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Organic acids and water-soluble phenolics produced by *Paxillus* sp. 60/92 together show antifungal activity against *Pythium vexans* under acidic culture conditions

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Abstract Ectomycorrhizal fungi can produce antifungal compounds in vitro as well as in symbiosis with the host plant that can reduce root diseases. The objective of this study was to isolate antifungal compounds from culture filtrate of *Paxillus* sp. 60/92, which can form mycorrhizas with *Picea glehnii* seedlings. Culture filtrate of *Paxillus* sp. 60/92 showed antifungal activity against *Pythium vexans* at pH 3–4 but not at pH 5–10, although sterile MMN-b liquid medium (pH 3–10) did not show antifungal activity. Upon separation of antifungal compounds in the culture filtrate, antifungal activity was detected in the organic acid and water-soluble phenolics fractions adjusted to pH 3. Although antifungal activity of individual fractions was lower than that of the culture filtrate, a mixture of these fractions showed antifungal activity similar to that of the culture filtrate. Furthermore, antifungal activity of oxalic acid, which is known to be produced by *Paxillus involutus*, was increased by mixing with the water-soluble phenolic fraction. Our findings indicate that *Paxillus* sp. 60/92 produces organic acids and water-soluble phenolics that together show antifungal activity at pH 3–4 against *P. vexans*.

Keywords *Paxillus* sp. · Antifungal compound · *Pythium vexans* · *Picea glehnii*

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Introduction

Ectomycorrhizal fungi are essential in maintaining the vigor of conifer seedlings. Many researchers have investigated disease-resistance mechanisms of conifers associated with ectomycorrhizal fungi; for example, production of antifungal compounds by either the mycosymbiont or host plant, a barrier effect of the fungal sheath around roots, and nutrient competition between ectomycorrhizal and pathogenic fungi (Zak 1964; Marx 1972). Several researchers have reported the chemical protection of ectomycorrhizas from pathogenic infection (Krupa and Fries 1971; Melin and Krupa 1971; Krupa and Nylund 1972; Krupa et al. 1973; Sylvia and Sinclair 1983). (For a review of chemical protection, see Duchesne and Peterson 1987.) Not only ectomycorrhizas but also ectomycorrhizal fungi in vitro have been reported to produce antifungal compounds. Duchesne et al. (1988, 1989) found that *Paxillus involutus* Fr. produced an antifungal compound, oxalic acid, against *Fusarium oxysporum* Schlecht. emend Snyd. & Hans f. sp. *pini* and that production was induced by exudates from *Pinus resinosa* Ait. seedlings. Kope and Fortin (1989, 1990) found that cell-free culture medium of *Pisolithus tinctorius* (Pers.) Coker & Couch inhibited the mycelial growth of 17 phytopathogens, and that liquid culture caused hyphal lysis and inhibited conidial germination and mycelial growth of a range of phytopathogens. Antifungal compounds produced by *Pisolithus arhizus* (Scop.: Pers.) Rauschert [syn. *P. tinctorius* (Pers.) Coker & Couch] were isolated and identified as *p*-hydroxybenzoylformic acid (pisolithin A) and (*R*)-(−)-*p*-hydroxymandelic acid (pisolithin B) (Kope et al. 1991). In contrast, Rasanayagam and Jeffries (1992) found that *P. involutus* (Batsch. ex Fr.) Fr. inhibited the growth of *Pythium ultimum* Trow on MMN medium, but this effect was eliminated by maintaining the culture medium at pH 6 with a biological buffer. They also found that *P. involutus* produced a large amount of acid and concluded that acidification of the medium was the cause of the inhibition.

Picea glehnii (Fr. Schm.) Masters is a slow-growing and dominant spruce in Hokkaido, northern Japan, where it grows naturally on infertile serpentine soil, volcanic ash soil or in areas of bogs and gravel (Tatewaki 1958; Matsuda 1989). *P. glehnii* is better adapted to serpentine soil than *Picea jezoensis* Carr. and *Picea abies* Karst., at least in part because of its greater needle life span and higher needle nutrient concentrations (Kayama et al. 2002). Phytopathogens (*Racodium*, *Pythium*, *Fusarium*, and *Rhizoctonia* species) pose a threat to planted and naturally regenerated *P. glehnii* seedlings (Endo et al. 1985; Jinno 1986). Kasuya (1995) examined the antifungal activity of 22 ectomycorrhizal fungi that can form mycorrhizas with *P. glehnii* seedlings in vitro against pathogens (*Pythium vexans* de Bary, *Fusarium roseum* Link ex Gray and *Rhizoctonia solani* Kühn) on MMN-a agar. All ectomycorrhizal fungi inhibited the growth of *P. vexans*, 18 inhibited the growth of *R. solani* and 14 inhibited the growth of *F. roseum*. Yokota (1995) also confirmed that *Paxillus* sp. 60/92 (one of the strains examined by Kasuya) inhibited mycelial growth of *Pythium vexans*, *F. roseum* and *R. solani* on MMN-b agar. Kasuya (1995) and Yokota (1995) suggested that antifungal compounds produced by *Paxillus* sp. 60/92 inhibit the growth of pathogens as reported by Duchesne et al. (1988, 1989), Kope and Fortin (1989, 1990) and Kope et al. (1991). However, according to the report of Rasanayagam and Jeffries (1992), it is important to consider whether or not acidification of *Paxillus* sp. 60/92 culture filtrate causes antifungal activity against pathogens.

The objective of this study was to isolate antifungal compounds against *Pythium vexans* from culture filtrate of *Paxillus* sp. 60/92. A second aim was to study the effect of pH on antifungal activity of the culture filtrate. Although we could not identify antifungal compounds, we analyzed the antifungal activity of separate fractions extracted from the culture filtrate.

Materials and methods

Fungi

Paxillus sp. 60/92 was isolated from tissues of basidiocarp in Asahikawa, northern Japan, and can form mycorrhizas with *Picea glehnii* seedling roots in vitro (Kasuya 1995). The basidiocarp of *Paxillus* sp. 60/92 was similar to that of *Paxillus filamentosus* Fr. and *P. involutus*. Analyses of 28S rDNA D1, D2 region of *Paxillus* sp. 60/92 showed high similarities to *Paxillus vernalis* and *P. involutus* (with 99.5 and 99.2% similarity, respectively). A damping-off fungus, *P. vexans*, isolated from seedlings in a nursery (Kasuya 1995) was grown on MMN-b agar (1.0% glucose, 2% agar, Kottke et al. 1987) in the dark at 25°C and used for the antifungal tests.

Culture of *Paxillus* sp. 60/92

Three mycelial disks (8 mm in diameter) of *Paxillus* sp. 60/92 grown on MMN-b agar (Kottke et al. 1987) for 10 days in the dark at 25°C were inoculated into MMN-b liquid medium (100 ml) in an Erlenmeyer flask (300 ml). The culture was incubated in the dark at 25°C on a rotary shaker (100 rpm) for 8, 12, 16, 20, 27 or 31 days with three replicates per incubation time. Mycelial dry weight and pH value in the culture were measured. Culture filtrate of 8, 12, 16, 20, 27 or 31 days was concentrated 14-fold in vacuo at 40°C to use for antifungal tests because the original culture filtrate showed low antifungal activity. Culture filtrate concentrated 14-fold and then adjusted to pH 3 with 1 M HCl (original pH of culture filtrate, see Fig. 2a), was also used for antifungal tests to examine whether or not culture pH influenced antifungal activity.

Measurements

Mycelial dry weight

The culture medium was filtered through no.1 filter paper (Advantec, Tokyo, Japan) in vacuo and mycelia were rinsed three times with de-ionized water. The filter paper with mycelia was dried in an oven at 160°C for 5 h. The mycelial dry weight was calculated as the difference between the dry weights of filter paper with and without mycelia.

pH

The pH of sterile MMN-b liquid medium and culture filtrate was measured with a pH microprobe (ISFET pH meter, Beckman Coulter, Fullerton, Calif.).

Antifungal test

An antifungal test was used to evaluate antifungal activity of (1) concentrated sterile MMN-b liquid medium and culture filtrate with or without adjustment to various pH values, (2) fractions and mixtures of several fractions obtained by separation of antifungal compounds, and (3) a mixture of oxalic acid and fraction *e* (Fig. 1). A mycelial disk (8 mm in diameter) of *P. vexans* grown on MMN-b agar for 2–3 days was inoculated onto the center of an MMN-b agar plate (90 mm in diameter). Samples (250 μl) were filtered through a 0.22 μm syringe filter (Advantec, Tokyo, Japan) and used to aseptically fill a sterile ring (10×6 mm i.d., made of 1 mm thick stainless steel) placed 16 mm from the margin of the *P. vexans* disk. After 36-h incubation in the dark at 25°C, the inhibition zone (mm) was measured from the center of the ring to the margin of the mycelium. Each test was conducted three times and the values were averaged.

Antifungal activity of concentrated sterile MMN-b liquid medium and 2-fold concentrated culture filtrate of *Paxillus* sp. 60/92 at different pH values

Antifungal tests of concentrated and then pH-adjusted sterile MMN-b liquid medium were conducted to examine whether concentration and pH influence antifungal activity. Sterile MMN-b liquid medium (pH 6.33) was concentrated 2, 4, 6, 8, 10 or 14 times in vacuo at 40°C. Concentrated medium was adjusted to pH 1, 2, 3, 4 or 5 with 1 M HCl and used for antifungal tests. Sterile MMN-b liquid medium concentrated 2-fold did not show antifungal activity influenced by concentration, therefore 2-fold culture filtrate of *Paxillus* sp. 60/92 31 days after the start of incubation was also used for antifungal tests to examine whether pH influences antifungal activity of the culture filtrate. The culture filtrate was concentrated twice in vacuo at 40°C and then adjusted to pH 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 with 1 M HCl and 1 M NaOH. The antifungal result of the 2-fold culture filtrate adjusted to each pH was compared with that of 2-fold sterile MMN-b liquid medium also adjusted to each pH. Three replicates were prepared per treatment.

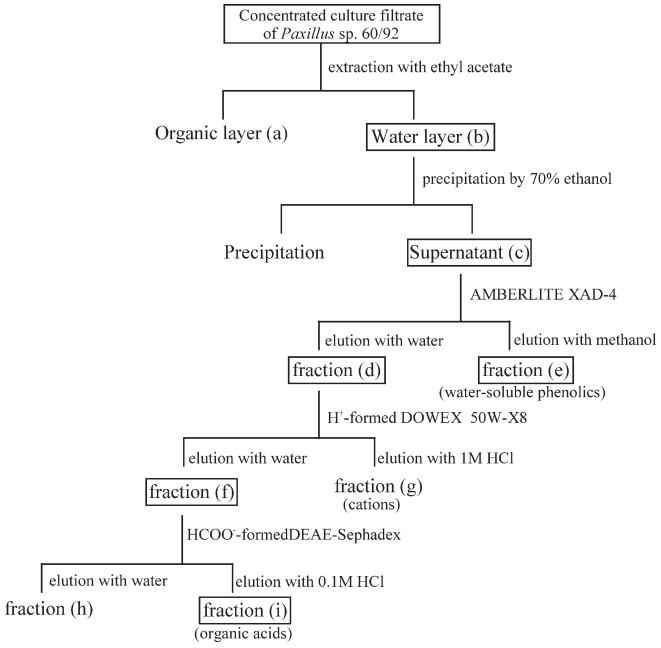

Fig. 1 Procedure for separation of antifungal compounds in culture filtrate. Seven-fold concentrated fractions (equivalent to 6 mg mycelial dry weight) were used for antifungal tests. Fractions in rectangles showed antifungal activity

Separation of antifungal compounds in the culture filtrate

Antifungal compounds in the culture filtrate were separated according to the procedure shown in Fig. 1. The filtrate of the culture grown for 31 days (1,100 ml, 5.30 g dry weight) was concentrated to 500 ml and extracted twice with ethyl acetate (250 ml). The organic layer (*a*) was dried over Na_2SO_4 followed by vacuum concentration (0.09 g). The water layer (*b*) was dried (5.20 g), dissolved in 70% ethanol (200 ml) and then centrifuged (3,000 rpm, 10 min). The supernatant (*c*) was dried in vacuo at 40°C (4.49 g). Part of the latter (4.27 g) was dissolved in de-ionized water and applied to an AMBERLITE XAD-4 column (160×80 mm in diameter) (Rohm and Haas, Philadelphia, Pa.) pre-equilibrated with de-ionized water for further fractionation. Fraction *d* was eluted with de-ionized water (3 l) and dried in vacuo at 40°C (3.11 g). Thereafter, fraction *e* was eluted with methanol (3 l) and dried in vacuo at 40°C (1.23 g). AMBERLITE XAD resin is a polymeric adsorbent (polystyrene) whose internal surface can adsorb phenols in water (Thurman et al. 1978; Maggi et al. 1989); therefore fraction *e* contained water-soluble phenolics. Part of fraction *d* (2.69 g) was dissolved in de-ionized water and applied to an H^+-formed DOWEX 50W-X8 column (100×50 mm in diameter) (The Dow Chemical Company, Mich.) pre-equilibrated with de-ionized water. Fraction *f* was eluted with de-ionized water (3 l) and dried in vacuo at 40°C (2.30 g). Fraction *g* was then eluted with 1 M HCl (2 l) and dried in vacuo at 40°C (0.52 g). The H^+-formed DOWEX 50W-X8 resin can adsorb cations in water (The Dow Chemical Company 2002); therefore fraction *g* contained cations. Part of fraction *f* (1.97 g dry weight) was dissolved in de-ionized water and applied to formic acid ion-formed DEAE-Sephadex A-50 column (100×50 mm in diameter) (Amersham Biosciences, Piscataway, N.J.) pre-equilibrated with de-ionized water. Fraction *h* was eluted with de-ionized water (1.5 l) and dried in vacuo at 40°C (0.24 g dry weight). Fraction *i* was then eluted with 0.1 M HCl and dried in vacuo at 40°C (1.18 g dry weight). Formic acid ion-formed DEAE-Sephadex resin is a weakly basic anion exchanger (Amersham Biosciences 2003), therefore it can adsorb weak acidic compounds in water, so fraction *i* contained organic acids.

Antifungal activity of fractions and mixtures of fractions

All fractions (*a*–*i*) separately and mixed fractions (*d*+*e*), (*f*+*g*), (*h*+*i*) were examined for antifungal activity. The effect of fraction *e* on the activity of fractions *f* and *i* was also examined. All fractions and the mixed fractions (equivalent to 6 mg mycelial dry weight, 7-fold concentrated sample) were adjusted to pH 3 for the antifungal test. As a control, sterile MMN-b liquid medium that had been shaken at 25°C in the dark for 31 days was fractionated by the same separation method and 7-fold concentrated fractions adjusted to pH 3 were used for antifungal tests. When testing the organic layer (*a*), the following antifungal test (paper disk method, Yamaji et al. 1999) was used. Concentrated organic layer (25 µl) equivalent to 6 mg mycelial dry weight was aseptically applied to a paper disk (8 mm in diameter). The paper disk was dried in vacuo to remove the solvent and placed 16 mm from the margin of the *P. vexans* disk. After a 36-h incubation in the dark at 25°C, the inhibition zone (mm) was measured from the center of the disk to the margin of the mycelium. Each test was conducted three times and the values were averaged.

Antifungal activity of oxalic acid and fraction *e*

Oxalic acid (Wako, Tokyo, Japan) was dissolved in de-ionized water at 200, 500, 1,000 and 2,000 µg/ml. Oxalic acid was also dissolved in fraction *e* (equivalent to 6 mg mycelial dry weight, 7-fold concentrated sample) at the same concentrations as the oxalic acid solutions described above. These solutions (250 µl) were adjusted to pH 3 for the antifungal test. Because fraction *e* adjusted to pH 3 produced a 4-mm inhibition zone, we subtracted 4 mm from the measured inhibition zones when examining the antifungal activity of the mixture of oxalic acid and fraction *e*.

Preliminary analysis of oxalic acid in fraction *d*

After excess sodium hydrogen carbonate was added into fraction *d* (250 mg dry weight, ~300 mg mycelial dry weight), fraction *d* was dried in vacuo at 40°C. The dried fraction was dissolved in 2 M HCl, dried again in vacuo and dissolved in methanol. The methanol solution was dried in vacuo, dissolved in diazomethane solution (1 ml, 0.17 mmol) to form dimethyl oxalate and then analyzed by gas chromatography-mass spectrometry (JEOL JMS-AX500, JEOL, Tokyo, Japan) equipped with an electron impact ionizer detector on a capillary column DB-WAX (60 m ×0.32 mm in diameter, Agilent Technologies, Wilmington, Del.). The column temperature was set at 40°C to 200°C at a rate of 4°C/min. The injector temperature was 170°C and flow rate of carrier gas (He) was 1 ml/min. The detector interface temperature was set at 150°C, with actual temperature in the MS source reaching approx. 200°C, and the ionization voltage was 70 eV. The mass spectrum of dimethyl oxalate in fraction *d* was identified by comparison with an authentic standard (Kanto, Tokyo, Japan).

Statistical analysis

One-factor ANOVA was used to compare antifungal activity between fractions. Two-factor ANOVA was used to compare antifungal activity between oxalic acid with or without fraction *e*. Differences were considered significant at $P<0.05$.

Results

Antifungal activity of culture filtrate

All concentrated sterile MMN-b liquid media showed antifungal activity against *P. vexans* at pH 1 and 2

Table 1 Inhibition zone of concentrated sterile MMN-b medium and 2-fold concentrated culture filtrate at pH 1–5 or pH 1–10. Culture filtrate of *Paxillus* sp. 60/92 grown for 31 days was used for the antifungal test. A 2-fold concentrated culture filtrate was equivalent to 1.7 mg mycelial dry weight. Original culture filtrate without pH adjustment (equivalent to 0.86 mg mycelial dry weight) caused an inhibition zone of ~2 mm. Results were expressed as mm±SE

Samples	pH 1	pH 2	pH 3	pH 4	pH 5* or pH 5–10**
2× Sterile MMN-b	20±0	15±0.6	0±0	0±0	0±0**
4× Sterile MMN-b	20±0	15.3±0.7	0±0	0±0	0±0*
6× Sterile MMN-b	20±0	17.5±1.5	0±0	0±0	0±0*
8× Sterile MMN-b	20±0	17.0±0.6	0±0	0±0	0±0*
10× Sterile MMN-b	20±0	14.7±0.3	2.7±1.3	0±0	0±0*
14× Sterile MMN-b	20±0	17.0±0.6	4.7±0.7	0±0	0±0*
2× Culture filtrate	20±0	16±0.3	5.5±0.8	1.3±1.3	0±0**

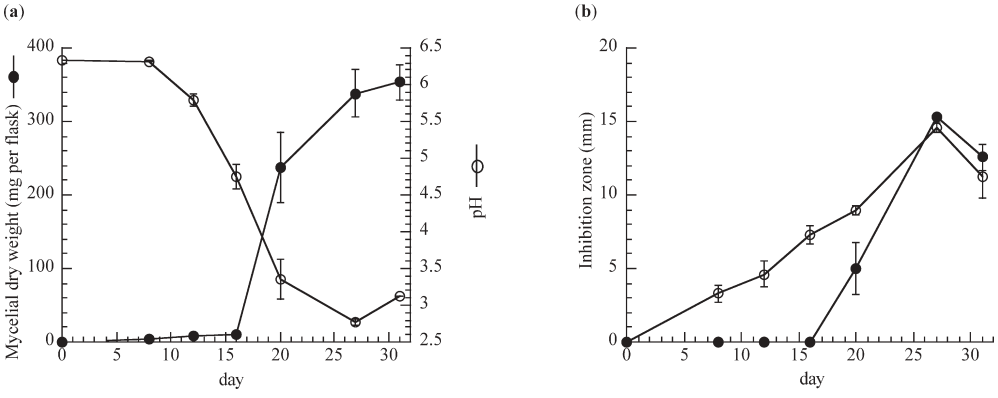

Fig. 2a, b Culture of *Paxillus* sp. 60/92 during a 31-day incubation. **a** *Closed circles* Mycelial dry weight (mg), *open circles* pH±SE. **b** Antifungal activity of 14-fold concentrated culture filtrate: *closed circles* without adjusting pH, *open circles* pH adjusted to pH 3. A 14-fold concentrated sterile MMN-b medium caused a 4.7-mm inhibition zone at pH 3 (Table 1), therefore 4.7 mm of inhibition zone was subtracted from the inhibition zone of concentrated culture filtrate of 20, 27 and 30 days (pH 3.4, 2.8 and 3.1, respectively, see Fig. 2a) and concentrated culture filtrate adjusted to pH 3. Results were expressed as inhibition zones (mm)±SE

(Table 1); 10- and 14-fold concentrated medium also showed antifungal activity at pH 3 (2.7 mm and 4.7 mm inhibition zones, respectively). None of the concentrated media showed any antifungal activity at pH 4 or 5 (Table 1). A 2-fold concentrated sterile MMN-b liquid medium did not show antifungal activity at pH 3–10 (Table 1). Therefore, we concentrated 2-fold and then adjusted the culture filtrate pH to various values to examine influence of pH on antifungal activity. The concentrated culture filtrate and sterile MMN-b liquid medium showed antifungal activity at pH 1 and 2, but not at pH 5–10 (Table 1). The concentrated culture filtrate showed antifungal activity at pH 3 and 4, while the concentrated sterile MMN-b liquid medium did not (Table 1).

Mycelial dry weight, pH and antifungal activity of culture filtrate during a 31-day incubation

Mycelial dry weight increased after day 16 of incubation and the pH decreased to less than 4 after day 20 (Fig. 2a). The 14-fold concentrated culture filtrate without adjustment of pH showed antifungal activity on day 20 for the first time and this continued until day 31 (Fig. 2b). In contrast, culture filtrate adjusted to pH 3 showed antifungal activity from day 8 to day 31 (Fig. 2b).

Antifungal activity of fractions

Antifungal activity of all fractions and the mixed fractions (equivalent to 6 mg mycelial dry weight, 7-fold concentrated sample) adjusted to pH 3, was examined. The organic layer (*a*, see Fig. 1) did not show antifungal activity. The water layer (*b*) and supernatant (*c*) showed antifungal activity similar to that of culture filtrate (Fig. 3, $P>0.05$). After separation with AMBERLITE XAD-4 resin, antifungal activity was detected mainly in fraction *d*, but it was lower than that of the culture filtrate (Fig. 3, $P<0.01$). Although fraction *e*, containing water-soluble phenolics, showed significantly lower antifungal activity than the culture filtrate, fractions *d*+*e* showed antifungal activity similar to that of the culture filtrate (Fig. 3, $P>0.05$). After separation with H^+-formed DOWEX 50W-X8 resin, fraction *f* showed antifungal activity resembling that of fraction *d* (Fig. 3, $P>0.05$) and the activity was lower than that of the culture filtrate (Fig. 3, $P<0.01$). Fraction *g*, containing cations, showed no antifungal activity, and fractions *f*+*g* showed lower antifungal activity (inhibition zone, 4±0 mm) than fraction *f*

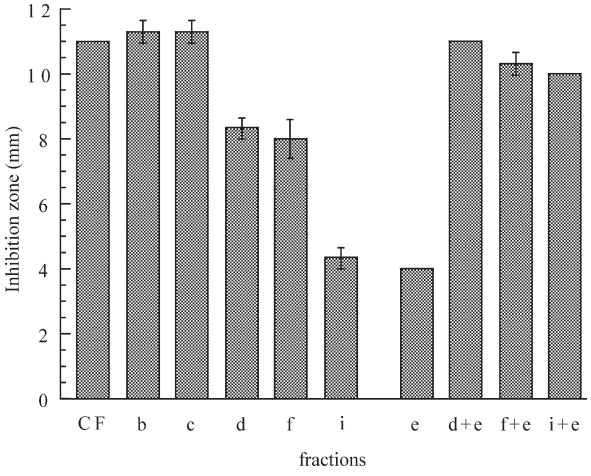

Fig. 3 Antifungal activity of fractions and mixtures of fractions. *CF* Culture filtrate. Seven-fold concentrated fraction (equivalent to 6 mg mycelial dry weight) was adjusted to pH 3 for antifungal test. Results were expressed as inhibition zones (mm)±SE. Inhibition zones ±SE of fractions *g*, *f+g* and *h* were 0±0, 4±0 and 0±0, respectively. No fractions of sterile MMN-b liquid medium shaken for 31 days caused inhibition zones

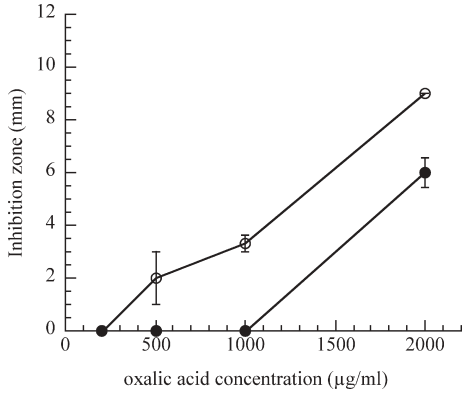

Fig. 4 Antifungal activity of oxalic acid with (*open circles*) or without (*closed circles*) fraction *e*. Fraction *e* equivalent to 6 mg mycelial dry weight (7-fold concentrated fraction) was used. Solutions were adjusted to pH 3 for antifungal tests. Since fraction *e* adjusted to pH 3 caused a 4-mm inhibition zone, 4 mm was subtracted from the inhibition zone of the mixture of oxalic acid and fraction *e*

($P<0.01$). In contrast, fractions *f+e* had antifungal activity similar to that of the culture filtrate (Fig. 3, $P>0.05$). After separation with formic acid ion-formed DEAE-Sephadex resin, antifungal activity was observed mainly in fraction *i*, containing organic acids, but it was significantly lower than that of the culture filtrate (Fig. 3, $P<0.01$). Fraction *h* did not show any antifungal activity, and the activity of fractions *h+i* was lower than that of fraction *f*. In contrast, fractions *i+e* had the same antifungal activity as the culture filtrate (Fig. 3, $P>0.05$). Fraction *e* was chromatographed on a TLC plate (0.25 mm, Kieselgel 60 F254, Merck) with *n*-butanol-acetic acid-water (4:1:2) and several spots were detected by phenolics-detecting reagents, Gibbs' reagent and silver nitrate solution (data not shown). None of the fractions (7-fold concentrated fractions adjusted to pH 3) obtained by the same procedure using sterile MMN-b liquid medium shaken for 31 days in the dark showed any antifungal activity.

Increase in antifungal activity of oxalic acid by mixing with fraction *e*

Oxalic acid showed antifungal activity at 2,000 μg/ml, but not at less than 1,000 μg/ml (Fig. 4). The antifungal activity of oxalic acid at 1,000 and 2,000 μg/ml was significantly increased by mixing with fraction *e* (Fig. 4, $P<0.001$ and 0.05, respectively).

Preliminary result of oxalic acid in fraction *d*

The peak of dimethyl oxalate in reacted fraction *d* with diazomethane was detected at 37 min after injection. Other peaks were also detected after 37 min, but they could not be identified.

Discussion

The 2-fold concentrated culture filtrate of *Paxillus* sp. 60/92 showed antifungal activity against *Pythium vexans* under acidic conditions (pH 3–4, Table 1). Although highly concentrated (10- and 14-fold) culture filtrate would be expected to show antifungal activity because of some components in the MMN-b liquid medium, 2-fold concentrated culture filtrate would not be expected to show antifungal activity influenced by concentration at pH 3–4 (Table 1). Therefore, we conclude that the antifungal activity of the 2-fold concentrated culture filtrate at pH 3–4 can be attributed to the effect of antifungal compounds. Rasanayagam and Jeffries (1992) found that the antifungal activity of *P. involutus* was due to acidification of MMN agar by production of a large amount of acid. However, our results indicate that antifungal compounds in the culture filtrate of *Paxillus* sp. 60/92 exhibit antifungal activity at pH 3–4. Our results also indicate that *Paxillus* sp. 60/92 produces antifungal compounds during the early period of growth (3.98 mg mycelial dry weight on day 8), but that decrease in pH of the culture filtrate is necessary to show antifungal activity. Serpentine soil in *Picea glehnii* forest in the northern part of Hokkaido is acidic (pH 3.9 at a depth of 0–5 cm, Usui 1997; pH 4.98 at a depth of 0–10 cm, Kayama et al. 2002). Although our results are based on an in vitro experiment and we do not know if the same antifungal compounds are produced in the field, the ability of *Paxillus* sp. 60/92 to produce antifungal compounds showing activity under acidic conditions may be an adaptation for the *Picea glehnii* forest environment.

We attempted to isolate antifungal compounds by the procedure shown in Fig. 1 without success. Our results indicate that organic acids and water-soluble phenolics would be possible antifungal compounds that act together to show activity in the culture filtrate. Kope et al. (1991) reported that hyphal lysis of a pathogenic fungus, *Truncatella hartigii* (Tubeuf.) Stey. was caused by phenolic compounds produced by *Pisolithus arhizus*. In the inhibition zones caused by the culture filtrate, fraction *e* (containing water-soluble phenolics) and fraction *i* (containing organic acids), hyphal swelling and branching of *Pythium vexans* were observed at the edge of the mycelium adjacent to the zone (data not shown). Organic acids and water-soluble phenolics produced by *Paxillus* sp. 60/92 would work together to cause morphological damage to *Pythium vexans*.

Paxillus involutus is known to produce oxalic acid (Duchesne et al. 1988, 1989; Lapeyrie 1987), and the antifungal compounds in fraction *d* seem to be organic acids. Therefore, we examined the antifungal activity of oxalic acid mixed with fraction *e*. Fraction *e* clearly increased the antifungal activity of oxalic acid (Fig. 4) and this supports our hypothesis that organic acids and water-soluble phenolics showed antifungal activity together in the culture filtrate. Preliminary analysis indicated that fraction *d* contained a small amount of oxalic acid. In a control experiment, oxalic acid (5 mg) dissolved in methanol reacted with diazomethane (1 ml, 0.17 mmol), but at a rate of 65%. Therefore, we could not conclude that oxalic acid was one of the antifungal compounds in the culture filtrate because of inaccurate quantification.

Cipollini and Stiles (1992) reported the influence of organic acids and phenolics in ripe ericaceous fruits on antifungal activity. They suggested that interactions with organic acids at pH 2–4 may increase the activity of small phenolics by decreasing their level of ionization, pH-dependent oxidation and substrate-binding reactions. Citrinin (not a phenolic compound but a polyketide compound) produced by *Penicillium oxalicum* is known to show high antifungal activity at acidic pH (pH 3–4) against *Saccharomyces cerevisiae* because uptake of citrinin to *S. cerevisiae* cells increases as the pH decreases (Haraguchi et al. 1987). Complex interactions between organic acids and water-soluble phenolics will also influence *Pythium vexans* cells under acidic conditions in our research. Generally, phenolics and organic acids originating from plant materials or microbial decomposition products accumulate in forest soil (Flaig 1971; Rice 1984; Jones 1998). The antifungal activity exerted by phenolics and organic acids together may act on some microorganisms.

The results of the present study indicate that (1) culture filtrate of *Paxillus* sp. 60/92 shows antifungal activity at pH 3–4 but not at pH$\geq$5, and (2) organic acids and water-soluble phenolics may act together to inhibit growth of *Pythium vexans*. Further studies are underway to clarify the involvement of organic acids and water-soluble phenolics and to elucidate the antifungal system in *Paxillus* sp. 60/92.

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