Nontransmissibility to Regenerants from Protected Tobacco Explants of Induced Resistance to *Peronospora hyoscyami*

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ABSTRACT

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Tobacco plants, *Nicotiana tabacum*, were protected against blue mold, caused by *Peronospora hyoscyami* f. sp. *tabacina*, by injection of the lowest stem internode with a sporangial suspension of the pathogen. Shoot-tip and leaf explants from protected tobacco were taken for rapid propagation through tissue culture, or used to establish callus lines. Regenerants obtained from protected tissues by rapid propagation were as susceptible to foliar challenge with sporangial inoculum of the fungus as similar regenerants derived from unprotected control explants. Regenerants produced by organogenesis from callus lines were also highly susceptible, although spore production per square centimeter of leaf area was significantly less in the case of protected regenerants. These results suggest that the expression of systemic induced resistance to blue mold depends mainly upon an active lesion being present in protected plants rather than an irreversible change in the inherent resistance expression of tobacco tissues.

Studies on several host genera have shown the feasibility of protecting plants against microbial disease through prior inoculation with virulent and avirulent pathogens, or nonpathogens (5,6). In tobacco, stem-base inoculation with the blue mold fungus, *Peronospora hyoscyami* de Bary f. sp. *tabacina* (Adam) Skalicky, protects emerging leaves against subsequent attack by the same pathogen (3). This protection is manifested on challenged plants as a reduction in lesion number, size, and subsequent sporulation. Leaves acquire resistance 17–21 days after induction, and when treatment is fully effective, disease severity following a challenge inoculation is reduced by more than 90%, compared with unprotected control leaves (2). Once acquired, resistance operates throughout the life of the leaf. It is also effective under field conditions, and compares favorably with currently available fungicidal control treatments (12).

The mechanism of induced resistance to tobacco blue mold is

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currently unknown, although evidence suggests that the restriction of pathogen takes place in internal tissues rather than prior to penetration of the host (2).

The aim of this study was to assess whether induced resistance to blue mold is retained in tobacco plants propagated through tissue culture. Three different procedures were used to obtain regenerants: shoot-tip culture, rapid propagation from leaf explants, and organogenesis from callus lines originally derived from protected leaf tissues. Comparison of these three different populations of regenerants from protected plants with similar populations derived from unprotected controls, provided evidence of the durability or otherwise of induced resistance in tissues.

MATERIALS AND METHODS

Plant material. Burley tobacco, *Nicotiana tabacum* L. 'Ky14' and 'B21,' were used. Plants were grown in a greenhouse (25–34 C in the summer, 22–28 C in the fall and winter) without supplementary lighting in UC mix compost (50% blow sand, 50% peat moss, plus 2.2 kg of dolomite, 148 g of KNO_3, and 148 g of K_2SO_4 per cubic meter (1) in 11-cm-diameter fiber pots. Plants were repotted in 16-cm-diameter plastic pots prior to the inducing inoculation.

Maintenance of pathogen. The KPT 1 isolate of *Peronospora hysocyami* f. sp. *tabacina* used in this study was originally collected in Georgetown, KY in 1979 (2). The fungus was maintained on 6- to 8-wk-old Burley tobacco by transfer at weekly intervals.

Inoculum for both inducing and challenge inoculations was freshly collected from sporulating lesions by gently brushing the abaxial leaf surface with a soft camel's-hair brush soaked in cold distilled water. The viability of sporangia was checked by incubating inoculum droplets on blocks of 1.5% (w/v) tapwater agar overnight in a petri plate lined with damp filter paper.

Inoculation methods. Resistance-inducing inoculations were carried out on plants at the five- to seven-leaf stage by injecting 1 ml of a 5×10^5 sporangia per milliliter suspension into the cambial layer on either side of the lowest stem internode. Excess inoculum was dripped onto the compost around the base of the stem. Control plants were similarly injected with sterile distilled water. As an adequate supply of nitrogen is required to maintain the development of stem-inoculated tobacco (7), all plants were watered at weekly intervals with a 0.5% (w/v) solution of NH_4NO_3.

Three different methods were used for challenge inoculations. In initial experiments plants were uniformly sprayed with a suspension containing 5×10^4 sporangia per milliliter. Alternatively, 6-mm-diameter filter paper disks previously dipped in a suspension containing 5×10^5 sporangia per milliliter were placed on the adaxial leaf surface in interveinal areas. Unless stated otherwise, all regenerant plants were challenged by applying eight 50-μl droplets of a suspension containing $2–5 \times 10^5$ sporangia per milliliter to interveinal areas of the adaxial surface of each fully expanded leaf.

Following challenge inoculation, plants were placed in an illuminated mist chamber ($17\ \mu E \cdot m^{-2} \cdot s^{-1}$, 16 hr day) at 22–25 C for 24 hr. They were then maintained in the greenhouse until chlorotic lesions developed.

Disease assessment. Disease severity was assessed 9–12 days after inoculation. Plants were transferred to the mist chamber for 24–48 hr prior to assessment to induce sporulation. When separate lesions developed, the number of lesions was determined; where lesions coalesced the percentage leaf area covered was estimated. The number of spores produced per lesion, leaf, or unit area of leaf was determined by excising infected areas of leaves, cutting them into small ($\sim 3\ cm^2$) pieces, and shaking vigorously for 30 sec with 25 ml of distilled water in a 250-ml Erlenmeyer flask. The concentration of the sporangial suspension was then estimated in a hemacytometer.

Culture media. All culture media contained Murashige and Skoog (8) major and minor salts and vitamins (Flow Labs. Ltd., Irvine, Scotland, UK) and 3% (w/v) sucrose, supplemented for specific media as follows: 1) shoot-tip propagation medium (MSAd): 170 mg/L $NaH_2PO_4 \cdot H_2O$, 80 mg/L adenine sulfate, 2 mg/L indole-3-acetic acid, and 2 mg/L kinetin (10); 2) leaf explant propagation medium (MSBAP): 1.1 mg/L 6-benzyl-aminopurine (4); 3) callus induction medium (CIM): 0.3 mg/L 2,4-D, 0.1 mg/L kinetin, 0.25 mg/L nicotinic acid, and 0.25 mg/L pyridoxine-HCl; 4) callus regeneration medium (CRM): 2 mg/L indole-3-acetic acid, 1 mg/L kinetin, and 1 mg/L 6-benzyl-aminopurine; 5) rooting medium (MSP2): 0.1 mg/L 1-naphthylene-acetic acid. All media were adjusted to pH 5.7 with KOH before autoclaving and were solidified with 0.8% (w/v) Difco Bacto-agar.

Culture techniques. Twenty-one days after the inducing stem-base inoculation, tissue explants from protected and control plants were removed for culture. Leaf pieces ($\sim 3 \times 1$ cm) for rapid propagation (4) or callus initiation were excised from leaves 4 and 6 of Ky14 plants. For callus initiation, 1-cm petiole sections were also taken from the same leaves. Tissue explants were surface sterilized for 15 min in a 10% commercial bleach solution (0.5% sodium hypochlorite) containing a drop of detergent. Explants were then washed in five changes of sterile distilled water and transferred to either MSBAP or CIM culture medium in 90-mm-diameter petri plates.

Rapid clonal propagation from shoot-tips was carried out by the method of Shabde-Moses and Murashige (10). Shoot-tips of B21 plants were excised ~2 cm below the stem apex, any small leaves were trimmed off, and the explants were surface sterilized as above. The apical 1-cm of the explant was then aseptically removed with leaf primordia intact, and transferred to MSAd propagation medium in containers consisting of two sterile clear plastic 180-ml tumblers joined together at the rims with Parafilm (American Can Co., Greenwich, CT).

Leaf and shoot-tip explants for rapid propagation were incubated in a culture room at 25 C under Sylvania Gro-Lux and General Electric cool white 20W fluorescent lights ($43\ \mu E \cdot m^{-2} \cdot s^{-1}$). Callus cultures were kept at low light intensity on an unilluminated shelf in the same room. For regeneration, callus pieces were transferred to CRM medium and placed under lights as above.

Regenerating shoots from each type of culture were aseptically removed, transferred to 25×150-mm glass culture tubes containing MSP2 rooting medium, and maintained in the light. Once roots formed, the regenerant plantlets were transferred to UC mix compost in 5-cm-diameter fiber pots and placed in a humid polyethylene chamber on the greenhouse bench. After 5 days the plants were removed to the open bench, grown on, and re-potted into 11-cm-diameter fiber pots prior to challenge inoculation.

RESULTS

Induction of resistance. Two separate groups of plants at the five- to seven-leaf stage were stem-base inoculated and maintained in the greenhouse for 21 days. Purple-brown necrotic lesions in the lowest internode were first observed between 1 and 2 wk after inoculation, and these lesions extended 5–10 cm up the stem of each plant by the time of challenge inoculation. No lesions were observed in water-injected control plants, apart from localized browning of tissues around the injection sites. In parallel with the study conducted by Cohen and Kuć (2), resistance induced by stem inoculation lasted through flowering and seed set in our experiments. Protected source plants of Ky14 and B21 developed flowers approximately 2–3 mo after induction.

Assessment of induced resistance. Table 1 presents the results of challenge inoculation. Leaves 4 and 6 of each of these plants were removed just prior to challenge and used for rapid propagation from lamina explants, and to establish callus lines from petiole and lamina tissues. In the control plants, 33–83% of inoculation sites gave rise to chlorotic sporulating lesions; in equivalent protected plants, none of the inoculation sites sporulated.

Protected and control plants were also used for shoot-tip propagation. Shoot-tips were excised from protected and unprotected control plants. Four plants of each treatment were then challenged by spraying with a sporangial suspension. Table 2 presents detailed results for one control and one protected plant in this group; symptom severity was similar in the remainder of the plants. The only signs of infection in leaves 1–6 of protected plants

were numerous small chlorotic flecks 1–2 mm in diameter; none of these restricted lesions produced spores. A few larger sporulating lesions were observed on the older and partially senescent leaves 7–9. In contrast, control plants had sporulating lesions 1–2 cm in diameter on all leaves, and on the older lower leaves these lesions coalesced and covered 30–75% of the leaf area.

These results, obtained by using three different inoculation procedures, indicated that all of the protected plants used as a source of explants for tissue culture were highly resistant to the blue mold pathogen, while unprotected controls were susceptible.

Time course of regeneration. Shoot-tip explants proliferated side shoots within 3–6 wk of transfer to MSAd medium. Leaf lamina explants placed on MSBAP medium regenerated small shoots over a period of 4–6 wk. Petiole and leaf lamina explants on CIM medium differentiated callus within 4–6 wk. Small pieces of rapidly growing friable callus were subcultured on fresh CIM plates for a further 4 wk, then transferred to CRM medium. Shoot formation from callus was observed within 4–8 wk.

Small plantlets with three to five leaves were excised and placed in tubes of MSP2 rooting medium. Root formation took place in 2–5 wk. All rooted regenerants were then transferred to potting mix and grown in the greenhouse until five to eight fully expanded leaves had formed. This took approximately 4 wk. Regenerants at the same stage of development were then challenged with fungal inoculum.

To summarize, the average time between removal of explants from the original source plants to testing of the regenerant populations was 12 wk for shoot-tip plants, 14 wk for regenerants from leaf explants, and 23 wk for callus-derived regenerants.

TABLE 1. Disease severity in representative control and protected Ky14 tobacco plants challenged by disk or droplet inoculation 21 days after stem-base inoculation with the fungus *Peronospora hyoscyami* and used for leaf explant and callus regeneration

Plant	Inoculation sites (no.)	Chlorotic lesions (no.)	Sporulating lesions (no.)	Inoculation sites sporulating (%)	Mean sporangia production per lesion[z] (no.)
Disk inoculation					
Control 1	6	5	2	33	
2	6	6	5	83	8.6×10^4
3	10	10	7	70	
Protected 1	6	1	0	0	
2	6	0	0	0	0
3	10	2	0	0	
Droplet inoculation					
Control 4	20	15	13	65	
Protected 4	20	0	0	0	

[z]Assessed for control and protected plants 1 and 2 only.

TABLE 2. Disease severity in one control and one protected B21 tobacco plant challenged by spray inoculation[x] and used for shoot-tip propagation

Leaf no.	Sporulating lesions (no.)		Sporangia (no. per cm^2 of leaf)	
	Control	Protected	Control	Protected
1	19	0		
2	16	0	4.2×10^4	0
3	8	0		
4	13	0		
5	30%[y]	0		
6	60%[y]	0		
7	75%[y]	1	6.7×10^4	2.9×10^3
8	S[y,z]	1		
9		7S[z]		

[x]Inoculum concentration was 5×10^4 sporangia per milliliter of *Peronospora hyoscyami*.
[y]Where lesions coalesced the figure given is percent leaf area covered.
[z]S indicates that leaf is senescent.

Disease assessment in regenerant populations. Due to the variable rate of development of regenerants it was not possible to simultaneously challenge all the plants in each population with fungal inoculum. Instead, regenerants of the same size and age derived from unprotected control and protected plants produced by the same tissue culture route were assessed in groups. Comparisons of disease severity were made within, rather than between, these groups, to minimize variation due to differences in quality of the inoculum and the greenhouse environment. The data therefore represent results for single groups of plants challenged at one time. Overall at least 12 control and 12 protected plants were assessed from each of the three populations of regenerants.

Table 3 shows results for one typical group of regenerants grown from leaf explants. The results provided no evidence for significant transfer of resistance in regenerant plants originally derived from protected leaf tissues. Sporulating lesions developed similarly on plants regenerated from both protected and unprotected sources. The mean spore production per lesion in this experiment was higher for control plants, but this pattern was not consistently repeated in other challenge inoculations with leaf explant regenerants.

Table 4 presents data for one group of regenerants obtained by shoot-tip propagation. Infection on this occasion was severe and lesions coalesced to cover up to 95% of the leaf area. There was no difference in disease severity between control and protected populations.

Unlike regenerants produced directly from shoot-tip and leaf explants, those from callus showed a high incidence (~50%) of morphological variation. Somaclonal variation among plants regenerated from tissue culture is a well-documented phenomenon and may affect disease resistance (9). Infection was severe in both control and protected populations following challenge with the fungus (Table 5). Spore production per square centimeter of leaf area in this group was significantly less in protected versus control callus regenerants, although sporulation was high in both cases.

TABLE 3. Disease severity for a single group of regenerant Ky14 tobacco plants derived from leaf explants and challenged by droplet inoculation[x]

Original plant source and leaf position	Regenerant plants				
	Leaf 4		Leaf 5		Leaf 6
	Lesions[y]	Sporangia[z]	Lesions	Sporangia	Lesions
Control 3 leaf 4	8	1.1×10^6	7	1.2×10^6	7
Control 3 leaf 4	8	1.5×10^6	8	2.0×10^6	8
Control 3 leaf 4	8	3.2×10^5	8	2.7×10^5	9
Protected 3 leaf 4	8	1.3×10^5	8	6.9×10^5	8
Protected 3 leaf 4	8	1.7×10^5	8	2.6×10^5	8
Protected 3 leaf 4	8	8.1×10^5	8	3.2×10^5	8

[x]Each leaf received eight 50-µl droplets of a 6×10^5 sporangia per milliliter suspension of *Peronospora hyoscyami*.
[y]Number of sporulating lesions per leaf.
[z]Sporangia production per lesion.

TABLE 4. Disease severity in a group of regenerate tobacco plants derived from shoot-tip explants

Original plant source	Regenerate plants leaf area chlorotic (%)[a]				
	Leaf 3	Leaf 4	Leaf 5	Leaf 6	Leaf 7
Control 1	10	55	60 (8.0×10^5)[b]	80	75
Control 2	25	45	60 (1.4×10^6)	60	80
Protected 1	15	60	80 (1.7×10^6)	85	85
Protected 4	0	50	65 (2.5×10^6)	90	95

[a]Each leaf received eight 50-µl droplets of a suspension containing 3×10^5 sporangia of *Peronospora hyoscyami* per milliliter.
[b]Data in parenthesis represents conidia produced per leaf.

TABLE 5. Disease due to blue mold in a single group of regenerant Ky14 tobacco plants derived from callus cultures[x]

Callus source	Regenerated plant no.	Sporangia per cm^2 of leaf 3	Percent leaf area chlorotic			
			Leaf 3	Leaf 4	Leaf 5	Leaf 6
Control 2 leaf 4	1	(6.1×10^4)	90	80	90	90
	2	(1.5×10^5)	90	90	90	80
	3	(7.9×10^4)	80	70	90	60
Control 1 leaf 4	1	(2.9×10^3)	90	15	20	50
Mean		1.2×10^5 a[y]				
Protected 3 leaf 4	1	(4.2×10^3)	15	10	20	40
	2	(9.7×10^3)	80	70	80	30
	3	(3.3×10^3)	90	80	90	70
	4	(2.8×10^4)	70	80	90	S[z]
Mean		1.1×10^4 b[y]				

[x]Inoculum concentration was 5×10^4 sporangia per ml of *Peronospora hysocyami*, which was sprayed on both leaf surfaces.
[y]Values followed by different letters are significantly different ($P = 0.05$).
[z]S indicates that leaf was senescent.

Somaclonal variation had no consistent effect on the expression of resistance in callus lines derived from protected or control plants.

DISCUSSION

The results of this study suggest that systemic induced resistance to *P. hyoscyami* f. sp. *tabacina* is largely lost in tobacco clones obtained via tissue culture. The rationale for using three different routes to obtain regenerants was to permit comparisons between plants produced via rapid clonal propagation, and others obtained more slowly from disorganized callus tissues through organogenesis. No evidence for significant transfer of resistance was found in any of the populations produced via rapid clonal propagation. Populations derived from callus of protected plants via organogenesis did show slightly reduced sporulation, but the level of protection was much less than that observed for the original plants protected by stem injection.

In the original protected plants a high level of resistance was detected under greenhouse conditions by using either spray, droplet, or filter paper disk inoculation methods. If high levels of resistance had been retained in regenerant plants then this should have been detected by the methods used. A complication in the assessment of clones regenerated from callus lines was the high incidence of somaclonal variation. Such variation is believed due to genetic modifications in cultured cells, and may affect disease resistance in addition to morphological characters (9). However, all the callus-derived regenerants tested were equally susceptible to challenge inoculation with the fungus.

It might be argued that the original plants from which explants were taken were not sufficiently well-protected for resistance to persist through a tissue culture cycle, but this is not supported by the experimental data presented in Tables 1 and 2. A more likely explanation is that protection of newly emerging leaves of plants stem-inoculated with the blue mold fungus requires the presence of an active lesion in stem tissues below those leaves. No evidence exists for an irreversible "conditioning" of leaf tissues once removed from the physiological influence of such a lesion. This conclusion is supported by the observation of Cohen and Kuć (2) that suckers formed from roots beneath a stem lesion are fully susceptible to foliar infection by the pathogen. These suckers could be rendered resistant by stem-base inoculation with the pathogen. Similarly in our experiments, susceptible callus-derived regenerants were protected to the same degree as protected source plants by an inducing inoculation in the lowest stem internode.

If a stem lesion is a prerequisite for the expression of induced resistance to blue mold in tobacco, then further studies on the mechanism should focus on products of the necrotic lesion that are translocated in the stem and affect changes in emerging leaves. It would also be of interest to determine whether tobacco regenerated from callus cultures infected with the fungus (11) show any signs of enhanced resistance to the disease.

LITERATURE CITED

1. Baker, K. F., ed. 1957. The U. C. System for Producing Healthy Container-Grown Plants. University of California, Division of Agricultural Sciences, Agricultural Experiment Station, Extension Service. 322 pp.
2. Cohen, Y., and Kuć, J. 1981. Evaluation of systemic resistance to blue mold induced in tobacco leaves by prior stem inoculation with *Peronospora hysocyami* f. sp. *tabacina*. Phytopathology 71:783-787.
3. Cruickshank, I. A. M., and Mandryk, M. 1960. The effect of stem infestation of tobacco with *Peronospora tabacina* Adam on foliage reaction to blue mold. J. Aust. Inst. Agric. Sci. 20:369-372.
4. Flick, C. E., and Evans, D. A. 1984. Tobacco. Pages 606-630 in: Handbook of Plant Cell Culture. Vol. 2. Crop Species. W. R. Sharp, D. A. Evans, P. V. Ammirato, and Y. Yamada, eds. MacMillan, New York and London. 644 pp.
5. Kuć, J. 1982. Induced immunity to plant diseases. Bioscience 32:854-860.
6. Kuć, J. 1983. Induced systemic resistance in plants to diseases caused by fungi and bacteria. Pages 191-221 in: The Dynamics of Host Defence. J. A. Bailey and B. J. Deverall, eds. Academic Press, Sydney, Australia. 233 pp.
7. Mandryk, M. 1962. The relationship between acquired resistance to *Peronospora tabacina* in *Nicotiana tabacum* and soil nitrogen levels. Aust. J. Agric. Res. 13:10-16.
8. Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissues. Physiol. Plant. 15:473-497.
9. Scowcroft, W. R., Larkin, P. J., and Brettell, R. I. S. 1983. Genetic variation from tissue culture. Pages 139-162 in: Use of Tissue Culture and Protoplasts in Plant Pathology. J. P. Helgeson and B. J. Deverall, eds. Academic Press, Sydney, Australia. 194 pp.
10. Shabde-Moses, M., and Murashige, T. 1979. Organ Culture. Pages 40-51 in: Nicotiana, Procedures for Experimental Use. R. D. Durbin, ed. U.S. Dep. Agric. Tech. Bull. 1586. 124 pp.
11. Trigiano, R. N., Van Dyke, C. G., Spurr, H. W., Jr., and Gray, D. J. 1984. Infection and colonization of tobacco callus by *Peronospora tabacina*. Phytopathology 74:280-285.
12. Tuzun, S., Nesmith, W., and Kuć, J. 1984. The effect of stem injections with *Peronospora tabacina* and metalaxyl treatment on growth of tobacco and protection against blue mold in the field. (Abstr.) Phytopathology 74:804.