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Isolation, Identification and Characterization of *Phytophthora katsurae*, Causing Chestnut Ink Disease in Korea

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Since July 2005, survey of chestnut ink disease was carried out in chestnut stands located at southern parts of Korea. Dead chestnut trees showing inky ooze on necrotic trunks were found in two different locations. In order to isolate and identify the causal fungus, infected tissues and soil samples around dead or dying trees were collected and placed on *Phytophthora*-selective medium. Rhododendron and chestnut tree leaves were used as a bait to isolate the fungus from soil samples by attracting zoospores in soil suspensions. On V-8 culture medium, the isolates produced homothallic oogonia with protuberances (34.0-46.2×21.9-26.7 μm) abundantly, but did not produced sporangia. Mass production of sporangia was possible by immersing agar plugs with actively growing mycelium in the creek water at 18°C for 3 days. Sporangia were papillate, and ovoid to obpyriform (17.0-38.9×14.6-29.2 μm) in shape. Comparison of the ITS sequences revealed that the isolates had 100% identity to the *P. katsurae* isolates from Japan and New Zealand and 99.6% identity to other *P. katsurae* isolates. All of the examined isolates from Korea were completely identical to each other in ITS sequence. Numerous sporangia were formed in filtered as well as unfiltered creek water, but no sporangia formed in sterilized distilled water. Light induced sporangia formation, but has no influence on oospore formation. Amendments of β-sitosterol in culture media have no significant effect on mycelial growth but significantly stimulate oospore and sporangia formation.

Keywords : amphigynous antheridium, chestnut ink disease, homothallic oogonium, *Phytophthora katsurae*, sporangia

Chestnut tree (*Castanea* spp.) has been one of the most commonly planted and cultivated tree species in Korea since the late 1970's because of high market price of the produced chestnuts, which are mostly consumed up in domestic markets and partly exported to Japan, China, and United States. So far, in chestnut plantations in Korea, chestnut blight caused by *Cryphonectria parasitica* has been known as the most serious disease and a major limiting factor for the chestnut cultivation. However, in many European countries, chestnut ink disease has been known as the more serious problem than chestnut blight. Chestnut ink disease in Europe and United States is usually caused by *P. cambivora* and *P. cinnamomi*. *P. cinnamomi* and *P. cambivora*, well known soil-borne pathogens of mainly woody hosts, were probably introduced into Europe early in the nineteenth century from the Pacific Celebes-New Guinea region (Brasier, 1996). These oomycetes entered North America in the mid-1800s and caused the recession of American chestnut (*Castanea dentata* (Marsh.) Borkh.) from large areas in the Gulf and Atlantic states in the United States prior to 1900 (Anagnostakis, 1995). In southern Australia, *P. cryptogea* cause the disease on chestnut. These pathogenic fungi cause brownish-black lesions on the roots that exude an inky-blue stain, hence the name ink disease. Trees die when the root collar is girdled, or when most of the roots are killed.

In Japan, *P. castaneae* has been reported on Japanese chestnut (*Castanea crenata*) as a trunk rot fungus in 1969. This soil-borne fungus may infect the root system, causing the wilting and death of chestnut trees. However, the most lesion appeared on the trunk at from 10 to 100 cm high from the ground. It colonized bark and cambial tissues reaching up the stem for dozens of centimeters. The infected tree usually died within 3 to 4 years after infection (Katsura, 1976). Thereafter, a new name, *P. katsurae*, was designated for *P. castaneae* (Ko and Chang, 1979). *P. katsurae* was also reported from coconut and cocoa in tropical areas, Hawaii and Ivory Coast (Liyanage and Wheeler, 1989).

Thus, the purpose of this study was to find chestnut ink disease caused by *Phytophthora* species in Korea, to identify the species, and to elucidate the factors involved in vegetative growth and reproduction of the pathogen. Several discolored wood tissues under the water-soaked bark with exudating inky ooze were collected in chestnut plantations located at the southern locations, Hapcheon and Hadong, and then two isolates of the pathogenic fungus was isolated

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in 2006. We again tried to get more isolates from both infected tissues and infested soil samples in 2008 to carry out genetical and ecological studies. We now report the typical symptoms appeared on the infected chesnut trees, isolation of the pathogenic fungus from the infected tissues and soil samples around dead or dying chestnut trees, and identification of the fungus by morphological characterisitcs and molecular analysis. In addition, we present the effects of factors such as water properties, light, and β-sitosterol on mycelial growth, oospores and sporangia formations of the pathogenic fungus.

Materials and Methods

Collection of infected chestnut tissues and soil samples. Dead or dying chestnut trees showing inky ooze on necrotic trunks were surveyed in two different locations, Hapcheon and Hadong in Korea. In order to isolate and identify the causal fungus, infected tissues and soil samples around dead or dying trees were collected in early August, 2008.

Isolation of the pathogen from infected tissues. After removing bark with inky symptoms, discolored wood tissues were collected, and small pieces were placed on Phytophthora-selective CARP medium (17 g/l cornmeal agar, 200 μg/ml ampicillin, 10 μg/ml rifampicin, 10 μg/ml pimaricin). After incubation at 25°C for 3 days, actively growing mycelium at the edge were transcultured and incubated for 1 week on CMA (17 g/l cornmeal agar, 20 μg/ml β-sitosterol) to get pure culture.

Isolation of the pathogen from soil samples. Soil samples were collected from three points around dead or dying chestnut trees at a distance of about 30 cm and depth of 20 cm from the root collar. Detached leaves from rhododendron and chestnut tree were used as a bait. After these leaves were submerged in soil suspensions for 5 days at room temperature, discolored parts of baiting leaves were sliced and placed on CARP$^+$ medium. After incubation at 25°C for 3 days, actively growing mycelium at the edge were transcultured and incubated for 1 week on CMA (17 g/l cornmeal agar, 20 μg/ml β-sitosterol) to get pure culture.

Identification of the fungal isolates. Oospore formation were examined under light microscope after incubation on V-8 agar medium at 25°C in the dark. Sporangia formation was induced by immersing agar plugs with actively growing mycelium in the creek water at 18°C for 3 days. Ribosomal DNAs of the 22 fungal isolates (Table 1) were extracted by DNeasy Plant Mini Kit (Quiagen, Germany), and ITS regions were amplified using the universal primers ITS1 and ITS4. ITS sequences of the fungal isolates were

Table 1. List of *Phytophthora* isolates used in the comparison of ITS sequences of rDNA

No.	isolates No.	Source	Location	Year	Culture No.
1	TPML 08001	Chestnut (*C. crenata* × *C. mollissima*)	Hapcheon, Korea	2008	
2	TPML 08002	soil (chestnut)	Hadong, Korea	2008	
3	TPML 08003	soil (chestnut)	Hadong, Korea	2008	
4	TPML 08004	Chestnut (*C. crenata* × *C. mollissima*)	Hapcheon, Korea	2008	
5	TPML 08005	Chestnut (*C. crenata* × *C. mollissima*)	Hapcheon, Korea	2008	
6	TPML 08006	Chestnut (*C. crenata* × *C. mollissima*)	Hapcheon, Korea	2008	
7	TPML 08007	Chestnut (*C. crenata* × *C. mollissima*)	Hapcheon, Korea	2008	
8	TPML 08008	Chestnut (*C. crenata* × *C. mollissima*)	Hapcheon, Korea	2008	
9	TPML 08009	Chestnut (*C. crenata* × *C. mollissima*)	Hadong, Korea	2008	
10	TPML 080010	Chestnut (*C. crenata* × *C. mollissima*)	Hadong, Korea	2008	
11	TPML 080011	Chestnut (*C. crenata* × *C. mollissima*)	Hadong, Korea	2008	
12	TPML 080012	Chestnut (*C. crenata* × *C. mollissima*)	Hadong, Korea	2008	
13	TPML 080013	Chestnut (*C. crenata* × *C. mollissima*)	Hadong, Korea	2008	
14	TPML 080014	Chestnut (*C. crenata* × *C. mollissima*)	Hadong, Korea	2008	
15	TPML 080015	Chestnut (*C. crenata* × *C. mollissima*)	Hadong, Korea	2008	
16	TPML 080016	Chestnut (*C. crenata* × *C. mollissima*)	Hadong, Korea	2008	
17	TPML 080017	Chestnut (*C. crenata* × *C. mollissima*)	Hadong, Korea	2008	
18	TPML 080018	Chestnut (*C. crenata* × *C. mollissima*)	Hadong, Korea	2008	
19	TPML 080019	soil (chestnut)	Hapcheon, Korea	2008	
20	TPML 080020	soil (chestnut)	Hapcheon, Korea	2008	
21	TPML 080021	soil (chestnut)	Hapcheon, Korea	2008	
22	TPML 080022	soil (chestnut)	Hadong, Korea	2008	

Table 1. Continued

No.	isolates No.	Source	Location	Year	Culture No.
23	AF266771	Coconut (*Cocos nucifera*)	Ivory Coast		IMI 360596
24	P1372	Coconut (*Cocos nucifera*)	Hawaii, USA		ATCC 60184
25	P1373	Coconut (*Cocos nucifera*)	Hawaii, USA		ATCC 60182
26	P3146	Coconut (*Cocos nucifera*)	Ivory Coast		
27	P3389	Monkey Puzzle Tree (*Auracaria* sp.)	Papua New Guinea		IMI 198426
28	P3638	Coconut (*Cocos nucifera*)	Hawaii, USA		ATCC 58426
29	P6920	Coconut (*Cocos nucifera*)	Hawaii, USA		
30	P6921	Coconut (*Cocos nucifera*)	Hawaii, USA		
31	P6924	Coconut (*Cocos nucifera*)	Hawaii, USA		
32	P6925	Coconut (*Cocos nucifera*)	Hawaii, USA		
33	P7785	Cacao (*Theobroma cacao*)	Ivory Coast		
34	P8271	Coconut (*Cocos nucifera*)	Ivory Coast		
35	P8274	Coconut (*Cocos nucifera*)	Ivory Coast		
40	P8275	Coconut (*Cocos nucifera*)	Ivory Coast		
41	P8276	Coconut (*Cocos nucifera*)	Ivory Coast		
42	22H6	Japanese chestnut (*Castanea crenata*)	Japan	2007	Virginia Tech.
43	Korea 11	Chestnut (*C. crenata* × *C. mollissima*)	Hapcheon, Korea	2006	
44	Korea 12	Chestnut (*C. crenata* × *C. mollissima*)	Hadong, Korea	2006	
45	AF266770	Rubber (*Hevea brasiliensis*)	Malaysia		ATCC 16700
46	P1000	Avocado (*Persea americana*)	Guatemala		
47	P1102	Avocado (*Persea americana*)	Guatemala		
48	P3428	Rubber (*Hevea brasiliensis*)	Malaysia		
49	P3604	Cacao (*Theobroma cacao*)	Malaysia		
50	P3947	Soil	USA		
51	P6246	Azalea (*Rhododendron* sp.)	USA		
52	P6247	Azalea (*Rhododendron* sp.)	USA		
53	P8240				
54	P10604	Soil	USA		
55	P10660	Forest soil	China		
56	EF067922	Kauri (*Agathis australis*)	New Zealand		ATCC 32256

searched by BLAST search in NCBI databank. ITS sequences of other *P. katsurae* isolates and *P. heveae* isolates were obtained from NCBI databank and Phytophthora database. ITS sequences of the isolated fungus, *P. katsurae* isolates, and *P. heveae* isolates were compared and analyzed by TreeView software (Roderic D. M. Page, Scotland, UK).

Effects of water properties, light and β-sitosterol on mycelial growth and sporulation of the pathogen. In order to understand the physiology of the isolated pathogen, effects of the water properties, light, and β-sitosterol on the vegetative and reproductive processes of the pathogen were investigated. Fresh creek water was collected from the creek nearly located at chestnut plantations in Gamjung-Ri, Dongsan-Myun, Chuncheon, Korea. Filtered creek water were obtained by filtering through 1.2 and 5 μm membrane filters. Light conditions for the sporangia and oospore formations of the isolated pathogen were adjusted at 500, 1000 lux or dark. β-sitosterol was added to the culture medium at the concentration of 20 μg/ml. Mycelial growth and oospore formation were checked after incubation at 25°C in the dark. Agar discs containing mycelium were taken from the edge of actively growing *P. katsurae* colony on V-8 juice agar. These were tansferred to the center of empty petri dish, and water was added to the level until the upper surface of agar discs was just submerged. The plates were kept at 18°C in the dark to induce sporangia formation. The obtained results were analyzed by Tukey's test.

Results and Discussion

Isolation of the pathogen from infected tissues and soil samples. Among 27 infected tissue samples collected, Fungal isolates from 16 samples were confirmed as *P. katsurae*. Isolation frequencies of the pathogen from Hapcheon and Hadong were 54.5 and 62.5%, respectively.

Table 2. Isolation frequency of the pathogenic fungus from infected tissues and infested soils at Hapcheon and Hadong areas

Location sample	Hapcheon	Hadong	Average(%)
Infected tissue	54.5	62.5	59.3
Infested soil	25.0	10.5	16.1
(Rhododendron leaf)	(66.7)[a]	(40.0)	(53.3)
(Chestnut leaf)	(10.0)	(10.0)	(10.0)

※ Infected tissues and infested soil samples were collected in April and August, 2008.
※ Isolation of the pathogenic fungus from the infected tissue and infested soil was tried on the *Phytophthora*-selective CARP medium (17 g/l cornmeal agar, 200 μg/ml ampicillin, 10 μg/ml rifampicin, 10 μg/ml pimaricin) and CARP$^-$, respectively.
[a]Isolation frequencies from the bait materials were the percentages of the pathogen from the soil samples infested with the pathogenic fungus.

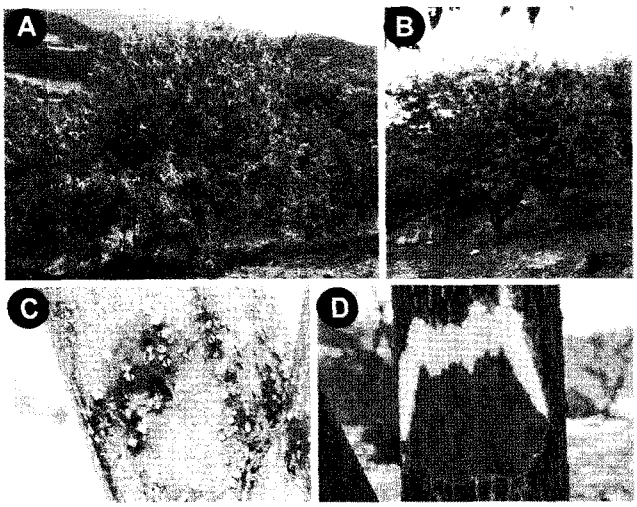

Fig. 1. (A) Early symptoms on chestnut tree infected with *Phytophthora katsurae*, causing chesnut ink disease, (B) dead chestnut trees, (C) inky ooze from soaked areas of the infected trunk, and (D) dark brown discolored wood after bark removal around the inky oozing area.

Among 31 soil samples collected, Fungal isolates from 5 samples were confirmed as *P. katsurae*. Isolation frequencies of the pathogen from Hapcheon and Hadong were 25.0 and 10.5%, respectively. Isolation frequency of the pathogen from the infected tissues was much higher than that from the infested soils. The lower isolation frequency from soil samples may due to the limited saprophytic ability of *Phytophthora* species, not growing and competing in soils with other microorganisms. When fresh rhododendron and chestnut leaves were used as a bait to isolate the fungus by attracting zoospores from the infested soil suspensions, the isolation frequency from rhododendron and chestnut leaves were 53.3 and 10.0%, respectively. Thus rhododendron leaf was more effective bait than chestnut leaf for the isolation of the pathogen (Table 2).

Formation of oospore and sporangia. Fungal isolates from both infected tissues and soil samples produced homothallic oogonia (34.0-46.2×21.9-26.7 μm) with protuberances and amphigynous antheridium abundantly, but

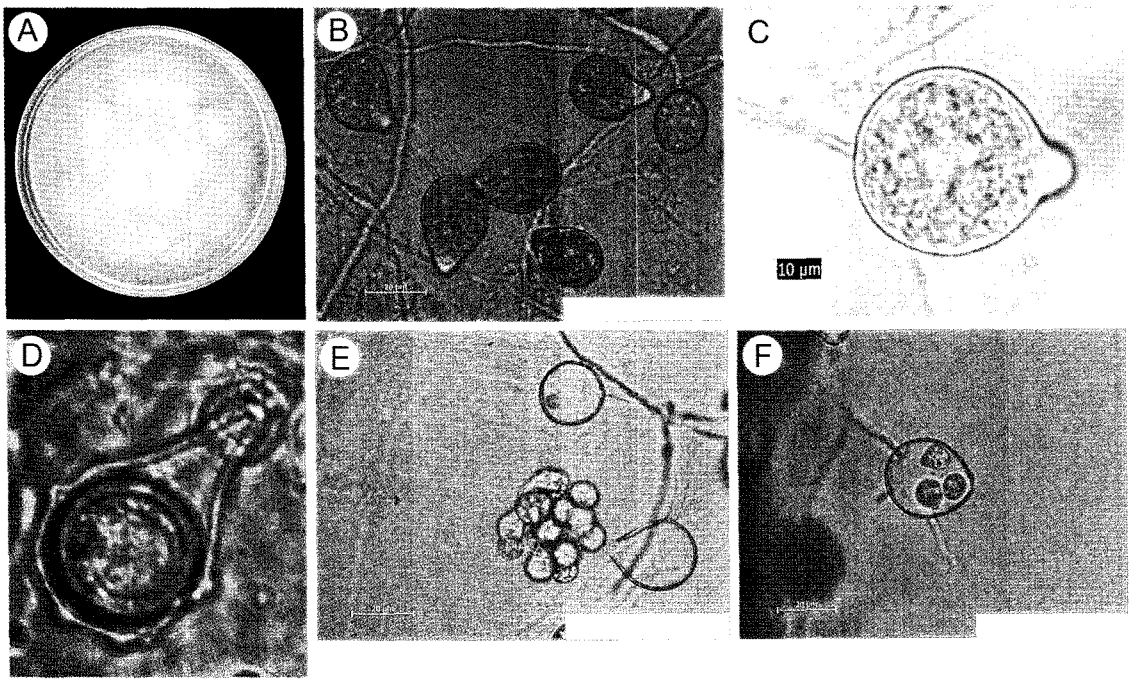

Fig. 2. (A) Colony morphology of *P. katsurae* on V-8 agar, (B) ovoid, obpyriform sporangia, (C) papillate sporangia, (D) oogonia with protuberances and amphigynous antheridium, (E) emitting zoospores from sporangia, and (F) zoospores left in sporangia.

did not produced sporangia on V-8 agar medium. Mass production of papillate sporangia was induced by immersing agar plugs with actively growing mycelium in the creek water at 18°C for 3 days. Sporangia were papillate, and ovoid to obpyriform (17.0-38.9×14.6-29.2 μm) in shape (Fig. 2). In terms of oogonia and sporangia size, oogonia was in the same range with the same species described in other papers, but sporangia was a little bit smaller (Ko and Chang, 1979; Ho et al., 1995).

Identification of fungal isolates. The rDNA ITS sequences of the 22 isolates from both infected chestnut tissues and soil samples around dead or dying trees showed 100% similarity with *P. katsurae* isolates, Korea 11, Korea 12 (Chestnut, Korea), 22H6 Virginia (Chestnut, Japan) and Phap NZ EF067922 (Kauri tree, New Zealand) (Beever et al., 2007), and 99.6% similarity with other *P. katsurae* isolates by showing differences in three base pairs. All isolates from different locations in Korea were completely identical in ITS sequence of rDNA (Fig. 3). Thus the pathogen of chestnut ink disease in Korea was identified as *Phytophthora katsurae* based on morphological characteristics and molecular analysis (Oh et al., 2008). *P. katsurae* closely resembles *P. heveae* in morphological characteristics with the exception of its verrucose oogonial wall (Ho et al., 1995; Stamps et al., 1990). It is suggested that *P. heveae* developed directly from *P. katsurae* by loss of protrusions. The high level of ITS sequence similarity between two species support the possibility of the recent development of one species from other species (Ko et al., 2006).

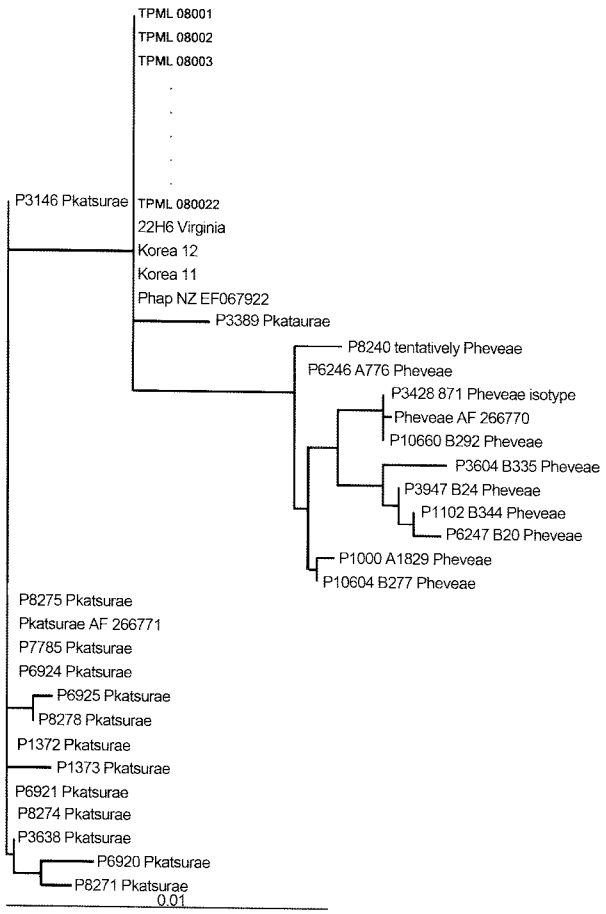

Fig. 3. Relationship between *P. katsurae* and *P. heveae* isolates based on similarity analysis of ITS sequences.

Table 3. Effects of water conditions on sporangia formation of *P. katsurae*

Treatments (18°C, dark)			5 days	10 days
Creek water	Unfiltered		++[(a)]	++++
	Filtered	5 μm	+	+++
		1.2 μm	+	++
Sterilized distilled water			–	–

※ 5 μm and 1.2 μm filtered creek water were obtained by filtering 5 and 12 μm membrane filters, respectively. [(a)]–: no sporangia formation, +: sporangia formed sparsely, ++: sporangia formed at the margin of agar discs, +++: sporangia formed at the margin and center of agar discs, ++++: sporangia formed abundantly.

Effects of water properties, light and β-sitosterol on mycelial growth and sporulation of the pathogen. Numerous sporangia of *P. katsurae* were formed in filtered as well as unfiltered creek water by 10 days' incubation at 18°C, but no sporangia formed in sterilized distilled water by the same periods of treatment. Unfiltered creek water was more effective than filtered water, and filtered water through 5 μm membrane filter was more effective than the water filtered through 1.2 μm filter (Table 3). Sporangia production in *Phytophthora* species is a complex process involving water potential, nutrients, sterols, aeration, light, temperature, cations such as Ca^{2+}, Fe^{3-}, Mg^{2+}, and K^{+}, age of culture, certain bacteria, root exudates, and soil extracts. The importance of each of these factors varies with the species. Water potential is perhaps the most significant factor influencing sporangium production of all *Phytophthora* species (Ribeiro, 1983). Thus it was conferred that chemical or biological components existed in creek water but not in distilled water, play somehow an important role in inducing sporangia formation of *P. katsurae*. In general, light enhanced, inhibited, or had no influence on sporangia production of *Phytophthora* species (Erwin and Ribeiro, 1996). Some species, e.g. *P. hibernalis* and *P. syringe*, sporulated better in the dark (Harnish, 1965). However, in the case of *P. katsurae*, light induced sporangia formation at the intensity of 500 and 1,000 lux, but it did not induce oospore formation. Light intensity did not give any differences in sporangia formation. No sporangia formed in sterilized unfiltered creek water in the dark condition, but light induced sporangia formation in the same water. In the

Table 4. Effects of light conditions on sporangia and oospore formations of *P. katsurae*

Incubation periods (day)		1		3		5		10	
Light/Dark	S/NS	SF	OF	SF	OF	SF	OF	SF	OF
1,000 lux	S	++	++	+++	++	+++	++	+++	++
	NS	–	++	++++	+++	++++	+++	++++	+++
500 lux	S	++	++	+++	++	+++	++	+++	++
	NS	–	++	++++	+++	++++	+++	++++	+++
Dark	S	–	+++	–	+++	–	+++	–	+++
	NS	–	+++	–	+++	–	+++	+	+++

S: Sterilization, NS: Non-sterilization of unfiltered creek water.
SF: Sporangia formation, OF: Oospore formation.

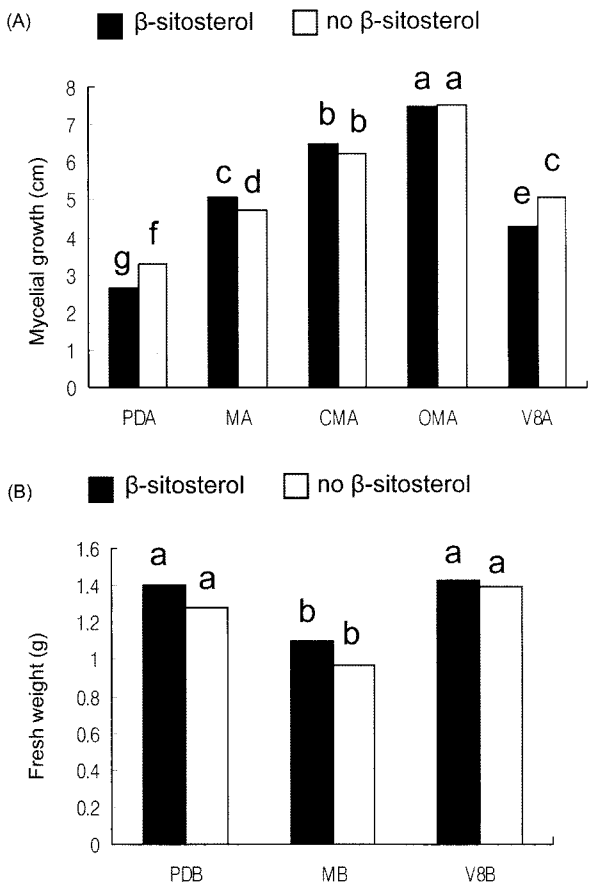

Fig. 4. Effects of β-sitosterol on (A) mycelial growth and (B) fresh weight of *P. katsurae* in various solid (PDA: potato dextrose agar, MA: malt extract agar, CMA: cornmeal agar, OMA: oatmeal agar, V8A: V-8 juice agar) and liquid (PDB: potato dextrose broth, MB: malt extract broth, V8B: V-8 juice broth) culture media. The same letters above the bars indicate no significant difference (p=0.05) by Tukey's test.

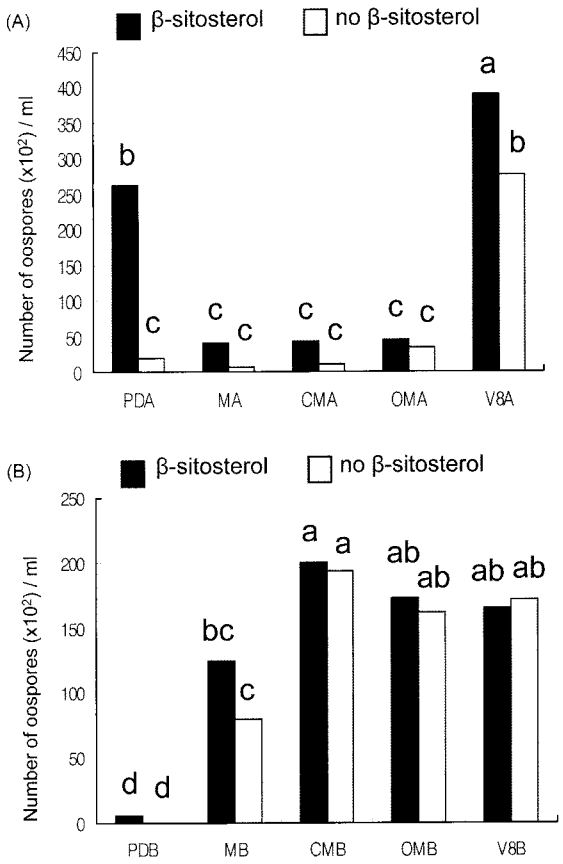

Fig. 5. Effects of β-sitosterol on oospore formation of *P. katsurae* in various solid and liquid culture media. Number of oospores (A) on solid media, respectively. The same letters above the bars indicate no significant difference (p=0.05) by Tukey's test.

light conditions, more sporangia and oospores were formed by non-sterilization than sterilization of unfiltered creek water. In the sterilized unfiltered creek water, oospores were produced slightly more than in the light condition. However, no differences were observed by light intensity in oospore formation (Table 4).

β-sitosterol slightly increased mycelial growth in both solid and liquid culture media as compared to the control without β-sitosterol, but the differences in fresh weight were not significant (Fig. 4). In contrast, oospore formation was stimulated on V-8 agar and PDA by the addition of β-sitosterol. Oospore formations on V-8 agar and PDA with β-sitosterol showed 1.3 to 14 times of increase as

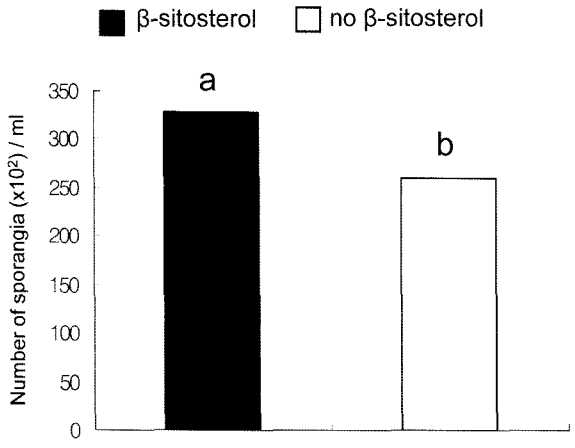

Fig. 6. Effects of β-sitosterol on sporangia formation of *P. katsurae* on V-8 juice agar. The same letters above the bars indicate no significant difference (p=0.05) by Tukey's test.

the control without β-sitosterol, respectively (Fig. 5). Sporangia formation was also increased by adding β-sitosterol on V-8 agar (Fig. 6). It had been reported that sporangium production of *P. lateralis* on a V-8 agar medium supplemented with β-sitosterol (10 μg/ml) and irradiated with light at 680 μW/cm^2 was approximately four times that obtained on sterol-free media incubated either in light or in the dark (Englander and Roth, 1980). It is known that sitosterol among all the β-hydroxysterols tested for their effects on production of oospores, sporangia, and chlamycospores, appears to be the most effective in promoting sporulation (Erwin and Ribeiro, 1996). Sitosterol induced oospore production by *P. cactorum* at 9 mg/l, and even lower condentrations (Nes, W.D. et al., 1979). Sterols are probably important as components of membranes and were also considered to be involved in fungal hormone biosynthesis (Elliott, 1977).

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