Excised Twig Assay for the Study of Apple Tree Crown Rot Pathogens In Vitro

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ABSTRACT

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Dormant, excised apple twigs were used to determine pathogenicity and relative virulence of pythiaceous fungi isolated from apple trees with crown rot symptoms. Twig segments were inserted vertically into a test isolate culture growing on cornmeal agar plus agar in a storage jar. After incubation, the twig segments were removed from the agar and stripped of their periderms, and the length of necrosis was measured. Dormant twigs were preferable to active twigs. Fungal contamination on the agar surface was minimized by amending the agar with pimaricin at 10 mg/L. The method can be used to determine the pathogenicity of different species of pythiaceous fungi, to compare relative virulence of isolates within a species, and possibly to determine relative resistance of different apple scion and rootstock cultivars.

Additional key words: collar rot, *Malus, Phytophthora* spp., *Pythium* spp.

Excised branch pieces from apple (*Malus pumila* Mill.) were used by Buddenhagen (7) to determine pathogenicity of *Phytophthora cactorum* (Leb. & Cohn) Schroet. in vitro. A similar assay developed by Sewell and Wilson (14) was modified by Borecki and Millikan (6) to reduce the incubation period and to minimize the amount of dormant wood needed for the assay. Excised twig assays have been widely used to determine virulence of *P. cactorum* associated with the crown and collar rots of apple trees (1-3,15). These assays have also been used to test pathogenicity of other crown rot fungi (5,11,12).

In our studies on crown rot in apple orchards in western New York (9), we first used the modified excised twig assay (6). This method produced such variable results that it was difficult to identify a pathogenic reaction. When comparing the effects of isolates, we often had problems measuring necrosis because the developing lesions tended to extend irregularly around the twigs or longitudinally in only one direction. This paper reports a modification of the excised twig assay for determining pathogenicity and for comparing relative virulence of pythiaceous fungi associated with crown rot in apple trees.

MATERIALS AND METHODS

Seventy milliliters of cornmeal agar plus agar (CMAA: 17 g of Difco cornmeal agar plus 5 g of Difco-Bacto agar per liter of distilled water) was dispensed into Pyrex jars to give an agar depth of about 10 mm. Each jar was seeded with an agar block of mycelium from a test culture and sealed with Parafilm to maintain a moist atmosphere. Jars were placed in plastic boxes containing wet paper towels at a temperature appropriate for the species being tested (25 C for *P. cactorum* and *P. megasperma*) until colony growth nearly covered the agar surface (about 5 cm diam). When we compared different species, all isolates were incubated at 25 C for 7 days. Control jars consisted of CMAA without fungus.

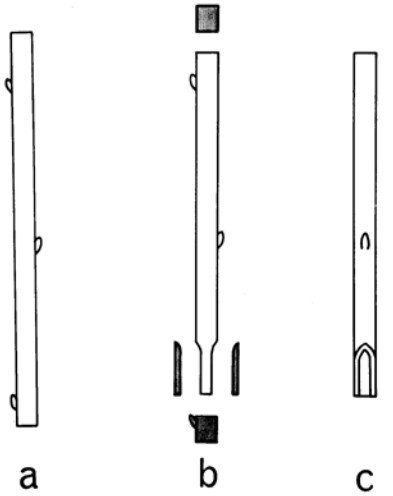

Fig. 1. Preparation of twig segments for in vitro assay of pathogenicity of pythiaceous fungi to apple. **(A)** 75-mm twig segment cut from the middle of a current season shoot before surface disinfestation. **(B)** Disinfested segment with both ends removed and tangential sections cut from opposite sides of the base. **(C)** The segment in B, turned 90° to show exposed inner tissues, ready for insertion into agar culture.

To compare the suitability of active and dormant apple tissue, we collected active shoots in July when they had ceased terminal growth and dormant shoots in March. Segments 75 mm long were cut from the central portion of the shoots, surface disinfested in 0.6% NaOCl for 5 min, rinsed three times in sterile water, and blotted dry. Twig segments were pooled, mixed, and then drawn at random. Disinfested pruning shears were used to remove about 5 mm from each twig end to eliminate any residual absorbed NaOCl.

The basal end of each twig segment was then pared by making tangential cuts 10 mm long and 1-2 mm deep on opposite sides with a sterile scalpel to expose the phloem-cambium region for infection by test isolates (Fig. 1) (6). Fifteen of these pared twig segments were inserted vertically, distal end up, into the agar medium in each jar at the periphery of the fungal colony. The jars were resealed with Parafilm, returned to the plastic boxes, and incubated at 25 C for 6-7 days (Fig. 2). Twig segments were then removed from the jars and stripped of their periderms with a sharp scalpel. The length of necrosis on each twig and the depth of agar in each jar were measured. By subtracting depth of agar from the total length of necrosis, we obtained a value for net necrosis length (NNL).

In one experiment, V-8 agar (200 ml of V-8 juice, 800 ml of distilled water, 2 g of $CaCO_3$, and 20 g of Difco-Bacto agar) was compared with CMAA using dormant

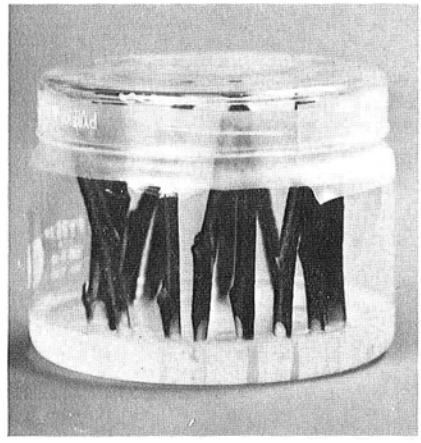

Fig. 2. Apple twig segments inserted into a culture of *Phytophthora cactorum* growing on cornmeal agar plus agar amended with pimaricin before incubation.

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and active twigs. Four jars of each medium were seeded with *P. cactorum* isolate 007, and four were left sterile as controls. All were incubated at 25 C for 7 days. Two jars of each medium were used to inoculate McIntosh twig segments. One jar contained eight dormant twig segments and the other contained active ones. Empire twig segments were inoculated similarly. Necrosis was measured after 4 days at 25 C.

Contamination of the agar surface was a problem after 4–5 days of incubation. Therefore, CMAA was amended with either the antibiotic pimaricin (PCMAA) (8) or the organic fungicide benomyl (BCMAA) (13), neither of which inhibits growth of pythiaceous fungi. The PCMAA was prepared by adding 0.4 ml of a 2.5% sterile suspension of pimaricin in distilled water, and the BCMAA by adding 20 mg of 50% benomyl to 1 L of CMAA after the autoclaved medium had cooled to 50 C, yielding a final pimaricin or benomyl concentration of 10 mg/L. Two jars each of CMAA, PCMAA, and BCMAA were made. One was seeded with *P. cactorum* isolate 007 and the other served as a control. After 7 days in the dark at 25 C, 15 dormant McIntosh twig segments were inserted into the medium in each jar and incubated for 6 more days in the dark at 25 C. We then measured NNL and observed the agar surface for contamination.

RESULTS

Length of necrosis differed according to the isolate used (Table 1). Necrosis tended to be uniform around each twig segment (Fig. 3), and the standard deviations of mean NNL were small enough to make meaningful comparisons among isolates. No necrosis developed on control segments.

The NNL on V-8 agar was not significantly different from that on CMAA (Table 2), although CMAA was preferred because it is simple to prepare and is relatively transparent. The NNL on McIntosh was not significantly different from that on Empire. With both media and both cultivars, however, dormant twig segments developed significantly longer lesions than did active twig segments (Table 2). Active twig segments had irregular lesions and tended to develop narrow necrotic strips on one side that made measurement difficult and inconsistent. Dormant twig segments, on the other hand, had lesions that developed uniformly around the segments. According to an analysis of variance, a significant F statistic was found only between active and dormant twig segments ($P = 0.01$).

No significant difference was found ($P = 0.05$) among the NNL on twig segments in CMAA, PCMAA, and BCMAA. However, the agar surface of PCMAA remained free of contamination, whereas BCMAA and CMAA were contaminated. This surface contamination in both BCMAA and CMAA resulted in small lesions on the twig segments.

Table 1. Length of necrosis on excised, dormant twigs of McIntosh apple inoculated with three pythiaceous fungi isolated from apple trees with crown rot symptoms[a]

	Length (mm) of necrosis induced by		
Replicate	*Phytophthora cactorum* isolate 007	*Phytophthora megasperma* isolate 029	*Pythium* sp. isolate 044
1	53	6	12
2	43	4	18
3	45	2	8
4	57	3	14
5	44	2	12
6	42	2	9
7	35	2	11
8	47	3	10
9	46	0	25
10	41	2	8
11	51	3	13
12	38	4	14
13	38	5	8
14	51	3	7
15	42	2	8
Mean	43.5	2.9	11.8
SD	5.4	1.5	4.8

[a] Length of necrosis was measured after 7 days of incubation in the dark at 25 C.

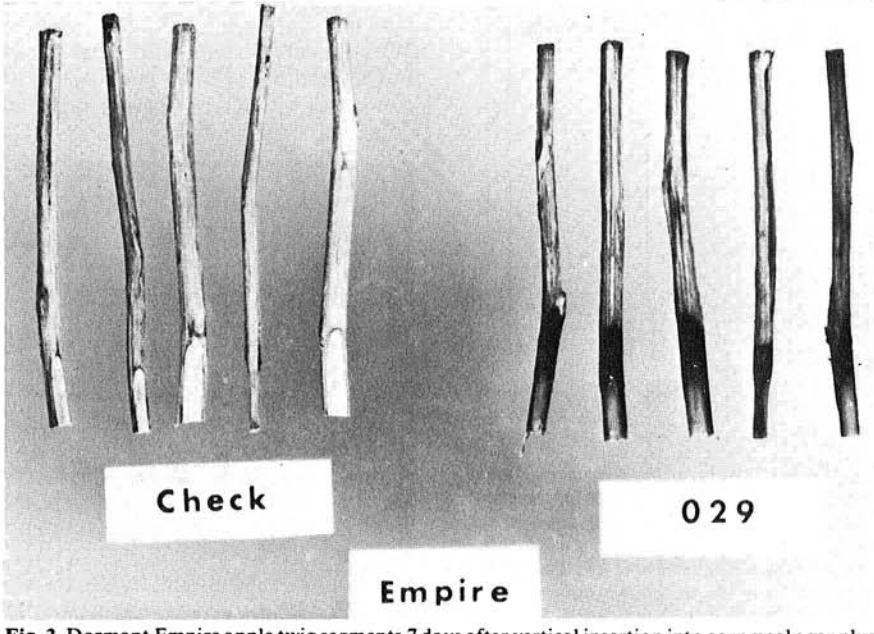

Fig. 3. Dormant Empire apple twig segments 7 days after vertical insertion into cornmeal agar plus agar amended with pimaricin without fungus (check) and with *Phytophthora megasperma* (isolate 029).

DISCUSSION

The assay of excised, dormant twigs described here is reliable and effective in determining the pathogenicity in vitro of different species of pythiaceous fungi and the relative virulence of isolates of the same species to apple. Problems originally encountered with the Borecki-Millikan method (6) have been overcome by eliminating much of the inherent experimental variability. Because of the uniform inoculation of the bases of the twig segments, lesions developed only upward rather than in several directions. Twig segments did not need identical wounding because measurement of necrosis began

Table 2. Length of necrosis on active and dormant excised twigs of McIntosh and Empire apple inoculated with *Phytophthora cactorum* isolate 007 growing on V-8 agar or cornmeal agar plus agar (CMAA)

	Length (mm) of necrosis[a]			
	McIntosh		Empire	
Agar	Active	Dormant	Active	Dormant
V-8	12.0	22.4	12.9	21.5
SD[b]	±2.7	±6.5	±7.1	±5.5
CMAA	11.4	22.1	9.8	21.8
SD	±5.2	±1.5	±2.1	±7.8

[a] Means of eight replicates per treatment. Necrosis was measured after 4 days at 25 C.
[b] Pooled standard deviation = 5.3 mm.

at the agar surface. Intimate contact of the inoculum and the twig was ensured by insertion of the twig into an agar culture of the fungus. The new method also allowed ample replication. Furthermore, isolates of pythiaceous fungi can be screened quickly at any time of the year under a controlled laboratory environment.

Twig assays have also been used in examining differences in resistance caused by host genotype (2,7,14), time of year (4,10,11), and age of inoculated wood (10). Results of a twig assay in vitro cannot always be applied to apple trees in the orchard. Bielenin (4) found that twig inoculations are inferior to direct inoculations of trees in situ for determining actual resistance of apple cultivars to *P. cactorum*. Kröber and Karnatz (10) noted that the degree of susceptibility determined in vitro is influenced by twig position on the tree.

A twig assay may be useful, however, in ranking or grouping cultivars based on their relative resistance, although this ranking does not necessarily correlate with absolute resistance expressed in the orchard. Our excised twig assay will be especially useful in such a study because it enables uniform and efficient screening of many isolates. The technique may also be used to compare reactions between individual cultivars and isolates on a quantitative basis.

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