Persistence of Induced Systemic Resistance to Blue Mold in Tobacco Plants Derived Via Tissue Culture

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ABSTRACT

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Injecting sporangia of *Peronospora tabacina*, the blue mold pathogen, into tobacco stem tissue external to xylem systemically protected foliage against blue mold. Plants derived via tissue culture from leaves and leaf midribs of parents injected with *P. tabacina* were also systemically protected against the disease. Protection was expressed as a reduction in sporulation on younger plants, whereas a reduction of symptom severity and sporulation were observed on older plants. Data were obtained for greenhouse and field experiments. Immunization was not transferred via seed of stem injected plants nor via seed from plants derived from tissue culture of stem-injected plants. Induced resistance increases with age of tissue culture-derived plants and, in this respect, resembles the increase in resistance to blue mold of nonstem-injected resistant and susceptible cultivars.

A reduction in the severity of blue mold was observed by Pont on naturally infected field-grown plants challenged with *Peronospora tabacina* Adam (8). Greenhouse and field experiments demonstrated elicitation of 90–95% protection against blue mold when plants were stem-injected with sporangia of *P. tabacina* or when sporangia were applied to the soil surface around stems of burley tobacco plants (1). A modified technique for stem inoculation with sporangia of *P. tabacina* increased the growth and marketable yield of induced plants and elicited > 95% protection, which lasted throughout the season (4,11,12,14). It was suggested that a graft-transmissible protection factor ("signal") was responsible for conditioning protection and fully grown plants derived from an immunized tip grafted on a nonimmunized rootstock developed into protected plants (12,13). This suggested the possibility of a permanent change in the genome or expression of the genome in plants stem-injected with *P. tabacina*. This study was conducted to investigate the transfer of systemic induced resistance via tissue culture and seeds from parents stem-injected with *P. tabacina* as well as seeds of tissue culture-derived plants. Preliminary results have been reported (13).

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MATERIALS AND METHODS

Plants. Burley tobacco plants (*Nicotiana tabacum* L. 'Ky 14') were grown in the greenhouse (20–26 C in fall and winter, 20–33 C in spring and summer, daylight supplemented with 16 hr of fluorescent and incandescent light) in 21 pots containing Pro-Mix Bx (Premier Peat Corp. Marketing, New York, NY). Pots were watered five times a week to saturation with a 0.1% 20:20:20 (N:P:K) fertilizer (Peters Fertilizer) solution. To test transfer of systemic protection via seeds of immunized plants, seeds from three different generations were collected from plants stem-injected with *P. tabacina* or water. Each generation was injected three times at weekly intervals, and tests were conducted with plants obtained from the seeds of third generation plants. Seeds were also obtained from plants regenerated via tissue culture. Seeds were collected either before or after challenge of regenerants, and plants from the seed were grown in the greenhouse. In the field experiments, plants were planted in a randomized complete block design on 2 August and 21 August 1985 in Fayette County, KY. Each planting comprised approximately 100 plants derived via tissue culture from parents injected with *P. tabacina*, 60 plants from parents injected with water, and 100 plants grown from seed of uninjected plants. The experiment was arranged into three blocks, and tissue culture-derived plants were obtained from three

different parents per treatment per planting.

Fungus and inoculations. Isolate 82 of *P. tabacina* was used for stem inoculations. The isolate was collected at Spindletop farm, Lexington, KY, in 1982. The fungus was maintained on 7–12-wk-old Ky 14 plants grown in growth chambers at 20 C, and leaves with sporulating lesions were stored in a freezer until used to prepare spore suspensions for stem injections. Isolate 79 of *P. tabacina* was used for challenge inoculations. The isolate was collected from a field near Georgetown, KY, in 1979 and was maintained as described above. Fresh sporangia of isolate 79 were used for challenge. Sporangia of both isolates elicited systemic resistance, and both were effective for challenge inoculations. The procedure followed was determined by convenience for the investigators. Sporangia were brushed from leaves with an artist's paint brush into distilled water, collected on a 3-μm Millipore filter, washed with distilled water, and resuspended in distilled water. The concentration of sporangia was determined with the aid of a hemacytometer. Approximately 1 ml of inoculum (5×10^5 sporangia per milliliter) of *P. tabacina* was injected into stems 5–10 cm above the soil line of 8–10-wk-old tobacco plants. The inoculum was injected with a hypodermic syringe into tissue external to the xylem as described earlier (11). Control plants were injected with distilled water.

Twenty-one days after stem injection with sporangia or water, the upper surfaces of the leaves of plants were uniformly sprayed with sporangial suspensions of *P. tabacina* (5×10^4 sporangia per milliliter) to determine the resistance of plants to be used as a source of regenerants. The same inoculum concentration was used to challenge plants grown from seeds of plants stem-injected with *P. tabacina* or water. Greenhouse-grown plants derived via tissue culture were challenged by using an inoculum concentration of 10^4 sporangia per milliliter. Different concentrations of sporangial suspensions of *P. tabacina* were used to challenge plants grown from seeds of tissue culture-derived plants according to the age of the plants as described in Table 1. After spraying sporangial suspensions on leaves, plants were covered with brown plastic bags whose inner surfaces were moistened and kept in the dark at 18 C for 18 hr. Plastic bags were then removed, and plants were kept in growth rooms (18–22 C, illuminated for 15 hr per day with cool-white lights supplemented with incandescent lights, 75 $\mu E/m^2/sec$). Plants were inoculated in the field by placing plants with sporulating lesions uniformly throughout the transplants at weekly intervals for 3 wk starting 21 September.

Tissue culture. Leaves and midribs from the growing tips of control plants and plants in which systemic resistance to blue mold had been induced were used to develop regenerants. The leaf and leaf midrib sections were rinsed for 30 sec with 70% ethanol, 10–15 min with a solution of 0.5% sodium hypochlorite containing one or two drops of Tween 20 per liter, and twice for 3 min with sterile distilled water. The leaves were cut into about 0.5-cm-diameter sections. Leaf and leaf midrib sections were placed in petri plates containing the Murashige-Skoog nutrient agar medium (3). Within 2 wk callus growth was observed, and plantlets developed approximately 4 wk later. Plantlets were cut and placed into tubes or jars containing a rooting medium (modified Murashige-Skoog nutrient agar containing 3-amino pyridine). Tissue sections and

TABLE 1. Symptoms of blue mold on tobacco plants derived from seeds of tissue culture-derived plants

		2-leaf stage[a]		3–5 leaf stage[b]		7–9 leaf stage[c]	
Parent[d]	Condition[e]	Disease severity[f]	Sporulation[g]	Disease severity[f]	Sporulation[g]	Disease severity[f]	Sporulation[g]
IMM 1	Challenged	0.8 ± 0.3	$2.2 \pm 2.2 \times 10^5$	3.9 ± 0.3	$2.7 \pm 1.9 \times 10^6$	3.7 ± 0.6	$1.8 \pm 0.7 \times 10^6$
IMM 2	Challenged	1.7 ± 0.6	$8.0 \pm 4.1 \times 10^5$	3.8 ± 0.4	$2.9 \pm 1.8 \times 10^6$	4.1 ± 0.3	$0.8 \pm 0.8 \times 10^6$
IMM 3	Challenged	1.3 ± 0.4	$5.0 \pm 2.0 \times 10^5$	4.1 ± 0.5	$4.8 \pm 2.6 \times 10^6$	3.4 ± 0.3	$1.3 \pm 1.1 \times 10^6$
CONT 1	Challenged	1.9 ± 0.6	$2.2 \pm 2.2 \times 10^5$	4.5 ± 0.2	$6.0 \pm 2.6 \times 10^6$	4.1 ± 0.7	$1.2 \pm 0.9 \times 10^6$
CONT 2	Challenged	1.9 ± 0.4	$8.1 \pm 4.2 \times 10^5$	4.1 ± 0.3	$4.8 \pm 2.3 \times 10^6$	4.0 ± 0.5	$1.2 \pm 0.9 \times 10^6$
CONT 3	Challenged	1.1 ± 0.3	$5.1 \pm 2.3 \times 10^5$	4.4 ± 0.4	$6.3 \pm 5.6 \times 10^6$	3.6 ± 1.0	$1.5 \pm 1.4 \times 10^6$
IMM 1	Unchallenged	1.1 ± 0.3	$4.1 \pm 2.7 \times 10^5$	3.9 ± 0.4	$4.8 \pm 3.2 \times 10^6$	3.2 ± 0.6	$0.9 \pm 0.9 \times 10^6$
IMM 2	Unchallenged	1.5 ± 0.4	$5.5 \pm 3.0 \times 10^5$	4.1 ± 0.1	$2.7 \pm 2.1 \times 10^6$	3.8 ± 0.4	$0.7 \pm 0.4 \times 10^6$
IMM 3	Unchallenged	1.3 ± 0.4	$8.8 \pm 3.4 \times 10^5$	3.9 ± 0.5	$4.2 \pm 2.9 \times 10^6$	3.3 ± 0.3	$1.5 \pm 0.8 \times 10^6$
CONT 1	Unchallenged	1.1 ± 0.5	$4.3 \pm 2.2 \times 10^5$	4.4 ± 0.3	$4.7 \pm 3.8 \times 10^6$	3.8 ± 0.5	$1.5 \pm 1.2 \times 10^6$
CONT 2	Unchallenged	1.1 ± 0.4	$5.4 \pm 2.5 \times 10^5$	3.9 ± 0.4	$4.7 \pm 3.0 \times 10^6$	3.6 ± 0.6	$0.6 \pm 0.3 \times 10^6$
CONT 3	Unchallenged	1.5 ± 0.3	$7.7 \pm 2.8 \times 10^5$	4.1 ± 0.5	$3.7 \pm 0.6 \times 10^6$	4.7 ± 0.3	$1.1 \pm 1.0 \times 10^6$
CONT	Nontissue culture	1.3 ± 0.3	$9.7 \pm 1.9 \times 10^5$	3.9 ± 0.2	$4.6 \pm 1.5 \times 10^6$	4.1 ± 0.7	$2.6 \pm 0.5 \times 10^6$

[a] Eight plants were used per treatment; plants were challenged by spraying with a suspension of 7×10^3 sporangia per milliliter.
[b] Six plants were used per treatment; plants were challenged by spraying with a suspension of 10^4 sporangia per milliliter.
[c] Four plants were used per treatment; plants were challenged by spraying with a suspension of 5×10^4 sporangia per milliliter.
[d] IMM indicates plants derived via tissue culture from parents stem-injected with *Peronospora tabacina*; CONT indicates plants derived via tissue culture from patents stem-injected with water.
[e] Parent plants were either challenged with *P. tabacina* and kept in the growth room or unchallenged and kept in the greenhouse until seeds were collected.
[f] Disease severity was determined by rating the area with blue mold symptoms according to a 0–5 scale (Fig. 1). Data are the means ± S.D. No significant differences were found among the treatments.
[g] Sporulation on two most infected leaves/plant (means ± S.D.). No significant differences were found among the treatments.

TABLE 2. Symptoms of blue mold on tobacco plants grown from seed or derived via tissue culture from parents stem-injected with sporangia of *Peronospora tabacina* or water in greenhouse tests

	Leaf stage of challenged plants[a]					
	2-leaf stage		3–4-leaf stage		7–8-leaf stage	
Source of plants	Disease rating[b]	Sporulation/ plant	Disease rating[b]	Sporulation/ plant	Disease rating[b]	Sporulation[c]
Leaves from parents stem-injected with *Peronospora tabacina*	1.6 A	2×10^5 B[d]	2.0 B	7×10^5 C	0.8 B	0.6×10^5 B
Leaves from parents stem-injected with water	2.1 A	8×10^5 A	2.9 A	34×10^5 B	2.8 A	25.4×10^5 A
Seed	1.7 A	14×10^5 A	3.2 A	59×10^5 A	...	...

[a] Plants challenged by spraying with a suspension of 10^4 sporangia per milliliter.
[b] Disease rating: 0 = no evidence of disease; 1 = 1–10% of leaf area with lesions; 2 = 11–40%; 3 = 41–75%; 4 = 76–100%. Both leaves were rated on plants in the two-leaf stage, and the three most severely diseased leaves were rated on plants in the three to four- and seven to eight-leaf stage.
[c] Sporulation/two most infected leaves/plant.
[d] Different letters indicate data significantly different ($P < 0.05$) by Duncan-Waller *K*-ratio *t* tests.

plantlets were kept in a culture chamber maintained at 26 C, illuminated 16 hr/day at about 45 $\mu E/m^2/sec$ from cool-white fluorescent lamps. Petri plates were sealed with Parafilm to minimize drying. Plantlets formed roots in approximately 1–2 wk. After adequate root development, they were placed in ProMix-containing peat pots. Plants were kept in moist chambers for 1 wk, then grown in a greenhouse as described (11). At least four plants were used per treatment per experiment (total plants tested: immunized leaf, 93 plants; immunized midrib, 46 plants; control leaf, 90 plants; and control midrib, 45 plants), and the experiment was repeated 11 times. Tissue culture-derived plants were obtained from 11 parents injected with fungus and from 11 parents injected with water. Because some variation was evidenced among the tissue culture-derived plants, only vigorous, uniform plants were chosen for the experiment.

Disease determination and statistical analysis. Disease was determined 7 days after challenge inoculations, unless otherwise indicated, in the greenhouse experiments and during the period of 15–17 October in the field. Disease was determined by rating (0–4 or 0–5 scale, see Table 2, footnote b, and Fig. 1), the percent leaf area with lesions on three or four leaves (usually starting with the first fully opened leaf below the bud), counting the number of infected leaves per plant, and counting the number of lesions per leaf and plant. To determine differences in time of symptom appearance, disease reactions in some experiments were determined each day after the first appearance of chlorotic lesions. To quantitate sporulation, usually the two most infected leaves of a plant from greenhouse plants and several highly infected leaves from field-grown plants were collected and kept in 100% humidity at 18 C in the dark for 18 hr. Spores were then brushed from the leaves into a known amount of distilled H_2O and counted with the aid of a hemacytometer. Plants in the two- and three- to four-leaf stage were tested by the double blind procedure. Data were analysed by using the Duncan-Waller *K*-ratio *t* test. Analyses of field experiments were performed on mean values from each plot.

RESULTS

The average disease rating of the parent plants injected with sporangia of *P. tabacina* was 0.4, whereas the average disease rating of plants injected with water was 3.7. This marked reduction in foliar symptoms on parent plants injected with *P. tabacina* was not observed on young (two-leaf stage) tissue culture-derived plants from parents injected with *P. tabacina* (Table 2). However, sporulation on these plants was significantly reduced when compared with tissue culture-derived plants from parents injected with water or plants derived from seed. At later stages of plant development, tissue culture-derived plants from parents injected with *P. tabacina* had significantly fewer foliar symptoms and sporulation than controls in greenhouse and field tests (Tables 2 and 3; Fig. 1). There were no significant differences between the source of tissue (leaf or midrib) or for parents (field tests) for tissue culture-derived plants, and in most experiments, therefore, only leaf tissue was used as a plantlet source. In three of 11 experiments, the appearance of disease was delayed on tissue culture-derived plants from parents injected with water as compared with plants derived from seed. However, ultimate severity of disease was generally not markedly different on plants from both sources in greenhouse tests. There was a significant reduction in number of lesions per plant on plants derived from tissue culture of control plants compared with seed-grown plants in the field tests; however, the reduction of disease was consistently and significantly greatest in regenerants from immunized parents. There were no significant differences between the plants derived from seeds of immunized and control plants (Table 4) nor between the plants derived from seed of regenerants derived from both sources (Table 1).

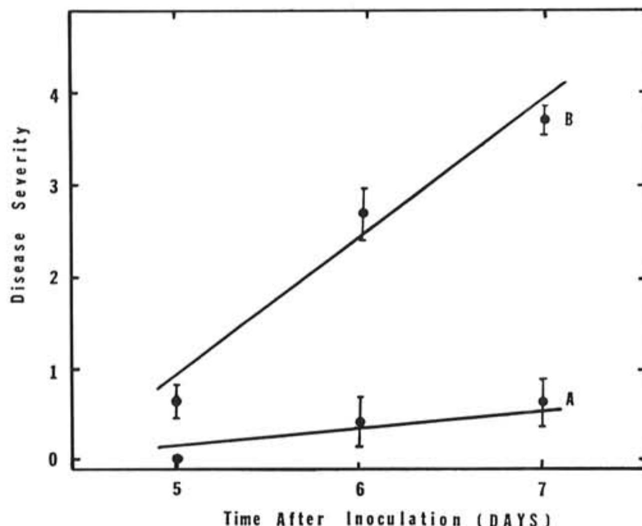

Fig. 1. Development of blue mold on tobacco plants derived via tissue culture from leaves of plants stem-injected with *Peronospora tabacina* (A) or water (B). Plants were at the 10–11-leaf stage and challenged with a sporangial suspension (10^4 sporangia per milliliter). Disease severity was determined according to a 0–5 rating scale where 0–4 was determined according to a scale described at Table 2 and leaves having 4 rating with severe necrosis were rated as 5. Bars indicate standard errors. Lines indicate regression lines and closed circles indicate means. Quadratic regression analyses indicated that rates of increase of symptoms were linear, and Student's *t* tests indicated that the rate of increase of symptoms derived from linear regression was significantly greater (< 0.01) for plants derived from parents injected with water compared with plants derived from parents injected with *P. tabacina*.

DISCUSSION

Results of these experiments indicated that systemic resistance to blue mold was transferred to regenerants via tissue culture. The resistance was expressed as a reduction in sporulation on younger plants, whereas a reduction in the area of leaves covered by lesions as well as sporulation was evident on older plants. This may be due to an increase in the resistance of plants with age (9), for even cultivars considered resistant to blue mold are highly susceptible in early seedling stages (*personal communication*, R. Rufty; *unpublished data*). Regenerants from immunized plants in the seven- to eight- and 10–11-leaf stage and those in the field experiments (Tables 2 and 3; Fig. 1) were as well protected against blue mold as plants stem-injected with *P. tabacina* observed in this and numerous other experiments (see Results; 1,4,11,12,14). The differences reported for sporulation, which were based on the two most heavily diseased leaves, are very conservative. Plants from protected parents have fewer heavily diseased leaves than plants from parents that were not protected. In addition, necrotization of diseased tissue occurs sooner on nonprotected plants and this

TABLE 3. Symptoms of blue mold on tobacco plants grown from seed or derived via tissue culture from parents stem-injected with sporangia of *Peronospora tabacina* or water in field tests[a]

Source of plants	Lesions/plant[b]	Sporulation/lesion[c]	Sporulation potential/plant[d]
Leaves from parents stem-injected with *Peronospora tabacina*	0.7 (0.3) C[e]	5.7×10^4 B	4.2×10^4 B
Leaves from parents stem-injected with water	15.5 (9.1) B	78.0×10^4 A	1.2×10^7 A
Seed	23.1 (18.4) A	119.0×10^4 A	2.7×107 A

[a] Challenge inoculum obtained from plants with sporulating lesions that were transplanted to the field (see text).

[b] Data in parentheses for plants transplanted to field 8/2/85. All other field data are for plants transplanted to field 8/21/85. Data for sporulation of plants transplanted 8/2/85 were not collected.

[c] Twenty-five lesions were excised per treatment and allowed to sporulate in the laboratory.

[d] Lesions/plant × sporulation/lesion.

[e] Different letters indicate data significantly different ($P<0.05$) by Duncan-Waller *K*-ratio *t* tests.

TABLE 4. Symptoms of blue mold on tobacco plants derived from seeds of plants stem-injected with sporangial suspensions of *Peronospora tabacina* or water[a]

Source of plants	Disease rating[b] (mean ± S.D.)	Sporulation[c] (sporangia/ml)
Plants derived from seed of plants stem-injected with water	3.0 ± 0.5	1.2×10^6
Plants derived from seed of plants stem-injected with *P. tabacina*	2.8 ± 0.2	1.7×10^6
Plants stem-injected with water	2.9 ± 0.3	2.1×10^6
Plants stem-injected with *P. tabacina*	0.4 ± 0.4	1.2×10^5

[a] Seeds of the plants stem-injected with *P. tabacina* or water were obtained after three generations of plants that were injected three times during the growing season. Plants were challenged with a sporangial suspension of *P. tabacina* (5×10^4 sporangia/ml) at the 10–11-leaf stage and six plants were used per treatment per experiment.

[b] Disease rating: 0 = no evidence of disease; 1 = 1–10% of leaf area with lesions; 2 = 11–40%; 3 = 41–75%; 4 = 76–100%. Both leaves were rated on plants in the three- to four- and seven- to eight-leaf stage.

[c] Sporulation for two most heavily infected leaves/plant.

reduces total sporulation on a heavily diseased leaf. Occasionally, the appearance of disease was delayed on tissue culture-derived plants from parents injected with water as compared with plants derived from seed of noninjected plants. Ultimate severity of disease was not different on plants from both sources in greenhouse tests. However, a significant reduction in symptoms was observed on regenerants derived from water-injected plants as compared with plants grown from seed in field tests. This may indicate development of some resistance due to stress caused by procedures of tissue culture. The age of the tissue culture and seed-derived plants, however, is not strictly comparable and data from both should be compared with caution. The level of resistance transferred via tissue culture from systemically protected plants was consistently and significantly greater than that transferred from nonprotected plants. Lucas et al concluded that systemic resistance to blue mold was generally not transmitted from plants stem-injected with *P. tabacina* to regenerants (5). However, they did report that sporulation was significantly reduced on regenerants obtained via callus from plants injected with *P. tabacina* as compared with those obtained from plants injected with water. It may be significant that their positive result was obtained when spores were sprayed on plants at a concentration of 5×10^4 sporangia per milliliter and not when regenerants were challenged by application of drops of inoculum (3 and 6×10^5 sporangia per milliliter). Greenhouse-grown regenerants in tests reported in this paper were challenged by spraying (10^4 sporangia per milliliter). The difference in method of challenge and inoculum concentration may be especially important for young greenhouse-grown plants in which resistance is not yet fully expressed.

Data reported in this paper indicate transfer of systemic protection against blue mold to plants differentiated via tissue culture from parents in which systemic resistance was induced. Transfer was indicated in greenhouse and field tests. It is possible that stem injection releases compounds that cause permanent changes in transmissible information or the expression of such information as related to blue mold resistance. The changes are carried over during differentiation in tissue culture, but not via seed, and become expressed as plants age. Instability of the plant genome is well established (6), and modifications in the genome are possible during stress. In this case, a specific and persistent low level of stress caused by injecting stems with *P. tabacina* might induce change. Experiments on the molecular level are in progress to explain the mechanism by which induced resistance is transferred to regenerants. It is unlikely that the transferred protection was due to infection of plantlets. Leaves used as a source of regenerants were obtained from healthy growing tips of induced parents. The leaves were far removed from the area of stem necrosis. Regenerated protected plantlets and fully grown plants from such plantlets did not develop symptoms of blue mold until challenged. Media used for tissue culture were apparently free of microorganisms. The transfer of protection was not observed on plants either derived from seeds of plants stem-injected with sporangial suspensions of *P. tabacina* over three generations or from seed of tissue culture-derived plants from parents stem-injected with *P. tabacina*. The testing of larger quantities of seed is advisable before ruling out seed transmission of induced resistance. Roberts (10) reported systemic acquired resistance of hypersensitive tobacco to tobacco mosaic virus (TMV) was seed transmitted if parent plants were repeatedly inoculated with TMV.

To the best of our knowledge, the experiments described are the first to demonstrate the consistent transfer of induced systemic resistance to a fungal plant disease via tissue culture. Transitory inhibition of TMV multiplication in virus-free plantlets derived via callus from dark-green island leaf tissue of plants infected with TMV has been reported (7). The procedure described in this manuscript may provide a technology for the protection of plants against disease that would minimize the dependence on chemical pesticides for plant protection and permit multiplication of disease resistant plants from existing high quality, high-yielding cultivars. The technology may also provide a tool to probe for an understanding of the mechanism(s) of induced resistance and varietal resistance on the genetic and molecular levels.

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