Organisms*

A mycelial strain of *Ph. spongiosus* was isolated from a paratype basidioma (specimen voucher CBM FB-38670 deposited in Natural History Museum and Institute, Chiba, Japan) on MMN (Modified Melin and Norkrans) medium (Marx 1969) and maintained on PDA medium [4g potato extract (Sigma Aldrich, MO, USA), 20g glucose (Wako, Tokyo, Japan), 15g agar (Difco, MI, USA) and filling to 1000 ml with distilled water] at 20±1°C, in darkness.

The bacterial strains were from National Biological Resource Center, National Institute of Technology and Evaluation (NITE – NBRC), Japan (Table 2). *Bacillus subtilis* subsp. *subtilis*, *Bacillus thuringiensis* and *Escherichia coli* were maintained on 802 broth (NBRC, NBRC website) [10g peptone (Difco, MI, USA), 2g yeast extract (Difco, MI, USA), 1g $MgSO_4.7H_2O$ (Wako, Tokyo, Japan) and filling to 1000ml with distilled water]. *Gluconobacter oxydans* and *Asaia bogorensis* were maintained on 804 broth (NBRC, NBRC website) [5g peptone (Difco, MI, USA), 5g yeast extract (Difco, MI, USA), 5g glucose (Wako, Tokyo, Japan), 1g $MgSO_4.7H_2O$ (Wako, Tokyo, Japan) and filling to 1000 ml with distilled water]. All were incubated at 30±1ºC in darkness.

**Table 2**

Bacterial strains used in antibacterial tests.

| ***Bacterial species*** | ***Strain number*** | ***Gram stain*** |
|---|---|---|
| *Escherichia coli* | NBRC 3301 | Negative |
| *Gluconobacter oxydans* | NBRC 14819 | Negative |
| *Asaia bogorensis* | NBRC 16594 | Negative |
| *Bacillus subtilis* subsp. *subtilis* | NBRC 13719 | Positive |
| *Bacillus thuringiensis* | NBRC 101235 | Positive |

The mycelial strain of *A. niger* (IFM55890), *P. citrinum* (IFM40616) and *T. viride* (IFM40938) were from the collection of Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Japan. All were also maintained on PDA medium at 20±1°C, in darkness.

### *In vitro interactions*

The *in vitro* interaction was examined on PDA plates (90 mm in diameter, 10 mm in high). Interaction between *Ph. spongiosus* with each mold was examined by 5 PDA plates.

A mycelial disk 4 mm in diameter of *Ph. spongiosus* was bored out using a cork borer, from sub-peripheral region of colony grown on PDA plates, and aseptically transferred to the side of new PDA plate. All inoculated plates were incubated at 25 ± 1ºC in darkness. After 72 hours, each mold (*A. niger*/*P. citrinum*/*T. viride*) was spot inoculated onto the opposite side of the plate. The distance between two inocula is about 40-50 mm. All plates were again incubated at 25 ± 1ºC, in darkness.

After several days, the plates were observed for check the interaction. After two colonies contacting together, all plates were

also kept a long time until the pattern of fungal interaction became stable. The patterns of interaction were described following Cooke and Rayner (1984).

***Culture broth extracting***

A mycelial disk 4 mm in diameter of *Ph. spongiosus* was bored out using a cork borer, from sub-peripheral regions of colonies grown on PDA plate, and aseptically transferred into 20ml of Ohta broth (Ohta 1990) in a 50ml conical flask (Pyrex Iwaki, Tokyo, Japan), corked with a sterilized Silicosen plug (Shin-Etsu Polymer, Tokyo, Japan).

After about 6 months, the culture broth was aseptically harvested by filtering two times with glass filter (17G3, Pyrex Iwaki, Tokyo, Japan) and membrane filter (pore size 0.2µm, mixed cellulose ester membrane, Advantec, Tokyo, Japan). Then, the culture (20 ml of culture broth in a 50ml flask) broth was condensed by freeze drying in sterile condition for collecting culture extract. The extract of each culture was collected and weighted.

***Antibacterial activity***

For each bacterium, 5 agar plates of maintain medium were prepared to test the antibacterial activity of fungal exudates by diffusion method. The stainless steel peni-cylinders (peni-cylinders, BioLogis Inc., Virginia, USA) of 6 mm in the inner diameter, 10 mm high were used for diffusing the fungal extract broth on the surface of agar plates.

Each 200 µl of bacterial suspension at a density of ca. $3x10^6$ cells/ml was aseptically spread on the surface of a agar plate by a glass spreader; the peni-cylinder was then placed in the center of each plate; a 200µl solution was poured into the peni-cylinder; then it was incubated at 30±1ºC in darkness, and observed after 24 hours. For the preliminary investigation, the water solutions of broth extract used in this study were 5% (50µg extract/ml) and 10% (100µg extract/ml). The solution of Ohta broth (1x Ohta broth) and 10 time concentration of Ohta broth (10x Ohta broth) were used as control.

**3. Results**

***In vitro interactions***

After about 48 hours of inoculation, the mycelia of *Ph. spongiosus* started to grow from inocula.

After about 3-4 days of inoculation, the colonies of *T. viride* were expanded and contacted with those of *Ph. spongiosus*. Then, the mycelia of *T. viride* infiltrated and invaded those of *Ph. spongiosus* (Fig. 1a). Finally, colonies of *T. viride* completely covered those of *Ph. spongiosus* after 2-3 weeks (Fig. 2b).

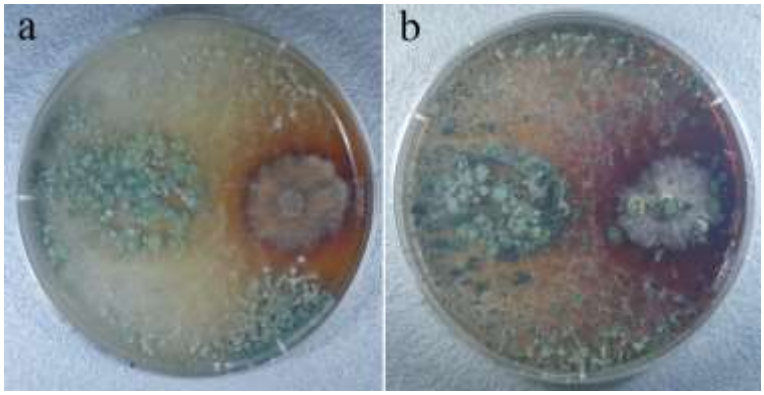


**Figure 1**. Interaction between *Phlebopus spongiosus* and *Trichoderma viride*.

a: The mycelium of *T. viride* infiltrated and invaded that of *Ph. spongiosus* after 5 days of inoculation of the mold;
b: Colony of *T. viride* completely covered that of *Ph. spongiosus* after 2-3 weeks of inoculation of mold.

In cases of *P. citrinum* and *A. niger*, their colonies were expanded and contacted with those of *Ph. spongiosus* after ca. 1 week. Both of their colony expansions were barred by colonies of *Ph. spongiosus* as deadlock interaction (Figs. 2a, 3a). These deadlock interactions did not change at 10 weeks of inoculation of mold. The interaction between *Ph. spongiosus* and *A. niger* was the deadlock antagonism and that between *Ph spongiosus* and *P. citrinum* was the deadlock competition.

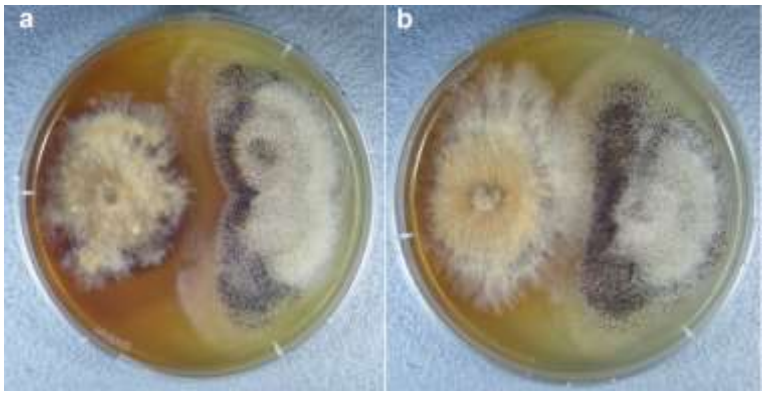


**Figure 2**. Interaction between *Phlebopus spongiosus* and *Aspergillus niger*. a: The deadlock antagonism interaction after 6 weeks of incubation between *A. niger* and *P. spongiosus* and had not changed until 10 weeks; b: The mycelium of *Ph. spongiosus* invaded and covered that of *A. niger* after 3 weeks of incubation and had not changed until 10 weeks.

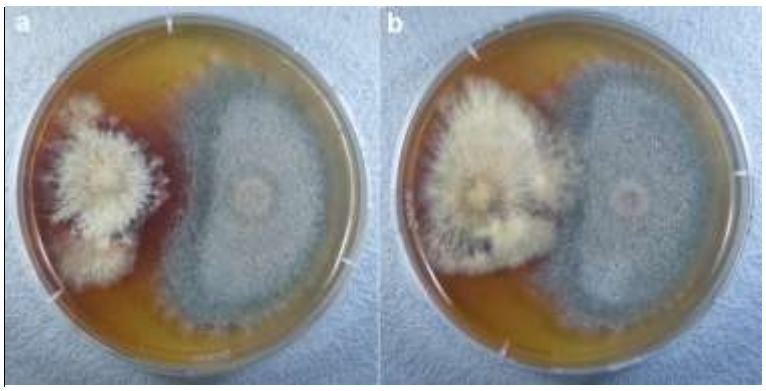


**Figure 3**. Interaction between *Phlebopus spongiosus* and *Penicillium citrinum*. a: The deadlock competition interaction after 6 weeks of incubation between *P. citrinum* and *Ph. spongiosus* and had not changed until 10 weeks; b: The mycelium of *Ph. spongiosus* invaded and covered that of *P. citrinum* after 3 weeks of incubation and had not changed until 10 weeks.

However, after 2-3 weeks, colonies of *Ph. spongiosus* in some plates invaded and covered those of *A. niger* and *P. citrinum* (Figs. 2b, 3b). These invasion interactions were also not change at 10 weeks of inoculation of mold.

***Culture broth extract***

After a few days of inoculation, mycelia started to expand from inoculum in all broths. Fungal colonies secreted exudates which blackened the medium after ca. 3 weeks. The culture broth changed to pure black after about 4 months (Figs. 4b, 5a). In a long time incubation, *Ph. spongiosus* formed primodia in both agar plate and broth, especially in Ohta medium (Fig. 4).

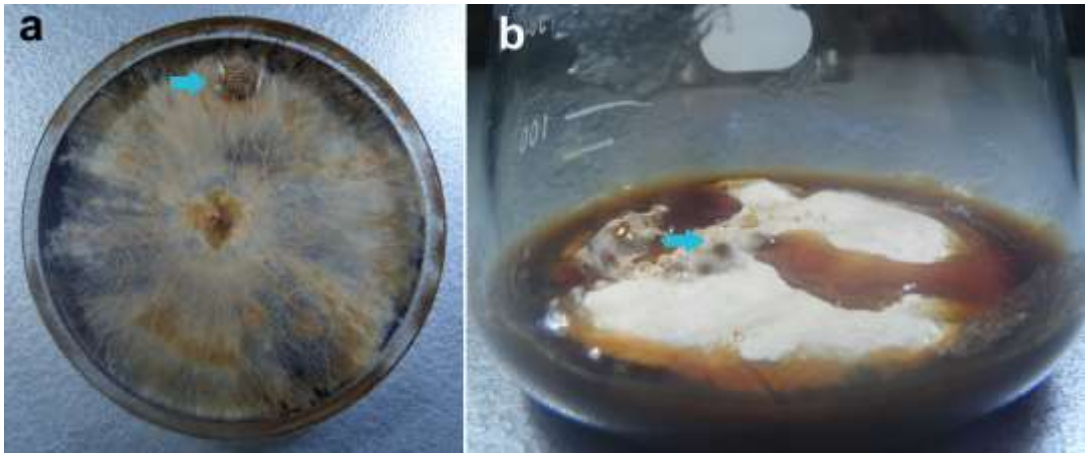


**Figure 4.** Cultivation *of Phlebopus spongiosus* in Ohta medium. Blue arrows indicated primodia.

a: On Ohta plates. b: In Ohta broth.

The culture broth extract (Fig. 5b) production was 0.30 ± 0.09 g per 20 ml culture broth. This extract was pure black, glutinous and soluble in water but insoluble in alcohol 95%.

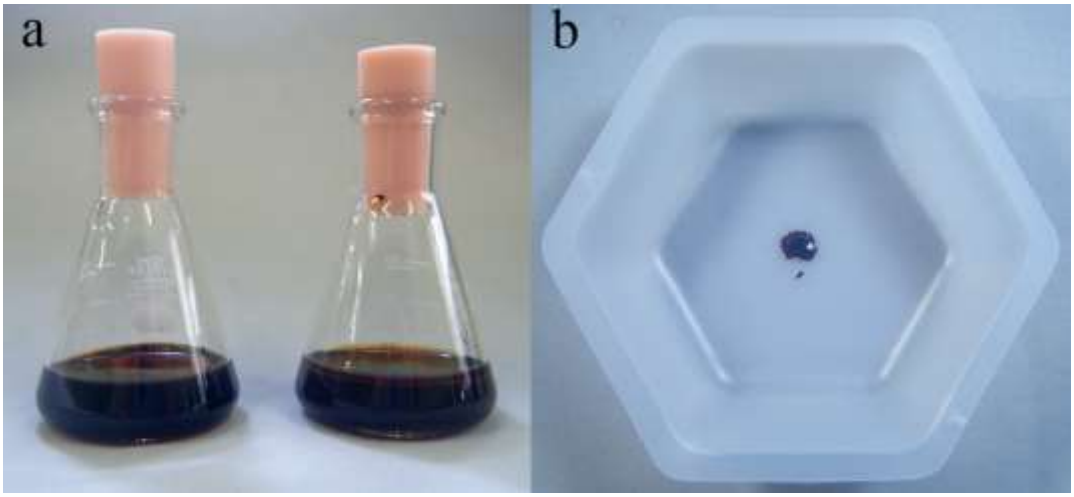


**Figure 5**. Culture broth and broth extract of *Phlebopus spongiosus*.

a: Fungal colonies of *Ph. spongiosus* blacked Ohta medium after 4 moths of inoculation.

b: Extract of culture broth from *Ph. spongiosus* culture on Ohta medium.

***Antibacterial activity***

The solutions of 1x Ohta broth and 10x Ohta broth have no effect in growth of all bacteria. However, these solutions of Ohta broth changed the pattern of *Bacillus* colonies, especially on *B. subtilis*, to more transparence comparing with normal pattern (Fig. 6). The 5% and 10% solutions of broth extract have no effect on the growth of Gram negative *E. coli* and *A. bogorensis* (Fig. 7). The 5% and 10% solutions of broth extract show the inhibition on growth of Gram positive *B. thuringiensis, B. subtilis* and Gram negative *G. oxydans* (Figs. 6, 7). The clear zone diameters in all inhibited effects are about 1-2 cm.

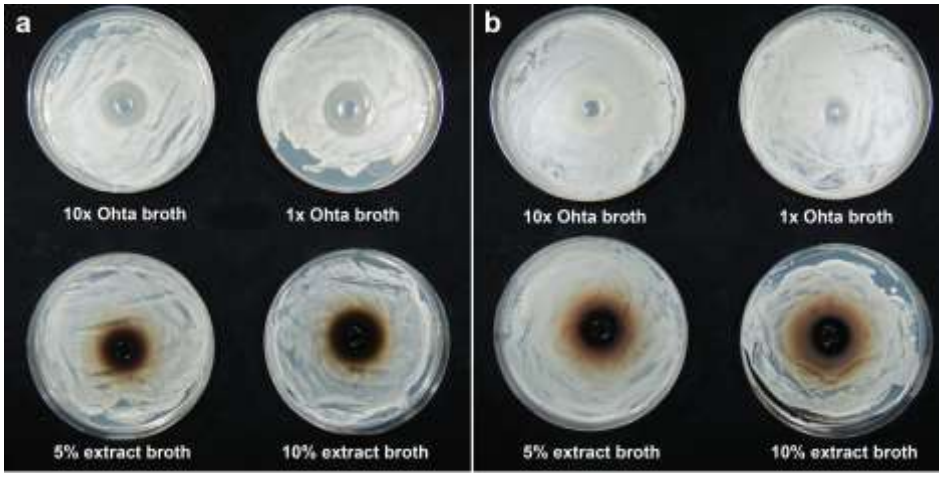


**Figure 6**. Antibacterial activities of fungal culture broth extract in Gram positive bacteria. The inside circle is derived from a contact of peni-cylinder on surface of plate; the outer circle indicates the clear zone. a: The inhibited effect on the growth of *Bacillus subtilis*. b: The inhibited effect on the growth of *Bacillus thuringiensis.*

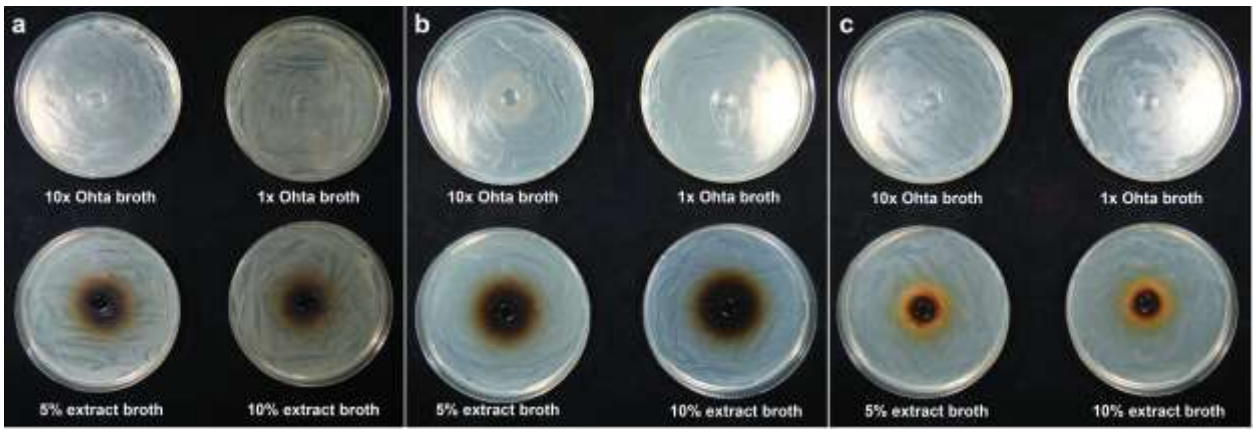


**Figure 7**. Antibacterial activities of fungal culture broth extract in Gram negative bacteria. The inside circle is derived from a contact of peni-cylinder on surface of plate; the outer circle indicates the clear zone. a: No effect on the growth of *Asaia bogorensis*. b: No effect on the growth of *Escherichia coli.* c: The inhibited effect on growth of *Gluconobacter oxydans*.

**4. Discussion**

Colonies of *Ph. spongiosus* were invaded and covered by those of *T. viride.* It should be derived from the property of mycoparasite of *T. viride* (Harman and Kubicek, 1998). It would be a rare phenomenon in edible mushrooms, i.e. colonies of *Ph. spongiosus* inhibited the expansion of *A. niger* colonies and *P. citrinum* colonies, and invaded colonies of both molds. The inhibition potential of *Ph. spongiosus* against *A. niger* and *P. citrinum* is suspected to be derived from the mycelial excretions which could have antimicrobial property. The various patterns presented in the interaction of *Ph. spongiosus* with *A. niger* and *P. citrinum* was ambiguous.

The inhibit potential of *Ph. spongiosus* on soil molds opened the applicability for using it as pathogenic antagonism. Combining with its ectomycorrhizal potential in *Citrus* spp., this fungus can be applied to increase harvest of several citrus crops in tropical areas. However, this study should be expanded to investigate interaction between *Ph. spongiosus* and several other plant pathogenic fungi in soil. Moreover, the further studies about the tri-partitions or

also tetra-partitions among ectomycorrhizal fungus *Ph. spongiosus*, its host plants, plant pathogenic fungi in soil and common soil fungi should be attended for clarifying multi-interaction inside mycorrhizosphere.

The culture broth of *Ph. spongiosus* presented the antibacterial activity not only on $Gr^+$ bacteria *Bacillus* spp. but also on $Gr^-$ bacteria *G. oxydans*. The no effect in control tests, even 10x Ohta broth, clearly indicated that the antibacterial activities were derived from the excretion of *Ph. spongiosus* in stock culture. However, the antibacterial activities looks like a weak effect with a small clear zone. It is suggested those activities should be from fungal compounds included in culture broth. There are many records about antibacterial activity of fungal extraction from mycelium/sporocarp/culture broth. Most of them are derived from extract of sporocarps or mycelium. Some are from compounds in sporocarps such as illudin S from *Omphalotus japonicus* syn. *Pleurotus japonicus* and *Clitocybe illuden* (Hara et al., 1987), and cordycepin from *Cordyceps* spp. (Sentenac et al., 1968). Some are from the extract of vegetative mycelia as study of Sasek and Musilek (1967) on several ectomycorrhizal fungi but only 4/16 species in this study showed the antibacterial activities. Moreover, the investigation of Alves et al. (2013) showed that phenolic compounds in wild mushrooms had antibacterial activy. In *Ph. portentosus* and *Ph. colosus*, phenolic compounds were determined (Kaewnarin et al., 2016, Liaotracoon and Liaotracoon, 2018). Therefore, exudates of *Ph. spongiosus* mycelia may be contain phenolic compounds. These compounds might cause the black colour of the cultured medium after ca. 3 weeks.

**5. Conclusion**

*Ph. spongiosus* colonies were infiltrated and invaded by the mycelia of *T. viride* which is a mycoparasitic fungus. However, this ectomycorrhizal fungus had the deadlock antagonism with a soil fungus *A. niger* and the deadlock competition with another soil fungus *P. citrinum*. Further field studies are required to clarify the interactions of *Ph. spongiosus* in mycorrhizophere of citrus with other organisms such as arbuscular mycorrhizae on *Citrus* spp., several harmful/profitable soil-born molds, soil fauna (including root aphids), and rhizosphere bacteria (including *Bacillus* spp.). The screening of antibacterial and antifungal activities of *Ph. spongiosus* under in vitro ectomycorrhization is necessary to be conducted in future.

The biological activity of broth culture of *Ph. spongiosus* is another conspicuous characteristic. Culture broth of this fungus showed the antibacterial activity on growth of both Gram positive and negative bacteria. The edibleness and antibacterial activity remarked this fungus to be a candidate for pharmaceutical application. However, the additional research is necessary for determining principal components for the activities of culture exudates■

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