Use of Detached Leaves to Evaluate Tobacco Haploids and Doubled Haploids for Resistance to Tobacco Mosaic Virus, *Meloidogyne incognita*, and *Pseudomonas syringae* pv. *tabaci*

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ABSTRACT

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Development of disease-resistant cultivars in self-pollinated crops like tobacco (*Nicotiana tabacum*) can be greatly accelerated by evaluating populations of haploid plants derived from F_1 hybrids resistant to various diseases. A limitation of haploid breeding is the need to assess reactions to multiple pathogens on single plants. To avoid confounding systemic or lethal effects from inoculations with multiple pathogens, detached leaves were inoculated separately with tobacco mosaic virus and *Pseudomonas syringae* pv. *tabaci*. Resistance to *Meloidogyne incognita* was identified by the associated reactions of detached leaves to the M^SN^R strain of potato virus Y. Detached leaves were maintained by immersing their petioles in water until symptoms appeared. Symptoms in detached leaves were similar to those in intact plants, and disease reactions corresponded with whole-plant determinations. The technique can also be used with doubled haploid or diploid populations segregating for disease resistance. The original intact plants with identified resistance may be evaluated for other traits, and susceptible genotypes may be discarded. Field evaluations for agronomic characteristics can then be performed on a population fixed for disease resistance genes.

Development of disease-resistant cultivars is one of the main objectives of tobacco (*Nicotiana tabacum* L.) breeding programs. Traditionally, the pedigree and backcross methods have been used for this purpose, but they require six to eight generations to obtain pure lines from a heterozygous source. Current techniques facilitate the production of large numbers of tobacco haploids that can be used efficiently in a breeding program (1,2). Advantages of evaluating germ plasm at the haploid level include direct selection for both dominant and recessive traits and the ability to produce completely homozygous lines upon chromosome doubling of selected genotypes. Haploid breeding has also been shown to be advantageous when the number of genes concerned is large and the frequencies of favorable alleles in the populations are small (6). Nevertheless, a serious disadvantage of selecting for disease resistance at the haploid level is the need to assess reactions to multiple pathogens on a single plant of a unique genotype, where replication is obviously not possible. Consequently, plant breeders have opted for doubling chromosome numbers of all haploids before evaluation. Because the process of doubling chromosome numbers is time consuming and labor intensive, it would greatly facilitate breeding programs if undesirable genotypes could be identified and discarded early. This study was conducted to determine if separate, detached leaves from a given haploid genotype (derived from F_1 hybrid heterozygous for disease resistance allelles) could be used to assess reactions to multiple pathogens. If so, confounding systemic or lethal effects of certain diseases would be avoided and the original intact plants with identified resistance could be evaluated for other traits.

MATERIALS AND METHODS

Two methods of haploid development were used:

1. The anther culture procedure (1,2) was used to produce androgenetic haploids in population 1. This population was obtained from F_1 hybrids derived from crosses Kentucky 14 (Ky 14) × Havana 307 and Jaraiz 1 × Havana 307 and segregated for resistance to tobacco mosaic virus (TMV).

2. The *N. africana* pollination procedure (3) was used to produce gynogenetic haploids in populations 2 and 3. Population 2 was obtained by pollinating the F_1 hybrid NC-528 × Ky 14 with *N. africana*, and population 3 was likewise derived from the F_1 hybrid NC-528 × Jaraiz 1. Haploids in populations 2 and 3 segregated for resistance to the root-knot nematode (*Meloidogyne incognita* (Kofoid & White) Chitwood). Haploids in population 2 also segregated for resistance to wildfire (*Pseudomonas syringae* pv. *tabaci*). All haploids were expected to segregate in a 1:1 ratio of resistant to susceptible plants for all diseases tested because resistance is controlled by a single, dominant gene in every case. Chi-square goodness-of-fit tests were conducted to determine whether detached leaf inoculations provided proper segregation ratios for resistance. Haploids were initially grown in a growth chamber at 25 C under a 16-hr photoperiod ($10W\ m^{-2}\ B^{-1}$ PAR) and transferred to a greenhouse (25–32 C) when primary leaves were about 5 cm long. Plants were then transplanted to 9-cm plastic pots containing Metro Mix.

TMV inoculation. Population 1 was evaluated for TMV resistance by detaching a single leaf 7–10 cm long from each haploid and inoculating it as described later. Detached leaves were placed in plastic boxes (24 × 30 × 6 cm) with hinged lids containing 2 cm of water at the bottom. Petioles were submerged in the water, and laminae were suspended above the water surface by wire mesh. Boxes were closed after inoculation and placed under a greenhouse bench at 25 C. Local lesions indicating TMV resistance developed within 4 days, whereas susceptible plants were free of symptoms. Controls included leaves of TMV-resistant Burley 21 plants as well as leaves of the parental cultivars (all diploids). Inoculum was prepared by grinding systemically infected leaf tissue in a mortar and pestle, using 1 g of tissue per 5 ml 0.05 M Na_2HPO_4-KH_2PO_4 buffer (pH 7.2). Carborundum (22 μm, 600 mesh) was added to the inoculum (10 mg of Carborundum per 1 ml of inoculum) to serve as an abrasive. Leaves were rubbed with a cotton swab dipped in inoculum, then rinsed with water.

Potato virus Y (PVY-M^SN^R) inoculation. Haploids in populations 2 and 3 were screened for resistance to root-knot

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nematodes using the M^SN^R strain of PVY (PVY-M^SN^R). This strain produces necrotic lesions at 23–26 C exclusively on root-knot-resistant plants because of the pleiotropic effects of a single gene (4,7).

One-third of the lamina of leaves 10–15 cm long was trimmed around the petiole, placed in 15-ml glass test tubes filled with water, and held on a greenhouse bench until symptoms developed. Inoculum was prepared from plants systemically infected with PVY-M^SN^R, and inoculations were done as described for TMV. Necrotic lesions appeared within 7–10 days in leaves from plants resistant to *M. incognita*.

Wildfire inoculation. Population 2 was also screened for wildfire resistance using the same procedure as with root knot. Leaves of resistant plants developed chlorotic lesions at the inoculation site, and leaves of susceptible genotypes developed necrotic lesions 7 days after inoculation. Leaves of cultivars Burley 21 (resistant) and Judy's Pride (susceptible) were included along with leaves of the parental cultivars as controls.

Cultures were initiated by macerating leaf tissue from the borders of lesions in a few milliliters of distilled water. The resulting suspension was streaked on nutrient agar plates to obtain distinct colonies and incubated for 24 hr at 25 C. Cream-colored colonies were selected from the plates and passed twice through a susceptible host to ensure virulence. Three to five loopfuls of bacterial suspension were placed in 50 ml of nutrient broth contained in 125-ml flasks and incubated for 18 hr at 15–25 C. Inoculum was diluted 1:4 (v/v) with sterile, distilled water before inoculation to provide a concentration of $1–3 \times 10^6$ cells per milliliter.

A self-feeding inoculator was devised by driving a thin nail through the inside of the metal cap of a small screw-cap container. The metallic protrusions on the outside of the cap served to wound the leaf. Inoculum suspension was placed in the inoculator and capped. Two leaves from each cultivar were held on a cheesecloth pad for support and wounded in the interveinal region in three or four areas.

Doubled haploids. The midvein culture technique (5) was used to double chromosome numbers in a random array of haploids from each of the three populations. The doubled haploids obtained were evaluated for their reactions to TMV, root-knot, or wildfire to compare disease reactions in detached leaves of haploids with reactions of whole plants of the same genotypes as doubled haploids in replicated trials. Known resistant and susceptible plants and detached leaves were included as controls in every case.

Haploid and diploid forms of standard burley cultivars with known reactions to wildfire were also inoculated with *P. syringae* pv. *tabaci*, using detached leaves to ascertain the validity of the inoculation method with this pathogen.

RESULTS AND DISCUSSION

A total of 613 haploids derived from the hybrid Jaraiz 1 × Havana 307 were evaluated for TMV resistance using detached leaves (Table 1). Five days after inoculation, 300 haploids were classified as resistant on the basis of presence of numerous necrotic local lesions similar to those normally observed in TMV-resistant plants (hypersensitive reaction). In the same population, 313 haploids were free of symptoms and were classified as susceptible. Of 413 haploids from the cross Ky 14 × Havana 307 similarly tested, 201 were classified as resistant and 212 as susceptible. Chi-square goodness-of-fit tests showed no significant deviation from the 1:1 segregation ratio of resistant to susceptible plants expected at the haploid level. Diploid controls, including parental cultivars as well as other resistant and susceptible cultivars, gave expected responses. These results indicate that single, detached leaves of either haploid or diploid plants may be used successfully to screen segregating populations for resistance to TMV.

Populations 2 and 3, derived from F_1 hybrids NC-528 × Ky 14 and NC-528 × Jaraiz 1, respectively, were screened for resistance to the root-knot nematode by detached-leaf inoculation using PVY-M^SN^R (Table 2). Of 57 haploids evaluated in population 2, 29 were classified as root-knot-resistant based on the presence of foliar necrotic lesions after PVY inoculation. Leaves of 28 haploids did not show symptoms and were thus classified as root-knot-susceptible. Similarly, of 20 haploids evaluated in population 3, 11 appeared resistant and nine susceptible. Diploid controls NC-95 (root-knot-resistant) and NC-2326 (root-knot susceptible) showed the expected reactions.

Twenty-three haploids from population 2 were also evaluated for wildfire resistance using single, detached leaves (Table 3). Wildfire resistance was characterized by development of chlorotic lesions and wildfire susceptibility by the development of severe, necrotic lesions surrounded by large, chlorotic halos. On this basis, nine haploids were classified as resistant and 14 as susceptible. Leaves of cultivars Burley 21 and Ky 14 were used as wildfire-resistant controls and Judy's Pride and Jaraiz 1 as susceptible controls. All controls showed the expected reactions. In all cases, chi-square goodness-of-fit tests indicated no significant deviation from the expected 1:1 segregation ratios.

Disease reactions for doubled-haploids at the whole-plant level were compared with those of parental haploids using detached

TABLE 1. Evaluation of tobacco haploids (population 1), diploid controls, and diploid parents for resistance to tobacco mosaic virus by detached-leaf inoculation

	Number of plants				
	Total	Resistant	Susceptible	χ^{2a}	*P*
Source of haploids					
Jaraiz 1 × Havana 307	613	300	313	0.28	0.75–0.5
Ky 14 × Havana 307	413	201	212	0.29	0.75–0.5
Diploid controls					
Jaraiz 1	10	10	0		
Ky 14	11	11	0		
Havana 307	31	0	31		
Burley 21	81	81	0		
NC-2326	15	0	15		

[a]Chi-square value based on the hypothesis of 1:1 segregation ratio of resistant to susceptible plants.

TABLE 2. Evaluation of tobacco haploids (populations 2 and 3) and diploid controls for resistance to *Meloidogyne incognita* by detached-leaf inoculation using strain M^SN^R of potato virus Y

	Number of plants				
	Total	Resistant	Susceptible	χ^{2a}	*P*
Source of haploids					
NC-528 × Ky 14 (population 2)	57	29	28	0.018	0.95–0.9
NC-528 × Jaraiz 1 (population 3)	20	11	9	0.20	0.75–0.5
Diploid controls					
NC-95	10	10	0		
NC-2326	10	0	10		

[a]Chi-square value based upon the hypothesis of 1:1 segregation ratio of resistant to susceptible plants.

TABLE 3. Evaluation of tobacco haploids (population 2), diploid controls, and diploid parents for resistance to *Pseudomonas syringae* pv. *tabaci* by detached-leaf inoculation

	Number of plants				
	Total	Resistant	Susceptible	χ^{2a}	*P*
Source of haploids					
NC-528 × Ky 14	23	9	14	1.09	0.5–0.25
Diploid controls					
Burley 21	5	5	0		
Judy's Pride	5	0	5		
Ky 14	2	2	0		
NC-528	1	0	1		

[a]Chi-square value based on the hypothesis of 1:1 segregation ratio of resistant to susceptible plants.

leaves. Reactions to TMV were identical on detached leaves and whole plants. Predicted disease reactions for *M. incognita* corresponded in 49 of 50 determinations. One genotype classified as root-knot-susceptible by detached-leaf inoculation was later classified as resistant in whole-plant evaluations. Failure to detect a resistant genotype was probably an inoculation escape in the initial screening. For wildfire, disease reactions corresponded in 21 of 23 cases. Two haploids gave a mixed reaction when single, detached leaves were inoculated and erroneously classified as susceptible. Necrosis can develop in resistant plants if leaves are severely wounded or if the bacterium penetrates the vascular system. Consequently, great care must be exercised during inoculation to avoid excessive injury. To avoid the possibility of making erroneous determinations with this disease, we recommend that two or three leaves of a given genotype be used in the screening process.

As a final test for the validity of detached-leaf inoculations with wildfire, several burley tobacco cultivars with known reactions to that disease were evaluated. Leaves from field-grown haploid and diploid versions of these cultivars were used in this test. All cultivars responded as expected (Table 4). These results demonstrate that detached-leaf inoculations are effective for classifying genotypes for wildfire resistance and that greenhouse or field-grown plants may be used as the source of leaves.

The detached-leaf inoculation techniques reported here should be advantageous to any tobacco breeding program emphasizing disease resistance whether the main objective of the program is population improvement or development of pure lines to be used as cultivars. The procedure outlined can be used equally well for selecting disease-resistant genotypes at either the haploid or diploid level, but it is particularly useful in a haploid breeding program. Evaluation at the haploid level offers the unique advantage that it corresponds directly with gametic evaluation, and thus, identification of desirable gametes can take place before they enter the reproductive cycle. Selection at the gametic level is especially useful when traits of interest are simply inherited as are many disease resistance reactions.

Narrow-sense heritabilities for disease reaction conditioned by dominant genes will be 1.0 in haploid arrays but less than 1.0 in diploid populations because of dominance variance in the latter. Nevertheless, a serious drawback limiting the scope of haploid breeding for disease resistance is the inability to evaluate haploids for resistance to multiple pathogens simultaneously, because only a single plant is available from every genotype. Inoculation of detached leaves eliminates some of these problems and permits evaluation of selected multiple-disease-resistant genotypes for other traits, because pathogens have not been introduced systemically.

Chromosome-doubling efforts can be directed only toward desirable genotypes, and a large number of undesirable genotypes can be eliminated. The breeding population may thus be fixed for disease resistance genes before field evaluation for agronomic characteristics. Disease-resistant selections may be evaluated as lines per se for yield, quality, and other traits or intermated in a recurrent-selection fashion to produce new populations in which to practice further selection. Breeding efforts should be facilitated because there will not be segregation for those disease-resistance factors already fixed, and population sizes would have been reduced to a manageable level. Furthermore, seed production, processing, and storage are limited to desirable genotypes. The overall result represents a considerable savings of time, effort, and resources.

TABLE 4. Comparison of known reactions of burley tobacco cultivars to *Pseudomonas syringae* pv. *tabaci* with reactions of haploid and diploid forms of the same cultivars using detached-leaf inoculations

Cultivar	Known reaction[a]	Ploidy	Detached-leaf reaction[a]
Burley 21	R	Diploid	R
		Haploid	R
Burley 49	R	Diploid	R
		Haploid	R
Ky 10	S	Diploid	S
Ky 14	R	Diploid	R
		Haploid	R
Ky 15	R	Diploid	R
Ky 16	S	Diploid	S
Ky 17	R	Diploid	R
		Haploid	R
Ky 35	S	Diploid	S
Greenville 131	R	Diploid	R
		Haploid	R
Greenville 136	R	Diploid	R
		Haploid	R
Va 528	R	Diploid	R
		Haploid	R
Jaraiz 1	S	Diploid	S
MB (J)	S	Diploid	S
NC-528	S	Diploid	S

[a] R = resistant and S = susceptible.

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