Theor. Appl. Genet. 52, 119-123 (1978)

The Influence and Possible Recombination of Genotypes on the Production of Microspore Embryoids in Anther Cultures of *Solanum tuberosum* and Dihaploid Hybrids

E. Jacobsen and S.K. Sopory[1]

Max-Planck-Institut für Züchtungsforschung (Erwin-Baur-Institute), Abteilung Straub, Köln (Federal Republic of Germany)

Summary. In addition to physical and chemical factors, genotype appears to be a very important factor influencing success in anther culture. Recombination by making crosses with selected responding clones has been introduced as a possible helpful method to positively influence the success and response type via the factor genotype. From the progeny of such a cross, one genotype could be selected, producing in 30 to 40 percent of the cultured anthers, fully developed embryoids and plantlets, which are a mixture of polyploids, dihaploids and monohaploids.

Further, a pleiotropic marker 'embryo spot' visible as a 'nodal band' in the plant stage, has been used to confirm the microsporic origin of dihaploids and polyploids and to prove their homozygous nature. This marker also shows potential use in confirming the origin of calli from individual microspores.

Key words: Potato – Anther culture – Monohaploid embryoids – Genetic marker – Recombination

Introduction

The extraction of dihaploids from the tetraploid potato is now routinely done with the aid of parthenogenesis in interspecific crosses with the selected pollinators of *S. phureja* carrying a homozygous pleiotropic marker (Hermsen and Verdenius 1973). Using the same approach it is also possible to obtain monohaploids (van Breukelen et al. 1977; Jacobsen 1978). In spite of the availability of this method, anther culture would be an additional and more attractive technique to obtain monohaploids since many more different microspore genotypes than macrospore genotypes are produced which are potentially available per flower to grow until the plant stage.

Production of monohaploid plants through pollen callus differentiation has been reported (Foroughi-Wehr et al. 1977). Recently, we communicated (Sopory et al. 1977) on the effects of the stage of microspore development and chemical factors on the production of direct embryoids, avoiding any callus phase, in a selected dihaploid clone of potato. In the present communication, we report on the influence of genotype on the successful regeneration of embryoids and plantlets. We also show that it is possible to recombine the ability of embryoid formation in order to obtain clones which are more efficient in the development of embryoids. A genetic marker was used in order to confirm the origin and homozygous nature of regenerated plants of higher ploidies.

Materials and Methods

Of the existing interdihaploid (H^2) clones, 41 were tested during the year 1976 on the media reported earlier (Sopory and Rogan 1976). From these, 16 clones were retested in 1977 on optimized media, P 58 and P 59, the composition of which is described in Sopory et al. (1977). The origin of these clones has been described earlier (Ross and Jacobsen 1976). Further, a cross between the interdihaploids, H^2 236 and H^2 439, which showed microspore divisions and microspore callus differentiation respectively, and crosses between these and a third interdihaploid, H^2 560, with *S. phureja* were made and studied (Table 1). All F_1 progeny of these crosses were heterozygous for at least the *B* gene for the pleiotropic marker 'embryo spot' visible as a 'nodal band' in the plantlet stage (hereafter termed 'nb') which is inherited by two complementary genes *BBPP* (Hermsen and Verdenius 1973). The female parents were homozygous recessive for at least one gene (*B*).

Cytological studies of the anthers were made with aceto carmine to check up microspore divisions. Staining of embryoids and root tips of plantlets for chromosome analysis was made according to Tjio and Levan (1950).

Results

Table 2 shows the different types of response obtained

1 On leave from Jawaharal Nehru University, New Delhi (India)

Table 1. The number of anthers cultured from different crosses between interdihaploids and interdihaploids with *S. phureja* carrying the dominant marker 'embryo spot' controlled by two complementary genes, one of which is *BB*

Female parent	X	Male parent	Total No. of anthers cultured
1. H^2 236 (md; E')		H^2 439 (md cd)	18 172
2. H^2 236 (md; E') bb		76. 163/53, *S. phureja* BB	6 061
3. H^2 439 (md cd) bb		IvP 35, *S. phureja* (E'') BB	3 265
4. H^2 560 (md) bb		IvP 35, *S. phureja* (E'') BB	2 060

md = microspore divisions, md cd = microspore divisions and microspore callus differentiation, E' = embryoids (< 0.1%), E'' = embryoids (< 0.3%)

after cytological examination of the cultured anthers in the 41 clones tested during 1976. In 31 clones (75,1%), no microspore divisions were observed (response type 1 and 5), in 6 clones there were microspore divisions (response type 2) and in five the divisions proceeded till the globular embryoidal stage (response type 3). Callus either originated from the microspores (response type 4) or from the filament region (response type 5). The differentiation of callus, whether from microspores or from filament was achieved on Murashige and Skoog (1962) (MS) + zeatin riboside (10^{-5}M). In either case only diploid and tetraploid plants could be regenerated.

During 1977, 16 clones were retested on P 59 and P 58 media which had been shown to support embryoid formation (Sopory et al. 1977). As shown in Table 2 (see underlined and double underlined clones), the response of most of the clones did not change except in H^2 236, H^2 258 and H^2 630 (all belonging to response type 3) where sometimes fully developed embryoids and plantlets were produced. This suggests a strong influence of genotype on the type of response.

In order to improve the response, crosses between interdihaploid clones, which showed response type 2, 3 and 4 (Table 2), were tested. One cross, between H^2 236 and H^2 439, showed a positive response and was studied in detail. As is clear from Table 3, the parents tested on P 59 and P 58 media responded mostly by showing microspore divisions. Rarely, as mentioned above did H^2 439 give callus formation. However, of the ten clones from the progeny tested, 6 showed microspore divisions and 4 of them developed until embryoids and plantlets resulted. In one clone, H^3 703, about 35-40% of the cultured anthers routinely produced embryoids on P 59 medium. Although exact quantitative studies to correlate the frequency of microspore divisions and embryoid formation has not yet been made, in clones H^3 701 and H^3 703 many more pollen divisions could be observed.

These results, in comparison to those mentioned above (Table 2), where responding clones were retested on P 59 and P 58 media, gives the impression that the ability for microspore divisions and/or embryoid formation is recombinable. The data in Table 4 further supports this. Here, 3 F_1 progenies of crosses between interdihaploids and *S. phureja* were studied. The response of almost all the parents was known (Table 1). Compared to the parents, the capacity of embryoid formation clearly increased in cross 2 and 3, suggesting, therefore, the possibility of positively recombining the parental abilities. The results in Table 3 and 4 also show a better effect of P 59 medium on embryoid formation.

The transfer of embryoids to MS + zeatin riboside (5 x 10^{-6}M) + Coconut milk (10%) showed the best development of embryoids. On an average, 12% of the embryoids in H^3 703, 15% of progeny 2 and 8% of progeny 3 could be developed further into plants. Cytological examination of ploidy level of 22 embryoids of clone H^3 703 revealed 7 monohaploids, 14 diploids an 1 triploid. However, examination of 40 plants from the same clone showed only 2 monohaploids, 34 diploids and 4 tetraploids. One monohaploid died after being transferred to soil and others grew slowly due to some chlorophyll deficiency. From this plant, leaf tissue culture started to diploidize and tetraploidize immediately. This is also done with other leaf materials in potato (Jacobsen 1977). The presence of triploids is an indication that diploids and polyploids can originate from reduced microspores.

The marker 'nb' was used as a tool to test for the origin of regenerated diploid and tetraploid plants. Anthers from F_1 progenies 2, 3 and 4, which all carried this marker, were cultured in order to obtain 'nb' free plants. From 36 regenerated plants of progeny 2, with H^2 236 as the mother parent, 19 were with and 17 were without the marker. The occurrence of 'nb' free plants indicates the origin of plants from reduced microspores and their homozygous nature.

Occasionally when progeny 2 was in the P 58 medium, besides embryoid even callus formation occurred. Interestingly, even at this stage it was possible to distinguish calli with deep pink colour, which we have never observed in anther cultures from interdihaploids alone, and pigment free calli, both originating from the same anther. These calli underwent differentiation when transferred to MS +

Table 2. Qualitative response of different dihaploid clones

Type	Responses	Clones	
1	nil	H^2	87, 54, 59, 110, 140, 260, 345, 73, 423, 424, 438, 432, 519, 451, 454, 455, 473, 548, 631, 633
2	microspore divisions upto 8-16 cells	H^2	437, 439, 408, 578, 580, 596
3	microspore divisions upto globular embryoidal stage	H^2	236, 258, 560, 601, 630
4	microspore callus	H^2	258* 439* 493, 596* 630*
5	callus from somatic origin	H^2	79, 63, 408* 411* 409, 252, 479, 488, 486, 578, 633

Underlined and double underlined clones were retested on P 59 and P 58 media; double underlined clones showed embryoid formation on P 59 media with less than 0.1% frequency; clones marked * are those where differentiation into plants was achieved in MS + zeatin riboside (10^{-5} M) medium. Note some clones showed two different response types

Table 3. Recombination of genotypes for microspore divisions and embryoid formation in a dihaploid family. Ten seedlings of cross between H^2 236 X H^2 439 were tested on P 58 and P 59 media.

Genotype		Response	
		P 58 medium	P 59 medium
H^2 236 (♀ parent)		md	md E + P′
H^2 439 (♂ parent)		few md	md + callus
Progeny	H^3 690	md	md
	H^3 692	E + P′	E + P′
	H^3 697	nil	nil
	H^3 701	E + P (2%)	E + P (0.3%)
	H^3 703	E + P (20%)	E + P (35%)
	H^3 709	nil	nil
	H^3 718	nil	nil
	H^3 719	E + P′	E + P′
	H^3 722	nil	nil
	H^3 728	md	md

md = microspore divisions, E = embryoids, P = plantlets, E + P′ = rarely, % = percent of cultured anthers producing embryoids

zeatin riboside (5 x 10^{-6}M). From pink calli grew only plants with the marker 'nb' and from green calli, plants without 'nb'. This shows that the marker 'nb' expresses itself even at the callus stage. Further, it indicates the origin of calli from single microspores since in such cases so far no mixed calli were observed, which would indicate multimicrosporic origin. An early selection in seperating calli of different origin is therefore possible.

Discussion

Earlier studies have shown that success in anther culture depends on the genotype (Nitzsche and Wenzel 1977). Irikura (1975) observed that out of 46 species of *Solanum*, from which 118 clones and 9 interspecific hybrids were tested, only 19 species and 4 interspecific hybrids were able to produce plants that were of microsporic origin. In the present studies, we also found a strong influence of genotype on the successful response in different clones of *S. tuberosum* (Table 2). It was also interesting to note that out of the 9 clones that fell into response type 2 and 3, 5 clones were the result of the same cross between the primary dihaploids H 171 x H 165. Similarly, progenies of other crosses either didn't show any response or only showed callus formation from the filament. Although the influence of parental genotype in anther cultures has recently been suggested in rye (Wenzel et al. 1977) and potato (Simon and Peloquin 1977), our results suggest the possibility of recombining (from breeding point of view) 'weakly responding' genotypes in order to produce and select 'highly responding' ones. This was

Table 4. The influence of recombination of genotypes between *S. tuberosum* interdihaploids and *S. phureja* on embryoid production. All F_1 progenies carried the genetic marker 'nb' E′ = embryoids rarely (< 0.3%)

Progeny number	Cross studied	P 58 medium			P 59 medium		
		Number of anthers cultured	Number of anthers producing embryos	Percent response	Number of anthers cultured	Number of anthers producing embryos	Percent response
2	H^2 236 X 76.163/53 *S. phureja*	987	80	8.10	1020	141	13.82
3	H^2 439 X IvP 35 E′ *S. phureja*	732	42	5.73	1182	73	6.17
4	H^2 560 X IvP 35 E′ *S. phureja*	613	5	0.81	437	11	2.5

demonstrated from the crosses we made between clones showing response type 2, 3 and 4 and then selecting one cross to get a highly responding clone, H^3703 (Table 3). These observations suggest that the genes which positively influence the success rate in microspore divisions and embryoid formation can be accumulated and selected. It is also possible that the regenerating ability of the callus of H^2439 did positively influence this development or it could be that the repression of the ability for embryoid formation was derepressed by recombination in some genotypes.

The results, based on the total recovery of plants obtained compared to the total number of embryoids produced, and their cytological analysis, showed that few embryoids developed further and that the ratio of monohaploids to diploids and polyploids changed during growth of the embryoids to plants. The probable explanations are:

i) the heterozygous nature of the dihaploid potato. This could allow the expression of lethal factors in monohaploids more than in dihaploids and polyploids since they could be of a heterozygous nature because of their origin from diplandroid microspores produced via parallel spindle mechanism (Jacobsen 1976);

ii) chromosome doubling during development of embryoids to plants;

iii) a negative selection pressure on monohaploid embryoids because of the ploidy level. It might be possible to select and further recombine genotypes with better regenerating ability to overcome the present difficulties.

The segregation of the marker 'nb' is a genetical proof for the microsporic origin of the dihaploid and tetraploid plants and for the homozygosity of at least the *B* gene. Because of the complementary nature of 'nb', the regenerated 'bandless' plants can possess different genotypes. It would be better, therefore, to test the regenerated diploids with nodal bands together with the donor clones of progeny 2 (Table 4) by crossing with the homozygous recessive genotype (*bbpp*). This would prove homozygosity, not only for gene *B*, but also for the complementary gene *P*. This test would give a better indication of complete homozygosity of the regenerated dihaploids and tetraploids and a better genetical description of the F_1 progenies tested here. Such a proof is essential since in breeding it is the homozygous diploids and tetraploids that are needed, and not the monohaploids, for crossing after selection.

Genetic markers have been used earlier to test the homozygous nature of the regenerated plants (Melchers 1975; Corduan 1975). However, it would be better to have other methods to prove the homozygous nature more generally and less specifically. The use of isoenzymes as markers, shown to be stable in tubers of potato (Stegemann and Loeschcke 1976), could be of help in this direction.

In conclusion, the present studies support the earlier observations on the influence of genotype in anther cultures. They further suggest that improvement may be made by selecting and recombining the existing genotypes in addition to manipulating the media and cultural conditions even though the latter are essential to help express the ability of the right genotype.

Acknowledgement

We thank Prof. H. Ross, Dr. G. Wenzel and Prof. W. Nitzsche for their discussing and critical reading of the manuscript; Dr. Ir. J.G.Th. Hermsen for kindly providing *S. phureja* material and Mrs. Stevelmanns for culturing the anthers. The research was supported partly by grants from 'Deutsche Forschungsgemeinschaft' (DFG) and Gemeinschaft zur Förderung der privaten deutschen landwirtschaftlichen Pflanzenzüchtung' (GFP)

Literature

van Breukelen, E.W.M.; Ramanna, M.S.; Hermsen, J.G.Th.: Parthenogenetic monohaploids (2n=x=12) from *Solanum tuberosum* L. and *S. verrucosum* Schlechtd. and the production of homozygous potato diploids. Euphytica **26**, 263-272 (1977)

Corduan, G.: Regeneration of anther derived plants of *Hyoscyamus niger L.* Planta **127**, 27-36 (1975)

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

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

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

Jacobsen, E.: Cytological studies on diplandroid production in a dihaploid potato clone and its correlation with seed set in 4x X 2x crosses. Z. Pflanzenzüchtung **77**, 10-15 (1976)

Jacobsen, E.: Doubling dihaploid potato clones via leaf tissue culture. Z. Pflanzenzüchtung **80**, 80-82 (1978)

Jacobsen, E.: Haploid, diploid and tetraploid parthenogenesis in interspecific crosses between *S. tuberosum* interdihaploids and *S. phureja* Potato Res. In Press (1978)

Melchers, G.: Genetik und Pflanzenzüchtung mit mikrobiologischen Methoden. Planta med. Suppl., 5-34 (1975)

Murashige, T.; Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Pl. **15**, 473-497 (1962)

Nitzsche, W.; Wenzel, G.: Haploids in plant breeding. Berlin and Hamburg: Parey 1977

Ross, H.; Jacobsen, E.: Beobachtungen an Nachkommenschaften aus Kreuzungen zwischen dihaploiden und tetraploiden Kartoffelformen; Samenzahl, Ploidiestufen, sowie Spaltungsverhältnisse des Gens für extreme Resistenz gegen das X-Virus (Rxacl). Z. Pflanzenzüchtung **76**, 265-280 (1976)

Simon, P.W.; Peloquin, S.J.: The influences of paternal species on

the origin of callus in anther culture of *Solanum* hybrids Theor. Appl. Genet. **50**, 53-56 (1977)
Sopory, S.K.; Rogan, P.G.: Induction of pollen divisions and embryoid formation in anther cultures of some dihaploid clones of *Solanum tuberosum*. Z. Pflanzenphysiol. **80**, 77-80 (1976)
Sopory, S.K.; Jacobsen, E.; Wenzel, G.: In vitro production of monohaploid embryoids and plantlets in cultured anthers of *Solanum tuberosum*. Submitted (1977)
Stegemann, M.; Loeschcke, V.: Index of European potato varieties. Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft, Berlin-Dahlem **168** (1976)
Tjio, J.H.; Levan, A.: The use of oxyquinoline in chromosome analysis. An. Estacion exp. Aula Dei **2** (21-64 (1950)
Wenzel, G.; Hoffmann, F.; Thomas, E.: Increased induction and chromosome doubling of androgenetic haploid rye. Theor. Appl. Genet. In Press (1977)

Communicated by G. Melchers
Received December 20, 1977

Jr. Evert Jacobsen
Max-Planck-Institut für Züchtungsforschung
(Erwin-Baur-Institute) Abteilung Straub
D-5000 Köln 30