A Dominant Gene Conferring Disease Resistance to Tobacco Plants is Expressed in Tissue Cultures

J. P. Helgeson, G. T. Haberlach, and C. D. Upper

Research Plant Physiologist, Research Assistant Plant Physiologist and Research Chemist, respectively, Agricultural Research Service, U.S. Department of Agriculture, Plant Disease Resistance Unit, Department of Plant Pathology, University of Wisconsin, Madison 53706.

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Mimeographed tables giving the results of comparisons of each individual plant and its callus are available from the authors upon request.

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ABSTRACT

Rooted cuttings and pith callus tissues from 185 tobacco plants were compared directly for their resistance to *Phytophthora parasitica* var. *nicotianae,* the causal agent of black shank of tobacco. Two plants, one homozygous resistant and the other homozygous susceptible, were used as parents. Plants tested were clonal cuttings from the resistant parent (resistant in 14 tests), clonal cuttings from the susceptible parent (susceptible in 14 tests), 13 progeny from the selfed susceptible parent (all susceptible), 19 progeny from the selfed resistant parent (all resistant), 42 F_1 progeny from a cross of the resistant and the susceptible parent (all resistant), 61 F_2 progeny from selfed F_1 individuals (45 resistant and 16 susceptible), 30 F_3 progeny from crosses of homozygous susceptible F_2 plants with heterozygous resistant F_2 plants (14 resistant and 16 susceptible), and 18 progeny from an outcross of an F_1 plant with susceptible *Nicotiana tabacum* 'Wisconsin 38' (11 resistant and 7 susceptible). In each case, plants that yielded resistant cuttings yielded only resistant callus, and plants that yielded susceptible cuttings yielded only susceptible callus. These results indicate that the single, dominant genetic factor which conferred disease resistance to intact tobacco plants was expressed in tobacco pith callus cultures. To our knowledge, our results constitute the first direct test of whether or not a gene for disease resistance in intact plants is also expressed in tissue cultures.

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Resistance to race 0, but not to race 1, of *Phytophthora parasitica* Dast. var. *nicotianae* (Breda de Haan) Tucker, the causal agent of black shank disease of tobacco, appears to be controlled in some cultivars by a single dominant genetic factor (3). This resistance factor was obtained from *Nicotiana plumbaginifolia* Viv. by crosses with *Nicotiana tabacum* L. (1, 2). Plants of *N. tabacum* type were then crossed with the cultivar Hicks. Five backcrosses to Hicks resulted in lines that are theoretically 97% isogenic. A selected plant was then selfed and progeny were carried through two more selfings to derive closely related, homozygous susceptible and homozygous resistant seed families (3).

Previously, we reported the development of a tissue culture system to study the interaction of this fungus and *Nicotiana tabacum* L. (5). Two plants, one homozygous resistant and the other homozygous susceptible, were used in these earlier studies. These plants have since been maintained clonally. Tissue cultures from the resistant plant showed less rapid colonization by race 0 of the fungus than did cultures from the susceptible plant. The results suggested that differential colonization rates were due to the genetic expression of resistance in callus tissues. Accordingly, we postulated that the resistance to race 0 of *P. parasitica* var. *nicotianae* in tobacco callus tissues is controlled by the same gene that governs resistance of the intact plant to the fungus.

If our hypothesis is correct, then in segregating progeny derived from crosses of the parental clones and selfs or backcrosses of the F_1 plants, each resistant plant should yield resistant callus and each susceptible plant should yield susceptible callus. Any deviation from this exact correspondence of plant and callus reaction would indicate that the same gene did not determine resistance under both conditions. We are now reporting the initial test of this hypothesis.

MATERIALS AND METHODS.—Direct comparisons of rooted cuttings and pith callus tissues derived from each test plant were used to test the hypothesis. Each test plant was grown to about 1 m in height. The terminal bud was then excised. Two weeks later, the shoots resulting from elongation of the axillary buds were removed and rooted. At the same time, pith was removed from the stem and induced to form callus. The decapitated mother plant, from which additional axillary buds would sprout, was returned to the growth chamber for crossings or for retests if necessary. Twenty eight days later, for simultaneous evaluation of resistance or susceptibility, rooted cuttings and the callus from each plant were inoculated on the same day with inoculum

derived from the same source.

Propagation of plants.—Plants were grown in a growth chamber (28 C, 12-hour photoperiod at 12,000 lx) in 15-cm diameter pots containing silica sand. Three times a week, the plants were watered with Hoagland's solution. Distilled water was used on the other days.

Propagation of cuttings.—The excised shoots were treated with "Rootone" and planted in aluminum pans containing water-saturated Perlite. The pans were then enclosed in a clear plastic bag and placed in a growth chamber (28 C, 12-hour photoperiod approximately 5,000 lx) for 2 weeks. The plastic bags were then removed and the cuttings were placed in a growth chamber at 28 C days, 20 C nights, and a 12-hour photoperiod

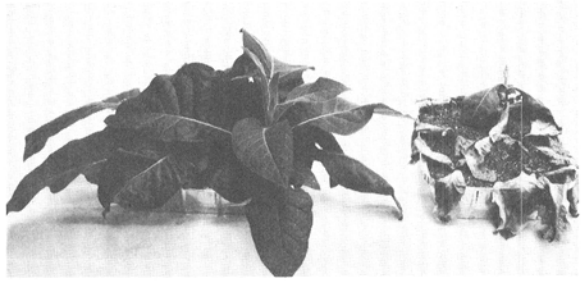

Fig. 1. Appearance of tobacco shoots from resistant (left) and susceptible (right) parent plants 14 days after inoculation with race 0 of *Phytophthora parasitica* var. *nicotianae*.

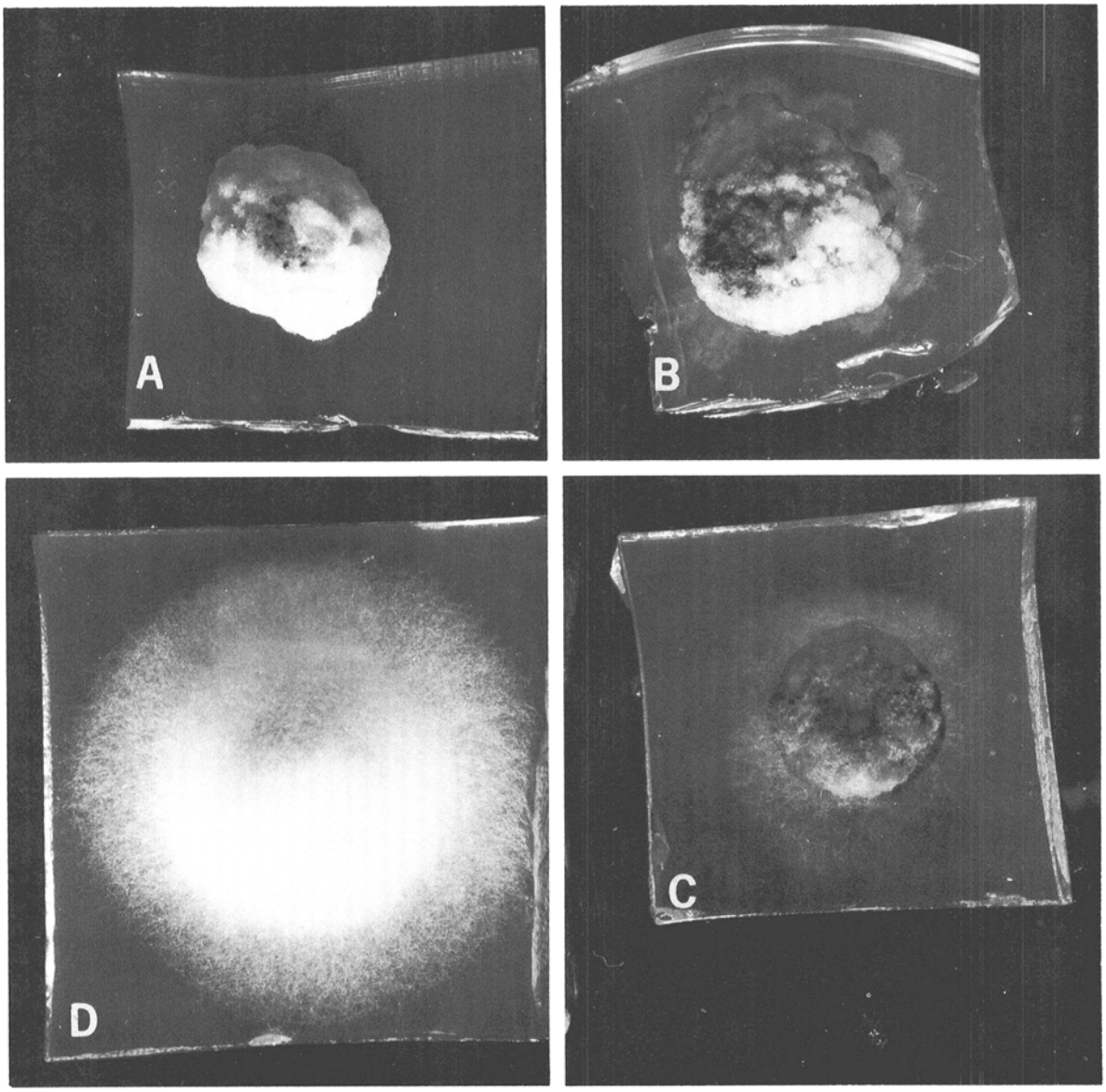

Fig. 2. Appearance of callus tissues from resistant (A and B) and susceptible (C and D) tobacco plants 7 days after inoculation. Disease ratings were: A = 0, B = 1, C = 2, D = 3. The rating system was: 0 = no visible fungus; 1 = fungus on the medium surrounding the piece but no visible aerial mycelium; 2 = visible aerial mycelium that did not cover the top of the piece; 3 = aerial mycelium completely covering the outside surface of the piece.

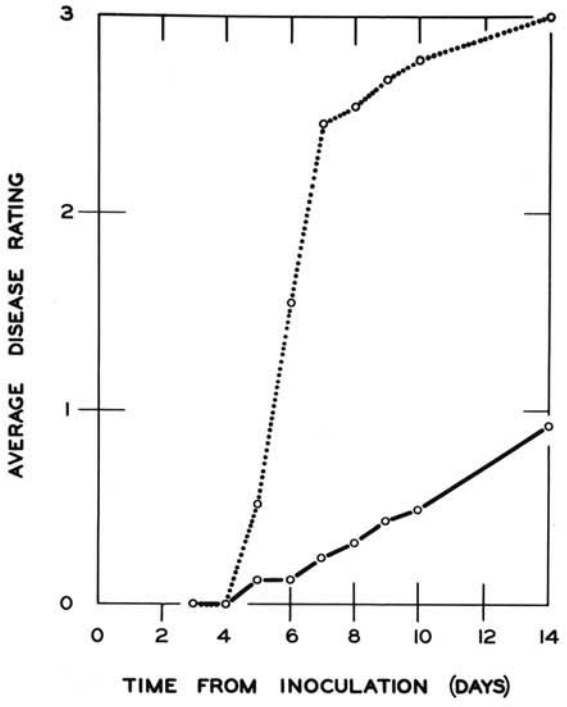

Fig. 3. Disease ratings of tissues from susceptible (dotted line) and resistant (solid line) parent tobacco plants at various times after inoculation with race 0 of *Phytophthora parasitica* var. *nicotianae*. Each point represents the average of results from at least eight experiments. The rating system was: 0 = no visible fungus; 1 = fungus on the medium surrounding the piece but no visible aerial mycelium; 2 = visible aerial mycelium that did not cover the top of the piece; 3 = aerial mycelium completely covering the outside surface of the piece.

(approximately 5,000 lx) for an additional 2 weeks. This procedure usually yielded two well-rooted cuttings from each test plant.

Growth of pith callus.—Immediately after removal of the lateral shoots, the main stem of the plant was cut off 10 cm above the surface of the sand in the pot. This stem was cut into 4- to 6-cm sections. The sections were surface-sterilized (95% ethanol or 1% sodium hypochlorite) and pith tissues were removed with a sterile No. 1 or No. 2 cork borer. Pith explants were cultured in petri plates (100 × 20 mm) on Linsmaier and Skoog's medium (9) containing 11.5 μM indole-3-acetic acid (IAA) and 1 μM kinetin. Tissues were grown in a dark incubator for 27 days at 28 C and for one additional day at 20 C in the dark prior to inoculation. Usually, four to six plates, each containing six pieces of callus, were obtained from each test plant.

Inoculum preparation and inoculation procedures.—Zoospores of *P. parasitica* var. *nicotianae* were obtained by the method of Gooding and Lucas (4) from stock cultures maintained on oatmeal agar. Spore counts were made with a hemacytometer after which the suspensions were diluted with water to give the desired concentration of spores. For inoculation of tissue cultures, a small (5-mm ID by 2-mm high) ring of sterile Tygon tubing was placed on top of each piece of callus and one drop of inoculum containing about 10 zoospores was placed inside each ring. Inoculated callus tissues were incubated in the dark at 20 C. Unless otherwise noted, only race 0 of the fungus was used for inoculations. Plants were inoculated by flooding the rooting medium with a homogenate of the mycelial colony from which spores had been obtained to inoculate the tissue cultures (the mat from one 100 × 15-mm petri plate was blended with 200 ml water in a Waring Blendor). The inoculated cuttings were then returned to the growth chamber (28 C days, 20 C nights, 12-hour photoperiod 5,000 lx) and incubated for 14 days.

Rating procedures.—Typically, the disease responses of rooted cuttings and callus tissues from up to 16 test plants were tested in each experiment. Cuttings from the original clonal resistant and susceptible parents were included with test plants in each aluminum pan. Also, pith callus cultures from stems of these parents were used as controls in each experiment.

Resistance or susceptibility of rooted cuttings was determined by whether or not the cuttings were killed within 14 days after inoculation (Fig. 1). Two separate ratings of each petri plate of tissue, one numerical and the other subjective, were used to judge the susceptibility or resistance reaction of pith callus tissues. Fungal growth on each piece of tissue was rated numerically on days 3, 4, 5, 6, 7, 10 and 14 after inoculation. The rating system was: 0 = no visible fungus (Fig. 2A); 1 = fungus on the medium

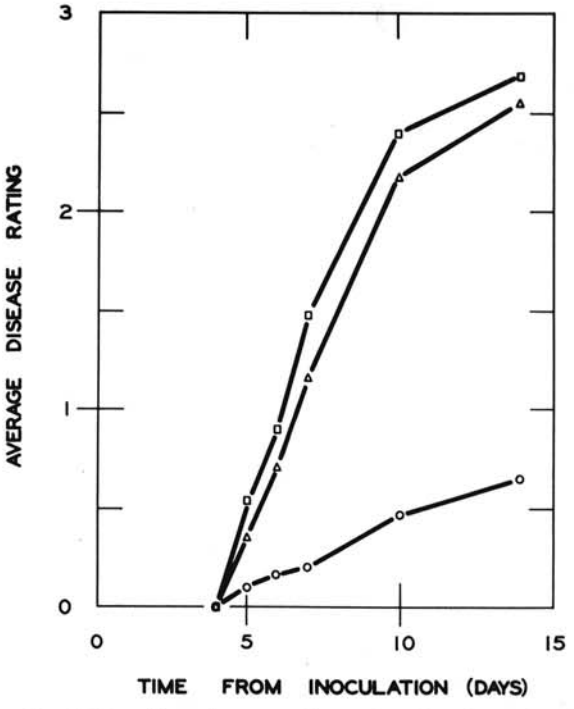

Fig. 4. Numerical disease ratings of callus from clonal controls (susceptible = □, resistant = o) and plant 25-27 (△). Data are an average from two experiments. The rating system was: 0 = no visible fungus; 1 = fungus on the medium surrounding the piece but no visible aerial mycelium; 2 = visible aerial mycelium that did not cover the top of the piece; 3 = aerial mycelium completely covering the outside surface of the piece.

TABLE 1. Summary of plant and callus tissue reactions to race 0 of *Phytophthora parasitica* var. *nicotianae*

Test plants	Plants (no.)	Callus numerical rating (av.)	Susceptible plants (no.)
Resistant parent	1[a]	0.33 ± 0.07[b]	0[c]
Susceptible parent	1[a]	1.93 ± 0.09	1[c]
Selfed resistant parent	19	0.22 ± 0.05	0
Selfed susceptible parent	13	2.03 ± 0.08	13
F_1 (resistant × susceptible)[d]	21	0.10 ± 0.02	0
F_1 (resistant × susceptible)	21	0.15 ± 0.03	0
F_2 Susceptible	16	1.94 ± 0.12	16
F_2 Resistant	45	0.35 ± 0.04	0
F_3 Susceptible[e]	16	1.96 ± 0.11	16
F_3 Resistant[e]	14	0.48 ± 0.08	0
Backcross susceptible[f]	7	2.30 ± 0.09	7
Backcross resistant[f]	11	0.23 ± 0.09	0

[a]Clonal parents were included in each test. The results given are for 14 separate tests on these two parents.

[b]The rating system was: 0 = no visible fungus; 1 = fungus on the medium surrounding the piece, but no visible aerial mycelium; 2 = visible aerial mycelium that did not cover the top of the piece; 3 = aerial mycelium completely covering the outside surface of the piece.

[c]A total of 128 of 130 resistant plant cuttings survived; 119 of 122 cuttings from the susceptible parent were killed.

[d]The F_2 lines were derived from selfed individuals from this cross.

[e]The F_3 lines were derived from crosses of susceptible F_2 plants with heterozygous resistant F_2 siblings (as determined by progeny testing).

[f]Progeny from a cross of an F_1 plant (1-4) with *Nicotiana tabacum* 'Wisconsin 38.'

surrounding the piece, but no visible aerial mycelium (Fig. 2B); 2 = visible aerial mycelium that did not cover the top of the piece (Fig. 2C); 3 = aerial mycelium completely covering the outside surface of the piece (Fig. 2D).

Subjective ratings of "susceptible" or "resistant" were also made for callus cultures. In general, callus tissues from the resistant clone, if colonized at all, resembled the piece shown in Fig. 2B. In contrast, the first visible fungus growth on callus tissues from the susceptible clone usually resembled that shown in Fig. 2C and colonization usually progressed quite rapidly to the level seen in Fig. 2D. Thus, tissues could be rated "R" or "S" on the basis of the appearance and extent of growth of the fungus. The subjective ratings were done at the termination of the experiment and sometimes also on the 7th day after inoculation.

As a precaution against bias in rating of callus tissues, all petri plates were renumbered randomly by a person who took no part in the disease rating. The inoculations and ratings were done subsequently by a person who had no knowledge of the source of the tissue. In each experiment, both numerical and subjective ratings were recorded for each plate. On the 14th day, when all numerical and subjective ratings had been recorded, the key for the relation between plate number and the plant from which the tissue was derived was given to the person doing the rating. All plates from each plant were then compared and the ratings summarized.

RESULTS AND DISCUSSION.—*Tests of clonal parents.*—Numerical ratings obtained with callus from resistant and susceptible parents are shown in Fig. 3. Each point represents the average of at least eight different experiments. At about 7 days after inoculation, the difference between numerical disease ratings of tissues from the resistant and susceptible plants appeared to be maximal. Tissues from the resistant clone showed an average 7-day numerical rating of 0.33 (range 0.01-0.90) whereas tissues from the susceptible clone showed an average numerical rating of 1.93 (range 1.29-2.39). Variation between experiments appeared to be small enough that the numerical rating for colonization could be used as one means of judging the resistance or susceptibility of the callus tissues. In each case, callus tissues from susceptible parent plants were given subjective ratings of "susceptible" and callus tissues from resistant parents were rated "resistant." Only two small cuttings of 130 from the resistant clone were killed. In contrast, 119 of 122 cuttings from the susceptible clone were killed. Because the decapitated mother plant was retained it was possible to test additional cuttings if necessary.

Tests of selfed parent plants.—Since one of our parent plants was homozygous susceptible, and the other was homozygous resistant (J. L. Apple, *private communication*), selfing resistant parents should yield only resistant progeny whereas selfing susceptible parents should yield only susceptible progeny. Of the 19 progeny from the resistant plant tested, all were resistant both as plants and in callus culture; all 13 progeny of the susceptible plant were susceptible as plants and as callus.

Tests of F_1 progeny.—All F_1 progeny from crosses of the homozygous resistant parent and the homozygous susceptible parent should be resistant. Reciprocal crosses were made to test this point. A total of 21 progeny from each cross was tested. As expected, the crosses yielded only resistant F_1 plants which, in turn, yielded only resistant callus tissue. Since we detected little difference between the two lots with different parent pollen donors, it appeared that cytoplasmic inheritance was not important in determining resistance, at least when the dominant factor for resistance is present.

Tests of F_2 progeny.—Our hypothesis requires that the resistance of callus tissues and plants should segregate

together in the F_2 generation. A total of 61 plants, derived from three selfed F_1 individuals, was tested. Sixteen susceptible and 45 resistant plants were obtained (expected ratio for 1:3 segregation, 15:46). Each of the 45 resistant plants yielded tissue that was judged resistant by the subjective rating procedure. The numerical ratings of callus from only three plants (ratings of 1.03, 1.07 and 1.23) were outside the range (0.01 to 0.90) obtained with callus from the original resistant plant in 14 experiments. When callus from these three plants was tested a second time, however, all of the subjective ratings were again "resistant", and the numerical ratings of 0.20, 0.83 and 0.57 indicated that callus derived from these plants is clearly resistant. Thus, callus from each of the 45 resistant plants was rated resistant by both procedures.

Of the 16 susceptible F_2 plants, callus from 14 was "susceptible" by both the subjective and numerical rating systems. Callus from the remaining two had a low numerical rating (1.00). Also, although most plates were rated "susceptible," some plates were borderline, and one plate out of six from each plant was actually rated "resistant" in the blind test. Callus from these two plants was retested.

This time, all callus tissues from both plants were rated "susceptible" subjectively. In addition, the numerical rating of callus tissue from one plant was 2.34, clearly in the susceptible range, while that of callus from the other plant was still low (1.29 compared with 1.42 for the susceptible control) but higher than the 0.02 for the resistant tissue control. When the time course of the fungus growth on callus from this second plant was examined, it was typical of that for susceptible plants, although the major "explosion" of aerial mycelium from the tissues was delayed by about 1 day (Fig. 4). Thus, we concluded that callus tissues from each of the 61 F_2 plants has the same overall disease ratings as the plant from which it was derived.

Test of crosses to susceptible parents.—Only 25 percent of the F_2 plants obtained by the selfing of heterozygous F_1 plants were susceptible. Our results with these F_2 plants suggested that susceptible plants in a segregating population might be the most likely source of the individual that would conclusively disprove our hypothesis. To increase the proportion of susceptible individuals, we used crosses between known heterozygous plants and susceptible plants to obtain a 1:1 segregation (theoretical) of resistant to susceptible individuals. Two crosses were between susceptible and heterozygous resistant (as determined by seedling progeny testing) F_2 siblings. In all, 30 plants were tested, 16 were susceptible, and 14 were resistant (expected ratio, 15:15). Each of the 16 susceptible plants yielded susceptible callus by both the numerical and subjective rating systems, and callus from each of the 14 resistant plants was rated resistant by the subjective procedure. Callus from 13 of the F_3 resistant plants was clearly resistant on the basis of numerical ratings procedure; but tissue cultures from one resistant plant gave a numerical rating of 1.11, somewhat higher than the normal range. However, two additional tests with this plant have given results similar to the resistant control.

Susceptibility to race 1 of Phytophthora parasitica var. nicotianae.—The dominant factor for resistance to race 0 of *P. parasitica* var. *nicotianae* does not impart resistance to race 1 of this fungus (2). Thus, if our hypothesis is correct, rooted cuttings and callus tissues that are resistant to race 0 should be susceptible to race 1.

A cross of the susceptible cultivar Wisconsin 38 with a resistant F_1 plant provided material for this test. Of the 18 progeny tested, 11 were resistant, and seven were susceptible to race 0 (expected ratio, 9:9). As expected, each resistant plant yielded resistant callus tissues, whereas each susceptible plant yielded susceptible callus tissues when inoculated with race 0. Fourteen of these plants were then retested against both race 1 and race 0. All rooted cuttings and callus tissues were susceptible to race 1. With race 0, the seven plants which were resistant to race 0 in the previous test again yielded resistant callus and cuttings whereas the seven plants which were susceptible to race 0 in the previous test again yielded susceptible callus and cuttings. Thus, the resistance we have observed in callus appears to be specific for race 0 of this fungus, as with the plants from which the callus is derived.

A tissue culture system could avoid many of the physiological, morphological, and environmental problems of disease resistance studies with intact plants and rapidly senescing excised plant parts. However, if the results with tissue cultures are to be extended to intact plants, it should be demonstrated that the same gene conditions resistance in both tissue cultures and intact plants. Our results, summarized in Table 1, indicate that this is probably the case with our system. In all, 53 susceptible and 132 resistant plants have been tested. Callus from each susceptible plant has been rated "susceptible" and callus from each resistant plant has been rated "resistant." Thus, we have been unable to find any evidence for independent segregation of the factor that conditions resistance in callus from the factor that determines resistance in intact plants.

Several attempts have been made to use tissue culture systems for studying host-pathogen interactions (7, 8, 10). However, these studies have had only very limited success (6). To our knowledge, our results constitute the first direct test of whether or not a gene for disease resistance in intact plants is also expressed in tissue cultures. Since the same gene appears to be expressed in both plants and callus, the tissue culture system would appear to be a useful, valid, system for the study of molecular events associated with the expression of the resistance gene.

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