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Detection of Cellulolytic Activity in *Ophiostoma* and *Leptographium* species by Chromogenic Reaction

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To understand the ability of producing cellulolytic enzyme activity in the sapstaining fungi, four species of *Ophiostoma* and two species of *Leptographium* were investigated in the culture media containing each of cellulose substrates such as CM-cellulose, Avicel and D-cellobiose and each of chromogenic dyes such as Congo-Red, Phenol Red, Remazol Brilliant Blue and Tryphan Blue. When the fungi were grown for 5~7 days at 25°C, the formation of clear zone by chromogenic reaction around the margin of the fungal colony was demonstrated in all the culture media Congo-Red containing CM-cellulose. There was difference in the formation of clear zone among the dyes. Only *Ophiostoma setosum* and *Leptographium* spp. showed cellulolytic activity to the three substrates. Overall, the results of this study show that ophiostomatoid sapstaining fungi can produce cellulolytic enzymes.

KEYWORDS: Cellulolytic enzyme, Chromogenic reaction, *Leptographium* sp., *Ophiostoma* sp., Sapstaining fungi

Sapstain present in wood is a cosmetic problem and reduces the value of wood resulting in economic loss in the forest products industry. Sapstaining fungi are the causal agents of sapstain. They colonize sapwood tissues by growing from cell to cell primarily through pits, and in some instances by penetrating wood cell walls (Eaton and Hale, 1993). Ophiostomatoid fungi belonging to Ascomycota are one of major sapstaining fungal groups. To protect wood from these worldwide problematic fungi, it is vital to understand its biology such as nutrient physiology and colonizing mechanism. The fungi are known to utilize non-structural components in wood such as lipids, proteins and soluble carbohydrates, by excreting a large number of other different hydrolases (Abraham *et al.*, 1998). The production of cellulases by ophiostomatoid fungi was reported from *Ceratocystis minor* (Rösch *et al.*, 1969) and the Dutch elm disease pathogens, *Ophiostoma ulmi* and *O. novo-ulmi* (Berkman 1956; Elgersma, 1976; Przybye *et al.*, 2006). However, no detectable extracellular cellulase activity has been reported from other sapstaining fungi. Thus, questions still have been remained on their ability of producing extracellulases.

In this study, we report new evidences that sapstaining species belonging to *Ophiostoma* and *Leptographium* genera can produce the cellulolytic enzymes. For the *in vitro* detection of the enzyme activity, different kinds of chromogenic dyes were also compared.

Seven fungal isolates including four *Ophiostoma* species and two *Leptographium* species were used in this study and their species name and source are given in Table 1. The two *Leptographium* isolates from Seo-chun, Chungnam, Korea, have not completely identified yet, but they have different morphological properties, thus they are treated as separate species. All the fungal isolates in Table 1 were cultured on 2% potato dextrose agar or 2% malt extract agar (Difco, USA) containing streptomycin (50 μg/*ml*) and cycloheximide (100 ug/*ml*) for 5 days at 25°C. For observation of fungal extra cellulase activity, agar cores (3 mm in diameter) of the fungal cultures on grown malt extract agar (MEA) were transferred onto chromogenic substrate medium and grown for 5 days. For the chromogenic substrate medium, 0.1% yeast nitrogen base (Difco, USA) and 1.5% agar powder was used as basal medium. As a carbon source, each of carboxymethyl-cellulose (CMC, Sigma, USA), D-cellobiose (Sigma, USA), and microcrystalline cellulose (Avicel, Fluka, Ireland) was added into the basal medium with combination of each of 0.5% chromogenic dyes such as Remazol Brilliant Blue, Phenol Red, Congo Red, and Tryphan Blue (Sigma, USA). The presence of cellulolytic activity was detected by observing the formation of clear zone (plaque) around the fungal colonies due to the radial diffusion of the

Table 1. Sapstaining fungal isolates used in this study

Species	Isolate no.	Host	Origin
Ophiostoma piceae	AU55-3	Spruce	Canada
O. piceae	TP-1	*Pinus thunbergiana*	Korea
O. floccosum	DS1/3B-2	Unknown	Canada
O. quercus	H1039	Oak	U.K.
O. setosum	AU160-25	Unknown	Canada
Leptographium sp.	JH-1	*Pinus thunbergiana*	Korea
Leptographium sp.	JH-2	*Pinus thunbergiana*	Korea

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Table 2. Detection of cellulolytic enzyme activity from the sapstaining fungi by chromogenic reaction with four different dyes

Species	Congo Red			Phenol Red			Remazol Brilliant Blue			Tryphan Blue		
	CMC	Cel	Avi	CMC	Cel	Avi	CMC	Cel	Avi	CMC	Cel	Avi
Trichoderma reesei	+	+	−	−	+	−	−	−	−	−	−	−
O. piceae AU55-3	+	−	−	−	−	−	−	−	−	−	−	−
O. piceae TP-1	+	−	−	−	−	−	−	−	−	−	−	−
O. floccosum	+	+	−	−	−	−	−	−	−	−	−	−
O. quercus	+	+	−	−	−	−	+	−	−	−	−	−
O. setosum	+	+	+	−	−	−	+	−	−	−	−	−
Leptographium sp. JH-1	+	+	+	−	−	−	−	−	−	−	−	−
Leptographium sp. JH-2	+	+	+	+	−	−	+	+	−	−	−	−

CMC: CM-cellulose, Cel: D-Cellobiose, Avi: Avicel. +: activity detection. −: no activity detection.

enzyme excreted to the medium. *Trichoderma reesei* ATCC56765, a known cellulase producing fungus, was used as a positive control.

Clear zone-based enzyme activity was observed in the chromogenic substrate medium containing Congo Red, Phenol Red, and Remazol Brilliant Blue (Table 2), while no clear zone was observed with Tryphan Blue. The highest number of positive detection of enzyme activity was found in the medium contained Congo Red. This result shows that the selection of chromogenic dye is important for the detection of enzyme activity by the *Ophiostoma* and *Leptographium* species. Recently, Onsori *et al.* (2004) also successfully used Congo Red in the detection of celluloytic enzymes in *Aspergillus* species.

Regarding carbon substrate, all the tested fungal species produced activity in all the Congo Red medium containing CMC (Table 2). We did not detect activity with negative control species such as *Epicoccum nigrum* (Fig. 1). This result suggests that the medium with Congo Red and CMC is proper to use as a choromogenic substrate medium for the detection of extra-cellulases by the *Ophiostoma* and *Leptographium* species. As like the well

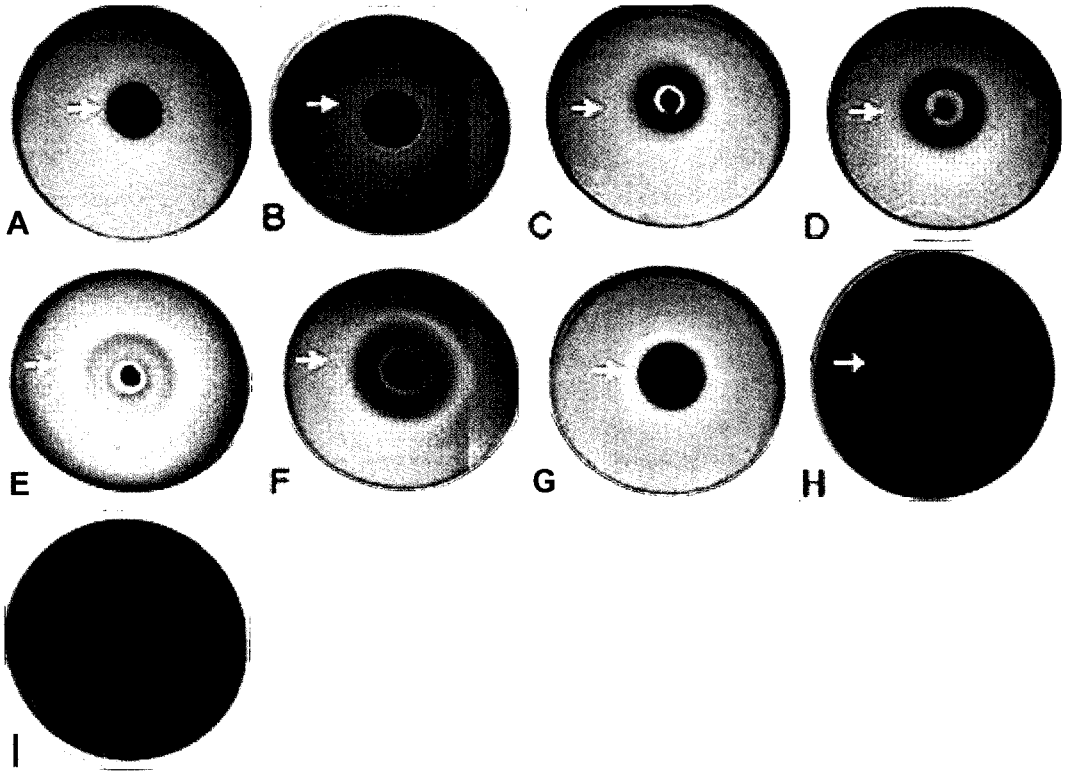

Fig. 1. Clear zone formed in the chromogenic medium containing CM-cellulose and Congo Red by cellulolytic enzyme activity of the sapstaining fungi. A: *O. piceae* AU55-3, B: *O. piceae* TP-1, C: *O. floccosum*, D: *O. quercus*, E: *O. setosum*, F: *Leptographium* sp. JH-1, G: *Leptographium* sp. JH-2, H: *Trichoderma reesei* ATCC56765 (positive control), and I: *Epicoccum nigrum* (negative control). Arrows indicate clear zones. Dark or gray circle inside clear zone is the periphery of fungal colony.

known cellulose-producing isolate *Trichoderma reesei* ATCC56765, all the tested *Ophiostoma* and *Leptographium* species produced activity clearly. Since CMC was used as a sole carbon source, our results suggest that the ophiostomatoid sapstaining fungi have the ability of using cellulose. Cellulases can be classified into three enzyme types, endoglucanase (CMCase), exoglucanase (cellobiohydrolase) and β-glucosidase. The detection of activity in the chromogenic media with CMC and D-cellobiose by *O. floccosum*, *O. quercus*, *O. setosum*, and *Leptographium* spp. suggest that these fungi can produce CMCase and cellobiohydrolase. Considering the fact that *Ophiostoma ulmi* and *O. novo-ulim* are cellulose producers (Elgersma, 1976; Przybye *et al.*, 2006) and taxonomically members of *Ophiostoma piceae* complex that include *Ophiostoma piceae*, *O. floccosum*, *O. quercus*, and *O. setosum*, it has been postulated that all the members of *O. piceae* complex may have cellulose-utilizing ability. Thus, several efforts for detecting cellulase activity in the sapstaining *Ophiostoma* fungi have been made, but no detection report has been available yet (Zink and Fengel, 1998; Schirp *et al.*, 2003). Consequently, the present work is the first report on the detection of cellulolytic enzyme activity in the sapstaining *Ophiostoma* fungi.

Detection of cellulases in fungi is not easy, thus it is still needed to develop better detection methods. Fungal cellulases are produced only in the presence of cellulose (Suto and Tomita, 2001). Recent advance in the analysis of fungal genes using molecular biological tools has been allowing us to find the genes encoding diverse cellulases. In this study we could not detect enzyme activity with D-cellobiose by *O. piceae*. But when we comparatively analyzed the genes expressed in *O. piceae* mycelium grown on different carbon media, we could find homologous sequences that highly matched with known fungal cellobiohydrolase genes (unpublished data). The results mean that although we could not detect cellobiose degrading activity, *O. piceae* fungus has the gene that can degrade cellobiose. Together with this molecular data, the detection of CMC degrading activity in this study shows that *O. piceae* is also able to produce cellulolytic enzymes. Further work will be needed to better understand the roles of cellulolytic enzymes in the ophiostomatoid sapstaining fungi.

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