THE ANTIFUNGAL COMPOUND TOTAROL OF *Thujopsis dolabrata* var. *hondai* SEEDS SELECTS FUNGI ON SEEDLING ROOT SURFACES

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Abstract—Hinoki-asunaro (*Thujopsis dolabrata* Sieb. et Zucc. var. *hondai* Makino) is a tree endemic in Japan whose seeds produce several terpenoids. We hypothesized that antifungal compounds in seeds might select fungi on the root surfaces of *T. dolabrata* var. *hondai* seedlings; that is, antifungal compounds in seeds may influence seed and soil fungi, and those fungi tolerant of antifungal compounds may grow on the seedling root surfaces. We examined seed fungi, soil fungi, the sensitivity of seed and soil fungi to methanol extracts of the seeds, the fungi on root surfaces of seedlings grown in Kanuma pumice (a model mineral soil) and nursery soil, and the frequency at which each fungus was detected on the seedling root surface. We then calculated correlation coefficients between the fungal detection frequency on root surfaces and the fungal sensitivity to the seed extract. We also isolated from the seeds the antifungal compound totarol that selected fungi on root surfaces. Species of *Alternaria*, *Cladosporium*, *Pestalotiopsis*, and *Phomopsis* were the most frequently isolated fungi from the seeds. *Mortierella* and *Mucor* were the dominant fungi isolated from Kanuma pumice, whereas *Umbelopsis* and *Trichoderma* were the main fungi isolated from nursery soil. *Alternaria*, *Cladosporium*, *Mortierella*, *Pestalotiopsis*, and *Phomopsis* were the dominant fungi isolated from root surfaces of seedlings grown in Kanuma pumice, and *Alternaria*, *Cladosporium*, *Pestalotiopsis*, *Phomopsis*, and *Trichoderma* were the main root-surface fungi isolated from seedlings grown in nursery soil. The fungal detection frequencies on root surfaces in both soils were significantly and negatively correlated with fungal sensitivity to the seed extract. That is, those seed and soil fungi tolerant of the seed extract grew on the seedling roots. A similar correlation was found between the fungal

detection frequency on the root surfaces and the fungal sensitivity to totarol. We conclude that totarol is one factor that selects fungi on root surfaces of *T. dolabrata* var. *hondai* in the early growth stage.

Key Words—*Thujopsis dolabrata* var. *hondai*, seed fungi, soil fungi, rhizosphere, antifungal compound, totarol.

INTRODUCTION

Hinoki-asunaro (*Thujopsis dolabrata* Sieb. et Zucc. var. *hondai* Makino; also called hiba or ate) is an endemic tree species in Japan. It is distributed from the Oshima Peninsula (42°10'N) on Hokkaido to Tochigi Prefecture (36°47'N) on Honshu (Kobayashi and Asakawa, 1981). The seeds (Hasegawa and Hirose, 1980), leaves (Nagahama et al., 1996; Takahashi et al., 1981), trunk, and branches contain characteristic secondary metabolites. In particular, essential oil extracted from *T. dolabrata* var. *hondai* wood, which contains sesquiterpenes and hinokitiol-related compounds, has antimicrobial activity against bacteria (Okabe et al., 1994; Saito et al., 1997; Morita et al., 2001), wood-rotting fungi (Inamori et al., 2000), and plant pathogens (Morita et al., 2004) and is insecticidal against termites (Miyazaki, 1996; Lee et al., 1997; Ahn et al., 1998). Houses made of the timber are thus free of decay and infestation for a long period, making *T. dolabrata* var. *hondai* a commercially important tree species in Japan.

The seedlings show physiologically and ecologically characteristic germination. Because of its slow growth, several nursing systems for *T. dolabrata* var. *hondai* have been developed (Aoki, 1936; Fujimori, 1958; Sakurai and Mori, 1985a, 1985b; Itoya and Shimoda, 1995, 1999; Nakano, 2000; Aomori Forestry Research Institute, 2004; Mori et al., 2003). In naturally regenerated forests, seedlings grow in mineral soil without humus (Mori et al., 2003). Tohoku Research Center of the Forestry and Forest Products Research Institute (FFPRI) in Japan has developed a nursing system that

imitates conditions in natural forests. The seedlings require 6–30% relative light intensity to germinate and grow, even though *T. dolabrata* var. *hondai* is a shade-tolerant tree (Sakurai and Mori, 1985a, 1985b; Itoya and Shimoda, 1995). Therefore, the nursery bed is set 20-cm lower than the surrounding ground and is covered with plastic shading nets (Itoya and Shimoda, 1999). Instead of the humic nursery soil generally used for the seedbed (Aomori Forestry Research Institute, 2004), Kanuma pumice is used as a model mineral soil of the naturally regenerated forest (Mori et al., 2003).

Plant roots interact with symbiotic, pathogenic, or epiphytic microorganisms in the rhizosphere, and root exudates (amino acids, nucleotides, sugars, and secondary metabolites) influence microbial populations in the rhizosphere (Brimecombe et al., 2001). Flavonoids and isoflavonoids exuded from roots act as organic signals in legume-rhizobia symbiosis (Werner, 2001). For instance, species of *Rhizopus* and *Fusarium* in the rhizosphere of Brassicaceae plants are selected by secondary metabolites (isothiocyanates) exuded from the roots (Ishimoto et al., 2000). Seeds of *T. dolabrata* var. *hondai* have resin cavities on the seed surface and contain several diterpenes and lignans (C_6C_3 dimers; Hasegawa and Hirose, 1980), and terpenoids and phenolics are generally antifungal (Waterman and Mole, 1994). Nursery beds of *T. dolabrata* var. *hondai* smell like terpenoids after germination, and the germination gradually continues for 5 months after sowing (Sakurai and Mori, 1985a; Itoya and Shimoda, 1995; K. Yamaji, unpublished data).

Based on these previous findings, we hypothesized that antifungal compounds of

seeds might select fungi on root surfaces of *T. dolabrata* var. *hondai* seedlings in the early growth stage. That is, antifungal compounds of seeds influence seed and soil fungi, and those seed and soil fungi tolerant of antifungal compounds may grow on seedling root surfaces. In this study, we examined seedlings growing in Kanuma pumice and nursery soil because different soil fungi and nutrient conditions may influence which fungi will grow on the root surfaces. We identified seed fungi and soil fungi and examined the sensitivity of seed and soil fungi to methanol extracts of the seeds. In addition, we identified fungi on the seedling root surfaces and calculated the fungal detection frequency (FDF, %). We then examined the relationship between FDF on root surfaces and the fungal sensitivity to the seed extract. Finally, we isolated from the seeds the antifungal compound totarol, which selected fungi on root surfaces in the early growth stage.

METHODS AND MATERIALS

Seeds, *Soil*, *and Seedlings. Thujopsis dolabrata* var. *hondai* seeds were randomly collected from approximately 20 felled trees in natural forests in Nakasato, Aomori Prefecture, northern Honshu, Japan (40°58'N, 140°28'E) in October 2002. *Thujopsis dolabrata* var. *hondai* trees have good, normal, and poor harvest years, and the harvest (3.23 g fresh weight [FW], 1000 seeds) was poor in 2002. Seeds were kept at 4°C in a paper bag until use.

Kanuma pumice, a volcanic andesitic rock and highly weathered, was purchased (Kyodo-Jirushi, Kanuma, Japan). Fifteen 18-l bags of Kanuma pumice were mixed

uniformly and used for the experiments. Humic nursery soil having andic and allophanic properties was collected in a *T. dolabrata* var. *hondai* nursery in Hiranai (50 × 100 m), Aomori Prefecture, Japan (40°53'N, 141° 00'E) on 16 April 2003. Approximately 18 l of nursery soil was collected from 15 sites (0–10 cm depth) at the nursery and was uniformly mixed. Table 1 shows the general characteristics of the Kanuma pumice and the nursery soil.

Fifty nonsterilized seeds were sown per plastic pot (90-mm height × 100-mm diameter) containing Kanuma pumice or nursery soil on 25 April 2003. The seeds were not covered with soil (Itoya and Shimoda, 1995). The pots were kept in a greenhouse of the Tohoku Research Center, FFPRI, Morioka, Japan (39°46'N, 141°08'E). Twenty pots per soil were placed in a block, and 10 blocks were prepared for replications of each soil. After 1 month, the pots were transferred from the greenhouse to a nursery covered with plastic shading nets that allowed 32% of photosynthetically active radiation, as evaluated by using an LI-1400 datalogger with a quantum sensor (Li-Cor, Lincoln, Nebraska, USA). The seeds were watered every 2 or 3 days, and the position of the blocks was randomized every month. On 1 July 2003, five seedlings were randomly selected from pots of each soil type. The heights and basal diameters of the seedlings were measured before the collection. The seedling roots were rinsed with water and dried on filter paper. The fresh weights of the aboveground parts and roots of each seedling were measured separately (Table 2). The roots of these seedlings were used to isolate fungi on the root surfaces.

Fungal Isolation from Seeds. Forty-five seeds were randomly selected and used to isolate seed fungi. Two seeds were placed separately per plate (90-mm diameter) of potato dextrose broth (10 ml; Wako, Osaka, Japan) containing 2% agar (PDA) and were incubated for 10 days in the dark at 25°C. Fungal colonies were detected using a microscope (×40 and ×100, Nikon Eclipse E400, Tokyo, Japan) and were inoculated onto acidic PDA (pH 3) containing 0.025% citric acid to separate fungi from bacteria. The fungi were again transferred onto acidic PDA, cultivated for 7 days in the dark and then transferred and cultivated on normal PDA. Fungal genera were identified based on Watanabe (2002) and Barnett and Hunter (1998). The fungal detection frequency on the seeds was calculated as:

$$\text{FDF on seeds (\%)} = (N_d/N_t) \times 100 \quad (1),$$

where N_d is the number of seeds from which the fungus was detected and N_t is the total number of seeds (= 45).

Fungal Isolation from Soil. Soil fungi were isolated using the method of Warcup (1950). Four boxes (240 × 120 × 100 mm high) each were prepared to hold the Kanuma pumice and the nursery soil. The soil in each box was fully mixed, and water was added to the boxes until it poured from the bottom. The soil was incubated for 2 days at room temperature. The water contents of the Kanuma pumice and nursery soil after the incubation were 43.3% and 48.6%, respectively. Ten milligrams of Kanuma pumice was

collected and crushed in sterile demineralized water (1 ml) using sterile tweezers on a plastic plate (90-mm diameter). Liquid PDA (10 ml, approximately 40°C) was poured into the plate and mixed fully with the crushed soil. The plate was incubated for 10 days in the dark at 25°C. Ten milligrams of nursery soil was collected and shaken with 10 ml of sterile water in a screw-topped test tube (120-mm height × 25-mm diameter) for 5 min under reciprocal rotation at 250 rpm. A 1-ml sample was then placed in a plastic plate (90-mm diameter) and melted PDA (10 ml, approximately 40°C) was poured into the plate. The plate was incubated for 10 days in the dark at 25°C. Eight replications were prepared for each soil type. Fungal isolation methods and identification of genera were the same as for fungal isolation from seeds. The FDF in the soil was calculated as:

$$\text{FDF in soil (\%)} = (N_d/N_t) \times 100 \quad (2),$$

where N_d is the number of plates from which the fungus was detected and N_t is the total number of plates (= 8).

Fungal Isolation from Seedling Roots. Mycelia were isolated from root surfaces of seedling growing in Kanuma pumice and nursery soil using the method described by Harley and Waid (1955). Roots were rinsed twice with demineralized water and transferred to a screw-topped test tube (120-mm height × 25-mm diameter) containing sterile demineralized water (7 ml). The test tube was reciprocally shaken at 250 rpm for 3 min, and then all the water was discarded and sterile demineralized water (7 ml) was

aseptically added to the test tube. The test tube was shaken again at 250 rpm for 3 min; this procedure was repeated nine times. The roots were then placed on a sterile filter paper (no. 1, Advantec, Tokyo, Japan) to remove extra water and were cut into 5-mm pieces on a glass plate. The pieces were separately placed onto PDA plates and incubated for 10 days in the dark at 25°C. Fungal isolation methods and identification of genera were the same as for fungal isolation from seeds. The FDF on roots was calculated as:

$$\text{FDF on roots (\%)} = (N_d/N_t) \times 100 \quad (3),$$

where, N_d is the number of root pieces from which the fungus was detected and N_t is the total number of root pieces. The FDF values for roots of five seedlings were averaged and used for correlation analyses.

Fungal Sensitivity to Seed Extract. The following strains were isolated from seeds and Kanuma pumice and nursery soil and were used for the fungal sensitivity test: the seed fungi *Alternaria* (isolate nos. 1a, 26c), *Cladosporium* (isolate nos. 5a, 10e), *Pestalotiopsis* (isolate nos. 8b, 17b2, 35b), and *Phomopsis* (isolate nos. 3a, 6c); the Kanuma pumice fungi *Mortierella* (isolate nos. C, D) and *Mucor* (isolate nos. A, F); and the nursery soil fungi *Trichoderma* (isolate nos. 4-5, 3-10) and *Umbelopsis* (isolate nos. 9-3a, 10-3). These were the most frequently isolated genera. *Mucor* and the other fungal genera were identified to the species level by rDNA sequence analysis using primer

pairs NL1/NL4 (O'Donnell, 1993) and ITS1/ITS4 (White et al., 1990), respectively.

Kanuma pumice or nursery soil (800 g) in 800 ml of demineralized water was autoclaved at 121°C for 20 min. The liquid was stored overnight at 4°C and filtered *in vacuo*. The filtrate was adjusted to 800 ml, 0.16 g of K_2HPO_4 and 1.6 g agar were added, and the mixture was autoclaved at 121°C for 20 min to produce Kanuma pumice extract agar (KPA) or nursery soil extract agar (NSA).

To produce seed extract, seeds (n = 150, totaling 490 mg FW) were soaked in methanol (10 ml) and cut into pieces with a scalpel. The mixture was left for extraction overnight in the dark at room temperature. The extract was evaporated and was stored at –20°C for later use.

The sensitivities of seed fungi and Kanuma pumice fungi were examined on KPA, and those of seed fungi and nursery soil fungi were examined on NSA. Methanol (50 μl) containing seed extract equivalent to 0.2, 0.4, or 0.8 seeds was added to a plastic plate (60-mm diameter) and was mixed with melted KPA or NSA (5 ml, approximately 40°C). As a control, only methanol (50 μl) was used. A piece (2 × 2 mm) cut from the edge of freshly grown mycelia on PDA was inoculated at the center of a soil extract agar plate (60-mm diameter). The plate was incubated in the dark at 25°C until the mycelial diameter on the control plate was approximately 30 mm for slow-growing fungi (*Alternaria*, *Cladosporium*) or approximately 40 mm for fast-growing fungi (*Pestalotiopsis*, *Phomopsis*, *Mortierella*, *Mucor*, *Umbelopsis*, *Trichoderma*); two mycelial diameters at right angles to each other were measured using a digital caliper square (Shinwa Rules Co., Ltd., Nigata, Japan). Four replications were prepared for

each seed extract concentration, and the eight mycelial diameters were averaged.

The average mycelial growth on control plates was defined as 100%, and the relative growth rates on plates containing seed extract were calculated. Regression analysis was performed to examine the relationship between the percentage of mycelial growth and seed extract concentration. All regression coefficients ($P < 0.05$) were negative, so the absolute value was used as an index of fungal sensitivity (Fig. 1). High fungal sensitivity values indicate that fungal growth was strongly inhibited by the seed extract. The fungal sensitivity values for the same species were averaged and used to calculate correlations with the FDF on root surfaces of the seedlings.

Antifungal Compounds of Seeds. A thin-layer chromatography (TLC) bioautography assay was used to test the antifungal compounds in the seed extract against *Mucor* (strain A), which showed the highest sensitivity to the seed extract. Seed extract equivalent to 1.6 seeds was charged on a TLC plate (0.25 mm, Kieselgel 60 F_{254}, Merck, Darmstadt, Germany) and was developed in chloroform-methanol (9:1 v:v). A spore suspension in nutrient solution was sprayed over the developed TLC plate, which was then incubated at 25°C for 3 days (Homans and Fuchs, 1970). Inhibitory zones were detected under UV light by binding with the fluorescent brightener Calcofluor White M2R (Woodward and Pearce, 1985).

To isolate the compounds, 370 seeds (total = 1.2 g FW) were soaked in methanol (20 ml) and cut into pieces with a scalpel, and the mixture was left for extraction overnight in the dark at room temperature. The extract (16.5 ml) was evaporated to

produce an oily residue (246.5 mg), which was dissolved in ethyl acetate. The precipitate (45.8 mg) was removed on a filter paper and the filtrate was evaporated to give an oily residue (220.6 mg). This residue (183.8 mg) was applied to a silica gel column (Wakogel C-60, 13 g, Wako, Tokyo, Japan) to elute the constituents with chloroform-methanol (9:1) and fractions 13–17 (2 ml each) were concentrated together (90.6 mg). This residue (72.4 mg) was applied to a silica gel column (Wakogel C-60, 10 g) to elute with chloroform and fractions 29–42 (1 ml each) were concentrated to 10.5 mg of antifungal compound.

To identify the compounds, we used ^{1}H and ^{13}C NMRspectra and 2D NMR (H-H COSY, HMQC, HMBC, NOESY and HOHAHA) with a JEOL Alpha-500 spectrometer (JEOL Ltd., Tokyo, Japan) to clarify the carbon sequences of the compound. The solvent was $CDCl_3$ and chemical shifts were recorded relative to the TMS (^{1}H) and solvent peaks (δ□□, ^{13}C). An EI-MS spectrum was obtained using a JMS-DX303HF mass spectrometer (JEOL Ltd., Tokyo, Japan) to clarify the fragmentation.

Quantification of Totarol. Gas chromatography analysis was performed using an Auto System XL Gas Chromatograph (Perkin Elmer, Inc., Waltham, USA) with a split-splitless injector (1:10 ratio, 230°C) and FID detector (230°C). Nitrogen gas was used as a carrier gas at 1 ml min^{-1}. Hicap series CBP1 25 m × 0.25 mm column (Shimadzu, Kyoto, Japan) was used for the analysis. The temperature program was 100–200°C at 10°C min^{-1}. The analysis was repeated three times and the values were averaged. Standard curves were prepared as follows: totarol (0.138, 0.275, 0.55, 1.1 μg

in 2 ml acetone; Sigma-Aldrich, St. Louis, MO) was analyzed three times and values were averaged. The amount of totarol in the seed extract was read from the standard curve.

Examination of Fungal Sensitivity to Totarol. Methanol (50 μl) containing totarol equivalent to 0.2, 0.4, or 0.8 seeds (5.75, 11.5, and 23 μg, respectively) was added to a plastic plate (60-mm diameter) and was mixed with KPA or NSA (5 ml, approximately 40°C). As a control, only methanol (50 μl) was used. Fungal inoculation and calculation of fungal sensitivity were the same as described for the fungal sensitivity to seed extract.

Statistical Analyses. The StatView 5.0 (Macintosh) computer package (SAS Institute, Inc., Cary, NC, USA) was used for statistical analyses. Fungal sensitivity was obtained by regression analysis. The relationship between FDF (%) on root surfaces and fungal sensitivity to seed extract or totarol were examined using Pearson's correlation test. Differences were considered significant at $P < 0.05$.

RESULTS

Seed Fungi. Penicillium (several species; FDF 46.7%), *Alternaria* (40.0%), *Cladosporium* (35.6%), *Pestalotiopsis* (17.8%), and *Phomopsis* (8.9%) were the main isolates. *Alternaria* (isolate nos. 1a, 26c), *Cladosporium* (isolate nos. 5a, 10e), *Pestalotiopsis* (isolate nos. 8b, 17b2, 35b), and *Phomopsis* (isolate nos. 3a, 6c) were

selected to examine fungal sensitivities to the seed extract and the antifungal compound totarol. *Papulospora* (FDF 6.7%), *Aspergillus* (2.2%), *Mortierella* (2.2%), *Phoma* (2.2%), *Sclerotium* (2.2%), and *Trichoderma* (2.2%) were also isolated.

Soil Fungi. *Penicillium* (several species; FDF 100.0%), *Mucor* (75.0%), *Cladosporium* (62.5%), *Mortierella* (50.0%), *Alternaria* (12.5%), *Aspergillus* (12.5%), *Aureobasidium* (12.5%), *Gliocladium* (12.5%), and *Ulocladium* (12.5%) were isolated from Kanuma pumice. These *Cladosporium* and *Alternaria* species were different species from the seed fungi in these genera. *Mortierella* (isolate nos. C, D) and *Mucor* (isolate nos. A, F) were selected to examine fungal sensitivities to the seed extract and totarol.

Penicillium (several species; FDF 100.0%), *Umbelopsis* (87.5%), *Trichoderma* (62.5%), *Truncatella* (50.0%), *Pestalotiopsis* (25.0%), *Mucor* (12.5%), and *Stagonospora* (12.5%) were isolated from the nursery soil. The *Pestalotiopsis* species was different from the seed fungus in this genus. *Trichoderma* (isolate nos. 4-5, 3-10) and *Umbelopsis* (isolate nos. 9-3a, 10-3) were selected to examine fungal sensitivities to the seed extract and totarol.

Fungi on Roots. *Penicillium* (several species; FDF 38.4%), *Phomopsis* (24.4%), *Cladosporium* (21.8%), *Pestalotiopsis* (16.7%), *Alternaria* (15.6%), *Gliocladium* (11.3%), *Coniothyrium* (10.9%), *Staphylotrichum* (5.4%), and *Mortierella* (4.4%) were isolated from the seedling roots growing in Kanuma pumice. The *Mortierella* and *Gliocladium* species were the same as the Kanuma pumice fungi in these genera.

Several different *Penicillium* species originated from the seeds and soil.

Cladosporium (FDF 26.0%), *Penicillium* (several species; 22.4%), *Trichoderma* (13.7%), *Phomopsis* (10.1%), *Pestalotiopsis* (9.0%), *Humicola* (6.2%), *Fusarium* (4.7%), *Alternaria* (4.2%), *Aspergillus* (4.2%), *Torula* (2.9%), *Papulaspora* (1.8%), *Geotrichum* (1.3%), and *Paecilomyces* (1.3%) were isolated from the seedling roots growing in nursery soil. The *Alternaria*, *Cladosporium*, *Pestalotiopsis*, and *Phomopsis* species were the same as the seed fungi in these genera, and *Trichoderma* was the same species as the nursery soil fungus. Several different *Penicillium* originated from the seeds and soil.

Fungal Sensitivity to Seed Extract. Fungal sensitivities to the seed extract on KPA were 45.6 for *Alternaria* (seed fungi), 25.0 for *Cladosporium* (seed fungi), 46.4 for *Phomopsis* (seed fungi), 45.6 for *Pestalotiopsis* (seed fungi), 80.0 for *Mortierella* (Kanuma pumice fungi), and 84.0 for *Mucor* (Kanuma pumice fungi). Fungal sensitivities to the seed extract on NSA were 57.5 for *Alternaria* (seed fungi), 22.1 for *Cladosporium* (seed fungi), 44.4 for *Pestalotiopsis* (seed fungi), 58.1 for *Phomopsis* (seed fungi), 41.4 for *Umbelopsis* (nursery soil fungi), and 38.2 for *Trichoderma* (nursery soil fungi).

Correlation Results. Significant and negative correlations were found between the FDF on seedling root surfaces and fungal sensitivity to the seed extract in both soils ($P < 0.05$; Fig. 2a, b). Correlation coefficients (r^2) for Kanuma pumice and nursery soil were

–0.915 (P = 0.007) and –0.834 (P = 0.038), respectively.

Relationship between FDF on Root Surfaces and Fungal Sensitivity to Totarol. Based on analyses of ^{1}H and ^{13}C NMR data and EI-MS data, we identified the antifungal compound in the seeds of *T. dolabrata* var. *hondai* as totarol (Fig. 3), which agreed fully with the NMR findings of Ying and Kubo (1991).

Fungal sensitivities to totarol on KPA were 68.6 for *Alternaria* (seed fungi), 55.1 for *Cladosporium* (seed fungi), 64.4 for *Phomopsis* (seed fungi), 62.2 for *Pestalotiopsis* (seed fungi), 75.2 for *Mortierella* (Kanuma pumice fungi), and 90.6 for *Mucor* (Kanuma pumice fungi); those on NSA were 70.2 for *Alternaria* (seed fungi), 40.5 for *Cladosporium* (seed fungi), 57.5 for *Pestalotiopsis* (seed fungi), 55.9 for *Phomopsis* (seed fungi), 56.7 for *Umbelopsis* (nursery soil fungi), and 54.1 for *Trichoderma* (nursery soil fungi).

Significant and negative correlations were found between the FDF on seedling root surfaces and fungal sensitivity to totarol in both soils ($P < 0.05$; Fig. 4a, b). Correlation coefficients for Kanuma pumice and nursery soil were –0.898 (P = 0.01) and –0.814 (P = 0.049), respectively.

DISCUSSION

The FDF values on root surfaces in both soils were significantly correlated with the fungal sensitivity to the seed extract and totarol, an antifungal compound of seeds. Our results indicate that seed and soil fungi tolerant of totarol can grow on the root surfaces

of *T. dolabrata* var. *hondai* seedlings. Totarol has been isolated from *T. dolabrata* var. *hondai* seeds (this study; Hasegawa and Hirose, 1982) as well as from *Pilgerodendron uviferum* (D. Don) Florin bark extracts, from which it was shown to be antifungal against *Rhizoctonia solani* and *Phragmidium violaceum* (Solís et al., 2004). In our study, totarol was also detected in Kanuma pumice and nursery soil (0.38 ± 0.08 μg g^{-1} soil FW and 0.61 ± 0.16 μg g^{-1} soil FW, respectively) in which 2-month-old *T. dolabrata* var. *hondai* seedlings were growing. The seedling roots also contained totarol (0.45 ± 0.06 μg g^{-1} root FW grown in Kanuma pumice and 0.84 ± 0.16 μg g^{-1} root FW grown in nursery soil, respectively), and therefore totarol would select fungi on root surfaces of the seedlings in the early growth stage. α- and β-Eudesmol have been isolated from *T. dolabrata* var. *dolabrata* seeds (Hasegawa and Hirose, 1982). β-Eudesmol has been extracted from amboyna wood and is antifungal against the wood-rotting fungus *Pleurotus pulmonarius* (Kusuma et al., 2004). In our study, mixtures of α-, β-, and γ-eudesmol (2:2:1) were isolated from *T. dolabrata* var. *hondai* seeds; these compounds were identified by analyses of ^{1}H and ^{13}C NMR data, which agreed fully with those of Raharivelomanana and Bianchini (1995). However, the amount of eudesmols in the seeds was too low to be antifungal against the tested fungi, except for *Mucor* (4.95 μg $seed^{-1}$). Therefore, we conclude that totarol is the main antifungal compound in the *T. dolabrata* var. *hondai* seeds.

Soil nutrient conditions influence mycelial growth, and the rate of mycelial growth seems to be one factor that selects fungi in the rhizosphere. We also examined the rates of mycelial growth of seed and soil fungi on both KPA and NSA. However, the

mycelial growth rates were not related to the FDF on the roots (r^2 = –0.11, P = 0.85 for Kanuma pumice; r^2 = 0.05, P = 0.93 for nursery soil). This result indicates that the nutrient differences between Kanuma pumice and nursery soil did not affect which fungi grew on the seedling root surfaces.

The seed extract would include amino acids and vitamins that stimulate fungal growth. Therefore, the correlation between FDF and fungal sensitivity to the seed extract was different from that between FDF and fungal sensitivity to totarol (Figs. 2, 4). Complicated interactions between chemical compounds (including antifungal compounds and nutrients) and fungal growth would be expected.

Our method (Fig. 1) did not allow us to compare fungal sensitivity values with the minimum inhibitory concentration (MIC). In order to examine the interaction between an antifungal compound and fungal growth in a natural environment, our fungal sensitivity values seem to be better than MIC, which is calculated using an impractically high amount of antifungal compounds against tolerant fungi. Because our fungal sensitivity values reflect the actual amount of antifungal compounds in plant tissues, these values should more accurately estimate the influence of antifungal compound on fungi.

Seeds were collected directly from felled *T. dolabrata* var. *hondai* trees, and therefore seed fungi would be epiphytic on trees. *Alternaria*, *Cladosporium*, *Penicillium*, and *Trichoderma* have been isolated from various conifer seeds (Watanabe, 2002) and thus are not specific to *T. dolabrata* var. *hondai* seeds. *Pestalotiopsis* has weak pathogenicity to conifer seedlings, and *Phomopsis* causes cankers of conifer trees and

seedlings (Ito, 1974). Future work should examine the influences of these fungi on *T. dolabrata* var. *hondai* seedlings.

Based on the findings of this study, we conclude that the antifungal compound totarol that is produced by *T. dolabrata* var. *hondai* seeds is one factor in the selection of fungi on root surfaces in the early growth stage.

ACKNOWLEDGMENTS

We thank the Hiranai Hiba Forestry Research Group for providing us with nursery soil; the Forestry Technology Center, Tohoku Regional Forest Office, for helping with the seed collection; and Mr. N. Shimoda for technical help with our work in the nursery. Financial support was provided in part by Research Fellowships of Japan Society for the Promotion of Science for Young Scientists and by a research grant of the Forestry and Forest Products Research Institute, Japan.

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Figure captions

Fig. 1. Fungal sensitivity value. Regression analysis was performed between the percentage of mycelial growth and the concentration of the seed extract. All regression coefficients were negative ($P < 0.05$), so the absolute value was used as an index of fungal sensitivity. High fungal sensitivity values indicate that fungal growth was strongly inhibited by the seed extract.

Fig. 2. Relationship between the fungal detection frequency (FDF) on the root surfaces and fungal sensitivity to seed extract: (a) Kanuma pumice ($r^2 = –0.915$, $P = 0.007$); (b) nursery soil ($r^2 = –0.834$, $P = 0.038$). The origin of each fungus is shown in parentheses.

Fig. 3. Chemical structure of totarol.

Fig. 4. Relationship between the fungal detection frequency (FDF) on the root surfaces and fungal sensitivity to totarol. (a) Kanuma pumice ($r^2 = –0.898$, $P = 0.01$). (b) Nursery soil ($r^2 = –0.814$, $P = 0.049$). The origin of each fungus is shown in parentheses.

TABLE 1. General characteristics of Kanuma pumice and nursery soil (0-10 cm depth)

Characteristic	Kanuma pumice	Nursery soil
pH (KCl)	4.79±0.01	4.25±0.02***
Exchangeable Na (cg/kg)	22.9±0.90	22.3±1.09
Exchangeable K (cg/kg)	18.0±0.99	27.6±1.28**
Exchangeable Mg (cg/kg)	11.7±0.97	16.5±0.09**
Exchangeable Ca (cg/kg)	120.9±4.47	126.6±1.58
Available P (mg/kg)	trace	22.2±2.21***
Total C (%)	0.13±0.01	9.34±0.07***
Total N (%)	0.01±0.00	0.56±0.00***
C/N	28.0±7.83	16.6±0.16

Results are expressed as value ±S.E.. Differences between values for Kanuma pumice and nursery soil were evaluated by using *Student's t-test*.

***, $P < 0.001$. **, $P < 0.01$. *, $P < 0.05$.

TABLE 2. Fresh weight, height, and basal diameter of seedlings used for fungal isolation on the root surface

Seedlings	Total FW (mg ± SE)	FW of leaves and stem (mg ± SE)	FW of roots (mg ± SE)	Height (mm ± SE)	Basal diameter (mm ± SE)
Kanuma pumice	28.3±1.83	20.2±1.55	8.04±0.73	17.9±0.63	0.78±0.04
nursery soil	34.4±3.10	25.6±1.75	8.76±1.61	19.6±1.06	0.88±0.07

No significant difference was detected between the soils for any variable (*Student's t-test*, $P>0.05$).