

## Effects of Co-culturing with Live and Autoclaved *Bacillus subtilis* on Antagonistic Activity of Marine Fungi against Plant Pathogens

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### Abstract

The co-culturing of the fungi with *Bacillus subtilis* resulted in increased antifungal activity of *Eurotium chevalieri* and *Emericella foveolata* extracts but decreased the effects of *Talaromyces tratensis* and *T. stipitatus* extracts. Meanwhile, the co-culturing of marine-derived fungi with autoclaved *B. subtilis* resulted in decreased antifungal activity of *E. foveolata*, *T. stipitatus* and *T. tratensis* but slightly increased activity of *E. chevalieri*. A significant effect of the co-culture of *E. chevalieri* and *B. subtilis* resulted in an increased antifungal activity compared to the axenic cultures with complete inhibition of mycelial growth of all the plant pathogens tested, whereas the extract from fungus alone displayed percentage inhibition ranging from 33.11-100% at 10 g L<sup>-1</sup> concentration. Overall, the effect of co-culturing with live or autoclaved *B. subtilis* had positive, negative or no effect on the antifungal activities of the tested marine-derive fungi against plant pathogens *in vitro*.

**Keywords:** Microbial co-cultivation, dual induction, fungal metabolites, antagonistic activity, plant pathogens

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## INTRODUCTION

Microorganisms have been proved to be one of most important sources for bioactive metabolites useful for pharmaceutical, industrial and agrochemical applications (Wakefield et al., 2017). The compounds produced by microorganisms in an interaction known as antibiosis are a defense mechanism against other microorganisms competing for space, nutrients, and other resources in natural habitats (Netzker et al., 2015; Oppong-Danquah et al., 2018). In a microbial environment, microorganisms living in close proximity interact with each other via chemical communication pathways resulting in mutualistic, synergistic or antagonistic interactions (Shtark et al., 2012; Ghorchiani et al., 2018; Tomm et al., 2019). Many reports of antibiosis have been recorded in which antagonist microorganisms exert antagonistic actions on plant pathogens (Zhang et al., 2013; Akone et al., 2016).

During the past few decades, co-culturing of either fungus and fungus or fungus and bacterium has been shown to be a method that stimulates the regulatory systems by activation of the silent biosynthesis of a broad diversity of bioactive metabolites and also enhances production of constitutive fungal compounds which are not isolated in axenic cultures *in vitro* (Ola et al., 2013; Liu et al., 2017; Wakefield et al., 2017; Kamdem et al., 2018; Qadir et al., 2018; Tomm et al., 2019). The co-culturing of different microorganisms from various habitats has been intensively studied in many media to investigate the production of unexplored bioactive metabolites, enzymes or other constituents (Ola et al., 2013; Moussa et al., 2020). Most reports are concerned with studies of the co-culturing of live microorganisms. For instance, Serrano et al., (2017) reported that the co-culturing as a dual culture of fungal strains and a plant pathogenic fungus, *Botrytis cinerea* triggered the production of new bioactive compounds that suppressed the mycelial growth of the test plant pathogens, *Colletotrichum acutatum*, *Fusarium proliferatum* and *Magnaporthe grisea* and the human pathogens, *Candida albicans* and *Aspergillus fumigatus*. There is only one report concerned with the effect of co-cultivation of a fungus and an autoclaved bacterium (Ancheeva et al., 2017).

Microorganisms from marine environments have also been reported as a great source of bioactive compounds which are not only useful in pharmaceutical applications but also have proved to be promising biological control agents for plant protection (Dethoup et al., 2018). Many previous reports showed the antagonistic activity of marine microorganisms against many plant pathogens of economically important crops (Rongbian et al., 2009; Manilal et al., 2010; Shen et al., 2014; Dethoup et al., 2015). Our group reported the marine fungus, *Talaromyces tratensis* KUFA 0091 in controlling rice diseases in plants and fruit rot in mango (Dethoup et al., 2018; Suasa-ard et al., 2019). Moreover, during the study of bioactive metabolites from marine fungi collected from Thai waters, our research group has isolated novel secondary metabolites, some of which possessed either cytotoxic or antimicrobial activities (Kumla et al., 2014; 2017; Buttachon et al., 2016).

To date, application of fungicides is still the convenient approach for crop protection. However, their residues and toxicity affect human and animal health as well as the environment (Rizzati et al., 2016; Holka, 2017; Brasil et al., 2018; Turull et al., 2018). Biological control agents (BCAs) present an attractive alternative to fungicides. However, the screening of antagonistic activities of microorganisms from various habitats requires time to assess their potential *in planta* (Terao et al., 2017; El-Mougy & Abdel-Kader, 2018).

Thus, the co-culturing of marine microorganisms might be a promising approach to combat plant diseases. Studies about the impacts of the co-culturing of marine fungi and marine bacteria against plant pathogens are still limited. Recently, Oppong-Danquah et al., (2018) found that plant pathogens triggered chemical signals in selected marine fungi resulting in production of novel metabolites and increased the production of compounds in co-cultures although no enhanced antagonistic activity against plant pathogens were reported.

The objectives of this study was to determine the effect of the co-culturing of both live and dead cells of a marine-derived bacterium, *B. subtilis*, with five marine-derived fungi on the dynamics of their antagonistic activity against plant pathogens *in vitro*.

## MATERIALS AND METHODS

### Fungal and bacterial strains

The marine-derived fungi and bacterium used in this study were isolated from marine sponges collected at different coral reefs at Chonburi and Phang Nga provinces during April 2010 - February 2015 as shown in Table 1. The sponge samples were collected by SCUBA diving and kept in plastic bags with sea water. The isolation of the fungi from marine sponge samples was conducted as described previously by Dethoup et al., (2015).

For isolation of the bacterium, sponge samples were surface sterilized and cut into small pieces. After drying on sterile filter papers, the samples were mashed in a sterile mortar and pestle. After this, sponge sap was collected with a sterile loop, cultured on nutrient agar (NA), and incubated at room temperature for 2 days. The single bacterial colony was sub cultured onto NA slants for further identification.

### Identification of the Fungi and the Bacterium

The marine fungi were identified using ITS primers, ITS1 and ITS4 (White et al., 1990). DNA was extracted from young mycelia obtained from 3-day-old colonies (Murray & Thompson, 1980). PCR reactions were performed on a thermal cycler as described previously by Dethoup et al., (2016) and the accession numbers of their ITS gene sequences are shown in Table 1.

The isolated bacterium was cultured in nutrient broth on a rotary shaker at 120 rpm for 24 h, and then, 1 mL of culture broth was collected and put into a micro tube (1.5 mL) and centrifuged at 13,500 rpm for 10 min. The bacterial cells were kept, and the DNA was extracted following a modified Murray and Thompson method (Murray and Thompson, 1980). Primer pairs fD1 and rP2

(Weisburg et al., 1991) were used for 16S rDNA gene amplification. PCR reactions were performed on a thermal cycler as described previously (Dethoup et al., 2016).

### Metabolite Extraction

The individual fungal metabolite extraction was conducted as described previously (Dethoup et al., 2016).

### Fungus-*B. subtilis* co-culturing

The *B. subtilis* was cultured on NA for 3 days by the streak method and a single colony was transferred into 10 mL of NB in a test tube and incubated on a rotary shaker for 2 days at 28°C. Each fungus was cultured in solid rice in a flask as described above for 10 days. Then, each fungal culture was inoculated with 10 mL of bacterial broth per flask and incubated further for 20 days at 28°C. After this, ethyl acetate (500 mL) was poured to each flask, macerated for 7 days and then filtered. The filtrate was evaporated using rotary evaporator to furnish the crude extracts of each fungus-*B. subtilis* co-culture crude extract.

### Fungus- autoclaved *B. subtilis* co-culturing

A single 3 day-old colony of *B. subtilis* was cultured in 10 mL of NB in a test tube, incubated on rotary shaker for 2 days at 28°C, and then autoclaved at 121°C for 15 min. Each fungus was cultured in solid rice in a flask as described above for 10 days, then inoculated with 10 mL of autoclaved bacterial broth per flask, and then incubated further for 20 days at 28°C. The crude extract of each fungus-autoclaved *B. subtilis* co-culture, was obtained as described above with ethyl acetate.

### Evaluation of single culture and co-culture extracts against plant pathogenic fungi

The antifungal activity of the ethyl acetate crude extract of fungal single culture and

**Table 1.** Marine-derived fungi and bacterium used in this study

Marine sponge-associated fungus/ bacterium	KUFA	Accession No.	Marine sponge	Location
<i>Eurotium chevalieri</i>	0464	KY942148	<i>Rhabdormia</i> sp.	Similan Island, Phang Nga
<i>Emericella foveolata</i>	1003	KY041869	<i>Xestospongia testudinaria</i>	Samaesan Island, Chonburi
<i>Myrothecium verrucaria</i>	0991	MH992143	<i>Xestospongia testudinaria</i>	Samaesan Island, Chonburi
<i>Talaromyces tratensis</i>	0091	KT728350	<i>Mycala</i> sp.	Samaesan Island, Chonburi
<i>Talaromyces stipitatus</i>	0207	KU500028	<i>Stylissa flabelliformis</i>	Samaesan Island, Chonburi
<i>Bacillus subtilis</i>	0097	MH992142	<i>Mycala</i> sp.	Samaesan Island, Chonburi

co cultures with either *B. subtilis* or autoclaved *B. subtilis* was determined using the dilution plate method against ten economically important plant pathogenic fungi *in vitro* (Table 2) as described previously by Dethoup et al., (2015). Each plant pathogen was identified by molecular techniques and submitted into the Genbank (Table 2). They were maintained on PDA for 7 days before evaluation. The antagonistic activity of each extract against plant pathogen was evaluated using dilution plant method at the final concentrations of 1 and 10 g L<sup>-1</sup> and then calculated as the percentage of inhibition (Dethoup et al., 2018). Each treatment was conducted with five plates (replications) and separately repeated three times.

## RESULTS

### Effects of crude extracts of the marine fungi cultured alone and co-cultured with either live or dead cells of *B. subtilis* against plant pathogenic fungi

#### *Eurotium chevaleri* (KUFA 0464)

At the highest tested concentration (10 g L<sup>-1</sup>) of *E. chevaleri* crude extract from a solitary culture displayed complete mycelial growth inhibition against *R. solani* and showed moderate antagonistic activity against *A. brassicicola*, *P. palmivora* and *S. rolfsii* with 88.61-95.11% mycelial growth inhibition (Fig. 1A). However, the crude extract showed moderate inhibition against *P. palmivora*, with 76.67% mycelial growth inhibition at 1 g L<sup>-1</sup>. Meanwhile, at 1 g L<sup>-1</sup>, the crude extract from the co-culture showed a higher inhibitory activity against the tested plant pathogens than *E. chevaleri* extract from solitary culture against the mycelial growth of six pathogens (Fig. 2A).

**Table 2.** Plant pathogenic fungi used in this study

Plant Pathogenic fungus	Accession number
<i>Alternaria brassicicola</i>	MH749465
<i>Bipolaris oryzae</i>	MG914430
<i>Colletotrichum capsici</i>	MH749466
<i>Colletotrichum gloeosporioides</i>	MK271274
<i>Fusarium oxysporum</i>	MK271275
<i>Lasiodiplodia theobromae</i>	MH724312
<i>Phytophthora palmivora</i>	MH749467
<i>Pyricularia oryzae</i>	MH749468
<i>Rhizoctonia solani</i>	MH749469
<i>Sclerotium rolfsii</i>	MH749470

Moreover, the extract obtained from co-culturing with *B. subtilis* increased the antifungal activity against all plant pathogenic fungi except on *B. oryzae* that caused a complete mycelial growth inhibition at 10 g L<sup>-1</sup> (Fig. 1A).

Meanwhile, the crude extract obtained from the co-culturing of the marine-derived fungus with autoclaved *B. subtilis* also exhibited higher inhibition against all plant pathogens except for *C. gloeosporioides* at 10 g L<sup>-1</sup> than the crude extract from solitary culture (Fig. 1A). However, at 1 g L<sup>-1</sup>, the extract showed a lower activity against the plant pathogens than the activity of *E. chevaleri* extract (Fig. 2A).

#### *Emericella foveolata* (KUFA 1003)

*Emericella foveolata* crude extract displayed a great inhibitory effect against tested plant pathogenic fungi at 10 g L<sup>-1</sup>. Inhibition of mycelial growth remained effective even at 1 g L<sup>-1</sup>, causing more than 50% mycelial growth inhibition of six out of the 10 plant pathogenic fungi tested (Fig. 2B). The co-culturing of the *E. foveolata* and live *B. subtilis* extract resulted in increasing the antifungal activity against seven of the tested plant pathogens but showed lower inhibition against *F. oxysporum*, *P. palmivora* and *R. solani* than *E. foveolata* crude extract from solitary culture at 1 g L<sup>-1</sup> (Fig. 2B).

The crude extract obtained from co-culturing the fungus and autoclaved *B. subtilis* exhibited higher inhibitory activity toward four of the plant pathogens and showed lower activity against five plant pathogens at 1 g L<sup>-1</sup> when compared with *E. foveolata* crude extract from solitary culture. Moreover, the extract showed lower antifungal activity against *B. oryzae* and *R. solani* than *E. foveolata* crude extract from solitary culture at 10 g L<sup>-1</sup> (Fig. 1B).

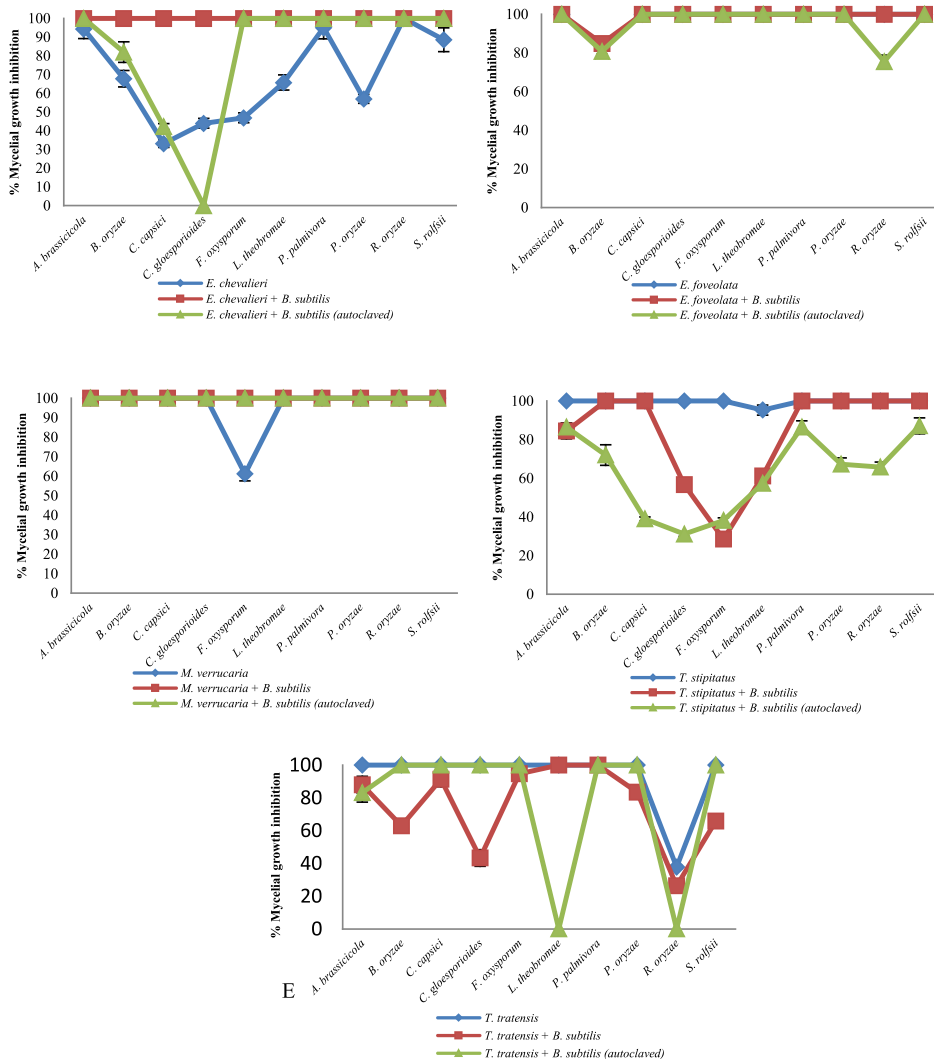
#### *Myrothecium verrucaria* (KUFA 0991)

The results of the antifungal evaluation of *M. verrucaria* crude extract revealed a potent antifungal activity in inhibiting all the plant pathogens at 10 g L<sup>-1</sup> (Fig. 1C). This extract showed strong activity against all tested plant pathogens except for *F. oxysporum* but showed low activity at 1 g L<sup>-1</sup>, causing 42% and 44.44% inhibition against *P. palmivora* and *R. oryzae*, respectively (Fig. 2C). The crude extract from the co-culturing of the *M. verrucaria* and *B. subtilis* crude extract exhibited a slightly higher activity against *P. palmivora*, *S.*

*rolfsii* and *R. oryzae* at 1 g L<sup>-1</sup> compared to *M. verrucaria* crude extract and caused complete mycelial growth inhibition of all plant pathogens at 10 g L<sup>-1</sup>, same as the effect of *M. verrucaria* solitary culture crude extract.

The crude extract obtained from the co-culturing of the fungus and autoclaved *B. subtilis* exhibited the antifungal activity similar to that of the extract from co-culturing of the *M. verrucaria* and non-autoclaved *B. subtilis*, which caused

complete mycelial growth inhibition of all plant pathogens at 10 g L<sup>-1</sup> (Fig. 1C). However, at 1 g L<sup>-1</sup>, the extract from co-culturing of the *M. verrucaria* and autoclaved *B. subtilis* exhibited higher antifungal activity against *B. oryzae*, *P. palmivora*, and *S. rolfsii* when compared with the effect *M. verrucaria* solitary culture crude extract but showed lower activity against *R. oryzae* mycelial growth at the same concentration (Fig. 2C).



**Fig. 1** Inhibitory effect of crude extracts obtained from marine-derived fungi and co-culturing with *B. subtilis* against plant pathogenic fungi at 10 g L<sup>-1</sup>.

- A. *Eurotium chevalieri* KUFA 0464,
- C. *Myrothecium verrucaria* KUFA 0991,
- E. *Talaromyces stipitatus* KUFA 0207

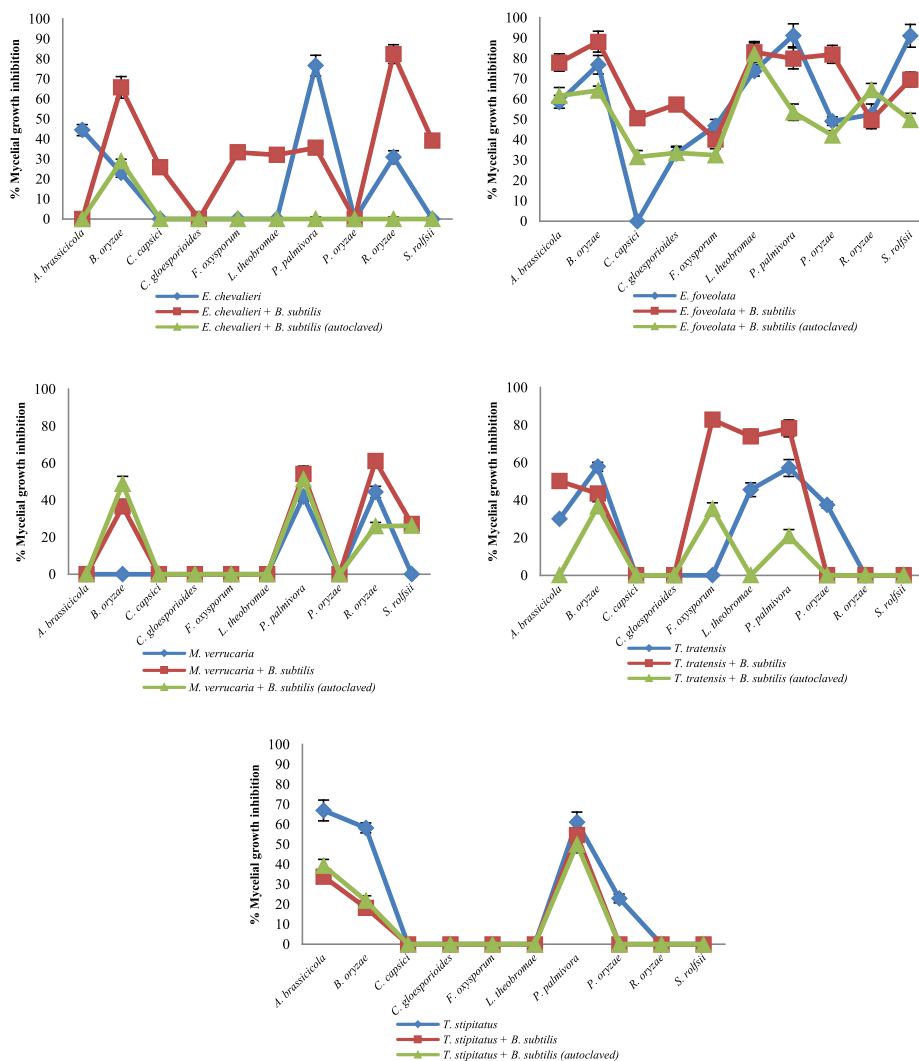
- B. *Emericella foveolata* KUFA 1003
- D. *Talaromyces tratensis* KUFA 0091

**Talaromyces tratensis (KUFA 0091)**

At 10 g L<sup>-1</sup> concentration, *T. tratensis* extract displayed potent antifungal activity against all plant pathogens tested except *L. theobromae* and *R. solani* at 10 and 1 g L<sup>-1</sup>, a moderate effect against *B. oryzae* and *P. palmivora*, causing 57% of mycelial growth inhibition. The extract from the co-culturing of the fungus with *B. subtilis* displayed lower inhibitory effects against all plant pathogenic fungi except *P. palmivora* and *L. theobromae* than the crude extract of *T. tratensis*

solitary culture at 10 g L<sup>-1</sup> (Fig. 1D). However, at 1 g L<sup>-1</sup>, the antagonistic activity from co-culturing crude extract with *B. subtilis* exhibited higher activity against *A. brassicicola*, *F. oxysporum*, *P. palmivora* and *L. theobromae* than did *T. tratensis* solitary culture crude extract (Fig. 2D).

Meanwhile, the crude extract obtained from the co-culturing of the fungus with autoclaved *B. subtilis* showed similar inhibition against plant pathogenic fungi tested when compared with the effect *T. tratensis* solitary culture crude extract.



**Fig. 2** Inhibitory effect of crude extracts obtained from marine-derived fungi and co-culturing with *B. subtilis* against plant pathogenic fungi at 1 g L<sup>-1</sup>.

- A. *Eurotium chevalieri* KUFA 0464,
- C. *Myrothecium verrucaria* KUFA 0991,
- E. *Talaromyces stipitatus* KUFA 0207

- B. *Emericella foveolata* KUFA 1003
- D. *Talaromyces tratensis* KUFA 0091

However, at 1 g L<sup>-1</sup>, the crude extract from the co-culturing showed significantly lower antifungal activity against *B. oryzae*, *L. theobromae* and *P. palmivora* than that *T. tratensis* solitary culture crude extract (Fig. 2D).

#### ***Talaromyces stipitatus* (KUFA 0207)**

At the highest concentration tested, *T. stipitatus* crude extract exerted great antagonistic activity against plant pathogenic fungi tested except for *L. theobromae* and *R. oryzae* at 10 g L<sup>-1</sup> (Fig. 1E). Meanwhile, at 1 g L<sup>-1</sup>, the crude extract displayed a moderate antifungal activity against *A. brassicicola*, *B. oryzae*, and *P. palmivora* resulting in 58.22-66.94% mycelial growth inhibition (Fig. 2E). However, the effectiveness of crude extracts from the co-culturing of the fungus with either live or autoclaved *B. subtilis* resulted in reduced antifungal activity against plant pathogenic fungi. The extract from co-culturing of the fungus with *B. subtilis* displayed lower mycelial growth inhibition of *A. brassicicola*, *C. gloeosporioides*, *F. oxysporum* and *L. theobromae* than did *T. stipitatus* solitary culture crude extract at 10 g L<sup>-1</sup> (Fig. 1E). Likewise, the activity of the extract from co-culturing of the *T. stipitatus* and autoclaved *B. subtilis* exhibited lower antifungal effect against all plant pathogenic fungi than *T. stipitatus* solitary culture crude extract at all concentrations tested.

## **DISCUSSION**

During the past decade, co-culturing has shown to be a successful method to induce microbial communications that can stimulate the silent pathways resulting in enhancement of metabolites and production of new compounds (Ola et al., 2013; Netzker et al., 2015; Zhang et al., 2017). Hence, we have applied this method to determine the silent antagonistic activity of five marine-derived fungi on mycelial growth of plant pathogenic fungi by co-cultivation with a marine-derived bacterium, *B. subtilis*.

The results in this study exhibited that the crude extract from solitary culture of *T. tratensis* exerted the best antifungal activity against the tested plant pathogens followed by *E. foveolata*, *M. verrucaria*, *T. stipitatus*, and *E. chevalieri*, respectively. The results were in accordance with many previous reports which found that many species belonging to genus *Talaromyces* acted as potent antagonists against various plant diseases

such as *T. falvus*, *T. trachyspermus*, *T. tratensis* and *T. wortmannii* (Naraghi et al., 2012; Kakvan et al., 2013; Dethoup et al., 2015; Bahramian et al., 2016). Recently, *T. tratensis* was reported as a potent antagonist against rice diseases even *in planta* (Dethoup et al., 2018). However, the antagonistic activities of *T. stipitatus* and *E. foveolata* against plant pathogenic fungi have not previously been reported.

The co-culturing of a fungus and a bacterium has been investigated for determining the chemical production dynamics in several fungi and might lead exploration of novel antimicrobial compounds against pathogens (Kamdem et al., 2018; Qadir et al., 2018; Abdel-Wahab et al., 2019). However, the results obtained from this study showed the extracts from co-culturing of marine-derived fungi with live or autoclaved *B. subtilis* resulted in positive, negative and even no effect on antifungal activities against plant pathogens *in vitro*. Moreover, the inhibitory effect of the co-culture extracts was dependent on the plant pathogen and the concentration. The co-culturing extracts of the fungi with live *B. subtilis* resulted in increased antifungal activity of *E. chevalieri* and *E. foveolata* extracts but decreased the effects of *T. tratensis* and *T. stipitatus* extracts against the plant pathogens tested. These findings are consistent with results of previous studies, although co-cultivation may lead to enhancement of known metabolites and production of new compounds, not all of the detected compounds showed potent antimicrobial activities.

The only report of a positive effect due to the co-culturing of a fungus and a bacterium is that of Ola et al., (2017). The authors found that the co-culturing of the fungal endophyte, *Fusarium tricinctum* with a bacterium, *B. subtilis* enhancing the production of secondary metabolites, of which four compounds and three new natural products. Three of the isolated metabolites showed strong antibacterial activity on *B. subtilis*, *E. faecalis*, *S. aureus* and *S. pneumonia*.

A significant positive effect of the co-cultivating between a marine bacterium and fungus in this study was observed in the co-culturing of *E. chevalieri* and *B. subtilis*, which led to complete mycelial growth inhibition of all of the plant pathogens whereas the extract from *E. chevalieri* solitary culture displayed mycelial

growth inhibition ranging from 33.11-100% at 10 g L<sup>-1</sup>.

In contrast, Wakefield et al., (2017) reported that the co-culture of *Aspergillus fumigatus* with *Streptomyces leeuwenhoekii*, a hyper-arid desert bacterium led to the production of new metabolites as well as induced the amount of known compounds in extract of axenic culture but no antimicrobial activities were reported. Similarly, many investigations have reported that the induce metabolites even novel compounds through co-cultivation of fungal strains and *B. subtilis* did not show increased antifungal activity against human pathogens tested (Akone et al., 2016; Liu et al., 2017; Kamdem et al., 2018).

Meanwhile, the co-culturing of marine-derived fungi with autoclaved *B. subtilis* resulted in decreased antifungal activity of *E. foveolata*, *T. stipitatus* and *T. tratensis* but slightly increased activity of *E. chevalieri*. The co-cultivating of marine-derived fungi with either live autoclaved *B. subtilis* had no effect on antagonistic activity of *M. verrucaria*. Ancheeva et al., (2017) is the only study reporting the effect of the co-culturing of a fungus with an autoclaved bacterium. These authors found that the co-culture of *Chaetomium* sp. with autoclaved *Pseudomonas aeruginosa* led to increase production of fungal metabolites. However, the isolated compounds from the co-cultivation did not show antimicrobial activity.

Although, co-culturing of a fungus and a bacterium may lead to enhance chemical production, the effect of this on increasing antibiosis is still in doubt. However, the microbial interaction will lead to antibiosis production for resources competition (Netzker et al., 2018; Qadir et al., 2018). Hence, many researchers have also evaluated the effect of co-culturing between fungi with plant pathogens. Opong-Danquah et al., (2018) reported the effects of co-culturing marine fungi with phytopathogenic bacteria (*Pseudomonas syringae*) and fungi (*Magnaporthe oryzae* and *Botrytis cinerea*) on agar plates. They found that the co-cultivation of the fungi with plant pathogens tested did not show significant increase in antifungal activity of the marine fungi on plant pathogens although the overall chemical richness was increased in all co-cultures. However, Serrano et al., (2017) reported the increasing antifungal activities and new metabolites from

the co-culture extract of *Botrytis cinerea* with three phytopathogens including *C. acutatum*, *F. proliferatum* and *M. grisea*. They found that the co-cultivation induced antifungal activities and that the new metabolites were only observed in the extracts from the co-cultures.

The effects of crude extract from the co-cultivation of a marine fungus with autoclaved *B. subtilis* in this study, showed a significant decrease in the antagonistic activity of marine fungi against plant pathogens. Moreover, the crude extract from the co-cultivation of a marine fungus with autoclaved *B. subtilis* showed lower antifungal activity than that of the crude extract from the co-culturing of a marine-derived fungus with *B. subtilis*. These results indicate that the use of the dead bacterium resulted in reduced antagonistic activity of marine-derived fungi and hence is not a promising approach to induce antagonistic activity of fungi.

In conclusion, the results from this study showed co-culturing with a marine bacterium, *B. subtilis* had positive, negative, or no effect on the antifungal activity of the marine fungi. Meanwhile, the co-culturing with autoclaved *B. subtilis* significantly reduced the antagonistic activity of the tested marine-derived fungi. To the best of our knowledge, this is the first report of the effects of five marine fungi with *B. subtilis* on antagonistic activity against plant pathogenic fungi.

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#### CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

#### AUTHORS' CONTRIBUTION

All authors designed the experiments. U. M. performed the experiments. T. D. prepared the manuscript and analysed the data. All authors read and approved the manuscript.

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### ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

### DATA AVAILABILITY

All datasets generated or analysed during this study are included in the manuscript.

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