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Protoplast Fusion in Sugar Beet (*Beta vulgaris* L.)

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Abstract: In this study, attempts were made to fuse the suspension-derived protoplasts of a sugar beet breeding line (ÇBM315) with the mesophyll-derived protoplasts of three breeding lines (M114, ELK345 and M1017), which were obtained from our previous experiments. Combinations of three different PEG concentrations (20%, 25% or 30%) and three different treatment durations (15, 20 or 25 min) were tested. The fusion efficiency was determined by measuring the viability, heterokaryon frequency and plating efficiency (PE) of the fused protoplasts after four weeks of culture. In general, 25% PEG combined with 20 min treatment duration produced the highest fusion frequency, but the highest rate of viability was obtained at 20% PEG with 25 min treatment duration. The capacity of heterokaryons to form cell colonies was highest when lower PEG (20 or 25%) was used for shorter treatment durations (15 or 20 min). No macrocallus or regeneration from the fused protoplasts was achieved.

Key Words: Sugar beet, protoplast fusion, PEG, breeding lines

Şeker Pancarında (*Beta vulgaris* L.) Protoplast Füzyonu

Özet: Bu araştırmada, daha önceki denemelerimizden elde edilen ÇBM315 adlı şeker pancarı ıslah hattına ait süspansiyon-kökenli protoplastlar ile yine üç şeker pancarı ıslah hattına (M114, ELK345 ve M1017) ait mezofil-kökenli protoplastların füzyonuna çalışılmıştır. Üç farklı PEG (%20, %25 ve %30) ile üç farklı uygulama süresinin (15, 20 veya 25 dakika) değişik kombinasyonları denenmiştir. Füzyon etkinliği; canlılık, hetereokaryon sıklığı ve füzyona uğrayan protoplastların PE (plating efficiency) değerlerinin dört haftalık kültürden sonra ölçülmesi ile belirlenmiştir. Genel olarak, %25 PEG ile 20 dakikalık uygulama süresinin kombinasyonunda en yüksek füzyon sıklığı elde edilmiş fakat en yüksek canlılık oranı %20 PEG ile 25 dakikalık uygulama süresi sonunda elde edilmiştir. Hetereokaryonların hücre kolonisi oluşturma kapasitesi, düşük PEG düzeylerinin (%20 veya %25) kısa süreli uygulamalarla (15 veya 20 dakika) kombine edilmesi halinde daha yüksek olmuştur. Füzyona uğrayan protoplastlardan makrokallus veya bitki rejenerasyonu ise elde edilememiştir.

Anahtar Sözcükler: Şeker pancarı, protoplast füzyonu, PEG, ıslah hatları

Introduction

Transformation techniques are valuable tools for genetic engineers because they allow the introduction of specific genes into plants. At the same time, this general strategy offers little immediate hope for the manipulation of polygenic traits because even if all the component genes influencing a quantitative character could be identified and isolated a large amount of DNA would have to be transferred and the individual genes presumably would have to be expressed in a coordinated manner. Protoplast technology has provided an alternative approach to this problem, in the non-sexual fusion of both nucleated protoplasts and enucleate cytoplasts, to generate hybrid cells with novel combinations of both nuclear and cytoplasmic genes. Interest in this technique stems from a gained ability either to overcome the limitations of conventional breeding (e.g. natural crossing barriers) or to provide a more rapid alternative to traditional breeding methods (1).

Cell to cell fusion is possible by using either electrical fusion or chemical fusions induced by (a) sodium nitrate, (b) high pH-high Ca^{+2}, (c) polyethylene glycol (PEG) or (d) a combination of (b) and (c) (2,3). With these techniques, protoplasts are first brought into contact, after which their plasma membranes can be induced to fuse together to form ultimately a single cell. In sugar beet, high fusion

frequencies can be obtained using electrical methods, though with a definite detrimental effect on subsequent cell development in certain materials. In contrast, PEG fusion techniques had a stimulatory effect on plating efficiencies. Using this method, approximately 5% of the cells available for plating out were found to be heterokaryon products (1). When PEG is diluted at high pH in the presence of a high concentration of Ca^{+2}, a relatively high frequency of fused protoplasts can be obtained (4).

The identification and selection of hybrids from a background of like parental (homocaryotic) fusion products can be carried out at early stages *in vitro*, such as by metabolic complementation, fluorescence-activated cell sorting or identification of molecular markers or at a whole plant level, where morphological, biochemical and molecular markers of hybridity can be employed (5). Narasimulu et al. (6) identified the hybrids of *Brassica carinata* and *Camelina sativa* by looking at their morphology (the hybrid leaves combine features of both parental species), cytology (leaf tip mitosis of two hybrid shoots was carried out to determine the chromosome number of the hybrid, which is the sum of its parental chromosomal constitution) and molecular analysis (RFLP was used in the molecular confirmation of the hybrid nature of regenerated shoots as well as the origin of their organelles).

With the aim of developing a reliable method for protoplast fusion in several sugar beet varieties, we attempted to fuse the suspension-derived protoplasts of a sugar beet line (ÇBM315) with the mesophyll-derived protoplasts of three lines (M114, ELK345 and M1017), which were obtained from our previous experiments (7). The fusion efficiency was determined by measuring the viability, heterokaryon frequency and PE of the fused protoplasts after four weeks of culture. The described protocol will then be employed in our on-going breeding studies in sugar beet.

Materials and Methods

The whole process of protoplast fusion initiation, PEG treatment, incubation time, treatment of high pH-high Ca^{2+}, washing steps and culture conditions are summarized in Table 1. Suspension-derived protoplasts of line ÇBM315 and mesophyll-derived protoplasts of lines M114, ELK345 and M1071 were isolated as described previously (7). A combination of PEG and high pH-high Ca^{2+} methods (2) was applied for protoplast fusions. The contents of the PEG solution were PEG 6000 (at 20, 25 or 30%), 4% sucrose and 0.147% $CaCl_2.2H_2O$. The PEG solution was autoclaved for 15 min and pH was adjusted to 5.7 before use. The high pH-high Ca^{2+} solution contained 0.375% glycine, 5.4% glucose

Table 1. An experimental outline for the polyethylene glycol (PEG) fusion method.

Stage	Process
1. Initiation	Addition of equal volume of suspension (colourless) and mesophyll (green) protoplasts from each parent
	↓
2. PEG treatment	Addition of 300 µl PEG solution dropwise into the tube
	↓
3. Incubation time	Leaving undisturbed for 15, 20 and 25 min
	↓
4. High pH-Ca^{2+}	Addition of 800 ml high pH-Ca^{2+} solution from one side of the tube
	↓
5. Incubation time	Leaving undisturbed for 20 min
	↓
6. First washing	Addition of washing medium A and centrifugation at 700 rpm for 5 min
	↓
7. Second washing	Addition of washing medium A and centrifugation at 700 rpm for 5 min
	↓
8. Third washing	Addition of washing medium B and centrifugation at 700 rpm for 5 min
	↓
9. Culture	Resuspending in liquid MS medium containing 0.23 mg/l BAP and cultured on agarose medium as 0.1 ml drops

and 0.735% $CaCl_2.2H_2O$. This solution was filter-sterilised and pH was adjusted to 10.5 before use. Fusion experiments were carried out in test tubes at room temperature (ca. 22ºC) under sterile conditions.

Isolated protoplasts were suspended in CPW (10x) solution containing 13% mannitol to yield a density of $3x10^5$ protoplasts ml^{-1}. Equal volumes of suspension- and mesophyll-derived protoplasts were gently mixed in a centrifuge tube (i.e., to make mixtures of ÇBM315+M114, ÇBM315+ELK345 and ÇBM315+M1017) and then 300 µl PEG solution was introduced dropwise and left undisturbed during incubations of 15, 20 and 25 min. After the incubation period, 800 µl high pH-high Ca^{2+} solution was added to the mixture along the sides of the tubes and left again undisturbed for 20 min. The content of the tubes was gently shaken before adding 8 ml Washing Medium A (Table 2) in order to remove the fusion solutions (i.e., PEG and high pH-high Ca^{2+}) by centrifuging at 700 rpm for 5 min. This washing step was repeated once more and followed by a final wash using Washing Medium B (Table 2). The protoplasts were then transferred to liquid MS (Murashige and Skoog, 1962) medium containing 0.23 mg/l BAP and 3% sucrose. Samples were taken for the determination of viability of protoplasts (%), and finally, 0.1 ml drops of the liquid medium containing the mixed protoplasts were transferred onto MS medium supplemented with 0.6% agarose, 0.23 mg/l BAP and 3% sucrose and incubated in the dark at 27 ± 2 ºC. Heterokaryon products were identified by the presence of green chloroplasts from mesophyll and cytoplasmic strands from suspension protoplasts (colourless). Using three samples for each treatment (0.1 ml), the frequency (%) of heterokaryons was determined after 12-16 h of incubation, and the percentages of protoplasts which developed into cell colonies (i.e., plating efficiencies, PEs) were recorded after 4 weeks of incubation in culture medium.

Table 2. Contents of the preplasmolysis solution and two different washing media used for protoplast isolation.

Washing Medium A: (pH 5.8) (Filter sterilised)	Mannitol (9%) npG (0.1 mM) CPW salts
Washing Medium B: (pH 5.8) (Filter sterilised)	Sucrose (15%) npG (0.1 mM) CPW salts

Results

Viability of Protoplasts During Fusion

Different combinations of protoplast mixtures behaved differently in their responses to varying incubation times and PEG concentrations. The mixture of protoplasts from ÇBM315+M114 produced the highest viability rate when protoplasts were treated with 20% PEG (82.3% viability). For treatment durations, 20 min produced the highest viability rate (82.4%) and was significantly different from the 15 and 25 min treatment durations (Table 3). For the mixture of protoplasts from ÇBM315+ELK345, 20% PEG was significantly more effective than 25% and 30% PEG for the production of viable protoplasts. For example, viability was 84.3% in 20% PEG treatment while it was 82.6% in 30% PEG treatment. In terms of treatment durations, 15 and 25 min resulted in higher rates of viability than 20 min. In the mixture of ÇBM315+M1017, 25% PEG treatment was found to be more effective on viability (90.3%) than 20% and 25% PEG concentrations. The highest viability rate (90.5%) was obtained from 25 min incubation when compared to 15 and 20 min (Table 3). It was also evident that the combination of ÇBM315+M1017 produced the highest rate of viability (88.6%) - while others produced 82.4% and 80.4% - when the overall means of all treatments were taken into account. For example, viability varied from 80.4% in ÇBM315+M114 to 88.6% in ÇBM315+M1017. Likewise, when different treatment durations and PEG concentrations were compared in terms of their overall means (Table 4), the combination of 20% PEG (84.6% viability) and 25 min (85.5% viability) treatment duration was superior to the treatment of higher concentrations of PEG (25% and 30%) and the lower treatment time durations (15 and 20 min). However, it should be considered that the optimum combination of the treatments could vary since the interactions between parameters (line, level of PEG and incubation durations) were found to be significant (Table 5).

Fusion of Protoplasts

Heterokaryon products could be identified by the presence of green chloroplasts of mesophyll-derived protoplasts and cytoplasmic strands of the suspension-derived protoplasts (Figure 1a). Since homofusions could not be identified by visual observations, only heterokaryons could be scored. Most of the heterokaryons involved two protoplasts only (Figure 1a) but, in some cases, as many as five protoplasts could be fused (Figure 1b).

Table 3. Effects of different concentrations and treatment durations of PEG on % viability of protoplast mixtures during fusion. Means followed by the same letter are not significantly different at $p \leq 0.05$.

% Viability Mixtures	PEG (%)	% Viability 15 min	% Viability 20 min	% Viability 25 min	Means of PEG[2]	Means of Lines[3]
ÇBM315+M114	20	74.5 ± 0.3	89.3 ± 0.3	83.3 ± 0.2	82.3 a	
	25	78.3 ± 0.3	76.4 ± 0.2	78.4 ± 0.6	77.5 b	
	30	83.8 ± 2.1	84.7 ± 2.3	81.8 ± 0.5	81.5 a	80.4 c
	Means[1]	78.0 c	82.4 a	80.8 b		
ÇBM315+ELK345	20	74.1 ± 0.1	87.8 ± 0.3	91.1 ± 0.1	84.3 a	
	25	88.9 ± 0.1	72.5 ± 0.3	79.1 ± 0.1	80.2 c	
	30	87.3 ± 0.4	79.4 ± 0.2	81.1 ± 0.1	82.6 b	82.4 b
	Means[1]	83.5 a	79.9 b	83.8 a		
ÇBM315+M1017	20	88.7 ± 0.2	86.3 ± 0.6	86.6 ± 0.5	87.2 c	
	25	91.0 ± 0.1	86.7 ± 0.5	93.3 ± 0.2	90.3 a	
	30	84.1 ± 0.2	89.0 ± 0.2	91.7 ± 0.6	88.3 b	88.6 a
	Means[1]	87.9 b	87.4 b	90.5 a		

[1] Means of treatment durations
[2] Means of PEG concentrations
[3] Means of breeding lines

Table 4. Overall comparisons of different PEG concentrations and treatment durations of PEG on % viability of protoplasts. Values are the means of all protoplast mixtures regardless of genotypes, and means followed by the same letter are not significantly different at $p \leq 0.05$.

PEG (%)	% Viability 15 min	% Viability 20 min	% Viability 25 min	Means
20	79.0	87.7	87.1	84.6 a
25	86.1	78.5	83.4	82.7 c
30	84.3	83.5	84.7	84.1 b
Means	83.1 b	83.2 b	85.0 a	

Table 5. Analysis of variance for viability (%) of fused protoplasts.

Source	DF	Mean Squares
Replication	2	1.49
Line	2	493.94**
PEG	2	27.62**
Time	2	47.45**
Line x PEG	4	31.01**
Line x Time	4	39.85**
PEG x Time	4	158.61**
Error	60	11.3

** Significant at $p \leq 0.01$

Heterokaryon protoplast frequencies were significantly higher in the protoplasts derived from ÇBM315+ELK345 (49.8%) as well as from ÇBM315+M114 (46.2%) than in the protoplasts from ÇBM315+M1017 (10.8%) (Table 6). In the mixtures of ÇBM315+M114 and ÇBM315+M1017, 25% PEG was superior to other concentrations (20% and 30%) while in the mixture of ÇBM315+ELK345, 30% PEG produced more viable heterokaryon protoplasts than other PEG concentrations. In terms of the treatment durations, 20 min treatment period yielded 54.7% viable heterokaryon protoplasts in ÇBM315+M114 and 54.4% in ÇBM315+ELK345, whereas the mixture of ÇBM315+M1017 produced more heterokaryons (12.8%) at 25 min than at 15 min (9.7%) or 20 min (9.8%), although the heterokaryon frequency was already significantly lower in this mixture than in the other two. When the overall means were considered, 25% PEG and 20 min treatment duration were the most suitable combination for induction of viable heterokaryon protoplasts in sugar beet (Table 7). However, since the interactions between the parameters (i.e., line x PEG, line x time, PEG x time) were significant, the best combination of the treatments can be expected to vary from genotype to genotype or from lower concentrations of PEG to higher and from shorter to longer treatment durations (Table 8).

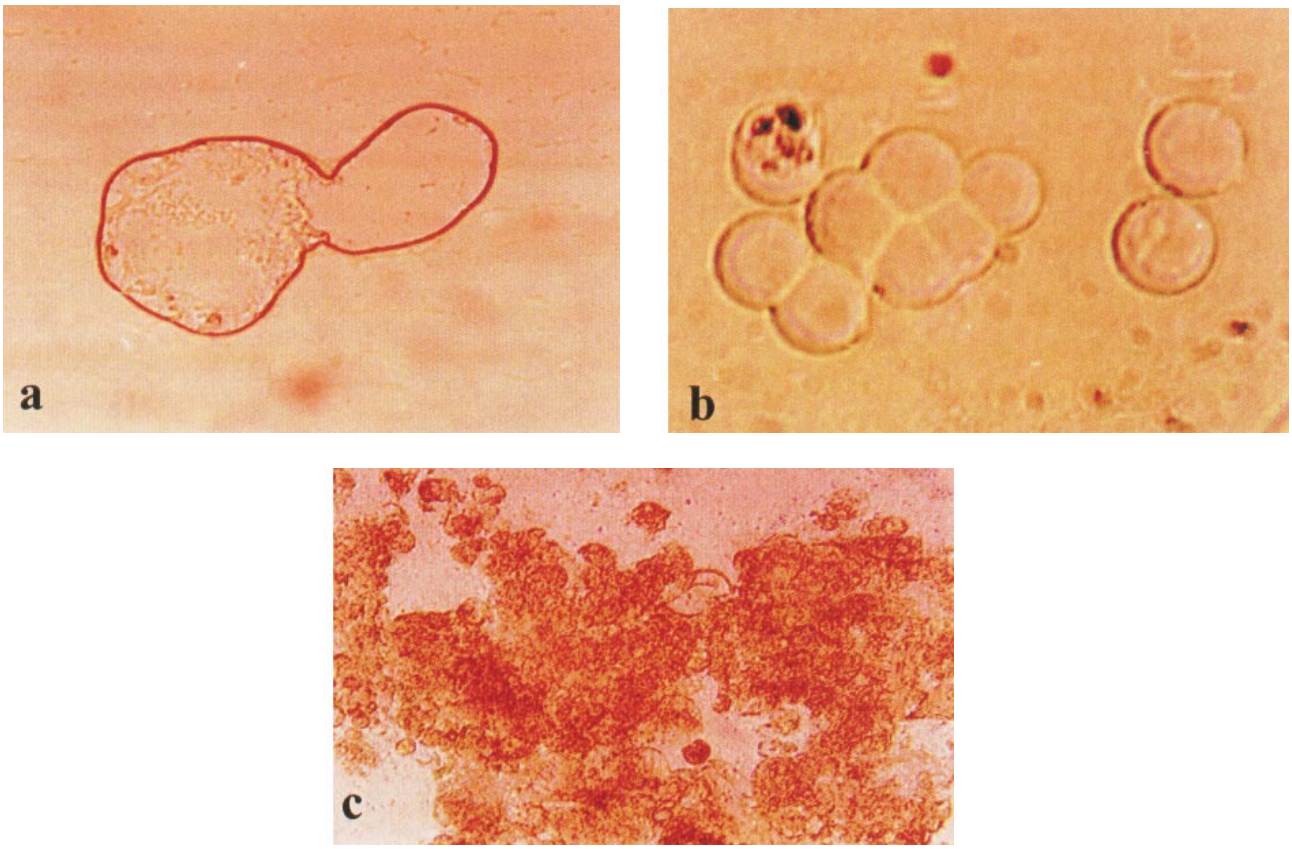

Figure 1. a) A typical heterokaryon involving a mesophyll-derived protoplast of line M114 (left) and a suspension-derived protoplast of line ÇBM315 (right) (x1500). b) An unusual heterokaryon involving more than two protoplasts (x800). c) Cell colonies developing from the fused protoplasts of ÇBM315+M114 after 4 weeks in culture (x800).

Table 6. Effects of different concentrations and treatment durations of PEG on % viability of heterokaryon protoplasts. Means followed by the same letter are not significantly different at $p \leq 0.05$.

Protoplast Mixtures	PEG (%)	% Viability			Means of	
		15 min	20 min	25 min	PEG[2]	Lines[3]
ÇBM315+M114	20	27.9 ± 0.1	58.8 ± 0.1	65.1 ± 0.2	50.6 b	
	25	44.6 ± 0.2	59.8 ± 0.3	50.0 ± 0.6	51.5 a	
	30	31.7 ± 0.3	45.5 ± 0.3	32.6 ± 0.1	36.6 c	46.2 b
	Means[1]	34.8 c	54.7 a	49.2 b		
ÇBM315+ELK345	20	33.5 ± 0.8	54.6 ± 0.3	41.0 ± 0.0	43.0 c	
	25	55.5 ± 0.2	57.5 ± 0.0	45.5 ± 0.3	52.8 b	
	30	61.3 ± 0.2	51.1 ± 0.2	47.9 ± 0.1	53.5 a	49.8 a
	Means[1]	50.1 b	54.4 a	44.8 c		
ÇBM315+M1017	20	7.4 ± 0.0	9.2 ± 0.0	13.0 ± 0.0	9.9 b	
	25	12.1 ± 0.0	10.5 ± 0.1	14.6 ± 0.3	12.4 a	
	30	9.8 ± 0.2	9.5 ± 0.1	11.0 ± 0.0	10.1 b	10.8 c
	Means[1]	9.7 b	9.8 b	12.8 a		

[1] Means of treatment durations
[2] Means of PEG concentrations
[3] Means of breeding lines

Table 7. Overall comparisons of different concentrations and treatment durations of PEG on % viability of heterokaryon protoplasts. Values are the means of all protoplast mixtures regardless of genotypes, and means followed by the same letter are not significantly different at $p \leq 0.05$.

PEG (%)	% Viability 15 min	20 min	25 min	Means
20	22.9	40.9	39.7	34.5 b
25	37.4	42.6	36.7	38.9 a
30	34.3	35.4	30.5	33.4 c
Means	31.5 c	39.6 a	35.6 b	

Table 8. Analysis of variance for viable heterokaryon protoplasts frequency (%).

Source	DF	Mean Squares
Replication	2	1.49
Replication	2	0.793**
Lines	2	12550.489*
PEG	2	229.771**
Time	2	362.478**
Line x PEG	4	441.019**
Line x Time	4	376.277**
PEG x Time	4	309.096**
Error	60	13.1

* Significant at $p \leq 0.05$, ** Significant at $p \leq 0.01$

Table 9. Effects of different concentrations and treatment durations of PEG on % plating efficencies (PEs) of protoplasts following the chemical fusion. Means followed by the same letter are not significantly different at $p \leq 0.05$.

% Viability Mixtures	PEG (%)	% Viability 15 min	20 min	25 min	Means of PEG[2]	Lines[3]
ÇBM315+M114	20	1.9 ± 4.6	2.3 ± 0.7	13.0 ± 1.5	11.5 a	
	25	15.7 ± 3.2	22.0 ± 3.1	4.7 ± 0.7	14.1 a	
	30	3.0 ± 1.0	7.0 ± 1.0	3.0 ± 1.2	4.3 b	10.2 a
	Means[1]	12.6 a	10.4 ab	6.9 b		
ÇBM315+ELK345	20	4.3 ± 2.4	3.0 ± 1.2	4.3 ± 0.7	3.9 a	
	25	2.7 ± 0.3	2.3 ± 0.7	3.0 ± 0.6	2.7 a	
	30	2.3 ± 1.2	3.3 ± 0.9	3.0 ± 1.0	2.9 a	3.2 b
	Means[1]	3.1 a	2.9 a	3.4 a		
ÇBM315+M1017	20	8.3 ± 4.3	4.7 ± 0.3	5.0 ± 1.6	6.0 a	
	25	4.3 ± 2.8	4.3 ± 0.3	3.0 ± 1.0	3.9 a	
	30	4.0 ± 0.0	5.0 ± 1.5	3.7 ± 0.7	4.2 a	4.7 b
	Means[1]	5.6 a	4.7 a	3.9 a		

[1] Means of treatment durations
[2] Means of PEG concentrations
[3] Means of breeding lines

Formation of Cell Colonies

Following the fusion, protoplasts regenerated cell walls and divided further to yield cell colonies. In heterokaryon products, initial cell divisions were noticed after 5-10 days in culture and cell colonies could be observed after 20-25 days of incubation (Figure 1c). Fused protoplasts (heterokaryons) failed to develop into macroscopic colonies when cultured in droplets on agarose-solidified medium containing 0.23 mg/l BAP and 3% sucrose.

The mixture of ÇBM315+M114, 20% and 25% PEG produced greater amounts of cell colonies (11.5% and 14.1%, respectively) (Table 9). For durations, 15 min was more effective than the other durations (20 and 25 min). However, in other combinations of the sugar beet lines used for fusions, there were no significant differences between the levels of PEG or durations. A genotypic variation was again evident as the mixture of ÇBM315+M114 produced significantly more cell colonies (10.2%) than the other two mixtures (2.2 and 4.7%) (Table 9). A comparison of the overall means showed that PEG concentrations lower than 25% and duration times less than 20 min seemed to be more effective for viable heterokaryon colonies (Table 10). These comparisons should, however, be evaluated in line with the significant interactions which occurred among the parameters evaluated, especially line x PEG, and PEG x duration (Table 11).

Table 10. Overall comparisons of different concentrations and treatment durations of PEG on % plating effciencies (PEs) of protoplasts. Values are the means of all protoplast mixtures regardless of genotypes, and means followed by the same letter are not significantly different at $p \leq 0.05$.

PEG (%)	% Viability			Means
	15 min	20 min	25 min	
20	11.1	3.3	7.4	7.3 a
25	7.6	9.6	3.6	6.9 a
30	3.1	5.1	3.2	3.8 b
Means	7.3 a	6.0 ab	4.7 b	

Table 11. Analysis of variance for PE of heterofused protoplasts.

Source	DF	Mean Squares
Replication	2	1.49
Replication	2	1.15
Line	2	364.78**
PEG	2	97.82**
Time	2	42.82**
Line x PEG	4	77.93*
Line x Time	4	25.93*
PEG x Time	4	94.41**
Error	60	15.8

* Significant at $p \leq 0.05$
** Significant at $p \leq 0.01$

Discussion

In principle, protoplast fusion allows us to bring together any desirable plant traits (e.g. disease resistance, salt tolerance and high yield) in combinations not possible by sexual means. In other words, it is a technique which allows natural incompatibility barriers operating at the whole-plant level to be by-passed. Although fragile in comparison with an intact cell wall, protoplast membrane still has a relatively high degree of structural stability (9). Protoplast fusions could be induced by both chemical and electrical methods. Since 1974, PEG has achieved widespread acceptance as a fusogen of plant protoplasts because of the reproducible high frequency heterokaryon formation and low cytoxicity to most cell types (9). Another merit of PEG-induced fusion is the formation of a high proportion of binucleate heterokaryons (10). A few minutes after being immersed in 20-40% (w/v) PEG of molecular weight 1500-6000, virtually all protoplasts exhibit adhesion (3). However, actual fusion occurs upon dilution of the PEG with a high Ca^{2+} and high pH eluting medium, which has been shown to neutralize the normal surface charge thus allowing the membranes of agglutinated protoplasts to come in intimate contact (3).

A higher frequency of heterokaryon after 20 min incubation in PEG was obtained, while longer treatment duration (25 min) reduced the heterokaryon rate significantly. It was also evident that higher concentrations of PEG were inhibitory to the development of heterokaryons since the treatment of protoplast mixtures with 30% PEG reduced the heterokaryon rate significantly. This inverse relationship between heterokaryon frequency and concentration of PEG was clear when viability rates and heterokaryon frequencies were compared in terms of the overall means of PEG and treatment durations. Twenty percent PEG combined with 25 min duration was the best combination for viability rates, while it was the combination of 25% PEG with 20 min duration for heterokaryon frequencies. This finding suggests that higher PEG becomes toxic to the cells as it increases the frequency of protoplast fusion. Our results are supported by Kao et al. (11), who reported that PEG concentration and treatment duration were critical for protoplast fusion. Prolonged incubation in PEG solution reduces heterokaryon formation (10) and increasing the duration of PEG treatment resulted in the reduction of the fusion products (12), which is in agreement with our findings.

Following treatment with PEG, the suspension-derived protoplasts continued to divide, resulting in cell colonies, whereas the mesophyll-derived protoplasts died shortly after treatment. PEG-mediated fusion may also be useful in sugar beet as it eliminates unfused mesophyll protoplasts. Therefore, only one marker would be needed to distinguish between hybrid and parental cell colonies (12).

All of the isolation and culture media used in the present work contained n-propylgallate (nPG) since its promoting effect on sustained divisions and subsequently regeneration from fused protoplasts is known (13). It was postulated that the formation of toxic peroxides by lipegenases is inhibited by nPG, thus reducing oxidative damage to the membrane.

Fusions of suspension- and mesophyll-derived protoplasts failed to develop into macroscopic callus, from which adventive shoots or somatic embryos would have been expected to develop. The known recalcitrance of the genus Beta remains a significant barrier to its further exploitation for biotechnological applications. The reasons, however, remain unknown. It appears that genotypic variation played a significant role and the use of a large number of different materials might be a necessity to achieve not only regeneration from fused cells, but also a obtain heterokaryons at high frequencies. Further research should clarify which type of protoplast is competent for regeneration. A better insight into the precise origin of callus types (micro or macro) and the reasons for their contrasting morphologies would benefit us in determining suitable modifications to enhance plant regeneration from beet protoplasts.

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