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Plant regeneration from in vitro culture of anthers of *Solanum chacoense* Bitt. and interspecific diploid hybrids *S. tuberosum* L. × *S. chacoense* Bitt.

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Summary. Production of plants from cultured anthers of *Solanum chacoense* clone IP 33, of its interspecific diploid hybrids with *S. tuberosum* clones IP 354 and IP 372, and of a complex *Solanum* hybrid containing in its genome *S. ajanhuiri* is reported. Genotypic differences were found to influence both the induction phase and the regeneration process. Hybrids derived from clone IP 354 of *S. tuberosum* were much more responsive in culture than hybrids from clone IP 372. Altogether, 507 plants were regenerated and 309 were cytologically analyzed. Of these, 52% were haploid, 47% diploid and 1% mixoploid or tetraploid. A number of diploid plants probably originated from unreduced microspores and some genetic consequences of this event are discussed.

Key words: Anther culture – Microspore – Regenerative capacity – Haploid – Interspecific hybrids

Introduction

The use of haploids for the introduction of desirable traits from wild diploid species of tuber-bearing *Solanum* into the germplasm of the cultivated tetraploid potato *Solanum tuberosum* L. (2n = 4x = 48) was discussed at the First International Conference on Haploids in Higher Plants (Mendiburu et al. 1974). *Solanum chacoense* Bitt. (2n = 2x = 24) with such valuable traits as disease and pest resistance, high solids, high protein content in the tubers, adaptation and variability, is an obvious choice (Hawkes and Hjerting 1969).

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Attempts to introduce such valuable germplasm into the cultivated potato by recurrent selection at the level of the species among its hybrids with selected dihaploid *tuberosum* lines, have met with little success (Leue 1980; Leue and Peloquin 1982). Naturally occurring haploids of *S. chacoense* are not known and their in vitro production has so far been unsuccessful (Irikura 1975; Mix 1982). Haploids produced from interspecific diploid hybrids between *S. chacoense* and *S. tuberosum* would be even more useful for potato breeding. These haploids are expected to be genetically different from one another and to carry different combinations of paternal and maternal chromosomes. After diploidization, homozygotes can be selected having desirable traits of both cultivated and wild species (de Nettancourt and Devreux 1977). Anther culture has been valuable in recent years in the development of both mono and di-haploids from certain genotypes of *S. tuberosum* or other *Solanum* species most closely related to it (Irikura 1975; Sopory et al. 1978; Wenzel et al. 1979; Mix 1983). It has also been used successfully in interspecific hybrids to produce plants having virus and nematode resistance in the homozygous condition (Wenzel and Uhrig 1981).

Finally, the production of haploids in species characterized by self-incompatibility of the gametophytic manofactorial type, could be utilized to investigate the mechanism of generation of new S-specificities (Cappadocia and Cheng 1982).

The present paper reports on the production of plants from anthers of self incompatible *S. chacoense,* of diploid hybrids *S. tuberosum* × *S. chacoense* and of a complex *Solanum* hybrid.

Materials and methods

Observations were made on anthers from ten genetically unrelated dihaploid clones of *Solanum tuberosum* L.; two diploid hybrid clones of *S. tuberosum* × *S. phureja*; a diploid clone (IP 33) of *S. chacoense* Bitt. noted for its resistance to nematodes, potato virus A, potato virus Y and verticillium wilt; a diploid complex hybrid *S.* (*ajanuhiri* × *stenotomum*) × (*phureja* × *stenotomum*) clone IP 56 characterized by frost tolerance and earliness; and two series of diploid hybrids

between *S. tuberosum* and *S. chacoense*. In both series, the *chacoense* male parent was clone IP 33. The *tuberosum* parents were clones IP 354 and IP 372. Hybrids derived from clone IP 354 are referred to below as series A and hybrids from clone IP 372 as series B. Series A was composed of 37 genetically different clones and series B of 24 genetically different clones. Each clone was propagated by cuttings and cultivated both indoors (greenhouse, for crosses and maintenance) and outdoors (field in San Carlos, California, for anther culture) during late April–September 1981 and 1982. Low temperatures for these months ranged from 5° to 14 °C (average 7–10 °C) while high temperatures ranged from 16° to 37 °C (average 22–27 °C).

Flower buds were harvested when about 4–6 mm in length, still unopened and sterilized. Anthers were dissected out and cultured with the filament removed. The developmental stage of the pollens was assessed on one test anther from each bud. Culture was restricted to anthers having microspores undergoing the first pollen division (mitotic stage) or slightly younger anthers (late uninucleate stage). The anthers were first floated on a liquid medium (A5, designated the induction medium), and then transferred to an agar medium (A57, the regeneration medium). For the induction phase, 50 mm plastic petri dishes were used containing 3 ml aliquots of culture medium and 20 anthers per dish. Dishes were sealed with parafilm. The cultures were then incubated at 30 °C for 2 days in darkness and then transferred to light at 27 °C (5 Klx, Sylvania Gro Lux tubes, 16 h day). After 7–10 days, the anthers were plated on regeneration medium (60 anthers in each 100 mm petri dish) and incubated under the same conditions. Macroscopic structures emerging from the anthers were transferred to another agar medium (A57-4, designated maintenance medium) to promote root and shoots. When the plantlets had developed good root and shoot systems they were transferred to sterile soil in pots and cultivated in the greenhouse.

Culture media composition

Medium A5. Basal constituents of Murashige and Skoog (1962) but with sucrose at 6% w/v. To this medium was added myo-inositol 500 mg/l, L-glutamine 200 mg/l, 6-benzylaminopurine and indole-3 acetic acid both at 1 mg/l. The pH was adjusted to 5.5 with IN NaOH and the medium sterilized by filtration.

Medium A57. Basal constituents of Nitsch (1969) with sucrose at 2% w/v. To these were added active charcoal (Sigma) 0.5% w/v and agar (Difco-Bacto) 0.6% w/v. The pH was adjusted to 5.8 and the medium sterilized by autoclaving.

Medium A57-4. As for A57 but without charcoal. Indolebutyric acid was added at 0.1 mg/l. The pH and sterilization procedures were the same as for A57.

Cytological analysis

Microspores and developing embryos were stained with lactoacetic orcein. Chromosome counts were made on root tips placed in a saturated solution of 1-bromonaphthalene for one hour, fixed overnight, hydrolyzed for 30 min at room temperature in 6N HCl, washed and placed in Schiff's reagent for 2 h in the dark. Finally they were squashed and examined in lactoacetic orcein. Ploidy was also assessed indirectly from chloroplast counts made on stomatal guard cells (Frandsen 1968). Finally, pollen fertility and size were determined on mature samples stained with 1% acetocarmine.

Results

Induction of growth

Induction was studied by direct microscopic observation of the microspores after 5 days of culture on A5 liquid medium. In preliminary experiments with clone IP 33, about twice as many anthers contained dividing microspores when cultured from field plants compared to those obtained from greenhouse plants. Therefore we conducted all our experiments with vigorous plants grown in the field. No significant differences in response were detected between microspores at the late uninucleate and those at the mitotic stage. Therefore, in further experiments microspores at these two stages were utilized without distinction.

In the case of the interspecific hybrids *S. tuberosum* × *S. chacoense* dividing microspores were mostly observed in anthers of series A. Among 37 genotypes tested in this series, 31 showed dividing pollen. In contrast, dividing microspores were observed only in 6 out of the 24 clones examined in series B.

Plant regeneration

Macroscopic structures emerged from the anthers after about 5–7 weeks on the regeneration medium. Some already possessed redimentary shoots and roots at emergence. Others were still embryonic, at the globular or torpedo stage. Although as many as 35% of the pollen grains (as in the case of the complex hybrid clone IP 56) were induced into division, few survived and emerged as macroscopic structures. In general there was only one, sometimes several structures per responsive anther. However, not all were successfully reared to maturity. The overall data concerning the responding genotypes, the number of macroscopic structures and plants regenerated are given in Table 1. It can be seen that in clone IP 33 an average of 7.45 plants for every 100 anthers cultured was obtained, while clones of series A yielded an average of 1.66 plants. There were marked genotypic differences in response among the series A hybrids (Table 2). Among the 31 responding genotypes, two hybrids (A23 and A41) performed better than the rest, giving more than 10 plants on average for every 100 anthers cultured.

In total 507 plantlets were regenerated in the course of this study and the ploidy was established for 309 (Table 1). Chromosome counts were made for 155 plants regenerated from clone IP 33 and 20 randomly selected plants regenerated from series A. Since in all cases the ploidy assessed by chromosome count, and the ploidy estimated by counting the chloroplast number of the guard cells were the same, the latter method was routinely used to quickly determine the ploidy status of the regenerants. Stomata (i.e. a pair of guard

Table 1. Frequencies of macroscopic structures and plantlets from cultured anthers of responsive clones and ploidy levels of regenerated plants

Genotype	No. of anthers cultured	No. of responsive anthers	No. of macroscopic structures (per 100 anthers cultured)	No. of plants regenerated (per 100 anthers cultured)	No. of plants analyzed	Haploids	Diploids	Other
IP 33 *S. chacoense*	2,645	288	327 (12.36%)	197 (7.45%)	190	141 (74%)	47 (25%)	2 (1%)[a]
IP 354 × IP 33[c] *S. tuberosum* × *S. chacoense*	18,258	921	1,220 (6.68%)	303 (1.66%)	116	19 (16%)	95 (82%)	2 (2%)[b]
IP 372 × IP 33[d] *S. tuberosum* × *S. chacoense*	9,796	10	12 (0.12%)	3 (0.03%)	2		2	
IP 56 *S.* × *(ajanuhiri* × *stenotomum)* × *(phureja* × *stenotomum)*	4,351	106	121 (2.78%)	4 (0.09%)	1	1		

[a] Mixoploids (n and 2n)
[b] Tetraploids
[c] 31 responsive clones out of 37 genetically different clones tested
[d] 6 responsive clones out of 24 genetically different clones tested

Table 2. Clones A (*S. tuberosum* IP 354 × *S. chacoense* IP 33) responding best to anther culture

Genotype	No. of anthers cultured	No. of responsive anthers	No. of macroscopic structures (per 100 anthers cultured)	No. of plants regenerated	No. of plants per 100 anthers cultured
A 5	43	4	4 (9.30%)	3	6.98
A 17	787	52	77 (9.78%)	26	3.30
A 22	571	32	45 (7.88%)	14	2.45
A 23	896	109	154 (17.19%)	95	10.60
A 28	502	24	34 (6.77%)	22	4.38
A 41	98	19	28 (28.57%)	16	16.33
A 44	298	21	24 (8.05%)	11	3.69
A 45	400	10	18 (4.59%)	26	6.50

cells) of haploid plants had a mean number of chloroplasts of 6.0 ± 1.3, while diploids had 11.1 ± 2.3.

Pollen fertility was very high in all the clones used as anther donors and ranged from 80 to 98% as assessed by staining mature pollen with acetocarmine. Unreduced pollen was never observed in *S. chacoense* clone IP 33 but was indeed present in variable amounts in its hybrids with dihaploid *S. tuberosum*. Haploid pollen diameters measured 22.8 ± 0.4 μm while unreduced pollen measured 32.1 ± 0.5 μm.

Discussion

To the list of species in which haploids are available it is now possible to add *S. chacoense*, its hybrids with *S. tuberosum* and a complex hybrid containing in its genome *S. ajanhuiri*, a valuable source of frost tolerance (Huaman et al. 1982).

Although highly successful with anthers of *S. chacoense*, the induction medium A5 has not proved universally applicable to other *Solanum* genotypes. Thus, when used on anthers of 10 different *tuberosum* clones, in those of hybrids between *S. tuberosum* × *phureja* or *S. stenotomum* × *phureja* and most of the clones of series B, morphogenetic divisions were not induced. Medium A5, on the other hand, proved exceedingly inductive for most of the hybrids of *S. tuberosum* × *chacoense* belonging to series A.

Genotypic differences in anther culture responsiveness are well documented (Guha-Mukherjee 1973; Wenzel et al. 1977; Chaleff 1981) and some evidence for the heritability of regenerative capacity has already been presented in potato (Jacobsen and Sopory 1978; Simon and Peloquin 1977; Wenzel and Uhrig 1981; Uhrig 1983). Although we lack information about the regenerative behavior of the two *tubero-*

sum clones IP 354 and IP 372, results obtained with hybrids of series A indicate that one can transfer the regenerative capacity via breeding and recover highly responsive genotypes (Table 2). However, the data presented here also suggest that this holds true only in specific genotypic interactions (series A vs series B). This view is further supported by the variations found within the clones belonging to the same responding series, (a few clones in series A did not respond at all).

The cultured anthers of the parent clone *S. chacoense* yielded a preponderance of haploids (Table 1). The origin of the diploid plants from haploid microspores of this clone was confirmed genetically by using the S-allele test (van Bruekelen 1981) and the details of this work will be presented in another communication.

In hybrids from the cross *S. tuberosum* × *chacoense* the observed preponderance of diploids was consistent with other findings on cultured anthers of dihaploid *tuberosum* (Jacobsen and Sopory 1978; Wenzel and Uhrig 1981). In *S. chacoense* unreduced microspores were apparently not present as judged by the uniformity of size of the spore population. On the other hand, unreduced microspores, easily recognized by their larger size, were observed, in variable amounts in the hybrids of series A, so that a number of diploids obtained in this material may have originated from them. The genetic consequences of such an event are important (Peloquin 1983; Ramanna 1979) and can be summarized as follows. Unreduced microspores may originate via mechanisms that are equivalent of a first division restitution (FDR) or a second division restitution (SDR) (Mok and Peloquin 1975). In the case of FDR, all loci from the centromere to the first crossover and one half the loci between the first and second crossovers will be heterozygous in the spores if they were heterozygous in the parent. By contrast, in the case of SDR, all loci from the centromere to the first crossover will be homozygous in the spores if these loci were heterozygous in the parent, and all the loci between the first and second crossovers will be heterozygous in the spores if they were heterozygous in the parent. At present we are investigating by which mechanism unreduced microspores are formed in our material (in progress). Should they arise from the SDR, the resulting plantlets are expected to be "genetically equivalent to sexually selfed progeny" as indicated by Ramanna (1974). In this case, in addition to the advantage of obtaining via anther culture "selfed" progeny in self-incompatible plant material, it will also be interesting to analyze such pollen-derived plants for gene mapping purposes. Some diploids could also have arisen by chromosome duplication or nuclear fusion as shown in other species by Sunderland (1974).

In conclusion, the procedure outlined above led to the production of pollen derived plants from *S. chacoense* so that the introduction of desirable gene combinations from this wild species into the germplasm of the cultivated potato is now possible. In this regard, we are particularly encouraged by the results obtained with some of the interspecific hybrids *S. turberosum* × *chacoense*. Since one person can cultivate up to one thousand anthers per day, given a highly responsive genotype like A23 and A41, it would be possible to raise pollen plants at a rate of 100–150 per day, which should be a scale attractive to plant breeders.

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