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Male fertility and freezing tolerance of hybrids involving *Solanum tuberosum* haploids and diploid *Solanum* species[1]

M. TUCCI[1], D. CARPUTO[1], G. BILE[2] and L. FRUSCIANTE[2]

[1]Research Centre for Vegetable Breeding, C.N.R., Parco Gussone, via Università 133, 80055 Portici (NA), Italy
[2]Department of Agronomy and Plant Genetics, University of Naples, via Università 100, 80055 Portici (NA), Italy

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Summary

Male sterility of (haploid x species) hybrids often hampers the exploitation of wild *Solanum* species in breeding for cold tolerance. In a study aimed at finding ways round these problems, we found that one accession of the cold tolerant wild species *S. sanctae-rosae* gave progenies segregating for male fertility in crosses with *tuberosum* haploids. Moreover, a source of male fertility restorer gene(s) was hypothesized in the (haploid x species) hybrid UP88-C80 (*tbr* x *chc*). A relatively high freezing tolerance was found in the progenies coming from *tuberosum* haploid x *sanctae-rosae* crosses and some hybrids showed a freezing killing temperature as low as -5.4 °C. Although the most tolerant clones did not shed pollen, at least one clone (Sr6, *tbr* x *sct*) combined male fertility, 2n pollen production and good freezing tolerance (-3.9 °C) and might be directly used in crosses with tetraploid *S. tuberosum* in order to introgress resistance to low temperature in *tuberosum* form.

Introduction

Diploid (2n=2x=24) tuber-bearing *Solanum* species have been widely used in potato breeding to broaden the genetic basis of the cultivated tetraploid *S. tuberosum*. In fact, wild species represent an important source of useful traits lacking in the cultivated potato. Several authors (Ross, 1986; Hanneman & Bamberg, 1986; Hanneman, 1989) reported that resistance to biotic and abiotic stresses as well as tuber quality traits have been found in several wild species. The easiest way to incorporate diploid wild species in *tuberosum* form is to produce hybrids between *S. tuberosum* haploids and the wild species of interest, provided that there are no crossing barriers. The (haploid x species) hybrids obtained can be used either as male or as female parents to synthesize tetraploids by sexual polyploidization. Indeed, if they form unreduced gametes, they can be easily used in 4x x 2x and 2x x 4x crosses, where the 4x parent is a cultivated variety and the 2x parent is a diploid hybrid which

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forms 2n pollen and 2n eggs, respectively. Tetraploids can be also produced by 2x x 2x crosses when both parents produce unreduced gametes.

Among the useful traits possessed by wild species, much attention has been given to frost tolerance. *S. tuberosum* is susceptible to freezing stress, displaying a frost killing temperature (LT50) of about -2 °C (Mendoza & Estrada, 1979). Cold tolerance might be of great interest in areas such as the temperate Mediterranean, especially in view of an anticipation of planting and/or a shortening of the growth cycle of early potatoes. Resistance to freezing stress has been found in several wild and primitive *Solanum* species, *S. acaule* (killing temperature -6.0 °C), *S. sanctae-rosae* (-5.5 °C) and *S. commersonii* (-4.5 °C) being the most tolerant (Li et al., 1979).

Unfortunately, barriers to interspecific crosses may hamper the exploitation of these wild species in potato breeding programmes. Due to endosperm abortion in the hybrid seed, *S. commersonii* (2n=2x=24) cannot be crossed with *tuberosum* haploids and other diploid species, and for the same reason *S. acaule* (2n=4x=48) cannot be used in crosses with tetraploid *S. tuberosum* (Hanneman, 1994). *S. sanctae-rosae* (2n=2x=24) can be crossed with *tuberosum* haploids, but then the main limitation resides in the nuclear-cytoplasmic male sterility of the offspring when *S. sanctae-rosae* is used as male parent (Hermundstat & Peloquin, 1985). The reciprocal crosses (when *S. sancta-rosae* is used as female parent) are fertile, but they are less useful from a breeding point of view both because *tuberosum* cytoplasm in the hybrids is preferable to wild cytoplasm and because male fertile *tuberosum* haploids are rare (Carroll & Low, 1976). Nuclear-cytoplasmic male sterility is widespread in *Solanum* spp. (Hermundstat & Peloquin, 1985) and has been well documented in crosses between *tuberosum* haploids and *S. phureja*, where it was explained by the presence of nuclear male sterility genes in the wild species interacting with sensitive factors in *tuberosum* cytoplasm (Ross et al., 1964; Hanneman & Peloquin, 1981).

The possibility of finding *tuberosum* haploids with a fertility restorer gene (Rt) (Iwanaga et al., 1991) and/or accessions of wild species that do not have the genes causing nuclear-cytoplasmic male sterility may represent an opportunity to circumvent the male sterility of (haploid x species) hybrids.

In view of this, we crossed three accessions of the frost resistant species *S. sanctae-rosae* with either *tuberosum* haploids or fertile (haploid x species) hybrids as female parents. Furthermore, in order to check for the presence/absence of fertility restorer genes in the female parents, we crossed them with one accession of *S. phureja* which has the dominant genes interacting with *tuberosum* cytoplasm to give male sterility (Hanneman & Peloquin, 1981). In this paper we present data regarding male fertility, 2n pollen production and freezing resistance of the hybrids produced.

Materials and methods

Plant material consisted of ten male fertile *Solanum* species and clones, including two *tuberosum* haploids (2n=2x=24), three accessions of the wild species *S. sanctae-rosae* (2n=2x=24), one accession of the primitive diploid cultivar *S. phureja* and four (haploid x species) hybrids (2n=2x=24) which were male fertile. The origin and male fertility of the material used is listed in Table 1.

Table 1. *Solanum* species and clones (2n=2x=24) used to produce interspecific hybrids.

Code	Pedigree	Pollen stainability
W730[b]	*tbr* hapl. from 4x clone Wis 231	64.9
W457[b]	*tbr* hapl. from cv. White Rose	52.2
SCT1	*sct*, unknown PI number	75.3
SCT2	*sct*, unknown PI number	22.5
SCT3	*sct*, unknown PI number	51.8
PI 1.22	*phu*, PI 225682	73.8
T710[b]	Kathadin hapl. US-W3304 x chc (PI 265566 x PI 275138)	82.0
UP88-C80	Chippewa hapl. US-W1831 x *S. chacoense* PI 230582	28.5
P100.1[b]	Merrimack hapl. x *S. tarijense*, unknown PI number	80.0
P116.4[b]	W730 x *S. tarijense*, unknown PI number	74.6

[a] *tbr, S. tuberosum; sct, S. sanctae-rosae; phu, S. phureja; chc, S. chacoense; tar, S. tarijense*
[b] Accessions belonging to Dr. S.J. Peloquin collection

Ten hybrid combinations were produced using the *tuberosum* haploids and the (haploid x species) hybrids as female parents, and the diploid species *S. sancta-rosae* and *S. phureja* as male parents (Table 2). Crosses were performed in Camigliatello Silano, southern Italy, during the Summer of 1991 using screenhouse-grown plants. Six weeks after pollination, berries were collected and allowed to ripen at room temperature before extracting seeds. In 1992, seedlings grown from true seed were transplanted into a screenhouse and screened for ploidy level by counting the chloroplasts in stomata guard cells. 104 diploid hybrids tuberized and were planted in Camigliatello Silano the following year to evaluate male fertility and tuber yield.

Table 2. Total number of plants analyzed, ratio between fertile and sterile plants and ratio of 2n pollen to non-2n pollen producing plants in hybrid families from 2x x 2x crosses.

Species combination	Cross	Plants analyzed no.	Fertile:sterile plants no.	2n:non-2n pollen producing plants no.
tbr x phu	W730 x PI 1.22	13	1:12	1:0
tbr x phu	W457 x PI 1.22	4	0: 4	–
tbr x sct	W730 x SCT1	15	11: 4	10:1
tbr x sct	W730 x SCT2	7	0: 7	–
tbr x sct	W730 x SCT3	4	0: 4	–
(tbr x chc) x sct	UP88-C80 x SCT3	8	4: 4	3:1
(tbr x chc) x phu	UP88-C80 x PI 1.22	10	5: 5	3:2
(tbr x chc) x phu	T710 x PI 1.22	11	0:11	–
(tbr x tar) x phu	P100.1 x PI 1.22	13	0:13	–
(tbr x tar) x phu	P116.4 x PI 1.22	19	2:17	1:1
Total		104	23:28	18:5

Pollen samples from at least five flowers of the diploid hybrids were stained using 1% acetocarmine. Pollen stainability was calculated using at least 200 pollen grains per flower, and genotypes with less than 5% stainable pollen were considered male sterile. In addition, the hybrids were scored for 2n pollen production, considering 2n grains those having a diameter 1.2–1.3x that of the n pollen. Genotypes having 2n pollen frequency higher than 1% of the stainable pollen were classified as 2n pollen producers.

Based on tuber/plant characteristics, 35 hybrids were selected to evaluate freezing tolerance. Leaves were collected from three field grown plants six weeks after planting and subjected to a controlled freezing cycle. Six terminal leaflets of different ages were folded in damp Miracloth™ tissue (Calbiochem) and placed in test tubes in a water bath. Temperature was lowered by 1 °C per hour and ice crystals were added to each test tube when the bath temperature reached 0 °C to initiate freezing of the leaflets. Leaf samples were collected at 1 hr intervals and allowed to thaw at 4 °C for 12 hrs. Viability of the leaf tissues was then assessed by the electrolyte leakage test of Sukumaran & Weiser (1972). After complete thawing, 30 ml of double distilled deionized water were added to each test tube and tubes were shaken for 1 hr at room temperature. The conductivity of the leachate was measured using a Toptronic conductivity meter type X74174. After leaves were killed by freezing at -80 °C, tubes were shaken for 1 hr at room temperature before determining the total leachable solutes with a second conductivity measurement of the solution. The freezing temperatures causing 50% loss of total leachate from three independent experiments were averaged and the resulting mean was designated as the killing temperature (LT50). Since the frost killing temperature of *S. tuberosum* is -2 °C (Mendoza & Estrada, 1979), clones showing an LT50 below -3 °C were considered freezing tolerant.

Results and discussion

Pollen stainability. Data on pollen stainability and 2n pollen production of 138 hybrids, grouped by cross combinations, are presented in Table 2. *Tuberosum* haploid x *phureja* families gave male sterile progenies, with one exceptional fertile clone found in W730 x PI 1.22 family. These results were expected since nuclear-cytoplasmic male sterility in *tuberosum* haploid x *phureja* hybrids has already been reported (Ross et al., 1964; Carroll, 1975). When the haploid clone W730 (*tbr*) was crossed with three accessions of *S. sanctae-rosae*, two families (W730 x SCT2 and W730 x SCT3) were completely male sterile, while one (W730 x SCT1) contained 11 male fertile and 4 male sterile plants. These results may indicate that the accessions SCT2 and SCT3 of *S. sanctae-rosae* possess the dominant gene(s) responsible for nuclear-cytoplasmic male sterility. On the contrary, the accession SCT1 which gave fertile progenies can be heterozygous for the gene(s) involved in nuclear-cytoplasmic male sterility, and segregated for this character. Results from reciprocal crosses would confirm the existence of nuclear-cytoplasmic male sterility.

It is also possible that W730 (*tbr*) has a nuclear-cytoplasmic male sterility restorer gene. Indeed, this haploid comes from the advanced selection W231, which has been

reported to be simplex (Rtrtrtrt) for the nuclear-cytoplasmic male sterility restorer gene (Iwanaga et al., 1991). Hermundstat (1986) obtained similar results in families from crosses between accessions of *S. sanctae-rosae* and *tuberosum* haploids. The author hypothesized that the accessions of *S. sanctae-rosae* used were homozygous for the genes which cause nuclear-cytoplasmic male sterility, but that the presence of a restorer gene in *tuberosum* haploids gave fertile plants.

Interesting results came from crosses involving clone UP88-C80 (*tbr* x *chc*) as female parent. Indeed, crosses between this clone and the accessions SCT3 (*sct*) and PI 1.22 (*phu*) gave fertile and sterile progenies in a 1:1 ratio, while when SCT3 and PI1.22 where crossed to other female parents the progenies were male sterile. These results may be explained assuming that UP88-C80 has a gene which restores male fertility. Clone T710, coming from the same cross combination as UP88-C80 (Table 1), produced only male sterile plants, so it probably does not have a restorer gene for nuclear-cytoplasmic male sterility.

2n pollen production. Among male fertile plants from the different cross combinations, some 2n pollen-producing clones were identified. Within these clones, 2n pollen frequency ranged from 1.5% to 48.8% (two UP88-C80 x PI1.22 clones) ((*tbr* x *chc*) x *phu*) (data not shown). Conicella et al. (1996) performed microsporogenesis analysis of some of the hybrids presented in this paper and found that 2n pollen production was mainly due to the parallel orientation of spindles at Metaphase II of meiosis. This meiotic mutation is caused by a single recessive gene (*ps*) which is widespread in both *tuberosum* and wild species (Watanabe & Peloquin, 1989). Due to the small sample size within each cross combination we could not deduce the genotype of the parents. However, the results from W730 x SCT1 (*tbr* x *sct*) (10 2n pollen:1 non 2n pollen producers) could indicate that both parents are recessive homozygous for *ps*, confirming the results by Conicella et al. (1996). The presence of a non 2n pollen producer within this progeny is probably due to the incomplete penetrance and expressivity of *ps* (Watanabe & Peloquin, 1989).

Frost tolerance. Thirty-five hybrid clones were evaluated for freezing tolerance. While clones which had *S. phureja* as the male parent were all susceptible (Table 3), hybrid families deriving from crosses of *tuberosum* haploids and (haploid x species) hybrids with the three accessions of cold tolerant *S. sanctae-rosae* showed different degrees of freezing tolerance (Fig. 1). When crossed with the *tuberosum* haploid W730, accessions SCT1, SCT2 and SCT3 gave progenies which segregated for cold tolerance. In fact, out of 16 hybrids analyzed, 9 survived temperatures below -3.0 °C and were classified as freezing tolerant. By contrast, accession SCT3 gave a susceptible progeny when crossed with UP88-C80 (*tbr* x *chc*).

A recent study on potato (Stone et al., 1993) pointed out that the different components of freezing tolerance, namely the hardening ability and the freezing survival in the non-acclimated status, are under distinct genetic control. Using the wild species *S. sanctae-rosae*, which is freezing tolerant but unable to cold harden, we focused only on cold resistance in the absence of acclimation, evaluated as cell injury through the electrolyte leakage test (Sukumaran & Weiser, 1972). When freezing

Table 3. Freezing tolerance (LT50, °C) of (haploid x species) hybrids.

Hybrid		Clones analyzed	LT50*(°C)	Clones with LT50<-3°C
W730 x PI1.22	*(tub x phu)*	5	-2.3±0.6	0
W457 x PI1.22	*(tub x phu)*	1	-1.5	0
W730 x STC1	*(tub x phu)*	6	-2.6±0.8	2
W730 x STC2	*(tub x phu)*	7	-3.6±1.3	4
W730 x STC3	*(tub x phu)*	3	-3.8±0.8	3
UP88-C80 x STC3	*((tub x cha) x stc)*	4	-1.6±0.7	0
UP88-C80 x PI1.22	*((tub x cha) x phu)*	1	-1.7	0
T710 x PI 1.22	*((tub x cha) x phu)*	1	-2.3	0
P100.1 x PI 1.22	*((tub x tar) x phu)*	3	-2.4±1.1	0
P116.4 x PI 1.22	*((tub x tar) x phu)*	1	-2.1±0.7	0
Total		35		9

* LT_{50} evaluated according to Sukumaran and Weiser (1972).

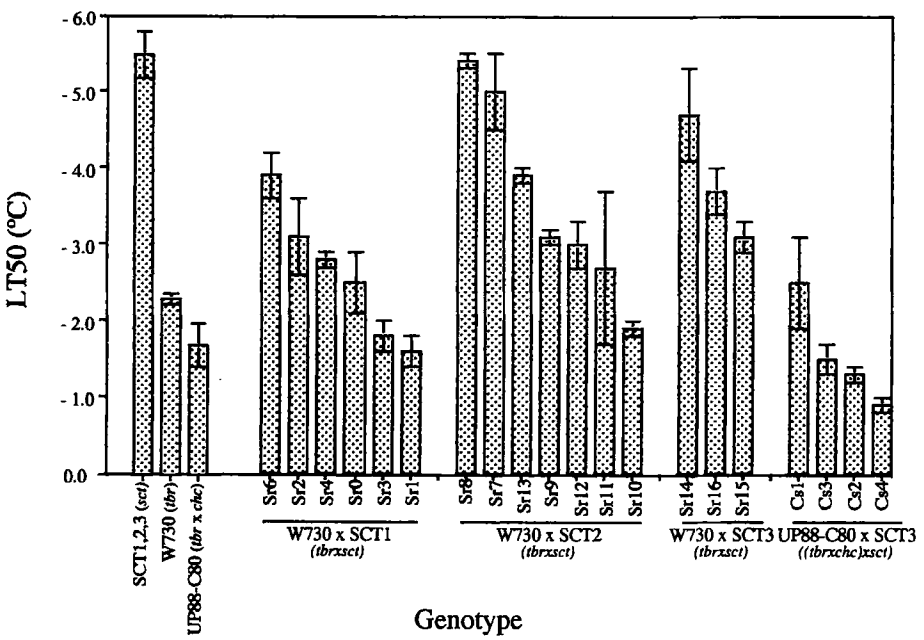

Fig. 1. Frost killing temperature (LT50), ± standard deviation, of parentals and hybrid families from crosses involving the wild cold tolerant species *S. sanctae-rosae*.

tolerance data of the three hybrid populations from W730 (*tbr*) x *S. sanctae-rosae* crosses were pooled ($\chi 2$ for homogeneity of variances not significant), the results seem to suggest a polygenic recessive control for this trait. In fact, the average cold tolerance of the hybrid families (-3.3 °C) was intermediate between the two parents, but closer to the susceptible one, namely W730. Although a larger population and detailed segregation analysis would be necessary, our partial results confirm those from Stone et al. (1993) in a cross between *S. commersonii* and *S. cardiophyllum*, who found that a major portion of genotypic variance for freezing tolerance in the non-acclimated status can be explained by a few partially recessive genes, although additive effects were also evidenced. Other reports in wheat (Brule-Babel & Fowler, 1988) and pea (Liesenfeld et al., 1986) indicate that freezing tolerance of acclimated plants is either dominant or under additive genetic control. It remains an open question whether these contrasting results are due to the different species, the different physiological status or the method for cold tolerance evaluation.

Among the hybrids, it was possible to identify clones, namely Sr7, Sr8 and Sr14, showing a LT50 very close to the tolerant parent. However, only diploid hybrids not

Table 4. Pollen stainability and 2n pollen frequency of 20 interspecific diploid hybrids from 2x x 2x crosses involving 3 accessions of *S. sanctae-rosae* as male parents.

Hybrid	Pollen stainability (%)	2n pollen (%)
W730 x SCT1 (tbr x sct)		
Sr0	54.7	8.4
Sr1	63.0	18.8
Sr2	–	–
Sr3	43.2	19.0
Sr4	71.9	0.8
Sr6	63.2	17.4
W730 x SCT2 (tbr x sct)		
Sr7	–	–
Sr8	–	–
Sr9	–	–
Sr10	–	–
Sr11	–	–
Sr12	–	–
Sr13	–	–
W730 x SCT3(tbr x sct)		
Sr14	–	–
Sr15	–	–
Sr16	–	–
UP88-C80 x SCT 3 ((tbr x chc) x sct)		
Cs1	84.0	19.0
Cs2	83.2	15.0
Cs3	94.6	0.5
Cs4	84.4	6.5

–, did not shed pollen

at the extremes of freezing tolerance were male fertile and produced 2n pollen (Table 4). For instance, clone Sr6 associated a LT50 of -3.9 °C with a 2n pollen production of 17.4%. Therefore, it will be possible to use this diploid clone as male parent in 4x x 2x crosses with *S. tuberosum* to transfer the resistance to low temperature into tetraploid varieties. Larger cells of tetraploid hybrids should not cause a lower freezing tolerance; Palta & Li (1979) reported that cell size is a minor factor in cold resistance. Moreover, cold resistant genotypes have been found among diploid (*S. sanctae-rosae*, *S. commersonii*), triploid (*S. juzepczukii*) and tetraploid (*S. acaule*) species.

All the diploid hybrids which had a LT50 lower than -3.0 °C are being evaluated for 2n egg production, with the aim of using them as female parents in crosses with cultivated varieties.

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