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Fertile *Solanum tuberosum* + *S. commersonii* somatic hybrids as sources of resistance to bacterial wilt caused by *Ralstonia solanacearum*

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Abstract The wild potato relative *Solanum commersonii* is reported to carry resistance to bacterial wilt disease caused by *Ralstonia solanacearum.* To overcome sexual incompatibilites due to differences in ploidy and endosperm balance numbers, somatic hybrids were made that combine the *S. tuberosum* and *S. commersonii* genomes. The resulting somatic hybrid plants are vigorous, but their disease resistance level and their fertility was unknown. We therefore tested the *S. commersonii* and *S. tuberosum* source material cv Superior, potato cv Atlantic and six somatic hybrid lines for resistance to a virulent strain of *R. solanacearum* (race 3, biovar 2) at 28°C. As expected, *S. commersonii* was significantly more wilt-resistant than the cultivated potatoes. In five of the six somatic hybrid lines, disease resistance levels were similar to that of the resistant *S. commersonii* parent. The resistance level of the sixth somatic hybrid was intermediate, significantly different from both *S. commersonii* and *S. tuberosum.* In controlled crosses, the somatic hybrids in this study proved both to be male- and female-fertile and were self-compatible. More importantly, the somatic hybrids can be crossed with *S. tuberosum* to produce viable seeds.

Key words EBN (endosperm balance number) · Somatic hybrids · Potato · Brown rot · *Pseudomonas solanacearum*

Introduction

Solanum commersonii is a wild potato species from southeastern South America. As early as 1930, it was found to be frost-resistant (Reddick 1930) and has since been used as a model species to study frost resistance and cold tolerance. This tuber-bearing species reportedly possesses several desirable traits, including high specific gravity (Sawicka 1971) and resistance to biotic stress, notably to the nematode *Ditylenchus destructor* (Stefan 1980), to the fungus *Alternaria solani* (Alam 1985), and to potato viruses X and Y (Sklyarova 1970; Tozzini et al. 1991). Recently, *S. commersonii* was also reported to carry resistance to bacterial wilt disease (Bamberg et al. 1994) caused by *Ralstonia solanacearum* (Smith) Yabuuchi et al. (formerly *Pseudomonas* and more recently *Burkholderia solanacearum*).

Bacterial wilt of potatoes, also known as brown rot, is widespread in the tropics and sub-tropics. This disease has recently gained prominence because of the increased demand for potatoes within its geographical range. In developing countries, potatoes are a particularly desirable crop because of their high per acre yield in calories and nutritive value compared to cereals and pulses (Niederhauser 1993), and because they can be used both as food and as a cash crop. However, in the tropics and subtropics, where potatoes are potentially an important resource, bacterial wilt is a serious constraint to potato production. Warm temperatures favor disease development, provoking symptoms that include rapid wilting and plant death. Bacterial wilt can cause total crop failure. Thus potato production is limited to the cooler higher altitudes, to temperate regions, and to fields free from the pathogen (Hayward

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1991). Recently, the disease has also become a threat to potato production in temperate regions. *R. solanacearum* has demonstrated its ability to overwinter in temperate Sweden (Olsson 1976) and has caused losses in outbreaks in the Netherlands. In 1995, Dutch authorities detected the disease in 24 potato cultivars and over 100 farms were subsequently placed under plant quarantine (Janse 1998). Since 1992, *R. solanacearum* has been detected in Belgium, France, Italy, Portugal, Spain and the United Kingdom. International plant quarantine regulations have been toughened in an effort to restrict the pathogen's spread and to eradicate it from Western Europe (Elphinstone 1996).

There is no chemical control for this disease. *R. solanacearum* persists in soil, in plant debris, in weed rhizospheres and in alternate hosts, and is spread in infected planting material (Hayward 1991) and in irrigation water (Olsson 1976; Elphinstone 1996; Janse et al. 1998). Use of disease-resistant crops is a cost-effective and environmentally benign form of control, but there are few sources of wilt resistance in the germplasm available to potato breeders. *Solanum phureja* has been a valuable source of wilt resistance but, unfortunately, this resistance is temperature-sensitive and is effective only at higher elevations or in cooler climates (French and De Lindo 1982).

Bacterial wilt resistance from *S. commersonii* has not, to our knowledge, been explored in potato breeding programs. *S. commersonii* is a diploid species with an endosperm balance number (EBN) of 1 (Johnson and Hanneman 1981) and is sexually isolated from the tetraploid 4EBN *Solanum tuberosum* (Johnson et al. 1980). Crosses between 4*x* *S. tuberosum* and hybrids from 2*x* *S. commersonii* and 2*x* *S. tuberosum* di-haploids (2EBN) have repeatedly failed (Carputo et al. 1997). Several schemes have been proposed to overcome this incompatibility involving the use of 2n gametes or chromosome doubling in conjunction with the use of bridge species (Carputo et al. 1997; Ehlenfeldt and Hanneman 1988 a; Masuli et al. 1992). These approaches are laborious and have low success rates. Another method used to overcome sexual incompatibility is somatic hybridization. In this approach, the full array of favorable epistatic genes may be maintained, and linkage drag from bridge species is avoided. Kim et al. (1993) produced somatic hybrids between *S. commersonii* and *S. tuberosum* using electrofusion. This material is examined further in the present study. Cardi et al. (1993) and Nyman and Waara (1997) produced *S. tuberosum* + *S. commersonii* somatic hybrids and have demonstrated that frost tolerance can be recovered in these hybrids. In other interspecific fusion studies, desirable traits of the wild species were recovered in the sexual progeny from the somatic hybrid × *S. tuberosum* cross (Austin et al. 1988; Brown et al. 1996; Helgeson et al. 1998). Thus, somatic hybridization may become a useful tool in accessing wild germplasm.

In this paper, we report on the potential of these somatic hybrids for bacterial wilt resistance breeding programs. Their usefulness is examined based on the following criteria: resistance must be stable through somatic hybridization, and somatic hybrids must be fertile with *S. tuberosum* in order to allow eventual introgression of desirable traits through conventional breeding. To this end, we tested the resistance of a promising series of *S. commersonii* + *S. tuberosum* somatic hybrids, and characterized their fertility. The somatic hybrid lines examined in this study were selected because they are vigorous in the field, flower prolifically and were observed to set berries (Fig. 1).

Materials and methods

Plant material

The somatic hybrids in this study are derived from two different fusions (Kim et al. 1993). *S. commersonii* ssp. *Commersonii* Dunal, line LZ3.2 (plant introduction number PI320266, Bamberg et al. 1994) contributed the wild potato germplasm in all the fusions. The HB09 series is a fusion product of LZ3.2 and clone R4♯2 (PI203900), a tetraploid *S. tuberosum* L. line used as a late-blight-race differential. HB09-2 and HB09-3 were derived from shoots off the same fusion callus and have 2n ploidies of 70 and 72 respectively (Kim et al. 1994). The HA series are fusion products of LZ3.2 and PT56, a di-haploid from cv Superior (US-13122, provided by S. J. Peloquin, University of Wisconsin, Madison, Wis.). The HA06 and HA31 series were derived from shoots off two separate fusion calli. They are tetraploid, or near tetraploid, with chromosome counts ranging from 42 to 55 (Kim 1994).

Disease resistance assay

We tested the bacterial wilt resistance of six somatic hybrids (HA06-1, and HA06-3–HA06-7), the *S. commersonii* fusion parent (LZ3.2), and two potato cultivars: cv Atlantic and cv Superior. Cultivar Superior is the source material for the di-haploid *S. tuberosum* fusion parent, but the di-haploid is a weak plant that could not be reliably tested and was therefore not included in the wilt resistance assay reported here.

All clones were maintained and multiplied in tissue culture on Murashige and Skoog (MS) medium (Murashige and Skoog 1962). Plantlets were transplanted from tissue culture to 5-cm-diameter cups containing a 55–65% peat-vermiculite mixture, and were then transferred to a 28°C growth chamber. After 12 days, the plants were inoculated with UW276, a virulent Mexican strain of *R. solanacearum* (race 3, biovar 2). Washed bacteria from an overnight liquid culture grown in CPG medium (Hendrick and Sequeira 1984) were suspended in distilled water to a concentration of 5×10^7 colony forming units (cfu) per ml. Plants were inoculated with 1 ml of suspension without root wounding, as described by Montanelli et al. (1995). Thus, each plant received 5×10^7 cfu, the equivalent of about 3×10^6 cfu per g of growing medium. Control plants received plain water.

Plant disease was scored using a standard five-point scale: 0 = no wilted leaves, 1 = up to 25% wilted, 2 = 26% to 50% wilted, 3 = 51% to 75% wilted and 4 = more than 75% wilted. An experimental unit consisted of four plants, each in its own transplant cup. One data point represented the average of the four plants in a unit. The layout was fully random, and the experiment was blind to clonal-line identity and to treatment. At the end of the experiment,

Fig. 1 Somatic hybrid line HA06-4, a fusion product of 2n = 2x = 24 *S. commersonii* and a 2n = 2x = 24 di-haploid derived from cv Superior. The lines in the HA06 series are vigorous and morphologically similar to each other

disease resistance data were analyzed by ANOVA. Clonal differences were tested in terms of the least significant difference (LSD).

Controlled crosses and germination assays

Pollen was obtained by collecting mature flowers and electronically vibrating the stamens to dislodge pollen into gel capsules. Pollen from several flowers was bulked in each gel capsule and stored at −20°C. As an indication of the potential for male fertility, pollen was tested with the vital stain acetocarmine, and the percent stainability was calculated on a minimum of 250 pollen grains. Crosses were performed by emasculating flowers at the closed bud stage and applying pollen to the stigma of mature flowers. We performed the following crosses: somatic hybrid × *S. tuberosum*, reciprocal cross *S. tuberosum* × somatic hybrid, and somatic hybrid × somatic hybrid (selfed). The *S. tuberosum* lines we used in the crosses were cv Atlantic, line A89804-7, and line B0718-3. Breeding lines A89804-7 and B0718-3 were kindly provided by Dr. J. Pavek, ARS Potato Breeding Program, Aberdeen, Idaho, and Dr. K. Haynes, ARS Beltsville, Maryland, respectively.

Seeds were harvested from mature berries approximately 2 months after fertilization, dried, counted and stored at room temperature. As part of the germination process, seeds were surface-sterilized, dipped first in 95% ethanol, then soaked in 1% sodium hypochlorite for 10 min followed by two 15-min rinses in sterile distilled water. Sterile seeds were then incubated overnight on a filter paper soaked with 1000 ppm of filter-sterilized gibberellic acid, transferred to agar plates (6 g/l) supplemented with 1.3 g/l of Peters fertilizer (15-5-15), and incubated at room temperature until germination. Seedlings were transferred and maintained in MS medium.

Results

Disease resistance assay

S. commersonii was significantly more wilt-resistant than the two cultivated potato lines. Of the 48 inoculated plants of *S. commersonii* fusion parent LZ3.2, only one succumbed to disease. In contrast, cv Superior and cv Atlantic were much more susceptible to disease; about half of the inoculated plants died. Figure 2 illustrates the bacterial wilt disease progress on the tested lines over time. None of the water-inoculated plants expressed disease symptoms.

We selected six somatic hybrids, all from the HA06 series, for the wilt resistance assay. They are the fusion products of *S. commersonii* and the cv Superior dihaploid PT56, derived from the same fusion callus. Chromosome counts have established that they are tetraploid or hypo-tetraploid (Kim et al. 1993). All six somatic hybrid lines expressed significantly less disease than the *S. tuberosum* source material, cv Superior. In five of the six somatic hybrids (HA06-1 to HA06-6), there was no significant difference in resistance compared to the *S. commersonii* fusion parent. However, the disease response of the sixth somatic hybrid (HA06-7) was intermediate; it was significantly more resistant than the *S. tuberosum* cultivars, yet more susceptible than the *S. commersonii* fusion parent.

Fertility

All the hybrids in this study produced pollen, though HA31-4 and HA31-7 produced very little. What pollen we obtained from the latter was used for selfing, so we did not perform the acetocarmine vital stain test on these two lines. Pollen stainability was high in all the hybrid lines we tested (Table 1) and all the somatic hybrids in this study produced seeds from selfing (see Table 2).

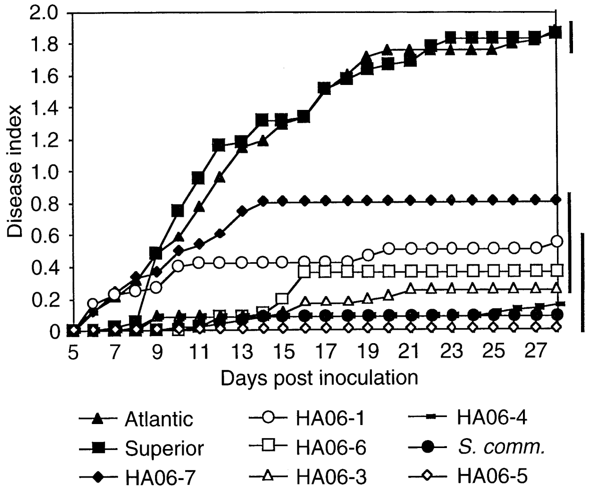

Fig. 2 Disease response of somatic hybrids (HA06 series), *S. tuberosum* cultivars (Atlantic and Superior) and *S. commersonii* (LZ3.2). For each line, the average disease rating of all 12 inoculated experimental units (four plants per unit) was calculated daily. *Vertical bars* indicate LSD groupings

Table 1 Somatic hybridization fusion parents and their expected ploidy, somatic hybrid lines, and pollen stainability as determined using acetocarmine

Fusion parents	Somatic hybrid clonal line	Pollen stainability
LZ3.2 + PT56	HA06-1	85%
2n = 4*x* = 48	HA06-3	89%
	HA06-4	87%
	HA06-5	94%
	HA06-6	95%
	HA06-7	96%
LZ3.2 + PT56	HA31-4	Not determined
2n = 4*x* = 48	HA31-7	Not determined
LZ3.2 + R4	HB09-2	77%
2n = 6*x* = 72	HB09-3	79%

We attempted crosses of the somatic hybrids with various *S. tuberosum* lines. Seeds were obtained from all the crosses we attempted (Table 2). The average seed-per-berry yield with A89807-4 as pollen donor was 142.4 for the HA06 series and 76.9 in the HB series. Seed yields were lower in crosses using cv Atlantic as the pollen donor: on average, 20.9 seeds-per-berry in the HA06 series, and 54 seeds-per-berry in the HB series. The stainability of frozen pollen from cv Atlantic was low: 5.4% as compared to 58.4% and 15.5% for similarly frozen A89804-7 and B0817-3 pollen, respectively. The seeds were variable in color and size; most seeds were cream coloured, but some had thick pigmented teguments. Endosperms were commonly degenerated; plump cream seeds were few.

Over 90% of the seeds from selfed HA06-4 and HA06-6 germinated. The germination rates of seeds from crosses of these somatic hybrids with *S. tuberosum* were much lower, ranging from 1.6% to 8.1% (Table 3). In contrast with the seeds from crosses with *S. tuberowum*, these seeds were evenly plump and large. Both HA06-4 and HA06-5 were crossed with cv Atlantic as female parent, and each gave viable seeds with 38% and 63% germination rates, respectively, higher than the average germination rate of seeds from crosses using cv Atlantic as the pollen donor (1.6%). The majority of these seeds were plump, but small. Seeds from selfed HB09-3 and from HB09-3 × *S. tuberosum* were large, plump, and had good germination rates (Table 3).

Discussion

Results from this study suggest that *S. commersonii* + *S. tuberosum* somatic hybrids may be useful as

Table 2 Fertility of the *S. commersonii* + *S. tuberosum* somatic hybrids. The somatic hybrids were crossed with various *S. tuberosum* parents and were selfed; n/a indicates that the cross was not attempted

Female	Male							
	Atlantic		A89804-7		B0718-3		Self	
	Berries	Seeds/ berry	Berries	Seeds/ berry	Berries	Seeds/ berry	Berries	Seeds/ berry
HA06-1	4	6.3	7	196.4	n/a	n/a	1	73.0
HA06-3	9	23.0	10	105.9	n/a	n/a	3	6.3
HA06-4	4	33.3	5	177.4	n/a	n/a	3	24.3
HA06-5	8	14.3	2	111.0	n/a	n/a	3	4.3
HA06-6	7	23.6	9	109.4	3	76.0	3	8.7
HA06-7	4	25.3	6	154.3	2	155.0	5	12.2
HA31-4	n/a	n/a	1	27.0	n/a	n/a	2	17.0
HA31-7	n/a	n/a	4	29.0	n/a	n/a	2	8.0
HB09-2	5	42.4	5	57.8	5	52.0	10	21.7
HB09-3	2	65.5	6	96.0	5	58.4	6	28.0

Table 3 Germination rates of seeds from crosses with *S. commersonii* + *S. tuberosum* somatic hybrids. The first column of each cross indicates the number of seeds that was tested for germination, the second column indicates the percent germination for those seeds; n/a indicates not attempted

Female	Male							
	Atlantic		A89804-7		B0718-3		Self	
	Seeds tested	% Germ.	Seeds tested	% Germ.	Seeds tested	% Germ.	Seeds tested	% Germ.
HA06-4	122	1.6	887	2.7	n/a	n/a	73	90.4
HA06-6	172	1.7	985	3.2	135	8.1	26	96.2
HB09-3	60	93.3	118	94.1	106	71.7	74	86.5

sources of bacterial wilt resistance in potatoes. Our study confirms that the *S. commersonii* fusion parent is significantly more wilt-resistant than the *S. tuberosum* cultivars, cv Atlantic and cv Superior. All these somatic hybrids were significantly more resistant than the potato cultivars. In five of the six somatic hybrids, the level of wilt resistance was statistically indistinguishable from that of the *S. commersonii* fusion parent. The sixth somatic hybrid, HA06-7, had intermediate wilt resistance; significantly more resistant than the potato lines, but less than the resistant *S. commersonii*. The di-haploid *S. tuberosum* fusion parent was derived from cv Superior, which proved quite susceptible to bacterial wilt in this study. Thus, resistance in the somatic hybrids was apparently conferred by the *S. commersonii*, rather than the *S. tuberosum*, fusion parent.

We selected the HA06 series of somatic hybrids for wilt resistance studies because they are vigorous, flower prolifically and continuously until senescence, and were observed to set berries. Since all the somatic hybrids tested for wilt resistance were derived from the same fusion callus, the intermediate resistance response of somatic hybrid line HA06-7 is interesting. Initial mitotic events after protoplast fusion can be unstable, resulting in differences in ploidy among the *S. tuberosum* + *S. commersonii* somatic hybrids (Kim 1993). It is possible that one or more genetic elements were lost in HA06-7 but remain in the other somatic hybrids, or that chromosomal rearrangements led to differences in wilt resistance. HA06-7 may be of some value in molecular studies on the genetics of bacterial wilt resistance.

Wilt resistance was assayed without artificial root wounding, and the inoculation level was about 3×10^6 cfu per g of potting mix, consistent with that of bacterial populations found in naturally infested soil at the onset of disease (Devi et al. 1982; Moffet and Wood 1984). The assay was conducted at 28°C to optimize bacterial growth and disease progress. The significant wilt resistance of *S. commersonii* and of the somatic hybrids under these conditions is encouraging; however, the stability of resistance under different field conditions and temperature regimes remains unknown. *R. solanacearum* is a very heterogeneous species consisting of five races, with high variation within some races (see Hayward 1991 for a review). We have, as yet, no information on strain specificity of the *S. commersonii* wilt resistance. In this study, we used a race 3 *R. solanacearum* strain. Race 3, sometimes referred to as the potato race, is a remarkably homogeneous group, thought to have originated in the Andes mountains, at the center of potato genetic diversity (Cook et al. 1989;

Smith et al. 1995). In contrast, race 1, which can cause disease on potatoes as well as many other species, is a highly heterogeneous group, common in the low-land tropics. The *S. commersonii* accession used in this study was collected in Uruguay at sea level, where race 1 strains should be predominant. It will be interesting to determine whether these somatic hybrids are also resistant to race 1 strains of *R. solanacearum*.

To be useful for potato breeding programs, plant material must be fertile to *S. tuberosum*. To this end, we performed some studies on the fertility of several somatic hybrids. All the somatic hybrids were both male- and female-fertile and, with the exception of HA31-4 and HA31-7, shed abundant pollen. Somatic hybrid pollen had good stainability when tested with the vital stain acetocarmine. The HA06 series performed especially well, with stainability ranging from 85% to 96%.

Theoretically, the HA group of somatic hybrids is tetraploid with an EBN of 3 (a product of the fusion of the 1EBN 2*x**S. commersonii* with the 2EBN di-haploid 2*x**S. tuberosum*). Based on interspecific crosses of *S. commersonii* and *S. chacoense*, Ehlenfeldt and Hanneman (1988 b) proposed that EBN is under the control of three additive loci, and that a slight EBN imbalance can be better tolerated where the maternal, rather than the paternal, contribution is excessive. Consistent with their results on the tolerance of endosperm imbalance, where the tetraploid (3EBN) somatic hybrid was used to pollinate *S. tuberosum* cv Atlantic (4EBN), the germination rate was greater than for seeds from the crosses where *S. tuberosum* (4EBN) was used as the staminate parent. The fact that these somatic hybrids are male- and female-fertile may have advantageous practical consequences in breeding programs. Most of these somatic hybrid lines can produce abundant supplies of pollen, and breeders are allowed the flexibility of choosing the cytoplasmic parent.

The HB group of somatic hybrids are theoretically hexaploid with an EBN of 5 (a product of the fusion of the 1EBN 2*x**S. commersonii* with the 4EBN 4*x**S. tuberosum*). As argued by Carputo et al. (1997), we reasoned that in a pentaploid BC1 population from this material, gene exchange may not be enforced, thus impeding rapid introgression into the cultivated potato. Further, the HB series poses the challenge of restoring the 48 chromosome number in its progeny; thus, we concentrated our bacterial wilt resistance research on the HA somatic hybrids. However, the high germination rate of the HB09-3 × *S. tuberosum* crosses is interesting. In these crosses, the 6*x*HB09-3 (5EBN) parent provides the slight excess maternal contribution to the endosperm but, at this higher ploidy, departure from the 2 : 1 maternal to paternal ratio is less pronounced. This may, in part, explain why germination rates of seeds from crosses to *S. tuberosum* are comparable to those of selfed seeds (Table 3). Resistance of the HB series and its progeny will be evaluated in a future study.

Results from this study are encouraging and indicate that further study on the genetics and nature of resistance in *S. commersonii* is warranted. Our observations suggest that wilt resistance is stable through somatic hybridization. Preliminary work on a population from the first backcross to *S. tuberosum* indicates segregation for resistance. Further, many clones of the BC_1 population are fertile and we have obtained BC_2 populations. Characterization of bacterial wilt resistance in these populations is ongoing. Somatic hybridization has thus rapidly provided material that can be crossed directly with the cultivated potato and without the use of bridge species. The population of somatic hybrids employed in this study is remarkably fertile and offers the tools to exploit desirable agronomic traits in *S. commersonii*, notably novel resistance to bacterial wilt disease.

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