A Tissue Culture System for Studying Disease Resistance: the Black Shank Disease in Tobacco Callus Cultures

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ABSTRACT

Tobacco tissue cultures were obtained from two genetically similar plants, one resistant and the other susceptible to race 0 of *Phytophthora parasitica* var. *nicotianae*. Under certain defined conditions, tissue cultures from the resistant plant were colonized less rapidly and less extensively by race 0 of the pathogen than tissue cultures from the susceptible plant. Race 1 of the fungus, to which both plant clones were equally susceptible, colonized tissue cultures of both lines to a similar extent. Tissue colonization rates increased as incubation temperature was increased from 15 to 32 C. The colonization differential was most apparent from 20-24 C, and negligible at 27-32 C. With the tissue culture line derived from the resistant plant, colonization rates were varied over a wide range, from relatively slow, to colonization rates much faster than in callus tissue from susceptible plants by modification of the hormonal regime of the growth medium. The tobacco tissue culture, *P. parasitica* var. *nicotianae* system appears suitable for studying the nature of disease resistance under strictly controlled conditions.

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We have developed a tissue culture system for studying the interaction of a fungus, *Phytophthora parasitica* Dast. var. *nicotianae* (Breda de Haan) Tucker, and its host, *Nicotiana tabacum* L. This system avoids many of the problems of the physiological and morphological complexity of intact plants and rapid senescence of excised plant parts.

Tissue culture systems for studying host-pathogen relationships have generally been limited to those employing obligate parasites (2, 11). With such systems, it is difficult to study the physiology of the pathogen. In 1965, Ingram & Robertson (12) reported that potato tissue cultures from resistant plants supported less growth of *Phytophthora infestans* than cultures from susceptible plants. Ingram (10) attributed this difference to the expression of the hypersensitive ("R" gene) reaction in cultures from resistant plants. Similar results have been obtained with tomato tissue cultures (25). Although this work indicated that systems employing a facultative saprophyte are feasible, the potato and tomato tissue culture systems are poorly defined at present.

Tobacco tissue cultures were chosen as host tissues because of the extensive metabolic and physiological studies performed with them. They have been used extensively in bioassays for cytokinins (18, 21) and in studies on physiology of differentiation (22), and have also provided material for metabolic studies (3, 13, 15, 20). Tobacco tissue cultures can be grown on a defined medium (16, 19) on which total growth is limited by the carbohydrate supply (24). Logarithmic growth rates can be controlled by the concentration of cytokinin in the medium (6, 7) and modified by the addition of exogenous gibberellic acid (8).

Resistance to *P. parasitica* var. *nicotianae* race 0 was transferred from *N. plumbaginifolia* into *N. tabacum* (1), and Goins & Apple (4) found that resistance was conditioned by a single, dominant genetic factor. They developed highly similar plant lines resistant and susceptible to race 0 of the black shank pathogen by making five backcrosses and selections from their interspecific cross (*N. tabacum* 'Hicks' × *N. plumbaginifolia*).

The availability of these tobacco lines and the ability to regulate growth of tobacco callus tissue on defined media made it feasible for us to explore the possibility of using this host-pathogen combination to study the nature of disease resistance.

MATERIALS AND METHODS.—Tobacco cultures and clonal plants were derived from two genetically similar plants, one resistant (line 46-8) and one susceptible (line 49-10) to race 0 of *P. parasitica* var. *nicotianae* (1, 4). These lines are susceptible to race 1, the other known race of the pathogen.

The derivation of tobacco materials for this study is outlined in Fig. 1. The initial plants were grown to ca. 1 m in height and detopped. Pith tissues were isolated and induced to form callus. Buds from the bases of the detopped plants were rooted to give clonal lines of intact plants. Tissue cultures were grown by standard procedures (7, 8, 24) on Linsmaier & Skoog's medium (16) containing 11.5 μM indole-3-acetic acid (IAA) and the various cytokinin regimes indicated below. Pith tissues were planted on medium containing 1.0 μM kinetin; "loose" callus tissues were grown on medium containing 0.2 μM N^6-(3-methyl-2-butenylamino)purine (2iP); "tight" callus tissues were grown on media containing 1.0 μM

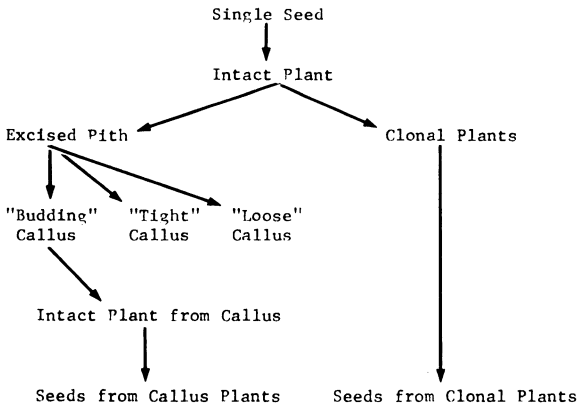

Fig. 1. Method of derivation of materials for study. All tobacco plant materials were derived from two plants, one resistant and the other susceptible to race 0 of *Phytophthora parasitica* var. *nicotianae*.

kinetin. Tissues were induced to bud on Linsmaier & Skoog's medium containing 11.5 μM IAA, 10 μM 2iP, and Smith & Murashige's (23) supplement of 160 mg/liter adenine sulfate, 340 mg/liter, NaH_2PO_4 and 100 mg/liter of L-tyrosine. Buds were removed from the cultures, rooted, and tested for resistance or susceptibility to the fungus as indicated below. To minimize light-induced degradation of the auxin, 1 μM 2,4-dichlorophenoxyacetic acid (2, 4-D) rather than IAA (11.5 μM) was used in the tight tissue medium in experiments in the Wisconsin Biotron.

Apple's isolates 1452 (race 1) and 1156 (race 0) of *P. parasitica* var. *nicotianae* were maintained on lima bean or oatmeal agar in the dark at ca. 20 C. Zoospore suspensions were obtained by the method of Gooding & Lucas (5), counted with a hemocytometer, and diluted to give the desired concentration for inoculation (usually 330 spores/ml). Callus tissues were inoculated by placing a single drop (ca. 30 μliter) of spore suspension on top of a tissue piece with a sterile calibrated Pasteur pipette. Suspensions were checked for bacterial contamination by addition of a drop of the inoculum to a flask containing nutrient broth. Results reported are only for those experiments where the inoculum was shown to be free of bacteria.

The influence of light and temperature on the interaction of *P. parasitica* var. *nicotianae* and callus tissue was studied in the cross-gradient room of the Wisconsin Biotron. Four temperatures (15, 22, 27, 32 C, first experiment, and 20, 24, 27, and 31 C, second experiment) and four light intensities (1,100, 450, 83, and 30 ft-c) were employed to give 16 different treatments. Deviation from the average temperature or light intensity was a maximum of 7% for each individual treatment. Materials tested for each treatment were 15 petri plates (six pieces/plate) each of tight callus from resistant and susceptible plants, two deep dishes each of seedlings of resistant and susceptible genotypes growing on agar-solidified Hoagland's solution, and three lima bean agar plates to which a drop of inoculum was placed inside a Tygon ring (4 mm internal diam).

Cultures were equilibrated in the cross-gradient room for 4 days, then inoculated with a single drop (10 zoospores/drop) of a zoospore suspension (race 0) placed in a small ring of Tygon tubing on top of each piece. Previous tests with the tubing indicated that it was not toxic to the zoospores or tissue cultures, but retained the drop on the tissue and away from the culture medium. After inoculation, each piece of tissue was examined visually each day and rated for tissue colonization according to the following system: 0 = no visible fungus; 1 = fungus on medium surrounding piece but no aerial mycelium; 2 = some aerial mycelium on piece but not covering the top of the piece; 3 = aerial mycelium completely covering the outside surface of the piece; and 4 = growth of aerial mycelium completely obscuring the tissue piece. As each plate contained six pieces of tissue, a maximum rating of 24 for any one plate was possible. A rating of 18/plate thus corresponded to 100% in the rating system used in Table 1.

RESULTS.—Addition of 3 to 33 spores/piece of "tight" callus tissue from a resistant or susceptible plant gave a differential rate of colonization (Fig. 2). These low numbers of zoospores were also sufficient to infect susceptible seedlings. Colonization in early experiments (Fig. 2, Table 1) was scored as positive when aerial mycelium was observed covering the callus piece. Race 0 of the fungus grew poorly on tissue cultures derived from resistant plants and vigorously on cultures derived from susceptible plants. Both lines of tissue culture were rapidly colonized by race 1; however, tissues from resistant plants were not colonized as rapidly as those from susceptible plants (Table 1).

When we inoculated tissue cultures by flooding the surface of the medium with race 0 or race 1 spore suspensions (1 or 2 ml containing ca. 1×10^4 spores/ml), all cultures were quickly and equally colonized by the fungus.

Colonization of different tissue forms.—Different morphological forms of tissue gave different interactions with the fungus (Table 1). In this test, a minimum of 20 pieces of each tissue type was inoculated with either three spores/piece of race 0 or 10 spores/piece of race 1. "Tight" and "budding" tissues from resistant plants supported less growth of race 0 than of race 1. However, "loose" tissues were colonized nearly equally by both races. Freshly isolated or 8-day-old pith tissues from resistant and susceptible plants showed no differential with race 0 or, in other experiments, with either race 0 or race 1. However, pith tissue grown for longer periods of time on a medium containing 11.5 μM IAA and 1 μM kinetin did show differential colonization. For example, in one test, 100% (24/24 in each case) of both fresh pith or 7-day cultured pith tissues from both resistant and susceptible plants became colonized 7 days after inoculation. Another set of pith tissues from the same isolation were grown for 29 days on identical culture medium. Eight days after

TABLE 1. Colonization of tobacco tissues of various morphological forms with races 0 and 1 of *Phytophthora parasitica* var. *nicotianae*

Tissue type	Race of fungus	% Tissue pieces colonized[a] 4 days R[b]	4 days S[b]	6 days R	6 days S	8 days R	8 days S	11 days R	11 days S	13 days R	13 days S
New pith[c]	0	28	45	28	45	52	75	89	90	93	100
8-Day pith[d]	0	25	26	30	33	55	67	75	100	85	100
Loose[e]	0	15	20	40	32	55	60	90	100	100	100
	1	10	10	30	30	48	60	72	90	100	100
Tight	0	0	4	0	24	8	64	20	96	45	100
	1	20	28	32	64	76	100	100	100	100	100
Budding	0	4	15	8	25	8	70	16	95	20	100
	1	8	55	32	75	64	100	92	100	100	100

[a] The tissue piece was considered to be colonized when aerial mycelium covered the callus piece.
[b] Tissue cultures derived from tobacco plants resistant (R) or susceptible (S) to race 0 of *P. parasitica* var. *nicotianae*.
[c] Pith freshly isolated and inoculated immediately after implanting on medium.
[d] Pith isolated and grown on medium for eight days prior to inoculation.
[e] "Loose", "tight", and "budding" tissues produced as described in text.

inoculation, these older tissues had become "tight" tissues and showed differential colonization (25% or 6/24 for tissues from resistant plant, and 100% or 24/24 for tissues from the susceptible plant; incubation temperature, 21 C).

Plants obtained from callus tissues exhibited the same specificity toward the fungus as did the clonal plant lines. Thus, plants from callus originally derived from the resistant plant and plants clonally derived from the resistant plant were resistant to race 0 but not to race 1 of the fungus. Plants from callus derived from the susceptible plant and plants clonally derived from this plant were susceptible to both races of the fungus. Rooted shoots from callus tissues or rooted cuttings from the clonal plants were grown in deep petri dishes (Pyrex 3250) on agar-solidified Hoagland's solution and inoculated by placing a drop of spore suspension (10 zoospores) immediately adjacent to the seedling. Larger plants grown in a growth chamber gave the same reaction as their parent plant when inoculated with mycelial fragments from lima bean agar plates.

Light and temperature in relation to fungal colonization.—The results shown in Fig. 2 were obtained in the dark at 18 C. Differential colonization, similar to that shown in Fig. 2, was obtained with a duplicate set of cultures at 22-23 C under fluorescent lights. Thus, at least at that temperature, light did not seem important for obtaining the differential response. In other preliminary tests, differential colonization was also obtained at 16, 20, and 24, but not at 28 C. Results from the first experiment conducted in the cross-gradient room of the Biotron indicated that little fungal colonization occurred at 15 C. Tissues from the resistant plant were colonized at a slower rate than tissues from the susceptible plant at 22 C, but differential colonization of these tissues was not obtained at 27 and 32 C. The four different light regimes had little effect on the results obtained at these temperatures except where colonization was decreased when severe drying of plates occurred with high light and temperature regimes.

In the second Biotron experiment, differential rates of colonization occurred at 20 and 24 C between tissues from the resistant or susceptible plant, but not at 27 and 31 C. Representative results (for 20 C, and 27 C at the low light regime, 30 ft-c) are shown in Fig. 3-A. In both tissues, colonization was increased by increasing temperature. However, at 20 C, the rate of colonization of tissues from the resistant plant was slower than that of the tissues

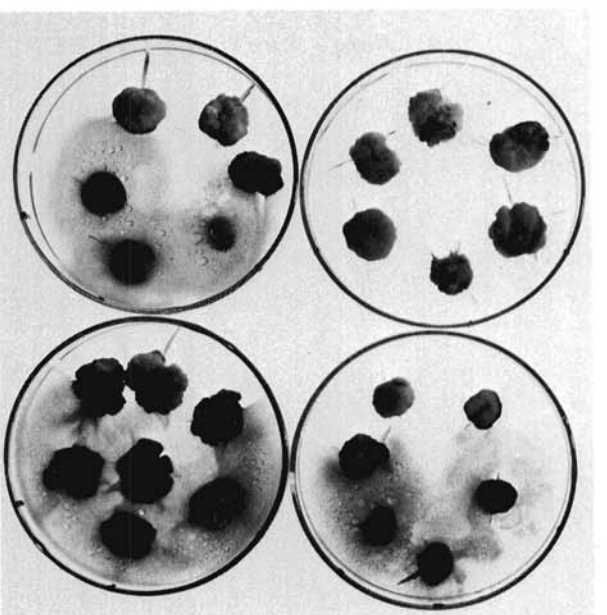

Fig. 2. Growth of *Phytophthora parasitica* var. *nicotianae* on "tight" tissue cultures derived from resistant and susceptible tobacco plants. Tissues and fungal lines were (upper left) "resistant" and race 0; (lower left) "susceptible" and race 0; (upper right) "resistant" and race 1; (lower right) "susceptible" and race 1. Only the three pieces on the right of the petri dish were inoculated (33 spores/piece). Cultures were incubated at 18 C after inoculation. Pictures were taken 7 days after inoculation.

from the susceptible plant. Again, light had little effect on the pathogen-tobacco tissue interaction except at 32 C and 1,100 ft-c, where severe drying of media occurred and, perhaps as a result, little colonization of plant tissues ensued. No fungal growth occurred on lima bean agar plates subjected to the two higher light intensities. At lower light intensities (30 and 90 ft-c), fungal growth was maximum at 24 and 27 C with slightly lower growth rates at 20 and 31 C.

None of the seedlings of resistant genotype had visible black shank symptoms. Except at 32 C, the seedlings of the susceptible genotype were severely affected and symptoms were seen as early as 2 days after inoculation.

Tissue colonization and growth substance regimes.—In initial experiments, when budding of the tissue cultures was induced, the resulting tissues exhibited differential colonization by the fungus (Table 1). If, however, 0.5 μM 2,4-D was substituted for the 11.5 μM IAA in the budding medium, no budding of the tissues resulted and the tissues derived from resistant plants were colonized more rapidly than were any other materials tested in 15 out of the 16 environmental regimes. The one exception was again at 32 C and 1,100 ft-c, where extreme drying and negligible colonization of the materials occurred.

A comparison of disease development at 20 and 27 C for these cultures from resistant and susceptible plants is given in Fig. 3-B. The rate of colonization was increased with increasing temperature for both sets of tissue cultures. However, the colonization rate was as great at 20 C for tissues from resistant plants as for the tissues from susceptible plants at 27 C.

DISCUSSION.—Under controlled conditions, tissue cultures from resistant plants show less rapid colonization by race 0 of *P. parasitica* var. *nicotianae* than do cultures from susceptible plants. As this suppressed colonization only occurs with the fungal race to which the intact plants are resistant and not with race 1, the race to which both plant clones are susceptible (9), we feel that the differential in colonization rates is due to the genetic expression of resistance in callus tissues. The similarity of temperature effects on callus tissues and intact plants (17) further supports this view.

Differential colonization of tissue cultures from resistant and susceptible plants was influenced by many factors. Temperature, inoculum concentration, morphological form of tissue cultures, hormonal content of the tissue culture medium, and the genotype of the callus tissue and pathogen all

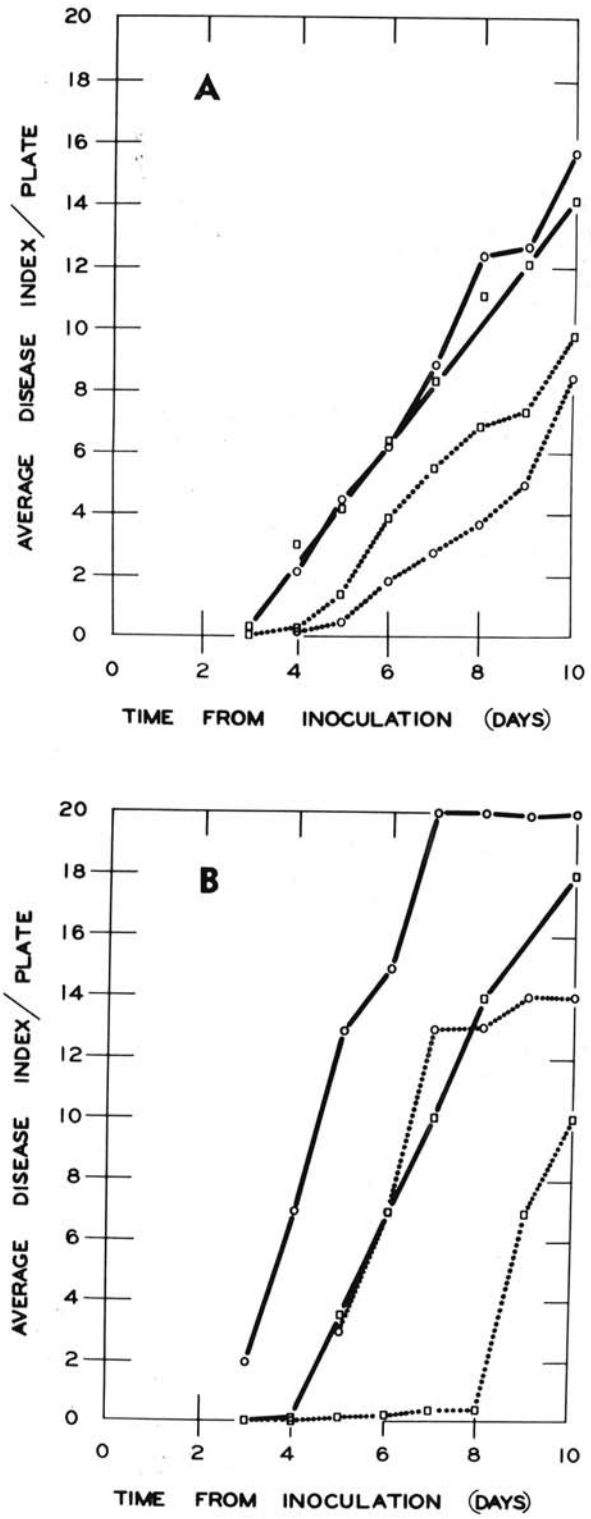

Fig. 3. Colonization of "resistant" (○) and "susceptible" (□) tobacco callus tissue by race 0 of *Phytophthora parasitica* var. *nicotianae* at 20 C and 30 ft-c (dotted line) and 27 C and 33 ft-c (solid line). The following tissue colonization rating was used: 0 = no visible fungus; 1 = fungus on growth medium but no aerial mycelium; 2 = some aerial mycelium; 3 = aerial mycelium completely covering the outside surface of the piece; 4 = aerial mycelium completely obscuring the tissue piece. Each plate contained six pieces. **A)** Tobacco callus grown on 1 μM 2,4-D, 1.0 μM kinetin. **B)** Tobacco callus tissues grown on Linsmaier and Skoog's medium containing 0.5 μM 2,4-D, 10 μM 6-(3-methyl-2-butenylamino)purine, 160 mg/l adenine sulfate, 100 mg/l L-tyrosine and 340 mg/l NaH_2PO_4.

appeared to affect the results obtained. The light regimes tried did not affect the interaction. The morphological form of the tissue was a critical factor in determining differential colonization. "Loose" callus and recently isolated pith tissues from resistant plants were colonized as readily as tissues from susceptible plants, whereas tight and budding tissues from resistant tobacco plants were colonized more slowly than corresponding tissues from susceptible plants. Perhaps more significantly, different colonization rates were obtained with tight tissue of similar morphology but grown on different hormonal regimes. This latest finding may provide the possibility of controlling resistance within a single line of tissues, thus allowing work with truly isogenic materials.

The tobacco tissue culture, *P. parasitica* var. *nicotianae*, system offers several distinct advantages for studying mechanisms of resistance: (i) The resistant mechanism is expressed in morphologically and physiologically similar cells. The complexity of whole plants and thus physiological differences can be avoided. (ii) Complication of the interaction by contaminating organisms is eliminated. (iii) The host material can be grown under defined, repeatable growth conditions. (iv) The resistance response of the tissue from the resistant genotype can be varied from highly susceptible to resistant by modification of tissue morphology (loose versus tight callus) or by modification of the hormonal regime in the growth media which support similar morphologies of callus tissue. (v) The resistance to race 0 appears to be a dominant monogenic factor (4), and thus biochemical expression of the resistant genotype might be easier to elucidate than with multigenic resistance.

Since many factors may influence the interaction of tissue cultures and pathogens, the rejection of such a system by Keen & Horsch (14), based on experiments with a single, rather high (1 mg/l or 4.65 μM) cytokinin concentration and a single, unspecified spore concentration, is of doubtful validity. In fact, our results would suggest that the conditions used by Keen & Horsch (14) would not lead to a differential.

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