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A Trial for Biological Control of a Pathogenic Fungus (*Fusarium solani*) by Some Marine Microorganisms

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Abstract: To address the problem of heavy losses in aquaculture and mariculture, a trial to develop a safer, more effective and less expensive biological fungicide was studied. So the aim of the present study was to evaluate the biological control of the two marine fungi (*Aspergillus japonicus* HK, *Trichoderma viride*) and three marine Chlorophyceae (*Chlorella salina*, *Tetraselmis chuii* and *Nannochloropsis oculata*) exudates on the growth of the pathogenic fungus (*Fusarium solani*). The different exudates were prepared in concentrations of 5, 10, 25 and 90 % and their effects on the isolated pathogenic fungus were studied. Results revealed high efficiency of the two fungal and three algal exudates. The highest exudates concentration of *T. viride* and *A. japonicus* HK, *T. chuii* and *C. salina* (90%) showed a good potency as antifungal (reductions of the pathogen colony diameter were, 95.3, 93.2, 92.7 and 90.5 %), respectively after 7 days as compared to control. On the other hand, the highest concentration of *N. oculata* exudates was less effective on the growth of *F. solani* (colony reduction = 63.3 %). There were negative correlations between the pathogen dry weight and the five different exudates concentrations ($P<0.01$). Based on this study, we concluded that the exudates of marine *T. viride* and *A. japonicus* HK have mycotoxics activities as well as those of marine algae *C. salina, T. chuii* and *N. oculata* and they can be exploited as potential antimicrobial candidates against the opportunistic pathogen *F. solani*.

Key words:*Fusarium solani* · *Aspergillus japonicus* HK · *Trichoderma viride* · Chlorophyceae · Exudates · Biological control

INTRODUCTION

Diseases and deterioration of environmental conditions often occur and result in serious economic losses [1]. Now, scientists have to look for methods which are ecologically friendly, safe and specific for controlling pathogens. Nature has continuously provided mankind with novel pharmaceuticals [2].

Black gill disease (fusariosis) caused by *Fusaruim* spp. (*F. solani* and *F. moniliforma*) which are opportunistic pathogens for shrimps and lead to high mortalities [3]. Ramsamy *et al*. [4] reported mortalities (90 %) in *Penaeus monodona*. *Fusaruim solani* causes the sever root-rot damping-off and wilt disease of *Vicia faba* [5]. Also, it was isolated from cork as a wood surface contaminant [6] and lead to loss in wooden parts reaches 60% [7,8]. Degradation of wood by fungi is one of the most important limiting factors of wood utilization [9, 10]. *Fusarium* spp. is one of the most drug-resistant fungi and *F. solani* in general tends to be most resistant of all [3]. Recently, there are many reports about the application of antagonistic fungi in controlling plant disease such as the use *Trichoderma* species by Soytong *et al.* [11]. Schiehser [12] and El–Kassas [13] demonstrated that marine fungi may form important resource for unique metabolites. The previous reports showed that *A. japonicus* produce a group of fungal metabolites which may have allelochemical effects [14-16].

Marine photoautotrophic micro-organisms collectively termed 'micro-algae' are known to produce a wide range of secondary metabolites with various biological actions. Some of them termed allelopathic

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compounds. Allelopathy defined as inhibitory effects of secondary metabolites against either competitors or predators. Rice [17] included microorganisms (bacteria, fungi and micro-algae) in this definition. Microalgae can produce complicated eukaryotic proteins after post-translational modification [18]. Certain free fatty acids produced by algae exert inhibitory effects on a variety of aquatic organisms [19,20]. Wu *et al.* [21] suggested that fatty acids primarily affect the plasma membranes. Some of the main contributions, concerning the production of chemicals from *Chlorella vulgaris*, are presented by Pratt [22,23]. *Tetraselmis suecica* when used as a food supplement for fish, the algal cells inhibited laboratory induced infection in Atlantic salmon [24]. The microalga *Nannochloropsis oculata* is an essential phytoplankton used as live feed for fish larvae. Li and Tsai [25] attempted to culture *Nannochloropsis oculata* in a way that would provide an organism against pathogenic infections. De Caire *et al*. [26] reported that extra-cellular products from *Nostoc muscorum* are promising as a biological control of soybean seedlings damping off. Kulik [27] mentioned that a filtrates or cell extracts from cyanobacteria applied to seeds as protectants against damping-off fungi such as *Fusarium* sp., *Pythium* sp. and *Rhizoctonia solani,* the substances produced by them *in vitro* may prove useful in controlling fungal fish pathogens.

Therefore, this study's goal was to confirm and evaluate the biological control aptitude of marine fungal as well as green micro algal exudates against the opportunistic pathogen *Fusarium solani.*

MATERIALS AND METHODS

Fungal Strains: Three strains named *Aspergillus japonicus* HK, *Trichoderma viride* and *Fusaruim solani* were isolated from Mediterranean coastal water, Alexandria, Egypt. *Aspergillus japonicus* HK and *Trichoderma viride* were used as antagonistic fungi, while *Fusaruim solani* was a pathogenic one.

Media Used for Cultivation of Fungi: Potato Dextrose Broth Medium (PDB): Different isolated fungi were cultivated on PDB medium, gl^{-1}. Potato Starch 4.0, Dextrose 20.0 and distilled water 1L. Wide spectrum antibiotic was added to avoid bacterial contamination [28]. The medium was autoclaved for 20 min. Fungal mycelium was removed from the medium by filtration under aseptic conditions and the fungal filtrates were obtained, fifteen grams of agar were added to (PDB) medium for solidification.

Algal Strains: Three algal species were obtained from National Institute of Oceanography and Fisheries, Alexandria, Egypt. The tested algae belong to Chlorophyceae and were *Chlorella salina* (Butcher), *Tetraselmis chuii* (Butcher) and *Nannochloropsis oculata* (Droop).

Meduim Used for Cultivation of Algae: The three algal strains were cultured in sterile $f/_2$-inreached seawater medium [29] in 1 liter capacity of Erlenmeyer flasks at 25±1°C, pH 7.5 with continuous light. After twelve days of incubation (exponential growth phase), the cells were removed from the culture growth medium by filtration through a sterilized Whatman glass fiber filters (GF/C nominal pore size 47 μm) and the exudates containing different extra cellular metabolites of algae were obtained.

Pathogenic Fungal Growth Measurements

Linear Growth Method: The exudates of the two antagonistic fungi and three Chlorophyceae were taken under sterilized conditions and added to autoclaved PDA media to give concentrations 0, 5, 10, 25 and 90% (v/v). The plates were inoculated with 5 mm disc from 7 days-old PDA fungal cultures. The plates were incubated at 25±2°C. The growth of fungus was measured every day by determining the mean of colony growth diameters (mm) and expressed as percentage reduction [6].

Dry Weight (DW) Method: 100 ml of the medium amended with different filtrate concentrations, in 250 mL Erlenmeyer flask were inoculated with 5 mm discs. The flasks were incubated at 25±2°C for 7 days. The fungal mats were removed by filtration and dried at 60±3°C to constant weight and their dry weights were recorded as g.

Efficacy of Antagonistic Fungi on Growth of Pathogen by Bi-culture Test: The antagonistic fungi and the pathogen were cultured on PDA media for 7 days at room temperature (28-30°C). Then, 5mm diameter of antagonistic fungi colony was cut and transferred to the opposite colony of pathogen and further incubated at room temperature for 7-10 days. Three replicates were taken in each of the experiments.

Statistical Analysis: All values were expressed as mean ± SD, n = 3 and the results of the effect of different exudates concentrations on the colony reduction of *F. solani* were compared by analysis of variance (one-way ANOVA), P= 0.01 was considered statistically

significant. Correlation was performed on the relationship between dry weigh of *F. solani* and different exudates concentrations at the level of significance at P=0.01. Statistical evaluation was carried out using SPSS version 10.0 for windows [30].

RESULTS

Growth Reduction of *Fusarium solani* as Affected by *Aspergillus japonicus* Hk and *Trichoderma viride* Exudates: The exudates of *T. viride* and *A. japonicus* HK showed a good potency of an antifungal against the growth of *F. solani* (Table 1). It was clear that all concentrations had unexpected inhibitory effect on the fungal growth and caused appreciable reduction in the colony diameter of the pathogenic fungus. The reduction of colony diameter increased with the increase in concentration of both fungal exudates. The highest concentration of *T. viride* and *A. japonicus* exudates (90%) revealed a significant (P<0.01) reduction in colony diameter of the pathogen (95.3% and 93.2%, respectively). Even at low dose (10%), the results showed a high efficiency of *A. japonicus* HK and *T. viride* exudates to suppress the growth of the pathogen where the reduction percentages were nearly 78.4 and 76%, respectively.

Growth Reduction of *Fusarium solani* as Affected by *Chlorella salina*, *Tetraselmis chuii* and *Nannochloropsis oculata* Exudates: The percentage reduction in colonial growth of the isolated fish pathogenic fungus (*Fusarium solani*) cultivated under the effect of different exudates concentrations of *Chlorella salina, Tetraselmis chuii* and *Nannochloropsis oculata* was recorded in Table (2). The data revealed a

Table 1: Growth reduction of *Fusarium solani* as affected by different concentrations of marine *Aspergillus japonicus* HK and *Trichoderma viride* exudates.

	Days						
Exudate conc.	1	2	3	4	5	6	7
Trichoderma viride							
0%	0	0	0	0	0	0	0
5%	40.4±0.6	49.4±0.7	52.7±1.2	56.3±0.7	60.3±0.8	65.3±0.9	69.6±1.3
10%	43.5±1.2	49.3±0.7	54.4±0.9	58.5±0.6	63.4±0.9	69.5±1.3	75.6±0.7
25%	44.2±1.0	52.4±0.9	58.3±0.9	64.2±0.7	68.1±1.0	74.3±0.9	88.3±0.8
90%	53.3±0.7	63.3±0.6	75.3±0.7	82.3±0.7	86.2±0.9	90.3±0.9	95.3±0.7
Aspergillus japonicus HK							
0%	0	0	0	0	0	0	0
5%	38.2±0.8	46.4±0.6	50.3±0.7	55.3±0.9	58.4±0.6	61.4±0.6	64.3±0.8
10%	40.2±0.8	49.5±1.0	50.3±0.7	59.4±0.8	62.2±0.8	67.4±0.6	78.4±1.0
25%	43.4±0.7	52.6±1.1	57.2±1.1	70.3±0.9	73.4±0.7	78.3±0.9	87.3±0.7
90%	50.2±0.9	64.3±0.7	75.2±0.8	81.3±0.7	84.4±0.6	89.3±0.7	93.2±0.8

Table 2: Growth reduction of *Fusarium solani* as affected by *Chlorella salina, Tetaselmis chuii and Nannochloropisis oculata* exudates.

	Days						
Exudate conc.	1	2	3	4	5	6	7
Chlorella salina							
0%	0	0	0	0	0	0	0
5%	37.5±0.9	43.3±0.7	49.4±0.7	54.6±1.0	60.4±0.8	69.6±1.2	74.6±1.0
10%	41.2±0.8	47.6±1.2	51.2±0.9	57.2±0.8	63.5±1.2	72.6±1.0	77.4±0.7
25%	45.3±0.8	52.4±1.1	55.2±0.8	62.2±1.1	69.3±0.7	83.4±0.9	88.4±0.7
90%	48.6±1.0	57.3±0.7	61.3±1.0	69.3±0.8	75.4±0.9	89.4±0.6	92.7±1.1
Tetraselmis chuii							
0%	0	0	0	0	0	0	0
5%	20.4±0.7	37.3±0.9	43.2±0.8	56.4±0.6	57.1±0.9	60.3±0.7	64.4±0.7
10%	40.5±0.7	50.4±0.7	50.5±1.2	57.4±0.6	70.2±0.9	43.3±0.7	55.2±0.9
25%	61.3±0.6	66.4±0.7	57.2±1.0	57.4±0.8	59.1±1.0	61.3±0.7	66.4±0.6
90%	60.2±0.8	65.34±0.6	70.4±0.8	74.4±0.8	80.3±0.7	85.4±0.6	90.5±0.9
Nannochloropsis oculata							
0%	0	0	0	0	0	0	0
5%	15.3±0.9	23.4±0.7	25.1±0.9	31.4±0.9	33.3±1.2	30.5±0.7	30.2±1.1
10%	15.4±0.8	25.3±0.9	30.3±0.8	30.3±0.7	35.4±0.9	50.2±0.8	50.5±1.0
25%	15.6±1.1	31.3±0.7	35.4±0.8	35.6±1.2	37.5±0.9	50.7±1.1	50.8±1.0
90%	25.3±0.7	33.3±0.7	41.2±1.1	52.2±1.1	57.2±0.7	62.3±0.6	63.3±0.6

Fig. 1: Effect of algal exudates (90%) on the growth of *Fusarium solani*, A: *Chlorella salina*, B: *Tetraselmis chuii*, C: *Nannochloropsis oculata* and D: control.

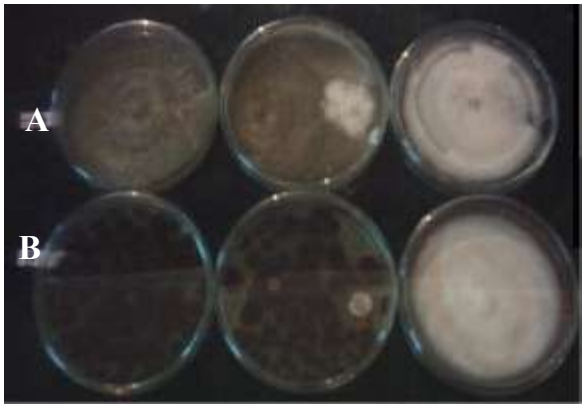

Fig. 2: Effect of antagonistic fungi on the growth of the *Fusarium solani*, A: *Trichoderma viride.* B: *Aspergillus japonicus* HK.

significance increase ($P<0.01$) in colony growth reduction of *F. solani* with increasing different algal exudates concentrations. The highest exudates concentration of *Chlorella salina* and *Tetraselmis chuii* (90%) caused a higher reduction in colonial growth of the tested fungus as compared to control (Fig. 1). *Chlorella salina* and *Tetraselmis chuii* exudates succeeded to inhibit the growth of *F. solani* by 92.7 and 90.5 %, respectively after 7 days of cultivation (Table 2). On the other hand, higher *Nannochloropsis oculata* exudates was less effect on the growth of *F. solani* (colony reduction = 63.3 %) after 7 days. Statistical analyses showed that this effect was significant ($P<0.01$) (Table 3).

Table 3: One way analysis of variance for different exudates influencing the growth of *Fusarium solani*

Exudates conc.	Sum of Sq.	df	Mean Sq.	F	p-value
Trichoderma viride	27614.9	4	6903.7	57.5	0
Aspergillus japonicus HK	29211.1	4	7302.8	56.6	0
Chlorella salina	26973.9	4	6743.5	42.4	0
Tetraselmis chuii	43219.7	4	10804.9	190.5	0
Nannochloropsis oculata	73559.7	4	18389.9	179.3	0

Table 4: Reduction of the pathogen dry weight as affected by different exudates concentrations

Dry weight (g)					
Exudates conc.	*T. viride*	*A. japonicus* HK	*C. salina*	*T. chuii*	*N.oculata*
0%	0.85±0.01	0.85±0.01	0.85±0.01	0.85±0.01	0.85±0.01
5%	0.68±0.02	0.73±0.02	0.73±0.02	0.76±0.02	0.75±0.01
10%	0.44±0.01	0.47±0.02	0.54±0.02	0.65±0.01	0.68±0.02
25%	0.37±0.01	0.38±0.01	0.41±0.01	0.43±0.02	0.51±0.01
90%	0.13±0.01	0.15±0.02	0.22±0.01	0.29±0.01	0.34±0.02
Correlation	-0.858	-0.862	-0.876	-0.858	-0.916

Reduction of the Pathogen Dry Weight (g): The different exudates concentrations led to a reduction in the mat dry weight of the pathogenic fungus after 7 days of incubation (Table 4). The data revealed a progressive decrease in the fungal mat dry weight with increasing concentrations of the applied exudates. The remarkable reduction by *T. viride* and *A. japonicus* HK exudates were recorded, where about 84.7 and 82.4 % reduction was enhanced by 90% for the two fungi, respectively. There were a significant ($P<0.01$) negative correlation between *T. viride* and *A. japonicus* HK exudates and *F. solani* mat dry weight (Table 4). As regards to algal exudates, the reduction in the fungal dry weight was increased with increase the different exudates concentration. The reduction in the fungal mat dry weight by the exudates of *Chlorella salina*, *Tetraselmis chuii* and *Nannochloropsis oculata* was 74.2, 65.9 and 60%, respectively. The correlation coefficient were negative and mostly significant ($P<0.01$) indicating the progressive decrease of the fungal dry weight with increasing algal exudates of the three algae (Table 4).

Antagonistic Ability of *Aspergillus japonicas* Hk and *Trichoderma viride:* The two isolates of *T. viride* and *A. japonicus* HK were found to display antagonistic effects against the test pathogen by suppressing its growth to varying degrees (Fig. 2). *Aspergillus japonicus* HK was found to be more effective in suppressing the

growth of *F. solani*. *Trichoderma viride* and *Aspergillus jponicus* HK overgrew the test pathogen when grown together in a single plate. When the tested pathogen was exposed to the trapped atmosphere from cultures of *A. japonicus* HK and *T. viride*, its growth was greatly inhibited.

DISCUSSION

Allelopathy is a common phenomenon in nature. It has been defined by the International Allelopathy Society in 1996 [31] as any process involving secondary metabolites produced by plants, micro-organisms, viruses and fungi that influence the growth and development of agricultural and biological systems (excluding animals), including positive and negative effects. However, the growth of *Fusarium solani* in general, was seriously affected when subjected to the studied two fungal and three algal exudates. In the current study, the significant unexpected inhibitory effect on the pathogen growth and the appreciable reduction in the colony diameter and dry weight (95.3% and 0.13 g, respectively) of the pathogenic fungus caused by the exudates of *T. viride* could be attributed to the fact that members of the genus *Trichoderma* have high reproductive capacity, efficient utilization of nutrients, strong aggressiveness against other phytopathogens and the production of trichotoxins that could inhibit plant pathogen and promote plant growth [32,33]. The genus *Trichoderma* produced a wide range of antibiotic substances and parasitizes other fungi. Furthermore, they inhibit or degrade pectinases and other enzymes that are essential for plant-pathogenic fungi, *Trichoderma* species exert a property that is known as rhizosphere competence [34,35]. Also, Gachomo and Kotchoni [33] revealed the production of volatiles by *Trichoderma* species against the pathogenic microorganisms. Regarding *Aspergillus japonicus* HK exudates, it was clear that, the colony diameter of pathogenic fungus was significantly decreased even by low dose. It caused a supreme inhibition of both the colony diameter and fungal dry weight of *F. solani* (93.2%. and 0.15 g, respectively) particularly at 90% concentration. In this respect, it was reported that the major mycotoxin allelochemical of *A. japonicus* was identified by spectroscopic methods as secalonic acid D (SAD) [15]. Many workers reported that *A. japonicus* produce a wide variety of enzymes which may be involved in antifungal activity [16].

In this study, greater inhibition of fungal growth was observed at higher concentrations of the exudates of *Tetraselmis chuii*, *Chlorella salina*. The inhibitory effect of the algal exudates might be attributed to the presence of antifungal compounds. The higher exudates concentration (90 %) of *C. salina* and *T. chuii* caused a higher reduction in colonial growth (92.7 and 90.5 %, respectively) of the tested fungus after 7 days as compared to control. The present study as well as Austin *et al.* [24] suggested that there may be some bioactive compounds in the algal cells and there appears to be a significant role for *Tetraselmis* in the control of fish diseases. *Chlorella vulgaris* is able to produce a mixture of fats and hydrocarbons (named chlorellin) which exhibited antibiotic activity [22,23]. In the present study, *Nannochloropsis oculata* exudates was less effect on the growth of *F. solani* (colony reduction = 63.3 %). Li and Tsai [25] attempted to culture *Nannochloropsis oculata* in a way that would provide an organism against bacterial pathogenic infection. Bovine lactoferricin (LFB) from *Nannochloropsis oculata* is an antimicrobial peptide which can kill or inactivate many kinds of pathogens [25]. Various concentrations of the different algal exudates in this study had reduced the fungal mat dry weights. The reduction in the fungal mat dry weight by the exudates of *Chlorella salina*, *Tetraselmis chuii* and *Nannochloropsis oculata* was 74.2, 65.9 and 60%, respectively. De Caire *et al*. [26,36] found that the growth of the plant pathogens *Rhizoctonia solani* and *Sclerotinia sclerotiorum* was inhibited by extra cellular products of *Nostoc muscorum.* They reported an inhibited average growth of *R. solani* by extra cellular products to 77 % compared to control. Generally, the inhibitory effect may be due to the algal products have biologically active compounds [27].

In conclusion, the detection of antifungal activities of the exudates from marine fungal and algal isolates prove and reflect their potentialities to complete *in-vitro* strong inhibition of the growth of the pathogenic fungus *F.solani* as they are biologically based and environmental safe alternatives.

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