Theor Appl Genet (1992) 84:473–479

Anther culture as a probable source of resistance to tobacco black shank caused by *Phytophthora parasitica* var 'nicotianae'

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Received October 4, 1991; Accepted November 15, 1991
Communicated by Hu Han

Summary. Anther-derived doubled haploid (ADH) tobacco lines possessing a high level of resistance to tobacco black shank, *Phytophthora parasitica* (Dast.) var 'nicotianae' (B. de Haan) Tucker (Ppn), have been identified from a cross of two tobacco cultivars susceptible to this disease. The objective of this study was to investigate the origin of black shank resistance in ADH lines developed from two susceptible parental cultivars: 'Ovens 62' and 'Ky 15'. In addition to the ADH lines, sexually-derived $F_{2:8}$ lines were produced using the single seed descent (SSD) method. Seventy-five ADH lines and 75 SSD lines along with two black shank resistant and three susceptible controls (including parental cultivars) were evaluated under field conditions for resistance to Ppn in 1989 and 1990. Lines were assigned at random to five sets and were planted in a randomized complete block design with three replications/set. A disease index was computed from weekly stand counts of the number of surviving plants per plot for each tobacco line. The 1989 experiment revealed resistance to Ppn in 8 ADH lines, numbers 29, 31, 32, 40, 68, 69, 74, 76, and 1 SSD line, number 32. The 8 ADH lines continued to show resistance in 1990, but SSD line number 32 exhibited a susceptible reaction. There were no other resistant SSD lines.

A second field experiment was conducted in 1990 using the putative resistant ADH lines and SSD line nr. 32, which expressed resistance to black shank in 1989. In addition, 12 randomly selected lines from the original 150 ADH and SSD lines were evaluated along with the same five controls as in the previous experiment to further substantiate the resistance of the ADH lines. Lines were planted in a randomized complete block design with eight replications. ADH lines continued to express resistance. SSD line nr. 32 did not show any resistance nor did any other line derived through the single seed descent procedure.

These results support the hypothesis that anther culture can generate a high level of black shank resistance. The basis for this resistance is postulated to be due to gene amplification of factors that regulate host response to the black shank fungus.

Key words: Anther-derived doubled haploid – Single seed descent – Residual heterozygosity – Chromosome doubling – DNA amplification

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Introduction

Black shank of tobacco (*Nicotiana tabacum* L.) is caused by the soil-borne fungus *Phytophthora parasitica* (Dast.) var 'nicotianae' (Breda de Haan) Tucker (Ppn) (Lucas 1975). This disease causes necrosis of the roots and basal area of the stem, dramatically reducing the yield and quality of tobacco. In North Carolina estimated losses due to black shank from crop loss and control expenses in flue-cured tobacco were approximately $ 17 million in 1990 (Melton et al. 1991). Control of tobacco black shank is accomplished by using a combination of chemical treatment, cultural practices, and resistant cultivars (Melton et al. 1991).

Intraspecific breeding using the *N. tabacum* cv 'Florida 301' developed by Tisdale in 1922 and interspecific breeding using the wild species *N. plumbaginifolia* and *N. longiflora* have resulted in the development of black shank resistant cultivars (Tisdale 1931; Lucas 1975). Inheritance of resistance to black shank in 'Florida 301' has been reported to be dominant and oligogenic (Wernsman and Rufty 1987) and also recessive and simple (Clayton

1958). Resistance derived from *N. longiflora* is reported to be controlled by a single dominant gene, and resistance derived from *N. plumbaginifolia* is controlled by a single gene that is partially dominant (Chaplin 1962). Cultivars 'Burley 49', 'Tn 86', 'Va 509', 'Coker 371-Gold', 'Coker 258', 'Coker 206', 'NC 2326', 'K 326', and 'Speight G-80' are a few examples of black shank resistant burley and flue-cured tobacco cultivars available to growers (Melton et al. 1991). The development of resistant cultivars through intra- and interspecific breeding methods is laborious and time consuming; therefore, faster and easier methods for obtaining resistance are desired.

Nitsch and Nitsch (1969) produced haploid tobacco plants through an in vitro anther culture procedure, but found haploid plants and flowers to be smaller than those of diploids by about one-third. The chromosome complement of haploid tobacco plants can be doubled through a midvein culture procedure (Kasperbauer and Collins 1972) or a colchicine treatment to produce doubled-haploid lines. Chromosome-doubling results in complete homozygosity of tobacco lines in one generation. Schnell et al. (1980) stated that the use of the anther culture procedure to produce doubled haploids could lessen the inbreeding or genotype stabilization process of tobacco breeding by 3–4 years. Nevertheless, anther-derived doubled-haploid lines have generally been found to be significantly lower yielding and of inferior quality compared to inbred lines developed through sexual hybridization (Wernsman and Rufty 1987). Larkin and Scowcroft (1981) reviewed tissue culture-induced variation in several crops and concluded that the variation and vigor reduction among doubled haploids were not so severe as to negate the efficiency of doubled-haploid breeding.

Since Nitsch and Nitsch's investigations showing reduced size and vigor in haploid plants compared to the source cultivates, great variation in haploids and doubled haploids has been found (Collins et al. 1974; Burk and Matzinger 1976; Arcia et al. 1978; De Paepe et al. 1982). The utility of the anther culture procedure to produce agronomically useful lines has been demonstrated. Wark transferred resistance to two races of the blue mold fungus (*Peronospora tabacina*) to commercial varieties of flue-cured tobacco using the anther culture procedure (Wark 1977). Burk and Chaplin (1980) also used this procedure to obtain haploids from an F_1 hybrid anther source and found that some of the doubled-haploids lines yielded significantly more than the mid-parent value. Witherspoon et al. (1987) generated a doubled-haploid line resistant to a necrotic strain of potato virus Y through anther culture of the susceptible tobacco cv 'McNair 944'. An experiment using anther-derived doubled-haploid (ADH) lines was conducted in 1983 in Puerto Rico to select for resistance to tobacco blue mold (*Peronospora tabacina*) (Rufty 1989). The field was naturally infested with Ppn, and black shank developed; however, high levels of resistance were expressed by some of the ADH lines although they had been derived from two black shank susceptible parental cultivars, 'Ovens 62' and 'Ky 15'.

The objective of this study was to determine the origin of resistance among black-shank resistant ADH lines observed in Puerto Rico; i.e., to elucidate whether the anther culture procedure induced the resistance or whether it was due to gene complementation or epistasis between the susceptible parental cultivars. Experiments were designed to compare levels of resistance in lines derived through anther culture with those of $F_{2:8}$ lines derived through inbreeding from the same parental sources.

Materials and methods

Experiment 1

Two groups of tobacco lines were derived from the cross of 'Ovens 62' and 'Ky 15' (two cultivars highly susceptible to Ppn): (1) Anther-derived doubled haploids (ADH) and (2) $F_{2:8}$ inbred lines derived by the single seed descent method (SSD). Haploids were produced from culture of anthers from F_1 generation plants (Rufty 1989). Resulting haploids were chromosome doubled using a 0.4% colchicine solution or through an in vitro midvein culture technique (Kasperbauer and Collins 1972). Plantlets used for chromosome doubling with colchicine were removed from anther culture plates when the first true leaves were 3–5 mm in length and were immersed in the colchicine solution for 4 h. Fully expanded or mature tobacco leaves were used for chromosome doubling through the midvein culture technique.

Experiments with ADH and SSD lines were conducted in 1989 and 1990 at the Upper Coastal Plain Research Station, Rocky Mount, N.C. The field soil was comprised of Norfolk loamy sand (fine-loamy, siliceous, thermic, typic kandiudult 0–3% slopes), Goldsboro fine sandy loam (fine-loamy, siliceous, thermic, aquic paleudult 0–2% slopes), and Lynchburg fine sandy loam (fine-loamy, siliceous, thermic., aeric paleaquilt). Seventy-five SSD lines and 75 ADH lines were randomly selected for testing and subdivided into five sets with 15 ADH and 15 SSD lines in each set. Five control cultivars, 'Ovens 62', 'Ky 15', 'Hicks', 'Burley 49', and 'Coker 258', representing the two susceptible parental cultivars, an additional highly susceptible cultivar, and two highly resistant cultivars, respectively, were also added to each set giving a total of 35 entries in each set. Entries were replicated 3 times in each set.

Seed of each entry of each plot was sown in prepared plant beds. Seedlings were transplanted to a field naturally infested with Ppn in a randomized complete block design. The experiment consisted of 525, single-row, five-plant plots with 56 cm between plants and 122 cm between rows. Standard fertilization practices for flue-cured tobacco were followed. Total rainfall and irrigation from May through July was 56.13 cm in 1989 and 41.38 cm in 1990.

Starting in early June, when a complete stand was obtained, weekly counts of the total number of surviving plants per plot were taken for approximately 2 months. Plant death was ascribed to black shank when severe symptoms of the disease (wilting and browning of the leaves and basal black stem lesions) were evident in more than 75% of the plant tissue. A disease index was computed for each tobacco line as a weighted scale according to disease progression using the following formula

(Csinos 1983):

$$DI = \frac{\sum_{i=1}^{n} Xi\left[100 - (i-1)\frac{100}{n}\right]}{I}$$

where i = ordinal evaluation number, n = number of evaluations (excluding initial stand count), X = the number of dead plants since last stand count, and I = initial number of plants per plot. Plant death early in the season was weighted heavier than plant death later in the season. Data were pooled over years. Analysis of variance (ANOVA) was performed on the pooled data and contrasts of ADH versus SSD, ADH versus parents, SSD versus parents, resistant ADH versus resistant controls, and SSD versus resistant controls were performed using the Statistical Analysis System, SAS (SAS 1985).

Experiment 2

A second experiment was conducted in 1990 based on the results of the 1989 test, which revealed 8 ADH lines and 1 SSD line as having resistance to Ppn. Seven of the 8 ADH lines and 1 SSD line along with 12 randomly selected lines and the same five controls as in the previous experiment were tested to further characterize the resistance to Ppn observed in the other experiments. The experiment ultilized a total of 200, 22-plant plots arranged in a randomized complete block design with eight replications. Planting, fertilization, plant spacing, data collection, and analysis were conducted in the same manner as the previous experiments.

Results

The analysis of variance conducted over years showed all sources of variation to be significant (Table 1). To obtain a more accurate estimate of among-entry variation, analyses were conducted on ADH and SSD entries only, i.e., variation among control cultivars was excluded. The total variation among ADH lines was greater than twice the amount of variation found among SSD lines as indicated by mean squares. Using these analyses, we calculated components of variance, and genetic variances were estimated to be 256.20 and 78.75 for ADH and SSD lines, respectively. Thus, the genetic variation among ADH entries was greater than 3 times that observed among SSD entries.

Differences in the variation among the ADH lines and among SSD lines can be readily visualized in a frequency distribution histogram (Fig. 1). The range of mean disease indices for the ADH group is 2–79, whereas the range for the SSD group is 28–87.

Mean population disease indices for all entries in the sets, computed from weekly stand counts, are shown in Table 2. Lower disease indices were observed among ADH lines as compared to SSD lines. The lowest disease indices (indicating a high level of black shank resistance) were found in some ADH lines and the resistant control cvs 'Burley 49' and 'Coker 258'. Mean disease indices, averaged over years, for ADH lines nr. 29, 31, 32, 40, 68, 69, 74, and 76 were 16.0, 13.5, 2.5, 14.5, 14.0, 21,0, 14.0, and 16.0, respectively. Mean disease indices for the controls averaged over sets and years were: 5.0 for 'Burley 49' and 7.5 for 'Coker 258' (resistant controls); 31.4 and 74.9 for 'Ovens 62' and 'Ky 15' (susceptible parental cultivars), respectively; and 35.8 for 'Hicks' (susceptible control). In 1989 SSD line nr. 32 appeared to be resistant to Ppn with a disease index of 5.0, but no resistance was expressed in 1990 (disease index = 50.0). Disease indices were generally higher in 1989 than in 1990.

A contrast of the mean disease indices over years of ADH versus SSD entries showed the two groups to be significantly different at the 0.001 level (Table 3). The contrast of ADH entries versus the two susceptible parental controls, 'Ovens 62' and 'Ky 15', was also highly signifi-

Table 1. Analysis of variance over years of disease indices representing the response of anther-derived doubled haploids (ADH), single seed descent (SSD) $F_{2:8}$ lines, and control cultivars to *Phytophthora parasitica* var 'nicotianae'. Rocky Mount, N.C., 1989 and 1990

Source	*df*	SS [a]	MS [b]	*F* value	$P > F$
Year	1	198 749.62	198 749.62	966.04	0.0000
Set	4	39 396.20	9 849.05	47.87	0.0001
Year set	4	23 646.57	5 911.64	28.73	0.0001
Rep (year set)	20	29 363.14	1 468.16	7.14	0.0001
Entry (set)	170	367 116.83	2 159.51	10.50	0.0000
Among ADH	70	127 438.36	1 820.55	9.39	0.0001
Among SSD	70	55 645.70	794.94	3.37	0.0001
Year entry (set)	170	59 337.80	349.05	1.70	0.0001
Entry rep (set year)	680	136 403.17	205.74		
Total	1049				

[a] SS represents the sums of squares
[b] MS represents the mean squares

Table 2. Mean disease indices (DI)[a] of anther-derived doubled haploids (ADH), single seed descent (SSD) $F_{2:8}$ lines, and control cultivars in response to *Phytophthora parasitica* var 'nicotianae' under field conditions. Rocky Mount, N.C., 1989–1990

Disease index (DI)				Disease index (DI)			
Entry	1989	1990	Mean	Entry	1989	1990	Mean
Set 1				Set 4			
ADH	72	28	50.0	ADH	57	37	47.0
SSD	80	33	56.5	SSD	72	43	57.5
Ovens 62	64	18	41.0	Ovens 62	37	19	28.0
Ky 15	92	70	81.0	Ky 15	88	69	78.5
Burley 49	34	0	17.0	Burley 49	6	5	5.5
Coker 258	37	6	21.5	Coker 258	9	0	4.5
Hicks	52	26	39.0	Hicks	40	37	38.5
Set 2				Set 5			
ADH	45	23	34.5	ADH	48	19	33.5
SSD	65	33	49.0	SSD	65	36	50.5
Ovens 62	49	28	38.5	Ovens 62	30	13	21.5
Ky 15	80	63	71.5	Ky 15	80	65	72.5
Burley 49	1	4	2.5	Burley 49	0	0	0.0
Coker 258	4	0	2.0	Coker 258	10	0	5.0
Hicks	35	34	34.5	Hicks	33	15	24.0
Set 3							
ADH	64	37	50.5	LSD 0.05 = 7.3			
SSD	69	58	63.5	LSD 0.01 = 9.5			
Ovens 62	48	8	28.0				
Ky 15	89	53	71.0				
Burley 49	0	0	0.0				
Coker 258	9	0	4.5				
Hicks	47	39	43.0				

[a] $$DI = \frac{\sum_{i=1}^{n} Xi\left[100-(i-1)\frac{100}{n}\right]}{I}$$

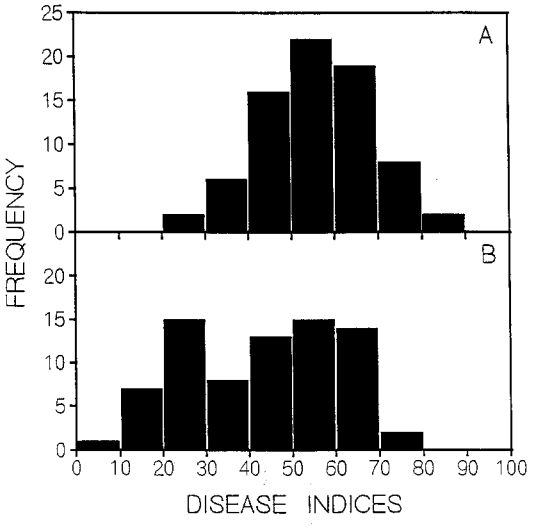

Fig. 1 A, B. Histogram of disease indices representing the response of single seed descent (SSD) $F_{2:8}$ **(A)** and anther-derived doubled-habloid (ADH) **(B)** lines to *Phytophthora parasitica* var 'nicotianae' under field conditions. Rocky Mount, N.C., 1989 and 1990. $n = 5250$

cant. No significant difference was found in the contrast of SSD entries versus the susceptible parental controls. Another contrast of interest was seen when the two groups of lines were compared with the resistant control cultivars. The SSD entries versus resistant control had a P value of 0.0001, but the ADH entries versus resistant controls had a P value of 0.031, indicating that the ADH lines as a whole are more like the resistant controls than SSD lines in their expression of resistance to Ppn.

Disease indices calculated for the second experiment conducted in 1990 again revealed greater variation among ADH lines than among SSD lines (Table 4). ADH lines nr. 29, 31, 32, 40, 68, 69, and 74 and the resistance controls 'Burley 49' and 'Coker 258' had disease indices of 4.5, 5.5, 1.8, 7.4, 9.8, 5.6, 7.4, 4.7, and 2.8 respectively. SSD line nr. 32 had a disease index of 55.8. Parental cvs 'Ovens 62' and 'Ky 15' had disease indices of 25.4 and 58.8, respectively. 'Hicks', the susceptible cultivar, had a disease index of 21.7. No SSD line appeared to have obtained any resistance to Ppn, whereas the ADH lines continued to express resistance. Contrasts of the mean

Table 3. Contrasts[a] of mean disease indices of anther-derived doubled haploids (ADH) versus single seed descent (SSD) lines, ADH versus parental cultivars[b], and SSD versus parental cultivars[b], which represent the host response to *Phytophthora parasitica* var 'nicotianae'. Data from Rocky Mount, N.C., 1989 and 1990

Contrast	*df*	SS[c]	*F* value	*P*>*F*
ADH versus SSD	5	35 592.95	101.97	0.0001
ADH versus parents	5	5 144.80	14.74	0.0002
SSD versus parents	5	453.91	1.30	0.2557

[a] Tests of hypotheses were constructed using the type III mean squares for year * entry (set) as an error term. Data were pooled over sets and years
[b] Parental cultivars are 'Ovens 62' and 'Ky 15'
[c] SS represents the sums of squares

Table 4. Mean disease indices (DI)[a] of anther-derived doubled haploids (ADH), single seed descent (SSD) $F_{2:8}$ lines, and control cultivars in response to *Phytophthora parasitica* var 'nicotianae' under field conditions. Data represent averages of eight replications ($n = 176$). Rocky Mount, N.C., 1990

Disease index (DI)		Disease index (DI)	
Entry	1990	Entry	1990
ADH-26	44.1	SSD-32	55.8
ADH-29	4.5	SSD-34	47.2
ADH-31	5.5	SSD-37	39.0
ADH-32	1.8	SSD-47	50.0
ADH-35	16.8	SSD-82	39.8
ADH-40	7.4		
ADH-44	41.5		
ADH-56	54.5	Ovens 62	25.4
ADH-66	10.7	Ky 15	58.8
ADH-67	50.3	Burley 49	4.7
ADH-68	9.8	Coker 258	2.8
ADH-69	5.6	Hicks	21.7
ADH-70	45.8		
ADH-74	7.4	LSD 0.05 =	1.6
ADH-77	55.3	LSD 0.01 =	2.1

[a] $DI = \dfrac{\sum_{i=1}^{n} Xi\left[100-(i-1)\dfrac{100}{n}\right]}{I}$

disease indices of the resistant ADH lines versus the susceptible parental cultivars and SSD lines versus the resistant controls showed the groups to be significantly different at the 0.001 level (Table 5). No significant difference was found in the contrasts of resistant ADH lines versus resistant controls and SSD lines versus the susceptible parental cultivars.

Discussion

Disease indices were greater in 1989 than in 1990 possible because there was more rain early in the season in 1989 than in 1990. Saturated soil greatly increases the levels of infection of tobacco by Ppn (Shew 1983). Even though the two seasons had very different environments for infection, 8 anther-derived doubled-haploid (ADH) lines expressed resistance to Ppn during both seasons of testing. The resistant ADH lines were generally symptomless and had a survival rate of approximately 100%. Low mean disease index values, denoting resistance, were found only in ADH lines and the resistant controls 'Burley 49' and 'Coker 258'. In particular, ADH line nr. 32 was as resistant as the resistant controls. One SSD line, nr. 32, appeared resistant to Ppn in 1989 but did not show resistance in either of the two tests conducted in 1990. The apparent resistance observed in 1989 was probably due to environmental conditions or other factors which allowed SSD nr. 32 to escape infection. Since no lines derived through conventional inbreeding (SSD) appeared to be resistant, the resistant reaction to tobacco black shank observed among ADH lines appears to have been obtained through the anther culture procedure. If genetic complementation or epistasis had been the basis for the resistance to tobacco black shank, then resistant lines would also have been found in the SSD group.

A wider range in phenotypic variation, as measured by disease indices, was observed among ADH lines as compared to SSD lines. The degree of genetic variation among ADH lines was also much higher in ADH than in SSD, as shown by the partitioning of the variance. In addition, the contrast of the ADH with the SSD group was highly significant, indicating that populations of lines derived through the two methods differ in their reaction to tobacco black shank. When both the ADH lines and the SSD lines were compared with the two susceptible parental cultivars, the ADH lines were significantly different from the parental lines, but the SSD lines were not;

Table 5. Contrasts of the mean disease indices of resistant ADH line numbers 29, 31, 32, 40, and 69 versus parental controls[a] and resistant controls[b], and SSD lines versus the parental controls and resistant controls, which represent the host response to *Phytophthora parasitica* var 'nicotianae'. Data from Rocky Mount, N.C., 1990

Contrast	*df*	Sum of squares	*F* value	*P*>*F*
Resistant ADH versus parents	1	15 832.01	260.44	0.0001
Resistant ADH versus resistant control	1	10.13	0.17	0.6836
SSD versus parents	1	204.16	3.36	0.0686
SSD versus resistant control	1	20 461.62	336.60	0.0001

[a] Parental controls were 'Ovens 62' and 'Ky 15'
[b] Resistant controls were 'Burley 49' and 'Coker 258'

i.e., there is resistance being generated only among ADH lines.

Experiment 2, in 1990, was consistent with the other two experiments. Resistant ADH lines continued to be resistant, no resistance was found in SSD lines, and greater variation was observed among ADH lines than among SSD lines in a much larger and highly replicated experiment. As before, contrasts revealed significant differences between resistant ADH lines and parental cultivars and between SSD lines and resistant controls, whereas no significant differences were found between resistant ADH lines and resistant controls and between SSD lines and parental cultivars. All results were consistent and provide evidence to support the hypothesis that the anther culture procedure has generated high levels of resistance to Ppn in lines derived from susceptible cultivars. Exactly where this change has occurred is not known, but it may be occurring in any part of the resistant pathway of the host plant. Explanations of genetic changes observed among ADH lines derived from cultivars have included loss of residual heterozygosity, mutational effects of the chromosome doubling procedures, and changes in the nuclear DNA.

Brown and Wernsman (1982) tested highly inbred cultivars, doubled haploids from these controls, and hybrids derived by crossing the doubled haploids and concluded that loss of heterozygosity cannot account for all of the variation found in ADH lines. Wernsman (1988 unpublished data) found the midvein culture technique used for chromosome doubling to be an insignificant factor in the variation of ADH lines. Similarly, chromosome doubling with colchicine has been shown to be of little importance (Collins et al. 1974; Burk and Matzinger 1976). The current explanation for the basis of the variability generated by the anther culture procedure is the amplification of nuclear DNA (De Paepe et al. 1982; Dhillon et al. 1983; Reed and Wernsman 1989).

Haploid tobacco plants produced from pollen (anther) culture arise from the vegetative cell of the binucleate pollen grain (Sunderland 1980). D'Amato et al. (1965) found that there is an increase in DNA without nuclear division in the vegetative nucleus of barley and tobacco. The vegetative cell is not involved in normal sexual fertilization. De Paepe et al. (1982) found heritable quantitative and qualitative changes in the nuclear DNA of anther-derived doubled haploids of *N. sylvestris*, the maternal ancestor of tobacco (Goodspeed and Clausen 1928). Dhillon et al. (1983) compared the tobacco cv 'Coker 139' and two doubled haploids produced from it using the anther culture procedure and found significantly higher amounts of DNA in the doubled haploids than the source cultivar. DNA synthesis of the vegetative nucleus may be the cause of variation found in anther-derived doubled haploids, but further investigation is necessary to determine if DNA amplification occurs elsewhere during the anther culture procedure. Nevertheless, results obtained in this study indicate that significant and useful changes have occurred during the anther culture procedure such as the apparent generation of resistance to Ppn among lines derived from susceptible parental cultivars.

The results presented in this study should be of great interest to tobacco breeders since in the past most of the variation found among ADH lines was undesirable. At present there are no other reports of resistance being generated by anther culture to soil-borne fungal pathogens. This information may also be useful to individuals involved in research at the molecular level pertaining to gene regulation and mechanisms of disease resistance. The value of the anther culture procedure needs to be explored further, particularly for those host × pathogen systems where there is no resistance available.

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