Theor Appl Genet (1993) 85:905–912

THEORETICAL AND APPLIED GENETICS



# Isolation and characterization of potato diploid clones generating a high frequency of monohaploid or homozygous diploid androgenetic plants

**R. Meyer, F. Salamini, and H. Uhrig**

Max-Planck-Institut für Züchtungsforschung, Carl-von-Linné-Weg 10, D-5000 Köln 30, Germany

Received April 6, 1992; Accepted June 3, 1992
Communicated by G. Wenzel

**Summary.** Cultured microspores of diploid potato clones lead with high frequency to diploid regenerants. In this paper we report on the genetic variability for in-vitro monohaploid production from anthers of diploid plants. Three diploid genotypes have been isolated which combine the capacity to regenerate monohaploid plants with outstanding embryoid production. A trait of the anther-donor clones associated with the generation of monohaploid plants is the low production of 2n pollen grains. When present in anthers of diploid genotypes, diploid unreduced microspores are, in fact, derived mainly from a first division restitution mechanism leading to high heterozygosity of the derived embryoids, a state which apparently supports superior growth in-vitro. Also, reduced microspores have been found capable of generating diploid regenerants and the adoption of the RFLP technique allowed the isolation of such diploid plants, which can be considered to be pure lines. Donor clones with a low capacity to generate monohaploids are, as expected, poor producers of homozygous diploid plants. The selection of an anther donor producing a sufficient number of monohaploid or homozygous diploid regenerants fulfills the requirements of the first part of the analytical breeding scheme, i.e., the production of homozygous diploid clones.

**Key words:** Androgenesis – Diploid potato – Monoploids – Potato pure lines

*Correspondence to:* F. Salamini

## Introduction

To improve the tetraploid gene pool of potato, breeding schemes based on diploid strains have been proposed (Chase 1963; Mendiburu et al. 1974; Uhrig and Salamini 1987). Wenzel et al. (1979) suggested the generation of homozygous doubled haploids through tissue culture, with subsequent sexual intercrossing to obtain heterozygous diploids. Protoplast fusion of such clones should then lead to tetraploid hybrids derived from four different haploid genomes.

The anther-culture response of diploid potato clones has been substantially improved leading to a large production of androgenetic embryoids and plants (Wenzel and Uhrig 1981; Johansson 1983; Uhrig 1985; Singsit and Veilleux 1989). One disadvantage of the in-vitro approach to the breeding of potato is still evident: a very high fraction of androgenetic plants derived from diploid anther-donor clones are also diploid (Wenzel and Uhrig 1981; Uhrig 1985).

In the present study, the cytological analysis of cultured microspores and embryoids reveals the possible origins of diploid regenerants: either from nuclear restitution during microsporogenesis or from the spontaneous doubling of monohaploid cells through fusion, endomitosis or endoreduplication. The RFLP technique was used to determine the genetic constitution of individual androgenetic plants. The ability to transmit the androgenetic capacity to their progeny marked a second breakthrough in the development of an efficient anther culture technology (Wenzel and Uhrig 1981; Uhrig 1985; Singsit and Veilleux 1989). However, one disadvantage of the in-vitro approach to the breeding of potato was still evident: a very high fraction of androgenetic plants derived from diploid anther-donor clones were themselves diploid (Wenzel

and Uhrig 1981; Uhrig 1985). It has been suggested that the majority of these diploid regenerated plants are of haploid origin but have doubled their chromosome number due to endomitosis or endo-reduplication during the process of regeneration (Wenzel and Foroughi-Wehr 1984). It is, however, known that nuclear restitution is a common process in the microsporogenesis of diploid *Solanum* species (Peloquin 1983). First-division restitution (FDR, Mok and Peloquin 1975a,b) during microsporogenesis generates 2n-pollen which is still heterozygous at a large fraction of genetic loci (Hermsen 1984). As a possible consequence, the in-vitro development of such microspores would produce partially heterozygous diploid androgenetic plants.

In the present study, the cytological analysis of cultured microspores and embryoids reveals two possible origins of diploid regenerants: either nuclear restitution during microsporogenesis or spontaneous genome doubling of monohaploid cells through fusion, endomitosis or endoreduplication during the embryogenic microspore development or the regeneration phase. The RFLP technique was used to determine the origin, and hence the genetic constitution, of individual diploid androgenetic plants. Diploid anther donors generating a high proportion of monohaploid androgenetic plants have been identified and characterized.

## Materials and methods

*Plant material*

Seventy-five diploid potato genotypes were used in this study. Five of them were EAPP-clones (*e*fficient *a*nther *p*lant *p*roducing clones) selected previously for outstanding anther-culture response (Uhrig 1985); 42 clones originated from five crosses between diploid anther-derived clones; 28 clones were selected from six crosses among diploid breeding lines not evaluated for their response in anther culture. The plants, grafted onto tomato root-stocks, were grown from seeds or tubers in the greenhouse at $22 \pm 2$ °C.

*Anther culture*

Anther culture was carried out under liquid medium conditions, essentially as indicated by Uhrig (1985). The Linsmaier and Skoog (1965) salt medium (1/2 LS) was supplemented with 0.05% active charcoal, 0.1 mg/l of IAA, 1.0 mg/l of BAP, $5 \times 10^{-5}$ M glutamine, $5 \times 10^{-5}$ M asparagine and 6% sucrose. Embryoids were harvested after 4–8 weeks of culture and plated for regeneration on 1/2 LS supplemented with 0.1 mg/l of $GA_3$, 2% sucrose and 0.8% agar. Regeneration and sub-culture of plants took place in a culture room with a 16 h light/8 h dark regime, at 20 °C and 70% relative humidity. The number of embryoids produced per 100 anthers after 8 weeks of culture, or the number of plants regenerated per 100 anthers, were taken as indices of anther culture response.

*Ploidy of plants and embryoids*

Plant ploidy was scored by chloroplast counting in stomatal guard cells (Frandsen 1968). Monohaploid or mixoploid plants were further evaluated by chromosome counting in leaf primordia pretreated with 0.029% 8-hydroxyquinoline for 4 h at room temperature. Carnoy fixation and an orcein staining technique (Dyer 1963) were adopted. In androgenic embryoids 50% formic acid was used as a fixative in order to remove starch grains (Gould 1984).

*Meiosis in pollen mother cells*

Cytological preparations for anther squash techniques were as described by Ramanna (1979) and Singh et al. (1988). Meiotic figures were analyzed in at least 100 pollen mother cells. A similar number of gametocytes was examined to determine the frequency of dyads and tetrads. Pollen vitality was based on staining with lacto-phenolic fuchsin. Unreduced pollen grains were evaluated from the size and the number of germ pores.

*Cytology of cultured microspores*

Anthers were removed from culture flasks at 2–7 day intervals for a period up to 6 weeks. After fixation over-night in Carnoy's fluid I, the microspores were carefully released from anthers, hydrolyzed for 15 min in 1N HCl at 60 °C, stained in orcein for at least 4 h and mounted in 1% acetocarmine.

*RFLP-analysis and probes*

Young leaves and shoots from greenhouse- and in-vitro grown plants were harvested for RFLP analysis. DNA-extraction was according to Doyle and Doyle (1990). Restriction digests, electrophoresis, blotting and hybridization procedures were as described in Gebhardt et al. (1989). Four restriction enzymes, *Rsa*I, *Taq*I, *Hin*dIII and *Alu*I, were employed.

## Results

### *Characterization of diploid clones with a superior response in anther culture*

The results from in-vitro cultivation of the anthers of the 75 clones considered are reported in Table 1. Twenty-six clones did not produce embryoids from microspores: among them the clones with parents not previously tested for the in-vitro androgenetic response prevailed. Seventeen additional clones were poorly adapted to anther plant formation, producing only embryoids (six clones) or less than ten plants per 100 anthers (16 clones). The best 27 clones gave rise to more than ten plants per 100 anthers. Five of these were already known as good anther plant producers (the 2x EAPP-clones: A83.2264/01/6, A83.2278/01/3, A84.2306/01/1, A84.2315/02/1, and A87.2315/89/1; Uhrig 1985; Uhrig and Salamini 1987). Twenty-three originated from crosses between clones derived from anther culture, and four from materials not previously selected in-vitro (86.3144/3, 86.3144/4, 86.3144/5 and H81.404/89).

The data of Table 1 indicate that a high rate of embryoid formation may not coexist with a high rate

**Table 1.** Frequency of embryoids and plants derived from in-vitro culture of anthers in the most responsive donors out of 75 diploid potato clones[a]

| Anther-donor clone | Embryoids per 100 anthers | Total no. of plants regenerated | Plants per 100 embryoids | Plants per 100 anthers |
|---|---|---|---|---|
| 87.2002/06 | 357.8 | 184 | 23.1 | 82.5 |
| A84.2306/01/1 | 313.3 | 537 | 22.1 | 69.3 |
| 87.2005/08 | 189.8 | 79 | 35.3 | 66.9 |
| 87.2004/04 | 261.4 | 80 | 17.4 | 45.5 |
| 87.2004/07 | 702.4 | 44 | 5.0 | 35.2 |
| 87.2002/03 | 655.6 | 57 | 5.4 | 35.2 |
| 87.2002/05 | 1263.5 | 52 | 2.6 | 33.3 |
| A84.2315/02/1 | 113.9 | 56 | 24.9 | 33.3 |
| 87.2004/09 | 277.7 | 90 | 10.9 | 30.4 |
| 87.2004/05 | 545.1 | 36 | 5.4 | 29.5 |
| 87.2003/05 | 1459.4 | 40 | 2.1 | 30.1 |
| 87.2001/07 | 523.8 | 69 | 5.2 | 27.4 |
| 87.2002/04 | 268.8 | 66 | 9.7 | 26.1 |
| A87.2315/89/1 | 76.0 | 68 | 32.1 | 24.4 |
| 86.3144/5 | 517.9 | 109 | 4.7 | 24.3 |
| 87.2002/02 | 56.5 | 39 | 37.5 | 21.2 |
| 87.2001/06 | 184.3 | 44 | 10.4 | 19.2 |
| 87.2004/06 | 1601.3 | 30 | 1.2 | 18.9 |
| 87.2005/04 | 313.5 | 51 | 5.8 | 18.1 |
| A83.2278/01/3 | 276.4 | 235 | 5.9 | 16.3 |
| 87.2005/05 | 49.3 | 33 | 33.0 | 16.3 |
| 87.2002/01 | 223.2 | 22 | 6.9 | 15.5 |
| A83.2264/01/6 | 180.0 | 92 | 8.3 | 15.0 |
| 87.2003/04 | 83.6 | 26 | 16.5 | 13.8 |
| 87.2001/04 | 873.6 | 20 | 1.5 | 13.5 |
| 87.2004/08 | 622.6 | 18 | 1.8 | 11.0 |
| 86.3144/4 | 192.2 | 55 | 5.6 | 10.7 |

[a] The other clones behaved as follows: 26 clones with no production of embryoids; six clones with 253.4 to 1.1 embryoids/100 anthers but no regenerated plants; 16 clones with less than ten plants/100 anthers

of plant regeneration from embryoids: a moderate response in terms of embryoid formation can be compensated by a high regeneration capacity of the embryoids. This leads to a number of regenerated plants per 100 anthers similar to that of a high-ranking embryoid producer with a low regeneration rate.

*Clones efficient in producing monohaploid derivatives*

In-vitro culture of microspores from diploid clones of *Solanum tuberosum* offers the opportunity to isolate monohaploid plants. It can be assumed that during the production of embryoids and the regeneration of plants, the genotypes with superior growing rates are more adapted to pass through the process. This approach to the isolation of monohaploids is, however, hampered by the high frequency of diploid androgenetic plants usually obtained from diploid clones. This fraction can derive from the doubling of monohaploids – a positive case with respect to the possibility of obtaining homozygous diploid potato lines – or from unreduced gametes originating from restitution mechanisms which still have a substantial degree of heterozygosity.

The identification of monohaploid-producing clones was carried out in 1143 anther-derived plants isolated from 29 different diploid donor clones. Eight hundred and seventy regenerants were diploid, whereas 124 were monohaploid (Table 2).

Significant differences in the production of monohaploid regenerants allow for the partitioning of the donor clones into four classes. Clones of class I produced more than 30% monohaploid regenerants, class II 20%, class III less than 10% while class IV clones did not produce any monohaploid plants.

The clones with the highest percentage of diploid regenerants (class IV) displayed either a very good anther culture aptitude (313.5 to 1459.4 embryoids per 100 anthers, Table 1) or a high regeneration rate (9.7 to 17.4 plants per 100 embryoids). In contrast, the monohaploid producers of class I showed either a low embryoid formation (clones 87.2002/01, 87.2002/10, 87.2005/01 and 86.3144/3) or a low regeneration rate (clones 87.2001/7, 87.2004/6 and 87.2005/3). An outstanding capacity for embryoid production was found in clones 87.2001/7, 87.2004/6 and 87.2005/3.

An experiment aimed at the improvement of the capacity of a clone to regenerate monohaploid plants

**Table 2.** Ploidy of plants regenerated from microspores of 29 diploid clones[a]

| Anther-donor clone | No. of plants scored | Fraction of total (%) | | | | |
|---|---|---|---|---|---|---|
| | | Monohaploid (x = 12) | Dihaploid (2x = 24) | Triploid (3x = 36) | Tetraploid (4x = 48) | Mixoploid |
| Class I | | | | | | |
| 87.2005/01 | 7 | 71.4 | 28.6 | | 0.0 | 0.0 |
| 87.2002/10 | 6 | 66.7 | 33.3 | | 0.0 | 0.0 |
| 87.2005/03 | 6 | 50.0 | 50.0 | | 0.0 | 0.0 |
| 87.2002/01 | 21 | 42.9 | 52.4 | | 4.8 | 0.0 |
| 87.2004/06 | 11 | 36.4 | 63.6 | | 0.0 | 0.0 |
| 87.2001/07 | 41 | 34.1 | 58.5 | | 2.4 | 4.9 |
| 86.3144/3 | 15 | 33.3 | 66.7 | | 0.0 | 0.0 |
| Class II | | | | | | |
| 2315/02/1 | 26 | 23.1 | 42.3 | | 26.9 | 7.7 |
| 2278/01/3 | 22 | 22.7 | 77.3 | | 0.0 | 0.0 |
| 87.2001/01 | 27 | 22.2 | 70.4 | | 0.0 | 7.4 |
| 86.3144/5 | 56 | 21.4 | 76.8 | | 0.0 | 1.8 |
| 87.2002/08 | 7 | 14.3 | 71.4 | | 0.0 | 14.3 |
| 2315/89/1 | 38 | 13.2 | 36.8 | | 50.0 | 0.0 |
| 87.2002/06 | 180 | 12.7 | 81.6 | | 1.7 | 3.9 |
| 2306/01/1 | 92 | 10.9 | 72.8 | | 13.0 | 3.3 |
| Class III | | | | | | |
| 87.2004/05 | 13 | 7.7 | 92.3 | | 0.0 | 0.0 |
| 86.3144/4 | 40 | 7.5 | 90.0 | | 0.0 | 2.5 |
| 87.2004/07 | 27 | 7.4 | 74.1 | | 3.7 | 14.8 |
| 2264/01/6 | 69 | 5.8 | 75.4 | 13.0 | 5.8 | 0.0 |
| 87.2002/02 | 41 | 2.4 | 68.3 | | 14.6 | 14.6 |
| 87.2001/06 | 42 | 2.4 | 76.2 | | 9.5 | 11.9 |
| Class IV | | | | | | |
| H81.404/89 | 9 | 0.0 | 100.0 | | 0.0 | 0.0 |
| 87.2002/03 | 47 | 0.0 | 85.1 | | 6.4 | 8.5 |
| 87.2002/04 | 51 | 0.0 | 88.2 | | 0.0 | 11.8 |
| 87.2002/05 | 21 | 0.0 | 81.0 | | 4.8 | 14.3 |
| 87.2003/05 | 30 | 0.0 | 86.7 | | 10.0 | 3.3 |
| 87.2004/04 | 59 | 0.0 | 79.7 | | 5.1 | 15.3 |
| 87.2004/09 | 74 | 0.0 | 89.2 | | 5.4 | 5.4 |
| 87.2005/04 | 36 | 0.0 | 94.4 | | 0.0 | 5.6 |
| 87.2005/05 | 30 | 0.0 | 83.3 | | 3.3 | 13.3 |
| Total | 1143 | 124 | 870 | 9 | 73 | 6 |

[a] Ploidy was based on chloroplast countings in stomatal guard cells

**Table 3.** Ploidy of androgenic regenerants after recurrent selection for monohaploid production

| Anther donor | 2264/01/6 | 2306/01/1 | 2315/02/1 | 2315/89/1 |
|---|---|---|---|---|
| Cycle no. | 0 | 1 | 2 | 2 |
| Ploidy of regenerants (% of total) | | | | |
| Monohaploid | 5.8 | 10.9 | 23.1 | 13.2 |
| Dihaploid | 75.4 | 72.8 | 42.3 | 36.8 |
| Tri/tetraploid | 18.8 | 13.0 | 26.9 | 50.0 |

by in-vitro recurrent selection is presented in Table 3. Anthers from cycle 0 clone A83.2264/01/6 regenerated 5.8% of monohaploid plants, 75.4% of diploids and 18.8% tri- and tetra-ploids. Among the anther-derived progenies selected in vitro during two recurrent cycles, the capacity to regenerate a high fraction of monohaploids was evident in clone A84.2315/02/1 (cycle 2), but was associated with an increase in the proportion of tetraploid derivatives. This was particularly evident in a second clone of cycle 2, A87.2315/89/1.

*Traits associated with the generation of monohaploid progeny*

The monohaploid-producing clones of class I had either a low yield of embryoids or a low regeneration rate of plants. Low percentages of unreduced male gametes were observed in these monohaploid producers (Fig. 1). The correlation between the frequency of vital 2n gametes and the percentage of monohaploid regenerants was, in fact, significantly negative ($r = -0.7$; $P < 0.01$). Exceptions were the clones 86.3144/5 and 87.2002/6 which had a significant fraction of diploid pollen grains, but also regenerated a considerable number of monohaploid plants. The negative correlation indicates that selection against unreduced gametes restricts the population in which monohaploid producers can be found.

Further cytological characterization (Table 4) revealed that clones showing the formation of dyads were most frequently associated with pollen mother cells with parallel spindles (FDR); two clones however, showed, premature cytokinesis (SDR). The observation that unreduced microspores begin to divide earlier and develop faster than the reduced ones (data not shown) and the finding of mechanisms such as FDR – which favour a high degree of heterozygosity of the unreduced microspores – explain why clones with a high percentage of unreduced pollen grains tend to regenerate preferentially diploid plants.

*Diploid regenerated plants: cytological origin and selection of homozygous doubled monohaploids*

Three different pathways followed in-vitro by potato microspores during the early development could be detected in our 2n material. In pathway A (Raghavan

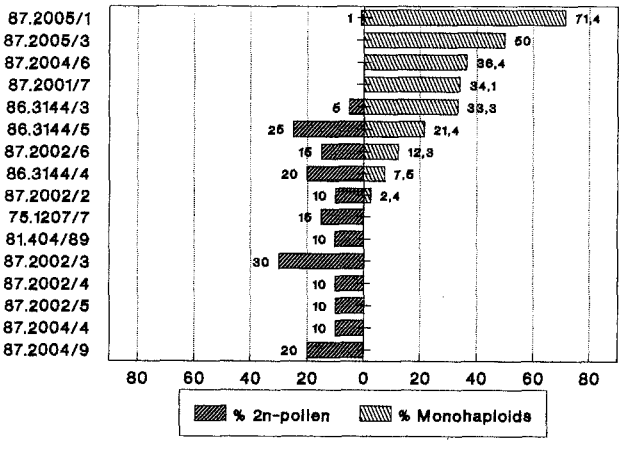


**Fig. 1.** Correlation between the production of 2n pollen by the anther donor and the capacity to regenerate monohaploid plants from anthers cultured in-vitro

**Table 4.** Presence of dyads and restitution mechanisms in a sample of anther-donor clones belonging to different classes of monohaploid producers[a]

| Anther donor | Class | % Dyads[b] | % FDR[c] | % SDR[d] |
|---|---|---|---|---|
| 87.2001/7 | I | 1.2 | 5.0 | 0.0 |
| 87.2004/6 | I | 5.2 | 6.1 | 0.0 |
| 87.2005/3 | I | 1.6 | 6.4 | 0.0 |
| 87.2002/6 | II | 20.5 | 19.0 | 2.0 |
| 87.2002/2 | III | 10.5 | nt | nt |
| 87.2002/3 | IV | 27.9 | 26.9 | 0.0 |
| 87.2002/4 | IV | 45.4 | 36.1 | 5.6 |
| 75.1207/7 | IV | nt | 25.8 | 0.0 |

[a] The definition of classes of monohaploid producers is given in the text
[b] Meiotic products after restitutional events; two diploid microspores
[c] First division restitution caused by parallel spindles in 'anaphase-II'
[d] Second division restitution by premature cytokinesis in 'telophase-I'
nt, not tested

1986), the normal first pollen mitosis is followed by a degeneration of the generative cell, whereas the vegetative cell divides mitotically; later on polynucleated stage become evident (Fig. 2A). This pathway leads mainly to polyploid plants (2n or higher). In pathway B (Raghavan 1986), instead of the normal first pollen mitosis, a symmetric division of the microspore takes place. One or both cells continue their division and generate multicellular microspores (Fig. 2B). This pathway was observed most frequently and, depending on the ploidy state of the microspores, generated haploid or diploid regenerants. Pathway '3' (Sunderland 1974) starts with a symmetric division of the microspore nucleus, but cytokinesis fails leading to polynucleated microspores (Fig. 2C). Up to six free nuclei per microspore could be detected in our material. This pathway leads to diploid or higher ploid plants.

The existence of cells containing free nuclei is a prerequisite for nuclear fusion, which occurs frequently following the simultaneous division of at least two nuclei. This process creates embryoids whose ploidy and homozygosity depend on the type of microspores (reduced or restituted) and on the number of nuclei involved. The observation that the monohaploid fraction decreased in the regenerated plants compared to that of embryoids (data not shown) suggests a lower vitality of the monohaploid embryoids. The frequent finding of free nuclei, however, suggested a further possibility: a spontaneous genome doubling which can lead to the regeneration of homozygous diploid

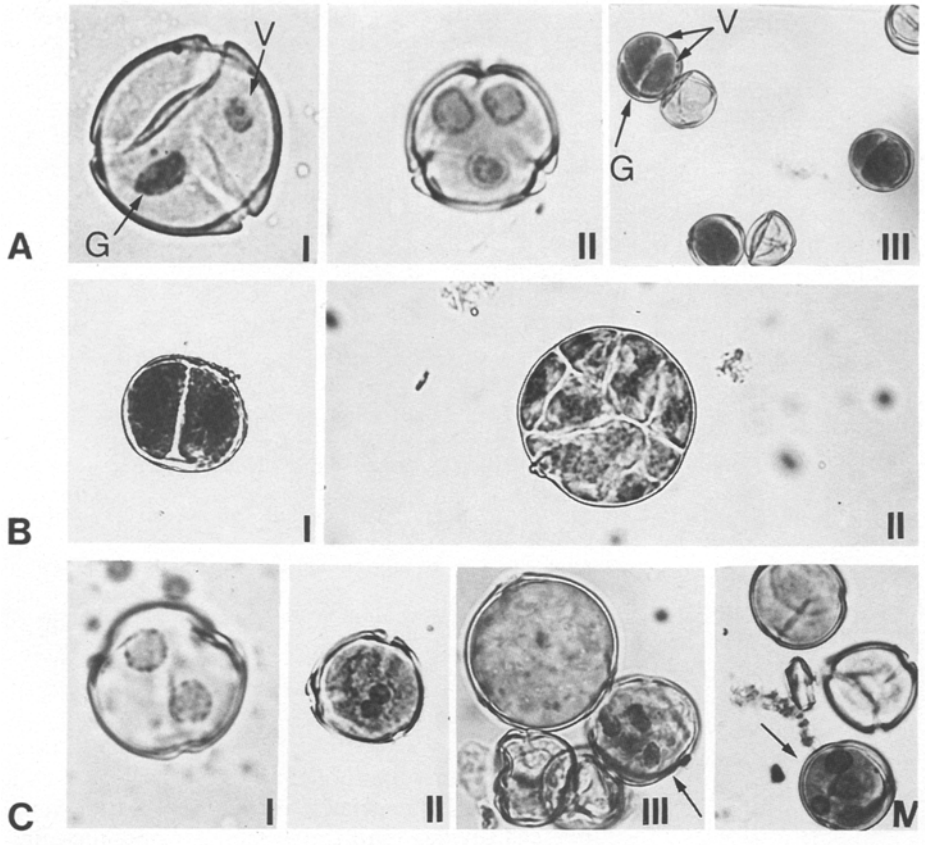


**Fig. 2A–C.** Most relevant pathways of potato microspore development found in anther culture. **A** Pathway A: *I*, asymmetrical first pollen mitosis (*V*, vegetative nucleus; *G*, generative nucleus). *II*, division of the vegetative nucleus. *III*, degeneration of the generative cell (*V*, cells originating from a division of the vegetative nucleus; *G*, generative cell). **B** Pathway B: *I*, symmetrical first division of the microspore. *II*, multicellular microspore with nine cells. **C** Pathway '3'. *I* + *II*, Microspores with two similar nuclei. *III* + *IV*, Microspores with four free nuclei

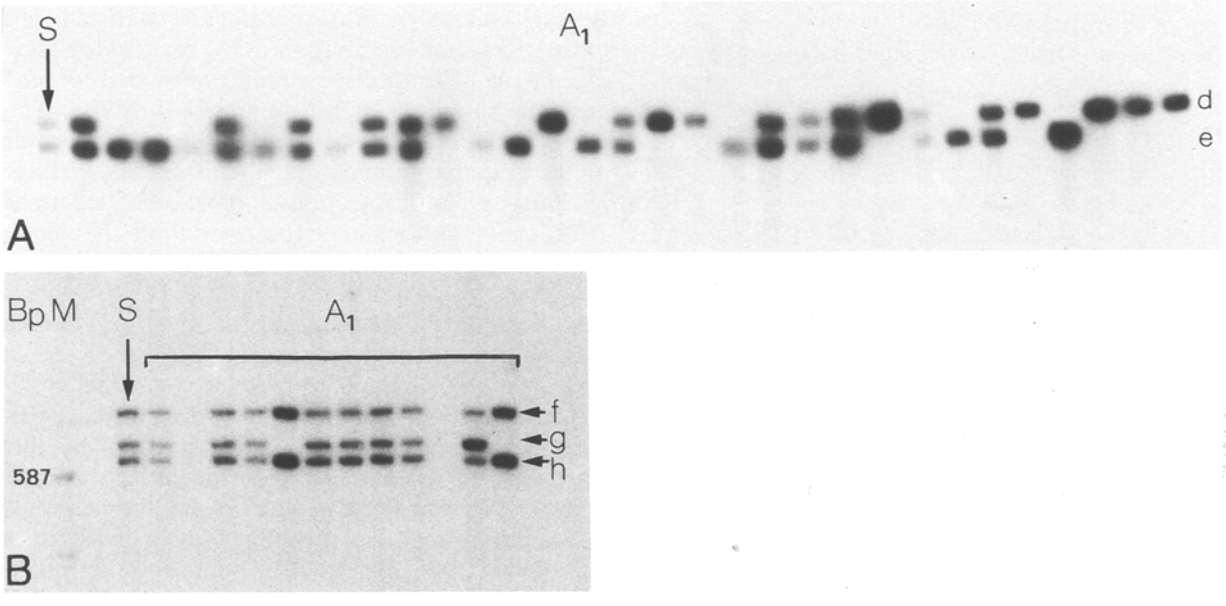


**Fig. 3A, B.** RFLP analysis of diploid anther-culture regenerants originating from clones 87.2002/6 and 87.2004/4. **A** Donor 87.2002/6. Genomic DNA digested with *Rsa*I; RFLP probe GP82 (Gebhardt et al. 1989). Eleven clones are heterozygous (two fragments), 20 clones are homozygous (one fragment) for this probe/enzyme combination. The state of complete homozygosity for a plant was established when it was characterized by only one allele at five different RFLP loci. **B** Donor 87.2004/4. Genomic DNA digested with *Taq*I; RFLP probe GP24 (Gebhardt et al. 1989). Eight clones are heterozygous (three fragments), two clones are homozygous (fragment '*g*' missing) for this probe/enzyme combination. *M*, DNA molecular weight marker; **S**, anther-donor clone; *A*, plants regenerated from anther culture; *d*, *e*; *f*, *g*, *h*, fragments analyzed for RFLP

**Table 5.** Percentage of homozygous diploid plants (pure lines) among 123 diploid regenerants from in-vitro anther culture

| Anther-donor clone | No. of plants analyzed | No. of homozygotes | % Homozygotes |
|---|---|---|---|
| 86.3144/3 | 5 | 1 | 20.0% |
| 86.3144/4 | 18 | 4 | 22.2% |
| 86.3144/5 | 21 | 5 | 23.8% |
| 87.2002/3 | 7 | 0 | 0.0% |
| 87.2002/6 | 56 | 4 | 7.1% |
| 87.2002/8 | 6 | 1 | 16.7% |
| 87.2004/4 | 10 | 0 | 0.0% |
| Total | 123 | 15 | 12.2% |

individuals. These are in fact present in the regenerated progenies together with heterozygous diploid plants.

The homozygosity of diploid anther-derived regenerants was determined by using the RFLP technique (Meyer and Uhrig 1990). A regenerant was considered homozygous at all loci when at least five RFLP probes, revealing a state of heterozygosity in the donor clone, marked the presence of only one of the two RFLP alleles (Fig. 3). In a sample of seven anther-donor clones the average proportion of homozygous diploid regenerants was 12.2% (Table 5): out of a total of 123 diploid regenerants considered, 15 were defined as diploid pure lines. The large majority of diploid regenerants, on the contrary, originated from unreduced microspores, or from nuclear fusion events, and were assumed to retain a variable degree of heterozygosity in their genetic constitution.

## Discussion

The results reported in this paper support the existence of genetic variability among diploid clones of potato for in-vitro monohaploid production (see also Sopory and Bajaj 1987). Three clones, 87.2001/7, 87.2004/6 and 87.2005/3, were isolated which combined a sufficient capacity to regenerate monohaploid plants with outstanding embryoid production. It was, however, noted that among clones which easily regenerated plants, several displayed an acceptable response in terms of embryoid formation, but these frequently did not produce monohaploid plants. Clones with a good embryoid production but a poor plant regeneration capacity were also observed. Agache et al. (1988) demonstrated in wheat that the development of microspores into embryoids and the capacity of the embryoids to regenerate plants are controlled by two different genetic systems. According to our results, the two steps leading to anther plant production in potato may also depend on different types of gene action.

Several traits of the anther-donor clones are associated with the aptitude to generate monohaploid progenies: either a low yield of embryoids or a low capacity to regenerate plants. Of particular interest is the low production of 2n pollen in the monohaploid-producing clones. In this respect, the cytological analysis of microspore development during anther culture confirmed the superior fitness of unreduced microspores due to their tendency for early division and their higher division rate. This supports the relationship between the degree of formation of unreduced microspores and their further development into embryoids and plants, as frequently found in the material considered in this study.

The cytological mechanisms originating unreduced microspores have been identified as FDR and, to a lesser extent, as SDR (definitions as in Mok and Peloquin 1975a, b; Ramanna 1979; Hermsen 1984). In potato, FDR- and SDR-gametes have 80% and 40%, respectively, of their loci which are still heterozygous compared to the nuclear state of the parent sporophyte (Hermsen 1984). This explains why a superior anther-culture response coexists with a high degree of heterozygosity of the diploid plants obtained (also found in diploid anther-culture regenerants of *Solanum chacoense*, Rivard et al. 1989).

Unreduced microspores are not the only route leading to high ploidy levels in anther-culture regenerants. During the regeneration phase, homozygous diploid regenerants may be formed through several mechanisms: nuclei fusion, endo-reduplication and endomitosis (Pijnacker et al. 1989). The same mechanisms can also lead to higher than 2x ploidy levels. Nuclear fusion generates diploid and triploid *Petunia* plants in-vitro (Gupta 1982). Out of the three developmental pathways followed by potato microspores in culture, pathways 'A' and '3' are characterized by a state of free nuclei which supports nuclear fusion leading to the formation of homozygous plants. The adoption of an RFLP technique allows the direct isolation of such homozygous *S. tuberosum* clones which are considered as pure lines.

The results of the recurrent selection based on the capacity to produce monohaploids from microspores cultured in-vitro suggest that lethal and/or sub-lethal alleles can be counterselected during anther culture, leading to the isolation of monohaploid clones with an improved viability. Results available supporting the presence of lethal or sublethal alleles in the potato genome are those of Gebhardt et al. (1991), who reported distorted segregations of RFLP-fragments in a backcross of *S. tuberosum*, most likely due to gamete or zygote elimination. Assuming that gene expression in gametophytes and sporophytes, as evaluated by isozyme analysis, is in a large part common (60% according to Zamir 1983), the development of breeding

schemes where selection in the haploid phase may produce positive correlated effects on the diploid can be suggested.

Our results indicate that anther culture may allow the reduction of the genetic load of the potato genome and, therefore, generate genotypes more useful in variety development. Diploid potato clones can be generated via anther culture in two ways: (1) the production and isolation of monohaploid plants followed by genome duplication and (2) the isolation, using RFLPs, of homozygous plants directly from a diploid anther-culture derived population.

## References


Agache S, DeBuyser J, Henry Y, Snape JW (1988) Studies of the genetic relationship between anther culture and somatic tissue culture abilities in wheat. Z Pflanzenzüchtg 100: 26–33

Chase SS (1963) Analytical breeding in *Solanum tuberosum* L. – A scheme utilising parthenotes and other diploid stocks. Can J Genet Cytol 5:359–363

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Dyer AF (1963) The use of lactopropionic orcein in rapid squash methods for chromosome preparations. Stain Technol 38:85–90

Frandsen NO (1968) Die Plastidenzahl als Merkmal bei der Kartoffel. Theor Appl Genet 38:153–167

Gebhardt C, Ritter E, Debener T, Schachtschabel U, Walkemeier B, Uhrig H, Salamini F (1989) RFLP analysis and linkage mapping in *Solanum tuberosum*. Theor Appl Genet 78:65–75

Gebhardt C, Ritter E, Barone A, Debener T, Walkemeier B, Schachtschabel U, Kaufmann H, Thompson RD, Bonbierbale MW, Ganal MW, Tanksley SD, Salamini F (1991) RFLP maps of potato and their alignment with the homeologous tomato genome. Theor Appl Genet 83:49–57

Gould AR (1984) Staining and nuclear cytology of cultured cells. In: Vasil IK (ed) Cell culture and somatic cell genetics of plants, vol 1. Academic Press, Orlando pp 698–711

Gupta PP (1982) Genesis of microspore-derived triploid petunias. Theor Appl Genet 61:327–331

Gupta PP (1983) Microspore-derived haploid, diploid and triploid plants in *Petunia violacea* Lindl. Plant Cell Rep 2:255–256

Hermsen JGT (1984) Mechanisms and genetic implications of 2n-gamete formation. Iowa State J Res 58:421–434

Johansson L (1983) Effects of activated charcoal in anther cultures. Physiol Plant 59:397–403

Linsmaier EM, Skoog F (1965) Organic growth requirements of tobacco tissue cultures. Physiol Plant 18:100–127

Mendiburu AO, Peloquin S, Mok D (1974) Potato breeding with haploids and 2n gametes. In: Kasha KJ (ed) Haploids in higher plants – advances and potential. University of Guelph, Ontario, pp 249–258

Meyer R, Uhrig H (1990) Feststellung von Homo- und Heterozygotie an diploiden, antherenbürtigen Nachkommen von *Solanum tuberosum* mittels RFLP. Vortr Pflanzenzüchtg 18:193–200

Mok DWS, Peloquin SJ (1975a) Three mechanisms of 2n pollen formation in diploid potatoes. Can J Genet Cytol 17:217–225

Mok DWS, Peloquin SJ (1975b) The inheritance of three mechanisms of diplandroid (2n pollen) formation in diploid potatoes. Heredity 35:295–302

Peloquin SJ (1983) New approaches to breeding for the potato for the year 2000. In: Research for potato in the year 2000, Lima, Peru: International Potato Center (CIP), pp 32–34

Pijnacker LP, Sree Ramulu K, Dijkhuis P, Ferwerda MA (1989) Flow cytometric and karyological analysis of polysomaty and polyploidization during callus formation from leaf segments of various potato genotypes. Theor Appl Genet 77:102–110

Raghavan V (1986) Pollen developmental biology in cultured anthers. In: Vasil IK (ed) Cell culture and somatic cell genetics of plants, vol 3. Academic Press, Orlando, pp 275–304

Ramanna MS (1979) A re-examination of the mechanisms of 2n gamete formation in potato and its implications for breeding. Euphytica 28:537–561

Rivard SR, Cappadocia M, Vincent G, Brisson N, Landry BS (1989) Restriction fragment length polymorphism (RFLP) analyses of plants produced by in-vitro anther culture of *Solanum chacoense* Bitt. Theor Appl Genet 78:49–56

Singh AK, Uhrig H, Salamini F (1988) Implications of chromosome pairing in a monohaploid and its colchidiploid of *Solanum tuberosum* (x = 12). Genome 30:347–351

Singsit C, Veilleux RE (1989) Intra- and inter-specific transmission of androgenetic competence in diploid potato species. Euphytica 43:105–112

Sopory SK, Bajaj YPS (1987) Anther culture and haploid production in potato. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, vol 3: Potato. Springer Verlag, Berlin Heidelberg, pp 89–105

Sunderland N (1974) Anther culture as a means of haploid induction. In: Kasha KJ (ed) Haploids in higher plants. University of Guelph, Guelph pp 91–122

Uhrig H (1985) Genetic selection and liquid medium conditions improve the yield of androgenic plants from diploid potatoes. Theor Appl Genet 71:455–460

Uhrig H, Salamini F (1987) Dihaploid plant production from 4x-genotypes of potato by the use of efficient anther plants producing tetraploid strains (4x EAPP-clones) – Proposal of breeding methodology. Z Pflanzenzüchtg 98:228–235

Wenzel G, Foroughi-Wehr (1984) Anther culture of *Solanum tuberosum*. In: Vasil JK (ed) Cell culture and somatic cell genetics of plants, Vol. 1, Academic Press, New York, pp 293–301

Wenzel G, Uhrig H (1981) Breeding for nematode and virus resistance in potato via anther culture. Theor Appl Genet 59:333–340

Wenzel G, Schieder O, Przewozny T, Sopory SK, Melchers G (1979) Comparison of single-cell culture derived *Solanum tuberosum* L. plants and a model for their application in breeding. Theor Appl Genet 55:49–55

Zamir D (1983) Pollen gene expression and selection: applications in plant breeding. In: Tanksley SD, Orton TJ (eds) Isozymes in plant genetics and breeding, Part A. Elsevier Sci Publications B.V., Amsterdam, pp 313–330