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Comparison of Single Cell Culture Derived *Solanum tuberosum* L. Plants and a Model for their Application in Breeding Programs

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Summary. The techniques of microspore and protoplast regeneration starting from dihaploid *Solanum tuberosum* plants has been improved to such an extent that the production of more than 2000 microspore derived A_1 plant lines and of several hundred protoplast derived plantlets has become possible. Further, from the dihaploid *Solanum* species *S. phureja* the regeneration of microspores to plants, and from the species *S. infundibuliforme, S. sparsipilum* and *S. tarijense* the regeneration of protoplasts to calluses, has been achieved. The plants descending from the two single cell culture systems are compared with reference to phenotypic markers and economic qualities. Some principles characteristic for either microspore or protoplast derived plants are examined and their significance is discussed. The results are compiled into an extended analytical synthetic breeding scheme based on a stepwise reduction of the autotetraploid to the monohaploid level and a subsequent controlled combination to a new synthetic completely heterozygous tetraploid potato.

Key words: Microspore culture – Protoplast culture – Fusion of protoplasts – Potato breeding

Introduction

Chase (1963) proposed an analytical breeding scheme for potatoes taking benefit of the possibility to reduce the chromosome number from the autotetraploid to the diploid (dihaploid) level. The reduction of chromosomes from 4x = 48 to 2x = 24 became a routine procedure when Houghas et al. (1964) detected the diploid primitive variety *Solanum phureja* to be a superior pollinator for the induction of parthenogenesis with an average rate of 4 dihaploids per 100 seeds. The applicability of the technique was further enhanced by Hermsen and Verdenius (1973) who brought the marker 'embryo spot' into *S. phureja* clones. This enabled an early detection of non-haploid hybrid seeds which could then be discarded without a further ploidy check. However, in potato this reduction from 4x to the 2x level is only a first step on the way to more essential practical potato breeding. Baerecke (Frandsen 1968) found a monohaploid plant possessing 12 chromosomes and Breukelen et al. (1975) increased the frequency of such monohaploids via a parthenogenetic procedure: the pollination with the *S. phureja* clones IvP 35 or IvP 48. The procedure is, however, still restricted to a few female genotypes; after *S. phureja* pollination a majority of females still produce monohaploid frequencies too low for applied purposes. Recently the production of androgenetic monohaploids was also successful via microspore culture within anthers isolated from dihaploid *S. tuberosum* clones (Foroughi-Wehr et al. 1977; Sopory et al. 1978; Wenzel and Sopory 1978). Furthermore, the culture and regeneration of protoplasts from dihaploid potatoes (Binding et al. 1978; Grun and Chu 1978; Melchers 1978) and fusion of protoplasts with subsequent hybrid plantlet regeneration (Melchers 1978; Melchers et al. 1978) could be achieved. By combining parthenogenetic, androgenetic and protoplast techniques, and by incorporating these techniques into the normal breeding procedure, potato should become one of the first crop plants exploiting the practical potential of such approaches. In addition to the results obtained in regenerating and comparing plants and tubers descending from either microspore or protoplast cultures, this paper will propose a scheme for an effective combination of the different conventional and cell culture techniques elaborated for potato.

Material and Methods

The experiments for the regeneration of microspore plantlets were concentrated on 5 dihaploid (2n = 2x = 24) *S. tuberosum* clones (H^2 258, H^2 260, H^2 630, H^3 701 and H^3 703), and the primitive

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variety *S. phureja* (Table 1). Three further hybrid families, including H I, H II, and H III, were heterozygous for the marker embryo spot/nodal band, enabling an early genetic analysis of the offspring (Jacobsen and Sopory 1978). Most experiments were carried out with the clone H^3 703, which is a hybrid of the interdihaploids H^2 236 and H^2 439. Both parents possess a good tissue culture and regeneration ability and contain potato leaf roll virus (PLRV) field resistance. Clone H^2 439 further contains resistance against potato virus X (PVX), and inherits the character high seed set (Ross and Jacobsen 1976).

For the protoplast regeneration experiments from 6 interdihaploid *S. tuberosum* clones (H^2 258, H^2 260, H^2 411, H^3 703, H I624 and H I/36) and the 3 *Solanum* species *S. infundibuliforme*, *S. sparsipilum* and *S. tarijense*, all possessing 24 chromosomes, protoplast regeneration could be achieved. The choice for these clones and species was predominantly guided by the knowledge available on how to handle these particular clones in cell and tissue culture. This paper will concentrate on the two interdihaploid *S. tuberosum* clones H^2 258 and H^2 260, both containing PLRV field resistance. Phenotypically the plants of H^2 258 contain a low level of anthocyanin, while the tubers are anthocyanin free (Fig. 1 c, d); the H^2 260 plants have deeply purple stained stems and a purple tuber skin (Fig. 1 b).

The donor material for protoplast isolation was cultured as shoot tip cultures as reported earlier (Binding et al. 1978) or as a suspension culture (Melchers 1978). For 3 clones greenhouse grown leaves were used as starting material (H^3 703, H I/24, HI/36). These latter plants were grown under the same conditions as the plants from which the anthers for the microspore culture were harvested: 16h light, extended during the winter season by Osram HQI-T 1000W lamps with a light intensity of 12 000 lux at the growing point of the plants; the temperature was kept below 18°C and additionally, the plants had to be grafted on tomato stocks (mostly the hybrid variety 'Supravite' was used).

The routine anther culture procedure has been reported previously (Sopory et al. 1978). In addition to the reported technique, anthers were routinely irradiated with 1000 rad γ rays (^{137}Cs) at the time of meiosis. This dose causes no visible alterations on the plants themselves or on the A_1 anther culture offspring, but probably acts as an unspecific shock, increasing the regeneration rate (Przewozny, Schieder, Wenzel, in preparation).

The regenerated plants were maintained under greenhouse conditions at temperatures of 20 ± 3°C. Immediately after transfer from in vitro culture the plantlets were covered with a beaker to prevent rapid dehydration. Determination of the ploidy level was performed roughly by counting plastids in the stomata; for an exact check the chromosomes were counted in root tips or young leaflet squashes (for technical details see review Nitzsche and Wenzel 1977). The vigour of the pollen was checked by staining either with aceto carmine or with lactophenol acid fuchsin. The crossing procedures were carried out on emasculated flowers of decapitated stems in a temperature and moisture controlled greenhouse (20 ± 3°C, 70-80% rel. humidity), or on grafted plants. The latter procedure was used mainly for selfings or crosses performed during September and October. A first trial under field conditions was run with potatoes grown from tubers descending from protoplast derived plants. This was performed in a field near the Baltic Sea at Weidefeld, Federal Republic of Germany, an area with very few aphids, to prevent virus contamination.

Results

Microspore Plantlets

The majority of the microspores cultured within anthers was harvested from the interdihaploid clone H^3 703. From this clone a total of more than 2 000 different androgenetic plant lines could be regenerated (Table 1).

Table 1. List of clones and hybrids of *Solanum tuberosum* subjected to microspore and/or protoplast culture, number of regenerated plants and their ploidy levels

2X donor clone	Starting material	No. of regenerated plants	Ploidy				
			No. of plants counted	1X	2X	4X	Aneuploid[a]
H^2 258	microspores	8	8		8		
H^2 260	"	5	5		5		
H^2 630	"	4	4		3	1	
H^3 701	"	5	5		5		
H^3 703	"	2 039	992		938	54	0.1%
F_1 H I[b]	"	221	124	2	118	4	1%
F_1 H II	"	53	41		40	1	
F_1 H III	"	3	2		2		
S. phureja IvP 35	"	11	8	8			
H^2 258	mesophyll protoplasts	142	49		1	48	4%
H^2 260	"	42	42			42	
H^2 411	"	10					
H^3 703	"	callus					
F_1 H I/24, 36	"	callus					
H^2 258	suspension culture protoplasts	211	106		4	102	10% (30%)[c]

[a] Routinely the ploidy level was estimated by plastid counts in stomata; the exact number of chromosomes was counted only for an aliquot
[b] F_1 H I : H^2 236 × *S. phureja* MPI 76.163/53; F_1 H II : H^2 439 × *S. phureja* IvP 35; F_1 H III : H^2 560 × IvP 35
[c] The amount in brakets is the percentage of aneuploids in mutagenically treated lines

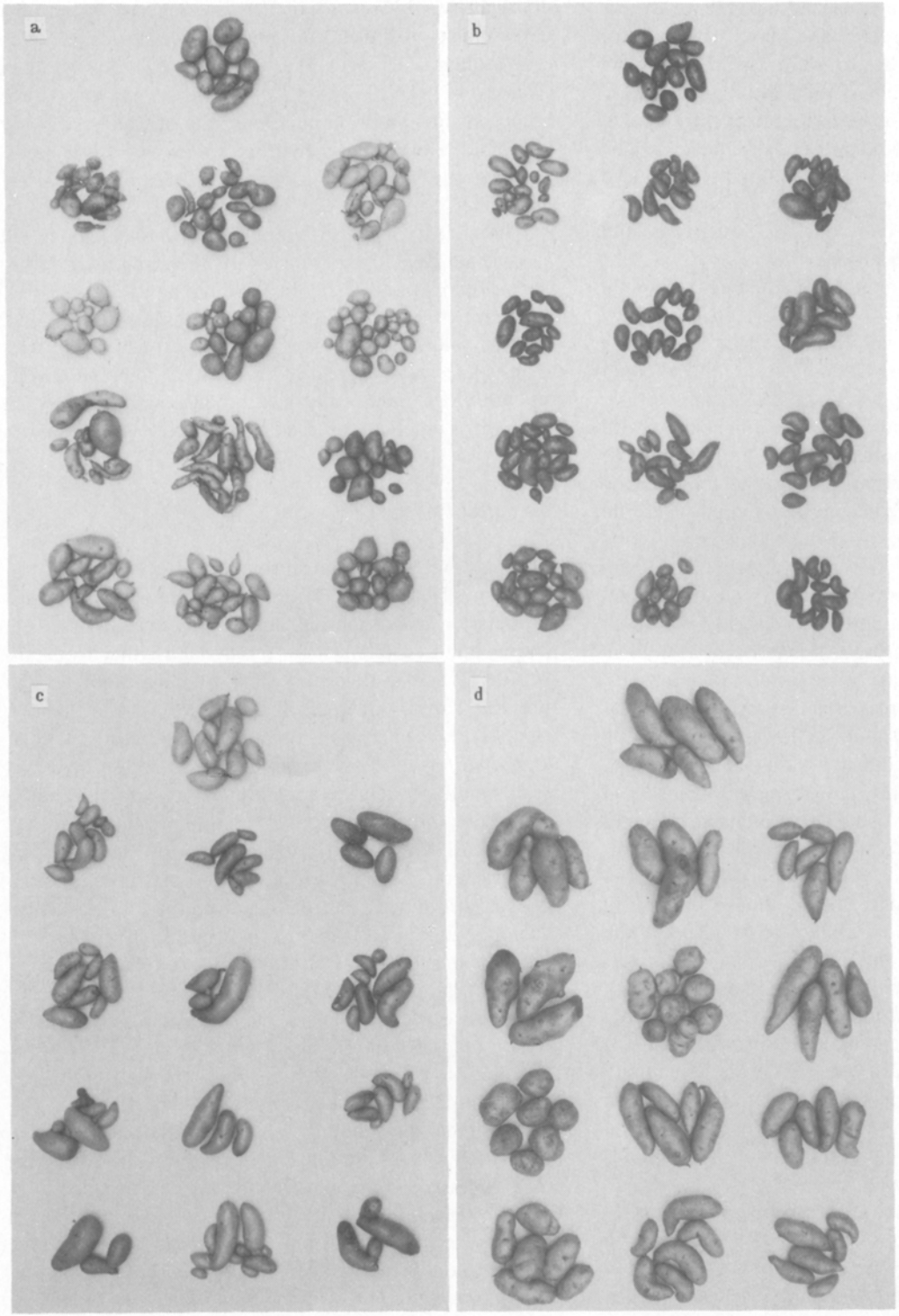


Fig. 1a-d. Groups of tubers from plants regenerated from microspores or protoplasts. The central tubers in the first row are tubers from the clones which served as the material source. **a** Pot-grown tubers from different A_1 plant lines. **b** Pot-grown tubers from regenerants of mesophyll protoplasts from the anthocyanin containing clone H^2 260, and **c** from the clone H^2 258. **d** Tubers from plants grown from tubers of regenerants from suspension culture protoplasts of clone H^2 258, harvested in the field.

The regeneration frequency reached 60 embryoids per 100 anthers plated during April and May. An average of 10% of the embryoids grew to plants which could be transferred to potting compost. The total regeneration rate reached a maximum of 6% (with an average of 4%) based on the number of plated anthers, a frequency which is comparable to the number of 2x plants produced parthenogenetically from 4x plants by crosses with *S. phureja*. Most microspore derived plants set flowers and tubers under greenhouse conditions.

Nearly all plants regenerated from anthers of dihaploid *S. tuberosum* (1189 from 1191 checked up until now, Table 1) were dihaploid again in mature state but during early stages of embryogenesis, monohaploid mitoses could be detected in vitro. Only 8 regenerants arising from *S. phureja*, which can be doubled by colchicin only with difficulty, remained monohaploid in the greenhouse (Table 1). Probably the internal phytohormone concentration together with the auxins in the medium cause an early spontaneous doubling of chromosomes quite common in tissue cultures. If this is simply a result of endomitosis giving rise to homodiploids it would be advantagous for most applied purposes. However, as the development of diplandroid microspores may occur as well, all non-haploid regenerants must be subjected to homozygosity screening. The phenotypic variability visible in the leaf shape, leaf colour and colour of the flowers, and the total vigour of the plants was indicative that at least a high proportion of the plants must have bypassed meiosis and must have originated from reduced, monohaploid microspores, or from diplandroid homozygous microspores arising from pollen mother cells with second division restitution. The shape and colour of the tubers also varied strikingly, as shown for a small aliquot of A_1 plants of the anther donor clone H^3 703 in Figure 1 a.

To verify the homozygous nature of the plants selfing was attempted. However, from the several genotypes only 20% of the A_1 plants descending from the F_1 hybrid H II (H^2 236 × *S. phureja* IvP 35) were self-fertile. The ploidy level, being in some plants immediately tetraploid, had no influence on the seed set, but the fertility of the tetraploids was decreased in comparison to that of dihaploids from the same anther origin.

The seeds, harvested after selfing from 7 self-fertile A_1 plants from the heterozygous donor F_1 H II with embryo spot, were sown and the properties of the A_2 plants were estimated. From these A_2 populations, three were homogeneous, expressing a recessive marker; three were homogeneous expressing a dominant marker; one was heterogeneous. This demonstrates that regenerated androgenetic plants are spontaneously doubled plants, arising from monohaploid microspores, as well as plants descended from diplandroid microspores. The latter can be either homozygous or heterozygous according to whether their mode of origin was by second or first division restitution. The development of diplandroid microspores is possible, as in rye, leading to homozygous and heterozygous A_1 lines (Wenzel et al. 1976, 1977). However, at least three homogeneous potato populations, one of which is already tetraploid, could be grown from homozygous true seeds, enabling the generative propagation of this vegetatively propagated crop.

The quality of the different anther derived clones will be checked in field experiments. As their number is rather large, homozygous dihaploids, combining PVX and PLRV resistance, should be expected. Self-sterile plants crossed with dihaploid clones possessing such valuable characters as resistance would yield hybrids which combine characters useful for tissue culture techniques with qualities important for practical breeding programs.

Protoplast Plants

Protoplast work was concentrated on the two *S. tuberosum* clones H^2 258 and H^2 260, which showed the highest protoplast regeneration rate amongst the 6 clones studied. Several hundred plants were regenerated either from suspension (H^2 258, Melchers 1978), or from shoot tip cultures (H^2 258, H^2 260, Binding et al. 1978). Additionally, from sterile, seed borne shoots of the *Solanum* species *S. infundibuliforme, S. sparsipilum* and *S. tarijense*, protoplasts could be isolated and regenerated into calluses; in these three primitive varieties plantlet regeneration has failed up till now. In all instances, plants which were rapidly regenerated from mesophyll protoplasts were homogeneous according to leaf, stem and flower colour, and the tuber populations of pot grown plants looked very uniform. Figures 1 b and c show samples of tubers harvested from protoplast regenerants of clone H^2 258 and H^2 260. The ploidy level of the plant lines regenerated from mesophyll protoplasts of the clone H^2 258 and H^2 260 was tetraploid in 90 of 91 plants (99%), checked by plastid counts. In 48 plants the chromosomes were counted also to determine the amount of aneuploids: only 2 plants (4%) had 47 or 49 chromosomes, respectively. As auxins are a necessary compound in the regeneration medium, the spontaneous doubling is most probably caused by them during *in vitro* culture. When the callus phase was extended, the frequency of aneuploids (checked in the callus) increased. The two aneuploid plants in the greenhouse were abnormal and sterile.

The situation for plants regenerated from protoplasts from suspension cultures of clone H^2 258 is slightly different. The variability of the phenotype is much higher and the frequency of aneuploids with 37 to 50 chromosomes is drastically increased. 96% of the regenerants were spontaneously tetraploid and 10% of the plants were

aneuploid (Table 1). This is probably due to alterations appearing during the suspension culture period as reported earlier (Melchers and Bergmann 1958; Melchers 1965). Further, the shape of the tubers was not as uniform, as in plants regenerated from the same clone but from shoot tip protoplasts. This variability could be increased when a mutagenic treatment was applied during the early regeneration phase (Schilde and Melchers, unpublished) but the variability never reached the variance expressed in the offspring of microspores.

In a second growing season tubers from protoplast derived lines of clone H^2 258 cell suspensions were grown to plants in the field. Figure 1 d demonstrates the variability of the field grown material. Like the first tuber generation their shape is intermediate to tubers of microspore derived plants (Fig. 1 a) and to tubers from mesophyll protoplasts (Fig. 1 c). These protoplast regenerants were untreated control plants of a large protoplast mutagenesis experiment (Schilde and Melchers, unpublished). The mutagenic treatment caused increased phenotypic variability and an increase in the number of aneuploids to 30% (Table 1).

Discussion

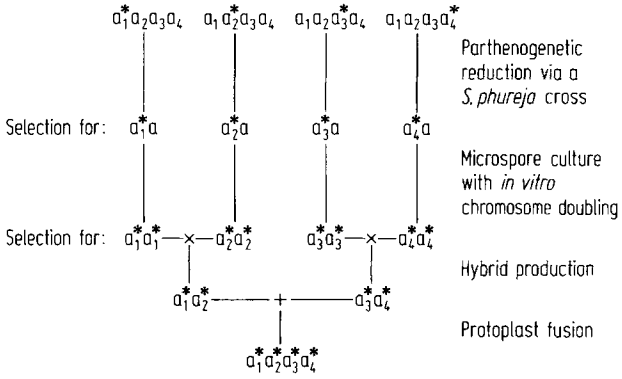
Microspore and Protoplast Plantlets

A comparison of regenerants arising either from microspores or from protoplasts clearly indicates the basic difference between the two procedures: plants regenerated from protoplasts show only slight alterations in geno- and phenotype and are dependent on the length of the callus phase and on the source of protoplasts. Such plants may serve as a means for a vegetative propagation combined with mitotic chromosome doubling. Microspore plants, however, express in the A_1 the full genetic power present in a heterozygous donor plant. The genome of the gametes is manifested at the total plant level. By the naturally occuring spontaneous chromosome doubling new homozygous lines are obtained. Further, the reduction from dihaploid to monohaploid means that all semi-lethal genes will cause lethality only when the lethal allele is present. As a consequence only those plants which possess a totally functional genotype can be regenerated from microspores. This means that the anther culture step is not only a technique for rapid construction of homozygous plant lines but also serves as a good and rapid selection for genotypes free of sublethal genes, overcoming the normal inbreeding depression. It is probably this selection which enabled the success in applied anther culture of tobacco and rice with just a few A_1 lines (Nakamura et al. 1974; Yin et al. 1976).

The rather high uniformity of plants regenerated from mesophyll protoplasts in our experiments is not in agreement with the results of Matern et al. (1978). These authors found many variants after mesophyll protoplast regeneration from the tetraploid potato variety 'Russet Burbank'. Matern et al. even reported different grades of resistance against crude extracts of *Alternaria solani* and recommended protoplast regeneration as a technique for intracultivar improvement. According to our results only cultures maintained in a prolonged callus phase and descending from suspension cultures or cultures receiving additional mutagenic treatment showed undirected aberrations. The use of the latter as a general effective breeding procedure is questionable. However, during protoplast culture of tetraploid material in tobacco a considerable variation of chromosome number was found in one case (Takebe et al. 1971), a very small variation was found in another (Nagata and Takebe 1971) and a useful mutation was found by Melchers (1974): one plant turned out to have changed from a day neutral into a short day type. There is a chance that the tetraploid starting material, which Matern et al. used and which was propagated over a rather long period vegetatively, was of chimeral structure, and segregated during protoplast isolation. If alterations are undesired, a rapid passage through the callus phase, or even better, direct embryogenesis, will be most effective. Starting with shoot tip cultures produces a higher proportion of euploid individuals than starting from suspension cultures. This rather stable nature of mesophyll protoplasts was also demonstrated for *Datura* regeneration (Schieder 1976).

Analytical Synthetic Breeding Scheme

The two cell culture techniques, together with the parthenogenetic reduction of potatoes from the 4x to the 2x level, combined with protoplast fusion techniques (Melchers et al. 1978) may allow a more defined potato breeding. The model presented in Figure 2 demonstrates how



a=Potato genome,
*=Favourable genes with qualitative inheritance (e.g. resistance)

Fig. 2. Scheme for an analytical synthetic breeding procedure combining tissue culture techniques and conventional breeding steps

the different procedures might be combined. Starting with at least four different autotetraploid clones, each of which has a positive qualitative inherited character, such as resistance to a particular disease, the dihaploid level is achieved parthenogenetically via a *S. phureja* cross. At the 2x level the presence of the desired genotype is selected for. If the character expressed is coded by a recessive allele, homozygosity for that allele is already achieved. However, dominant genes are more important in applied breeding work. To obtain dominant genes in the homozygous condition also, the dihaploids should be subjected to anther culture with in vitro chromosome doubling. As long as the A_1 plants are self-fertile, the resulting homozygous lines may be propagated via true seeds, overcoming most virus problems. From the different 4x starting clones one will receive different homozygous 2x clones which can be combined to well defined F_1 hybrids. They have to be doubled to the tetraploid level – at this particular time the adaptation of potatoes is much better at this level; further progress in dihaploid breeding work may result in dihaploid varieties, overcoming the need of a return to the 4x level. This doubling is performed most successfully via meiotic doubling with a 4x × 2x cross (Mendiburu et al. 1974). However, the female genome is mixed during meiosis, resulting in an undesired segregation. As there is normally only poor seed set, this doubling step is the bottle neck for procedures using dihaploids in applied potato breeding. The ideal procedure would be to fuse protoplasts of different dihaploid hybrids. The heterozygous dihaploids would be combined to a completely heterozygous tetraploid plant, without any meiotic segregation. Propagated vegetatively it could immediately become a stable variety. During this experimental step primitive varieties, or different species – most of which are diploid – may easily be incorporated, broadening the resources of resistance. *S. sparsipilum*, for instance, from which protoplasts could be regenerated, contains genes for resistance against the ill worm *Globodera pallida*.

The selection for the somatic hybrids may be performed by hybrid vigour, as Schieder (1978) demonstrated in *Datura* protoplast fusion experiments. Calluses with a high growth rate, expressing high vigour, were shown to be somatic hybrids. In applied breeding programs hybrids with a good combining ability and possessing such vigour, will be of interest. Experiments applying this scheme, which combines conventional and tissue culture techniques with the aim to increase the efficiency in potato breeding programs, are under examination.

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