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Somaclonal variation in celery: screening for resistance to *Fusarium oxysporum* f. sp. *apii*

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Summary. Two methods were used to screen putative *Fusarium*-resistant celery (*Apium graveolens* L.) plantlets from cell culture: placing plantlets on a mycelial mat for one month or planting them directly in *Fusarium*-infested soil. Resistant phenotypes were identified with both methods, but the plants grown on the mycelial mat died before they reached reproductive maturity. Four plants, K, T-2, T-3, and R-R_1, from the soil screen, survived and produced viable seed. Tests of self-pollinated progeny, in field and greenhouse conditions, showed that T-2, T-3, and R-R_1 were superior to the original cultivar, 5270R, with respect to disease resistance, as measured by vascular discoloration and plant height. Chi-square analysis of progeny scores for root and crown decay showed that the new variation was heritable and appeared to be conditioned by more than one locus.

Key words: *Fusarium oxysporum* f. sp. *apii* – Tissue culture – Celery – Somaclonal variation

Introduction

The potential of somaclonal variation for plant improvement first became apparent in sugarcane in which variation in morphological, cytogenetic and enzyme traits were reported as early as 1969 (Heinz and Mee 1969). The applications of tissue culture in crop improvement have increased dramatically to include selection for herbicide resistance, stress tolerance, amino acid overproduction, physiological mutants, and disease resistance (Larkin and Scowcroft 1981; Tomes and Swanson 1982; Evans and Sharp 1983; Chaleff and Ray 1984; Maliga 1984).

One area in which somaclonal variation could be particularly useful is in the selection of plants for disease resistance. Selection for increased tolerance to specific diseases has been achieved using known toxins or crude culture filtrates. Gengenbach et al. (1977) reported the selection of somaclonal variants from cultures of maize T-cytoplasm resistant to the toxin produced by *Helminthosporium maydis* race T and the disease it causes, southern corn blight. Increased resistance to two pathogens of tobacco, *Pseudomonas syringae* and *Alternaria alternata,* were reported by Thanutong et al. (1983) after selecting protoplast-derived callus on media containing the pathotoxins. Regeneration from callus of potato plants resistant to *Phytophthora infestans* culture filtrate and the pathogen were reported by Behnke (1979). Resistant plants and plants with reduced susceptibility to *Phoma lingam* were regenerated from callus and embryogenic cultures of haploid rape (Sacristan 1982) which survived exposure to a toxin produced by the fungus. Hartman et al. (1984) selected lines of alfalfa with increased resistance to both the culture filtrate and the pathogen, *Fusarium oxysporum* f. sp. *medicaginis.*

Other have reported increased resistance to specific pathogens by selecting variants from populations of regenerated plantlets. Examples include the development of sugarcane varieties with enhanced resistance to eyespot disease, Fiji disease and downy mildew (Heinz et al. 1977) and increased resistance to verticillium wilt in *Medicago sativa* L. related to increased ploidy levels (Latunde-Dada and Lucas 1983).

The incursion of *Fusarium oxysporum* f. sp. *apii* race 2 (FOA_2) into the prime celery growing regions of California is a major concern because the commonly

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cultivated varieties are highly susceptible. Symptoms of the disease are severe stunting, yellowing of the leaves, susceptibility to wilt, and vascular browning and decay extending from the root tips into the crown. FOA_2 is a soil borne pathogen that persists in soil for long periods of time. Therefore, aside from soil fumigation which is expensive, the only effective control is the use of resistant cultivars. Although progress has been made toward developing a FOA_2 resistant line by conventional methods (Orton et al. 1984b), this breeding line has not yet achieved stable marketable horticultural characteristics.

Because of its regenerative abilities from cell culture (Williams and Collin 1976; Chen 1976; Fujii 1982; Rappaport 1979–1980) and evidence of somaclonal variation in the regenerated plants (Rappaport 1980–1981; Browers and Orton 1982; Fujii 1982; Pullman and Rappaport 1983), it seemed appropriate to use cell culture to select for resistance to FOA_2 to complement traditional breeding. Here we report that somaclonal variants selected from the 'Tall Utah 5270R' line (hereafter referred to as 5270R) were shown to have increased tolerance to the pathogen in greenhouse and field tests.

Materials and methods

Celery tissue culture system

Compositions of the cell culture media are versions of a modified Murashige and Skoog (1962), hereafter referred to as MS salts. The vitamin stock used in all of the media contained the following: 10 mg thiamine, 50 mg nicotinic acid, 50 mg pyridoxine, and 200 mg glycine. These compounds were dissolved first in 1 ml DMSO and then added to 99 ml dH_2O. The solution was stored at 4 °C. Sterilized apical meristems or axillary buds of the cultivar 5270R (susceptible) were placed on callus initiation medium in a controlled temperature chamber at 23.5 °C for two to three weeks under fluorescent light. Callus initiation medium contained the following compounds: 965 ml dH_2O, 4.33 g MS salts, 20.0 g sucrose, 0.1 g myo-inositol, 2.0 mg benzyladenine, 1.0 mg 2,4-D, 9.0 g Nobel Agar, and 1 ml vitamin stock. The pH was adjusted to 5.8 with 0.1 N NaOH. After callus growth was established, small pieces (1 g/30 ml) were transferred to liquid suspension culture (same as callus initiation medium without agar, except benzyladenine was replaced by 0.1 mg kinetin) to increase the number of cells, or they were transferred to regeneration medium (same as callus initiation medium, minus the hormones). Cell suspension cultures were maintained under low intensity light, while plantlets were regenerated under plant lights (Sylvania-Lifeline, 40 w). After four to six weeks the plantlets were transferred to regeneration medium plus 2% charcoal to enhance root growth. The plantlets were then transferred to sterile vermiculite in small cups and covered with plastic-wrap. Holes were poked in the plastic-wrap after three days. The covering was completely removed about one week later.

Fusarium culture

1 Barley straw inoculum. Ten ml of FOA_2 microconidal suspension in Charudattan media (Charudattan and DeVay 1972) containing 10^6 spores/ml was used to prepare a stable inoculum following the method of Schneider (1984). Five or 10 g of inoculum were used per 1 kg soil.

2 Fusarium mycelial mat. A small piece of PDA agar with *Fusarium* mycelia (~5 mm) was placed in the center of a plate of modified water agar containing 4.33 g of MS salts (Murashige and Skoog 1962) and incubated for one month on a laboratory bench at room temperature.

Selection regime – laboratory and greenhouse

Two basic approaches for selection of tolerant phenotypes were used. Either the regenerated plantlets were transplanted directly into soil containing the barley straw inoculum (see *Fusarium* culture, above), or they were plated in petri dishes on a *Fusarium* mycelial mat (see *Fusarium* culture, above) followed by an additional three to four weeks on CRM media containing the commercial fungicide Benlate (Pullman and Rappaport 1983). In both procedures, the plantlets were acclimated in the greenhouse for one to two weeks prior to transfer to soil containing the barley straw FOA_2 inoculum.

Disease evaluation of regenerated plantlets

After ten weeks in the FOA_2 infested soil, plants were sliced longitudinally and the roots and crown tissue were evaluated for evidence of the disease. Depending upon the extent of root and crown rot present, the plants were rated on a scale of 0–7 in greenhouse tests and 1–5 in field tests. The numbers indicate a range from no root or crown discoloration to completely rotted and dead. Only those plants receiving a 0, 1, or 2 rating were saved for seed production by rinsing the roots in a solution containing Benlate and transplanting them to FOA_2 free soil. Survivors were self-pollinated. Plants that received a rating of two on a scale of 0–7 rarely survived to maturity; therefore, only 0 to 1 ratings were considered resistant.

Field evaluation of putative resistant plants

Transplants grown from seed of putative resistant plants (T-3 and $R\text{-}R_1$) were grown at several locations in California fields known to be infested with FOA_2; a total of 12 plantings were made on farms at Salinas, Arroyo Grande, Ventura, Oxnard and the South Coast Field Station in Orange County. At maturity, the plants were rated for the presence of the disease; root and crown discoloration and plant height (height of tallest leaf) were used as criteria for disease evaluation. Not all of the progeny were planted in every field, so the number of locations per progeny varied from six to eight for root scores and three to four for plant height. Control populations were seedlings of 5270R and 'Tall Utah 52-70HK' (5270HK), highly susceptible and moderately tolerant cultivars, respectively.

Statistical analysis

All statistical analyses were performed using the Stats Plus program for the Apple IIe (Human Systems Dynamics).

Results

Laboratory and greenhouse tests

A total of 65 potentially resistant individuals were obtained by prescreening 3,500 regenerated plantlets on

Table 1. Greenhouse soil test (10 g inoculum per kg soil) of plantlets prescreened on FOA_2 hyphal mats compared with the control population of non-prescreened regenerated plantlets

	No. of plants	Average rating[a]	% Resistant	*t*-test
Prescreened	65	5.23±1.37	3.0	4.58**
Control	182	6.17±0.54	0	

[a] Mean±sd
** Significant at the 1% level

Table 2. Summary of the greenhouse soil screen (5 g inoculum per kg soil) for plants regenerated from cultures of plant D, plant T, and plant E

Genotype	No.	Average rating[a]	% Resistant	*t*-test
Plant D	91	4.96±1.85	6.7	1.81 NS
Control	11	6.00±1.34	0	
Plant T	4	1.75±3.50	75.0	3.55**
Control	11	6.00±1.34	0	
Plant E	233	3.38±2.16	24.5	3.09**
Control	59	4.32±1.79	5.1	

[a] Mean±sd
** Significant at the 1% level

the fungal mycelial mat. They were subsequently inoculated a second time by planting them in soil containing *Fusarium*. Table 1 shows the results of this screen. Although the mean rating of the prescreened population was significantly lower than that of the control population (not prescreened), it was considerably higher than a mean rating of 1 or 2 expected for a truly resistant population. Furthermore, none of the plants in the prescreened resistant class (rating 0 and 1) survived to maturity.

The second approach, however, did provide useful material. A total of 505 plants regenerated from a cell suspension culture initiated from tissue of 5270R were tested in soil containing FOA_2 inoculum. Only one plant, called plant K, survived to maturity. The cell lineage was continued, however, by reestablishing cultures from three of the original potentially resistant mutants, plants E, D, and T with ratings of 2, 0, and 2, respectively. Plantlets regenerated from callus or cell suspension cultures of these individuals were tested for resistance in the greenhouse. Regenerated plantlets from suspension cultures of D were not significantly different in performance from the control population, 5270R, although there was a slight increase in the number of individuals in the zero (no root or crown discoloration)

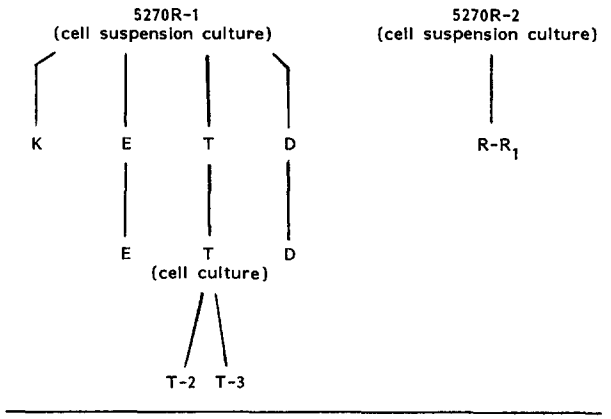

Fig. 1. Lineage of plants K, E, D, T-3, and R-R_1. 5270R-1, and 5270R-2 represent cultures derived from different individuals of 5270R

class (Table 2). Plantlets regenerated from suspension cultures of E were significantly more resistant than 5270R; 8.6% of the individuals showed no disease symptoms (Table 2). The most interesting results were obtained with the regenerates of T obtained from callus. Three of the four regenerates rated for root and crown discoloration received a 0 rating (Table 2). One plant, T-3 produced seed.

In addition to the plantlets regenerated from plants E, D, and T, 530 plantlets were regenerated from another culture of 5270R. A possible FOA_2 resistant mutant, called R-R_1, was isolated from this group. See Fig. 1 for a diagram detailing the origin of all selections discussed in this paper.

The progeny of plants T-2, T-3, R-R_1, and K were tested for disease resistance in the greenhouse soil inoculum. The progeny of T-2 were included in the evaluation, although T-2 was not screened for resistance. Table 3 shows that, on the basis of the t-test for comparison of mean ratings, the progeny of T-2, T-3, and R-R_1 were significantly more resistant to FOA_2 than 5270R, with 47%, 45%, and 35% of the individuals tested occurring in the resistant rating class, respectively. The progeny of plant K were not significantly different from the control population.

The lower disease rating scores of T-2, T-3, and R-R_1 appeared to be transmitted to their selfed progeny (as shown in Table 3). Chi-square analysis of the progeny scores indicated that the new variation could not be ascribed to a single dominant gene for any of the three selections ($P > 0.001$). However, the low numbers of progeny available prevented adequate testing of any two-locus models.

Table 3. The greenhouse soil screen (5 g inoculum per kg soil) for seed progeny of T-2, T-3, R-R_1, and plant K

Genotype	No.	Average rating[a]	% Resistant	*t*-test
T-2	77	1.94±1.80	47	8.09**
Control	39	4.70±1.67	0	
T-3	73	2.39±1.95	45	2.41*
Control	19	3.47±1.02	5	
R-R_1	83	2.60±1.87	35	2.65**
Control	23	3.70±1.19	4	
K	60	4.20±1.90	15	1.5 NS
Control	16	3.38±2.06	19	

[a] Mean±sd
* Significant at the 5% level
** Significant at the 1% level

Table 4. Mean values for root scores (rating scale 1–5) and plant height (cm) for T-3 and R-R_1 compared with the respective control population, 5270R (R) or 5270HK (HK) from field locations

No. of locations	T-3	R	R-R_1	R	T-3	HK	R-R_1	HK
	Root scores							
8	2.1	3.6						
4			2.6	4.0				
6					2.2	2.9		
4							2.6	2.8
	Plant height							
4	52.5	31.9						
3			44.1	29.8				
4					52.5	46.8		
3							44.1	39.3

Field tests

The progeny of T-3 were compared with 5270R and 5270HK plants for root and crown discoloration. T-3 had an overall mean disease score lower than either 5270R or 5270HK (Table 4) and the analysis of variance (Table 5) shows that the differences were significant. Hence, the progeny of T-3 were more resistant to FOA_2 than 5270R and 5270HK plants. In comparisons of T-3 with 5270R and T-3 with 5270HK there were highly significant effects of location on disease expression. This is not unexpected, because the fields differed in degree of fungal infestation as indicated by the response of neighboring commercial varieties. Also, there were significant genotype × location effects which indicated that T-3 responded differently than 5270R and 5270HK to different environments.

The progeny of R-R_1 had a significantly lower overall mean score for disease expression when compared to 5270R (Tables 4 and 5). However, when the progeny of R-R_1 were compared with the more tolerant line, 5270HK, there were no significant differences. Thus, the effect of location on disease expression was significantly different, but there were no significant genotype × location effects as was observed for T-3 (Table 5).

FOA_2-infected plants were noticeably stunted; therefore, plant height was included originally as a reflection of fungal infection. Unfortunately plant height cannot be used as the sole measure of disease because some cultivars are relatively tall, but nevertheless exhibit severe root rot, and vice versa. Nonetheless, height is of considerable importance for horticultural

Table 5. Two factor analysis of variance for root and crown discoloration and plant height for genotypes (G) compared across locations (L). T-3 and R-R_1 represent the progeny arrays of the two potentially resistant selections; 5270R (susceptible) and 5270HK (tolerant) are control populations. The numbers in parenthesis represent the number of field locations tested

Root scores				
	T-3 with 5270R (8)	R-R_1 with 5270R (4)	T-3 with 5270HK (6)	R-R_1 with 5270HK (4)
G	$P<0.001$**	$P<0.001$**	$P<0.001$**	$P=0.0834$ NS
L	$P<0.001$**	$P<0.001$**	$P<0.001$**	$P<0.001$**
G×L	$P=0.002$**	$P=0.2725$ NS	$P=0.002$**	$P>1$ NS
Plant height				
	T-3 with 5270R (4)	R-R_1 with 5270R (3)	T-3 with 5270HK (4)	R-R_1 with 5270HK (3)
G	$P<0.001$**	$P<0.001$**	$P<0.001$**	$P=0.009$**
L	$P<0.001$**	$P<0.001$**	$P<0.001$**	$P<0.001$**
G×L	$P<0.001$**	$P>1$ NS	$P<0.001$**	$P<0.001$**

** Significant at the 1% level
NS = no significant difference

Fig. 2. A typical T-3 progeny compared with the tallest 5270R plant in the plot. Both were harvested from a heavily infested field in Oxnard, California (June 1986)

evaluation. As shown in Tables 4 and 5, the progeny of T-3 and R-R_1 were significantly taller than individuals of the control populations. The height difference between T-3 and 5270R was often very large, as can be seen in Fig. 2. In addition, as with root scores, there were significant location effects and significant genotype × location effects, with the exception of R-R_1 with 5270R.

Discussion

One of the major limitations to utilizing somaclonal variation in a breeding program is the identification of desired mutant phenotypes. Ideally, to seek a desirable trait, selection pressure should be applied to a large population of cells. We used fusaric acid (a toxin produced by *F. oxysporum* spp.) in an attempt to select cells for increased resistance to the fungus (unpublished data). The regenerated plantlets prescreened on the media were not significantly more resistant than those not prescreened (prescreened $\bar{X} = 5.55$; not prescreened $\bar{X} = 6.17$). Thus, the principal mode of FOA_2 infection does not appear to involve a host specific toxin, a conclusion reached by other workers (Shahin 1986). Although *Fusarium* resistance in alfalfa was enhanced by regenerating plantlets on media containing a crude culture filtrate (Hartman et al. 1984), it is not clear whether the effect was due to the selection procedure, or to increased ploidy levels, a form of gene amplification which apparently occurred during cell culture.

Selecting FOA_2 resistant phenotypes at the level of regenerated plantlets is considerably more laborious and requires a great deal of space. However, a modified screening method that at first appeared to be very promising, was to expose regenerated plantlets to a mycelial mat for four weeks and planting the survivors in FOA_2 infested soil. After ten weeks in soil, two plants received a zero rating compared to none in the control population, but both succumbed to the disease before the plants could form seed. Therefore, despite the disadvantages, the soil inoculum screen was chosen as the principle selection method.

This selection procedure produced two individuals, T-3, and R-R_1, whose progeny were significantly more resistant to FOA_2 than individuals of 5270R, the line from which they were derived. It was also shown that in the field, the progeny of T-3 were more resistant to FOA_2 than the progeny of R-R_1, because unlike R-R_1 they exhibited less disease than individuals of 5270HK (a line with moderate resistance to FOA_2). The differences in disease response between T-3 and R-R_1 may be attributable primarily to two factors: 1) differences in their genetic background, and 2) the time spent in cell culture. Because celery is an outcrossing species, each individual is unique. Hence, two lines derived from different individuals may contain different alleles at loci conferring resistance. Furthermore, mutations occur spontaneously and apparently at very high rates in cell cultures (Evans and Sharp 1983). Therefore, a cell line kept in culture for a long time, or one such as T-3 which experienced two passages in culture, may conceivably contain several mutations.

In spite of the differences observed in degree of resistance, the patterns of transmission to selfed progeny of T-3 and R-R_1 were similar. The resistance appeared to be conferred by at least two genes whose expression was either codominant or recessive but no

definitive genetic model could be established. In addition, the expression of resistance was influenced by the environment, as indicated by the significant genotype × location effects (Table 5). These results are in contrast to previous reports about the inheritance of FOA_2 resistance in celeriac, which appears to be governed by a single major gene with modifiers (Orton et al. 1984a). The inheritance appears similar, however, to that seen in "OXN40", a moderately resistant selection derived from 5270R which has the horizontal (additive) type of resistance (Orton et al. 1984a). The exact origin of the genes contributing to resistance in T-3 and $R\text{-}R_1$ is unclear due to the factors mentioned previously. More than one mutational event could have occurred in these selections and might have included some form of gene amplification. Although this type of inheritance is not often reported, Carlson (1973) showed that the progeny of one methionine sulfoximine mutant exhibited a ratio indicative of more than one gene.

While the exact nature of the inheritance and the stability of transmission in T-3 and $R\text{-}R_1$ remains to be fully characterized, it nevertheless appears that we were able to increase the resistance to infection by FOA_2 in an otherwise susceptible genotype by generating new variation in cell culture. Efforts are being made to further increase the level of resistance by returning tissues of these line to culture with the aim of incorporating additional resistance genes.

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