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Antimicrobial activities of some microorganisms isolated from Moroccan Atlantic Coast

Activités antibactériennes de quelques micro-organismes de la côte atlantique du Maroc

Hinde Aassila*, Aziz Fassouane*, Marie-Lise Bourguet-Kondracki**, Michèle Guyot**[1]

*Laboratoire de biochimie marine, Département de biologie, Faculté des sciences, Université Chouaib Doukkali, BP 20, El Jadida, Maroc

**Laboratoire de chimie des substances naturelles, ESA 8041 du CNRS, Muséum national d'histoire naturelle, 63, rue Buffon, 75005 Paris, France

[1] Corresponding author (Fax: +33 1 40 79 31 35; e-mail: guyot@mnhn.fr)

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ABSTRACT

Aassila H., A. Fassouane, M.L. Bourguet-Kondracki, M. Guyot - Antimicrobial activities of some microorganisms isolated from Moroccan Atlantic Coast. Mar. Life, **12** *(1-2) : 3-10.*

Marine microorganisms isolated from seawater, sediments and marine invertebrates from the Moroccan Atlantic Coast have been tested against some bacteria, yeasts and filamentous pathogenic fungi. Among 46 isolated strains, 31 showed inhibitory activity towards at least one pathogen.

RÉSUMÉ

Aassila H., A. Fassouane, M.L. Bourguet-Kondracki, M. Guyot - [Activités antibactériennes de quelques micro-organismes de la côte atlantique du Maroc]. Mar. Life, **12** *(1-2) : 3-10.*

Des micro-organismes marins isolés d'eau de mer, de sédiments et d'invertébrés marins de la côte atlantique du Maroc ont été testés pour leur activité antimicrobienne et antifongique. Parmi les 46 souches isolées, 31 présentent une activité inhibitrice vis-à-vis d'au moins un pathogène.

INTRODUCTION

The sea is an immense and almost unexploited source of new, potentially useful biologically active substances (Faulkner, 1999). Recently, marine microorganisms have been found to produce a variety of chemically interesting and biologically significant secondary metabolites, some of them being expected to serve as lead compounds for drug development or pharmacological tools for basic studies in life sciences (Kobayashi, 1989; Jensen, Fenical, 1994).

Attention has been drawn to this field following the discovery over the years that microorganisms are the ultimate source of a number of natural products previously ascribed to invertebrates. The microorganism may be part of the diet of a macroscopic organism or grow on/or in the tissues of a host (Schmitz, 1994).

Many examples support this hypothesis. Okadaic acid initially isolated from the sponge *Tedania ignis* (Tachibana *et al.*, 1981), is the toxic component of marine dinoflagellates (Murakami *et al.*, 1982). The diketopiperazines isolated from the sponge *Tedania ignis* (Poecilosclerida, tedaniidae) (Schmitz *et al.*, 1983) were later found to be produced by a *Micrococcus* sp. isolated from the sponge (Stierle *et al.*, 1988). The brominated diphenyl ether, first reported from a *Dysidae* sp. (Dendroceratida, dysideidae), has now been demonstrated to be produced by a cyanobacterium isolated from the sponge (Unson, Faulkner, 1993). Tetrodotoxin, the toxin of pufferfish, is produced by

numerous bacteria (Do *et al.*, 1990), saxitoxin and neosaxitoxin dinoflagellate toxins are also synthesized by cyanobacteria (Shimizu, 1993). The structural similarity between discodermolide isolated from the *sponge Discodermia dissoluta* (Gunasekera *et al.*, 1990) and alteramide produced by an *Alteromonas* sp. isolated from the sponge *Halochondria okadai* (Shigemori *et al.*, 1992) suggests a bacterial source for the former.

Over the years, marine microorganisms of different origins have been found to produce substances with antibacterial, antifungal and antiviral activities (Fenical, 1993; Fenical, Jensen, 1993; Jensen, Fenical, 1994; Cuomo *et al.*, 1995). The great potential opened up by these discoveries is emphasized by the fact that these active substances could be produced by biotechnological processes.

In a program focussed on a search for bioactive compounds of marine origin, we investigated bacteria collected from different substrates on the littoral of El Jadida, Atlantic Coast of Morocco. We report here the results of a screening for antibacterial and antifungal activities performed on 46 strains of microorganisms isolated from seawater, marine sediments or marine invertebrates (clams, oysters, sponges, ascidians, eggs clumps) from the North-West Moroccan Coast. Results of assays of different solvents to determine which was the most suitable for extraction of the active principles are also given.

MATERIAL AND METHODS

Collection of strains and culture conditions

Samples from different marine environments: seawater, marine sediments or marine invertebrates (clams, oysters, sponges, ascidians, eggs clumps) were collected in the intertidal zone at several points of the Atlantic Coast near El Jadida city (North-West of Morocco):

- seawater: 10 mL seawater samples were taken near the coast, stored in sterile tubes and processed in the laboratory within 24 h;
- marine invertebrates were washed with sterile seawater. Surfaces were brushed with a sterile tube brush, and the swab immerged in sterile seawater. Invertebrates were then crushed in sterile seawater;
- sediments collected in the intertidal zone were immediately suspended in sterile seawater.

The resultant suspensions, were then diluted in ten fold series and seeded onto marine Sabouraud agar (glucose 20 g, peptone 10 g, agar 20 g, filtered (0.2 µm) sea water 1,000 mL), Marine agar 2216 (Difco), glucose enriched Zobell agar (seawater 750 mL, distilled water 250 mL, bacto-peptone 5 g, bacto-yeast extract 1 g, glucose 20 g, $FePO_4$ 0.01 g, gelose 20 g, pH 7.7) and Luria Bertani agar (bacto tryptone 10 g, yeast extract 5 g, NaCl 10 g, agar 20 g, distilled water 1,000 mL, pH 7.5). After six days of incubation at 27°C, all colonies with different morphology were isolated and maintained on the suitable agar slants at 4°C.

For experiments, these isolates were grown in the suitable media (6 mL): glucose enriched Zobell medium (seawater 750 mL, distilled water 250 mL, bacto-peptone 5 g, bacto-yeast extract 1 g, glucose 20 g, $FePO_4$ 0.01 g, pH 7.7), and Luria Bertani broth (Difco) for 24 h. Marine fungi were grown in marine Sabouraud broth (Sabouraud 30 g, filtered (0.2 µm) sea water 1,000 mL) for 24 h.

Test strains and culture media

The following two bacterial strains (*Staphylococcus aureus* IP6538, *Escherichia coli* IP8739), five representative yeasts and filamentous fungi: *Candida albicans* (IP884.65), *Cryptococcus neoformans* (IP960), *Arthroderma simii* (IP902.65), *Aspergillus fumigatus* (IP1025), *Aspergillus niger* (IP 218.53), from the Institut Pasteur Collection (Paris, France) and one yeast, *Saccharomyces cerevisiae* (Centre hospitalier universitaire de Poitiers, France), were used for antifungal and antibacterial bioassays.

Some extracts (T2, 2N1, 1X2 and 5P2) were tested against *Arthroderma simii, Candida albicans, Cryptococcus neoformans, Staphylococcus aureus* and *Vibrio anguillarum* (ATCC 19264), using Sabouraud agar, Sabouraud broth for yeasts and filamentous fungi, Luria Bertani agar, Luria Bertani broth (Difco) for bacteria. Marine agar 2216 (Difco) and marine broth 2216 (Difco) were used for *Vibrio anguillarum.*

For *Candida albicans* and *Cryptococcus neoformans*, a suspension (10^4cells.mL^{-1}) was prepared from a 24-hour culture on Sabouraud agar at 30°C. For filamentous fungi, suspensions of 10^5 conidia.mL^{-1} were used.

For *Staphylococcus aureus*, three colonies were suspended in 5 mL of sterilized sea water, and diluted at 1/100; for *Escherichia coli*, one colony was suspended in 5 mL of sterilized sea water and then diluted at 2/100; for *Vibrio anguillarum*, 20 colonies were suspended in 5 mL of sterilized sea water, and the suspension was diluted at 1/10.

Screening for antimicrobial and antifungal activities

The screening for antimicrobial and antifungal activities was carried out by using the paper disc method (Thompson *et al.*, 1985).

For each strain, 10 µL of whole culture were added to a sterile paper disc (diameter: 6 mm) placed on a nutrient agar plate (thickness: 5 mm)

Table I - Origin of strains, isolation medium and antibiotic spectrum. / *Origine des souches, milieu et spectre antibiotique.*

As: *Arthroderma simii*; Ca: *Candida albicans*; Cn: *Cryptococcus neoformans*; Sc: *Saccharomyces cerevisiae*; Af: *Aspergillus fumigatus*; An: *Aspergillus niger*; Sa: *Staphylococcus aureus*; Ec: *Escherichia coli*.
nd: not determined.
(1): marine Sabouraud; (2): Luria Bertani; (3): glucose enriched Zobell medium; (4): marine broth
* identified as *Microcosmus australis* by Françoise Monniot, Laboratoire des Invertébrés Marins, M.N.H.N., France.

		Inhibitory activity (inhibition zones in mm) against the following species							
Strain	Origin	As	Ca	Cn	Sc	Af	An	Sa	Ec
A (3)	sea water	31	nd	nd	nd	nd	nd	nd	nd
B (3)	sponge	25	-	-	-	-	-	-	-
C (1)	sponge	20	-	-	-	+	+	-	-
D (1)	ascidian*	18	-	-	-	-	-	-	-
E (3)	ascidian*	25	-	-	-	+	+	-	-
F (3)	ascidian*	30	-	nd	nd	nd	nd	nd	nd
H (3)	sea water	+	-	-	-	-	-	-	-
I (1)	marine sediment	+	-	-	-	-	-	-	-
J (3)	salina water	+	-	-	nd	-	-	-	-
K (3)	marine sediment	23	-	-	-	+	-	-	-
L (3)	marine sediment	+	-	-	-	+	-	-	-
M (1)	marine sediment	+	nd	nd	nd	nd	nd	nd	nd
2N1 (2, 4)	eggs of Muricidae	15	-	-	-	-	-	-	-
N2 (3)	eggs of Muricidae	15	-	-	nd	-	-	-	-
O1 (2)	sea water	29.5	-	-	nd	-	-	-	-
1P2 (2)	ascidian*	-	-	-	+	+	+	nd	-
4P2 (2)	ascidian*	-	-	-	+	+	+	nd	+
5P2 (2, 4)	ascidian*	17.5	-	-	-	-	-	+10	-
R1 (1)	sea water	63	-	-	nd	-	-	-	-
R2 (3)	sea water	18.5	-	-	nd	+/-	-	-	-
R3 (2)	sea water	19.5	-	-	nd	9	-	10	-
S2 (3)	oyster	24.5	-	-	nd	10	-	-	-
T1 (2)	clam	+	-	-	nd	-	-	-	-
T2 (1)	clam	17	20	17	+	13	nd	14	-
T3 (3)	clam	15	-	-	nd	10	-	-	-
U1 (2)	marine sediment	+	-	-	nd	-	-	-	nd
U2 (3)	marine sediment	+	-	-	nd	-	-	nd	nd
W2 (1)	red alga	28.5	-	-	nd	13	-	-	-
X1 (3)	mussel	17.5	-	-	nd	14	-	-	-
1X2 (2, 4)	mussel	20	-	30		20	-	10	-
X3 (1)	mussel	20	-	-	nd	-	-	-	-

containing a bacterium, yeast or fungus lawn. Inhibition was indicated by the appearance of a clear inhibition zone, the diameter of which was measured after incubation for 24 h to 48 h for yeasts and bacteria, 72 h for *Arthroderma simii* and 48 h for *Aspergillus fumigatus* and *Aspergillus niger*.

Antifungal assays were also performed using the MIH Micromethod (Fassouane *et al.*, 1995). 10 mL of Sabouraud agar (at 50°C) containing 1 mL of conidia (10^5) suspension were poured into a petri dish. After solidification, a disc (diameter: 4 mm) or a square (4x4 mm) agar slice was cut out and deposited on one of the eight circles of an immunofluorescent Microprint slide, then overflowed with 10 µL of extract. After an appropriate incubation time at 30°C, the microculture was examined under light microscope for growth inhibition, spore germination, hyphal elongation and morphological alterations.

Identification of strains

Strains exhibiting an antibiotic activity were identified using the API strips in the laboratory of microbiology, Centre hospitalier de Poitiers, France, and by DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen) and by J.L. Nicolas, Ifremer Brest, France, using 16S ribosomic RNA sequencing.

Bioassay of extracts

The four strains T2, 2N1, 1X2 and 5P2, which exhibited the most apparent activity and could be identified further, were grown in 250 mL batches under static conditions up to the stationary phase. The whole cultures were then extracted and later on concentrated under reduced pressure.

Several solvents with different polarity (methanol, ethanol, ethyl acetate) were used until we found the most effective one to extract

Table II - Morphological and physiological features of strain T2. / *Caractères morphologiques et physiologiques de la souche T2.*

Morphology

Colony on potato dextrose agar butyrous, smooth, creme coloured. Blastospores ellipsoidal.
No pseudomycelium; no sexual reproduction detected.

Utilization of C- and N- source

anaerobic:	Glucose	+		
aerobic:	Glucose	+	α-Methylglucoside	-
	Galactose	+	Salicin	-
	Sorbose	weak	Cellobiose	-
	Rhamnose	-	Maltose	-
	Dulcitol	-	Lactose	-
	Inositol	-	Melibiose	-
	Mannitol	+	Sucrose	+
	Sorbitol	+	Trehalose	+
	Glycerol	+	Inulin	-
	Erythritol	-	Melezitose	-
	D-Arabinose	-	Raffinose	+
	L-Arabinose	-	Starch	-
	Ribose	-	Xylitol	+
	D-Xylose	+	Gluconate	-
	L- Xylose	-	2-Keto-gluconate	-
	Adonitol	-	5-Keto-gluconate	-
	Nitrate	-		

+: utilized
-: not utilized

antimicrobial compounds. The whole culture was added to an equal volume of methanol, and stored at room temperature for 30 min until complete disruption of cells.

After evaporation of the methanol, the aqueous phase was extracted four or five times with 250 mL of ethyl acetate. The organic phases were pooled and ethyl acetate evaporated under reduced pressure. The extracts were stored at -20°C until testing for antimicrobial and antifungal activities.

Four extracts (T2, 2N1, 1X2 and 5P2) (1 mg/disc) were tested for *Arthroderma simii, Candida albicans, Cryptococcus neoformans, Staphylococcus aureus* and *Vibrio anguillarum*. The inhibition diameters were measured.

RESULTS

Isolation of strains and antibiotic spectrum

From a total of 18 samples, 46 strains were isolated and tested for antibiotic activity against eight different pathogens. 31 of these strains (67%) inhibited at least one of the test-pathogens (table I). All active strains inhibited the dermatophyte fungus *Arthroderma simii*, except two (1P2 and 4P2) (6.45%).

Sixteen (51.6%) of these strains exhibited a specific inhibitory activity against *Arthroderma simii*, whereas fourteen (48.4%) also inhibited other pathogens such as *Aspergillus fumigatus, Aspergillus niger, Candida albicans, Cryptococcus neoformans, Staphylococcus aureus,* and/or *Saccharomyces cerevisiae.*

In the disc agar diffusion test, the diameters of inhibition zones after appropriate time of incubation were from 0 to 63 mm for all bacteria, yeasts and fungi tested.

When antimicrobial and antifungal activities assays were carried out 6 months later, only two strains (B and H) (6.45%) out of 31 showed total lack of activity.

Identification of strains

The following microorganisms were identified: the yeast T2 as *Torulaspora delbrueckii*, 2N1 as *Enterococcus faecium* (Deutche Sammlung von Mikroorganismen und Zellkulturen GmbH), 1X2 as *Pseudomonas putida* (Laboratoire de microbiologie du Centre hospitalier de Poitiers, France) and 5P2 as *Pseudomonas doudorofii* (identification carried out by J.L. Nicolas, Ifremer Brest, France). Biochemical characteristics of these strains are given in tables II, III and IV.

Extraction of the bioactive strains

Four of the 31 active strains (T2, 2N1,1X2 and 5P2) were selected for extraction of active compounds

Table III - Morphological, physiological and growth characteristics of strain 2N1. / *Caractéristiques morphologiques, physiologiques et de croissance de la souche 2N1.*

Morphological and growth characteristics			
General description: Gram-positive cocci in pairs and chains, facultatively anaerobic, obligatory saccharoclastic, end product of glucose fermentation is lactic acid			
Motility	-		
Spore formation	-		
Catalase	-		
Gas from glucose	-	Gas from gluconate	nd
Growth at 10°C	nd	Growth at 45°C	+

Physiological characteristics			
Acid produced from:			
Ribose	+	Cyclodextrin	+
Mannitol	+	Glycogen	-
Sorbitol	-	Pullulan	-
Lactose	+	Maltose	+
Trehalose	+	Melibiose	-
Raffinose	-	Melezitose	-
Sucrose	-	Meth.-β-D-glucopyranoside	+
L-Arabinose	+	Tagatose	-
D-Arabitol			-
Arginine dihydrolase	+	β-Galactosidase	+
β-Glucosidase	+	Pyroglu-Arylamidase	+
β-Galacturonidase	-	N-Acetyl-β-glucosaminidase	+
β-Glucuronidase	-	Gly-Try-Arylamidase	+
α-Galactosidase	-	Hippurate hydrolysis	-
Alkaline phosphatase	-	β-Mannosidase	+w
Acetoin production	+	Urease	-
Ala-Phe-Pro-Arylamidase	-	Configuration of lactic acid:	nd
Peptidoglycan type:	nd		

nd: not determined
+: utilized, +w: weak or delayed reaction
-: not utilized or absence

with the different solvents (methanol, ethanol, ethyl acetate). Antimicrobial assays showed that ethyl acetate was the most effective solvent for extracting substances with antifungal or antibacterial activities.

The four extracts were also tested, by means of the disc agar diffusion test (1 mg extract/disc towards *Arthroderma simii, Candida albicans, Staphylococcus aureus* and *Vibrio anguillarum* (table V).

The diameters of the inhibition zones after appropriate time of incubation were from 0 to 21 mm with all bacteria, yeasts and fungi tested.

In growth inhibition assay performed on *Arthroderma simii* mycelium using the MIH micromethod, whereas untreated inoculum developed long hyphae (figure 1A), no visible hyphae elongation could be seen with T2, 1X2, 2N1, or 5P2 extracts (figure 1B for T2 extract). Moreover, observation under light microscope of mycelium untreated (figures 2A, 2C) and treated with these extracts, clearly indicated that the hyphae of *Arthroderma simii* were altered. The cytoplasm was contracted and void spaces appeared in the fungal cells (figure 2B for T2 extract) and protoplasm ejection (figure 2D for T2 extract) was sometimes noted.

Overall, these results led to the conclusion that the extracts of strains T2, 5P2, 2N1, and 1X2 contained highly active antimicrobial compounds.

DISCUSSION AND CONCLUSION

Within a limited geographical area, the El Jadida area coast, a large number of bacterial strains have been isolated: 46 strains (bacteria and fungi) were isolated on different media from a total of 18 samples collected from seawater, marine sediments and marine invertebrates. 31 strains displayed anti-infectious activity.

71% of these strains grew in seawater medium such as marine Sabouraud and glucose enriched Zobell medium, 19% grew in classical medium such as Luria Bertani broth or Sabouraud

Table IV - Utilization of carbon sources by strain 1X21 (oxydase positive gram negative bacilli). / *Utilisation des sources carbonées par la souche 1X21 (bacille gram négatif oxydase positive).*

Rhamnose	-	Valerate	+
N-Acetyl glucosamine	-	Citrate	+
D-Ribose	+	D-Lactate	+
D-Saccharose	-	Propionate	+
Maltose	-	Caprate	+
D-Melibiose	-	3-Hydroxybenzoate	-
L-Fucose	-	4-Hydroxybenzoate	+
D-Glucose	+	5-Ketogluconate	-
L-Arabinose	-	2-Ketogluconate	-
Mannitol	-	3-Hydroxybutyrate	+
D-Sorbitol	-	Glycogen	-
Inositol	-	Salicine	-
Itaconate	-	L-Serine	-
Suberate	-	L-Proline	+
Malonate	+	Histidine	+
Acetate	+	L-Alanine	+

+: utilized,
-: not utilized.

medium, and 10% were able to grow as well in seawater medium as in classical medium. Together with the fact that the samples were collected near the shore, these results suggest that some strains may have a terrestrial origin.

This preliminary work showed that some strains exhibit a broad activity spectrum, being able to inhibit both fungi and bacteria, but others showed a more specific activity on these groups.

In this preliminary study, antibacterial activity was evaluated only by the disk method in gelose medium, in spite of its limitations (solubility of active compounds). At this stage, determination of MIC would not be significant since molecular weight of active compounds is unknown. Hence, strains isolated from seawater or marine invertebrates exhibited significant antifungal activity, especially towards *Arthroderma simii*, while strains collected from marine sediments demonstrated weak activity. The strongest antifungal activity towards *A. simii* was shown by the strain R1, isolated from seawater.

Four strains R3, 5P2, T2, and 1X2 isolated from seawater, ascidians, clams and mussels respectively, displayed a broad antifungal spectrum of activity and antibacterial activity towards the Gram+ strain *Staphylococcus aureus* but did not exhibit any antibacterial activity towards the Gram- strain *Escherichia coli*.

Some of the strains that we have identified and which showed antimicrobial activity belong to the *Pseudomonas-Alteromonas* group, in agreement with previous reports (Gauthier *et al,*. 1975; Barja *et al.*, 1989).

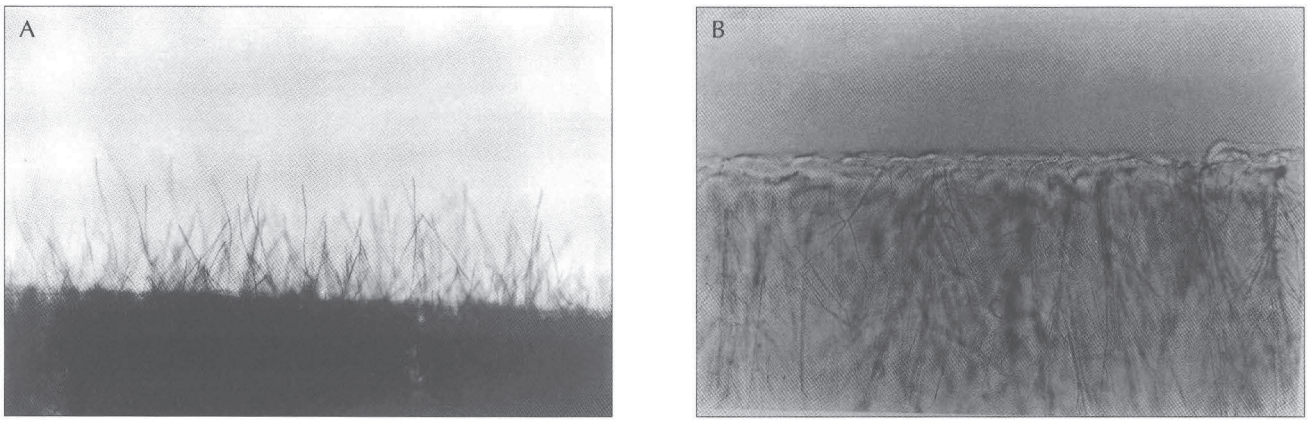

Figure 1 - Growth inhibition of *Arthroderma simii*, as shown by mean of the MIH micromethod, observed under light microscope. (A) and (B): square slices containing five day old hyphea of *Arthroderma simii* (A) untreated and (B) treated with 100 µg T2 extract. / *Inhibition de la croissance d'*Arthroderma simii, *observée en microscopie optique par la microméthode MIH. (A) et (B) : gels contenant des hyphes d'*Arthroderma simii *de cinq jours non traitées (A) et traitées (B) par 100 µg d'extrait T2.*

Table V - Antimicrobial activity of the extract of the four active species T2, 5P2, 2N1, 1X2 (inhibition zones in mm). / *Activité antimicrobienne des échantillons des quatre espèces actives T2, 5P2, 2N1, 1X2 (zones d'inhibition en mm).*

	Extracts of some species			
Test organism	T2	5P2	2N1	1X2
Arthroderma simii	21	17.5	15	16
Candida albicans	16	-	-	nd
Cryptococcus neoformans	17	-	-	nd
Staphylococcus aureus	14	10	-	10
Vibrio anguillarum	17	12	14	10

nd: not determined,
-: inactive.

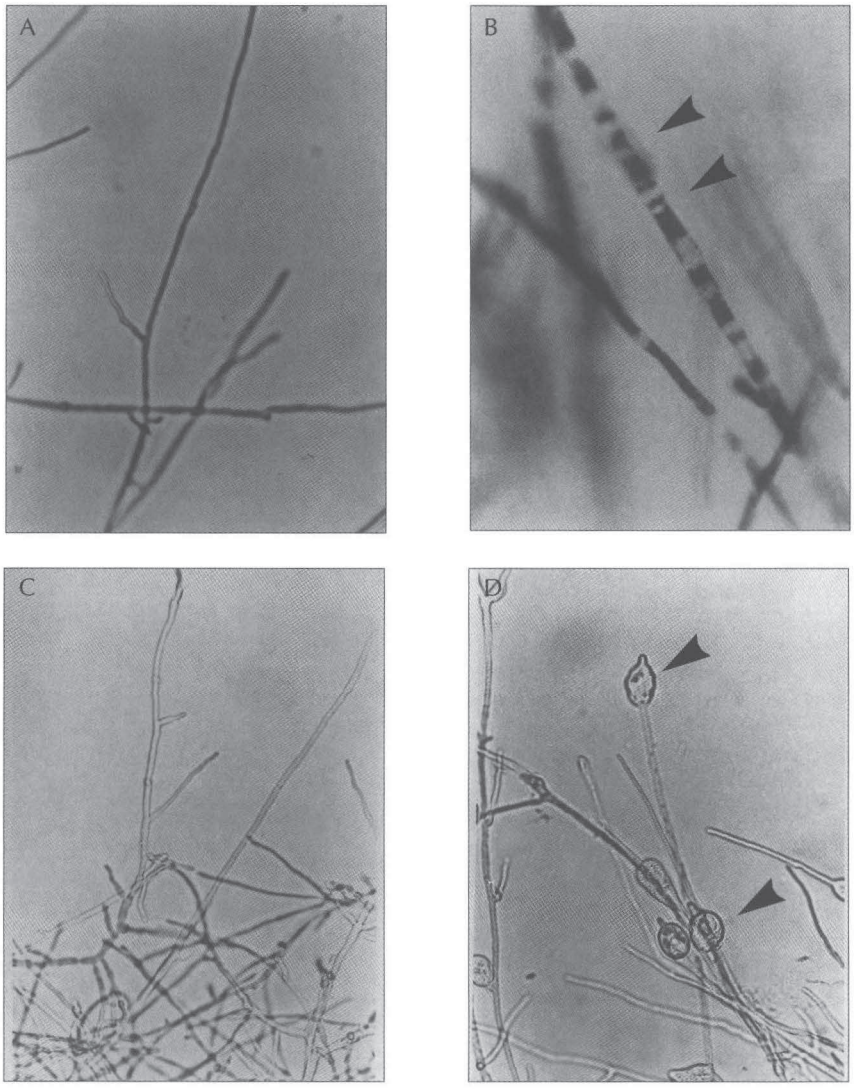

Figure 2 - Morphological alterations of *Arthroderma simii* hyphae caused by 100 µg of T2 extract observed by light microscopy. (A) and (C): untreated hyphae of *Arthroderma simii*; (B) and (D): inoculum treated for six hours with 100 µg of T2 extract. Cytoplasmic condensation (B) and protoplasm ejection (D) (arrows) were observed. / *Altérations morphologiques des hyphes d'*Arthroderma simii *provoquées par 100 µg d'extrait T2, observées en microscopie optique. (A) et (C) : hyphes non traitées, (B) et (D) : inoculum traité pendant six heures par 100 µg d'extrait T2. Des condensations du cytoplasme (B) et des éjections de protoplasme (D) (flèches) sont observées.*

This study demonstrates the great potential of the marine environment to isolate bioactive strains and clearly illustrates the fact that marine bacteria emerge as a significant resource for natural product drug development.

The bioactive extracts 5P2, T2 and 1X2 are currently being investigated to isolate the active compounds, to determine their chemical nature and to clarify their practical potential. Their antibiotic activity will be established by determination of the Minimum Inhibitory Concentration (MIC) in liquid medium.

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