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Antifungal activity of sodium chloride on *Saprolegnia diclina* and *Aphanomyces* sp.

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Sixteen identified and three unidentified species belonging to six genera of zoosporic fungi were isolated from forty water samples which were collected from different fish and fish hatcheries farms at Abbassa city, Sharkiya governorate, Egypt, using sesame seeds baiting technique at 20±2°C. *Saprolegnia* and *Achlya* contributed the broadest spectra of species diversity amongst the other genera of zoosporic fungi. *Saprolegnia diclina* and *Aphanomyces* sp. were the most prevalent species of zoosporic fungi. The abundance of zoosporic fungal species in these aquacultures was correlated with some physicochemical characteristics of the water samples. The two dominant species of zoosporic fungi were tested for their tolerance of NaCl solution and its impact on some morphological and metabolic activities of these fungi. *Saprolegnia diclina* tolerated concentrations of NaCl solution till 12000 μg/ml whereas the maximum resistance of *Aphanomyces* sp. was 8000 μg/ml. The examined morphological aspects of the two studied fungal species, which included the colony diameters, the vegetative hyphae, zoosporogenesis, zoospores discharge, sexual reproductive structures and gemmae formation, were generally affected depending upon the tested fungal species and the applied dose of NaCl solution. The low treatments of NaCl solution were significantly stimulative compared with the control for protease production by *S. diclina* but higher doses were significantly suppressive. A significant decline in protease activity at all applications was found when *Aphanomyces* sp. was treated with NaCl solution. The total free amino acids and total protein content of *S. diclina* and *Aphanomyces* sp. mycelia were almost significantly increased relative to untreated controls at the low dose of NaCl solution and they were significantly dropped at the higher concentrations by the two zoosporic fungi.

Key words: zoosporic fungi, salinity, morphogenesis and biochemical activities, sporulation

INTRODUCTION

Fish diseases constitute one of the most important problems and challenges confronting fish culturists. Aquaculture now represents more than 30 percent of total fish production for consumption (Delgado et al. 2003). The majority of global production comes from freshwater aquaculture (58%), followed by marine culture (36%) and brackish water (6%). Over the past ten years, aquaculture production has increased on average by 11% per year. Consequently it is the fastest growing sector of the world food economy (Van West 2006). There are a steady increase in fish farms in Egypt during the recent years as an alternative protein and a trial to solve the increase in meat prices. Outbreaks of waterborne fungal infections (saprolegniasis) on fish and fish eggs continue to cause problems among cultured fish. Oomycetes of the order Saprolegniales, such as *Saprolegnia*, *Achlya* and *Aphanomyces* species are endemic to all fresh water habitats around the world and they cause the saprolegniasis fish diseases. They are responsible for main types of fungal infections on fish and shellfish in aquaculture, fish farms and hobby fish tanks (Neish, Hughes 1980; Willoughby, Pickering 1977; Daugherty et al. 1998; Bruno, Wood 1999; Hussein, Hatai 2002). Oomycetes infections are second only to bacterial diseases in their impact and cause considerable economic losses in aquaculture (Meyer 1991; Bly et al. 1992; Bruno, Wood 1999). Disease also reduces hatchery efficiency and production, which in turn, increases costs and reduces profit.

Antifungal agents are essential for the maintenance of healthy stocks of fish and their eggs in intensive aquaculture operations. Sodium chloride has been recognized as a safe treatment for saprolegniosis in salmonid incubation and rearing (Schreck et al. 1990, 1992; Waterstrat, Marking 1995). This investigation devoted for studying the occurrence of zoosporic fungi in fish and fish hatcheries farms at Abbassa city, El-Sharqyia governorate in Egypt. The efficacy of NaCl as safe antifungal agent on the most prevalent species of zoosporic fungi in these aquacultures was evaluated. In addition, the morphological deviations and some biochemical activities of these zoosporic fungal species as affected by NaCl solution were taken into consideration during this study.

MATERIALS AND METHODS

Sampling sites and collection of water samples from aquacultures. Forty sites were chosen for this study during March 2007 where each small or big water basin was considered an aquaculture site. The forty investigated aquacultures located at Abbassa city, Sharkiya governorate, Egypt and they included both fish farms and fish hatcheries farms. One surface water sample was collected from each aquaculture (fish farm or fish hatcheries farm). Each water sample was collected in five brown and sterilized glass bottles (400 ml capacity each). Four of these bottles were used for baiting and recovery of zoosporic fungi from waters of aquacultures; where each bottle contained eight germinated and sterilized sesame seeds as capture material

of zoosporic fungi. These bottles were aerated at different intervals. The fifth bottle was kept without addition of sesame seeds for the determination of physico-chemical characteristics of aquacultures under investigation.

Physico-chemical analysis of water samples of aquacultures. Physical characteristics of water samples of both fish farms and fish hatcheries included the measurements of water temperature (ranged between 17-21°C) and pH *in situ* (between 7.85 and 11.03). Glass bottles containing water samples free of sesame seeds were used for quantification of total soluble salts and organic matter according to Jackson (1958).

Isolation of zoosporic fungi from fish and fish hatcheries farms (aquacultures). Previously prepared glass bottles containing germinated and sterilized sesame seeds were functioned and used for capture and recovery of the resident zoosporic fungi (Khallil 1984). Water samples in these bottles were poured under aseptic conditions into equivalent numbers of sterilized Petri-dishes (15 cm in diameter each). Petri-dishes were kept at 22°C during 24 hours for the colonization of sesame seeds by fungal propagules. Colonized sesame seeds were then transferred into other clean and sterile, but smaller, Petri-dishes (10 cm in diameter each) containing sterile distilled water to which crystalline penicillin (2000 units per liter of water) was added as indicated by Roberts (1963). Then, these dishes were incubated at 22°C for twenty days during which they were daily examined, and the recovered zoosporic taxa were identified. Most of the recovered zoosporic fungi were purified under aseptic conditions on glucose peptone agar medium (GP) as described by Willoughby and Pickering (1977). Some species of zoosporic fungi required specific media for isolation and purification. *Allomyces* species were purified on yeast peptone starch agar medium YpSs (Emerson 1941) whilst *Aphanomyces* species were purified on YP agar medium (Hatai, Egusa 1979). For assessing the total counts of the genera and species which were identified during this investigation, the fungal species appeared on one sesame seed was counted as one colony (isolate) relative to the seed numbers (24/water sample).

Identification of the recoverable taxa zoosporic fungi. Isolated zoosporic fungi were identified based on morphological characteristics according to Coker (1923), Johnson (1956, 1971), Sparrow (1960), Scott (1961), Karling (1977), Seymour (1970), Rattan et al. (1978).

Selected species of zoosporic fungi and stress solution. Two species of zoosporic fungi namely *Saprolegnia diclina* LAF47 and *Aphanomyces* sp. LAF38, which dominated in fish farms and fish hatcheries were chosen for further morphological and biochemical studies. These species of zoosporic fungi were preserved in the Herbarium of our Laboratory of Aquatic Fungi in pure cultures as water sesame seeds cultures and also maintained on slants at 4°C where they renewed every one-two months period. Purified NaCl was used as stress solution and tested to control the two selected fungal species at different treatments. NaCl solution was prepared under aseptic conditions to give different concentrations. These concentrations were 0.0 (control), 2000, 4000, 6000, 8000, 10000 and 12000 μg/ml of NaCl solution.

Media. Water sesame seeds cultures (Khallil 1984) were used for studying the impact of sodium chloride on the morphological aspects of *Saprolegnia diclina* and *Aphanomyces* sp. Glucose peptone (GP) agar medium (Willoughby, Pickering 1977) was used for purification of *Saprolegnia diclina* and in a broth form (without agar

addition) for estimation of the biochemical activities of the species as affected by the different treatments of NaCl solution. In case of *Aphanomyces* sp. glucose yeast extract (GY) agar medium (Hatai, Egusa 1979) was functioned either in a solid form for purification of the species or in a broth form for studying its biochemical activities under salinity (NaCl solution) stress. Glucose peptone (GP) and GY agar media impregnated with previously mentioned NaCl treatments were also used to determine the colony diameters as affected by the different salinity levels.

Morphological studies under salinity stress. Agar discs were excised at the periphery of actively growing mycelia of the two tested species of zoosporic fungi and transferred to Petri plates containing sterile distilled water supplemented with germinating and sterilized sesame seeds for the preparation of zoospores suspension. Sterilized Petri-dishes (12 cm in diameter) containing different concentrations of NaCl were enriched under aseptic conditions with sterilized germinating sesame seeds and 2 ml of encysted zoospores suspension of *Saprolegnia diclina* and *Aphanomyces* sp. The experiment was designed in triplicate for each fungal species at each treatment. Petri-dishes were then placed in an incubator at 22°C for two weeks during which they were daily examined starting from the second day of incubation using a light Olympus microscope provided with Camera. The morphological changes in *S. diclina* and *Aphanomyces* sp. deviated away from the normal shapes were monitored, recorded and were almost photographed. These morphological features included the vegetative hyphae, zoosporangial formation and discharge and sexual reproductive structures. The diameters of the vegetative colonies of the two zoosporic fungal species were measured (cm) by the end of experiment (15 days) at each treatment of NaCl solution including controls. Average numbers of zoosporangia, sporangial discharge and oogonia were assessed and counted per one colony (sesame seed).

Biochemical activities under salinity stress. Treatments, inoculation and incubation. Glucose peptone (GP) and glucose yeast extract (GY) broth media were dispersed into 100 ml Erlenmeyer conical flasks (20 ml each) and flasks were plugged and sterilized in an autoclave. Then, solutions within flasks were cooled until 40°C and they were adjusted to final concentrations of NaCl solution; 0.0 (control), 2000, 4000, 6000, 8000, 10000 and 12000 μg/ml. Conical flasks contained GP broth were inoculated under aseptic conditions with 8-d-old GP agar mycelial discs (1 cm) of *Saprolegnia diclina* whereas that contained GY broth were inoculated with 8-d-old GY agar mycelial discs of *Aphanomyces* sp. Thereafter, the conical flasks were incubated in an incubator at 22°C for two weeks. The mycelial mats were harvested by vacuum filtration at the end of the incubation period. Cultures filtrates were used for the estimation of protease production by the two zoosporic fungal species. The mycelia were washed three times with distilled water and then dried in oven at 75°C until constant weight. Dried mycelia were used for the determination of total free amino acids and total protein content.

Screening for protease activity by test tubes assay. The two species of zoosporic fungi were screened for their activity to secrete protease enzyme on solid medium impregnated with NaCl concentrations used in this study. The medium described by Paterson and Bridge (1994) was employed. The medium was mixed well to obtain miscible medium solution and autoclaved. Then, 10 ml volume of the medium were poured under aseptic conditions in test tubes which were kept vertically. The test tubes

were inoculated after the medium solidification with 0.5 agar discs taken from the periphery of the actively growing mycelia of each species of the two zoosporic fungi. Tri-replicates of test tubes were prepared at each treatment of NaCl. Test tubes were incubated at 22°C for two weeks after which the depth of clear zone (protease detection) starting from the surface fungal growth was measured and recorded in cm.

Estimation of protease production. For the determination of protease production, the method described by Kunitz (1947) was employed. The quantity of protease were calculated as mg casein/h × 20 ml culture filtrate.

Total free amino acids. The method of Lee and Takahashi (1966) was adopted for the determination of total free amino acids of mycelia. The data of amino acids were expressed as mg glycine/g dry weight of mycelia.

Total protein content. The total protein content was determined using the method of Lowry et al. (1951). Bovine serum albumin was used as a standard substance and the extinction was measured against appropriate blank at 700 nm. The data obtained were calculated as mg bovine serum albumin/g dry weight of mycelia.

Statistical analysis. One-way analysis of variance (ANOVA) was used to test effects of NaCl concentration on the biochemical activities of *S. diclina* and *Aphanomyces* sp. using PC Stat Computer Programme. The means at each concentration were compared with control values using LSD test at 5% significant level.

RESULTS

The physico-chemical characteristics of water samples which were collected from the fish and fish hatcheries farms were evaluated. The pH values of aquacultures water samples ranged from 7.85 to 11.03. The total soluble salts of the water samples were relatively low and varied from 6.4 to 53.76 mg/L. The organic matter contents of water samples fluctuated between 189.43 and 497.10 mg/L. The results indicated in table 1 show that sixteen identified in addition to three unidentified species appertaining to six genera of zoosporic fungi were isolated. Most of the isolated species during this study are known as fish pathogenic fungi. *Achlya* and *Saprolegnia* contributed the broadest spectra of species diversity (five species each) recovered from the investigated aquacultures. The most dominant species of zoosporic fungi were *Saprolegnia diclina* and *Aphanomyces* spp (highly occurred). The species of *Achlya dubia*, *Dictyuchus monosporus* and *Pythium rostratum* were considered as of moderate occurrence in these aquacultures. The rest of species of zoosporic fungi were either isolated in low or rare occurrence.

Saprolegnia was the dominant genus in the surface waters of fish and fish hatcheries farms and it was of high occurrence (32 out of 40 water samples). It was represented by five species and thus ranking with *Achlya* the broadest spectra of the isolated species. Of the isolated species; *S. diclina* was the most prevalent (highly occurred; 24 water samples) inhabiting waters of fish and fish hatcheries farms in the studied area. The remaining species were *S. ferax* (low incidence; 6 water samples), *S. furcata*, *S. glomerata* and the unidentified species (rare occurrence; 2-4 water samples). *Aphanomyces* came next after *Saprolegnia* and it was also of high occurrence (26

Table 1
Total counts, cases of isolations and occurrence remarks of zoosporic fungi collected from fish and fish hatcheries farms

Genera and species of zoosporic fungi	Number of isolates	Cases of isolations	Occurrence remarks
Achlya	156	23	H
A. diffusa Harvey ex Johnson	19	2	R
A. debaryana Humphrey	37	6	L
A. dubia Coker	72	12	M
A. racemosa Hildebrand	20	5	L
Achlya sp.	8	2	R
Allomyces macrogynus Emerson & Wilson	13	4	R
Aphanomyces	115	26	H
Aphanomyces laevis de Bary	6	2	R
A. scaber de Bary	17	6	L
Aphanomyces sp.	92	21	H
Dictyuchus	103	16	M
D. monosporus Leitgeb	58	11	M
D. sterilis Coker	45	5	L
Saprolegnia	257	32	H
S. diclina Humphrey	162	24	H
S. ferax (Gruith.) Thuret	45	6	L
S. furcata Maurizio	21	4	R
S. glomerata (Tiesenhausen) Lund	10	2	R
Saprolegnia sp.	18	2	R
Pythium	60	15	M
P. debaryanum Hesse	10	3	R
P. rostratum Butler	43	12	M
P. ultimum Trow	7	2	R
Total number of isolates	704		

Abbreviations: H – high occurrence; more than 20 water samples; M – moderate occurrence between 10-20 water samples; L – low occurrence; between 5-9 water samples; R – rare occurrence less than 5 samples.

water samples). This genus included three species of which the unidentified species (high incidence; 21 samples) were the most prevalent in the waters of aquacultures. The other two species namely; *A. scaber* and *A. laevis* were isolated in low to rare occurrence (6 and 2 water samples, respectively). *Achlya* was also of high occurrence and it contributed five species of which *A. dubia* was the commonest (moderately occurred; 12 water samples). The other species namely; *A. debaryana* and *A. racemosa* which were of low incidence (6 and 5 water samples) whilst *A. diffusa* and the unidentified species were of rare occurrence (2 samples). *Dictyuchus* was of moderate occurrence (16 water samples) and it was represented by two species namely *D. monosporus* (moderately occurred; 11 samples) and *D. sterilis* (low incidence; 5 water samples). *Pythium* was also moderately encountered from 15 water samples of aquacultures and it was included three species of which *P. rostratum* moderately prevailed (12 samples). *Pythium debaryanum* and *P. ultimum* regarded as of rare occurrence (2-3 water samples). *Allomyces* was of rare incidence (4 samples) in waters of aquacultures and it was represented by only one species (*A. macrogynus*).

Morphological studies. The data presented in table 2 show that the diameters of the vegetative colonies of both *S. diclina* and *Aphanomyces* sp. on the solid media were generally decreased with uprising the concentration of NaCl solution. The radial growth of *S. diclina* was greater than that of *Aphanomyces* sp. at controls and the parallel concentrations of NaCl solution.

Table 2
Effects of NaCl solution on some morphological characteristics of *Saprolegnia diclina* and *Aphanomyces* sp.

Zoosporic fungi	NaCl conc. µg/ml	Morphological characteristics				
		Colonies diameter (cm)	Sporangia formation	Sporangia discharge	Oogonia and antheridia	Gemmae formation
S. diclina	0.0	3.5	31 H	31 H	26 H	34 H
	2000	3.2	27 H	27 H	11 M	32 H
	4000	3.0	12 M	5 L	5 L	27 H
	6000	2.7	5 L	2 R	2 R	18 M
	8000	2.3	2 R	-	2 R	4 R
	10000	1.6	2 R	-	NA	3 R
	12000	0.9	-	-	NA	-
Aphanomyces sp.	0.0	1.1	40 H	40 H	-	-
	2000	1.0	43 H	43 H	-	-
	4000	0.8	18 M	15 M	-	-
	6000	0.6	4 R	2 R	-	-
	8000	0.5	-	-	-	-

Abbreviations: H – high number per one colony; more than 14 sporangia, > 12 oogonia, > 21 gemmae in case of *S. diclina* and > 20 sporangia for *Aphanomyces* sp.; M – moderate number per colony; 7-14 sporangia, 6-12 oogonia, 10-21 gemmae in case of *S. diclina* and 10-20 sporangia for *Aphanomyces* sp.; L – low number per colony; 3-5 sporangia, 3-6 oogonia, 5-9 gemmae in case of *S. diclina* and 5-9 sporangia for *Aphanomyces* sp.; R – rare number per colony; less than 3 sporangia, < 3 oogonia, < 5 gemmae in case of *S. diclina* and < 5 sporangia for *Aphanomyces* sp.; NA – means only non-functional antheridia appeared; – - means structures did not appear.

Saprolegnia diclina treated with 2000 µg/ml of NaCl solution show that both sporangial formation and discharge were little affected compared with that at controls (Fig. 1) and they were still in high numbers. Sexual reproductive structures (oogonia and antheridia) were of moderate numbers. Compared with the oogonia at controls (Fig. 2), the oogonia at this treatment expanded in their sizes and oospheres contents. The gemmae were of high number and were similar in shape to controls (Fig. 3). At 4000 µg/ml of NaCl solution, zoosporogenesis (sporangial and zoospores formation) was observed in moderated number whereas discharge was remarked in low number. Low number of oogonia and antheridia were observed at this concentration and oogonia showed mild symptoms of plasmolysis. The resting bodies of gemmae appeared in high numbers. At 6000 µg/ml of NaCl solution, zoosporogenesis occurred in low number and zoospores discharge rarely recorded. Rare number of plasmolysed oogonia (Fig. 4) and non-functional antheridia were shown at this treatment of NaCl solution. The resting structures (gemmae) were remarked in moderate number and expanded in their shapes (Fig. 4). At 8000 µg/ml of NaCl the vegetative hyphae were loosed in their appearance. Zoosporangia were observed in rare number and they displayed neither sporulation nor spores liberation. Rudimental oogonia (Fig. 5) were formed in rare numbers and antheridia were non-functional. Gemmae were also represented in rare numbers. At the application of 10000 µg/ml of NaCl solution the vegetative hyphae were thin compared with the control. Zoosporangia were of rare number and showed unusual small appendages appearance (Fig. 6). These sporangia never showed spores differentiation and liberation. The sexual apparatus was represented by non-functional filamentous antheridia and gemmae were of rare numbers. The maximum tolerance (sub-lethal concentration) of *S. diclina* for NaCl solution was 12000 µg/ml. At this treatment, the vegeta-

Table 3
Effect of different treatments of NaCl on total free amino acids (TAA, mg glycine/g dry weight of mycelia), total protein content (TP, mg bovine albumin/g dry weight of mycelia), and protease detection (PD; cm) and production (PP, μg bovine albumin/20 ml culture medium) by *S. diclina* and *Aphanomyces* sp.

Zoosporic Fungal species	Concs. (μg/ml)	Biochemical activities			Protease detection (PD; cm)
		TAA	TP	PP	
S. diclina	0.0	0.41	34.01	12.82	6.4
	2000	0.47*	41.22*	14.85*	5.7
	4000	0.39	31.01*	14.12*	4.6*
	6000	0.22*	24.84*	7.41*	1.8*
	8000	0.18*	20.70*	6.28*	1.2*
	10000	0.17*	16.69*	5.71*	0.5*
	12000	0.15*	13.18*	4.91*	-
LSD at 5% level		0.04	1.20	1.29	1.3
Aphanomyces sp.	0.0	0.44	21.48	18.36	6.7
	2000	0.45	22.20	15.23	4.1*
	4000	0.39	20.06*	11.01*	1.6*
	6000	0.17*	16.43*	8.00*	0.6*
	8000	0.14*	12.96*	7.12*	-
LSD at 5% level		0.09	0.77	3.27	1.5

Abbreviations: LSD – least significant difference; * significant difference compared with the control; - protease enzyme was not detected at this treatment.

tive hyphae (thin) showed only non-functional antheridial appendages and neither sporangia or sporangial discharge were observed.

Aphanomyces sp. was sensitive for NaCl solution treatment in comparison with *S. diclina* where its maximum tolerance was 8000 μg/ml. The vegetative hyphae of *Aphanomyces* sp. were of sparse lateral branching (Fig. 7) at controls and they were of condensed growth and profusely branched (Fig. 8) at 2000 μg/ml applications of NaCl solution. This dose was stimulative for zoosporogenesis and zoospores liberations (high numbers). At 4000 μg/ml of NaCl solution, the vegetative hyphae showed minor lateral short branches formation compared with the control. Sporangial formation as well as zoospores release were moderately observed. The vegetative hyphae at 6000 μg/ml of NaCl solution seemed loose and spiral in their structure and zoosporangial formation and discharges were of rare numbers. The sub-lethal treatment of NaCl solution was 8000 μg/ml at which only sterile and stunted vegetative hyphae appeared.

Biochemical studies. The results shown in table 3 reveal that, the total free amino acids content of *S. diclina* significantly raised compared with the control at the lowest application of NaCl solution (2000 μg/ml) and it slightly dropped (non-significantly) at 4000 μg/ml. With increasing NaCl solution until the sublethal treatment (12000 μg/ml), the mycelial amino acids of *S. diclina* were significantly decreased relative to untreated controls. In instance of *Aphanomyces* sp., the total free amino acids of the mycelia were increased non-significantly comparable to the control at the low supplement of NaCl solution (2000 μg/ml) and they relatively declined at 4000 μg/ml. The highest two applications of NaCl solution (6000 and 8000 μg/ml) were inhibitory (significantly) for the mycelial content of amino acids.

The data presented in table 3 indicate that, the total protein content of *S. diclina* mycelia was significantly promoted at the low treatment of NaCl solution (2000 μg/ml). All the other tested concentrations (4000-12000 μg/ml) were found inhibitory for the mycelial content of protein which was decreased with rising the concentration

of NaCl solution. Regarding *Aphanomyces* sp., the total protein content of the mycelia activated (non-significantly) only comparable with the control at the low dose of NaCl solutions (2000 μg/ml) and the other applied concentrations (4000-8000 μg/ml) significantly suppressed the protein content.

As presented in table 3, protease production by *S. diclina* was induced significantly at 2000 and 4000 μg/ml of NaCl solution. Thereafter, as the concentration of NaCl solution increased, the protease activity of *S. diclina* significantly suppressed comparable with the control. In case of *Aphanomyces* sp., protease activity was almost significantly inhibited at all the tested concentrations of NaCl solution. The quantity of protease enzyme by *Aphanomyces* sp. was greater than that of *S. diclina* at controls and at 2000, 6000 and 8000 μg/ml of NaCl solution.

The data presented in table 3 show that protease enzyme detection, as indicated by clear zone (Fig. 9), on solid medium was inhibited significantly at most different NaCl concentrations compared with the control by the two tested fungal species. No protease activity was detected at the sublethal dose of NaCl solution of each fungal species.

DISCUSSION

The pH values of the water samples were alkaline between 7.85 and 11.03 and they had no role on the occurrence of species of zoosporic fungi in the investigated aquacultures. The total soluble salts of aquacultures water samples were found low (6.4 to 53.76 mg/L) and they had no effect on the incidence of the isolated fungal species. The organic matter contents of aquacultures water samples fluctuated between 189.43 and 497.10 mg/L and the waters had high content of organic matter were the richest in the isolated species of zoosporic fungi. More or less similar results were obtained by Kiziewicz et al. (2004) who found that physicochemical parameters of waters had no important effect on the occurrence of zoosporic fungi isolated from bathing sites of the Suprasl River. In addition, Jeffrey et al. (1985) the production of viable zoospores (zoosporogenesis) by *Lagenidium giganteum* took place from pH 4.5 to 8.4 by three isolates and from 4.5 to 8 by two other isolates (±0.2). Also, Piotrowski et al. (2004) reported that isolates of the chytrid *Betrachochytrium dendrobatidis* grew and reproduced at pH 4-8 and the growth was maximal at pH 6-7.

Most of the recoverable species of zoosporic fungi (sixteen identified and three unidentified species) during this study are economically important as fish pathogenic fungi. It was found that *Saprolegnia diclina* and *Aphanomyces* spp were the highly occurred zoosporic fungi in the investigated aquacultures. The species of *Achlya dubia*, *Dictyuchus monosporus* and *Pythium rostratum* were also common but they were of less extent. The other isolated species were recovered either in low or rare occurrence.

In this regards, Ogbonna and Alabi (1991) isolated 24 zoosporic fungal species from the infected fish in a Nigerian freshwater fish pond. *Achlya racemosa*, *Aphanomyces laevis*, *Dictyuchus sterilis*, *Saprolegnia ferax*, *S. litoralis* and *S. parasitica* had 100% frequency of occurrence. There were similarities in the species of fungi

isolated from the infected fishes in the fish pond and those isolated from the hatchery. Also, Kiziewicz et al. (2004) identified thirty six fungi species in the bathing sites of the Suprasl River, among them fish pathogens *Achlya orion*, *Aphanomyces laevis*, *Dictyuchus monosporus*, *Saprolegnia ferax*, *Saprolegnia monoica* and *S. parasitica*. In addition, Khulbe et al. (1994) reported *Achlya debaryana* (Saprolegniales, Oomycetes) for the first time as a fish pathogen in a huge artificial reservoir and recognized fish production center in Naini Tal district, India.

The two tested fish pathogenic fungi varied in their tolerance rates of NaCl solution. *Saprolegnia diclina* tolerated against NaCl solution until 12000 μg/ml μg/ml whilst *Aphanomyces* sp. could resist only until 8000 μg/ml of NaCl solution. The diameters of the vegetative colonies of the tested oomycetous fungi decreased with increasing the dose concentration of NaCl solution. In this respect, several authors found that NaCl reduced growth and was the most effective in controlling of *Phytophthora cinnamomi* (Sterne et al. 1976), *Paecilomyces lilacinum* and *Aspergillus niger* (Mert, Dizbay 1977), *Saprolegnia diclina* (Taylor, Bailey 1979), *Saprolegnia* spp. (Martinez-Palacios et al. 2004; Khodabandeh, Abtahi 2006) and *Saprolegnia parasitica* (Ali 2005). However, Mert and Ekmekci (1987) reported that the vegetative growth of *Aspergillus flavus* and *Penicillium chrysogenum* increased with an increase in the NaCl content of the nutrient medium.

The vegetative hyphae of *S. diclina* appeared loose and thinner at the high concentrations of NaCl solution whereas that of *Aphanomyces* sp. were profusely branched compared with the control at the low applications and they displayed spiral at the high supplements. In accordance with these results, Katz and Rosenberg (1971) observed that numerous septa and branches are formed when *Aspergillus nidulans* is exposed to osmotic shock. More or less similar result was obtained by Ali (2005).

Zoosporogenesis and zoospores discharge in *S. diclina* were little affected at the low treatment of NaCl solution. They were dropped in numbers and deformed in shapes with increasing the dose concentration of NaCl solution while they switched off at the sublethal dose in case of the two tested species of zoosporic fungi. The low dose of NaCl solution was found to be inducible for zoosporogenesis and zoospores discharge in case of *Aphanomyces* sp. In agreement with these findings, some investigators found that relatively low concentrations of NaCl suppressed or eliminated zoosporogenesis and reproductive growth in *Trichophyton mentagrophytes* (Kane, Fischer 1973), *Saprolegnia* species (Harrison, Jones 1975; Smith et al. 1990), *Lagenidium giganteum* (Jeffrey et al. 1985), *Aspergillus flavus* (Mert, Ekmekci 1987) and *Saprolegnia parasitica* (Ali 2005). Kirvu et al. (2005) characterized sporulation and cyst formation of secondary zoospores of two isolates of *A. invadans* which prevalent in Atlantic menhaden, *Brevoortia tyrannus* at different salinities and found that sporulation occurred only at low salinities. From laboratory observations, they concluded that low salinities appeared crucial to transmission of the pathogen. The two isolates of *A. invadans* produced free-swimming secondary zoospores at salinities of 0, 1 and 2 psu (salinity unit = per thousand), but not at 4 psu or higher. Sporangial formation and zoospores release by the oomycete fish pathogen

The reproductive structures, oogonia and antheridia, produced by *S. diclina* responded variably and depended upon the dose concentration of NaCl solution. Oogonia declined in numbers but were enlarged in size and increased in the number of oospheres content at the low application of NaCl. They showed subsequent decrease

in their numbers and morphological alterations ranged between mild and severe plasmolysis, rudimental structure and appeared without their antheridial attach with rising the concentration of NaCl solution. Sexual structures were represented by only non-functional antheridia at the sublethal application of NaCl solution. In confirmation with these results, Kane and Fischer (1973) reported that the addition of 3–5% NaCl to the incubation medium inhibited the reproductive growth of *Trichophyton mentagrophytes*. Moreover, Harrison and Jones (1975) and Smith et al. (1990) claimed that oogonium production of pathogenic and non-pathogenic *Saprolegnia* species is suppressed by relatively low salt concentrations.

The gemmae bodies formation by *S. diclina* was activated at the low concentrations of NaCl solution where they were recorded abundant. It may attributed for unsuitable conditions being resulted from the increase of salinity level to tolerate them. In this regards, Faye et al. (2006) reported that sclerotial yield and sclerotial germination of five isolates of *Rhizoctonia solani* declined with decreasing osmotic potential on osmotically adjusted media. However, higher concentrations of NaCl solution were inhibitory for gemmae formation in *S. diclina* compared with that at controls.

The *S. diclina* and *Aphanomyces* sp. produced extracellular protease enzyme in the culture filtrates at control and treated cultures. The quantity of protease secretion varied between the two tested fungi where *Aphanomyces* sp. was nearly more potent than *S. diclina* in protease production at the parallel concentrations of NaCl solution. Protease screening by the two tested species of zoosporic fungi on solid medium showed a measurable reduction as the concentrations of NaCl increased. The ability of zoosporic fungi to produce protease was followed by Weiland (2004) who examined the production of protease by the sugarbeet pathogen *Aphanomyces cochlioides*, the legume pathogen *A. euteiches*, and the fish pathogen *Saprolegnia parasitica*. Protease activity was readily detected in supernatants of water cultures of each organism using autoclaved host tissue as a nutrient source. For the three oomycetes tested, abundant protease activity was present in culture supernatants; additionally, high protease levels were detected in host seedlings infected with the two phytopathogens. In inoculated sugarbeet seedlings, the protease activities were detected prior to or concomitant with the onset of disease symptoms and the activities were capable of digesting protein extracted from sugarbeet hypocotyls. Also, Soederhaell and Unestam (1975) found protease activity in culture filtrates from the crayfish plague parasite, *Aphanomyces astaci.* In addition, Dieguez-Uribeondo and Cerenius (1988) reported that species of *Aphanomyces* that attack fish (e.g., *A. invidans*) and crayfish (e.g., *A. astaci*) have been shown to produce abundant extracellular protease. The penetration of the crayfish cuticle by *Aphanomyces astaci* (Bangyeekhun et al. 2001) and of human epidermis by *Pythium insidiosum* (Ravishankar et al. 2001) is proposed to involve digestion by protease. However, Leger et al. (1986) found that several pathogenic isolates of *Metarhizium anisopliae, Beauveria bassiana*, and *Verticillium lecanii* when grown in buffered liquid cultures containing comminuted locust cuticle as composite carbon source, produced a variety of extracellular enzymes corresponding to the major components of insect cuticle which included proteases. Considerable variations occurred in levels of production between species and even within a species, but proteases were exceptional as production of them was high with all the isolates.

The low supplements of NaCl solution (2000 and 4000 μg/ml) were significantly stimulative for protease production by *S. diclina* compared with the control whereas

the higher concentration were significantly suppressive for the enzyme production. However, protease production by *Aphanomyces* sp., was almost significantly dropped at all doses of NaCl solution.

The elevated concentrations of NaCl solution (starting from 6000 μg/ml) significantly dropped the mycelial content of amino acids of the two tested species of zoosporic fungi comparable to untreated controls. Similar result was also obtained by Ali (2005). On the other hand, Wethered and Jenning (1985) found that amino acids within the mycelia of marine fungi increased with increasing salinity.

The total protein contents of *S. diclina* and *Aphanomyces* sp. mycelia were significantly decreased at the tolerant concentrations of NaCl solution (except at the low dose) as compared with the control. In agreement with this result, Ali (2005) found that the total protein content of *Saprolegnia parasitica* was inhibited at all tested concentrations of NaCl solution.

CONCLUSIONS

Several species of the isolated zoosporic fungi from the study zone are known as fish pathogenic fungi. This information gives a sign for possible infection of the cultured fish species in the investigated fish and fish hatcheries farms by these fungi. As a result problems can face culturists and investments in these aquacultures. The results of testing the effect of NaCl solution on the most two dominant zoosporic fungal species (known as fish pathogens) in these farms indicated that the high salinity levels were successful in controlling and suppressing the activities of these fungi.

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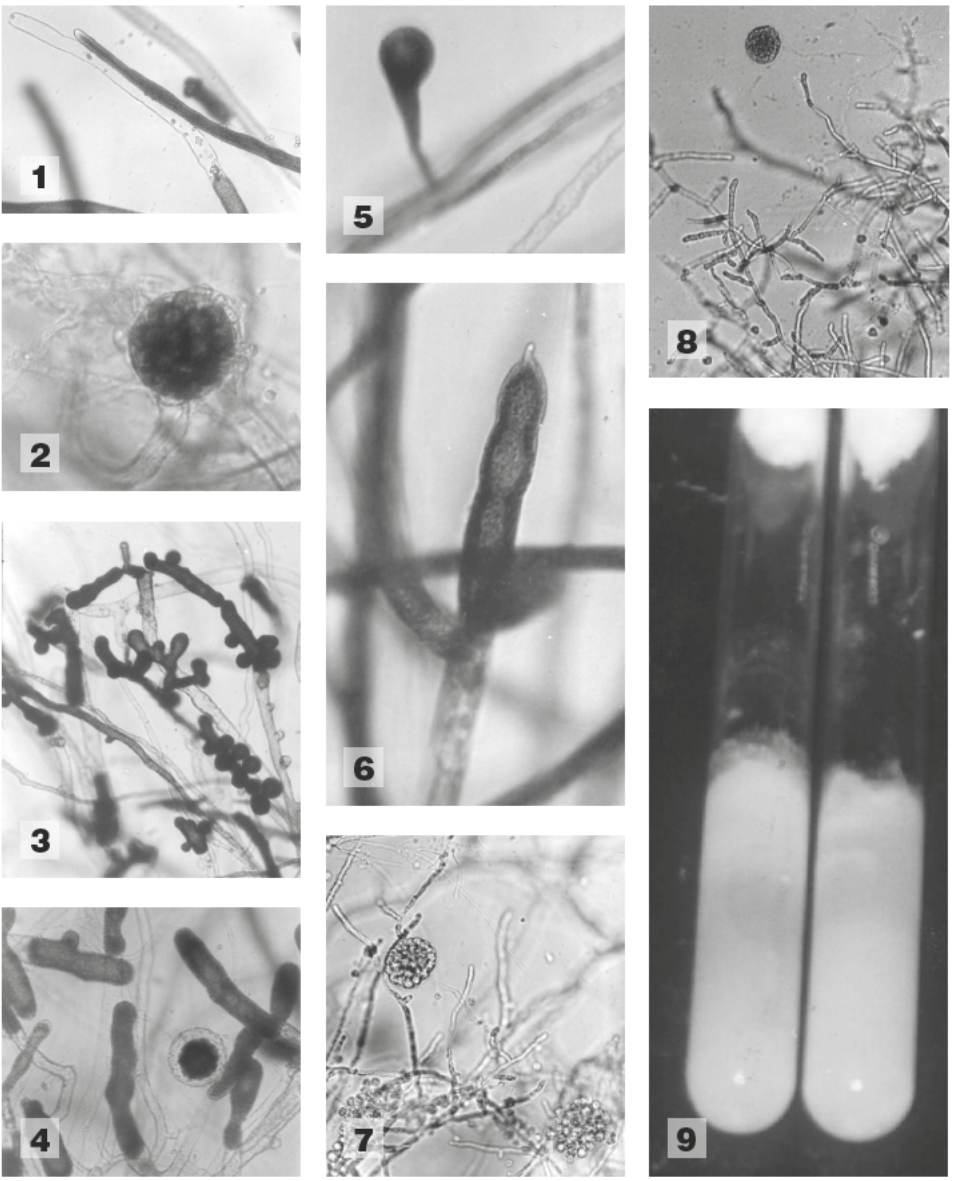

Figs 1-9. 1. *Saprolegnia diclina* showing sporangial proliferation and discharge at controls. 2. Normal shapes of oogonia and diclinous antheridia of *S. diclina* as appeared at controls. 3. Gemmae structures of *S. diclina* at controls. 4. Plasmolysed oogonia and expanded gemmae of *S. diclina* at 6000 µg/ml of NaCl solution. 5. Rudimental oogonia and non-functional antheridia of *S. diclina* as formed at 8000 µg/ml of NaCl solution. 6. Sporangium of *S. diclina* provided with small appendage at 10000 µg/ml of NaCl solution. 7. Vegetative hyphae and zoospores encystment (at the mouth of sporangia) of *Aphanomyces* sp. at controls. 8. Profusely branched vegetative hyphae and encysted zoospores of *Aphanomyces* sp. at 2000 µg/ml of NaCl solution. 9. Clear zones formation in test tubes indicates protease activity of *S. diclina* on solid medium at control (left) and at 4000 µg/ml NaCl concentration (right).