

Selective Medium for Isolating *Lasiodiplodia theobromae*

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ABSTRACT

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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 elliotii* 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 0.5 µg/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. elliotii* 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. elliotii* 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. elliotii* 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. elliotii* 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. elliotii* 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. elliotii* 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. elliotii* seeds were crushed and agitated in 5 ml of sterile water for 5 min, and the resultant

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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. elliotii* 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

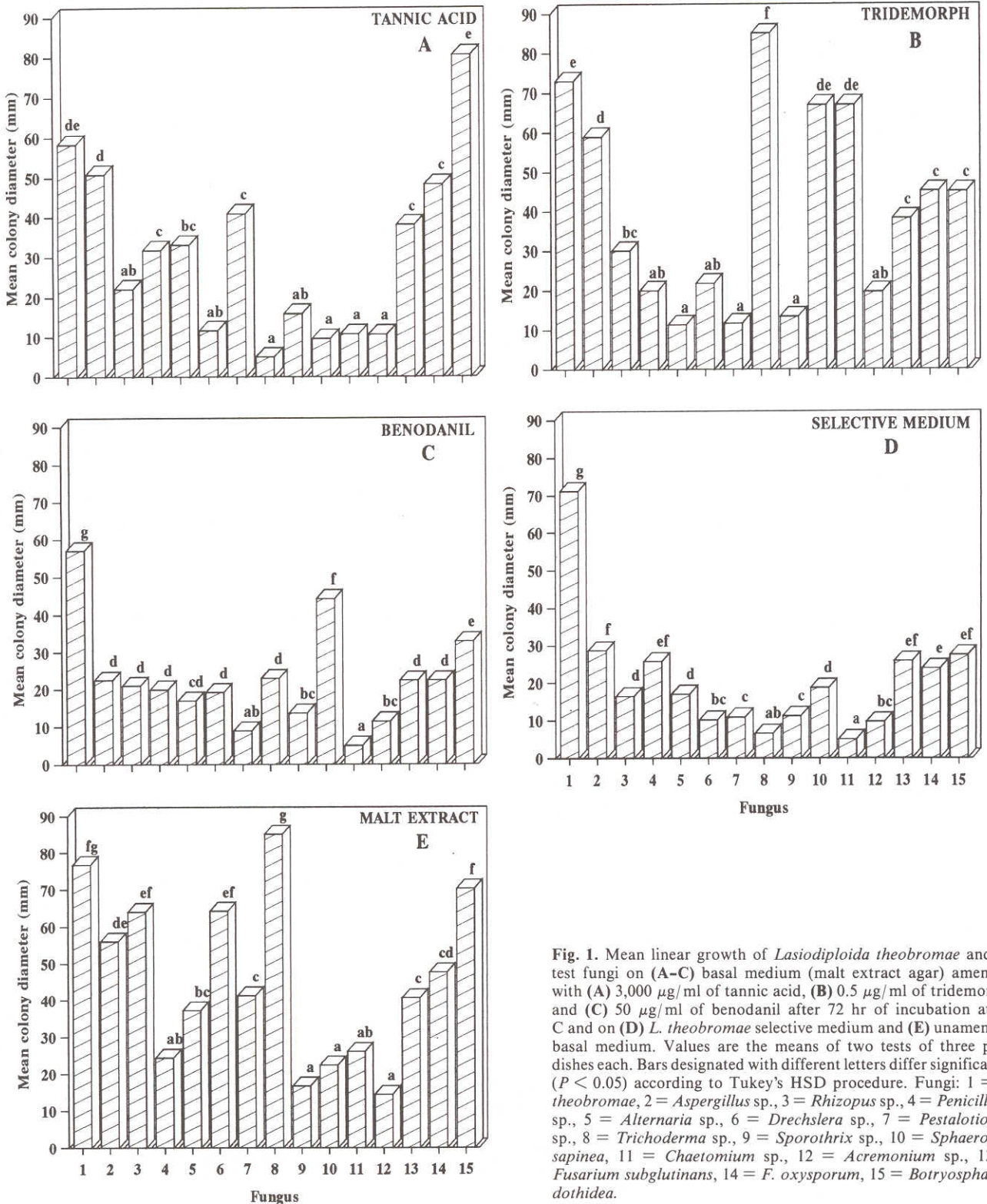


Fig. 1. Mean linear growth of *Lasiodiplodia theobromae* and 14 test fungi on (A-C) basal medium (malt extract agar) amended with (A) 3,000 µg/ml of tannic acid, (B) 0.5 µg/ml of tridemorph, and (C) 50 µg/ml of benodanil after 72 hr of incubation at 25 C and on (D) *L. theobromae* selective medium and (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*.

