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REGENERATION CAPACITY OF POTATO PROTOPLASTS ISOLATED FROM SINGLE CELL DERIVED DONOR PLANTS

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SUMMARY

Protoplast yield, survival, and regeneration was investigated in dihaploid (2n = 2x = 24) clones of potato. These clones consisted of unselected interdihaploids and of microspore or protoplast derived clones, particularly doubled haploids regenerated from the same anther donor clone, androgenetic clones regenerated in a second cycle from androgenetic clones, and protoclones. Significant differences were observed for all characters studied between donor plants without an *in vitro* history and the other groups, but also within the androgenetic clones, which underlines that they originated from different gametes. Non-significant differences among clones having passed twice through anther culture are indicative of the stability and the homozygosity of the androgenetic diphaploids. Differences between second cycle anther derived clones observed as well, indicated the induction of mutational events during microspore culture. Such variation of the regeneration parameters was also observed between protoplast cultures of an interdihaploid clone and of its protoclone. As in most experiments such variation is undesired, experiments were conducted to reduce the somaclonal variation. By optimizing the culture procedure, the undifferentiated callus phase could be reduced thus far, that the majority of the regenerants proved to be phenotypically uniform at the diploid or tetraploid ploidy level.

1. INTRODUCTION

The tetraploid nature of *Solanum tuberosum* (2n = 4x = 48) is one of the greatest obstacles in predictable potato breeding programs. One possibility to overcome this problem is the production of dihaploids (2n = 2x = 24) and their utilization in combination breeding (PELOQUIN et al. 1966). This approach may be further improved by formation of homozygous dihaploids via doubling of parthenogenetic or androgenetic monohaploids (2n = x = 12). Homozygosity of artificially or spontaneously doubled monohaploids was checked by analysis of F_1's and revealed some residual heterozygosity, probably as a result of the regeneration of unreduced gametes. The tetraploid level of the clones shall be regained via protoplast fusion (WENZEL et al. 1979). This is particularly necessary as many of the dihaploids have a reduced fertility, preventing the meiotic doubling technique (HANNEMAN & PELOQUIN 1968). The reproducible regeneration of plantlets from protoplasts is a prerequiste for this applied goal. However, even during normal generation a wide range of phenotypic variation due to gene and chromosomal mutations have been reported in protoplast derived potato

plants (SREE RAMULU et al. 1983). This variation is generally detrimental and should be reduced. Further, not all clones give good results in protoplast culture measured as the frequency of regeneration.

To overcome these problems different protoplast donor clones were analyzed for their regeneration capacity and their genotypic stability. The clones used, were either randomly selected or they had passed up to two microspore or protoplast in vitro regeneration cycles. The selective effect of such an in vitro culture step has been reported already for androgenetic procedures (WENZEL 1980, UHRIG 1985) and it should be checked, how far this selection was also beneficial for protoplast regeneration.

2. MATERIALS AND METHODS

One primary dihaploid, ten interdihaploid clones, seven microspore derived dihaploid clones, and thirteen protoclones from two different dihaploids were used in the present study. The clones and a partial pedigree is listed in *fig. 1.*

Protoplasts were isolated from axenic shoot cultures (BINDING et al. 1978), which were produced from meristem derived plants and placed on MS medium (MURASHIGE & SKOOG 1962) supplemented with 20 gl^{-1} sucrose, 1 gl^{-1} and 7.5

A) Primary dihaploid
H76.981/29

B) Interdihaploids

H236	H258	HH372
HH 411	HH 630	H77.417/9
H77.420/10		H78.1208/13

C) Interdihaploids related to B
H75.1207/7 (HH236 × H69.1382/14) and protoclones of this: P84.044
H78.551/1 (HH258 × H2086) and protoclones of this: P84.033

D) Pedigree of androgenetic dihaploid clones

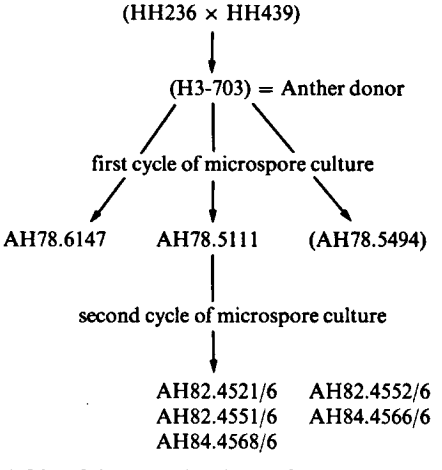

Fig. 1. List of clones used as donors for protoplast regeneration.

Table 1. Medium sequence for optimal growth of anther donor shoot cultures (basic medium: MS).

	time (weeks)	BAP mgl^{-1}	NH_4NO_3 mgl^{-1}
Initiation:	3	–	1650
Propagation 1:	3	0.5	1650
Propagation 2:	3	0.5	150
Preharvest:	3	–	150

gl^{-1} agar. For propagation this medium contained the normal NH_4NO_3 concentration but no cytokinin, in the second transfer 0.5 mgl^{-1} 6-benzylaminopurine (BAP) was added. In the next transfer the NH_4NO_3 concentration was reduced to 150 mgl^{-1} and three weeks before harvesting for protoplast isolation the BAP was omitted (*table 1*). The shoot cultures were maintained at 24 °C under a light intensity of 4000 Lux and a daylength of 16h.

Several grams of plant material, isolated from the upper halves of the shoots, were harvested and cut into small pieces. After 15 min. in 0.3 M mannitol solution to preplasmolyse the cells the source material was incubated in enzyme solution containing 1.5% cellulase 'ONOZUKA' R-10, 0.2% macerozyme R10 (both from Serva, Heidelberg) in lmM $CaCl_2$ and 0.6 M mannitol at a pH of 5.5. Bottles (100 ml volume) containing 10 ml of the enzyme solution per 1 g of plant material were placed on a roller (Rollacell-RC-42, New Brunswick Scientific Co. Inc.) for 3 h (2 rotations per min) at 24 °C. After enzyme treatment the material was filtered through sieves, the final filtrate was washed by centrifuging, and the viable protoplasts were separated from the dead protoplasts by resuspending the pellet in 60% Percoll in 0.6 M mannitol and centrifuging. The band of protoplasts on the surface of the Percoll layer was collected and washed. The protoplasts were diluted with V-KM medium (BINDING et al. 1978) at a titre of 5×10^4–10^5 per ml. After counting the number of protoplasts and viability determination by fluorescin diacetate staining (WIDHOLM 1972) protoplasts were plated in 3 cm plastic petri dishes taking 1.2 ml protoplast suspension in each plate. The plates were incubated at 25 °C under 800 Lux of illumination. The plating efficiency was defined as the number of dividing cells as a percentage of the initial number of plated protoplasts. After 14 days, the developing cell aggregates were cultured for another fortnight in a semi-solid V-KM medium (by diluting with an equal volume of a 40 °C V-KM medium supplemented with 0.4% agar at 550 mOsm).

The microcalli which formed, were transferred to medium 3 of AUSTIN & CASSELLS (1983) supplemented with 0.025 mgl^{-1} $CaCl_2$ and 16.9 mgl^{-1} $MnSO_4$ instead of $MnCl_2$ and incubated for 4 weeks in a growth chamber at 2000 Lux and 25 °C temperature. Subsequently the calli were transferred to MS medium supplimented with 800 mgl^{-1} NH_4NO_3, 0.8% agar, 3% sucrose, 0.01 mgl^{-1} napthylacetic acid (NAA), and 1.0 mgl^{-1} zeatinribosid, pH 5.7 (modified after ROEST & BOKELMANN 1983). After two weeks the calli were transferred to MS medium supplemented with 1% sucrose, 1.5% mannitol, 1 mgl^{-1} indoleacetic acid (IAA),

1 mgl^{-1} BAP, 10 mgl^{-1} gibberellic acid (GA_3) and 0.8% agar, pH 5.8 (modified after SREE RAMULU et al. 1983), where multiple shoots were generated. Individual adventitious shoots (approx. 1.5 cm in height) were removed by cutting them at their base and placed on MS medium containing 0.5% activated charcoal for root formation.

Protoplast isolation and culture was replicated three times with each clone. Statistical analysis for number of protoplasts per 1 g leaf material, survival percentage (percentage of protoplasts showing viability as judged by microscopic examination 9 or more days after isolation), division percentage (percentage of protoplast showing at least one cell division after plating) number of calli per plate after two months of isolation, and shoot percentage (percentage of those calli that formed shoots within 15–25 weeks) was done following a randomised design. Callus growth rate was determined in 13 randomly selected protoclones of H75.1207/7 by culturing leaf tissues in MS medium supplemented with 0.5 mgl^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). Data were taken on mg fresh weight after 14 days of transferring in the latter medium.

Three tubers of several protoclones from the clones H75.1207/7 and H78.551/1 were planted in the field at Grünbach and screened for their phenotype and yield per hill.

3. RESULTS

Protoplasts were obtained from all clones studied, and divisions occurred from most of them after third days of culture (*table 2*). Further divisions took place on and after the fifth day, and was followed by successive divisions resulting in the formation of masses of cells. Cell division was promoted by exclusion of NH_4NO_3 from the V-KM medium. After eight weeks of culture, most of the colonies were 1.0–2.0 mm in size, and were now ready for transfer onto shoot initiation medium. For most genotypes a prolonged culture on this medium was more successful measured in the regeneration percentage and number of shoots, than a transfer onto GA_3 containing medium of SREE RAMULU et al. (1983). Here numerous shoots developed and elongated upto 2–3 cm within four weeks. The shoots subsequently produced roots on MS medium within one week when they could be transferred to soil and grown in greenhouse.

From the non-selected clones only a few plantlets could be regenerated; among the other group analysis of variance for protoplast yield and survival, division of surviving cells and regeneration of calli showed highly significant mean squares ($p < 0.01$) indicating significant differences for all characters studied. The relative permanence of each clone having passed an *in vitro* preselection-cycle is presented in *table 3*. The clones AH78.5111, AH82.4521/6 and AH84.4568/6 were statistically identical with respect to all five characters. These three clones were superior to clone AH82.4551/6 for protoplast yield, number of calli per plate and percentage of shoots regenerated. Clones AH78.6147 and AH84.4566/6 exhibited statistically similar results for all characters except percentage of shoot regeneration for which the later was superior. These two clones,

Table 2. Results on the regeneration capacity of protoplasts from unselected and selected dihaploids.

1. Non-selected donor clones.
H76.981/29: No shoot culture induction achieved.
HH236: Limited protoplast regeneration, poor shoot formation.
HH258: Limited protoplast regeneration, poor shoot formation.
HH372: Very good protoplast regeneration to callus, but no shoots.
HH411: No protoplast divisions.
HH630: No protoplast divisions.
HH77.417/9: No regeneration of meristems.
HH77.420/10: Limited protoplast regeneration, poor shoot formation.
H78.1051/1: Limited protoplast regeneration, no shoot formation.

2. Combinations with the best clones of 1.
H75.1207/7: Good protoplast regeneration and shoot formation.
H78.551/1: Good protoplast regeneration and shoot formation.

3. Clones from single cell culture.
AH78.5111: Very good protoplast regeneration and shoot formation.
AH78.6147: Good protoplast regeneration and good shoot formation.
AH82.4521/6: Best protoplast regeneration and shoot formation.
AH82.4551/6: Average protoplast regeneration, very good shoot formation.
AH82.4552/6: Average protoplast regeneration, no shoot formation.
AH84.4566/6: Good protoplast regeneration and good shoot formation.
AH84.4568/6: Very good protoplast regeneration and shoot formation.
PH84.033: Good protoplast regeneration and shoot formation.
P84.044: Average protoplast regeneration and shoot formation.

Table 3. Protoplast yield, survival, cell division and regeneration of calli in nine cell culture derived clones.

Clone	Protoplast yield no. × 10^3/g leaf	Survival %	Division %	Calli/plate no.	Shoots %
AH82.4521/6	867 a*	33 b	11 b	335 b	65 ab
AH78.5111	825 ab	33 b	11 b	344 b	67 a
AH84.4568/6	817 ab	30 b	10 b	347 b	63 ab
H75.1207/7	696 abc	47 a	31 a	578 a	25 e
AH84.4566/6	600 cd	22 cd	7 b	303 bc	56 bc
AH78.6147	500 cd	23 c	8 b	266 c	45 d
P84.044	392 de	17 de	8 b	317 bc	25 e
AH82.4551/6	283 e	30 b	9 b	270 c	52 cd
AH82.4552/6	258 e	13 e	3 e	0 d	0 f

* = Figures followed by common letters are statistically identical at 5% level of significance.

however, were better than that of AH82.4552/6 for all traits studied. AH82.4552/6 was the poorest among all clones and did not form micro-calli and thus no shoot regeneration took place. Clone H75.1207/7, which was used as control, was statistically superior to its protoclone P84.044 for all traits studied except for percentage of shoot regeneration for which both clones were identical. The percentage of protoplast survival and division and number of calli per plate

Table 4. Correlation co-efficient for protoplast yield, survival, division and callus regeneration of the nine clones of *table 3*.

	Protoplast yield	Survival %	Division %	Calli/ plate
Survival %	0.63			
Division %	0.40	0.88**		
Calli/plate	0.56	0.85**	0.86**	
Shoots	0.68*	0.38	–0.04	0.38

* = $P<0.05$; ** = $P<0.01$.

was highest for the clone H75.1207/7. However, it was pooled down by percentage of shoot regeneration.

Correlation study among different characters indicated that protoplast yield had no correlation with protoplast survival and division and number of calli per plate (*table 4*.) This suggests that the genes or gene groups controlling these characters assort independently. Non-significant correlation co-efficients between shoot regeneration and all other characters except protoplast yield also suggest the similar conclusion. The genes or gene groups for protoplast yield and shoot regeneration might be linked or exhibit pleotropic effect since the correlation co-efficient is positive and significant. Positive and highly significant correlation co-efficient among the characters protoplast survival, protoplast division and number of calli per plate suggest that these characters are controlled by the genes or gene groups which are tightly linked or exhibit pleotropic effect.

Counting of chromosomes revealed two gross classes of ploidy levels and a number of aneuploids in the regenerants of the dihaploid clones, which is given for the protoclones regenerated from H75.1207/7 and H78.511/1 in *table 5*. The percentage of somaclonal variation was studied in one of the clones, H75.1207/7 for more parameters. Callus culture from thirteen protoclones exhibited callus growth rate from 74% to 142% of the parental clone. Out of these thirteen protoclones, eight exhibited statistically similar response as that of parental clone for callus growth. The rest of the protoclones, though having similar phenotype and chromosome number as that of parental clone, exhibited differential response for callus growth which might be due to mutation at gene level during protoplast isolation and culture. Regrowth of the tubers from the euploid clones in the field demonstrated uniform phenotypes, perfect flower formation (*fig. 1*) and good fertility (SCHUCHMANN 1985).

Table 5. Ploidy levels of some regenerated protoclones.

Clone No. of protoclones		2x	4x	aneuploid
H75.1207/7	53	11	37	5
H78.551/1	49	3	34	12

All parameters measured underlined that the amount of somaclonal variation could be reduced by the rapid induction of organogenesis. At the same time the procedure described allowed such high regeneration frequencies that selection e.g. for resistance using exotoxins could be started (SCHUCHMANN 1985).

4. DISCUSSION

Somaclonal variations are genetic alterations produced *in vitro* particularly during the unorganized growth of the callus. They appear in microspore regenerants (gametoclonal) as well as in protoplast (protoclonal) regenerants but can be minimized by speeding up the time till organo- or embryogenesis and/or reducing the growth hormones particularly 2,4-D in the culture media. Further, the media should not be too rich, to allow the survival of too many new spontaneous genotypes, which would not survive on a poor medium.

The homozygous dihaploids AH78.6147 and AH78.5111 were developed by anther culture from the dihaploid clone H3-703 which was again selected from a cross between the dihaploids HH236 and HH439 on the basis of its ability to produce large numbers of embryoids (SOPORY et al. 1978). Hence the significant difference between the clones AH78.6147 and AH78.5111 with respect to protoplast yield and survival percentage, number of calli per plate and percentage of shoot regerenation is logical since they originated from gametes with different genotypes. The three dihaploids AH82.4521/6, AH82.4551/6 and AH 84.4568/6 originated from the same androgenetic dihaploid AH78.5111. Hence these clones and the homozygous donor clone were supposed to be identical. This was confirmed in case of AH78.5111, AH82.4521/6 and AH84.4568/6 as they were statistically identical for all four traits studied. However, the clone AH82.4551/6 was not identical with the other three for protoplast yield, number of calli per plate and shoot regeneration frequency. This might be due to mutation (gametoclonal variation), which arose during the anther culture. This is also implied in case of AH82.4552/6 and AH84.4566/6 which originated from the same homozygous dihaploid AH78.5494 but gave different results.

The clone H75.1207/7 was developed from the cross HH236 × H69.1382/14. This clone was observed statistically non-different from the clone AH78.5111, derived androgenetically from the interdihaploid H3-703 (HH236 × HH439) for only protoplast yield per 1 g leaf material. This might be due to the fact that these clones shared some common genes controlling this character from the clone HH 236. However, clone H75.1207/7 was superior to AH78.5111 for percentage of protoplast survival and division and number calli per plate, but was inferior for shoot regeneration. In other words, the clone HH439 might possess the superior genes for shoot regeneration and H69.1382/14 for protoplast survival and regeneration. Hence, from the present study, it could be expected that protoplast yield and survival, division of surviving cells and their regeneration are controlled by different genes or gene groups, which could be altered at gene level when cultured *in vitro*. This is also evident from the results obtained in

the protoclone of H75.1207/7 and its protoclone P84.044, which was inferior to H75.1207/7 for all characters except shoot regeneration.

In conclusion it can be stated that selection for clones with a good regeneration capacity during protoplast culture is possible; a preselection either by microspore or protoplast culture is very beneficial. Probably in protoplast culture this is a simple somatic selection phenomenon, while in the haplophase of microspore culture genomes are cured from sublethal genes. During this first cycle of *in vitro* culture, however, not only a positive selection may take place, but also gametophytic or somaclonal variation, which may result in a reduced generation rate compared to the original genotype. The data given, further underline the hypothesis that regeneration capacity is heritable (WENZEL 1980), but probably not coded by just one gene.

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