Theor Appl Genet (1985) 71:455–460

Genetic selection and liquid medium conditions improve the yield of androgenetic plants from diploid potatoes

H. Uhrig

Max-Planck-Institut für Züchtungsforschung (Erwin-Baur-Institut), Abteilung für Pflanzenzüchtung, Egelspfad, D-5000 Köln 30 (Vogelsang), Federal Republic of Germany

Accepted July 9, 1985
Communicated by F. Salamini

Summary. *Solanum tuberosum* L. diploid strains with superior androgenetic capacity have been selected for from androgenetic progenies of unselected diploid material. The paper also demonstrates that the use of a liquid medium for culturing potato anthers, instead of the conventional solid agar plates, improves the yield of androgenetic embryoids. The new method, associated with two successive cycles of selection for superior androgenetic response, allows the induction and regeneration of microspore derived plants on a large scale. The best genotype (clone 21 in this paper) regenerates androgenetic plants with a frequency around 30 per each anther plated. Over 80% of the regenerated plants are diploid. It is suggested that the androgenetic embryoids mainly originate from unreduced microspores by a mechanism which maintains a heterozygous or a partly heterozygous genetic situation.

Key words: Anther culture – Embryoids – Potato – Diploid – Microspores

Introduction

A proposed breeding scheme for potato is based on the maximization of heterozygosity through fusion of diploid protoplasts deriving from F1 crosses between doubled haploid strains (Wenzel et al. 1979). Diploid *S. tuberosum* L. (2n = 2x = 24 chromosomes) may be obtained via parthenogenesis (Hougas and Peloquin 1957; Hermsen and Verdenius 1973), while true haploids (2n = 1x = 12) can be produced from diploid *S. tuberosum* by anther culture (Foroughi-Wehr et al. 1977; Sopory et al. 1978).

The main drawback of this breeding scheme is that the yield of androgenetic plants from anther culture of 2n *S. tuberosum* was, until recently, relatively poor. A moderate improvement of the androgenetic capacity of diploid potato was, however, achieved using superior clones from crosses of highly responding diploids (Wenzel and Uhrig 1981; Uhrig 1983).

A difficulty which further complicates this cellular approach to the breeding of *S. tuberosum* is that a very high fraction of androgenetic plants from the diploid *S. tuberosum* anther donors is again diploid. It has been suggested that these diploid androgenetic plants are of haploid origin but they double their ploidy during the process of regeneration from embryoids (Wenzel et al. 1979; Wenzel and Foroughi-Wehr 1984).

In this study a new technique for culturing 2n potato anthers is proposed which allows the production and collection of androgenetic embryoids on a large scale. Coupled with repeated selection of 2n strains with superior androgenetic capacity, this technique offers the opportunity of obtaining large populations of potato embryoids to which laboratory-based selection methods can be, if needed, applied. A major point remains unresolved: the majority of potato embryoids obtained still have a diploid chromosome number.

Materials and methods

Plant material

Diploid genotypes originally utilized in this experiment (clone no. 1 to 10) originated from complex crosses of *Solanum tuberosum* L. 2n-clones with several 2n-wild species bearing disease resistance (see Table 1). Original *S. tuberosum* 2n-clones were obtained by parthenogenesis (Baerecke et al., unpublished) following pollination of 4n-breeding clones, used

Table 1. Origin of clones utilized in this study as anther donors

Clone no.	Identification no.	Genomes involved in the generation the diploid clone[a]
1	H 78.2020/12	*tuberosum*^{6} × *vernei*^{2} × *sparsipilum*^{1}
2	H 81.2001/1	*tuberosum*^{7} × *vernei*^{2} × *sparsipilum*^{1}
3	H 81.2033/21	*tuberosum*^{7} × *vernei*^{2} × *sparsipilum*^{1}
4	H 79.2024/6	*gourlayi*^{1}
5	H 81.2004/6	*tuberosum*^{1} × *gourlayi*^{1}
6	H 78.2022/14	*tuberosum*^{3} × *vernei*^{1} × *sparsipilum*^{1}
7	H 81.2038/30	*tuberosum*^{4} × *vernei*^{1} × *sparsipilum*^{1}
8	H 81.2038/32	*tuberosum*^{4} × *vernei*^{1} × *sparsipilum*^{1}
9	H 81.2038/34	*tuberosum*^{4} × *vernei*^{1} × *sparsipilum*^{1}
10	H 78.2020/26	*tuberosum*^{6} × *vernei*^{2} × *sparsipilum*^{1}
11	A 83.2200/61	androgenetic descendent of clone 2
12	A 83.2200/70	androgenetic descendent of clone 2
13	A 83.2200/73	androgenetic descendent of clone 2
14	A 82.2250/1	androgenetic descendent of clone 2
15	A 83.2265/1	androgenetic descendent of clone 5
16	A 83.2264/1	androgenetic descendent of clone 7
17	A 83.2238/1	androgenetic descendent of clone 8
18	A 83.2278/1	androgenetic descendent of clone 9
19	A 81.2211/29	androgenetic descendent of clone 10
20	A 81.82.2238/6	androgenetic descendent of clone 10
21	A 84.2302/13	androgenetic descendent of clone 13
22	A 84.2308/3	androgenetic descendent of clone 14
23	A 84.2306/1	androgenetic descendent of clone 16
24	A 84.2306/2	androgenetic descendent of clone 16

[a] In the complex pedigree reported under this heading, a species participated in the multiple cross for the number of times indicated in its exponent

as female parent by *S. phureja* Jut. et Buk., P.I. 225 682.1 and P.I. 225 682.22 (Hougas and Peloquin 1957). As reported in the last column of Table 1 the *S. tuberosum* contribution to the diploid potatoes considered in this study largely prevailed.

Clones from 11 to 20 were derived from the preceding ones by selecting, after regeneration of plants via anther culture, 2n-plants with a superior capacity for generating embryogenic structures leading to complete plant regeneration. Clones 21 to 24 were derived from a second cycle of selection imposed on plants grown in vitro from clones 11 to 20.

Growing conditions of anther donors

Clones were grown from tubers under semicontrolled conditions in the greenhouse and grafted on tomato rootstocks, as described earlier (Wenzel and Uhrig 1981). Prior to anther culture, excised flower buds were kept for 3 days at 6 °C. Buds were surface sterilized for 1 min in alcohol (70%) followed by several washings in autoclaved tap water.

Anther culture and plant regeneration from microspores

Androgenesis was induced by two different methods. The method of Sopory et al. (1978) was originally used. Accordingly, anthers were plated on solid medium (0.8% agar) in Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 6% sucrose, 0.5% active charcoal and 1 mg/l 6-benzylaminopurine (BAP).

Induction of androgenesis was later achieved in liquid conditions using half strength Linsmaier and Skoog medium (Linsmeier and Skoog 1965 (1/2 LS)). The medium was supplemented with 0.05% active charcoal, 0.1 mg/l indole acetic acid (IAA), 2.5 mg/l BAP and 6% sucrose.

Usually up to 50 anthers per 25 ml of medium were placed in 50 ml Erlenmeyer flasks and shaken at 26 °C with 120 strokes/min in an incubator. Light intensity was dimmed to approximately 30 Lux. Induction of microspore divisions was controlled after 1 week of incubation through dissecting anther samples from the respective flasks under the microscope. After 2 to 3 weeks, growth of embryoids outside of the anthers on the bottom of the flasks was frequently observed. One week later, the embryoids were harvested and plated on 1/2 LS supplemented with 0.1 mg/l gibberellic acid (GA3) in order to assure a further development of the plantlets. The medium was solidified with 0.8% agar (Merck 1614) and the amount of sucrose was reduced to 2% to prevent extensive callus formation. The dishes were transferred to a culture room and kept in a 16 h light regime at a constant temperature of 24 ± 2 °C.

After induction of morphogenesis, the plantlets were subcultured once on 1/2 LS without growth regulators. Rooting was achieved in the greenhouse using cuttings of the plantlets from the aseptic dishes. The cuttings were placed in small pots with soil and covered with plastic bags to maintain high relative humidity during rooting and adaptation. From 5 to 10 cuttings per each original embryoid were usually grown in the greenhouse.

Level of ploidy

The ploidy level of regenerated androgenetic plants was routinely evaluated by counting the plastids in the guard cells of the stomata from the lower leaflet epidermis (Frandsen 1968). In doubtful cases the somatic chromosome number was scored using squashes from the terminal leaflets of the shoot apex stained with acetocarmine.

Results

Liquid anther culture

In Table 2 the number of anthers plated per genotype and per method of culture is given, together with numbers of embryoids and regenerated plants produced. A total of 45,143 anthers were plated on agar plates (solid medium technique), yielding 5,040 embryoids and 283 plants. In liquid anther culture 4,580 anthers were cultured (1/10 of the number plated on solid medium) yielding 16,629 embryoids; 1,918 plants have been regenerated from these embryoids so far. Efficiency of obtaining embryoids and plants, measured on the basis of 100 anthers plated, shows an overall superiority of liquid culture (517.9 embryoids and 18.7 plants per 100 anthers for clones 11–20 and 485.6 embryoids and 304.6 plants for clones 21–24) when compared to solid medium (12.7 embryoids and 0.7 plants per 100 anthers for clones 1–10 and 42.9 embryoids and 11.7 plants for clones 11–20). A more direct comparison between solid versus liquid medium can be obtained by considering only the data of clones 13, 14, 16, 17, 18, and 20 for which both sets of data are available. Here, the following numbers of embryoids and plants were obtained

Table 2. Number of anthers plated, embryoids formed, plants regenerated and efficiencies of various anther donor genotypes. Both solid and liquid media were used for plating anthers

Clone no.	Conditions for induction of androgenetic plants									
	Solid medium					Liquid medium				
	Anthers plated	Embryoids		Plants		Anthers plated	Embryoids		Plants	
		formed	/100 anthers	obtained	/100 anthers		formed	/100 anthers	obtained	/100 anthers
1	12,746	492	3.9	0	0					
2	2,377	1,460	61.4	121	5.1					
3	778	16	2.1	1	0.1					
4	5,114	13	0.3	0	0					
5	739	5	0.7	1	0.1					
6	6,286	1,081	17.2	31	0.5					
7	5,021	1,065	21.2	11	0.2					
8	3,112	387	12.4	12	0.4					
9	1,772	67	3.8	1	0.1					
10	6,281	243	3.9	62	0.6					
Total 1–10	44,226	4,829		240						
Mean 1–10			12.7		0.7					
11	10	16	160.0	5	50.0					
12	25	22	88.0	9	36.0					
13	105	117	111.0	17	16.2	80	975	1,218.8	44	55.0
14	30	10	33.3	1	3.3	196	1,408	718.3	22	11.2
15	20	4	20.0	1	5.0					
16	434	27	6.2	1	0.2	1,042	1,423	136.5	157	15.0
17	165	10	6.0	8	4.8	691	2,236	323.6	63	9.1
18	24	0	0	0	0	1,243	1,841	148.1	186	15.0
19						172	3,248	1,888.3	118	68.6
20	104	5	4.8	1	1.0	425	3,168	745.4	54	12.7
Total 11–20	917	211		43		3,849	14,299		644	
Mean 11–20			47.7		13.0			739.9		26.7
21						25	880	3,520.0	723	2,892.0
22	(1) Plant regeneration already started but is					23	229	995.6	(1)	(1)
23	delayed compared to other clones within a					397	881	221.9	393	99.0
24	comparable time interval					286	340	118.9	158	55.2
Total 21–24						731	2,330		1,274	
Mean 21–24								1,214.1		1,015.4

per 100 anthers cultured, respectively, on solid and in liquid media:

clone 13: 111.0 and 16.1 vs. 1218.7 and 55.0;
clone 14: 33.3 and 3.3 vs. 718.3 and 11.2;
clone 16: 6.2 and 0.2 vs. 136.5 and 15.0;
clone 17: 6.0 and 4.8 vs. 323.5 and 9.1;
clone 18: 0 and 0 vs. 148.1 and 15.0;
clone 20: 4.8 and 0.9 vs. 745.4 and 12.7.

From these results one may conclude that culturing anthers in liquid medium enhanced the yield of embryoids and, consequently, the number of plants regenerated (see also Fig. 1 which stresses visually the potential of the liquid anther culture for embryoid production).

Effect of genotype and selection for androgenesis in vitro

A clearcut difference in the capacity for producing androgenetic plants among anther donor potato clones was particularly evident in the unselected genotypes (clones 1 to 10; Table 2). Clone 2, for instance, was able to produce 5 plants per 100 anthers while clones 1 and 4 responded negatively, regenerating not even a single plant. It is worth noting that in some instances the

Fig. 1. Somatic embryogenesis in diploid potatoes obtained by culturing young anthers under liquid medium conditions

capacity for embryoid formation was not associated with the final regeneration of complete plants – this was the case of clone 1: not even one plant was obtained from 492 embryoids.

Data for clones 11 to 24 showed more stable and favorable genotypic effects as far as the capacity of generating androgenetic plants is concerned. In fact, these clones, deriving from selection based on capacity for generating androgenetic progenies, behaved more homogeneously, especially under liquid medium conditions. Partial exceptions were clone 18, which still was unable to produce plants under solid medium conditions, and clone 21 which was characterized by an extremely high capacity of regenerating complete plants (up to 29 per single anther). A possible variant genotype was clone 22, which although giving rise to a consistent number of embryoids, was unable to originate completely developed plantlets in a comparable time interval.

The benefit of repeated selection for androgenetic capacity in anther culture becomes evident if the number of regenerated embryoids and plants per 100 anthers are compared considering mean values of clones 1–10, 11–20, and 21–24. These three groups of clones, in fact, represent, respectively, an unselected population (C0) and the first (C1) and second (C2) cycle of selection for androgenesis in vitro. From cycle 0 to cycle 1 the comparison, possible only for results obtained from solid medium, shows a mean increase of embryoids per 100 anthers from 12.7 (C0) to 42.9 (C1) and of plants per 100 anthers from 0.7 to 11.7. The step from cycle 1 to cycle 2, at best evaluated in liquid medium conditions, did not reveal a substantial progress in the capacity of producing embryoids per anther (517.9 for C1 versus 415.6 for C2) while the production of plants was definitely improved (18.7 for C1 versus 304.6 for C2). The last figure presented is strongly influenced by the results of clone 21, which by far was the best producer of androgenetic plants with a mean value near 30 per anther cultured.

The behaviour of clone 21 emphasizes a remarkable fact. Though the production of androgenetic embryoids was enhanced by selecting superior clones (compare, for example, clones 2 to 11–13 or clones 3 to 14), after being selected, other clones were found without a particular increase or even with a slow decrease in yield of embryoids. This is the case when clones 7–10 are compared to 16–20 (solid medium) and when clone 16 is compared to 23 and 24 (liquid medium). This observation may suggest a partial sporophytic determination of the embryogenic capacity in androgenesis and may be responsible, in a few instances, both for the absence of correlation between the regenerating capacity of a donor plant and its progenies and, even more likely, for the noted difference between the capacity of producing embryoids and the subsequent ability to differentiate them into plants (the case of clone 22).

Ploidy level of regenerated plants

The ploidy level of a substantial fraction of regenerated plants is listed in Table 3. The total number of genotypes investigated was 313. From these, approximately 5.2% (=16 plants) exhibited variation in the ploidy

Table 3. Number of androgenetic plants with different ploidy level. The calculated percentage over the total plants considered is reported in brackets

Clone no.	Ploidy level				
	1x	2x	3x	4x	2x/4x
2		78 (84)		7 (8)	8 (8)
4				1 (100)	
6		23 (85)		4 (15)	
7		8 (100)			
8		6 (100)			
9		1 (100)			
10		60 (98)			1 (2)
11		3 (100)			
12		5 (100)			
13		13 (93)		1 (7)	
14		6 (100)			
15		1 (100)			
16	1 (4)	17 (65)	1 (4)	2 (8)	5 (19)
17		4 (100)			
18	1 (33)	2 (66)			
19		3 (100)			
20		7 (100)			
23		14 (82)		3 (18)	
24				25 (93)	2 (7)
Total	2 (0.6)	251 (80.2)	1 (0.3)	43 (13.7)	16 (5.2)

levels (last column in Table 3): both diploid and tetraploid somatic chromosome numbers were found in cuttings of the same regenerate or chimeric ploidy levels of 2x and 4x within the same plant were observed.

The majority of regenerated genotypes (80.2%) were diploid. Two genotypes proved to be haploid (2n = 1x = 12), one genotype was triploid (2n = 3x = 36) and 43 (= 13.7%) were tetraploid (2n = 4x = 48). From clone 24 almost exclusively 4n plants were regenerated. Both the haploid and the triploid plants derived from liquid anther culture conditions proved to be karyotypically stable during vegetative propagation in aseptic shoot cultures and as grafts on tomato rootstocks. Some of the regenerated diploids from microspores showed a phenotype so different from that of the donor parent as to be associable to Mendelian variants already described in the Solanaceae family. Variants with chlorophyll variegations were also selected.

Discussion

The results presented in Tables 2 and 3 emphasize that two components are necessary for a successful anther culture of diploid potatoes: first, a suitable method allowing for the collection and regeneration of androgenetic embryoids on a large scale and, second, suitable genotypes which express to a satisfactory level their androgenetic capacities. The combination of both conditions allows a considerable embryoid production together with high efficiencies of plant regeneration. The effectiveness of both genetic and methodological approaches was not clearly apparent from data of former experiments with diploid potatoes (Sopory et al. 1978; Wenzel and Uhrig 1981). Under those conditions, the limits for obtaining a high number of regenerants themselves hindered a precise judgement of the methodology.

The use of a liquid medium for culturing the anthers instead of plating them on solid agar may induce less competition among microspores for nutrients, leaving more space available for individual development of embryoids. This is clearly seen in Fig. 1, where the anther walls appear open allowing the developing microspores to diffuse into the surrounding fluid. Even under liquid culture, however, it seems beneficial that the initial divisions of the microspores occur within the anthers, since isolated microspores cultured in liquid medium develop preferentially into calli (data not presented).

The number and further development of the embryoids obtained under our growing conditions depends largely on the genotype used as the anther parent. This was, to some extent, evident from the results dealing with unselected materials (clones 1 to 10). Two direct cycles of selection for androgenetic capacity confirmed the role of the genotype. In this respect the data presented are in accord with the suggestion already made by several authors (Wenzel and Uhrig 1981; Uhrig 1983; Miller and Lipschutz 1984; Wenzel and Foroughi-Wehr 1984).

Androgenetic plants in several species have been associated with a reduction of the ploidy level from diploidy to haploidy (Reinert and Bajaj 1977). This has also been found in polyploids and diploid *Solanum* species (Irikura 1975). *Solanum tuberosum* L., however, seems to occupy an exceptional position. In fact, data from Wenzel and Foroughi-Wehr (1984) show an average yield of androgenetic n-plants of approximately 4%, independent of whether one starts from tetraploid (2n = 4x) anther donors or from diploids (2n = 2x). It is then evident that diploid *S. tuberosum* strains, when used as anther donors, manifest an abnormal behaviour. The results from our investigation (Table 3) confirm the predominant regeneration of diploids from diploid anther donor genotypes. Discussing this particular phenomenon it has been suggested that more than two thirds of 2n androgenetic plants from 2n *S. tuberosum* represent completely homozygous diploids, originating by a doubling of the ploidy number during the process of in vitro culture

(Wenzel and Foroughi-Wehr 1984). The results presented in this paper may, to some extent, suggest caution in accepting only this interpretation. It was, for instance, possible to improve considerably the overall production and viability of the embryoids in the second cycle of selection, a fact which is difficult to explain if one does not admit that selection acted on an heterozygous population of 2n regenerants.

A second striking observation in favor of heterozygosity of anther derived diploids is that in our second cycle of selection, plants were found expressing mutated traits, such as variegated leaves, zebra-necrotic leaves, necrotic leaf tips, etc. Such traits were not shown by the anther donor plants which already originated in vitro by androgenesis and which were then supposed to be heterozygous for such characters. Although in vitro culture is known to generate genetic variants (Scowcroft and Larkin 1983), the finding of the same phenotype in progenies with a common parent suggest that a certain degree of heterozygosity is still present in 2n androgenetic derived potato plants. Also a certain degree of uniformity of the plants regenerated from a particular genotype, and the fact that they are, to a large extent, similar to their anther parents, support the conclusion that in vitro the selection and regeneration of a large fraction of heterozygous microspores represent a common phenomenon. Meiotic abnormalities described for this species which are based on inheritable genetic situations and leading to unreduced microspores, such as first devision restitution (Mok and Peloquin 1975; Peloquin 1983), may be at the base of the maintenance of heterozygosity in at least part of the 2n androgenetic plants.

The origin of the diploid state in androgenetic potato plants remains, however, doubtful. Direct experiments by using simply inherited traits have been planned to clarify this item. It is of interest, in fact, to understand at which degree homozygosity is expressed before adopting the technique proposed in this paper for selecting useful variants from a large population of androgenetic regenerants.

Acknowledgements. The author is indebted to Professor Dr. F. Salamini for critical reading the manuscript and for helpful suggestions. The excellent technical assistance of Mrs. Sieglinde Effgen and of Ms. Iris Bürst is gratefully acknowledged.

References

Foroughi-Wehr B, Wilson HM, Mix G, Gaul H (1977) Monohaploid plants from anthers of a dihaploid genotype of *Solanum tuberosum* L. Euphytica 26:361–367

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

Hermsen JGTh, Verdenius J (1973) Selection from *Solanum tuberosum* group phureja of genotypes combining high frequency haploid induction with homozygosity for embryo-spot. Euphytica 22:244–259

Hougas RW, Peloquin SJ (1957) A haploid plant from potato variety Katahdin. Nature 180:1202–1210

Irikura Y (1975) Induction of haploid plants by anther culture in tuberbearing species and interspecific hybrids of *Solanum*. Potato Res 18:133–140

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

Miller SA, Lipschutz L (1984) Potato. In: Ammirato PV, Evans DA, Sharp WR, Yamada Y (eds) Handbook of plant cell culture, vol 3. McMillan, New York, pp 291–326

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

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497

Peloquin SJ (1983) Genetic engineering with meiotic mutants. In: Mulcahy DL, Ottaviano E (eds) Elsevier, North Holland, pp 311–316

Reinert J, Bajaj YPS (1977) Anther culture: haploid production and its significance. In: Reinert J, Bajaj YPS (eds) Applied and fundamental aspects of plant cell, tissue and organ culture. Springer, Berlin Heidelberg New York, pp 251–267

Scowcroft WR, Larkin PJ (1983) Somaclonal variation and genetic improvement of crop plants. In: Better crops for food. Ciba Found Symp 97, Pitmn Books, London, pp 177–193

Sopory SK, Jacobsen E, Wenzel G (1978) Production of monohaploid embryoids and plantlets in cultured anthers of *Solanum tuberosum*. Plant Sci Lett 12:47–54

Uhrig H (1983) Breeding for *Globodera pallida* resistance in potatoes. 1. Improvement of the androgenetic capacity in some resistant dihaploid clones. Z Pflanzenzücht 91: 211–218

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

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

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