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Relative performance of monohaploid potato clones and their diploid parents at plant level and after protoplast isolation and subsequent fusion

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Summary. Plant growth performance was studied in 118 potato monohaploids and in their diploid parents. Of these monohaploids 76 were also investigated at the protoplast level and eight of these were used in protoplast fusion experiments as well. No correlation was found between relative performance of greenhouse grown and in vitro grown plants. No or only weak correlations were found between different in vitro characteristics such as plant growth, protoplast yield per gram plant material, plating efficiency and callus growth. This indicates the unpredictability of these characters.

The protoplast fusion experiments indicated that only in some genotype combinations increased callus growth rates may be found. However, it is not clear whether such calli were hybrids or not. In protoplast monocultures only diploid and tetraploid regenerants were obtained. After fusion, tetraploids but also some triploids could be regenerated. The finding of triploids indicates that monoploid protoplasts were involved in fusion. Isozyme analysis and morphological assessment of the plants pointed out that the majority of the fusion regenerants were hybrids. The implications of these results are discussed.

Key words: Monohaploids – *Solanum* – Potato – In vitro-performance – Protoplast fusion

Introduction

The performance of plants is genotype dependent. Research in potato has made clear that also tissue culture ability is genetically determined and can be transferred after crossing to progeny plants (Wenzel et al. 1979; Uhrig 1985). Knowledge about the relation between relative performance of genotypes under greenhouse and under in vitro conditions is poor. Such knowledge is of great importance since, in general, plant material is tested under greenhouse conditions first, before it is used for in vitro experiments. In this study five diploid clones have been screened for their relative performance at the plant level in the greenhouse and at different levels of in vitro culture. In addition the relative performance of these diploids was compared with that of their gynogenetic monohaploid descendants (= gametic sample) in order to test the quality of their gametes.

The relative performance of monohaploids in the greenhouse and at different levels of in vitro culture has been investigated.

So far, only little report has been made about protoplast culture experiments in monohaploid potato. Regenerants obtained from monohaploid potato derived protoplasts all appeared to be tetraploids (Sree Ramulu et al. 1986; Uijtewaal et al. 1987b) and the only fusion experiment described resulted in tetraploid hybrids. In this study protoplast isolation and culture have been investigated of a large number of monohaploids and from some of them plants could be regenerated. In addition fusion experiments between monohaploids will be described. Since a better regeneration capacity of hybrid fusion products was observed in such combinations (Uijtewaal et al. 1987b) diploid, triploid and tetraploid hybrid regenerants could be expected. If this better regeneration capacity after fusion of monohaploids would be generally valid, then a useful selection criterion for hybrids would be available. In this study regenerants obtained after fusion of

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monohaploids were screened for hybridity using isozymes as genetic markers.

Materials and methods

Plant material

One hundred and eighteen monohaploids were investigated at plant level and 76 of them also at protoplast level (Table 1). They originated from five different (groups of) diploid *Solanum tuberosum* L. or *S. tuberosum* × *S. phureja* Juz. et Buk. hybrids:

i) M9 is a diploid hybrid, partly derived from *S. phureja.*

ii) The parthenogenetic diploid (PD) clones are derived from cv. Gineke

iii) Clone LGM-1 is a cross product of dihaploids of cv. Libertas (L) and cv. Gineke (G), and the diploid hybrid M9.

iv) The clones MGPG-2; -3 and -12 are cross products of M9 and clone GPG. The latter is derived from (G609 × *S. phureja*) × G609, where G609 is a dihaploid from cv. Gineke.

v) 7322 is an androgenetic monohaploid derived from the diploid clone H78.01. It has been obtained at the Max Planck Institute, Cologne, FRG. The detailed ancestry of the first four (groups of) genotypes has been presented in Uijtewaal et al. (1987a); that of 7322 in de Vries et al. (1987). The origin and number of monohaploid genotypes studied on plant level and in protoplast experiments have been included in Table 1.

The plant material was cultured in vivo in 24 cm pots in the greenhouse under normal daylight conditions and temperatures varying from 20–30 °C and in vitro in 15 cm high glass tubes or in 9 cm high glass containers in vitro, on solidified MS-medium with 2% sucrose (25 °C, 16 h daylight, 7,000 Lux). Plants grown in glass tubes were used for determination of relative vigour; those in glass containers were used for phenotypic classification and protoplast isolation.

The relative vigour is expressed in a scale from one to ten. For the greenhouse grown plants it was calculated from the height in cm (h) after 3–4 months of growth and the plant age in days (a), according to the formula $10 \times h/a$. Dependent on the degree of branching a value of at most two scale units was added to the calculated vigour. For the in vitro grown plants, relative vigour was calculated from the parameters h = height in cm after about one month of growth, la = leaf area as measured with a photo-electric area meter, both parameters being related to a = the age in days of the plants when the assessments were made. The calculation was according to the formula $(H + LA) \times 0.5$, where $H = 2 \times h/a$ and $LA = 0.4 \times la/a$.

The phenotypic classification is shown in Fig. 1.

Protoplast isolation and culture

Protoplasts were isolated from four weeks old axenic shoots. The procedures for protoplast isolation, culture and plant regeneration were according to Uijtewaal et al. (1987b).

The yield of protoplasts was determined per gram plant material. The plating efficiency was calculated as percentage of calli formed per number of cultured protoplasts. The callus growth was determined visually two months after protoplast isolation by estimation of the callus size, and classified from 0 to 4. All observations were carried out twice. The callus growth type classification is shown in Fig. 2 (Loose, loose/compact and compact).

Table 1. Source and number of gynogenetic (g) and androgenetic (a) monohaploids investigated on plant level and in protoplast experiments

Diploid source	Monohaploids	Investigated no.	
		on plant level	in protoplast experiments
M9	839–. . ; 849–. . g	36	9
PD23-2	851–. . g	52	52
PD23-26	854–. . g	16	6
PD23-28	852–. . g	7	2
LGM-1	855–. . g	4	4
MGPG-12	8522–. . g	2	2
H78.01	7322a	1	1
Total		118	76

Protoplast fusion

Protoplast fusion was carried out according to Uijtewaal et al. (1987b). All vital protoplasts still present after the fusion procedure (starting with 5×10^5 protoplasts of each clone) were cultured in 6 cm Petri dishes. Depending on the number of calli formed, the cultures were diluted in 9 cm Petri dishes to final colony concentrations of ca. 400 per Petri dish, i.e. ca. 30 colonies per ml.

Ploidy level determination in regenerants

Regenerants were checked for their ploidy level by counting the number of chloroplasts in the guard cells according to Frandsen (1968), and the number of chromosomes in the root tip cells according to Henderson and Lu (1968).

Isozyme analysis

For control of hybridity of the fusion products the isozymes 6PGDH and MDH were used. Sample preparation, polyacrylamide gel electrophoresis and staining procedure were according to Suurs and Jongedijk (1987).

Results

Genotype comparison for relative performance in the greenhouse and in shoot culture

Large differences in performance, both in the greenhouse and in vitro, were detected among monohaploids originating from one diploid and between groups of monohaploids originating from different diploids. In Table 2 the data are given for the diploid parental clones themselves as well as for their monohaploid derivatives.

It is apparent that the diploids M9 and LGM-1 have a better greenhouse performance than PD23-2, PD23-26 and PD23-28. M9 exceeded the PD-clones also under in vitro conditions. Unfortunately LGM-1 got virus infected and had to be removed before the in vitro performance could be determined. The largest variation (1–9) in relative greenhouse performance between monohaploids originating from one diploid

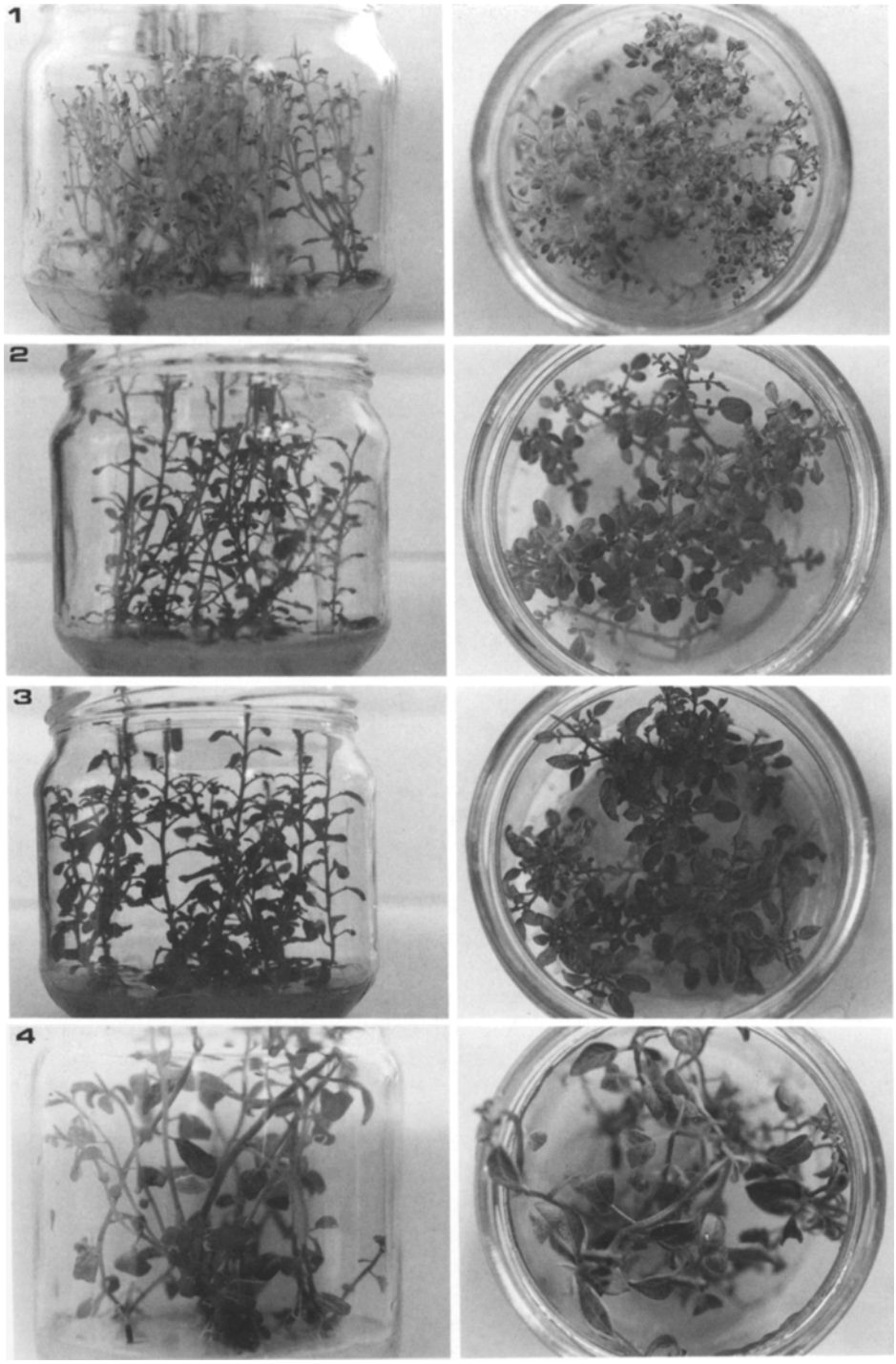

Fig. 1. Phenotypic classification (1 = small leaves, thin stems; 4 = big leaves thick stems) of monohaploids grown in shoot cultures in glass containers

was found for the monohaploids originating from M9, PD23-2 and PD23-26, and the smallest (1–3) for the monohaploids from LGM-1. The mean relative greenhouse performance was similar for the monohaploids from M9 and PD23-2, somewhat lower for the monohaploids from PD23-28 and much lower for those from LGM-1. The latter is in contrast with the results from the in vitro grown plants, where the mean performance of the monohaploids of LGM-1 exceeded that of the other diploids. No correlation was found between the

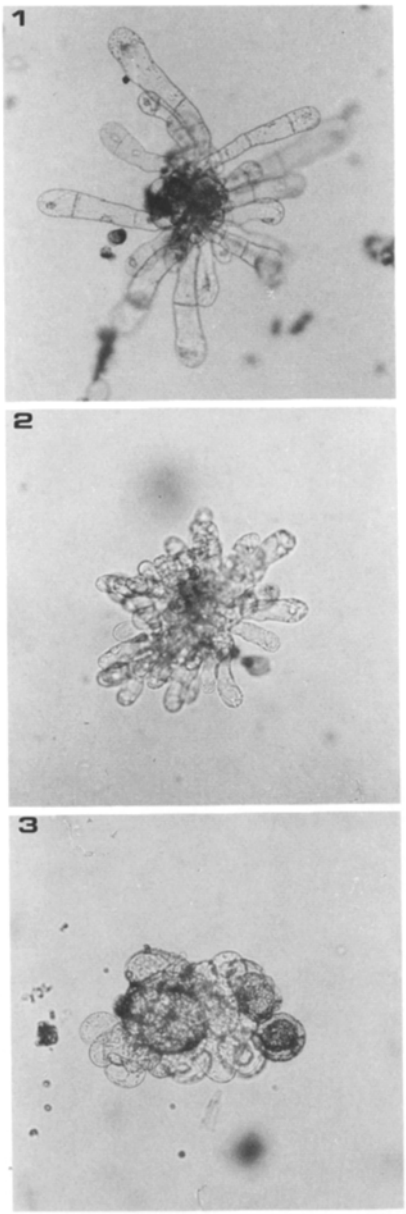

Fig. 2. Growth type classification of calli (1 = loose; 3 = compact) derived from protoplasts of monohaploid clones

growth performance of individual monohaploids in the greenhouse and in vitro (r = 0.00; $P = 0.48$; n = 115).

Genotype comparison for protoplast culture ability

All genotypes tested yielded protoplasts that started to divide within six days of culture. However, the number of protoplasts obtained per gram plant material varied greatly (Table 3). Large genotypic variation could be detected for the plating efficiency (PE: $0–3.10^{-3}$) and the callus growth rate (CGR).

It is apparent that the relatively vigorous diploid M9 had a much lower protoplast yield per gram plant material than PD23-2 and PD23-26. This can be due to the thicker stems of M9 as compared with the other diploids. The monohaploids from PD23-2 had a better general performance for PE than their diploid parent. Monohaploids from M9 and PD23-26 showed an equal or lower performance than their diploid parents. The callus growth rates of monohaploids and their respective diploid parents were comparable. Within each group of monohaploids individual genotypes were detected with an equal or better plating efficiency and callus growth than their diploid parent. Plants could be regenerated out of protoplast derived calli, of three monohaploids from M9 (43%) and of six monohaploids from PD23-2 (12%). This difference in regeneration percentage suggests a genotypic influence, but careful consideration of these results is required since the number of regenerants is low.

Within the group of monohaploids from PD23-2 coefficients of correlation have been calculated between the different plant and cell growth characteristics (Table 4). It is apparent that no correlation was found between plant growth in the greenhouse and different characteristics of in vitro growth ($P > 0.25$). Also, no correlation was found between shoot culture growth (including leaf area) and the yield of protoplasts per gram plant material ($P > 0.25$). The relatively high correlation between plant vigour in vitro and growth type (r = 0.74; $P = 0.00$) was expected, since the latter was more or less dependent on the personal interpretation of the former. Small but significant correlations were found between the protoplast yield and plating efficiency (r = 0.29; $P = 0.02$), between plating efficiency and callus type (r = –0.39; $P = 0.01$) and between callus type and callus growth (r = 0.34; $P = 0.02$). Also significant correlations were found between the callus growth and the in vitro plant vigour (r = 0.31; $P = 0.02$) and plant growth type (r = 0.42; $P = 0.00$).

Protoplast fusion

Fusion experiments have been carried out with 8 relatively vigorous monohaploid clones. In all fusion combinations the monohaploid 7322 was one of the fusion partners, because (i) protoplasts of monohaploid 7322 could not divide under the standard culture conditions used, and (ii) 7322 could be distinguished from the other monohaploids by its banding pattern for at least one of the isozymes used. In Table 5, the protoplast culture results of the monohaploids in monoculture (controls) and after fusion with 7322 are shown. From six out of the seven combinations plants were obtained; in three out of these six, plant regeneration did not occur in monoculture. It is apparent that in the combinations 7322 with 849-30 and 8522-9 plant regeneration occurred much sooner than in the monocultures.

Table 2. Data showing the greenhouse and in vitro performance of five different diploid clones and their monohaploid derivatives. *N* = number of monohaploid genotypes. The greenhouse data are the means of two measurements carried out during one season; the in vitro data are the means of three measurements carried out during two years of shoot tip propagation; standard deviations in parentheses. r = Coefficient of correlation for greenhouse and in vitro performance of individual monohaploids; p = one-sided critical level

Diploid source	*N*	Greenhouse			In vitro			Coeff. of correlation
		Max.	Min.	Mean	Max.	Min.	Mean	
M9	–			14			8	
M9	36	9	1	4.9 (2.4)	6	2	3.6 (1.5)	r = 0.11; p = 0.39
PD23-2	–			10			2	
PD23-2	52	9	1	5.2 (1.9)	7	2	3.7 (1.3)	r = 0.03; p = 0.42
PD23-26	–			10			3	
PD23-26	16	9	1	5.0 (2.7)	9	2	4.2 (1.6)	r = 0.31; p = 0.13
PD23-28	–			10			3	
PD23-28	7	8	1	4.1 (3.2)	4	2	3.0 (0.8)	r = 0.26; p = 0.21
LGM-1	–			14			no data	
LGM-1	4	3	1	2.0 (0.8)	9	3	6.3 (2.5)	r = 0.16; p = 0.42
Total	115			4.9 (2.2)			3.8 (1.4)	r = 0.00; p = 0.48

Table 3. Genotype comparison for protoplast culture ability of five diploid clones and their monohaploid derivatives. *N* = number of monohaploids; P = mean relative in vitro performance of the monohaploids. Standard deviations in parentheses. [a] Callus growth was classified from 0 to 4. n.d. = Not determined

Diploid source	P	*N*	Protoplast yield ($\times 10^5$)	Plating efficiency ($\times 10^{-3}$)	Callus growth[a]	No. of regenerants
M9	–	–	5	3.0	3	1
M9	4.3	7	7.7 (3.1)	1.5 (1.5)	2.9 (1.1)	3
PD23-2		–	15	1.0	3	–
PD23-2	3.7	52	7.7 (4.2)	1.5 (1.5)	2.8 (1.0)	6
PD23-26		–	11	1.0	3	–
PD23-26	5.0	6	9.2 (3.8)	0.5 (0.5)	2.4 (0.9)	–
LGM-1		–	n.d.	n.d.	n.d.	n.d.
LGM-1	6.3	4	7.5 (5.3)	0.5 (0.5)	2.3 (1.5)	–

The ploidy level of the plants regenerated from monocultures of different monohaploids was 4x or 4x and 2x. Also with 7322, cultured under other conditions (see footnote Table 5), only tetraploid regenerants were obtained. Some of the regenerants were screened for isozyme banding pattern but did not show any differences with the original monohaploid parent.

The ploidy level of the regenerants of five different fusion combinations was 4x, but from the combination 855-2 with 7322 also 3x regenerants were found (Table 5). The latter must have originated from either homo- or heterofusion.

Isozyme analyses of all first regenerants from the five combinations mentioned showed the hybrid banding pattern. For the combinations 7322 with 8522-9 and 855-2 also homozygous regenerants were found, after 17 and 19 weeks, respectively. Calli tested for banding pattern for 6 PGDH also showed the homozygous banding type. In no fusion experiment homozygous 7322 calli or regenerants could be obtained. In Fig. 3 the determination of the hybrid character of a regenerated plant in two particular combinations compared with that of the parents is shown. Morphological determination of a sample of the regenerants of the fusion 7322 with 849-30 and 8522-9 showed that all regenerants had intermediate leaf hairiness and flower colour; all showed a better greenhouse growth performance and male fertility than the homozygous tetraploid counterparts.

Discussion

Uijtewaal et al. (1987a) already pointed out for greenhouse plant vigour that for obtaining vigorous monohaploids not only the monohaploid production ability of a diploid, but also the genetic basis of its vigour is of importance. In other words, a vigorous diploid is not

Table 4. Coefficients of correlation (r) between different levels of plant and cell growth calculated for 52 monohaploids originating from the diploid clone PD23-2. n = Number of plants; p = one-sided critical level. Growth type and callus type classification as shown in Fig. 1 and 2, respectively

	1) Growth in vivo	2) Growth in vitro	3) Growth type	4) Yield protopl.	5) Plating eff.	6) Callus type	7) Callus growth
2)	r = 0.03 (n = 52) p = 0.42						
3)	r = 0.01 (n = 52) p = 0.48	r = 0.74 (n = 52) p = 0.00					
4)	r = 0.07 (n = 52) p = 0.32	r = −0.09 (n = 52) p = 0.25	r = −0.02 (n = 52) p = 0.43				
5)	r = −0.05 (n = 49) p = 0.37	r = 0.66 (n = 49) p = 0.33	r = −0.09 (n = 49) p = 0.27	r = 0.29 (n = 49) p = 0.02			
6)	r = −0.06 (n = 44) p = 0.35	r = 0.19 (n = 44) p = 0.11	r = 0.22 (n = 44) p = 0.08	r = −0.08 (n = 44) p = 0.31	r = −0.39 (n = 44) p = 0.01		
7)	r = 0.11 (n = 42) p = 0.25	r = 0.31 (n = 42) p = 0.02	r = 0.42 (n = 42) p = 0.03	r = −0.08 (n = 42) p = 0.31	r = −0.12 (n = 42) p = 0.23	r = 0.34 (n = 42) p = 0.02	

Table 5. Data of 8 monohaploid clones for callus growth (scale 1–9), shoot regeneration time in weeks (regen.) and ploidy level of the regenerants (ploidy), in protoplast mono-cultures and after protoplast fusion. n = Number of regenerants

Clone	Monoculture				After fusion with 7322			
	callus	regen.	ploidy	n	callus	regen.	ploidy	n
7322	0	24[a]	4x[a]	10	–	–	–	–
839-79	4	30	4x	10	3	0	–	–
849-30	3	25	2x/4x	10	3	12	4x	20
855-1	1	0	–	–	4	19	4x	2
855-2	3	0	–	–	3	16	3x/4x	4
855-3	1	0	–	–	3	16	4x	8
8522-9	3	14	2x/4x	10	4	7	4x	20
851-22	4	24	2x/4x	10	3	20	4x	4

[a] Protoplasts of 7322 did not start first cell divisions under normal auxin concentrations (0.2 mg/l 2,4 D and 1 mg/l NAA) but did divide in the presence of high auxin concentrations (0.2 mg/l 2.4 D and 6 mg/l NAA) during the first 12 days of culture until a multicellular stage was obtained. After that, the high auxin concentration started to become toxic and had to be lowered to a concentration as used for the other genotypes after 12 days of culture (0.1 mg/l 2,4 D and 0.5 mg/l NAA). Callus culture and plant regeneration were carried out under the same conditions as used for the other genotypes

always able to produce relatively vigorous monohaploids when its vigour is mainly based on heterozygosity. In this paper it is shown that it also holds true for in vitro plant growth (Table 2). Furthermore no correlation was found between relative performance of greenhouse grown and in vitro grown plants. This has implications for the technique to be used for monohaploid production. Such a consideration should be based, not only on practical considerations, but also on the research objectives with the monohaploids obtained. So, it may be better to use the prickle pollination technique (in vivo) when practical breeding or gene dosage research with homozygous plants is the main objective. On the other hand, it may be recommendable to use the anther culture technique to produce monohaploids for in vitro research. In both cases it will be better to use both techniques in order to obtain monohaploids from sufficiently various diploid breeding material.

The fact that no correlation could be detected between in vivo plant growth, in vitro plant growth and the yield of protoplasts per gram plant material, and only weak correlations were found between the other

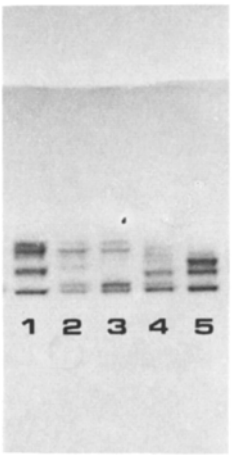

Fig. 3. 6PGDH banding patterns of parental clones and regenerants after protoplast fusion. 1 = 849-30; 2 = 7322 + 849-30; 3 = 7322; 4 = 7322 + 8522-9; 5 = 8522-9

in vitro growth characteristics underlines the unpredictability of these characters. The weak correlation found between in vitro plant growth and callus growth ($r = 0.31$; $P = 0.02$) and between plant growth type and callus growth ($r = 0.42$; $P = 0.03$) suggests that anther culture, which includes a callus phase, may imply a positive selection for in vitro performance. The weak positive correlation between the yield of protoplasts per gram plant material and the plating efficiency ($r = 0.29$; $P = 0.02$) may be genotypically determined but may also be a result of some co-protection or mutual stimulation during the protoplast isolation procedure. The weak negative correlation between plating efficiency and callus growth type (= compactness) suggests that the latter is a purely environmental effect based on mutual competition in the first weeks of culture. However, there appeared to be a weak positive correlation between the compactness of the calli and the callus growth rate. The lack of a negative correlation between plating efficiency and callus growth rate excludes the possibility that the callus growth rate is purely an effect of the lower number of calli developing in the first weeks of culture.

The protoplast fusion experiments indicated that only in some genotype combinations increased callus growth rates were found. However, it is not clear whether such calli were hybrids or not.

It has already been mentioned in literature that starting with monohaploid material the majority of explant or protoplast derived shoots are diploid or tetraploid and only rarely octoploid (Karp et al. 1984; Tempelaar et al. 1985; Sree Ramulu et al. 1986). This suggests that the diploid and the tetraploid level are near to optimal for plant regeneration under the culture and regeneration conditions applied. Probably this is also the case for the material and culture conditions discussed here. The in vitro culture period was probably too long to enable monohaploid regeneration.

This may also explain the regeneration of mostly tetraploid plants after protoplast fusion. Presuming, in the population of freshly isolated protoplasts, a ratio 1C:2C of 2:1 with a neglectable percentage of 4C (Uijtewaal 1987) and an equal chance to take part in fusion for both, the number of C+C fusions will be four times higher than the number of 2C+2C fusions. This suggests that at least part of the tetraploid hybrids are the result of somatically doubled C+C fusion products. The appearence of some triploid regenerants, probably originating from 2C+C fusion products, suggests that the triploid level is stable enough to pass the callus phase without doubling. The only possibility to check for the occurrence of multiple fusions (C+C+C or C+C+C+C) to result in triploids or tetraploids is to use three or more fusion partners, all with a different dominant marker, presuming the regeneration percentage is high enough to detect the multiple hybrids. In these experiments the majority of the fusion products appeared to be hybrids. This is remarkable since no such reports are known from literature. The fact that homozygous regenerants occurred later than the heterozygous hybrids and that a lot of calli tested showed the homozygous 6PGDH banding pattern suggests that after fusion the regeneration capacity of homozygous clones is influenced more negatively than that of heterozygous hybrids. This seems likely since homofusions carried out in other experiments showed a microcallus development comparable to that of unfused controls, but showed less and delayed plant regeneration.

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