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NOTE

First record of *Pythium grandisporangium* in Japan

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Abstract *Pythium grandisporangium* was isolated from roots of common reed and seawater in a saline marsh in Osaka Bay for the first time in Japan. Morphological features and rDNA ITS sequences were described on isolates of the species. The isolates grew on corn meal agar containing 0–9% sea salts, with 1–3% being the ideal range for growth. The isolates produced no disease on roots of common reed, but they colonized root surfaces in in vitro inoculation studies.

Keywords Common reed · *Phragmites australis* · Saline marsh · Seawater

Pythium grandisporangium Fell and Master is a pythiaceous oomycete that inhabits saline marshes (van der Plaats-Niterink 1981). It has only been reported from fallen leaves in mangroves in the USA (Fell and Master 1975; Newell 1992) and marine algae in Canada and the Netherlands (de Cock 1986). *P. grandisporangium* is most closely related to *P. insidiosum* de Cock, L. Mend., A. A. Padhye, Ajello and Kaufman based on sequence similarity of the ribosomal DNA internal transcribed spacers (rDNA ITS), and they form a monophyletic clade in the phylogeny of *Pythium* (Schurko et al. 2003; Lévesque and de Cock 2004). However, there are few morphological similarities between them (Lévesque and de Cock 2004). *P. grandisporangium* forms pyriform sporangia and plerotic oospores but the latter produces filamentous non-inflated sporangia and aplerotic oospores (de Cock 1986; de Cock et al. 1987). Antheridium longitudinally applied to the oogonium is the only morphological trait that these two species have in common. Despite the unique taxonomical status of *P. grandisporangium*, the characterization of this species is limited to a few isolates (Fell and Master 1975; de Cock 1986).

In November 2006 and March 2007, *P. grandisporangium* was isolated from roots of common reed, *Phragmites australis* (Cav.) Trin. ex Steud., and seawater (salinity was a minimum of 1.8% and a maximum of 2.7%) from a saline marsh in Osaka Bay, Japan. The objectives of this study were to characterize the Japanese isolates of *P. grandisporangium* by morphology, by identifying the sequences of the rDNA ITS, and by testing its pathogenicity to *P. australis*.

Isolates OPU795 and OPU796 recovered from a saline marsh in Sennan City (E135°17′, N34°22′), Osaka were used in this study. Isolate OPU795 was recovered from a root of common reed in November 2006. Isolation was performed on *Pythium* selective medium (Morita and Tojo 2007) composed of 17 g corn-meal agar (CMA) (Becton, Dickinson and Company, Franklin Lakes, USA), 10 mg nystatin (Sigma-Aldrich, St. Louis, USA), 250 mg ampicillin (Sigma-Aldrich), 10 mg rifampicin (Sigma-Aldrich), 1 mg miconazole (Sigma-Aldrich), 15 g sea salts (Sigma-Aldrich) and 1 l distilled water (PSM). Three matured plants were collected from the saline marsh, and a total of 20 root pieces were obtained. The root pieces were approximately 1 mm in diameter and 20 mm long. They were washed in tap water to remove soil particles, placed on PSM, and incubated at 25°C in darkness for 3 days. Mycelia that appeared on PSM were transferred to water agar composed of 15 g agar (Wako, Osaka, Japan), 15 g sea salts and 1 l distilled water (SWA). Single hyphal tips of the developed mycelia on SWA were transferred to sea

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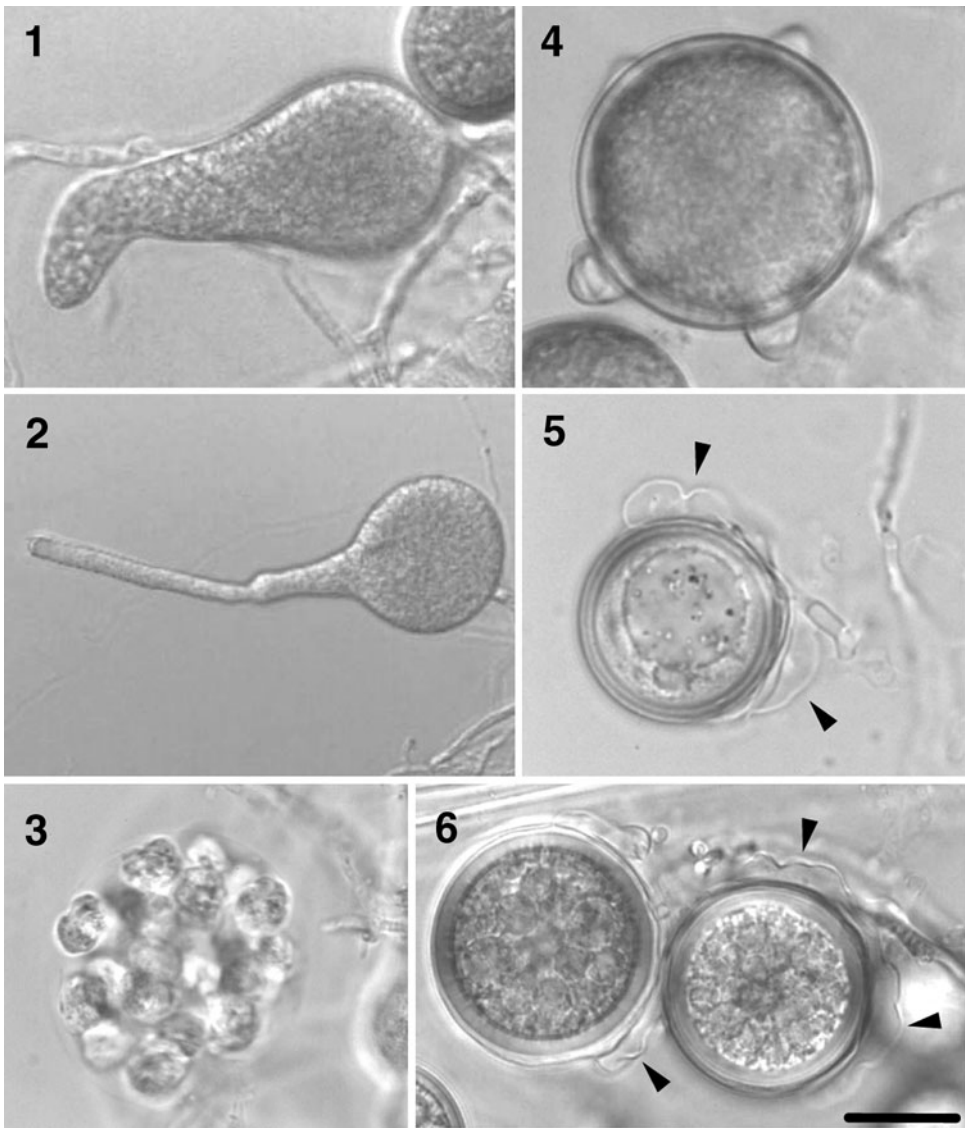
salt CMA composed of 15 g CMA, 15 g sea salts and 1 l distilled water (CMA-S) and maintained at 25°C in darkness until use. Isolate OPU796 was recovered from seawater with leaves of garden pea (*Pisum sativum* L.) as a substrate in March 2007. Ten grams of the leaves were placed in a mesh bag (2 mm mesh size) and floated in seawater in the saline marsh for 3 days under field conditions. The leaves were then placed on PSM. Emerging mycelia were isolated and maintained as described above. Leaves of common reed were used as bait, but *P. grandisporangium* was not isolated. Both isolates have been deposited at the Microbiological GeneBank, National Institute of Agrobiological Sciences, Ibaraki Prefecture, Japan (MAFF), with reference numbers MAFF 240866 (OPU795) and MAFF 240867 (OPU796).

The morphologies of the isolates OPU795 and OPU796 were examined on grass-leaf water culture (van der Plaats-Niterink 1981) at 20°C using distilled water containing 15 g/l of sea salts. Both isolates were identified as *Pythium grandisporangium* based on morphology. A detailed description is provided for isolate OPU795.

Main hyphae were up to 6 μm in diameter. Sporangia were terminal, ellipsoidal or limoniform, 24.2–72.1 (mean 51.6) μm wide × 43.1–152.7 (mean 104.8) μm long, and often papillate (Figs. 1, 2). Sporangia produced single discharge tubes and a terminal vesicle in which zoospores differentiated (Fig. 3). Zoospores were formed at 17–22°C. Encysted zoospores were 6.6–8.2 (mean 7.7) μm in diameter. Oogonia were globose, smooth, terminal, and 30.0–45.0 (mean 37.5) μm in diameter. Antheridia were monoclinous or diclinous, 1–5 (mostly 1–2) per oogonium (Fig. 4). Antheridium cells longitudinally applied to the oogonium (Figs. 5, 6). Oospores were plerotic, globose, smooth 29.0–43.0 (mean 35.5) μm in diameter, one per



Figs. 1–6 Morphological characteristics of *Pythium grandisporangium* isolate OPU795. **1** Terminal sporangium. **2** Germinating sporangium. **3** Developed vesicle with zoospores. **4** Young oogonium with four antheridia. **5**, **6** Mature oogonia. Antheridial cells (*arrowheads*) longitudinally applied on the oogonia. *Bar* 20 μm

oogonium (Figs. 4–6). The thickness of the oospore wall was 2.0–4.0 (mean 3.4) μm.

The molecular approach to the identification of *Pythium* isolates was based on sequences of rDNA ITS following Uzuhashi et al. (2008). The sequences of the isolates OPU795 and OPU796 were 831 bp in length. A single base substitution at position 146 of ITS1 was observed from adenine in isolate OPU795 to guanine in isolate OPU796. Interestingly, isolates CBS286.79 from the USA and CBS211.85 from the Netherlands showed exactly the same base substitution as our isolates OPU795 and OPU796. Furthermore, the ITS sequences of isolates CBS286.79 and CBS211.85 were identical to those of isolates OPU795 and OPU796, respectively. Despite the worldwide distribution of *P. grandisporangium*, the present results regarding its morphological and molecular characteristics indicate that it is a distinctive species with little intraspecific variation. The sequences have been deposited in GenBank under the accession nos. AB444071 and AB444140 for isolates OPU795 and OPU796, respectively.

The cardinal temperatures for hyphal growth of the isolates OPU795 and OPU796 were determined on plates of potato–carrot agar (PCA) (van der Plaats-Niterink 1981) in darkness at 4, 7, 10, 13, 17, 19, 22, 25, 28, 31, 34, 37, and 40°C. The effect of sea salt concentration on hyphal growth was determined on CMA plates amended with 0–9% (w/v) sea salts at 25°C in darkness. Three replicates were used for each isolate at each temperature or sea salt concentration. The cardinal temperatures for hyphal growth on PCA had similar patterns in both isolates, although isolate OPU795 showed a slower growth than isolate OPU796. For isolate OPU795, the minimal, optimal and maximal temperatures for growth were 4, 25 and 31°C, with daily growth rates of 0.5, 6.9 and 4.4 mm, respectively. Hyphae grew on CMA containing 0–9% of sea salts in CMA and were near optimal at 1–3% sea salts in both isolates (Fig. 7). Although morphological features were very similar for the two isolates, their growth rates differed. This corresponds with a previous observation (de Cock 1986), where variation in growth rate among isolates of *P. grandisporangium* was observed.

To test pathogenicity, isolates OPU795 and OPU796 were inoculated into newly developed roots of *P. australis* grown in a greenhouse in water containing 1.5% sea salts. The root pieces used were approximately 1 mm in diameter and 15 mm long. A 10 mm agar plug of *P. grandisporangium* grown at 25°C for 24 h on CMA-S was the inoculum. The root of *P. australis* was surface disinfected with 70% ethanol and inoculated with an agar plug into the unwound surface of each root. The inoculated roots were placed on sterile filter paper in petri dishes (90 mm) and moistened with 10 ml of sterile water with 1.5% sea salt. Nine roots were inoculated for each isolate, while nine roots with agar only were employed as the controls. The roots were kept in a growth chamber at 25°C in darkness for 10 days and checked for the development of symptoms. The root tissue was also examined by microscopic examination and by reisolating from the tissue with PSM. Experiments were performed in duplicate. Isolates OPU795 and OPU796 did not produce symptoms on *P. australis* roots 10 days after inoculation. The isolates were consistently reisolated from inoculated plant roots but not from the noninoculated roots. Microscopic observations showed that *P. grandisporangium* hyphae grew on the surfaces of the roots. This hyphal growth was not observed on the surfaces of the roots of the noninoculated plant. *Pythium* species can act as a pathogen in *P. australis* (Nechwatal et al. 2005), but the present results suggest that *P. grandisporangium* is not pathogenic to this plant under the conditions tested. The species likely acts as a saprophyte on dead epidermal tissue on living or decaying plants in saline marsh seawater.

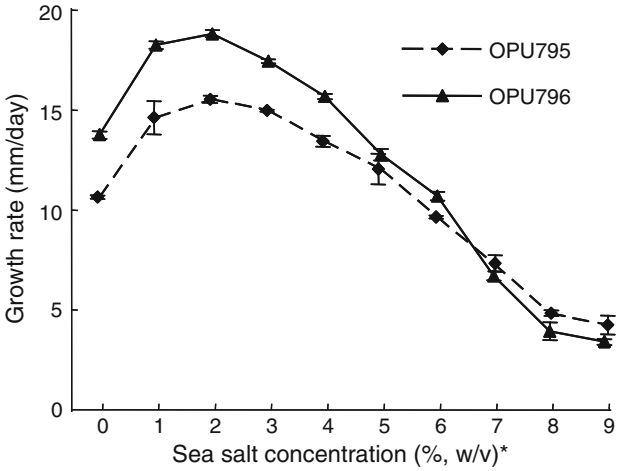


Fig. 7 Effect of sea salt concentration on growth in two isolates of *Pythium grandisporangium* OPU795 and OPU796 on corn meal agar. Each point represents the average of 3 colonies with the 3 longest measurements taken per colony. *Bar* represents standard deviation. Asterisk indicates that a sea salt product from Sigma-Aldrich was used

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