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Note

Physiological characteristics of the trunk sap rot pathogen *Fomitiporia* sp. on the "Sanbu-sugi" cultivar of *Cryptomeria japonica*

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ABSTRACT

An unidentified *Fomitiporia* sp. initially identified as *Fomitiporia punctata*, causes severe trunk sap rot on *Cryptomeria japonica* cultivar "Sanbu-sugi". We investigated the physiological characteristics of the mycelia of the causal fungus (F2, F6, and F43), in comparison with *F. punctata* (Fp) as a reference, in eight different experiments. The three unidentified isolates showed similar tendencies in change in mycelial growth during incubation, optimal growth temperatures (25 °C), optimal pH range (pH 5–6), glucose to yeast extract ratio (45), utilizable carbon sources (amylose, CM-cellulose, and pectin), utilizable nitrogen sources (yeast extract and polypepton), and water potential (−1.7 Mpa).

An unidentified *Fomitiporia* sp. causes severe sap rot with elongated cankers on the trunks of the economically important cultivar "Sanbu-sugi" (Okizumi 1993; Yoshimaru et al. 1995; Kondo and Watanabe 2005) of Japanese cedar, *Cryptomeria japonica* D. Don (Fig. 1A, B; Imazeki 1960; Aoshima et al. 1964). Aoshima et al. (1964) named this causal fungus from Chiba Prefecture, Japan, "Cha-ana-take-modoki" in Japanese, trunk sap rot, and identified it as *Fomitiporia punctata* (Pilát) Murrill. This fungus is a known pathogen of grapevine (Cortesi et al. 2000) and other angiosperm trees (Dai et al. 2007). Hattori et al. (2009, 2010) morphologically and phylogenetically analyzed the preserved fruit bodies and mycelial collections of this fungus in the Forestry and Forest Product Research Institute, Tsukuba, Japan, and found it to be distinct from *F. punctata* (Wagner and Fischer 2002; Cony et al. 2007; Dai et al. 2008), but a sister species within the genus. The fungus was observed to cause white rot on Sawara cypress, *Chamaecyparis pisifera* (Siebold & Zucc.) Endl., Hinoki cypress, *Ch. obtusa* Siebold & Zucc., and Japanese pear, *Pyrus pyrifolia* var. *culta* (Makino) Nakai (Kaneko et al. 2011). Ota et al. (2009, 2010) reported that the fungi isolated from *Cr. japonica*, from trunk rot of *Ch. pisifera*, and from *P. pyrifolia* var. *culta* were phylogenetically conspecific and distinct from *F. punctata*.

In previous research, trunks of living "Sanbu-sugi" trees were artificially inoculated and cankers were formed (Hayashi et al. 1980; Abe and Hattori 1991). Studies of the physiological characteristics of the fungus are important for understanding invasion strategy and prevention. Physiological studies, however, have not been reported. Previously there is no physiological characteristic study on the polypore. The aim of this study was to clarify the physiological characteristics of

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Fig. 1 – (A) Sap rot with elongate canker on the trunk of the "Sanbu-sugi" cultivar of *Cryptomeria japonica*, caused by *Fomitiporia* sp., Sammu, Chiba. (B) Fruit body of *Fomitiporia* sp. formed on trunk of a "Sanbu-sugi" cultivar. (C) Mycelia of isolates F2 (*Fomitiporia* sp. – left) and Fp (*Fomitiporia punctata* – right).

the mycelia of the causal fungus in preparation for ecological studies of invasion strategies and prevention.

Mycelia of three isolates of the causal *Fomitiporia* sp., F2, F6, and F43, and those of one isolate of *F. punctata* (designated as Fp hereafter), as a reference, were used (Fig. 1C, Table 1). The mycelia of F2 isolated from basidiospores of a fruit body developed on a "Sanbu-sugi" trunk in Matsuo, Sammu area, Chiba, Japan, were obtained from the Ministry of Agriculture, Forestry and Fisheries (MAFF). Mycelia of F6 were isolated from decomposed sapwood just under the bark on which fruit bodies of the fungus had developed on "Sanbu-sugi" in Sammu, Chiba, Japan, and mycelia of F43 were isolated from decomposed wood just under the bark on which *Fomitiporia* sp. fruit bodies had developed on a *P. pyrifolia* var. *culta* tree in Ichihara, Chiba. The wood was placed in potato dextrose agar (PDA, Tanabe, Tokushima, Japan) medium using sterilized cork borers (8 mm in diameter). These three isolates showed the same phylogenetic clade (Hattori et al. 2009; Ota et al. 2009). The isolate Fp originated from Fujisan, Yamanashi, and was obtained from MAFF; it was known to belong to a different phylogenetic clade from the above tree isolates (Hattori et al. 2009, 2010). Mycelia were grown on PDA medium in 90 mm Petri dishes, and 4 mm agar disks of mycelia were taken from the peripheries of the dense colonies and used as inocula.

Eight kinds of experiments were undertaken to analyze weight change during incubation, optimal temperature, optimal pH, glucose to yeast extract ratio (GY ratio), carbon sources, nitrogen sources, amino acid assistance, and water potential, for growth under the conditions shown in Table 2.

For the weight change experiment, mycelia were incubated from 0 to 70 days, and weighed at 7 days intervals. Media after incubation were filtered with membranes (0.45 μm in pore diameter) and glucose concentrations were determined by enzymatic method using an F-Kit for D(+)-glucose (Roche Diagnostics, Tokyo, Japan), measuring light absorbance at 340 nm using a double beam spectrophotometer (U-2900, Hitachi High Technologies Co., Tokyo, Japan). For the different temperature experiment, incubation temperatures (5–45 °C) were set at 5 °C intervals. For the different pH experiment, a pH range (ca. 3–9) was created by adjustment with sterilized 1 N-KOH or 1 N-HCl solution, and actual initial pH values after sterilization and final pH values after incubation were recorded. For the GY ratio, the media were adjusted to a ratio from 0 to100 with glucose (0–100 g/l) and yeast extract (1 g/l, Difco, Tokyo, Japan). For the carbon sources, each of 17 different carbon sources (30 g/l) was combined with yeast extract (1 g/l). The insoluble carbon sources were put into 100 ml Erlenmeyer flasks with distilled and deionized water (water hereafter) prior to sterilization. For the nitrogen sources, each of 10 different nitrogen sources (1 g/l) was combined with glucose (30 g/l). In the amino acid assistance experiment only the mycelia of F6 were used in comparison with the reference fungus. Each of 18 amino acids (0.4 g/l) was added to glucose (30 g/l) and of yeast extract (0.6 g/l). For the insoluble amino acids, 1 N-HCl was added and dissolved. Media without carbon, nitrogen, or amino acids were used as controls for the carbon and nitrogen source experiments and the added amino acid experiment. For the water potential experiment, to the media were added different concentrations of polyethylene glycol 6000 (0, 1, 2, 3, 4, 5, 10, 20, 30, 40, and 50% (w/v)); the water potential of the media measured using a vapor-pressure osmometer (Aqualab CX-2, Decagon devices, Inc., Washington, USA) at 25 °C was −0.69, −0.76, −0.83, −0.87, −0.92, −0.96, −1.10, −1.66, −2.49, −4.32, and −8.34, respectively.

Grown mycelia were collected using a nylon cloth (125 mesh), rinsed with water, dried at 95 °C for more than 24 h to constant weight, and weighed. The data were statistically

Table 1 – List of fungal mycelia used.

Isolate no.	Scientific name	Original name/number	Location	Host	Isolated section
F2	*Fomitiporia* sp.	MAFF420111, Pa46f	Matsuo, Chiba	*Cryptomeria Japonica*	Basidiospores
F6	*Fomitiporia* sp.		Sammu, Chiba	*C. japonica*	Decomposed sapwood
F43	*Fomitiporia* sp.		Ichihara, Chiba	*Pyrus pyrifolia* var. *culta*	Decomposed wood
Fp	*Fomitiporia punctata*	MAFF420211, WD-568	Fujisan, Yamanashi	Unknown	Fungal tissue of *F. punctata* fruit body

Table 2 – Experiment design.

Experiment	Medium				Cultivation condition	
	Kind	Concentration (g/l)	Amount (ml/100 ml – Erlenmeyer flask)[a]	Initial pH	Temperature (°C)	Incubation period (day)
Weight change during cultivation	PDB[b]	24	20	5.0	26	0–70
Temperature	PDB	24	10	5.0	5–45	14
pH value	PDB	24	10	3.0–9.3	26	14
Glucose to yeast extract ratio	Glucose	0–100	10	5.0	25	14
	Yeast extract	1				
Carbon source	17 kinds of carbon sources[c]	30	10	5.0	26	14
	Yeast extract	1				
Nitrogen source	Glucose	30	10	5.0	26	14
	10 kinds of nitrogen sources[d]	1				
Amino acid assistance	Glucose	30	10	5.0	26	14
	Yeast extract	0.6				
	18 kinds of amino acid[e]	0.4				
Water potential	PDB with PEG6000 (0–50%, w/v)[f]	24	10	5.0	26	14

a $n = 8$.
b Potato dextrose broth (Tanabe, Tokyo).
c The list of the sources are listed in Fig. 6.
d The list of the sources are listed in Fig. 7.
e The list of the sources are listed in Fig. 8.
f Polyethylene glycol with 6000 molecular weight.

analyzed using Tukey's post hoc test ($p < 0.01$) for the results of each experimental item for each fungus.

The mycelial weights of F2, F6, and F43 increased linearly from day 7 to 14, 21, and 35, respectively, then showed gradual increase, and were constant thereafter (Fig. 2A). Mycelia of Fp showed the same tendency as those of F43. The quantities of remaining glucose in the media of F2, F6, and F43 rapidly decreased from day 7 to 21 (Fig. 2B), while the quantity of glucose in the medium of Fp rapidly decreased from day 7 to 14.

The mycelia of F2 and F43 grew between 10 and 35 °C, and those of F6 between 5 and 45 °C. There was statistically no difference between the growth of F2 at 25 °C and that at 30 °C, and between the growth of F6 at 25 °C and that at 30 °C (Fig. 3). F43 grew best statistically at 25 °C. Therefore, the significantly best optimum temperature for *Fomitiporia* sp. was 25 °C. The mycelia of Fp grew also between 5 and 40 °C, and there was statistically no difference between the growth at 25 °C and that at 30 °C.

The best mycelial growth of F2 was obtained in medium of pH4, and there was no significant difference between pH4 and pH6 (Fig. 4A). The best mycelial growth of F6 was also obtained

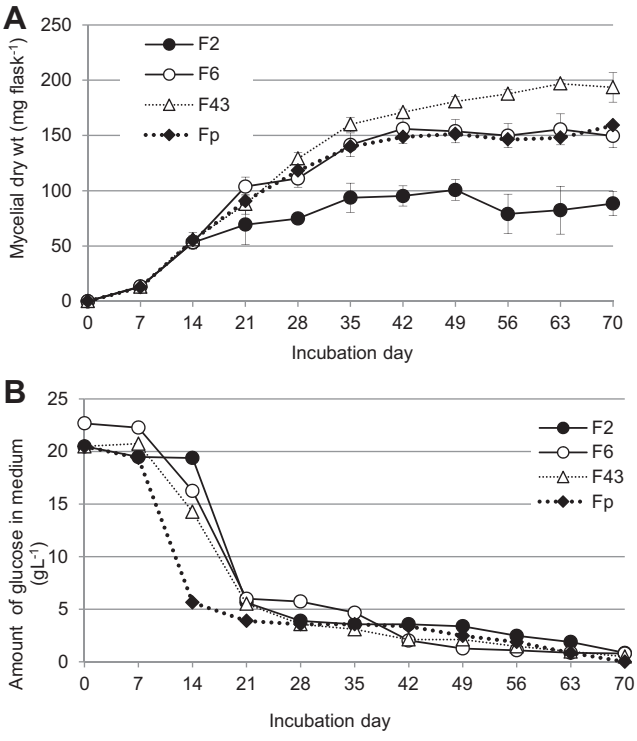

Fig. 2 – Changes in mycelial growth (A) and remaining glucose (B) after inoculation of three isolates of the causal *Fomitiporia* sp., F2, F6, and F43, and one isolate of *F. punctata*, Fp.

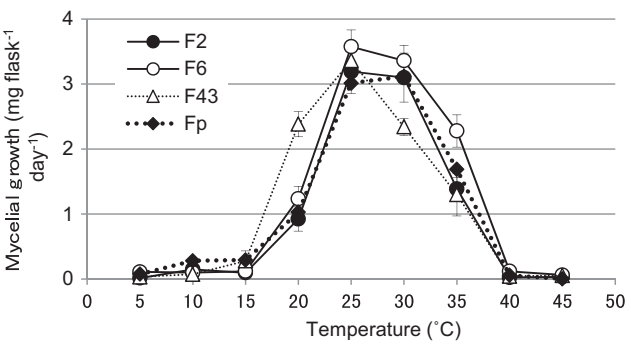

Fig. 3 – Influence of temperature (5–45 °C) on mycelial growth of three isolates of the causal *Fomitiporia* sp., F2, F6 and F43, and one isolate of *F. punctata*, Fp.

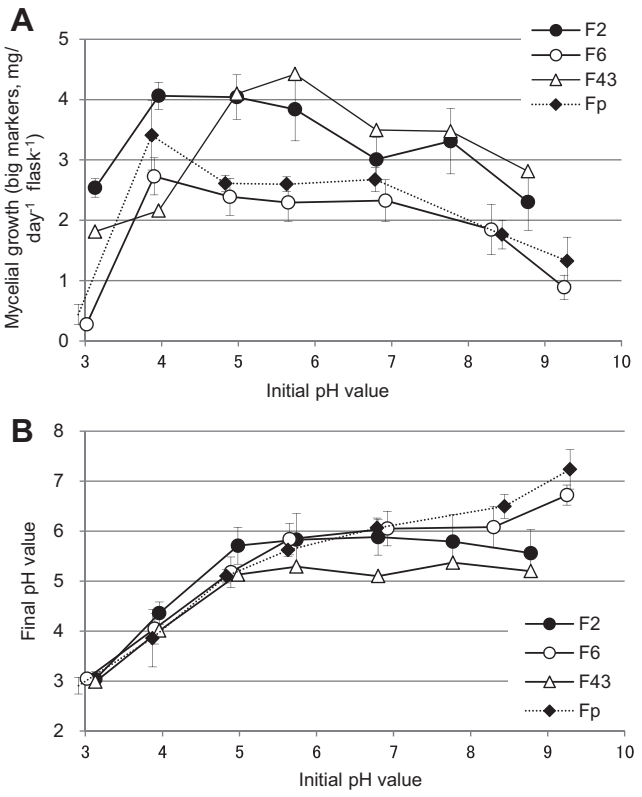

Fig. 4 – Influence of initial pH value (3–9) in media on mycelial growth (A) and final pH value (B), of three isolates of the causal *Fomitiporia* sp., F2, F6, and F43, and one isolate of *F. punctata*, Fp.

in medium of pH4, and there was no significant difference between pH4 and pH7. Mycelia of F43 grew best in medium of pH6, and there was no significant difference between pH5 and pH 6. Thus, the best mycelial growth of *Fomitiporia* sp. was obtained in medium of pH 5 and 6. Mycelia of Fp grew best in medium of pH 4 and pH 8, and there was no significant difference between pH 5 and pH 7. The final pH values after incubation of F2 and F6 were close to the initial pH values in the media with initial pH values between 3 and 6 (Fig. 4B). The final pH values after incubation of F43 were close to the initial pH values in the media with initial pH values between 3 and 5. The pH values of media with initial pH of 7, 8, or 9, in which F2 and F6 were grown, showed a decrease in pH to around 6 or 7, and that of the media with initial pH between 6 and 9, in which F43 was grown, declined to around pH 5. The final pH values of Fp showed the same tendency as those of F2 and F6.

Among GY ratios of 0–100, the average mycelial growth of F2 was best in medium of ratio 40, and there was no significant difference between ratios 30 and 45 (Fig. 5). The F6 had the best mycelial growth in medium of ratio 80, but there was no significant difference between ratios 45 and 100. The best mycelial growth of F43 was obtained in medium of ratio 55, but there was no significant difference between ratios 10 and 80. Therefore, the *Fomitiporia* sp. grew best in medium of ratio 45. The mycelial growth of Fp increased according to the increment of the GY ratio, showing a difference from the other isolates, but there was no significant difference between ratios 60 and 100.

The mycelia of F2 significantly utilized amylose, and secondly pectin and galactan (Fig. 6). The mycelia of F6 significantly utilized amylose, and secondly carboxymethyl (CM)-cellulose and xylan. The mycelia of F43 significantly utilized CM-cellulose, and secondly pectin and xylan. Thus, the mycelia of *Fomitiporia* sp. utilized amylose, CM-cellulose and pectin. The mycelia of Fp utilized pectin, and secondly xylan, and galactan. The mycelia of all three isolates, F6, F2 and F43 were significantly best at utilizing yeast extract and polypepton (Nihon Pharmaceutical, Tokyo, Japan), followed by casamino acid (Fig. 7). The mycelia of Fp showed the same tendency. The mycelia of F6 grew significantly best aided by L-tyrosine, glycine, and L-proline, and those of Fp were significantly aided by L-serine, L-glutamic acid, and L-aspartic acid (Fig. 8).

Water potential expressed in the pressure unit, Mpa, has a negative value; the greater the negative value, the greater force a fungus must overcome to obtain water. The mycelia of F2 grew best when water potentials were −1.1, −1.7 and 2.5 (Fig. 9). The mycelia of F6 grew best when water potentials were −0.9, −1.1 and −1.7, and those of F43 −1.7 and −2.5. The best water potential for the growth of *Fomitiporia* sp. was

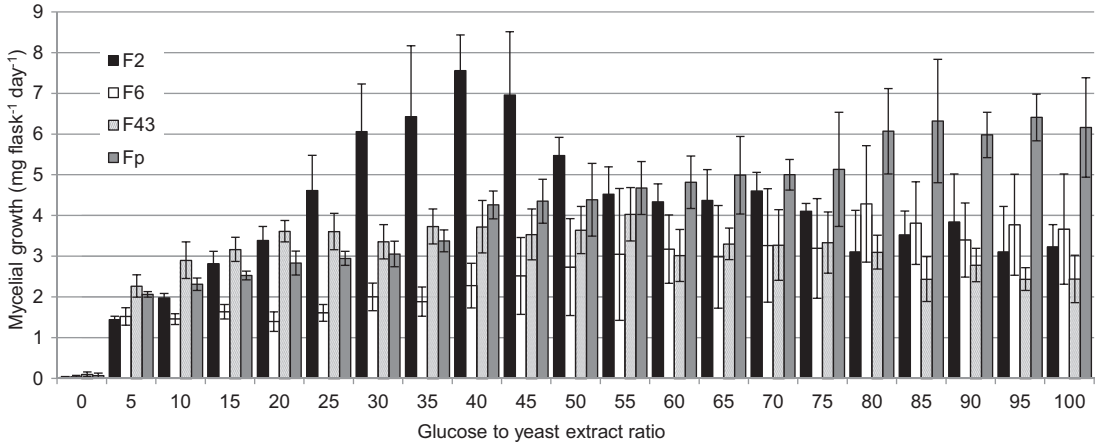

Fig. 5 – Influence of glucose to yeast extract ratio (0–100) in media on mycelial growth of three isolates of the causal *Fomitiporia* sp., F2, F6, and F43, and one isolate of *F. punctata*, Fp.

−1.7 Mpa. The mycelia of Fp grew best when water potentials were −2.5. All isolates grew in the media even when water potential was −8.3 Mpa.

Numerous factors of physical, chemical, or microbial nature may influence growth of pathogenic fungi in wood (Schwarze et al. 1999). Reactions to abiotic environmental factors, temperature, nutrients, pH, and water, vary among fungi and are obviously important in relation to colonization (Cook and Rayner 1984; Rayner and Boddy 1988; Schwarze et al. 1999). The three isolates of the causal fungus *Fomitiporia* sp., F2, F6 and F43, showed similar tendencies in change in mycelial growth after inoculation (Fig. 2), optimal temperature (25 °C, Fig. 3), pH value (pH 5–6, Fig. 4), glucose to yeast extract ratio (45, Fig. 5), suitable carbon sources (amylose, CM-cellulose and pectin, Fig. 6), suitable nitrogen sources (yeast extract and polypepton, Fig. 7), and suitable water potential (−1.7 Mpa).

The three isolates of *Fomitiporia* sp., F2, F6, and F43, were in a different phylogenetic clade from *F. punctata* (Hattori et al. 2009; Ota et al. 2009; Hattori et al. 2010; Ota et al. 2010). *Fomitiporia punctata* is found in Europe, North America, and temperate Asia (Cony et al. 2007), and is known to be

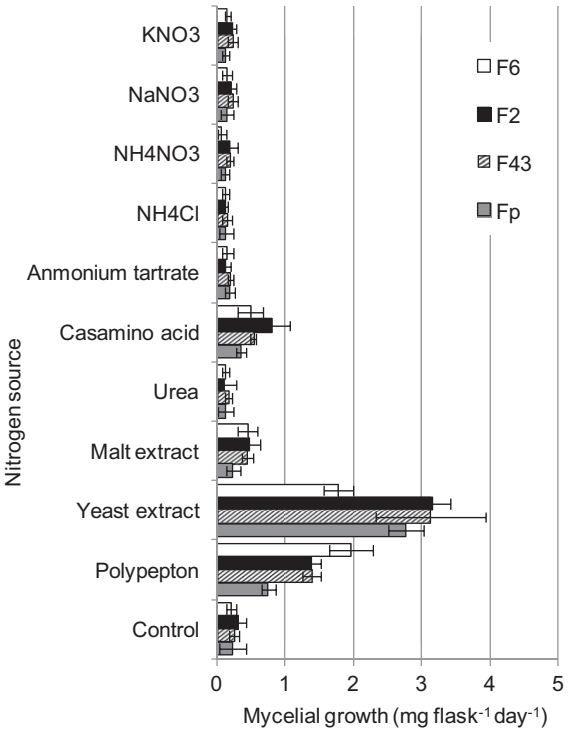

Fig. 7 – Influence of 10 different nitrogen sources in media on mycelial growth of three isolates of the causal *Fomitiporia* sp., F2, F6, and F43, and one isolate of *F. punctata*, Fp.

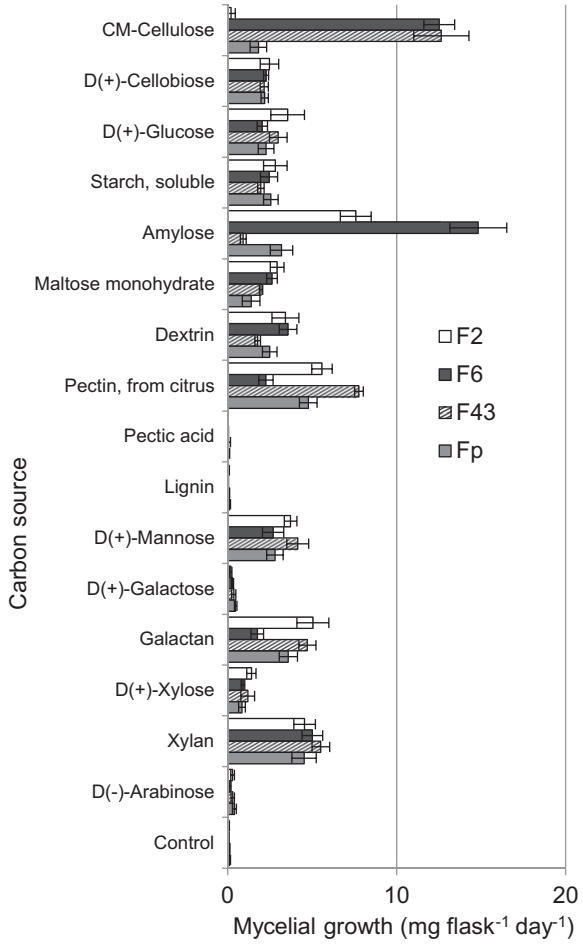

Fig. 6 – Influence of 17 different carbon sources in media on mycelial growth of three isolates of the causal *Fomitiporia* sp., F2, F6, and F43, and one isolate of *F. punctata*, Fp.

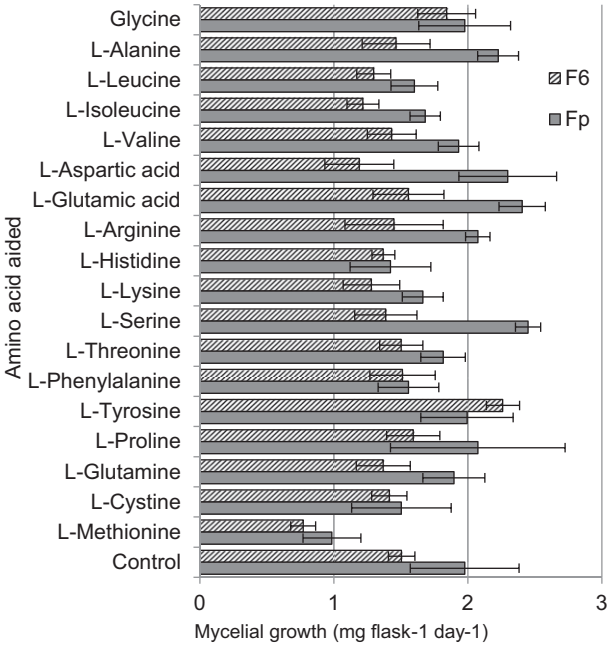

Fig. 8 – Influence of the addition of 18 different amino acids as nitrogen sources in media on mycelial growth of three isolates of the causal *Fomitiporia* sp., F2, F6, and F43, and one isolate of *F. punctata*, Fp.

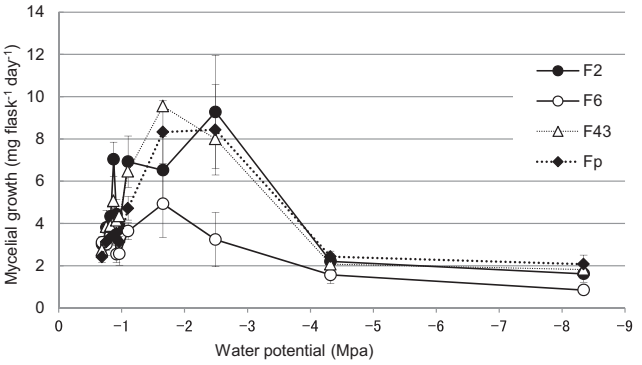

Fig. 9 – Influence of water potential of media on mycelial growth of three isolates of the causal *Fomitiporia* sp., F2, F6, and F43, and one isolate of *F. punctata*, Fp.

pathogenic to grapevines (Cortesi et al. 2000). Interestingly the isolate of *F. punctata* used in this study was obtained in Yamanashi, the high-elevated area in Japan (Table 1). The three isolates of *Fomitiporia* sp. showed no difference in optimal temperature from the reference isolate, Fp, derived from the area of lower temperature. *Fomitiporia torreyae* Y.C. Dai & B.K. Cui was originally described on living trees of *Torreya grandis* (Fortune ex Lindl.) from eastern China (Dai and Cui 2005), and later it was found on living trees of *Cryptomeria* (Dai 2012). Further study is needed to confirm whether *Fomitiporia* sp. on *Cr. japonica* is the same as *F. torreyae*.

The mycelia of *Fomitiporia* sp. utilized cellulose and saccharides, and components of hemicellulose (Fig. 6); these substances are suitable for cultivation of the causal fungus. Amylose is a disaccharide contained in living and dead plant tissues. Cellulose is a polysaccharide composed of microfibrillar aggregates of glucose polymers in plant cell walls. Pectin is polymer of galacturonic acid in plant primary cell walls and middle lamellae, and hemicellulose is a short-chain polymer of mannose, galactan, xylane, and others in plant cell walls (Cook and Rayner 1984).

The isolates of *Fomitiporia* sp. grew slightly, even in medium of −8.3 Mpa, although the critical water potential for most fungi has been reported as −2.5 to −6.0 (Boddy 1983; Dix 1985; Mswaka and Magan 1999).

The mycelia of F2, F6 and F43, when compared with the isolate of *F. punctata*, Fp, showed less ability to utilize glucose against yeast extract (Fig. 5). The isolates F2, F6, F43, and Fp showed differences in optimal pH (Fig. 4). *Fomitiporia* sp. grew best in media with pH 5–6, while Fp grew best in media with pH 8 as well as pH 4.

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