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FULL PAPER

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Phoma sp. FOM-8108, a producer of gentisylquinones, isolated from sea sand

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Abstract A fungal strain, FOM-8108, that was isolated from sea sand was found to produce gentisylquinone and chlorogentisylquinone, inhibitors of neutral sphingomyelinase. The fungus grew well under normal conditions, in the darkness, or on various agar media, but typical morphological characteristics were not observed to determine its taxonomy. Therefore, culture conditions were studied extensively, resulting in formation of a number of pycnidia by the fungus on the media containing seawater or on natural substrates such as hydrangea leaves, gardenia leaves, and rice straw under natural light or near-ultraviolet radiation exposure. Eventually, from the morphological characteristics of pycnidia, conidiogenous cells, and conidia, strain FOM-8108 was considered to belong to the genus *Phoma*. Culture studies showed seawater in the fermentation medium was necessary for the strain to produce gentisylquinones. Particularly, a full production of chlorogentisylquinone, which has a chloride ion in the structure, was performed in the medium with higher concentration (75%–100%) of seawater.

Key words Gentisylquinones · Light exposure · Marine fungus · *Phoma* sp. FOM-8108 · Pycnidia formation

Introduction

During the course of our search for new bioactive compounds from marine microoganisms, a fungal strain, FOM-8108, that was isolated from sea sand was found to produce inhibitors of sphingomyelinase, which is involved in important intracellular functions such as signal transductions, inflammation, and apoptosis (Hannun 1994; Levade and Jaffrezou 1999). Two structurally related inhibitors, a known compound, gentisylquinone, and a novel compound designated chlorogentisylquinone (Fig. 1), were isolated from the culture broth of strain FOM-8108 (Uchida et al. 2001). The producing fungus showed no typical morphological characteristics such as sexual or asexual organs to define its taxonomic position, even after 1 month of cultivation under normal conditions. Taking into consideration that the fungus originated from a marine environment, cultural characteristics of the strain were tested to form the morphogenesis of the fungus for taxonomic analysis. Finally, we could induce the fungus to produce asexual organs as pycnidia and found that the fungus belongs to the genus *Phoma* Sacc. Furthermore, we showed that the production of gentisylquinones by the fungus is dependent on the presence of seawater in the fermentation medium.

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Materials and methods

Strain

Fungal strain FOM-8108 was isolated from marine sand collected in Enoshima seaside, Kanagawa Prefecture, Japan. The strain was maintained on potato dextrose agar dissolved in seawater (PD/SW) slants composed of 19.5 g potato dextrose agar (PDA; Difco, Michigan, USA) and 7.5 g agar in 1 l 50% seawater (500 ml of natural seawater and 500 ml of distilled water) and Miura/SW slants composed 1 g glycerol, 0.2 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl, 2 g $NaNO_3$, 0.2 g yeast extract, and 15 g agar in 1 l 50% seawater.

Media

For morphological characterization, PDA, cornmeal agar (CMA; Difco), and malt extract agar (MEA) composed of 20 g malt extract (Difco), 1 g peptone, 20 g glucose, and 20 g agar in 1 l of water were used. To study the effect of seawa-

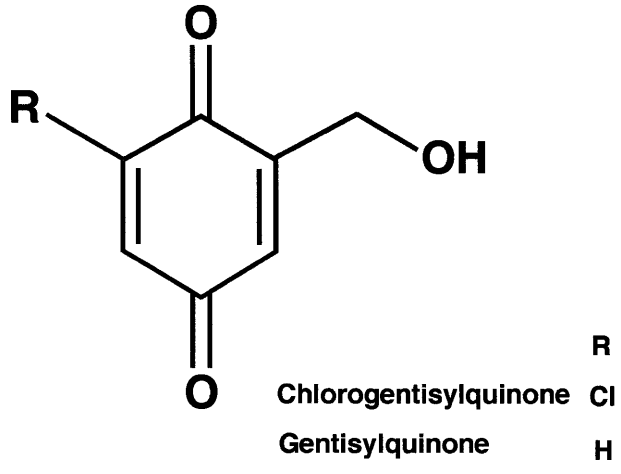

Fig. 1. Structures of gentisylquinones

ter concentration on cultural characteristics, each medium was dissolved in 0% (distilled water), 25%, 50%, 75%, or 100% seawater. Three plates were used in each condition.

Culture on media

Mycelial disks (4 mm in diameter) from PD/SW plate cultures of the strain were inoculated on the center of 90-mm glass dishes containing each agar medium and were incubated at 25°C for 7–30 day or longer.

Culture on substrates

The experiments on substrates were carried out according to the agar-leaf disk method (Kishi 1995). Hydrangea leaves, gardenia leaves, and rice straw were used as natural substrates. The strain was inoculated on the center of 90-mm glass dishes containing plain agar (15 g agar in 1 l distilled water), and the substrates (2–3 cm^2), boiled for 1 min, were placed around the inoculation point. Those plates were incubated at 25°C for 7–30 days or more. Pretreatment of substrates by boiling was sufficient for sterilization.

Light exposure

The dishes of the strain inoculated on media with 0%–100% seawater and on substrates were incubated in the darkness or under natural light or near-ultraviolet radiation (NUV) exposure. The experiment with NUV exposure was carried out under a black light blue (BLB) lamp (FL15 BLB; Toshiba, Tokyo, Japan) with 352 nm as a peak wavelength in a shaded box, respectively.

Fixation and sectioning of pycnidia

The pycnidia, which were produced on the rice straw, were fixed by immersion in 8% formaldehyde, frozen in Tissue-Tek O.C.T. compound (Sakura, Tokyo, Japan), and stored at −20°C until sectioning (Ishii et al. 1993; Kuenzi and Sherwood 1995). Frozen pycnidia were cut in 5-μm sections with the Cryostat (Leica, Nusslosh, Germany) at −20°C. Then cross sections were observed under a light microscope (Vanox-S AH-2; Olympus, Tokyo, Japan) and scanning electron microscopy (SEM).

Scanning electron microscopy

For SEM observation, spores and sectioned pycnidia were fixed with vapor from OsO_4 crystalline in a glass-stoppered flask for 1 h at room temperature. Sections were coated with gold by using the fine coater (JFC-1200; JEOL, Tokyo, Japan) and observed with the JSM 5600 (JEOL) operated at 15 kV.

Production of gentisylquinones

The seed media, containing 20 g glucose, 2 g yeast extract, 0.5 g $MgSO_4 \cdot 7H_2O$, 5 g polypepton, 1 g KH_2PO_4 and 1 g agar in 1 L of 0% (tap water), 25%, 50%, 75%, or 100% seawater, and the production media containing 24 g potato dextrose broth, 10 g $Mg_3(PO_4)_2 \cdot 8H_2O$ in 1 l 0%, 25%, 50%, 75%, or 100% seawater were used for gentisylquinones production. The strain on a PD/SW slant was inoculated into a test tube containing 10 ml of the seed medium, which was shaken at 27°C for 3 days. The seed culture (1 ml) was transferred to a 500-ml erlenmeyer flask containing 100 ml of the production medium, which was then shaken on a rotary shaker (210 rpm) at 27°C for 6 days. The pH of the culture broth (1 ml) was adjusted to 8–9 and gentisylquinones were extracted with ethyl acetate. The ethyl acetate layer was concentrated to dryness and the remaining material was dissolved in methanol to determine the amount of gentisylquinones.

Quantitative analysis of gentisylquinones by LC/UV

The productivity of gentisylquinones was analyzed by liquid chromatography with ultraviolet detection (LC/UV) (HP 1100 system; Hewlett Packard, CA, USA). Conditions were as follows: column, Symmetry C18/3.5 μm (i.d. 2.1 × 150 mm, Waters); mobile phase, a 20-min linear gradient from 0.05% phosphoric acid to 40% acetonitrile; flow rate, 0.2 ml/min; detection, UV at 254 nm. Gentisylquinone and chlorogentisylquinone were eluted as peaks with retention times of 11.3 and 7.2 min, respectively.

Results

Effects of seawater and light exposure on morphogenesis

Colony appearances of the cultures at 25°C for 14 days on PDA with different seawater concentrations or under different light exposures are shown in Fig. 2. Growth rates

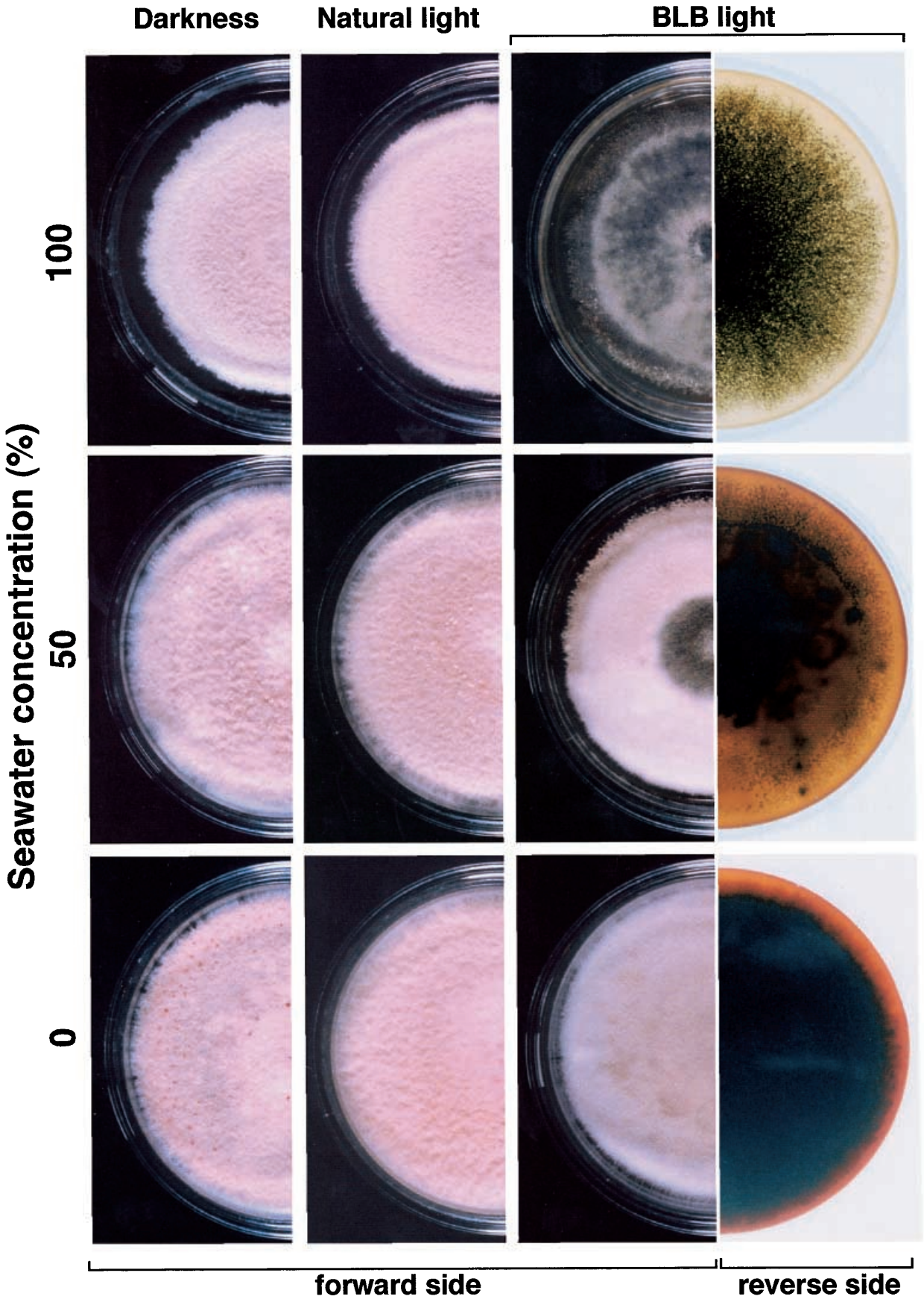

Fig. 2. Colony morphology of the strain FOM-8108 on potato dextrose agar (PDA) with different seawater concentrations, cultured in the darkness or under different light exposure, at 25°C for 14 days. Black light blue (BLB)

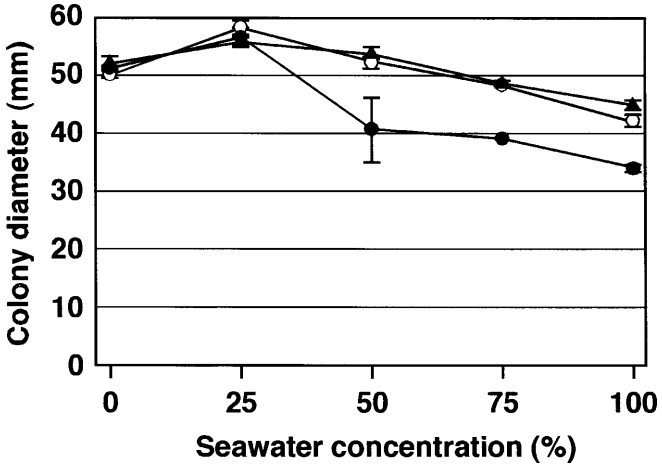

Fig. 3. Growth rate of FOM-8108 on PDA with different seawater concentrations, at 25°C for 14 days. Mean values of colony diameter ($n = 3$) after incubation in the darkness (▲), under natural light (●), or under BLB light (○) exposure

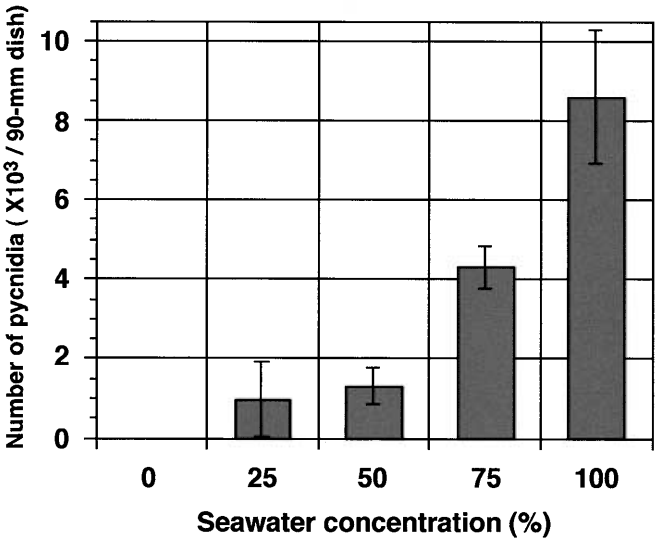

Fig. 4. Number of pycnidia of strain FOM-8108 formed on a cornmeal agar (CMA) plate with different seawater concentrations under BLB light exposure at 25°C for 14 days. The number of pycnidia was counted under a stereoscopic microscope (Olympus SZH10)

measured from the colony size on the media with 0%–25% seawater were almost the same, whereas growth rates on the media with greater than 50% seawater were slower (Fig. 3). Light exposure showed no effect on growth rate, as colony sizes were almost the same in the darkness and under natural light or BLB light exposure (Fig. 3). Similar results were shown on MEA and CMA media (data not shown). Under BLB light exposure, dark brown pycnidia were formed on the colony surface (Fig. 2), and the number of pycnidia increased dramatically according to the seawater concentration in the medium (Figs. 2, 4). A large number of pycnidia were formed on CMA with 100% seawater (Fig. 4). A very few pycnidia were formed under natural light after more than 30 days, and no pycnidia were observed under darkness even on the medium with 100% seawater after more than 30 days. Similar results were obtained on PDA and MEA media (data not shown).

Effects of substrates and light exposure on morphogenesis

Pycnidia formation was induced by incubating the strain on plain agar with each substrate under only BLB light exposure at 25°C for 14 days. The pycnidia were semiimmersed or immersed in the substrates (Fig. 5A). On the other hand, no pycnidia were observed under darkness and natural light exposure after more than 30 days.

Morphological characteristics of pycnidia

The dark brown pycnidia formed on the rice straw were cut in sections to observe the morphology under a light microscope. As shown in Fig. 5B, a number of slimy spores were formed abundantly within the pycnidia. The pycnidia were pseudoparenchymatous, thin-walled, glabrous or often with some semipilose, with a single ostiole, and not aggregated on a stroma. Some difference was observed in morphology of pycnidia formed on the agar media from those on the substrates. Pycnidia formed on the substrates were ventricose rostrate in form, whereas pycnidia on the agar media with seawater were of globose to subglobose form. Further, pycnidia on substrates were 50–200 μm in diameter whereas those on the media with higher seawater (75%–100%) was usually less than 50 μm. SEM observation of the section showed the conidiogenous cell was phialidic. Conidium-forming phialides are shown in Fig. 5C,D. The conidia were aseptate or rarely 1-septate, navicular to ellipsoidal, 2.5–5.5 × 1.5–2.0 μm, and hyaline (Fig. 5E). The chlamydospores were unicellular. Based on these characteristics, strain FOM-8108 was considered to belong to the genus *Phoma* (Domsch et al. 1993; Kobayashi et al. 1992, Kobayashi 1994; Sutton 1964). Furthermore, the strain was considered to be classified in *Phoma* Sacc. sect. *Phoma* with the dichotomous key to the sections based on characteristics in vitro (Boerema 1997).

Effect of seawater concentration on gentisylquinones production

The time course of gentisylquinones production in cultures of the strain FOM-8108 with different concentrations of seawater is shown in Fig. 6. Gentisylquinones were produced only in the presence of seawater in the culture medium. Almost full production of gentisylquinone (1800–2500 mg/l) was obtained at a wide range of seawater concentrations (25%–100%) whereas the incubation times for maximum production were slightly different (at day 2 for 25%–50% seawater vs. at day 3 for 75%–100% seawater). On the other hand, full production of chlorogentisylquinone (~340 mg/l) was found in the media with higher concentrations of seawater (75%–100%) at day 4, beyond the time for the maximum production of gentisylquinone.

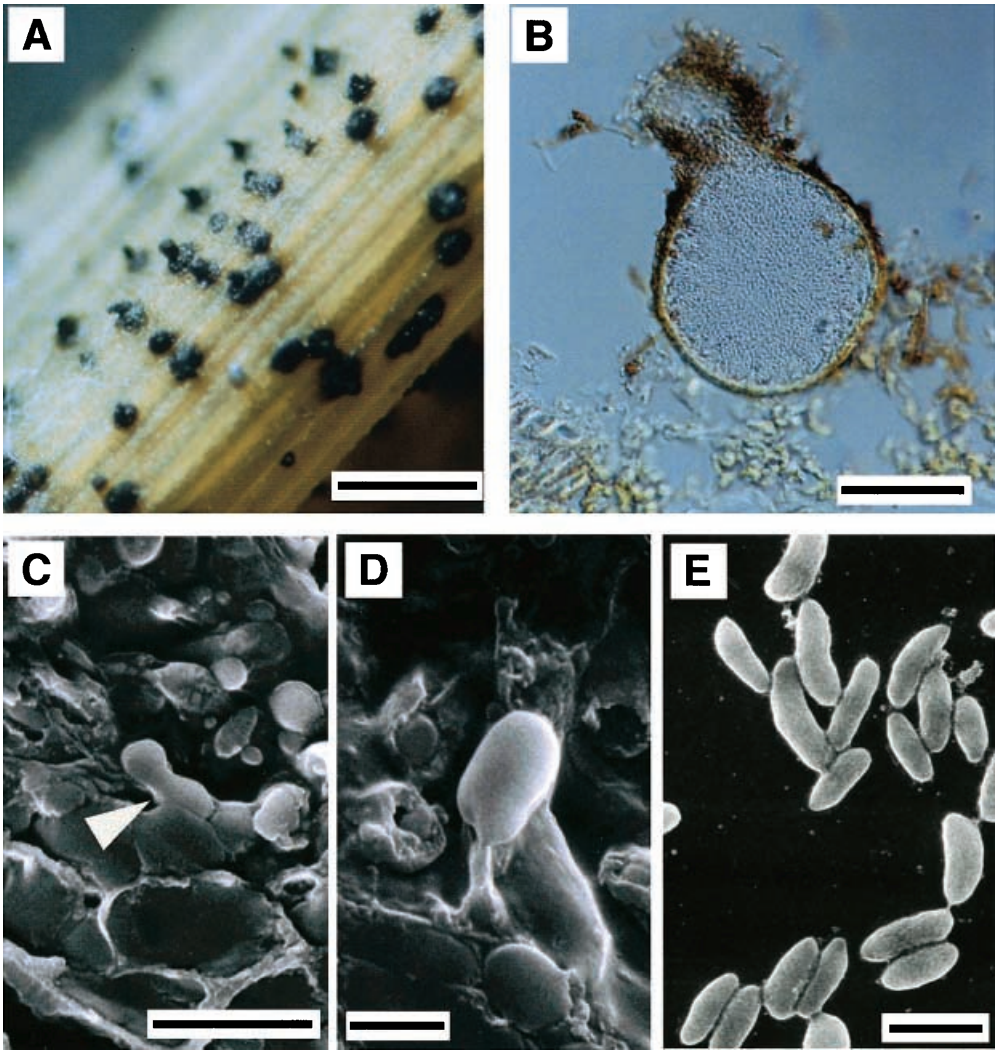

Fig. 5. Photomicrographs of FOM-8108. **A** Pycnidia formed on a rice straw under BLB light at 25°C for 14 days. **B** Cross section of pycnidia from a rice straw. **C** Scanning electron microscopy (SEM) of conidium forming a conidiogenous cell (*arrowhead*). **D** SEM of a phialide producing a conidium. **E** SEM of conidia. *Bars* **A** 500 μm; **B** 50 μm; **C**, **E** 5 μm; **D** 2 μm

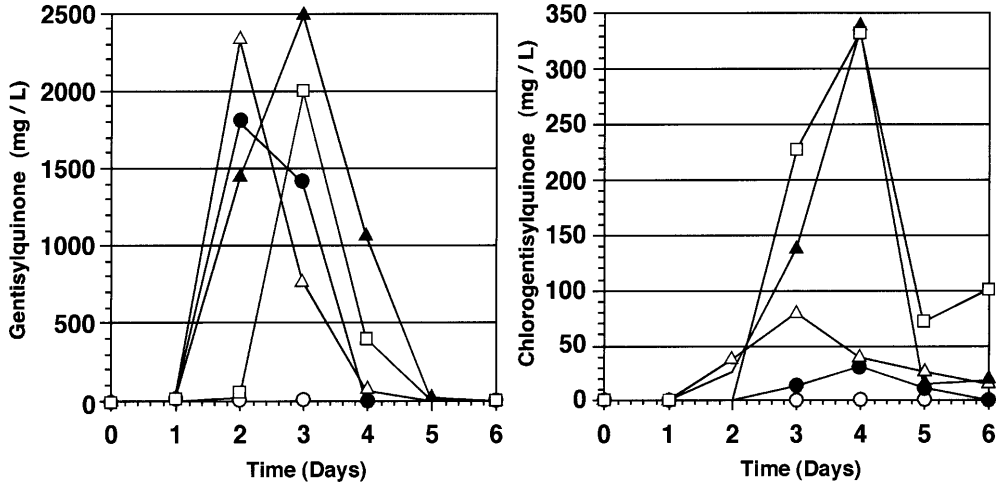

Fig. 6. Effect of seawater concentrations on gentisylquinones production: 0% (○), 25% (●), 50% (△), 75% (▲), and 100% (□)

Discussion

The growth and morphogenesis of reproductive organs in many fungi are affected by various environmental factors such as light exposure, temperature, humidity, and nutritional conditions. Under normal culture conditions, fungi such as strain FOM-8108 do not always show typical morphological characteristics such as the sexual or asexual organs that are necessary to identity their taxonomic position.

Therefore, to determine the taxonomic position of strain FOM-8108, various cultural conditions were studied as reported in this article.

We found that light exposure is necessary for pycnidia formation of strain FOM-8108. Especially, BLB light exposure was most effective for morphogenesis, while natural light exposure was effective only on the media with a high seawater concentration (see Fig. 2). The importance of light exposure for morphogenesis has been reported in many basidiomycetes, which need exposure to light for fruit body formation in the perfect stage (Kitamoto et al. 1968; Perkins and Gordon 1969; Morimoto and Oda 1973; Schwalb and Shanler 1974; Tan 1977; Eger-Hummel 1980; Geon et al. 1995), and in many ascomycetes, which also require light for sporulation in the imperfect stage (Gressel and Hartmann 1968; Kumagai and Oda 1969; Tan 1977). These fungal developments by light exposure may be related to their photoreceptors (Briggs 1976; Tan 1977).

Kishi (1995) reported that natural substrates were useful for morphogenesis of certain fungi that had shown no typical morphological characteristics on the usual media. Regarding strain FOM-8108, abundant pycnidia were formed on substrates such as rice straw under BLB light exposure (Fig. 5A), allowing taxonomic analysis of the strain. The genera *Phoma* and *Aposphaeria* Sacc. show very similar morphological characteristics (Clements and Shear 1931). To distinguish them, Kobayashi et al. (1992) described that the conidiomata of *Aposphaeria* are superficial on the substrates, whereas those of *Phoma* are semiimmersed or immersed. The strain FOM-8108 showed semiimmersed or immersed conidiomata on the substrates (Fig. 5A), leading to the conclusion that the strain belongs to the genus *Phoma*.

Effects of seawater concentration on morphogenesis were tested because the strain was isolated from the marine environment. Byrne and Jones (1975) reported that asexual reproduction of terrestrial fungi imperfecti was slightly inhibited by high salinities although their hyphal growth exhibited a broad tolerance to increasing salinities. In contrast, pycnidia formation of the strain FOM-8108 was enhanced by the presence of seawater in media under BLB light exposure, although the hyphal growth rate gradually decreased (Figs. 3, 4). Thus, the salinity response of this marine strain is completely different from that of terrestrial fungi imperfecti.

We isolated a number of fungal strains from marine environments including typical terrestrial fungi such as *Aspergillus* and *Trichoderma*. These fungi show better growth and metabolite production in the presence of seawater, whereas terrestrial isolates tend to show suppressed growth and metabolite production in the presence of seawater (Masuma et al. 2001). The marine isolate FOM-8108 produced gentisylquinones only in the presence of seawater (see Fig. 6). Seawater could be replaced with NaCl (4%) for gentisylquinones production (data not shown). Production of chlorogentisylquinones was enhanced at the higher concentrations (75%–100%) of seawater (Fig. 6). A high-salt condition usually causes a harmful effect on cells as stress. Several different mechanisms of tolerance against salt stress in microorganisms have been reported. *Saccharomyces cerevisiae* Meyen ex Hansen actively extrudes Na^+ through the P-type Na^+-ATPase under high salt stress (Park et al. 2001). In *Candida albicans* (Robin) Berkhout, cellular Cl^- concentration is regulated with Cl^- transport systems (channels and exchangers) (Northrop et al. 1997). Thus, it is possible that the strain FOM-8108 extrudes cellular Cl^- as a form of chlorogentisylquinone under higher salt condition.

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