Selective Medium for Isolating *Sphaeropsis sapinea*

W. J. Swart, M. J. Wingfield, and P. S. Knox-Davies

First and second authors, Plant Protection Research Institute, Private Bag X5017, Stellenbosch 7600, South Africa; third author, Department of Plant Pathology, University of Stellenbosch, Stellenbosch 7600, South Africa.
We gratefully acknowledge the assistance of E. Daniels and M. Ter Haar.
Accepted for publication 25 March 1987.

ABSTRACT

Swart, W. J., Wingfield, M. J., and Knox-Davies, P. S. 1987. Selective medium for isolating *Sphaeropsis sapinea*. Phytopathology 77:1387-1389.

Seventeen fungicides were tested in vitro for their effect on the growth of *Sphaeropsis sapinea*. Ten were selected for further evaluation on 14 contaminant fungi commonly associated with *S. sapinea* in host tissue. Benodanil and rose bengal suppressed all test fungi more than *S. sapinea*. Chlorothalonil and o-phenylphenol were the most effective inhibitors of *Trichoderma* sp. and *Mucor* sp., the two fastest-growing contaminants. A selective medium comprising Difco agar (20 g/L), Difco malt extract (10 g/L), rose bengal (50 μg/ml), benodanil (10 μg a.i./ml), chlorothalonil (1 μg a.i./ml), and o-phenylphenol (1μg a.i./ml) was more effective for isolating *S. sapinea* from woody tissue than malt extract agar (MEA) alone, or a previous MEA medium containing tannic acid.

Additional key words: *Diplodia pinea*, *Pinus* spp.

Sphaeropsis sapinea (Fr.) Dyko & Sutton (*Diplodia pinea* (Desm.) Kickx) is a serious pathogen of pines in many countries (1,4,7). Attempts to isolate the pathogen from host tissue are often impeded by fast-growing fungal contaminants. Vaartaja (10) used a malt-extract agar (MEA) medium containing tannic acid to isolate *S. sapinea* (as *Macrophoma sapinea* (Fr.) Petrak) from pine wood. We found Vaartaja's medium to be unsatisfactory because the tannic acid did not inhibit common contaminants sufficiently, and it softened and discolored the medium.

This paper describes a selective medium for the isolation of *S. sapinea* from diseased pine roots and tissue infested with pine insects and compares it with a nonselective medium and one containing tannic acid.

MATERIALS AND METHODS

Screening fungicidal activity. Seventeen fungicides (Table 1) were assayed for their effect on the radial growth of *S. sapinea*, with each compound being tested at concentrations of 0.1, 0.5, 1, 5, 10, 50, 100, and 500 μg a.i./ml. The basal medium (MEA) consisted of 10 g of Difco malt extract, 20 g of Difco Bacto agar, and 1 L of deionized water. The medium was cooled to 50 C and amended with appropriate volumes of a stock solution of each fungicide. The unamended MEA medium served as the control. Media were agitated for 2 min before approximately 20 ml of each medium was poured into each of five 90-mm petri dishes. Plates were inoculated with 5-mm disks from the periphery of a 3-day-old MEA culture of *S. sapinea* (PREM 48859—National Collection of Fungi, Pretoria). Plates were incubated at 25 C and the colony diameters (mm) were recorded after 92 hr as the mean of two measurements taken perpendicularly to each other. Each test was conducted twice.

Suppressing contaminant fungi. During the preliminary tests 10 compounds had an inhibitory effect on *S. sapinea* only at concentrations greater than 1 μg a.i./ml (Table 1). These were further screened at specific concentrations to determine their effect on 14 fungi commonly occurring together with *S. sapinea* in host tissue. The test fungi were: *Ceratocystis ips* Rumbold, *Leptographium serpens* (Goid.) Siem., *Trichoderma* sp., *Cladosporium* sp., *Penicillium* sp., *Pestalotiopsis* sp., *Gliocladium roseum* Bain., *Pestalotia* sp., *Lasiodiplodia theobromae* (Pat.) Griff. & Maubl., *Cytospora* sp., *Sclerophoma pythiophila* (Cda.) Höhn., *Curvularia* sp., *Rhizoctonia solani* Kühn, and *Mucor* sp. They were grown on MEA plates for 3 days at 25 C. A 5-mm plug of each fungus was then transferred from the periphery of the colony to each of five plates containing the compound to be screened. Colony diameters were recorded after 72 and 144 hr at 25 C, as the mean of two measurements perpendicular to each other.

Comparative efficiency of combined fungicides. Compounds either selective toward *S. sapinea* or highly inhibitory toward the fastest-growing fungi, especially *Trichoderma* sp., were combined in various concentrations in selective media and tested with *S. sapinea* and the 14 test fungi. Colony diameters were measured on the selective media, MEA, and the tannic acid medium (TAM).

To verify the efficacy of the final selective medium (SM), recovery percentages of *S. sapinea* from naturally infected and artificially inoculated host tissue were calculated. Pine needles and 25-mm-long stem sections from 1-yr-old *Pinus radiata* D. Don seedlings were autoclaved, placed on water agar that had been inoculated with *S. sapinea*, and incubated at 25 C for 30 days. The

TABLE 1. Effects of different fungicides on the growth of *Sphaeropsis sapinea*[a]

Compound	Lowest concentration causing > 50% reduction of growth (μg a.i./ml)	% Inhibition of *S. sapinea*[b]
Benomyl	0.1	98.90
Tridemorph	0.1	65.13
Etaconazole	0.5	88.12
Propiconazole	0.5	87.88
Cycloheximide	0.5	52.78
Iprodione	1	81.26
Imazalil	1	67.30
o-Phenylphenol	5	70.62
Chlorothalonil	5	50.32
Mancozeb	10	51.22
Copper oxychloride	50	98.30
Captab	50	60.00
Benodanil	50	65.79
Rose bengal	100	83.78
Zineb	500	60.92
Novobiocin	500	95.74
Streptomycin	>500	--

[a] Colony diameter on malt-extract agar (MEA) after 92 hr incubation at 25 C.
[b] Reduction in colony diameter compared with MEA.

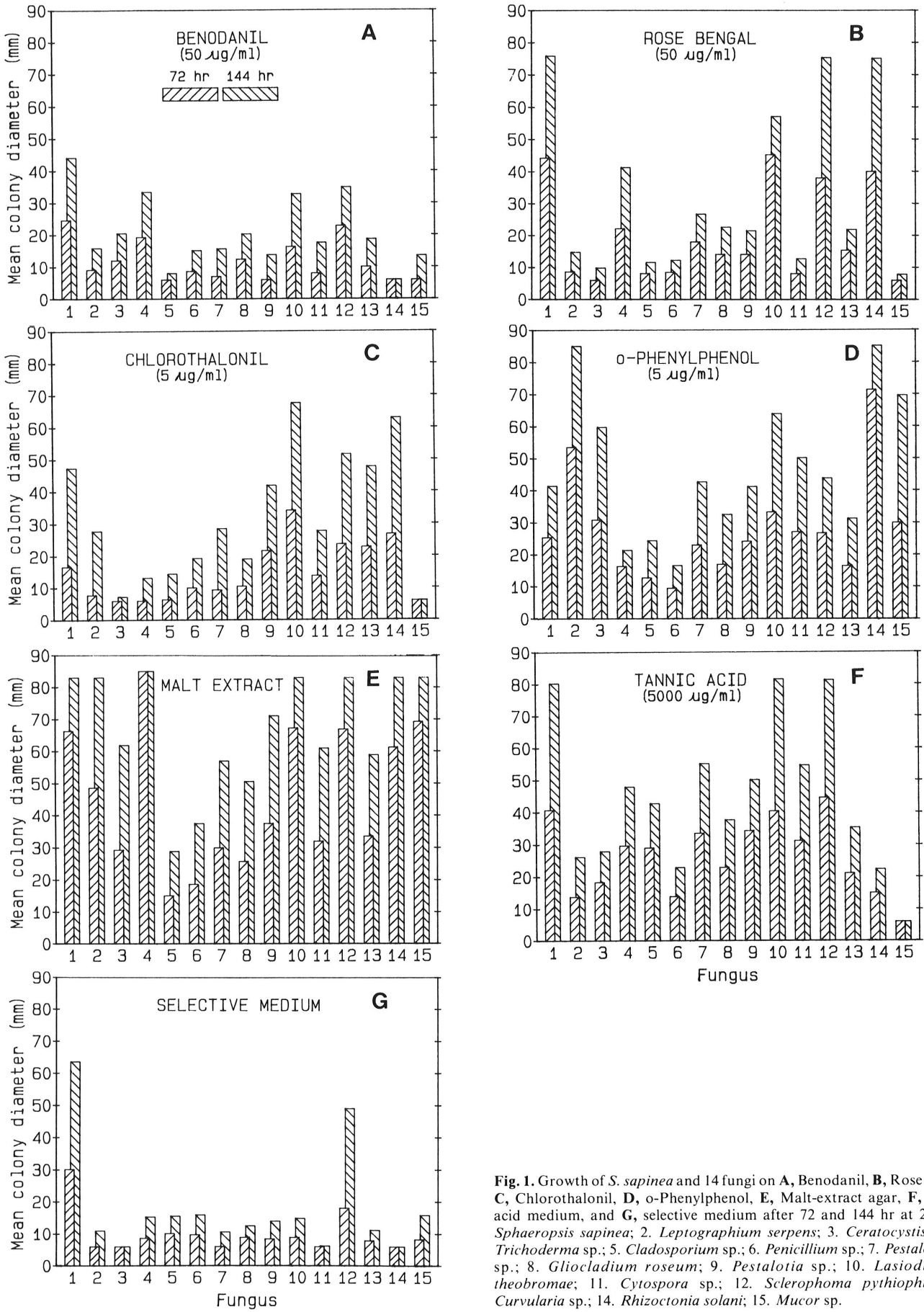

Fig. 1. Growth of *S. sapinea* and 14 fungi on **A,** Benodanil, **B,** Rose bengal, **C,** Chlorothalonil, **D,** o-Phenylphenol, **E,** Malt-extract agar, **F,** Tannic acid medium, and **G,** selective medium after 72 and 144 hr at 25 C. 1. *Sphaeropsis sapinea*; 2. *Leptographium serpens*; 3. *Ceratocystis ips*; 4. *Trichoderma* sp.; 5. *Cladosporium* sp.; 6. *Penicillium* sp.; 7. *Pestalotiopsis* sp.; 8. *Gliocladium roseum*; 9. *Pestalotia* sp.; 10. *Lasiodiplodia theobromae*; 11. *Cytospora* sp.; 12. *Sclerophoma pythiophila*; 13. *Curvularia* sp.; 14. *Rhizoctonia solani*; 15. *Mucor* sp.

colonized needles and stems were removed and buried in unsterilized forest soil for 10 and 20 days, respectively. Fifty small pieces of tissue were then plated on MEA, SM, and TAM.

P. radiata shoots, naturally infected with *S. sapinea*, were collected. Fifty pieces of tissue (2 mm^2) were placed on MEA, SM, and TAM without prior surface disinfestation.

SM was also used to isolate *S. sapinea* occurring in the presence of other pine fungi. Ten stem sections (25 mm long) from 1-yr-old *P. radiata* seedlings were placed on water agar plates that had been inoculated with a combination of *S. sapinea, L. serpens,* and *C. ips*. After 14 days, five 2-mm^2 pieces of tissue were cut from each section, plated on MEA, SM, and TAM and incubated at 25 C for 3 days.

RESULTS

Screening fungicidal activity. Growth of *S. sapinea* was inhibited by more than 50% by seven compounds at concentrations less than or equal to 1 μg a.i./ml (Table 1); these compounds were not used for further screening. Growth of *S. sapinea* was inhibited by more than 50% by the following 10 compounds only when higher concentrations were used: benodanil 150EC, captab 500WP, chlorothalonil 500SC, copper oxychloride 850WP, mancozeb 800WP, zineb 700WP, o-phenylphenol, novobiocin, streptomycin sulphate, and rose bengal. Thus *S. sapinea* was less sensitive to these 10 compounds than to the other seven.

Suppressing contaminant fungi. Each compound was screened for its effect on the 14 test fungi at the concentrations listed in Table 1. Rose bengal, however, was tested at a lower concentration (50 μg a.i./ml) because of its marked inhibitory effect on *S. sapinea* at the higher concentration. Although copper oxychloride and novobiocin were also inhibitory to *S. sapinea* at the given concentrations, rose bengal was chosen for further testing in preference to these substances because it has both antifungal and antibacterial properties (2,3,6,8,9).

Of the 10 compounds tested, only benodanil (50 μg a.i./ml) Fig. 1A) and rose bengal (50 μg/ml) (Fig. 1B) allowed *S. sapinea* to outgrow most of the contaminant fungi. Rose bengal inhibited *S. sapinea* less than benodanil and allowed more precise differentiation of *S. sapinea* from most test fungi. *Sclerophoma pythiophila* and *R. solani* grew almost as fast as *S. sapinea* on rose bengal, but benodanil reduced the growth of *Sclerophoma pythiophila* to less than that of *S. sapinea* and completely inhibited *R. solani* for up to 144 hr.

Chlorothalonil (5 μg a.i./ml) (Fig. 1C) and o-phenylphenol (5 μg a.i./ml) (Fig. 1D) caused the greatest reduction in the growth rate of *Trichoderma* sp. Both compounds were nonselective toward *S. sapinea*, though chlorothalonil inhibited *S. sapinea* less than o-phenylphenol compared with the test fungi and was also more inhibitory toward *Trichoderma* sp.

Comparative efficiency of combined fungicides. Various combinations of benodanil, rose bengal, chlorothalonil, and o-phenylphenol were used to obtain a medium highly selective for *S. sapinea*. A combination of rose bengal (50 μg/ml) and benodanil (50 μg a.i./ml) restricted the colony diameter of *S. sapinea* to 30 mm after 144 hr at 25 C. This was 14 mm less than on benodanil (50 μg a.i./ml) (Fig. 1A) and 46 mm less than on rose bengal (50 μg/ml) (Fig. 1B) alone. SM comprised of Difco agar (20 g/L), Difco malt extract (10 g/L), rose bengal (50 μg/ml), benodanil (10 μg a.i./ml), o-phenylphenol (1 μg a.i./ml), and chlorothalonil (1 μg a.i./ml).

The overall growth rates of *S. sapinea* and the test fungi were greater on MEA (Fig. 1E) than on TAM (Fig. 1F) and SM (Fig. 1G). *Trichoderma* sp., *R. solani, Mucor* sp., *L. theobromae*, and *S. pythiophila* grew as fast, or faster than *S. sapinea* on MEA. *L. theobromae* and *S. pythiophila* grew faster than *S. sapinea* on TAM. These and all other test fungi were inhibited on SM.

The recovery of *S. sapinea* from buried pine needles was 83, 72, and 44%, and from buried stems, 63, 23, and 13% on SM, TAM, and MEA, respectively.

Isolations from naturally infected pine shoots yielded 78% *S. sapinea* on SM, compared with 64 and 50% on TAM and MEA, respectively.

It was difficult to distinguish between *S. sapinea* and *L. serpens* or *C. ips* on MEA or TAM as their colonies had become completely intermingled. On SM, however, *C. ips* and *L. serpens* were clearly visible as small dense colonies in the center of the much larger *S. sapinea* colony.

DISCUSSION

The principle of selective inhibition (8) was used to develop this selective medium. The high level of rose bengal tolerated by *S. sapinea* justified its use as the major ingredient in the selective medium. Benodanil inhibited other contaminant fungi not suppressed by rose bengal.

In developing a selective medium it is important to restrict the growth of rapidly proliferating fungi that produce large masses of easily dislodged dry spores. *Trichoderma* spp. were commonly isolated together with *S. sapinea* from pine tissue. Therefore, o-phenylphenol (Fig. 1D), which Russell (5) found to be an effective suppressant of *Trichoderma*, and chlorothalonil (Fig. 1C) were used in the medium at concentrations sufficient to inhibit *Trichoderma* and other fast-growing fungi but not *S. sapinea*. Although *S. pythiophila* was less suppressed than *Trichoderma* in most cases, no attempt was made to suppress it further because it produces wet spores that do not contaminate plates as easily as dry spores.

When used in combination, synergism or antagonism between certain compounds can prevent successful isolation of the desired organism (8). Synergism between rose bengal and benodanil necessitated reducing the concentration of benodanil, the more inhibitory compound, when combined with rose bengal.

This selective medium provides an efficient means of isolating *S. sapinea* in association with microorganisms found in forest soil and woody pine tissue. In addition to its use for routine isolations, it is also a valuable tool for ecological studies of *S. sapinea*.

LITERATURE CITED

1. Gibson, I. A. S. 1979. Diseases of forest trees widely planted as exotics in the tropics and southern hemisphere. Part II. The genus *Pinus*. Commonw. Mycol. Inst., Kew, and Commonw. For. Inst., University of Oxford, UK. 135 pp.
2. Martin, J. P. 1950. Use of acid, rose bengal, and streptomycin in the plate method for estimating soil fungi. Soil Sci. 69:215-232.
3. Pady, S. M., Kramer, C. L., and Pathak, V. K. 1960. Suppression of fungi by light on media containing rose bengal. Mycologia 52:347-350.
4. Punithalingam, E., and Waterston, M. J. 1970. *Diplodia pinea*. No. 273 in: Descriptions of pathogenic fungi and bacteria: Commonw. Mycol. Inst., Kew, UK.
5. Russell, P. 1956. A selective medium for the isolation of Basidiomycetes. Nature (London) 177:1038-1039.
6. Smith, N. R., and Dawson, V. T. 1944. The bacteriostatic action of rose bengal in media used for the plate counts of soil fungi. Soil Sci. 58:467-470.
7. Swart, W. J., Knox-Davies, P. S., and Wingfield, M. J. 1985. *Sphaeropsis sapinea*, with special reference to its occurrence on *Pinus* spp. in South Africa. S. Afr. For. J. 35:1-8.
8. Tsao, P. H. 1970. Selective media for isolation of pathogenic fungi. Annu. Rev. Phytopathol. 8:157-186.
9. Vaartaja, O. 1960. Selectivity of fungicidal materials in agar cultures. Phytopathology 50:870-873.
10. Vaartaja, O. 1968. Wood inhabiting fungi in a pine plantation in Australia. Mycopathol. Mycol. Appl. 34:81-89.