Selective Medium for Isolating *Lasiodiplodia theobromae*

A. J. CILLIERS, Department of Microbiology and Biochemistry, W. J. SWART, Department of Plant Pathology, and M. J. WINGFIELD, Department of Microbiology and Biochemistry, University of the Orange Free State, Bloemfontein, 9300, South Africa

ABSTRACT

Cilliers, A. J., Swart, W. J., and Wingfield, M. J. 1994. Selective medium for isolating *Lasiodiplodia theobromae*. Plant Dis. 78:1052-1055.

Six chemicals were tested in vitro for their effect on the growth of *Lasiodiplodia theobromae*. Three were selected for further evaluation on 14 of the fastest growing fungi commonly associated with *L. theobromae* on *Pinus elliottii* seeds. Tannic acid suppressed *Rhizopus* sp., *Drechslera* sp., *Trichoderma* sp., and *Sphaeropsis sapinea*, four of the fastest growing test fungi. Benodanil and tridemorph suppressed most of the other fungi. The selective medium, consisting of 33.6 g/L of malt extract agar (MEA), 3,000 μg/ml of tannic acid, 50 μg/ml of benodanil, and 0.5μg/ml of tridemorph, was effective in suppressing all fungi selected for testing except *L. theobromae*. The selective medium was also more effective than MEA for isolating *L. theobromae* from soil, woody tissue, and *P. elliottii* seeds.

Lasiodiplodia theobromae (Pat.) Griffon & Maubl. has been associated with diseases of a wide range of host species, including *Pinus* spp. (5,6,9,10). More recently, *L. theobromae* has been associated with black discoloration and reduced germination of *P. elliottii* Engelm. seeds originating from clonal seed orchards in the United States (3) and South Africa (2). In contrast, the ubiquitous pine pathogen *Sphaeropsis sapinea* (Fr.:Fr.) Dyko & Sutton in Sutton, which has been associated with pine seeds elsewhere (7,8), and the closely related pathogen *Botryosphaeria dothidea* (Moug.:Fr.) Ces. & De Not. have not to date been associated with this phenomenon in South Africa.

In order to screen seed lots from clonal seed orchards of *P. elliottii* for extent of contamination by *L. theobromae*, it was necessary to isolate from seeds on agar medium, i.e., malt extract agar (MEA). Isolation of *L. theobromae* from diseased seeds is hampered, however, by the presence of numerous saprophytic fungi, bacteria, and yeasts that overgrow isolations onto MEA. A selective medium was therefore needed for the isolation of *L. theobromae* from symptomatic *P. elliottii* seeds. The procedure involved in developing and verifying the efficacy of such a medium is described.

MATERIALS AND METHODS

Screening fungicidal activity. In a previous study (12) in which a selective medium was developed and verified for the isolation of *S. sapinea* from pine tissue, *L. theobromae* was observed as being relatively tolerant of tannic acid, benodanil, rose bengal, tridemorph, chlorothalonil, and etaconazole. In preliminary tests, these six chemicals were tested individually for their inhibitory effect on 14 fungi usually associated with *L. theobromae* on pine tissue at the following concentrations: tannic acid, 5,000 μg/ml; benodanil, 50 μg/ml; tridemorph, 1 μg/ml; etaconazole, 0.1 μg/ml; chlorothalonil, 5 μg/ml; and rose bengal, 50 μg/ml. Fungi tested were *S. sapinea, B. dothidea, Aspergillus* sp., *Rhizopus* sp., *Penicillium* sp., *Alternaria* sp., *Drechslera* sp., *Pestalotiopsis* sp., *Trichoderma* sp., *Sporothrix* sp., *Chaetomium* sp., *Acremonium* sp., *Fusarium subglutinans* (Wollenweb. & Reinking) P.E. Nelson, T.A. Toussoun, & Marasas, and *F. oxysporum* Schlechtend.:Fr.

Stock solutions or suspensions of each chemical were made in distilled water. A basal medium (MEA, 33.6 g/L) was individually amended after autoclaving for 20 min with specific volumes of each stock solution. The unamended basal medium served as the control. The amended agar medium was agitated for 2 min to allow for even mixing of the chemical before approximately 20 ml of each medium was poured into each of three 90-mm culture dishes and allowed to solidify. Test fungi and *L. theobromae* were transferred to dishes containing the chemical from the periphery of actively growing colonies on 2% potato-dextrose agar (PDA) by placing a 5-mm agar plug in the center of each culture dish. The colony diameter on each petri dish was recorded as the mean of two perpendicular measurements after 72 hr of incubation at 25 C, and the mean value of three dishes was recorded.

Results of these preliminary tests indicated that tannic acid, tridemorph, and benodanil were the most suitable candidates for incorporating into a selective medium for the isolation of *L. theobromae*. In subsequent tests, the concentration of tannic acid was reduced to 3,000 μg/ml because the agar medium did not solidify at 5,000 μg/ml. The concentration of tridemorph was also decreased to 0.5 μg/ml because it inhibited the growth of *L. theobromae* at 1 μg/ml. Each chemical was then retested in vitro, as described above, for its effect on the growth of *L. theobromae* and the 14 test fungi. The experiment was repeated and an analysis of variance (ANOVA) was conducted with the pooled data to compare the growth of *L. theobromae* and the 14 test fungi.

Efficiency of combined chemicals. The three chemicals were tested for their combined effect on the growth of the 14 test fungi and *L. theobromae*. Chemicals were incorporated into the basal medium as described above, and the pH of the basal medium with and without the addition of the three chemicals was measured. Growth tests were subsequently conducted as described above with the unamended basal medium serving as the control. Each test was conducted twice, and an ANOVA was conducted with the pooled data. In order to test the effect of the medium on bacteria and yeasts, the three most commonly isolated bacteria and yeasts from symptomatic *P. elliottii* seeds were streaked onto five dishes each of the selective medium and the unamended basal medium and incubated for 20 hr at 25 C.

Verification using pine seeds. Forty discolored *P. elliottii* seeds were surface-sterilized for 5 min in 3.5% (m/v) NaOCl and evenly distributed among 20 dishes containing selective medium. Dishes were incubated at 25 C for 4 days and evaluated for the presence of *L. theobromae* by transferring a 5-mm agar plug from the peripheral portion of all developing fungal colonies to a 90-mm culture dish containing 1.2% water agar overlaid with sterile pine needles. The dishes were placed under near-ultraviolet light (black light), and resulting pycnidia were examined microscopically for the presence of *L. theobromae* spores. The unamended basal medium served as a control treatment for isolations from seeds. The experiment was repeated, and the mean percent recovery of *L. theobromae* from the selective medium and the control was then determined.

Fifteen symptomatic *P. elliottii* seeds were crushed and agitated in 5 ml of sterile water for 5 min, and the resultant

Accepted for publication 14 June 1994.

suspension was serially diluted to obtain 20, 40, 60, and 80% dilutions of the original suspension. Each dilution was plated out onto three dishes each of the selective medium and the unamended basal medium. Dishes were evaluated after 4 days of incubation at 25 C by transferring the peripheral portion of developing colonies to water agar and following the identification procedure for *L. theobromae* described above. The experiment was conducted twice, and the data were pooled for analysis.

Verification using pine tissue. The stems of 1-yr-old *P. elliottii* plants were artificially inoculated by removing a small strip of bark, placing a strip of cheesecloth (10 × 5 mm) previously colonized by *L. theobromae* on 2% PDA over the wound, and then wrapping the wound with Parafilm to prevent desiccation of the inoculum. After 2 wk, when cambial lesions were approximately 60 mm in length, stems were cut into 100-mm lengths and buried in unsterile forest soil for 5 days. Stems were then removed and surface-sterilized for 3 min with a 40% (v/v) solution of hydrogen peroxide. Two small pieces of infected tissue from each of 10 stems were placed on the selective medium, and the percent recovery of *L. theobromae* was evaluated as described above. The unamended basal

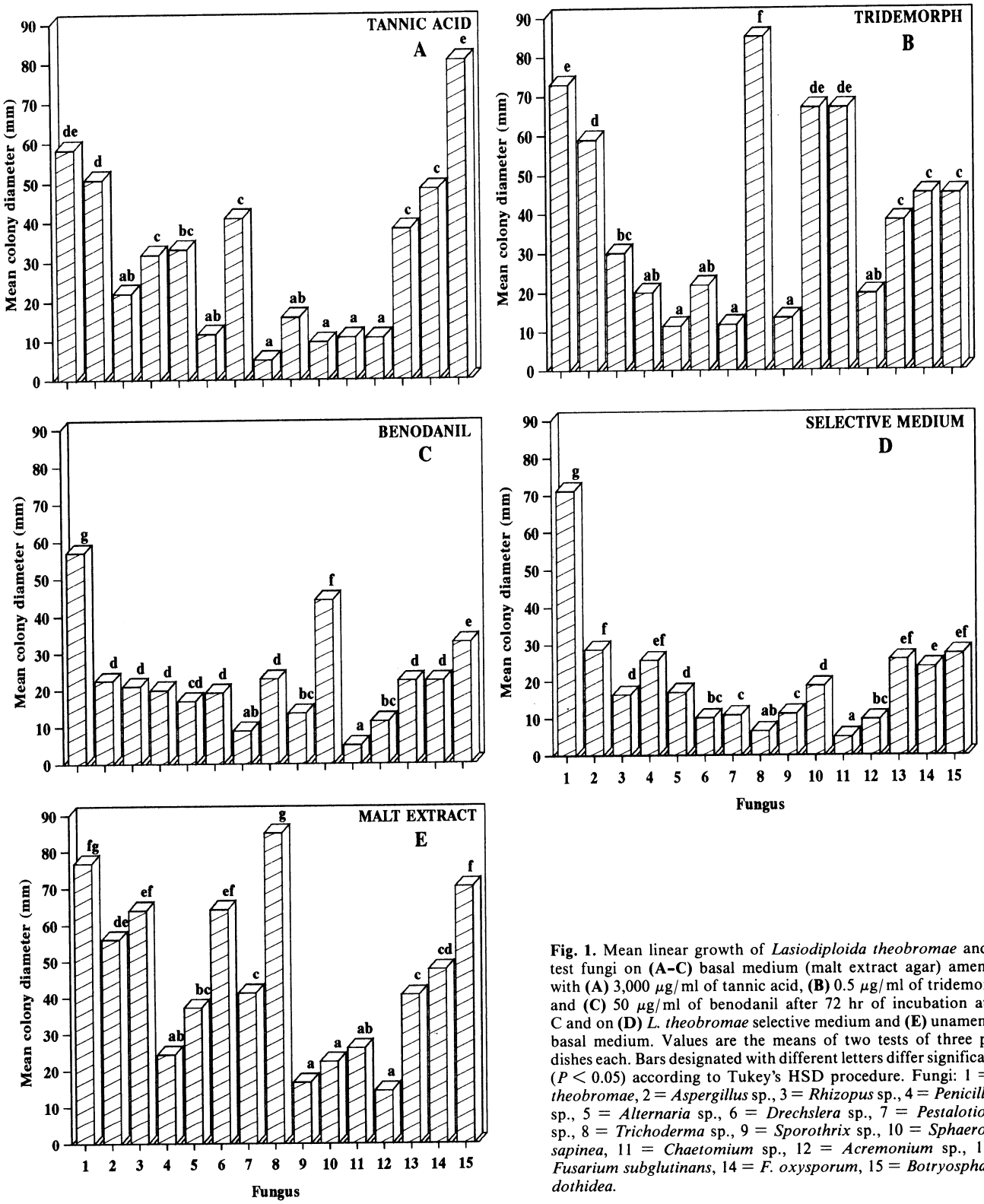

Fig. 1. Mean linear growth of *Lasiodiploida theobromae* and 14 test fungi on **(A-C)** basal medium (malt extract agar) amended with **(A)** 3,000 μg/ml of tannic acid, **(B)** 0.5 μg/ml of tridemorph, and **(C)** 50 μg/ml of benodanil after 72 hr of incubation at 25 C and on **(D)** *L. theobromae* selective medium and **(E)** unamended basal medium. Values are the means of two tests of three petri dishes each. Bars designated with different letters differ significantly ($P < 0.05$) according to Tukey's HSD procedure. Fungi: 1 = *L. theobromae*, 2 = *Aspergillus* sp., 3 = *Rhizopus* sp., 4 = *Penicillium* sp., 5 = *Alternaria* sp., 6 = *Drechslera* sp., 7 = *Pestalotiopsis* sp., 8 = *Trichoderma* sp., 9 = *Sporothrix* sp., 10 = *Sphaeropsis sapinea*, 11 = *Chaetomium* sp., 12 = *Acremonium* sp., 13 = *Fusarium subglutinans*, 14 = *F. oxysporum*, 15 = *Botryosphaeria dothidea*.

medium served as the control treatment. The experiment was conducted twice, and the data were pooled for analysis.

Verification using soil. *L. theobromae* was cultured on a mixture of sterile sand and maize meal (95:5, w/w) and 26 ml of water in 250-ml Erlenmeyer flasks at 30 C for 2 wk. The resulting inoculum was combined with unsterile forest soil in the following proportions: 0:100, 20:80, 40:60, 60:40, 80:20, and 100:0. A 5-mm layer of each inoculum combination was placed in a sterile 90-mm petri dish and covered with 15 ml of MEA cooled to 45 C. The agar was allowed to solidify, and four agar plugs from each of five dishes were removed with a 5-mm cork borer from each inoculum:sand combination, placed on the selective medium, and incubated at 25 C for 5 days. Unamended MEA dishes served as controls. Transfers were made from the periphery of each developing colony for recovery and identification of *L. theobromae*, using water agar overlaid with sterile pine needles as described above. The experiment was conducted twice, and the data were pooled for analysis.

L. theobromae, S. sapinea, and *B. dothidea* were cultured on a sand:maize meal mixture as described above. The inocula of *L. theobromae, S. sapinea*, and *B. dothidea* were combined in ratios of 100:0:0, 60:20:20, 40:30:30, 20:40:40, and 0:50:50, respectively. The isolation and evaluation procedure described above was followed.

RESULTS

Screening fungicidal activity. Most of the test fungi exhibited rapid growth on MEA. Each of the three chemicals tested significantly suppressed specific test fungi. Benodanil was the only chemical tested that allowed *L. theobromae* to outgrow all test fungi; however, *S. sapinea* grew almost as much. Tannic acid significantly suppressed growth of *S. sapinea* and also three of the four fastest growing fungi on MEA, i.e., *Rhizopus* sp., *Drechslera* sp., and *Trichoderma* sp.; the fourth, *B. dothidea*, grew faster on tannic acid than did *L. theobromae* (Fig. 1A). Tridemorph significantly suppressed *B. dothidea* as well as *Rhizopus* sp., *Alternaria* sp., and *Pestalotiopsis* sp. (Fig. 1B), and benodanil suppressed *B. dothidea, F. subglutinans, F. oxysporum*, and *Aspergillus* sp. (Fig. 1C).

Efficiency of combined chemicals. The selective medium comprising 33.6 g/L of MEA, 3,000 μg a.i./ml of tannic acid, 50 μg a.i./ml of benodanil, and 0.5 μg a.i./ml of tridemorph was the most selective for *L. theobromae* (Fig. 1D). The suppression of *Aspergillus* sp., *Sporothrix* sp., and *Acremonium* sp. was apparently due to the combined effect of tridemorph, tannic acid, and benodanil. The bacteria and yeasts tested exhibited very little or no growth on the selective medium (pH 3.4) but grew well on the unamended basal medium (pH 4.7).

Verification of selective medium. *L. theobromae* was the most frequently isolated fungus from the symptomatic pine seeds, infected pine tissue, and the unsterile soil:inoculum mixture. Isolation of *L. theobromae* from whole, discolored pine seeds was 20% greater and from crushed seed suspensions between 10 and 20% greater on the selective medium than on the unamended basal MEA medium (Table 1). Reisolation of *L. theobromae* from diseased pine tissue was 30% greater on the selective medium than on the basal medium alone (Table 1).

The mixture comprising 80% forest soil and 20% *L. theobromae* inoculum yielded a 70% recovery of *L. theobromae* on the selective medium and a 30% recovery on the unamended basal MEA medium (Table 1). Recovery of *L. theobromae* was generally between 25 and 40% less on the basal medium for soil:inoculum mixtures containing up to 80% *L. theobromae* inoculum. In a sand:maize meal mixture containing 20% *L. theobromae* inoculum, 40% *S. sapinea*, and 40% *B. dothidea*, a 75% recovery of *L. theobromae* was obtained on the selective medium compared with a 5% recovery on the unamended basal medium (Table 1). Isolations from other inoculum combinations containing 40% and 60% *L. theobromae* yielded 100% *L. theobromae* on the selective medium.

Table 1. Percent recovery of *Lasiodiplodia theobromae* on selective medium (SM) and basal medium (malt extract agar, MEA) from various substrates

Substrate	Percent recovery SM	MEA
Whole pine seeds[a]	70	50
Crushed pine seeds[b]		
20% suspension	25	15
40% suspension	35	15
60% suspension	55	35
80% suspension	60	45
100% suspension	60	40
Pine tissue[a]	80	50
Forest soil:*L. theobromae* (%)[c]		
100:0	0	0
80:20	70	30
60:40	75	45
40:60	93	65
20:80	100	75
0:100	100	100
L. theobromae:*Sphaeropsis sapinea*:*Botryosphaeria dothidea* (%)[c]		
0:50:50	0	0
20:40:40	75	5
40:30:30	100	0
60:20:20	100	0
100:0:0	100	100

[a] Means are averages of two tests.
[b] Means are averages of two tests with three replications per test.
[c] Means are averages of two tests with five replications per test.

DISCUSSION

The selective medium developed for *L. theobromae* provides an efficient means for isolating *L. theobromae* from diseased pine tissue. We used the principle of selective inhibition (14) in developing this medium. Swart et al (12) found this technique to be successful in developing a medium for the isolation of *S. sapinea* from pine tissue. Vaartaja (15) used a medium containing tannic acid for the isolation of *S. sapinea*, although we found tannic acid to inhibit the growth of *S. sapinea* and to have very little inhibitory effect on *L. theobromae*.

Fast-growing fungi such as *Trichoderma* sp., *Rhizopus* sp., and *Drechslera* sp. can hamper isolation of *L. theobromae* on rich media such as MEA if they are not effectively suppressed. These fungi were effectively suppressed by the selective medium and showed significant ($P < 0.5$) differences in radial growth to *L. theobromae*. The effective suppression of fungi such as *Penicillium* sp. and *Aspergillus* sp. was also important because of the production of dry spores by these fungi that increase the likelihood of contamination. The growth of these two fungi was effectively suppressed by benodanil and tridemorph, and their radial growth was significantly less than that of *L. theobromae*.

Numerous chemicals have been used in selective media to inhibit gram-positive and gram-negative bacteria (11,13,14). The low pH of the selective medium developed for *L. theobromae* succeeds in inhibiting the growth of bacteria and yeasts occurring on *P. elliottii* seeds without inhibiting the growth of *L. theobromae*.

Substrates were chosen for verification of the selective medium that would be used in pathogenicity studies of *L. theobromae*. Since *L. theobromae* has also been associated with *S. sapinea* and *B. dothidea* in various diseases of pines (1,4,5), the selective medium could serve to effectively distinguish among these closely related fungi in isolations from aboveground and belowground diseased pine tissue. It is for this reason that forest soil was used in verifying the medium. This selective medium provides an efficient means of isolating *L. theobromae*, in association with other microorganisms, from soil, pine seeds, and pine tissue and will also serve to facilitate ecological studies of the fungus.

LITERATURE CITED

1. Bega, R. V., Smith, R. S., Jr., Martinez, A. P., and Davis, C. J. 1978. Severe damage to *Pinus radiata* and *P. pinaster* by *Diplodia pinea* and *Lophodermium* sp. on Molokai and Lanai in Hawaii. Plant Dis. Rep. 62:329-331.
2. Cilliers, A. J., Swart, W. J., and Wingfield, W. J. 1993. A review of *Lasiodiplodia theobromae* with particular reference to its occurrence on coniferous seeds. South Afr. For. J. 166:47-52.
3. Fraedrich, S. W., and Miller, T. 1989. Cone harvesting practices affect the incidence of black seed rot of slash pine caused by *Lasiodiplodia theobromae*. (Abstr.) Phytopathology 79:1165.

4. Hodges, C. S. 1983. Pine mortality in Hawaii associated with *Botryosphaeria dothidea*. Plant Dis. 67:555-556.
5. Punithalingam, E. 1976. *Botryodiplodia theobromae*. No. 519 in: Descriptions of Pathogenic Fungi and Bacteria. Commonw. Mycol. Inst., Kew, England.
6. Punithalingam, E. 1980. Plant Diseases Attributed to *Botryodiplodia theobromae*. J. Cramer, Vaduz, Lichtenstein.
7. Rees, A. A. 1988. Infection of *Pinus caribaea* seed by *Lasiodiplodia theobromae*. Trans. Br. Mycol. Soc. 91:321-324.
8. Rees, A. A., and Webber, J. F. 1988. Pathogenicity of *Sphaeropsis sapinea* to seed, seedlings and saplings of some Central American pines. Trans. Br. Mycol. Soc. 91:273-277.
9. Rowan, S. J. 1982. Tip dieback in southern pine nurseries. Plant Dis. 66:258-259.
10. Shayesta, B., and Rahman, M. A. 1985. Needle cast of *Pinus elliottii* at Forest Research Institute campus, Chittagong. (Abstr.) Rev. Plant Pathol. 66:3052.
11. Smilanick, J. L., and Eckert, J. W. 1986. Selective medium for isolating *Penicillium digitatum*. Plant Dis. 70:254-256.
12. Swart, W. J., Wingfield, M. J., and Knox-Davies, P. S. 1987. Selective medium for isolating *Sphaeropsis sapinea*. Phytopathology 77:1387-1389.
13. Thomas, R. 1985. Selective medium for isolating *Termitomyces* from termite nests. Trans. Br. Mycol. Soc. 84:519-526.
14. Tsao, P. H. 1970. Selective media for the isolation of pathogenic fungi. Annu. Rev. Phytopathol. 8:157-186.
15. Vaartaja, O. 1968. Wood inhabiting fungi in a pine plantation in Australia. Mycopathol. Mycol. Appl. 34:81-89.