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Selection of peach cells for insensitivity to culture filtrates of *Xanthomonas campestris* pv. *pruni* and regeneration of resistant plants

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Summary. Individual callus cultures were initiated from 400 immature embryos of bacterial leaf spot-susceptible 'Sunhigh' peach. Each was subjected to several selection cycles of a toxic culture filtrate produced by *Xanthomonas campestris* pv. *pruni*, the causal agent of leaf spot of peach. Progressively higher concentrations of the filtrate were used in each cycle. Two calli survived, and two plants were regenerated from each of the surviving calli. Each of the four clones was propagated in vitro and tested for whole plant resistance to *X. c.* pv. *pruni*. Results from bioassays on greenhouse-grown plants indicated that two out of the four selected clones were significantly more resistant to *X. c.* pv. *pruni* than the parental cv 'Sunhigh'. In addition, one clone was significantly more resistant than the moderately resistant cv 'Redhaven'.

Key words: *Prunus persica* – *Xanthomonas campestris* pv. *pruni* – Tissue culture – Cell selection – Disease resistance

Introduction

Bacterial spot, caused by the bacterium *Xanthomonas campestris* pv. *pruni* (E. F. Sm.) Dows, is a serious disease of peach [*Prunus persica* (L.) Batsch] in warm, humid, temperate environments (Jones and Sutton 1984). Many high quality commercial cultivars are susceptible to this pathogen (Fogle et al. 1974). Chemical control is costly and often ineffective, and the use of disease resistant cultivars is the recommended approach to disease control (Werner et al. 1986). Recent field evaluations (Werner et al. 1986) in the southeastern United States indicated that several cultivars previously reported to be highly resistant (Fogle et al. 1974) were only moderately resistant. In addition, none of the 58 plant introductions evaluated exceeded the level of resistance currently found in commercial cultivars grown in the United States. The scarcity of germplasm with high levels of resistance suggests that alternative approaches to conventional breeding need to be employed to obtain resistant germplasm.

In vitro selection for somaclonal variants that are insensitive to toxic metabolites produced by plant pathogens is a viable approach to obtaining disease resistant plants (Daub 1986; Hammerschlag 1984b). The feasibility of this approach depends on demonstrating that (i) a toxic metabolite is produced by the pathogen, (ii) this metabolite is involved in disease development, and (iii) the metabolite is active at the cellular level, i.e., the level employed in cell culture. An additional prerequisite for successful in vitro selection is an efficient system for regenerating plants from callus or cell cultures. Hammerschlag (1984a) demonstrated that a toxic metabolite produced by *X. c.* pv. *pruni* is involved in bacterial spot development and is active at the cellular level. Subsequently, Hammerschlag et al. (1985) developed a reliable system for regenerating peach plants from embryo-derived callus. This report describes the selection of peach cells for insensitivity to a toxic metabolite produced by *X. c.* pv. *pruni* and the regeneration of disease resistant plants from these cells.

Materials and methods

Plant material

Immature peach embryos excised from fruit removed from open-pollinated, bacterial spot-susceptible 'Sunhigh' peach trees 56–70 days after full bloom (Hammerschlag et al. 1985) were used as a source of highly regenerative callus. Greenhouse-

grown bacterial spot-susceptible 'Sunhigh' and moderately resistant 'Redhaven' (Fogle et al. 1974) plants produced by micropropagation (Hammerschlag et al. 1987) were used to supply leaves for the detached-leaf bioassay.

Culture filtrate production

Culture filtrate of *X. c.* pv. *pruni* strain XP1 (highly virulent) was prepared as previously described (Hammerschlag 1984a; Hammerschlag et al. 1982), except that the crude culture filtrate was subjected to ultrafiltration through an Amicon YCO5 membrane with a molecular weight cut-off of 500 Da (Amicon Corporation, Lexington MA). The low molecular weight toxic filtrate (TF) was filter sterilized (0.22-μm filter) and freeze-dried.

Callus initiation, selection, and regeneration

Methods for the production of primary callus from immature peach embryos, the production of highly regenerative callus from primary callus, and the regeneration of plants from highly regenerative callus were described previously (Hammerschlag et al. 1985). Four hundred primary calli (approximately 15 mg each), initiated separately from 400 immature embryos, were each transferred to a 2.5 × 2.0-cm well of a 12-well plastic dish containing 4 ml of Murashige and Skoog (1962) salts, 555.1 μ*M* myoinositol, 3.05 μ*M* nicotinic acid, 2.43 μ*M* pyridoxine HCl, 1.18 μ*M* thiamine HCl, 0.27 μ*M* α-naphthaleneacetic acid, 2.2 μ*M* benzyladenine, 87.6 m*M* sucrose, and 0.6% agar [highly regenerative callus (HRC) medium], to which 0.7 mg freeze-dried TF/ml of HRC medium was added. Callus cultures were maintained at 25°C with a 16 h photoperiod of approximately 40 $\mu E \cdot m^{-2} \cdot s^{-1}$ irradiance provided by cool white fluorescent lights. Recurrent selection (at 3 week intervals) of surviving calli (Fig. 1a) and recovered growing sectors (Fig. 1b) was performed first on 3.5 mg TF/ml HRC medium, followed by exposure to 7.0 mg (TF/ml HRC medium, a predetermined sublethal level. Finally, surviving calli were grown on HRC medium without TF and then returned to 7.0 mg TF/ml HRC medium. Plants were regenerated from surviving calli (Hammerschlag et al. 1985), micropropagated, rooted, acclimatized (Hammerschlag et al. 1987), and grown in the greenhouse.

Modified detached-leaf bioassay

A detached-leaf bioassay (Randhawa and Civerolo 1985), that was modified by incorporating a lesion rating system, was used to evaluate greenhouse-grown clones. Briefly, leaves from the third below the terminal to the eighth on an actively growing plant were detached, surface sterilized in 70% ethanol for 2 min, and rinsed with sterile distilled water. Five-centimeter mid-section pieces were prepared and were placed abaxial side up on 3–4 layers of sterile paper towelling. Inoculum of *X. c.* pv. *pruni* or *X. c.* pv. *pelargonii* (1×10^6 colony-forming units/ml sterile distilled water) or sterile distilled water (control) was then infiltrated with a needle-less 1-ml syringe at 10 sites on the abaxial side. Infiltration was continued until at each site, a 2- to 4-mm diameter area of mesophyll tissue was water soaked (Fig. 2). The inoculated leaf pieces were placed with the abaxial side up on 0.5% water agar (15–20 ml per 100 × 15 mm plastic petri dish) and incubated at 25°C under fluorescent lights (40 $\mu E \cdot m^{-2} \cdot s^{-1}$, 16 h photoperiod). After 24 h, leaf pieces that still contained visible water-soaked areas were discarded. Symptom development at each inoculated site was evaluated after 2 and 3 weeks. Symptoms were rated on a 0–3 scale with 0 = no symptoms or slight chlorotic flecks, 1 = distinct chlorotic spot and/or slight necrotic flecks, 2 = distinct necrotic spot or greyish white lesion less than 2 mm in diameter, and 3 = distinct necrotic or greyish white spot greater than 2 mm in diameter, with or without chlorotic halo. Three leaves, replicated a minimum of three times, were inoculated per clone or cultivar. Analysis of variance was performed on data, and means were separated by Duncan's multiple range test.

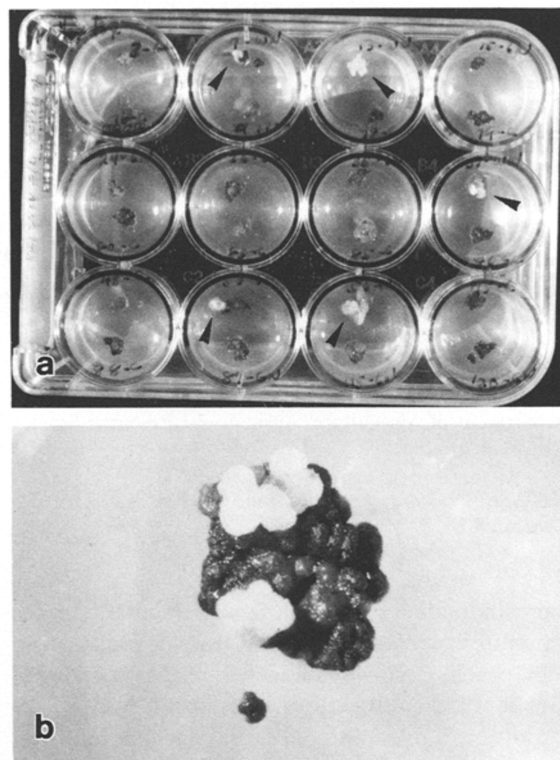

Fig. 1. a Calli (5 growing [*arrows*], 19 inhibited) 21 days after transfer to medium containing 0.7 mg freeze-dried culture filtrate/ml of medium; **b** toxic metabolite insensitive callus sectors growing out from metabolite sensitive callus

Fig. 2. Detached peach leaf exhibiting water-soaked lesions immediately following inoculation with either *Xanthomonas campestris* pv. *pruni*, *X. c.* pv. *pelargonii* or infiltration with sterile distilled water

Results

Cell selection, regeneration and in vitro propagation

From a total of 400 individual calli, two calli survived exposure to the three concentrations of TF of *X. c.* pv. *pruni*. Plants designated 13-2 and 13-9 and 19-1 and 19-2

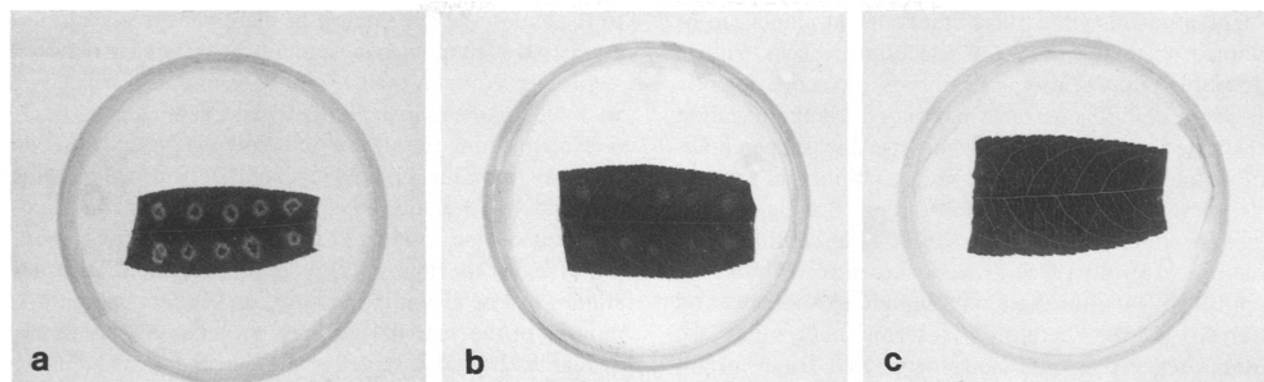

Fig. 3. **a** Necrotic spots surrounded by chlorotic halos on 'Sunhigh' peach leaf, 3 weeks after inoculation with *Xanthomonas campestris* pv. *pruni;* **b** chlorotic spots on clone 13-9, 3 weeks after inoculation with *X. c.* pv. *pruni;* **c** no symptom development on 'Sunhigh' peach leaf, 3 weeks after infiltration with sterile distilled water

Table 1. Reaction of detached leaves of four regenerant plants and two scion peach cultivars to *Xanthomonas campestris* pv. *pruni*

Clone or cultivar	Field reaction[a]	Average lesion rating[a] after inoculation	
		2 weeks	3 weeks
Sunhigh	Highly susceptible	1.4 a	2.4 a[d]
Redhaven	Moderately resistant	1.2 a	1.6 b
Sunhigh[c]	Not yet evaluated	0.7 b	0.8 c
13-2	–	1.4 a	2.4 a
13-9	–	0.8 b	1.1 c
19-1	–	1.0 ab	1.6 b
19-2	–	1.3 a	2.1 a

[a] Fogle et al. (1974) and Werner et al. (1986)
[b] Lesion rating: 0 = no symptoms or slight chlorotic flecks; 1 = distinct chlorotic spot and/or necrotic flecks; 2 = distinct necrotic spot or greyish white lesion <2 mm in diameter; and 3 = distinct necrotic spot >2 mm in diameter, with or without chlorotic halo. Control leaves infiltrated with water had a rating of 0 (Fig. 3c)
[c] Inoculated with *Xanthomonas campestris* pv. *pelargonii* nonpathogenic to peaches
[d] Each value represents the average of at least three replications with three leaves inoculated per replicate. Values in each column followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test

were regenerated from two of the calli, respectively. None of the clones responded well to micropropagation, multiplying very slowly and tending toward vitrification. Because of vitrification, only a limited number of plants of each clone were available to root, acclimatize, and grow in the greenhouse.

Modified detached-leaf bioassay

The results of the detached-leaf bioassay can be seen in Table 1. Lesion ratings for highly susceptible 'Sunhigh' and moderately resistant 'Redhaven' were not significantly different 2 weeks after inoculation. However, differences among clones and cultivars in infection were evident 3 weeks after inoculation with *X. c.* pv. *pruni.* Average lesion ratings for clones 13-2 and 19-2 were similar to the rating for susceptible cv 'Sunhigh', and symptoms typically seen were necrotic lesions, each surrounded by chlorotic halos (Fig. 3a). In contrast, the average lesion rating for clone 19-1 was significantly less than that for 'Sunhigh' and similar to that for moderately resistant cultivar Redhaven. The average lesion rating of 1.1 for clone 13-9 was significantly less than those for either 'Sunhigh' or 'Redhaven' and similar to that for 'Sunhigh' inoculated with nonpathogen *X. c.* pv. *pelargonii.* Symptoms typically seen on clone 13-9 were distinct chlorotic spots (Fig. 3b). The lesion rating on control plants was 0 (Fig. 3c).

Discussion

These results suggest that bacterial spot-resistant peach plants can be produced by regenerating plants from cultured callus that is insensitive to a toxic metabolite(s) produced by *X. c.* pv. *pruni.* Although a purified toxin may have been a more appropriate selective agent, our partially fractionated culture filtrate proved effective. A number of other investigators have also obtained successful selections using culture filtrates to select resistant material (Sacristan 1982; Hartman et al. 1984). To guard against selecting for resistance to nonspecific substances found in the culture filtrates, we separated the toxic components from any large molecular weight media components by ultrafiltration, and we determined that the response of cells of different cultivars to this low molecular weight fraction of the culture filtrate is correlated with the disease reaction of those host cultivars (Hammerschlag 1984a).

This study also illustrates that resistant plants can be obtained when the units for selection are cells from a susceptible plant genotype. Embryos, and consequently cells from embryos of open-pollinated 'Sunhigh' might be expected to be genotypically diverse due to open pollination, and therefore, not the same as 'Sunhigh' somatic cells. Nevertheless, in previous studies, 'Sunhigh' seedlings were either shown to be a source of susceptible plant material (Civerolo 1975) or were shown to be more susceptible to leaf spot than cv 'Sunhigh' (Randhawa and Civerolo 1986). Therefore, it seems likely that the variants selected in this study originated from variant cells not representative of most cells in the embryo explants. That a spot-susceptible plant was regenerated from each of the two surviving calli (clones 13-2 and 19-2) also suggests that the primary callus contained susceptible cells. These cells probably survived exposure to the toxic metabolites either by escaping full exposure or by somehow being protected by proximity to the resistant cells that gave rise to clones 13-9 and 19-1.

A simpler approach to in vitro selection might have been to isolate variants in the absence of selection. Some researchers have been successful in obtaining desired variants by screening unselected regenerants (Sacristan 1982; Hartman et al. 1984), whereas others have been unable to isolate resistant lines (Thanutong et al. 1983). In studies comparing results from unselected cultures with those obtained from cultures preselected with toxins, preselection significantly increased the frequency of obtaining resistant plants (Brettell and Thomas 1980; Sacristan 1982; Hartman et al. 1984). An explanation is that a much larger population can be evaluated at the cellular level than at the whole plant level. In the present study, the 400 calli used for selection contained an estimated 3×10^6 cells, and selection and evaluation was accomplished in 2 years. No more than 500 peach plants could be regenerated and screened for leaf spot resistance in the same amount of time.

Previously, a wick bioassay (Hammerschlag 1988) was found efficient for screening in vitro propagated shoots from leaf spot-resistant and -susceptible cultivars. This bioassay technique was less useful, however, in evaluating clones produced following cell selection. Many plants are needed for this bioassay, and both physiological and morphological abnormalities can influence the plant's response to the pathogen. Unfortunately, all of the four clones multiplied poorly, and many of the plants became vitrified. To avoid losing these clones, plants of each clone were rooted, acclimatized, and placed in the greenhouse. The few in vitro wick bioassays that were conducted did suggest that disease resistance was obtained (unpublished data), and this was confirmed by the detached-leaf bioassay. The differences among the clones and cultivars were much more evident in the detached-leaf bioassay than in the wick bioassay. This may be due to the superior performance of plants in the greenhouse compared with in vitro culture, or the difference between stems and leaves in their response to *X. c.* pv. *pruni*. The wick bioassay employs plantlet stems as the test issue. It is evident from the above that having more than one bioassay to evaluate plants regenerated from cell cultures is invaluable. A major advantage of the in vitro bioassay is that it can be conducted year-round. For this reason, putative disease resistant regenerants produced in future studies will be evaluated in vitro, especially during the 6 months of the year that actively growing plants are not available. The advantages of the detached-leaf bioassay are that the test plants are not sacrificed, that each plant produces many leaves that can be assayed, and most important, that the comparative susceptibility of detached leaves of different peach cultivars is directly related to susceptibility under field conditions (Randhawa and Civerolo 1985). For these reasons, the detached-leaf bioassay will also be used in future studies.

The potential impact of selection in vitro from spot-susceptible plant material is great, for many high quality peach cultivars are otherwise susceptible to leaf spot (Fogle et al. 1974; Werner et al. 1986). Many selections of this type will likely be conducted once a reliable system is developed for regenerating plants from cells derived from mature peach cultivars. Since the present system utilizes cells from heterozygous embryos, resistant plants produced in vitro must still be evaluated for fruit quality. Phenotypic evaluation of resistant clones is currently in progress. Tests of progeny will also be conducted to establish whether the disease resistant phenotype is heritable.

In conclusion, this study represents the first report of using in vitro selection to obtain disease resistance in a woody crop species. As such it offers a promising means of obtaining disease resistance in other woody crop species.

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