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Transformation of arsenic(V) by the fungus *Fusarium oxysporum melonis* isolated from the alga *Fucus gardneri*

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A fungus isolated from the macroalga *Fucus gardneri* was identified by using 28S rDNA sequence analysis, 99% similarity match, as *Fusarium oxysporum meloni*. The fungus was exposed to arsenic(V) (500 ppb) in artificial seawater to investigate the possibility that the fungus is the source of the metabolic activity that results in the presence of arsenosugars in the macroalga. High-performance liquid chromatography coupled with inductively coupled plasma mass spectrometry was used to identify the arsenic species in the fungus, and in the growth medium. The fungus was able to accumulate arsenic(V) and an increase in arsenite and dimethylarsinate was also observed. Some reduction of arsenate led to a small increase of arsenite in the growth medium. The fungus does not seem to be involved with the accumulation of arsenosugars by the *Fucus*. Copyright © 2002 John Wiley & Sons, Ltd.

KEYWORDS: arsenic; fungus; inductively coupled plasma; polymerase chain reaction (PCR); rDNA

INTRODUCTION

Arsenic is found in the natural environment and in living organisms in different chemical forms, sometimes at elevated levels.[1–3] It is the chemical form of the arsenic compound that ultimately determines its toxicity, and the organometallic forms of arsenic found in marine animals and plants are generally believed to be less toxic than the inorganic arsenicals found elsewhere in the environment.[1,3–5] Several studies have been undertaken to determine where and how these organometallic forms of arsenic are produced within the marine animals and plants.[5–8] In one of our previous studies, cultures of the alga *Fucus gardneri*, which we assumed to be axenic, were exposed to arsenate [arsenic(V)] in the growth medium. It was found that the alga was able to accumulate the arsenate and biotransform it into arsenite [arsenic(III)] and dimethylarsinate (DMA), but not into arsenosugars, the predominant arsenic species in *Fucus*.[4] However, during the course of these studies a fungus was observed to grow with the *Fucus* in spite of the strong treatment with antimycotics and antibiotics. This fungus has now been isolated in order to determine if it might be involved in the processes that result in the accumulation of arsenosugars by *Fucus*.[4,5]

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EXPERIMENTAL

Isolation and identification of the fungus

A fungus was observed to be growing on *Fucus gardneri* after the preparation of an axenic culture. The axenation involved multiple washings with an antimycotic/antibiotic solution[9] and acclimating the *Fucus* in autoclaved seawater (400 ml) and the antimycotic/antibiotic solution (4 ml).[9] The fungus was removed from the *Fucus* surface by washing the *Fucus* with sterile seawater and then collecting it from these washings. The collected fungus was plated on full strength potato dextrose agar (PDA; DIFCO dehydrated) and incubated at 15°C and in 800 lux light in a Conviron Environmental Chamber. After an incubation period of 10 days, five samples (1 cm in diameter) were cut from the agar dishes containing the fungus and added to a 2 l Erlenmeyer flask containing 600 ml of 1/10 strength potato dextrose

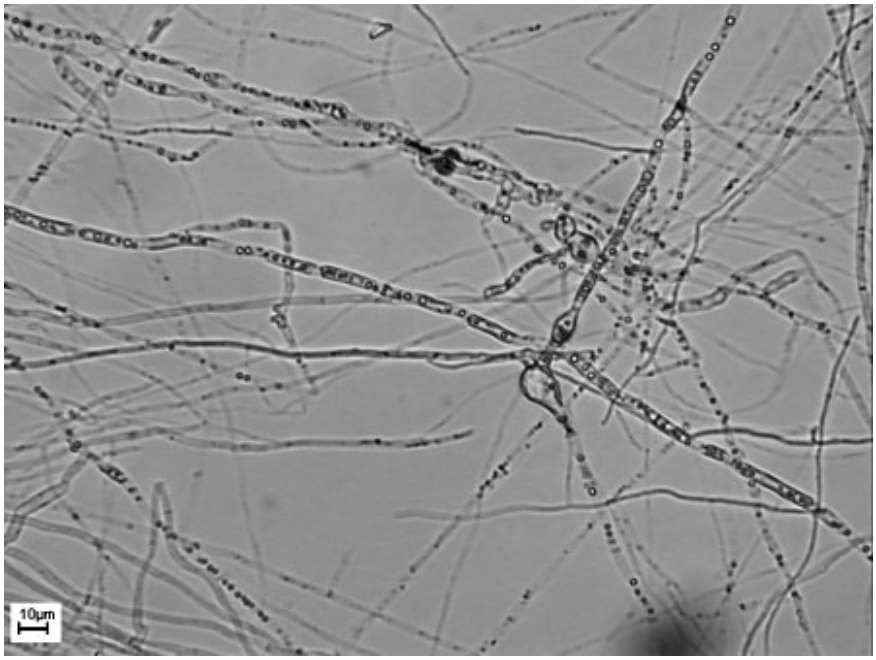

Figure 1. Micrograph of *Fusarium oxysporum melonis*.

broth (PDB; DIFCO dehydrated). Ten similar-sized pieces of agar without fungus made up from 1/2 strength PDA and an equal amount of bactoagar (DIFCO) was also added to the flasks. The plain pieces of agar were added to give the fungus a surface to grow on, and the broth made it easier to remove the fungus in order to collect enough biomass for the arsenic exposure experiments.

The fungus was allowed to grow in this broth for up to 8 weeks, at which time some of the fungus was removed from the broth, washed with artificial seawater (ASP6 F2) and then separated into two 1 l flasks, each containing 200 ml artificial seawater. To the remaining broth, sufficient 1/10 PDB was added to return the volume to 600 ml. This procedure of removing the fungus from the broth was repeated a total of three times. All the fungus in the artificial seawater was then combined into a 2 l flask with final volume of approximately 1 l for the arsenic exposure experiment. All steps performed with the fungus were done under sterile conditions. A micrograph of the isolated fungus grown in PDB is shown in Fig. 1.

DNA was extracted directly from the growing culture (1 year old) using a modified version of the sodium dodecyl sulfate (SDS) protocol described by Kurtzman and Robnett.[10] Some fungal culture (10 ml) was removed and resuspended in 30 ml of buffer (200 mM Tris–HCl (pH 8.5), 250 mM NaCl, 25 mM EDTA, 0.5% SDS). The resuspended cells were added to a Mini-Bead Beater (Biospec) containing 55 g of sterile zirconia beads (0.1 mm diameter). The cells were fractured in the bead beater for 2 min, then allowed to cool on ice for an additional 2 min. The fracturing and cooling steps were repeated a total of four times. 7 ml of the lysed cell culture was placed in a 15 ml Falcon tube and an equal volume of concentrated chloroform (99.8%, Fisher) was added. The tube was vortexed for 5 s, and then centrifuged for 10 min at 10000 rpm (Dynac Centrifuge). A 7 ml portion of the aqueous phase was removed to a new 15 ml Falcon tube and the DNA was precipitated out by the addition of isopropanol (0.54 ml isopropanol per 1 ml of aqueous phase). The tube was then centrifuged for 3 min at 10000 rpm and the resulting supernatant was discarded. The pellet was washed with 500 µl of 70% EtOH, centrifuged for 5 min at 13000 rpm (IEC Micromax), and dried in a Savant SpeedVac concentrator for 2 min at room temperature. The dried pellet was resuspended in 50 µl of sterile distilled deionized water rather than TE buffer (10 mM Tris–HCl, 1 mM EDTA); this avoids interference with the polymerase chain reactions (PCRs) resulting from chelation of the $MgCl_2$ in the PCR mixture. The pellet was dissolved by heating it in a water bath at 55 °C for 1 h and then it was stored at −20 °C.

All reagents to be used in the PCRs were tested for DNA contamination. Three PCRs were performed to ensure that at least one of the reactions would produce a good quality template. The PCR reactants, along with their concentration and the volume used in each reaction, are listed in Table 1. The genomic DNA was diluted in sterile distilled deionized water to achieve the desired concentration and then added to a 0.2 ml thin-walled tube (MJ Research). The remaining PCR reactants were added to each tube with the exception of the $MgCl_2$. All primers used in this experiment were prepared by the Nucleic Acid and Protein Sequencing (NAPS) laboratory

Table 1. Contents of PCRs

Reactants	Volume added
Genomic DNA[a]	Tube 1, 2.0 μl undiluted
	Tube 2, 2.0 μl 1:10 dilution
	Tube 3, 2.0 μl 1:100 dilution
Primer NL-1[b] (102 μM)	0.25 μl
Primer NL-4[c] (79.5 μM)	0.30 μl
10 × PCR buffer[d]	5.0 μl
dNTPs	0.40 μl
Taq DNA polymerase (5 U μl^{-1})	0.20 μl
Sterile distilled deionized water	40.35 μl
$MgCl_2$	1.5 μl
Total volume per tube	50.0 μl

[a] Diluted in sterile distilled deionized water.
[b] NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG).[10]
[c] NL-4 (5′-GGTCCGTGTTTCAAGACGG).[10]
[d] 10 × PCR buffer (200 mM Tris–HCl (pH 8.4), 500 mM KCl).

Table 2. Additives used for sequencing reactions

Reactants	Volume added
Big Dye[a]	4.0 μl
Primers	Tube 1, 0.6 μl NL-1
	Tube 2, 0.6 μl NL-2A[b]
	Tube 3, 0.6 μl NL-4
Template	4.2 μl
Sterile distilled deionized H_2O	11.2 μl
Total volume per tube	20.0 μl

[a] Big Dye (Applied Biosystems' Big-Dye Terminator Cycle Sequencing Reaction).
[b] NL-2A (268.86 μM) (5′-CCTGGTCGCTATCGGTCTC).[10]

at the University of British Columbia, Vancouver, BC. To avoid polymerization prior to the first denaturation step, two measures were taken. The first was to cool the tubes containing the reactants (except $MgCl_2$) to 0 °C before being transferred to the 94 °C preheated thermal cycler (MJ Research MiniCycler); the second was to allow denaturation of the template at 94 °C to occur, prior to the addition of the 1.5 μl of $MgCl_2$.

Amplification was performed for 30 cycles with denaturation at 94 °C for 45 s, annealing at 55 °C for 40 s, and elongation at 72 °C for 90 s. The cycle also included an initial 3 min at 94 °C and a final 10 min at 72 °C to ensure full denaturation and elongation respectively. Visualization of the amplified DNA was achieved by using a 0.8% agarose gel (1% strength, GibcoBRL) in 1 × TAE buffer, (2 M Tris–glacial acetic acid (pH 8.5), 0.1 M $Na_2EDTA{\cdot}2H_2O$), stained with 30 μl of ethidium bromide, and run at 85 v for 35 min. The brightest band was cut from the gel, and the amplified DNA was removed by using a QIAGEN QIAquick gel extraction kit. A second PCR was performed in an effort to increase the concentration of template with the extracted amplified DNA following the same procedure as above, including diluting the template (Table 1), with the only change being an increase in the number of cycles from 30 to 40. The amplified template was viewed under UV light and the best band was cut and removed from the gel. It was the amplified DNA from this second cut gel band that was used in the sequencing reactions.

Three sequencing reactions were set up employing the reactants listed in Table 2. The sequencing reactions were conducted in an MJ Research minicycler by using the 'Bigdye' fluorescent-labelled DyeDeoxy protocol established for the Perkin–Elmer Model 480. Amplification was performed for 25 cycles with denaturation at 96 °C for 30 s, annealing at 50 °C for 15 s, and elongation at 60 °C for 4 min. There was an initial denaturation of 1 minute at 96 °C. The excess DyeDeoxy terminators in each reaction were removed by using CENTRI-SEP Columns (Princeton Separations). The samples were then dried in a Savant Speed-Vac concentrator for 25 min at room temperature, and then sent to NAPS for sequence determination. Sequences were determined by using an automated Applied Biosystems DNA sequencer. Output from the sequencer was collected by software on a Macintosh computer during the electrophoresis run as it was generated.

The DNA sequences generated were aligned by using the NCBI website[11] and submitted to the BLAST database for a similarity match.

Exposure of the fungus to arsenic(V) and DMA

The artificial seawater used in the arsenic exposure experiments was prepared according to the recipe for ASP6 F2, which was previously used by Fries[12] to culture members of the family Fucaceae. The pH of the artificial seawater was adjusted to the same pH as the seawater that was collected with the algae by the addition of HCl.[4] The vitamin solution and the vitamin B_{12} solution were sterilized by using 0.22 μm sterile filters and then added to the autoclaved seawater. The artificial seawater with the vitamins was then stored at 4 °C.

Details are available of chemical sources, purity, and cleaning methodology.[4,5]

The fungus was removed from the PDB broth and placed into artificial seawater, washed with artificial seawater (500 ml), and then redistributed into six 1 l flasks each containing 200 ml of artificial seawater and either arsenic (V) or DMA (50 μl of 1000 ppm stock solutions), or neither. No antimycotic/antibiotic solution was added to the artificial seawater. A summary of the treatment is shown in Table 3.

All treatments of the fungus were carried out under sterile conditions and the flasks were sealed with sterile cotton-plugs. The flasks were incubated at 15 °C in the dark in a Conviron Environmental Chamber. From each flask, 1 ml samples of media (no fungus) were taken under sterile conditions. Samples were taken at intervals doubling in time (0, 2, 4, 8, 16 h, etc) with the exposure experiment lasting 45

Table 3. Growth conditions for *Fusarium oxysporum melonis* in the presence of arsenic species

Flask #[a]	ASP6 F2 medium[b]	Fungus	Arsenic
1	200 ml	*	0.50 ppm As(V)
2	200 ml	*	0.50 ppm DMA
3	200 ml	*	
4	200 ml		0.50 ppm As(V)
5	200 ml		0.50 ppm DMA
6	200 ml		

[a] All flasks were duplicated.
[b] Flasks did *not* contain antimycotic/antibiotic solution.

days. The flasks were swirled during sampling to ensure adequate dissolved oxygen in the medium.

All media samples taken during the exposure experiment were frozen (−20°C) immediately to preserve sample integrity until they were analysed by using anion-exchange high-performance liquid driomatography coupled with inductively coupled plasma mass spectrometry (HPLC–ICP-MS) (Table 4).[4,5]

The fungus samples were isolated by filtering the medium through a glass funnel lined with Whatman filter paper after 45 days of exposure to the arsenic compounds. The fungus samples were rinsed with sterile deionized water and then frozen, while embedded on the filter paper, and kept at −20°C until needed for extraction as described next. A filter paper without the fungus was used as a control.

The arsenic species were extracted from the frozen fungus samples (fungus and filter paper) by using a procedure similar to that described by Shibata and Morita.[13] (See Refs 4 and 5). Kelp powder (a laboratory standard), oyster tissue SRM (NIST-1566a) and *Fucus* sample (IAEA-140/TM) were similarly extracted as reference materials. The extracts were speciated for arsenic as described previously.[4,5]

RESULTS

The fungus identified as *Fusarium oxysporum melonis* (see below) grows in a variety of media in temperatures varying from 7 to 15°C. The fungus exhibits morphological changes as it is subjected to new environments. At the macro-level the most noticeable change is in colour. The fungus is white and

Table 4. Summary of experimental HPLC conditions

Conditions	Column	Mobile phase[a]	Flow rate (ml min^{-1})
Anion exchange (medium samples)	Hamilton PRP X 100	20 mM phosphoric acid, pH 6.0	1.5
Ion pairing (extract samples)	Inertsil ODS (GL Sciences, Japan)	10 mM tetraethylammonium hydroxide, 4.5 mM malonic acid, 0.1% MeOH pH 6.8	1.0

[a] The mobile phases were filtered through a 0.45 μm filter (Millipore) after they were made up.

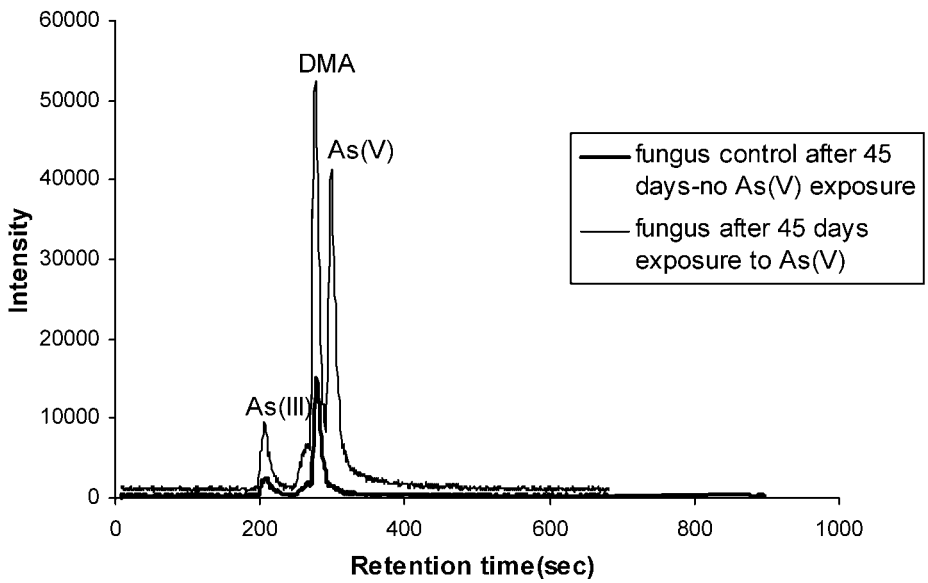

Figure 2. Arsenic species (HPLC–ICP-MS) in extracts of *Fusarium oxysporum melonis* after exposure to arsenic (V) in artificial seawater medium (ASP6 F2).

Table 5. Arsenic speciation of *Fusarium oxysporum melonis* extracts after As(V) exposure

	Arsenic species found (ppb, fresh weight)		
Fungus sample	As(III)	DMA	As(V)
Control, no As(V) exposure	1.08 ± 0.09[a]	5.0 ± 0.3	0
After exposure to As(V)	3.3 ± 0.2	14.7 ± 0.7	16.1 ± 0.8
After exposure to DMA	1.05 ± 0.09	5.3 ± 0.3	0

[a] Standard deviation from analytical results obtained with the calibration curve.

fluffy when collected from the *Fucus gardneri* exposure experiment.[5] It turns to a purple colour with white streaks once it starts growing on the petri dish with full strength PDA, it turns to a yellowish colour in 1/2 PDB and to a red/purplish colour in 1/10 PDB. On transfer to artificial seawater, the fungus is a light purple colour.

The fungus was isolated from *Fucus gardneri* after a 14 day growth period and identified by sequencing its 28S ribosomal RNA gene. Two PCRs were employed that used the primer pair NL-1 and NL-4 to amplify the 28S rDNA sequence. The PCR was performed on the amplified DNA in an effort to maximize the concentration of the template to be used in the sequencing reactions. The sequence of the 28S rDNA of the fungus strain was determined and analysed by using the BLAST system database. For 49 out of 50 sequences retrieved, similarity values of 96–99% established that the fungus strain was most closely related to members of the genus *Fusarium*. A similarity match of 99% was found to correspond with *Fusarium oxysporum melonis* for positions 1 to 585 of the 28S ribosomal-like gene.

Arsenic species in both the medium and the fungus were examined after the fungus was exposed to arsenic(V) and DMA in artificial seawater for 45 days. The predominant arsenic species found in the MeOH–H_2O extracts of the fungus after arsenic(V) exposure were arsenic(III), DMA and arsenic(V) (Fig. 2 and Table 5). Controls consisted of fungus samples maintained under the same conditions except that they were not exposed to any arsenic compounds, and these samples showed the presence of arsenic(III) and DMA species (Figure 2) at concentrations about three times less than found in the exposed fungus (Table 5). This indicates that there was an external source of arsenic(V); this was probably the culture medium, because *acid* digestion revealed that PDA contained ~0.33 ppm of arsenic, and PDB contained ~0.26 ppm, both on a dry weight basis.[14]

The medium samples from the *Fusarium* exposure experiments were analysed by using the anion-exchange HPLC–ICP-MS conditions described in Table 4. Essentially, the arsenate concentration changed little over the 45 days of the experiment (Figure 3). There was a slow increase in arsenite concentration up to a maximum of 10% reduction of the added arsenate.

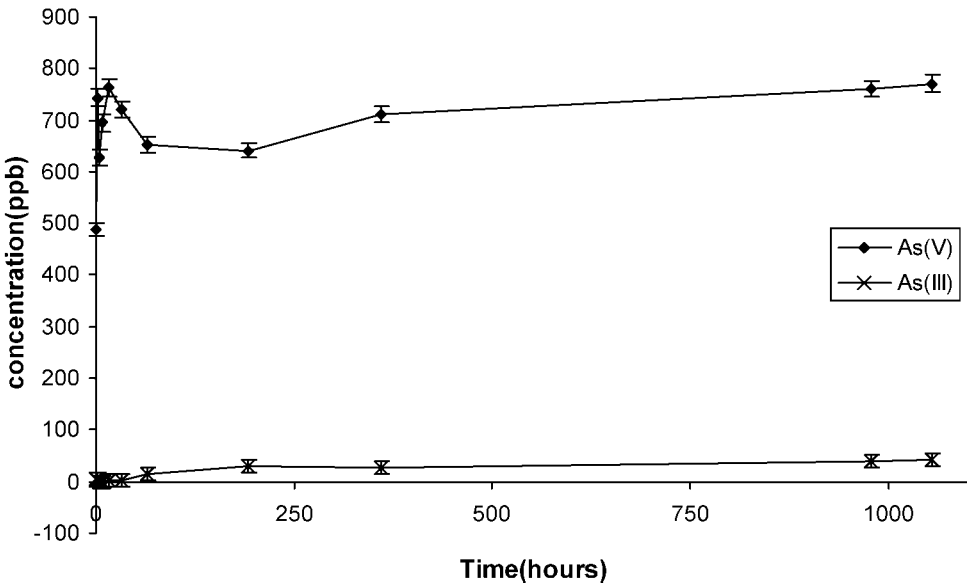

Figure 3. Time course of arsenic species (HPLC–ICP-MS) in arsenic (V)-enriched artificial seawater medium samples collected during the growth of *Fusarium oxysporum melonis*.

The DMA exposure experiment was also performed for 45 days; however, the *Fusarium* extracts after exposure were similar to the controls (Table 5) and the medium collected did not show any observable changes.

DISCUSSION

The *Fusarium* species are widely distributed in soil and on organic substrates and have been isolated from insects, running water, plants, permafrost in the Arctic, and from the sands of the Sahara.[15] They abound in cultivated soil, both in temperate and tropical regions, and are amongst the fungi most frequently isolated by plant pathologists.[15] As with many soil fungi, they are abundantly endowed with various means of survival, one of the mechanisms of which is the capacity for rapid change, often morphologically as well as physiologically, to a new environment. Thus they can survive on a wide range of substrates and have been isolated from many preserved foods, from stored chemicals and from aircraft fuel tanks.[15,16]

Fungi are often found growing with algae in a parasitic relationship, as has been recognized in Japanese 'Nori' farms since the 1940s. Fungi can affect the algal cells by competing for nutrients, by changing the physical state of the medium, and by releasing substances that inhibit the growth of or kill the host cells.[17,18] In the present case, *Fusarium oxysporum melonis* was isolated from the macroalga *Fucus gardneri* after the preparation of an axenic culture.[4,5] The *Fusarium* grows with the algal samples even after the algal samples undergo a strong cleaning treatment that requires the *Fucus gardneri* to be washed five to eight times in sterile seawater followed by an acclimation period of 14 days in seawater containing an antimycotic/antibiotic solution.[5] *Fusarium* has the ability to penetrate the vascular tissue of roots and stems in plants, which might account for the difficulty experienced in removing the fungus from the algae.[15] It is likely that a stronger decontamination treatment would have killed the *Fucus*.

Because macroalgae are well known to accumulate arsenic in the form of arsenosugars, and because the biological pathway for this accumulation is not known, there was the possibility that the *Fusarium* species was responsible for the transformation, and the macroalga simply acted as a store-house. This notion was tested by exposing the fungus to arsenate and DMA, both possible precursors to the arseno-sugars.

Fusarium oxysporum melonis is capable of accumulating arsenic(V) from the surrounding medium and transforming it into arsenic(III) and DMA; however, this metabolic activity does not seem to be high, because only a slight increase of DMA is seen (Fig. 2). This conclusion is reinforced by the very slow conversion of arsenate to arsenite in the growth medium, and the absence of any methyl arsenicals (Fig. 3). Organisms that biotransform arsenate invariably carry out this reduction to arsenite as a first step. The fungus does not metabolize DMA, which is another putative arsenosugar precursor. The fungus does not produce any arsenosugars from either of the arsenicals added.

On this basis, it can be concluded that the metabolism of arsenic by *Fucus*, the host macroalgae, is probably independent of *Fusarium*. However, this may not be true if the relationship between the two species is symbiotic and the production of the arsenosugars requires the participation of both species. In any case, the origin of the arsenosugars must remain obscure.

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