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Expressed Resistance to Black Shank Among Tobacco Callus Cultures*

W. R. Deaton, G. J. Keyes and G. B. Collins
Department of Agronomy, University of Kentucky, Lexington, Ky. (USA)

Summary. Quantitatively inherited resistance to the black shank pathogen (*Phytophthora parasitica* var. 'nicotianae') was expressed among callus tissue cultures of tobacco (*Nicotiana*). Tissue cultures of genotypes known to posses polygenic mechanisms for black shank resistance expressed that resistance in vitro when challenged by the viable pathogen. Callus of a susceptible cultivar was readily parasitized in culture. Furthermore, single gene resistance to the common pathogen race was also shown to operate in vitro. Nongenetic factors examined did not contribute significantly to the observed differences. Disease expression in vitro appeared to be highly correlated with its expression at the whole plant level.

Screening for quantitative disease resistance can be complicated at the whole plant level by variable host-pathogen reactions and by significant genotype × environment interactions. Since quantitatively inherited mechanisms of black shank resistance are expressed in tobacco callus cultures, an in vitro host-pathogen system may be useful in screening tobacco lines for black shank resistance.

Key words: Disease screening – *Phytophthora parasitica* var. 'nicotianae' – *Nicotiana* – Tissue culture – Cytokinins

Introduction

Black shank of tobacco is a major disease throughout the areas of flue-cured and burley production in the United States (Lucas 1975; Stokes and Litton 1966). The causal organism, *Phytophthora parasitica* (Dast.) var. 'nicotianae' (B. de Haan) Tucker, is a soil-born fungal pathogen which parasitizes the tobacco stalk at or near the soil level (Lucas 1975). The breeding of disease-resistant cultivars has played a major role in the control of black shank and may be considered the least expensive, most practical approach to combating this disease problem (Lucas 1975).

Genetic sources of resistance to black shank include the wild species *Nicotiana plumbaginifolia* Viv. and *N. longiflora* Cav. (Stokes and Litton 1966). These immune species from the section *Altae* possess a dominant monogenic factor which confers high resistance to race 0 (Ppn0) of the causal organism (Collins et al. 1971; Goins and Apple 1970). The resistance has been backcrossed into commercial tobacco types (Chaplin 1962; Valleau et al. 1960). Disadvantages of monogenic resistance to Ppn0 include the evolution of virulent races, such as Ppn1 (Apple 1962; Apple 1967), and the expression of undesirable leaf characteristics linked with the resistance factor (Collins et al. 1971). These considerations suggest that alternatives to the incorporation of alien monogenic resistance are desirable.

Field-level polygenic resistance to black shank has been identified in *N. tabacum* (Silber and Heggestad 1963; Tisdale 1931). Cultivars with moderate polygenic resistance to both Ppn0 and Ppn1 have been developed using Tisdale's "Florida 301" breeding line (Stokes and Litton 1966; Wills and Moore 1971). Quantitative resistance is less vulnerable to pathogen mutation. Further, such resistance is not linked with unfavorable leaf characteristics. For these reasons, it should be more efficient to raise the level of "Florida 301" type resistance already present in *N. tabacum* sources, than to rely upon interspecies hybridization with backcrossing (Apple 1962; Valleau et al. 1960).

Evaluation of disease resistance is the most difficult task in breeding black shank resistant varieties (Gooding and Lucas 1959). This is especially true of the quantitative-type resistance since there is not the clear demarcation between healthy and diseased plants typical of monogenic resistance systems. Wide variability in response among individuals within a cultivar and even

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among tissues of an individual plant indicates that polygenic resistance is a dynamic condition, dependent upon numerous genetic and environmental factors (Wills and Moore 1971). Nevertheless, the advantages of polygenic resistance, in terms of both stability and agronomic desirability, justify the greater effort required for effective evaluation.

Researchers have developed a number of nonfield techniques for evaluating black shank resistance under experimental conditions which reduce some components of environmental variability (Hendrix and Apple 1967; Litton et al. 1970; Taylor and Waggoner 1979; Valleau et al. 1960; Wills and Moore 1971). One novel approach to the study of host-parasite interactions in the tobacco-black shank system involves the infection of tobacco callus cultures with viable Ppn0 zoospores (Haberlach et al. 1978; Helgeson et al. 1976; Helgeson et al. 1972; Maronek and Hendrix 1978).

Caution must be exercised in interpreting the significance of host-parasite interactions in vitro. A number of examples can be cited to indicate that some in vitro systems may produce artifactual results irrelevant to the defense mechanisms operable in nature (Ingram 1969; Keen and Horsch 1972; Maheshwari et al. 1967). Nevertheless, a substantial body of evidence exists to indicate that the use of in vitro techniques to study the expression of black shank resistance in tobacco is a valid approach. Monogenic resistance to black shank has been expressed in tobacco callus cultures infected with zoospores of a virulent Ppn0 isolate (Haberlach et al. 1978; Helgeson et al. 1976; Helgeson et al. 1972; Maronek and Hendrix 1978). The authors of these reports concluded that the basic mechanism of monogenic resistance was fully expressed at the cellular level.

Two experiments are reported in this paper. Research was designed (1) to examine genetic and nongenetic factors contributing to black shank resistance among tobacco callus cultures; and (2) to consider whether the expression of quantitative resistance in culture is of sufficient magnitude to undertake recurrent selection for black shank resistant cultivars using disease resistance in vitro as one selection criterion.

Materials and Methods

Host Materials

Experiment 1

Host materials for the first experiment consisted of ten burley tobacco genotypes (*Nicotiana tabacum*) and *N. longiflora* with monogenic resistance to Ppn0 of black shank. Five true breeding pure lines were included among the burley genotypes. Kentucky 10 (Ky 10), Kentucky 14 (Ky 14), and Kentucky 16 (Ky 16) are susceptible to the black shank causal organism, *Phytophthora parasitica* var. 'nicotianae' (Ppn). Burley 37 (By 37) and Virginia 509 (Va 509) possess moderate field-level resistance to black shank. Five hybrid combinations were produced by crossing the pure lines onto Burley 49 (By 49). By 49 possesses high polygenic resistance to both races of the causal organism.

Experiment 2

Twelve *Nicotiana* genotypes were evaluated in the second experiment. Monogenic resistance to Ppn0 was represented by *N. longiflora* and L8, a burley breeding line with monogenic resistance obtained by crossing *N. longiflora* and *N. tabacum* followed by backcrossing to *N. tabacum*. Polygenic resistance to both races of Ppn was represented by several *N. tabacum* lines. Beinhart 1000 (B 1000) is a cigar-wrapper type with high polygenic resistance. Kentucky 17 (Ky 17), By 49, Va 509, and the By 49 × Va 509 hybrid combination are burley types with "Florida 301" resistance. Burley 21 (By 21), a susceptible *N. tabacum* genotype, was included as a check. Anther-derived haploids from the four burleys (By 21, By 49, Ky 17, Va 509) were also included to examine ploidy effects.

Callus of the experimental materials was induced from cotyledonary explants of seedlings or haploid plantlets on A-medium (Kasperbauer and Collins 1972) modified by a 10-fold increase in NAA. In both experiments, calli were initially cultured for 28 days and subcultured for an additional 21-day growth period prior to inoculation with the pathogen. Single callus colonies, about 200 mg, were transferred to 60 × 20 mm culture plates which contained a few drops of sterile H_2O and no culture medium. The medium was excluded so that the pathogen could obtain substrate only from the callus exudate or from direct parasitization of the tissue. Previous experiments in this lab indicated that callus colonies remained viable for up to 20 days when sealed in sterile plates containing only a few drops of sterile H_2O to maintain humidity.

Pathogen Cultures and Inoculum

Isolates of Ppn0 and Ppn1 were obtained from C. C. Litton, University of Kentucky. Cultures of both races were maintained on oatmeal agar. Only pathogen cultures 12–16 days old after subculturing were used as inoculum sources. Mycelial suspensions for inoculation were prepared by placing a small mycelial fragment (approximately 2 cm^2) into a sterile screw top vial containing approximately 10 ml of sterile H_2O. Agitation of the vial produced the mycelial suspension. Callus tissue in both experiments was inoculated with 1 drop of the suspension from a sterile syringe. All treatments within a replication were treated with the same suspension.

Experimental Treatments and Designs

Experiment 1

In addition to differences attributed to genetic lines, the first experiment was designed to examine the effects of pretreatment of callus with a crude extract of the pathogen. Previous research indicates that pretreatment of whole plant tissue with a nonviable extract of Ppn can give protection against challenge with viable fungus (McIntyre and Miller 1978). A crude extract of the pathogen was prepared by suspending 100 mg of mycelia in 100 ml of double-distilled H_2O using a Waring blender. One-half the extract was filtered through a millipore filter. The other half was repeatedly frozen and thawed to inactivate the fungus. These fractions were recombined and serially diluted to prepare four extracts; a base level, 5^0X, and 5^{-1}X, 5^{-2}X, and 5^{-3}X concentrations. Colonies were treated with about 0.2 ml of an extract and

allowed to incubate for one, three, or five days prior to challenge with viable inocula. Only Ppn0 was used as the inoculum source in the first experiment.

The experiment was a split-split plot design with five replications. Main plots consisted of the three incubation times following pretreatment with crude extract. Sub-plots consisted of the eleven genetic lines. Sub-sub-plots consisted of the four pretreatment doses.

Experiment 2

The second experiment was designed to evaluate effects attributable to differences in the callus media and pathogen races, as well as in the genetic lines. The effect of substituting 6-(3-methyl-2-butenylamino)purine (2ip) for kinetin as the cytokinin in the callus medium was examined. These cytokinins have demonstrated different effects on the black shank-tobacco interaction in vitro (Haberlach et al. 1978). In addition, both races of the black shank organism, Ppn0 and Ppn1, were compared.

Experiment 2 was a split-split plot design with ten replications. Main plots were the two cytokinins. Pathogen races constituted the subplots. Sub-sub-plots consisted of the twelve genetic lines.

Scoring Fungal Growth

After inoculation, callus colonies were maintained under diffuse, continuous lighting at 26 ± 1C. Colonies were visually rated for the degree of fungal growth three, four, five, and seven days following inoculation in Experiment 1. Data from the ninth day postinoculation were included in the second experiment. The scoring criteria in the second experiment were as follows:

0 – no visible mycelia
1 – a few aerial mycelia
2 – localized growth
3 – moderate colonization
4 – extensive colonization
5 – callus enveloped in a mycelial mat.

Fungal growth was scored with a 0–4 scale in the first experiment.

Results

Experiment 1

The rate at which callus colonies were parasitized by the pathogen was significantly influenced by genetic differences among lines (Table 1). Differences among genotypes were greatest on the fourth and fifth days following inoculation with the viable pathogen.

Fungal growth rates of the five burley hybrids and the monogenic resistant control are shown graphically (Fig. 1). The F_1 combinations were paternal half-sibs with By 49 as the common parent. Significant differences among the hybrid lines were present on all evaluation dates. Callus from the Va 509 × By 49 hybrid was the most resistant type in this study. As the postinfection period lengthened, differences among

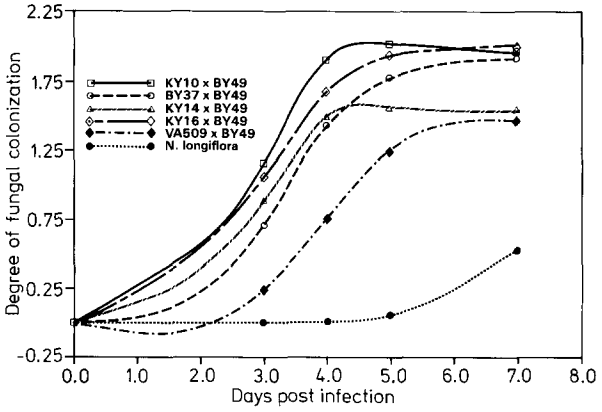

Fig. 1. Colonization of six tobacco callus lines by *Phytophthora parasitica* var. 'nicotianae', race 0, in Experiment 1 (S.E. on day 4 = 0.42)

Table 1. Analyses of variance on visually rated colonization of tobacco callus by *Phytophthora parasitica* var. 'nicotianae', race 0, in Experiment 1

Source	df	Mean squares for (N) days postinoculation			
		MS (3)	MS (4)	MS (5)	MS (7)
Replications	4	7.152	13.513	10.471	11.945
Duration of Pretreatment (A)	2	21.392	16.705	10.847*	11.620
Main Plot Error	8	9.539	5.702	2.129	3.631
Genetic Lines (B)	10	8.587**	19.623**	22.506**	17.221**
A × B	20	1.319	1.550	2.257	1.588
Split Plot Error	120	1.153	1.352	1.242	1.305
Pretreatment Concentration (C)	3	0.727	0.883	0.699	0.212
A × C	6	0.138	0.545	1.303	1.460
B × C	30	0.760	0.761	0.808	1.030
A × B × C	60	0.618	1.094	0.996	1.223
Split-Split Plot Error	396	0.669	0.884	0.980	1.026

*,** Significant difference at P = 0.05 and P = 0.01, respectively.

Table 2. Analyses of variance on visually rated colonization of tobacco callus by *Phytophthora parasitica* var. 'nicotianae' in Experiment 2

Source	df	Mean squares for (N) days postinoculation				
		MS (3)	MS (4)	MS (5)	MS (7)	MS (9)
Replications	9	15.389	17.390	14.219	7.813	7.193
Cytokinin (A)	1	5.002	14.876	14.352	8.008	1.463
Main Plot Error	9	2.960	3.504	3.457	1.742	1.657
Race (B)	1	7.500*	5.526	2.408	0.052	0.026
A×B	1	0.533	0.638	0.918	11.102	7.376
Split Plot Error	18	1.027	2.836	3.738	4.396	3.461
Genetic Lines (C)	11	3.132**	7.916**	6.657**	4.676**	2.621*
A×C	11	1.107	1.963	3.094*	1.169	0.704
B×C	11	2.652**	5.336**	7.350**	4.285**	2.926*
A×B×C	11	0.720	1.585	1.197	1.519	1.121
Split-Split Plot Error	396	1.017	1.520	1.618	1.599	1.283

*,** Significant difference at P=0.05 and P=0.01, respectively.

genetic lines were diminished. Resistance in the callus of the monogenic resistant *N. longiflora* was fully evident in the first five days postinoculation. However, fungal growth was evident even in this line by the seventh day.

Increasing duration of the pretreatment interval resulted in increased susceptibility on all dates, but this increase was significant only on the fifth day postinoculation (Table 1). Differences in the dose of crude extracts prepared from the deactivated pathogen were not significant on any evaluation date (Table 1).

Experiment 2

As in Experiment 1, the genetic lines examined supported growth of the fungus at significantly different rates (Table 2). The differences were most pronounced on the fourth and fifth days postinoculation as in the first experiment.

Monogenic resistance to Ppn0, present in *N. longiflora* and L8, was expressed in vitro (Fig. 2). Callus tissue from these lines was significantly more resistant to Ppn0 than either the susceptible control (By 21) or the polygenic-resistant line (By 49). However, *N. longiflora* and L8 were extensively pathogenized by Ppn1 while By 49 was equally resistant to both races.

Haploid-diploid genotypes did not differ significantly within a given genetic line on any evaluation date tested by a ploidy×line interaction term. Also, haploid and diploid means within a line were of similar magnitude (Fig. 3). By 49 supported less fungal growth than any of the other polygenic-resistant types (Fig. 3). All were more resistant in vitro than the susceptible control (By 21).

The effect of substituting 2ip for kinetin in the callus media was not significant on any of the

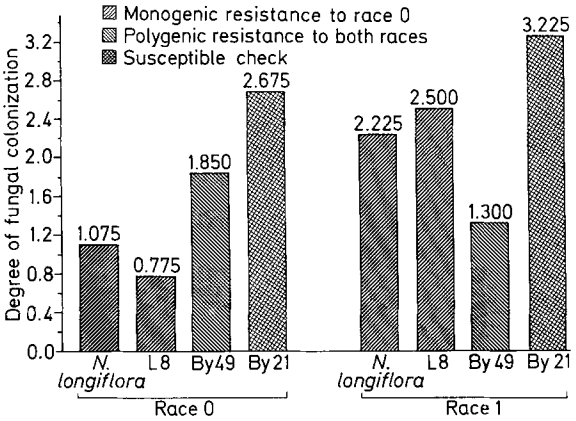

Fig. 2. Differential response of genotypes to colonization by two races of *Phytophthora parasitica* var. 'nicotianae' on day 4 of Experiment 2 (S.E. = 0.28)

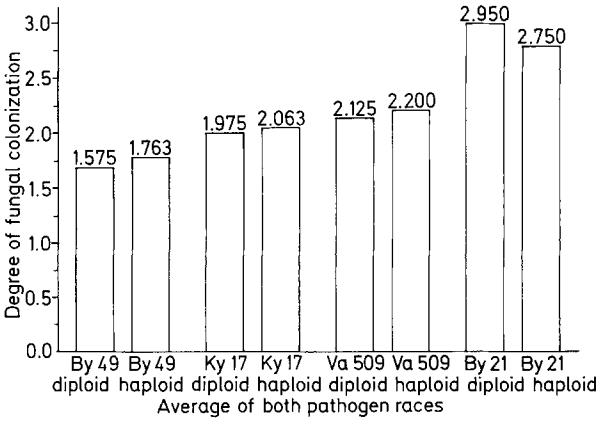

Fig. 3. Colonization of haploid and diploid tobacco callus by *Phytophthora parasitica* var. 'nicotianae' on day 4 of Experiment 2 (S.E. = 0.28)

evaluation days (Table 2). However, the cytokinins did interact significantly with the genetic line differences on the fifth day postinoculation.

Ppn0 and Ppn1 were significantly different only on the third day postinoculation (Table 2). Significant race × genetic line interaction was evident every day fungal growth data were collected.

Discussion

The expression of monogenic resistance to black shank has been demonstrated in tobacco callus cultures (Haberlach et al. 1978; Helgeson et al. 1976; Helgeson et al. 1972; Maronek and Hendrix 1978). Research reported in this paper substantiates these observations of monogenic resistance in vitro, extending those previous observations to include monogenic susceptibility to race 1. This paper further reports the first evidence for the in vitro expression of polygenic resistance to the black shank fungus. These results indicate that in vitro expression of resistance to black shank corresponds with whole plant expression.

Differences among genetic lines were significant in both experiments. In the first experiment, callus of closely related half-sib lines expressed differential susceptibility to colonization by the black shank causal organism. Callus of polygenic-resistant lines in Experiment 2 also expressed differences in their ability to resist colonization by Ppn. These results suggest that by using an in vitro system to reduce the magnitude of genotype × environment interaction, it may be possible to detect real genetic differences in black shank resistance, even when those differences are very small. Indirect selection for a character based on the performance of another correlated character has been suggested to overcome a low heritability of the first character due to large genotype X environment interactions (Falconer 1960). Therefore, polygenic resistance expressed at the cellular level in tobacco callus cultures may be useful in a recurrent selection program for the development of tobaco cultivars with increased resistance to black shank.

Since ploidy level did not interact significantly with genetic line performance, a selection scheme utilizing alternating haploid-diploid generations from a hybrid with superior polygenic resistance could produce valuable results. Haploids derived from this F_1 would segregate for polygenic resistance to black shank. Selection of superior haploid genotypes based on in vitro disease expression followed by doubling could produce fertile pure lines with improved polygenic resistance. This process could be repeated with haploids from crosses of the selected lines if more gene recombination is deemed necessary. Selection in this manner for an agronomic character based on the performance of a correlated character in vitro could provide both efficient and rapid advance in improving polygenic resistance to a major disease of commercial tobacco.

Kinetin and 2ip have demonstrated different effects on the expression of resistance to black shank in vitro (Haberlach et al. 1978). However, in this experiment, media with the two different cytokinins were not significantly different on any evaluation date. Furthermore, cytokinin effects did not interact with the expression of genetic lines except on the fifth day postinoculation. In the previously cited study, callus tissue remained on the medium throughout the processes of inoculation and fungal growth. This contact allowed pathogen-medium interaction. The general lack of cytokinin effect in the present study may be due to the removal of callus tissue from the medium prior to challenge with the pathogen.

Virulence of the isolates of the Ppn races did not differ significantly in this experiment. As expected, significant race × genotype interactions were recorded. These interactions result from the response of the monogenic-resistant types, which are resistant to Ppn0 but highly susceptible to Ppn1.

An experiment at the whole plant level has shown that pretreatment of both resistant and susceptible plants with a cell-free sonicate of black shank resulted in significant protection against subsequent inoculation with the viable pathogen (McIntyre and Miller 1978). In that study, protection against infection was greatest when the pretreatment preceded challenge by 24 hours. Protection decreased progressively with time and was less effective as the sonicate was diluted. In the present study, pretreatment of callus colonies with crude extracts of the pathogen did not trigger a resistance response regardless of the pretreatment dose or duration. It is assumed that in the black shank-tobacco system, callus tissue lacks the physiological capabilities either to recognize or to respond to such treatments.

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Mr. W.R. Deaton
Dr. G.B. Collins
Department of Agronomy
University of Kentucky
Lexington, Ky. 40546-0091 (USA)

Mr. G.J. Keyes
Cell Biology Group
Monsanto Company
800 N. Lindbergh Blvd.
St. Louis, Mont. 63166 (USA)