

Selective Medium for Isolating *Penicillium digitatum*

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ABSTRACT

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Media varying in nutrients, pH, and inhibitory compounds were evaluated for selective development of colonies of *Penicillium digitatum* and suppression of fungal contaminants in plates exposed to the atmospheres of citrus groves and packinghouses. Thirteen species of common airborne fungi were replica-plated to the suppressive media and incubated at 20 C. *o*-Phenylalanizole (OPA) and 2,3,4,6-tetrachloroanisole (TCA) were the most selective agents when added to potato-dextrose agar (PDA) enriched with 2 g each of neopeptone and yeast extract per liter, whereas PCNB and acidification to pH 3.5 were less effective. The enriched PDA medium adjusted to pH 5.5 and amended with OPA at 100 µg/ml and PCNB at 500 µg/ml greatly inhibited the contaminant fungi and fungi encountered in citrus packinghouses. This selective medium did not affect the colony-forming potential of spores of *P. digitatum* but restricted the colony size, permitting more efficient enumeration of colonies forming from airborne spores of these pathogens.

Penicillium digitatum Sacc. incites green mold of citrus fruits. Epidemiological studies of these diseases, especially those using culture media exposed to the atmosphere, have been impeded by

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contaminating microorganisms that overgrow or inhibit the development of colonies of *Penicillium*, making it difficult to identify and enumerate the latter. This problem is especially troublesome in citrus groves and packinghouses, where the number of propagules of other microorganisms may be large relative to the number of spores of *P. digitatum*. Citrus packinghouses are monitored at regular intervals for the level of fungicide resistance in the populations of *P. digitatum* (1,4,5). An enriched potato-dextrose agar (PDA) has been widely used because the colonies of *P. digitatum* that develop from airborne spores are characteristic on the medium (9). However, because the medium does not suppress the growth of the colonies, the number of spores collected and the incubation period may be critical to prevent coalescence of the colonies. The objective of this investigation

was to develop a selective medium for the collection of airborne spores that is superior to a dicloran-amended PDA medium (4) currently employed for this purpose.

MATERIALS AND METHODS

Media and amendments were screened for selective inhibition of microorganisms other than *P. digitatum* that frequently appear on nonselective culture media exposed to the atmospheres of citrus groves and packinghouses. The basic media were PDA (Difco) enriched with 2 g/L each of neopeptone and yeast extract (enriched PDA [9]), a sucrose-asparagine-salts medium (2), and media with one or more ingredients that might selectively favor the development of colonies of *P. digitatum*. These included orange peel extract, orange juice, ascorbic acid (12), and citrus pectin (sample A-7042, Sunkist Growers, Inc., Ontario, CA) as the sole carbon source. Potentially selective chemicals were added to the media: Tergitol NP-10 (Union Carbide Corp., New York, NY), *o*-phenylalanizole (OPA) (Eastman Chemical Co., Rochester, NY), 2,3,4,6-tetrachloroanisole (TCA) (synthesized in our laboratory by methylation of the parent phenol), dicloran, chloroneb, and PCNB 75WP.

The amendments were added to the sterilized medium after cooling to 50 C, and the pH was adjusted to a value between 4.5 and 5.9 depending on the medium tested. OPA and TCA were dissolved in 0.5 ml of 95% (v/v) ethanol containing 1 g of Tergitol XD per liter (6) or in 2 ml of 95% ethanol before addition

to 1 L of media. The concentration of a selective chemical that slightly inhibited colony development of *P. digitatum* but did not change colony color or morphology was evaluated further for selective suppression of potential "contaminants" that appear commonly on nonselective media: *Cladosporium* (two isolates), *Pithomyces* (one isolate), *Alternaria citri* (three isolates), *Aspergillus* (two isolates), *Epicoccum nigrum* (one isolate), *Dothiorella* (one isolate), *Ulocladium* (one isolate), *Volutella* (one isolate), and *Stemphylium* (one isolate). Two isolates each of *P. digitatum* were included in each test. The fungi were transferred with a replicating tool from a mother culture

with a colony of each fungus to each selective medium in a petri dish. A *Rhizopus* sp. insensitive to dicloran was tested independently because of its rapid growth rate. The cultures were incubated at 20 C and growth was assessed after 4 days. The development of discrete colonies from individual spores of nine isolates of *P. digitatum* was evaluated quantitatively on promising selective media.

RESULTS AND DISCUSSION

OPA (10–20 µg/ml) and TCA (100–500 µg/ml), added to either enriched PDA or the sucrose-asparagine-salts medium, greatly inhibited the surface growth of the

contaminant fungi but did not alter the characteristic morphology of the colonies of *P. digitatum*. The insensitivity of *P. digitatum* to OPA was reported previously (6). A lesser degree of selectivity was produced by acidifying the media to pH 3.0 and by adding PCNB. All other amendments and modifications of the media were ineffective. On the basis of these observations, two selective media were developed to identify and enumerate *P. digitatum*. Enriched PDA was amended with OPA (100 µg/ml) and PCNB (500 µg/ml). The defined sucrose-asparagine-salts medium was amended with OPA (10 µg/ml) and TCA (90 µg/ml). Both media were adjusted to pH 4.5 by adding HCl after autoclaving. Airborne bacterial contaminants were encountered in the atmospheres of packinghouses where these media were tested. These bacterial contaminants were controlled, without altering the development of *P. digitatum*, by adding chloramphenicol (100 µg/ml) and penicillin G (100 µg/ml) to the media.

The incubation temperature and time were not critical for the development of *P. digitatum* on the selective media. Colonies of these fungi could be identified and enumerated after the cultures were incubated at 20 C for 4–8 days. On both media, sporulating colonies of *P. digitatum* were Rayner's (13) greenish glaucous (no. 90) in the center surrounded by a narrow margin of white, sterile hyphae. The colonies appeared white when viewed from below. Although the sucrose-asparagine-salts medium was somewhat more selective than the enriched PDA medium, most of our surveys of *P. digitatum* in citrus groves and packinghouses used the latter medium because it was convenient to prepare and the ingredients were readily

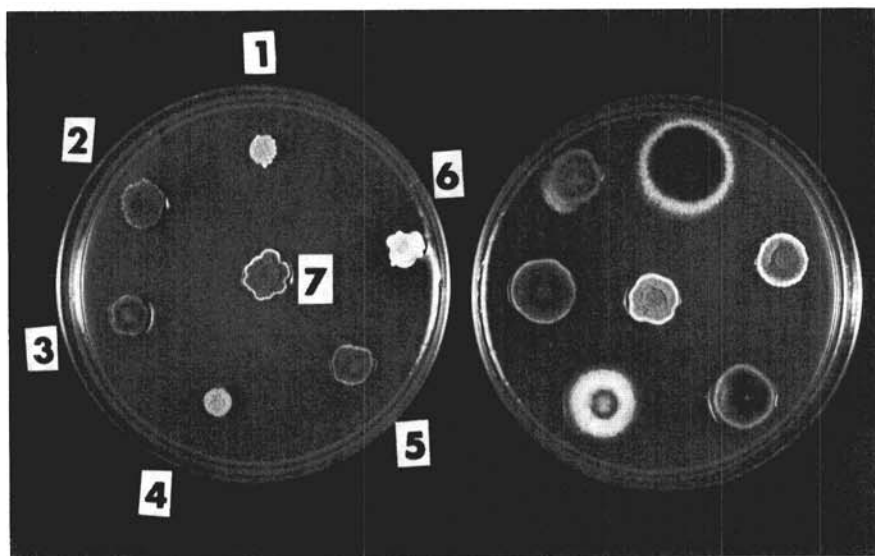


Fig. 1. Growth of *Penicillium digitatum* and six "contaminant" fungi after 5 days at 20 C on enriched PDA amended with (left) 3 µg/ml of dicloran and (right) 100 µg/ml of *o*-phenylanzole and 500 µg/ml of PCNB. 1 = *Epicoccum nigrum*, 2 = *Cladosporium* sp., 3 = *Stemphylium* sp., 4 = *Pithomyces* sp., 5 = *Alternaria citri*, 6 = *Penicillium expansum*, and 7 = *P. digitatum*.

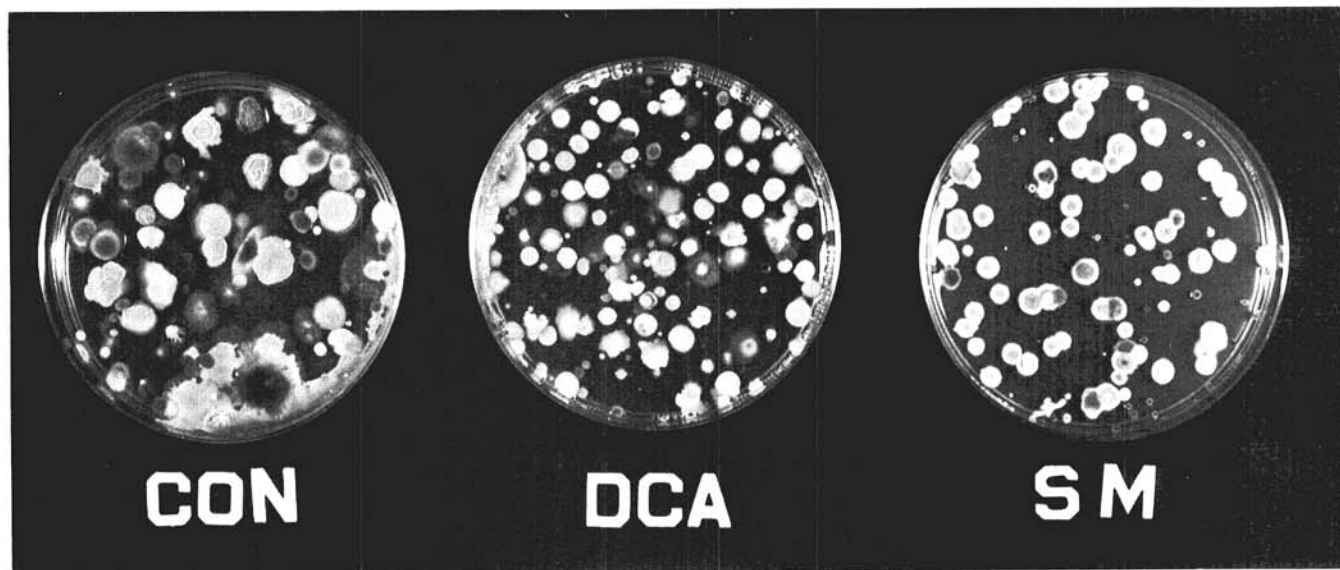


Fig. 2. Development of colonies of *Penicillium digitatum* and other fungi on three agar media: CON = control (PDA + neopeptone), DCA = CON medium amended with 3 µg/ml of dicloran, and SM = CON medium amended with 100 µg/ml of *o*-phenylanzole and 500 µg/ml of PCNB. Petri plates with media were exposed simultaneously for 10 min to the atmosphere of a lemon packinghouse in Yuma, AZ, and incubated at 20 C for 5 days. Note that the SM medium greatly inhibits the growth of other fungi while rendering the *P. digitatum* colonies compact and nondiffusive.

available.

Compact, easily identifiable colonies of *P. digitatum* developed on the enriched PDA medium containing OPA and PCNB (SM). The radial growth of both fungi on SM was reduced about 70% compared with enriched PDA alone. Some *o*-phenylphenate-resistant isolates, however, grew in an uninhibited, diffuse manner on SM, probably because these isolates were cross-resistant to OPA or PCNB. Positively correlated cross-resistance, where mutants obtained by selection on one compound are resistant to structurally related compounds, has been shown (8) with *P. digitatum* for various aromatic hydrocarbons. All colonies sporulated normally but about 1 day later than on enriched PDA alone. Growth and sporulation of most contaminant fungi was inhibited more than 95%, with the exception of *Cladosporium* and *Dothiorella*, which sporulated on the SM but their radial growth was reduced about 90% compared with that on enriched PDA. Growth of a dicloran-resistant *Rhizopus* sp. was reduced 70% on the SM.

Conidia of nine isolates of *P. digitatum* were streaked on the SM to determine their colony-forming potential. Conidia of all isolates germinated to the same extent as on enriched PDA and formed readily identifiable colonies. Addition of carbendazim (1 µg/ml) or *sec*-butylamine (500 µg/ml) to the medium to isolate fungicide-resistant races of *Penicillium* (7,14,15) did not alter the selectivity of the medium for *P. digitatum* or reduce the ability of spores of these species to form typical colonies.

The addition of dicloran (3 µg/ml) to media has been recommended to suppress the spread of *Penicillium* colonies and to inhibit the development of fungal contaminants such as *Rhizopus stolonifer* (3,10). Other *Rhizopus* spp. are

not sensitive to dicloran (11). The addition of OPA and PCNB to enriched PDA suppressed the radial growth of the *P. digitatum* to about the same extent (70% inhibition) as enriched PDA with dicloran, but the contaminant fungi were suppressed to a greater degree on the SM (Fig. 1).

The SM may be exposed to the atmospheres of citrus groves and packinghouses to identify and enumerate large numbers of colonies that form from spores of *P. digitatum* (Fig. 2) and is useful for reculturing contaminated stock cultures of these fungi. The medium may be amended with 1 µg/ml of carbendazim and 500 µg/ml of *sec*-butylamine for enumerating fungicide-resistant colonies in packinghouses with minimum interference by nonpathogenic contaminants. These concentrations are well above that found completely inhibitory to sensitive races of *P. digitatum* and would allow only those spores retaining a relatively high level of fungicide resistance to grow (14,15). For the detection of *o*-phenylphenate-resistant colonies, no additional selective amendment is required in a medium prepared with enriched PDA containing 20 µg/ml of penicillin G and chloramphenicol, because the broad-spectrum action of *o*-phenylphenate effectively suppresses contaminating fungi, thus allowing only spores with a relatively high level of resistance to grow.

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