Semiselective Media for the Isolation of *Elsinoë fawcettii* from Citrus Scab Pustules

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ABSTRACT

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Isolating *Elsinoe fawcettii* from citrus scab pustules or detecting viable propagules of this pathogen in them can be difficult or even impossible by conventional methods, particularly if the pustules are old. This slow-growing pathogen is confined to the pustule's stromatic portion, which is colonized by many fast-growing organisms including *Cladosporium* spp. and yeasts. *E. fawcettii* was isolated successfully by scraping small fragments from the pustules onto semiselective media in isolation plates. Potato-dextrose agar (PDA) was the preferred basal medium, because *E. fawcettii* formed a unique, compact, raised, and easily identifiable colony on it. Dodine was the only material found that suppressed yeasts and *Cladosporium* spp. sufficiently to permit use of PDA. The most useful medium developed contained 39 g of PDA, 100 mg of streptomycin sulfate, 100 mg of tetracycline hydrochloride, and 400 mg a.i. of dodine per liter of water. Chlorothalonil showed some potential for the selective isolation of *E. fawcettii*, but because of inactivity against yeasts, it had to be used in water agar to avoid abundant yeast growth.

Isolation of *Elsinoë fawcettii* Bitancourt & Jenkins from citrus scab pustules is difficult. This fungus grows slowly on agar media and produces a visible colony from a single spore or small mycelial fragment only after 4–6 days. Thus the colonies of *E. fawcettii* are frequently overgrown by faster-growing organisms before isolation is possible. Only the stromatic portion of a scab pustule contains the causal fungus, and this is usually heavily contaminated by other organisms. *Cladosporium* spp. and yeasts have proven particularly troublesome in this respect. Surface-sterilization with ethanol or sodium hypochlorite has reduced contamination only slightly and may even be detrimental because the scab pathogen is located on or near the pustule surface.

Previously reported techniques depended on the transfer of minute fragments of pustules to isolation plates, using pieces of stroma or conidiophore that were so minute that at least some of them might contain uncontaminated propagules of *E. fawcettii*. Winston (4) and Bitancourt and Jenkins (1) scraped the surfaces of scab pustules into petri dishes and poured agar media over them. Whiteside (2) cut thin tangential sections from scab pustules and mashed them in a sterile petri dish with a bent steel spatula to produce small fragments that were dispersed in cooled melted water agar poured into the dish. Within 3–4 days, before contaminants overran the plate, the fragments had to be viewed microscopically for any growth of *E. fawcettii* from their margins. These procedures were laborious and usually succeeded only if young pustules were available.

Accurate diagnosis of citrus scab and determinations of survival of *E. fawcettii* in scab pustules have been hindered by the inability to isolate this pathogen easily from diseased tissue. This paper reports on improved techniques for facilitating the isolation of *E. fawcettii* from citrus scab pustules.

MATERIALS AND METHODS

Source materials included naturally occurring scab pustules on leaves and fruit of Temple (*Citrus temple* Hort. ex Y. Tan.) and Murcott (hybrid of unknown origin), on leaves of rough lemon (*C. jambhiri* Lush.), and on pustules on grapefruit (*C. paradisi* Macf. 'Marsh') and sweet orange (*C. sinensis* L. Osbeck 'Valencia') fruit resulting from inoculations with *E. fawcettii* using a

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previously described technique (2). Diseased leaves and fruit were washed to remove surface detritus, avoiding excessive pressure that could cause disintegration of the stromatic portions of the scab pustules.

Transfer of propagules from the pustules was tested in three ways. 1) Tangential sections cut from the stroma were mashed into small fragments with a bent steel spatula in a petri dish. Melted agar at 40 C was poured into the dish, and the fragments were stirred into it. 2) The surfaces of the pustules were scraped above an open petri dish, and the fragments were allowed to fall into the dish. Melted agar media cooled to 40 C was poured into the plate, and the fragments were dispersed in it. 3) Fragments of stromata were scraped directly onto solidified media.

Various substances were tested for their ability to permit growth of *E. fawcettii* (Table 1). Some fungicides, such as chlorothalonil (Bravo 500), prochloraz (BTS 40542 40EC), iprodione (Rovral 50W), thiram (Thylate 65W), mancozeb (Dithane M-45 80W), and dodine (Cyprex 65W), were selected for study because they had shown little or no activity against scab in field tests (3). All materials were tested as amendments to water agar or potato-dextrose agar (PDA) (Difco). They were added to the melted basal media after it cooled to 45 C. Initially, each material was tested at different concentrations in water agar or PDA to determine its effects on the radial growth of *E. fawcettii*. Materials showing relatively little activity against *E. fawcettii* were tested later for their ability to suppress contaminating microorganisms present on or in scab pustules. For this purpose, the test media were seeded with scrapings from old scab pustules.

RESULTS AND DISCUSSION

Colonies of *E. fawcettii* arising from fragments of stromatic tissue from scab pustules were more distinct when growing on the agar surface than when submerged. Therefore, it was better to deposit the fragments scraped from scab pustules onto solidified agar in the isolation plate than to stir them into cooled, melted agar. Another objection to the latter procedure was the risk of pouring the agar medium while too hot and thereby possibly killing any viable propagules of *E. fawcettii*.

The first materials examined for potential use in a selective medium were streptomycin sulfate and tetracycline hydrochloride, which were added to the agar media only after cooling because they are thermolabile. *E. fawcettii* tolerated these materials well, and there was no suppression of this pathogen when they were used in agar at 400 μg/ml. Only 100 μg/ml of each of these materials was needed to prevent bacterial contamination; therefore, this concentration was used in all subsequent studies. All other materials investigated (Table 1) were tested in the presence of streptomycin and tetracycline.

Thiram, imazalil 68EC, prochloraz, pimaricin, cycloheximide (Acti-dione), fentin hydroxide (Du-Ter 48W), etaconazole (Vangard 10W), mancozeb, and hymexazol were too toxic to *E. fawcettii* to justify further study (Table 1).

The only materials that *E. fawcettii* tolerated relatively well were sodium metabisulfite, iprodione, chlorothalonil, dicloran (Botran 75W), and dodine. Sodium metabisulfite and iprodione showed little activity against *Cladosporium* spp. and yeasts. Chlorothalonil inhibited growth of *Cladosporium* spp. and many other fungi but not of yeasts. Some success was obtained with

Table 1. Effects of different materials, in combination with streptomycin and tetracycline,[a] on the growth of *Elsinoë fawcettii* on amended agar media

Material	Basal agar media[b]	Concentrations tested (μg a.i./ml)	Lowest concentration (μg a.i./ml) causing >50% reduction in radial growth
Thiram	PDA	1, 10, 100	10
	A	1, 10, 100	10
Chlorothalonil	PDA	1, 10, 100, 200, 400	>400
	A	50, 100, 200, 400	100
Imazalil	PDA	1, 10, 100	1
Prochloraz	PDA	1, 10, 100	10
Sodium metabisulfite	PDA	100, 200, 400	400
Pimaricin	PDA	1, 10, 100	10
Cycloheximide	PDA	50, 100, 200	50
Iprodione	PDA	100, 200, 400	>400
	A	50, 100, 200	100
Fentin hydroxide	PDA	1, 10, 100	>100
	A	1, 10, 100	10
Dicloran	PDA	1, 10, 100	>100
	A	1, 10, 100	100
Etaconzole	A	1, 10, 100	1
Hymexazol	PDA	5, 10, 100	5
Mancozeb	PDA	1, 10, 100	10
Dodine	PDA	100, 200, 400, 600	600
	A	50, 100, 200, 600	100

[a] The two antibiotics were included in all tests, each at concentrations of 100 μg/ml.
[b] PDA = potato-dextrose agar, A = 2% water agar.

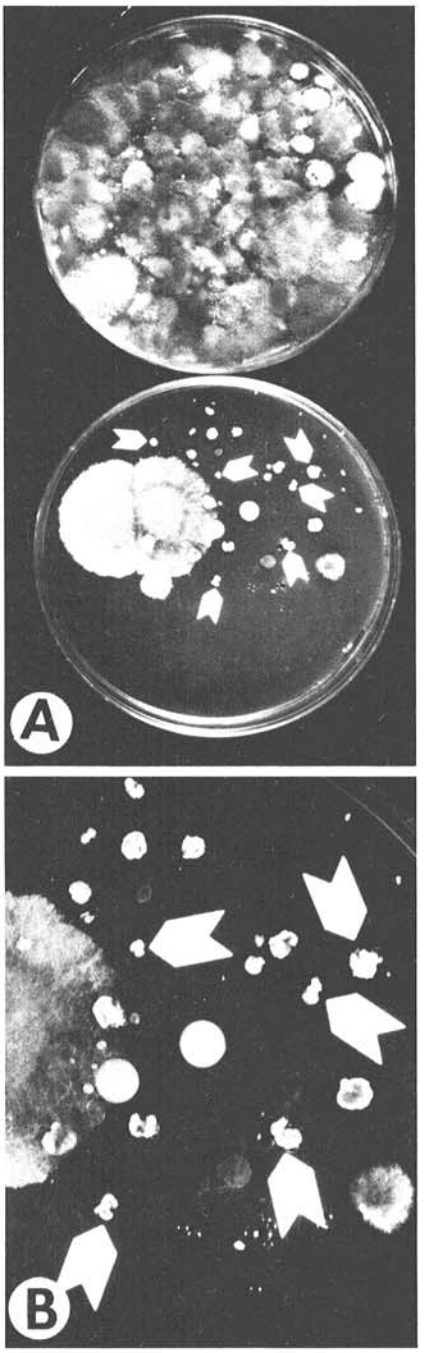

Fig. 1. Growth of *Elsinoë fawcettii* and contaminants 7 days after scraping fragments of stromata from citrus scab pustules on dried rough lemon leaves onto solidified agar media. **(A)** (Top) Heavy growth of fungal contaminants on potato-dextrose agar (PDA) amended with only streptomycin and tetracycline compared with (bottom) restricted contaminant growth and presence of visible colonies of *E. fawcettii* on a plate of PDA that contained dodine (400 μg a.i./ml) in addition to the two antibiotics. **(B)** Magnification of a part of the plate containing the dodine-amended PDA. Arrows point to a few of the colonies of *E. fawcettii* present.

Table 2. Numbers of colonies of *Elsinoë fawcettii* and amounts of contaminating fungal growth 7 days after seeding dodine-amended potato-dextrose agar[a] with fragments of stromata scraped from scab pustules

Concentration of dodine (μg a.i./ml)	Colonies per 90-mm-diameter dish (no.)			Area of medium covered by contaminants (%)
	E. fawcettii[b]	Yeasts	*Cladosporium* spp.	
0	ND[c]	ND	ND	95
50	12	ND	ND	57
100	13	ND	ND	28
200	25	118	34	20
300	26	89	11	21
400	27	50	2	8
500	13	35	1	7

[a] Potato-dextrose agar contained the antibacterial agents streptomycin and tetracycline, each at 100 μg/ml.
[b] Counts included only colonies that were discrete and not visibly contaminated.
[c] ND = no data because plates were too overrun by contaminating fungi.

chlorothalonil for the selective isolation of *E. fawcettii* when it was mixed at 50 μg/ml in water agar. It did not perform satisfactorily in PDA because of the heavy growth of yeasts on this medium. Dicloran did not inhibit *Cladosporium* spp. but was active against yeasts. Therefore, attempts were made to use dicloran plus chlorothalonil to suppress yeasts as well as *Cladosporium* spp. This combination proved too fungicidal to *E. fawcettii* even when the concentration of each material was reduced. For example, chlorothalonil at 50 μg/ml plus dicloran at 1 μg/ml in water agar was not suppressive enough to yeasts to be useful, and this mixture was highly toxic to *E. fawcettii*.

Dodine was the only material tested that was relatively nontoxic to *E. fawcettii* at concentrations that would suppress the major fungal contaminants, *Cladosporium* spp. and yeasts. On water agar, 200 μg/ml of dodine completely suppressed yeasts; however, to avoid reducing the growth rate of *E. fawcettii* excessively, the concentration was reduced to 50 μg/ml. In water agar, this concentration of dodine still reduced yeast growth by 75%.

PDA was a more satisfactory medium for the isolation of *E. fawcettii*, because the colonies on it were more easily identified than those on water agar. Use of PDA had one disadvantage in that yeasts grew vigorously on this high-nutrient medium, and dodine was only partially effective in inhibiting their growth at concentrations that would not suppress *E. fawcettii* excessively.

Tests were conducted to determine the best compromise concentration for dodine in PDA. To estimate the relative usefulness of each concentration of dodine for producing discrete, uncontaminated colonies of *E. fawcettii* on the agar surface, it was necessary to ensure as much uniformity of deposition of stromatic fragments as possible within each replicate. Therefore, several passes with the scrapings were made over each dish. A different group of scab pustules was scraped for each of the eight replicates used. After 1 wk, counts were made of the colonies of *E. fawcettii*, *Cladosporium* spp., and yeasts present, and an estimate was made of the total area of the plates covered with contaminants. Results of one test are shown in Table 2. The average numbers of uncontaminated, discrete colonies of *E. fawcettii* present per dish were similar when dodine was used at 200, 300, and 400 μg/ml but decreased at 500 μg/ml. Therefore, the highest rate at which dodine could be used without substantially reducing the number of pure colonies of *E. fawcettii* formed was 400 μg/ml. Some fungi were not well suppressed by dodine at 400 μg/ml, including *Colletotrichum gloeosporioides* and species of *Fusarium*, *Curvularia*, and *Choanephora*.

In other tests, variations in pH of the medium from 5.0 to 7.0 had little or no effect on the efficiency or selectivity of the dodine-streptomycin-tetracyline (DST)-PDA medium. This medium performed as well after storage for 30 days in sealed bags at 25 C as it did when prepared and poured immediately before use. The fungicidal activity of dodine was not affected by autoclaving. Nevertheless, it was more convenient to add it with the antibiotic solution after the PDA cooled.

Sometimes the colonies of *E. fawcettii* were contaminated by yeasts without this being visibly apparent. To ensure purity of the isolates, fragments from the colonies had to be crushed in a petri dish and stirred into cooled PDA, which was later poured into the dish. It was difficult to purify mixed colonies; therefore, any isolates found contaminated by yeasts were discarded.

Use of solidified DST-PDA medium facilitated isolation of *E. fawcettii* (Fig. 1) from all the cultivars tested. Generally, more colonies of this fungus formed on plates seeded with fragments of scab pustules from leaves than from fruit. This fungus was also successfully isolated from pustules on air-dried rough lemon leaves and Valencia orange fruit that had been left exposed in the laboratory for 5 and 2 mo, respectively. Without this selective medium, it usually proved impossible to isolate *E. fawcettii* from older scab pustules. DST-PDA now provides a tool for determining with greater certainty the period of survival of this pathogen in scab pustules, which should assist future studies on the epidemiology of this disease.

Tests that I made with *Sphaceloma perseae* Jenkins from avocado (*Persea americana* Mill.) fruit and *S. poinsettiae* Jenkins & Ruehl from poinsettia (*Euphorbia pulcherrima* Willd. ex Klotsch) leaves indicated that both of these pathogens tolerate relatively high concentrations of dodine. Therefore, the DST-PDA medium might be useful for isolating these fungal species and perhaps other *Elsinoë-Sphaceloma* fungi that are difficult to isolate by conventional methods.

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