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Fractionation of Plant Protoplast Types by Iso-osmotic Density Gradient Centrifugation

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Summary. A simple effective technique for the fractionation of protoplast populations is described. Protoplasts are separated by low-speed centrifugation in an iso-osmotic, discontinuous density gradient system on the basis of differences in their buoyant densities. At a constant osmolality of 660 ± 20 mOs/kg H_2O, the gradients provide a density range from 1.017 to 1.069 g/cm^3 at 20°C which corresponds to the buoyant densities of most protoplast types studied. Characteristics of the KMC/S-density gradient system and factors affecting the fractionation were investigated. Protoplasts were isolated from various tissues and cultivars of tobacco, barley, wheat, rye, oat and maize. Their density-dependent distribution profiles in KMC/S-gradients and their average buoyant densities were determined under standardized conditions. Great differences in the buoyant densities were found between protoplasts of different tissues. Mixed populations of two types of protoplasts, differing in buoyant density by about 15-20 mg/cm^3, were separated to give highly purified fractions. Factors affecting the buoyant densities of protoplasts have been investigated. Ploidy level and species differences did not significantly affect the fractionation profiles. However, an age-dependent variation in the average buoyant density of tobacco mesophyll protoplasts was observed. Fractionation of tobacco mesophyll protoplasts and their subsequent regeneration to plants demonstrates the practicability and physiological compatibility of the KMC/S-density gradient system under sterile conditions. The morphogenetic potential of protoplasts was not affected by the separation procedure or the gradient components.

Key words: Cell sorting – Cereals – Density gradient – Protoplasts – Selection

Introduction

The fractionation of mixtures of cells provides pure fractions for detailed biochemical and cell biological studies. Fractionation and cell sorting methods are now being used routinely in animal cell biology and medicine. The techniques include differential electrophoresis of cells, and numerous gradient procedures, employing either density gradient centrifugation or velocity sedimentation (for review see Harwood 1974; Mel and Ross 1975; Pretlow et al. 1975). No comparable system has been described for the separation of various types of isolated plant cells or protoplasts.

The essential properties of a gradient system designed for protoplast separation are: (1) suitable density range, corresponding to the buoyant density of the protoplasts; (2) iso-osmolality throughout the whole density range; (3) physiological compatibility of the gradient components, not interfering with the viability of the protoplasts, their division capacity and regeneration at any level of development. In the present paper we report on the development of a simple density gradient technique for the fractionation of protoplast populations which meets these basic requirements.

Materials and Methods

Protoplast Isolation

The methods for the isolation of large quantities of viable protoplasts from cereal leaves have been previously described (Lörz and Potrykus 1976). Young leaves of 2 or 6 week old greenhouse-grown plants were used. The following species and cultivars were used: *Avena sativa* cv. 'Tiger', *Hordeum vulgare* cv. 'Dura', 'Vogelsanger Gold' (winter types) and cv. 'Kiebitz', 'Villa', 'Zeisig' (spring types), *Secale cereale* cv. 'Perolo', *Triticum aestivum* cv. 'Diplomat' and *Zea mays* cv. 'Prior'. The same protocol was used for the isolation of mesophyll protoplasts from primary leaves of etiolated seedlings (9 d old, grown at 24°C in a growth chamber) of *Zea mays* cv. 'Prior', *Triticum aestivum* cv. 'Diplomat', and *Hordeum vulgare* cv. 'Dura'. Tobacco protoplasts were isolated from leaves of greenhouse-grown plants of cv. 'Samsun' and the two light-sensitive mutants 'virescent' (v) and 'sublethal' (s) (Melchers and Labib 1974), kindly provided by Professor Melchers. The leaves were cut into thin strips using razor blades (Potrykus 1971) and incubated at 22-25°C in a solution containing 1% Macerozyme R10, 1% cellulase (Onozuka R10, Kinki Yakult, Nishi-

0040-5752/78/0053/0057/$ 1.40

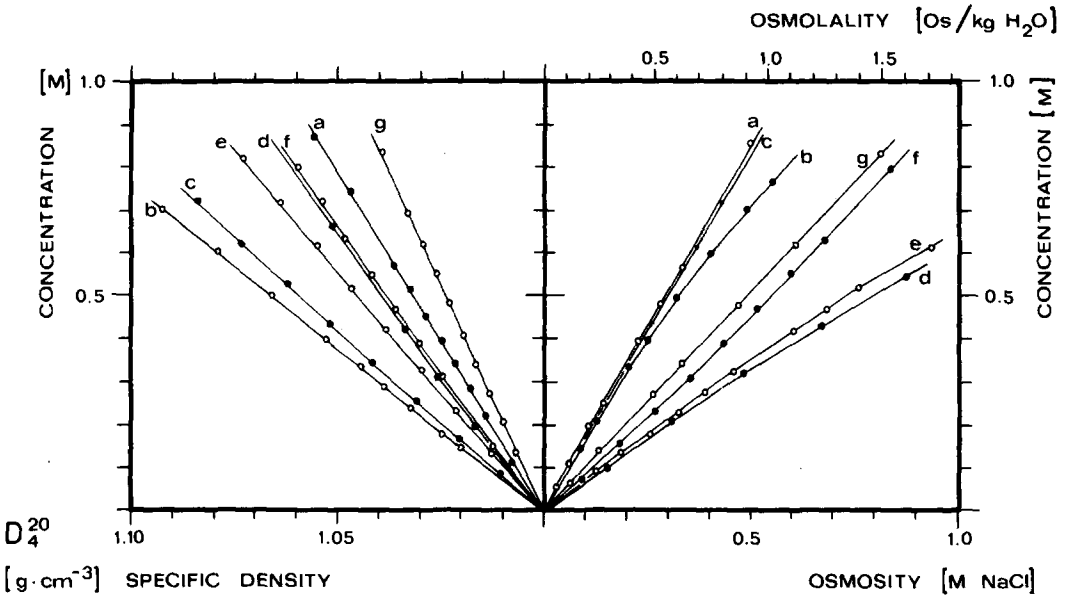


Fig. 1. Graph relating concentrations of aqueous solutions to their specific density at 20° C and osmolality.
left: specific density at 20° C (g/cm³)
right: osmolality (mOs/kg H_2O; upper abscissa)
osmosity (M NaCl; lower abscissa)

a mannitol, sorbitol
b sucrose, maltose
c $MgSO_4 \cdot 7\,H_2O$
d $MgCl_2 \cdot 6\,H_2O$
e $CaCl_2 \cdot 2\,H_2O$
f $(NH_4)_2SO_4$
g KCl

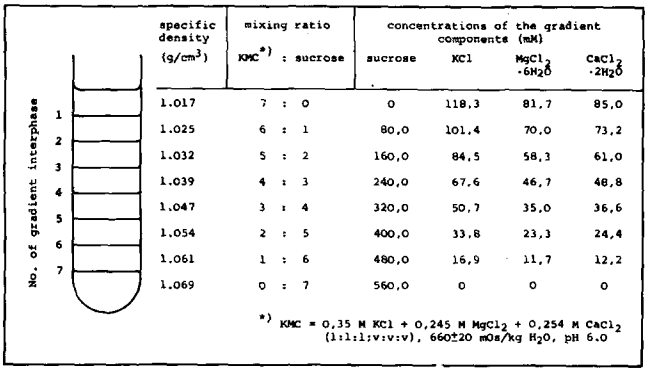
This graph was used to evaluate the concentrations required to give iso-osmotic solutions of gradient components

nomiya/Japan), 0,6 M mannitol and 5 mM $CaCl_2$ of pH 5,8. Alternatively, the enzyme concentrations were reduced by 90% when protoplasts were isolated overnight at 17° C. Epidermis protoplasts were recovered from the enzyme mixture by floating the protoplasts on 0,6 M mannitol/0,56 M sucrose (mixed 3:1) and then washed in 0,25 M $CaCl_2$. Mesophyll protoplasts were sedimented by low-speed centrifugation (50-100 xg). Protoplasts from maize stem internodes were isolated according to the methods described previously (Potrykus et al. 1977). The osmotic pressure of all solutions was determined in a freezing point depression osmometer (Roebling, Berlin) and, if necessary, adjusted to 660 ± 20 mOs/kg H_2O, corresponding to 0,6 M mannitol.

Preparation of Iso-osmotic KMC/S-density Gradients

Iso-osmotic concentrations of the solutions used to prepare the gradients were calculated from the data given in Figure 1 (right side), which were obtained from osmometer measurements. The left side of Figure 1 provides the respective specific density values of these solutions. A KMC solution, consisting of equal volumes of 0,35 M *K*Cl, 0,245 M *Mg*Cl_2 and 0,254 M *Ca*Cl_2 (pH 6,0), was mixed in various ratios with 0,56 M sucrose to produce the gradient media of the KMC/S-density gradient system described here. Mixtures, concentrations and specific densities of the gradient phases are shown in Figure 2. To form discontinuous gradients, 1,5 ml aliquots of the gradient media were layered in glass centrifuge tubes (100 X 13 mm i.d.). Care was taken in layering the gradient phases to prevent mechanical mixing of the layers. Protoplast samples, containing 0,8-5 X 10^5 protoplasts/ml, suspended in KMC solution, were layered as the uppermost phase onto the gradients. Gradients were run for 5-20 min at various centrifugal forces in a Hettich Rotofix II centrifuge equipped with a 4 X 12 ml swinging bucket head.

After fractionation, the protoplasts were carefully pipetted off the gradient interphases using pasteur pipettes. The density-dependent distribution profiles of the protoplasts resulting from their separation in the gradient were determined by counting the number of protoplasts from each of the bands in a haemacytometer. This number is expressed as percentage of the total number of protoplasts re-isolated from the whole gradient.



specific density (g/cm³)	mixing ratio KMC*) : sucrose	concentrations of the gradient components (mM) sucrose	KCl	$MgCl_2 \cdot 6H_2O$	$CaCl_2 \cdot 2H_2O$
1.017	7 : 0	0	118,3	81,7	85,0
1.025	6 : 1	80,0	101,4	70,0	73,2
1.032	5 : 2	160,0	84,5	58,3	61,0
1.039	4 : 3	240,0	67,6	46,7	48,8
1.047	3 : 4	320,0	50,7	35,0	36,6
1.054	2 : 5	400,0	33,8	23,3	24,4
1.061	1 : 6	480,0	16,9	11,7	12,2
1.069	0 : 7	560,0	0	0	0

*) KMC = 0,35 M KCl + 0,245 M $MgCl_2$ + 0,254 M $CaCl_2$ (1:1:1;v:v:v), 660±20 mOs/kg H_2O, pH 6.0

Fig. 2. Composition of the iso-osmotic KMC/S-density gradient: specific densities, mixing ratios and concentrations of the gradient components

Culture of Tobacco Protoplasts after Gradient Separation

To examine the culture of protoplasts separated on KMC/S-density gradients, tobacco protoplasts (cv. 'Samsun'; s; v) were isolated and fractionated under sterile conditions. After gradient fractionation, protoplasts from each of the interphases were collected, washed twice in culture medium (Nagata and Takebe 1971) containing 1,6 × 10^{-6} M p-chlorophenoxy acetic acid (pCPA) and 2,3 × 10^{-5} M kinetin (6-furfuryl-aminopurine). Cultures were kept at 12°C in the dark for 24 h and were then transferred to growth chambers at 25°C, illuminated with Osram L-Natura cool fluorescent light of 500-800 lx 16 h/d. Plants were regenerated from these cultures following the methods of Takebe et al. (1971) and Melchers and Labib (1974).

Results

Some Characteristics of the Iso-osmotic KMC/S-density Gradient System

A specific density increase from one phase to the next of about 8 mg/cm^3 was found optimal for both resolution and practicability reasons (Fig. 2). Quantities of 0,5-5 × 10^5 protoplasts could be fractionated per test tube in a single run. Theoretically the capacity of a continuous gradient at 100 xg should be as high as 8 × 10^6 particles of 20 μm radius (Mason 1976). In practice, and considering the discontinuity of the KMC/S-density gradients, 2-5 × 10^5 protoplasts per gradient were found to give optimal results. Higher concentrations of protoplasts tended to form clumps which, based on Stokes' Law (see Mel and Ross 1975), make fractionation unpredictable.

When carefully pipetted off, up to 90% (mean: 69%) of the protoplasts could be re-isolated from the gradient interphases without mixing the fractions. To demonstrate that complete separation according to the buoyant densities of the protoplasts was accomplished, single fractions were isolated and recentrifuged in a second gradient of the same composition. The photographs of Figure 3 clearly indicate that the separation was complete in the first run since the isolated fractions (interphase 5 in Fig. 3) reband in the same position when centrifuged for a second time (Fig. 3b). Rebanding percentages calculated from 15 experiments were between 85 and 98%.

In some experiments protoplasts were suspended in 0,56 M sucrose and layered at the bottom of the gradients rather than at the top. Gradients of both types were run similtaneously under identical conditions. The density-dependent distribution profiles of the protoplasts were almost identical in both types of gradients. This finding strongly supports the view that fractionation of protoplasts in the KMC/S-density gradient system is due to differences in buoyant density rather than to velocity sedimentation. However the size of the protoplasts, which is an essential factor in velocity sedimentation, also plays a role on the KMC/S-density gradient system. Tobacco mesophyll protoplasts were fractionated and protoplast diameters determined for each fraction. The results given in Figure 4 show that mean protoplast diameters increased as buoyant density decreases.

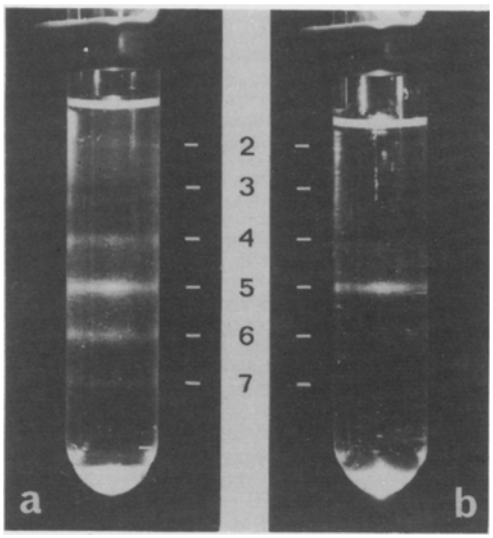


Fig. 3a, b. Reproducibility of the density-dependent fractionation in iso-osmotic KMC/S-density gradients as revealed by the rebanding of a single isolated fraction:
a density-dependent distribution profile of barley mesophyll protoplasts (*Hordeum vulgare* cv. 'Kiebitz')
b interphase 5 of the gradient shown in Fig. 3a was isolated, washed once with KMC solution and recentrifuged in a second KMC/S-density gradient of the same composition

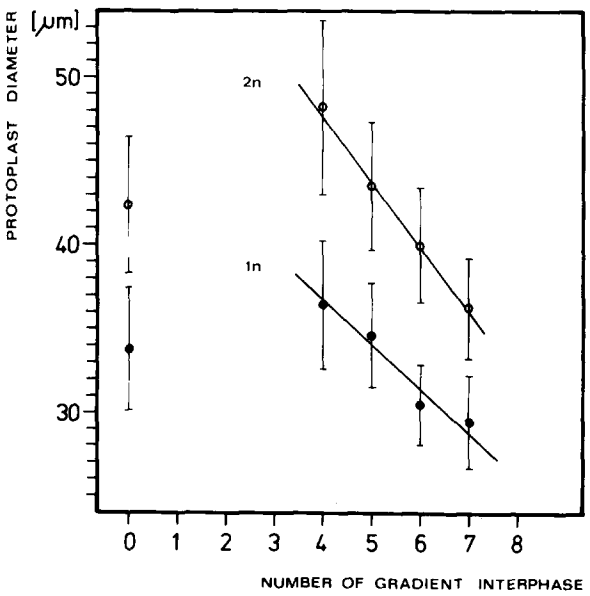


Fig. 4. Relationship between protoplast diameters and gradient position of tobacco protoplasts fractionated in iso-osmotic KMC/S-density gradients (protoplasts from *Nicotiana tabacum* 'sublethal', both amphidiploid and amphihaploid; diameters were determined for 500 protoplasts from each gradient interphase)

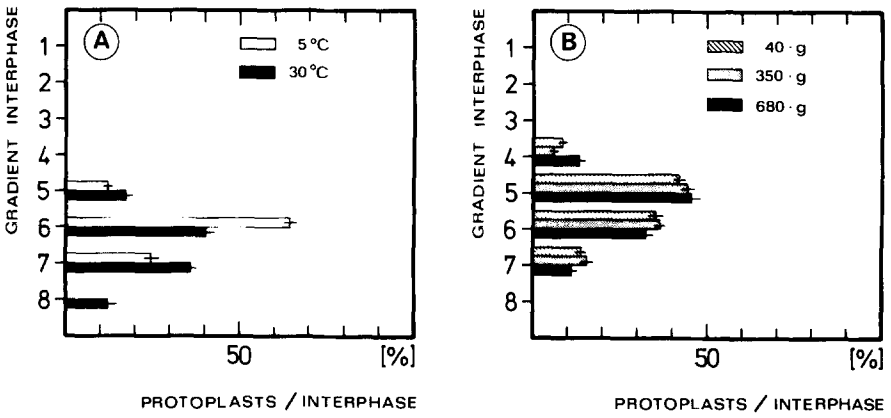


Fig. 5. A and B. Effect of gradient temperature on the density-dependent distribution profiles of tobacco mesophyll protoplasts (cv. 'Samsun', 2n) in iso-osmotic KMC/S-density gradients
B Effect of various centrifugal forces on the density-dependent distribution profiles of barley (cv. 'Dura') mesophyll protoplasts in iso-osmotic KMC/S-density gradients (centrifugal gradients (centrifugal forces given were calculated for geometrical mid of gradients)
abscissa: protoplasts recovered from each interphase as percentage of the total number of protoplasts recovered from the whole gradient; each experiment was repeated at least three times

Factors Affecting Protoplast Fractionation in Iso-osmotic KMC/S-density Gradients

The effects of various centrifugal forces, of the gradient temperature and of the duration of the run on protoplast fractionation have been studied. Figure 5 summarizes some results. As can be seen from the different distribution profiles in Figure 5A, there was a considerable effect of gradient temperature upon resolution and the quality of distribution in KMC/S-density gradients. At 30°C gradient temperature the protoplast distribution profiles broadened over a wider range of densities and the protoplasts also moved further towards zones of seemingly higher densities. There are two main reasons for this phenomenon. Firstly, the specific densities of the gradient phases decrease by about 1,8 mg/cm^3 when the temperature increases from 4°C to 20°C, and they are even lower at 30°C. Therefore, the protoplasts had to move further to find their adequate positions in the density gradient. Secondly, higher diffusion rates reduce the density increment at the interphases and thus resulted in disturbance of the interphases.

When KMC/S-gradients were run at centrifugal forces between 20 and 1000 xg, the density-dependent distribution profiles of the protoplasts remained constant, as is shown in Figure 5B, for the fractionation of barley mesophyll protoplasts at 40 xg, 350 xg, and 680 xg. In these experiments the spinning gradients were illuminated with stroboscopic light (Ministrob 2000, Bamberg & Bormann Electronic, Neheim-Hüsten) to allow direct observation of the fractionation process *in situ*. It was obvious from these observations that fractionation of the protoplasts was completed after 2-4 min at 50-100 xg. The protoplast distribution profile present at this time did not change upon further centrifugation for 10 min, thus indicating the density dependence of the fractionation achieved in these gradients. Later, diffusion and perturbation tended to destabilize the profiles. Based on these findings, the routine procedure in further experiments was centrifugation for 5 min at 100 xg.

Factors Affecting the Buoyant Densities of Protoplasts

Density-dependent distribution profiles and average buoyant densities have been determined, under standardized conditions, for a series of protoplast populations. Results are summarized in Table 1. The standard conditions used were: KMC/S-density gradient, iso-osmotic at 660 ± 20 mOs/kg H_2O, 20°C, centrifugation for 5 min at 100 xg and a protoplast load of 2-4 × 10^5 protoplasts per gradient. The figures given are representative of at least three gradients run separately; two samples of protoplasts from each interphase were counted. Both the density-dependent distribution profiles and the average buoyant densities indicate only minor variations between mesophyll protoplasts isolated from a wide range of species. No differences were observed between the density-dependent distribution profiles of several barley cultivars. Moreover, the profiles of mesophyll protoplasts from spring and winter types of barley were essentially the same. Different ploidy levels did not account for differences in buoyant density or in the density distribution profiles. This was demonstrated with mesophyll protoplasts from amphidiploid and amphihaploid tobacco (s) (Table 1). Since haploid and diploid protoplasts have significantly different sizes (see Fig. 4), the fact that they show essentially congruent density distribution profiles once more indicates

Table 1. Density-dependent distribution profiles and average buoyant densities of various protoplast types in iso-osmotic KMC/S-density gradients under standardized conditions (660 ± 20 mOs/kg H_2O, 20°C, centrifugation for 5 min at approx. 100 xg, protoplast load 2-4 X 10^5 protoplasts per gradient)

	Mesophyll							Etoilated primary leaf			Stem	Epidermis	
Species	Avena sativa cv. Tiger	Hordeum vulgare cv. Dura	Secale cereale cv. Perolo	Triticum aestivum cv. Diplomat	Zea mays cv. Prior	Nicotiana tabacum 'sublethal' (2n)	Nicotiana tabacum 'sublethal' (1n)	Hordeum vulgare cv. Dura	Triticum aestivum cv. Diplomat	Zea mays cv. Prior	Zea mays cv. Prior	Nicotiana tabacum 'virescent' (2n)	Nicotiana tabacum 'virescent' (1n)
No. of gradient interphase 1	--	--	--	--	--	--	--	--	--	--	--	--	--
2	--	--	--	--	--	--	--	--	--	--	21±7	40±6	18±4
3	--	--	--	--	--	--	--	25±3	15±2	15±4	72±6	21±5	45±3
4	5±4	22±7	12±6	9±3	4±2	8±4	12±4	52±4	46±4	37±3	7±3	26±4	29±6
5	35±4	45±7	34±4	35±3	21±4	65±7	52±6	23±3	39±4	30±3	--	13±4	8±4
6	39±4	25±6	36±4	40±4	48±5	22±3	30±4	--	--	18±4	--	--	--
7	21±4	8±5	18±6	16±6	27±4	5±3	6±4	--	--	--	--	--	--
Average buoyant density (g/cm³)	1.056	1.051	1.054	1.055	1.057	1.052	1.052	1.043	1.045	1.046	1.034	1.029	1.030

the density dependence of the fractionation in iso-osmotic KMC/S-density gradients.

Major differences in the density-dependent distribution profiles and average buoyant densities do, however, exist between protoplasts isolated from different tissues (Table 1). Taking advantage of these differences, protoplast mixtures, i.e. wheat mesophyll mixed with maize internode protoplasts, could be separated into fractions having only minor contamination with protoplasts of the other type. Mesophyll protoplasts isolated from etiolated primary leaves have density-dependent distribution profiles and average buoyant densities intermediate between those of mesophyll and epidermis protoplasts (Table 1), suggesting that the content of functional chloroplasts is a major factor contributing to the buoyant density of protoplasts. Thus, mixed protoplast populations isolated from chimeral leaf tissue could also be fractionated (Harms, unpublished data).

Table 2. Density-dependent distribution profiles and average buoyant densities of protoplasts isolated from leaves of different ages (as represented by their relative position on the plant) (figures represent protoplasts recovered from a particular interphase as percentage of the total number of protoplasts recovered from the whole gradient; the leaves of a greenhouse-grown tobacco cv. 'Samsun' plant were numbered from bottom to top)

	Relative leaf position				
No. of gradient interphase	basal 2.	6.	9.	14.	apical 17.
4	62±5	30±3	22±3	18±3	3±2
5	32±3	47±4	38±4	36±3	34±3
6	7±2	23±3	40±4	46±4	63±5
average buoyant density (g/cm³)	1.046	1.049	1.051	1.052	1.054

To study the effect of the age of the plant material, protoplasts isolated from different leaves of a single tobacco plant were fractionated. Table 2 summarizes the results obtained. The density-dependent distribution profiles indicate a negative correlation between the age (as represented by the relative leaf position) and the average buoyant density of the protoplasts.

Culture and Regeneration of Protoplasts Fractionated in Iso-osmotic KMC/S-density Gradients

To study the effect of the gradient media upon protoplast viability and development, samples of tobacco protoplasts were fractionated under sterile conditions. Protoplasts from each interphase were collected and incubated in their respective gradient phase for 0, 1, 5, and 24 h at 25°C. Thereafter they were washed twice in culture medium and cultured in liquid medium. In all experiments,

the plating efficiency was as high as the controls. Cell wall regeneration and regular cell division were observed after 2 and 3 days, respectively. No loss of viability and vitality was observed in any of the experiments even after 24 h incubation in the gradient media of high salt concentration. Cell colonies from the above experiments were grown to calluses which were induced to regenerate plants. The morphogenetic response of these cultures did not significantly differ from control cultures. A total number of 134 plants (cv. 'Samsun': 83; amphidiploid s: 10; amphihaploid s: 19; amphihaploid v: 22) were regenerated.

Discussion

The KMC/S-density gradient system described here meets the basic requirements for any gradient technique designed for protoplast fractionation. Iso-osmolality is easily achieved by the mixing, in various ratios (Fig. 2), of two iso-osmotic solutions whose concentrations can easily be determined for any osmolality between 0 and about 1500 mOs/kg H_2O from the data presented in Figure 1. Their respective specific densities can also be obtained from this diagram. The use of polymers, including various types of Dextran, Ficoll, Ficoll-Paque^R and Ludox in gradients was unsatisfactory (Harms, unpublished data).

The use of a stroboscope to follow the fractionation process *in situ* was found to be extremely valuable when evaluating optimal conditions for the performance of the gradients. The observations revealed that fractionation was complete within 2-4 min at 50-100 xg. The capacity of the gradients to fractionate 2-5 $\times$ 10^5 protoplasts per tube in a single run means that large quantities of protoplasts can be fractionated within a reasonably short time.

As can be seen from both the rebanding experiments (Fig. 3) and the standard deviations in Table 1, which were calculated from at least three different gradients each, the density-dependent distribution profiles are highly reproducible. However, the adequate choice of the plant material used for protoplast isolation is an important factor. When density-dependent distribution profiles and average buoyant densities of protoplasts from various plant species and tissues were studied, a great similarity between mesophyll protoplasts was found, irrespective of the species or cultivars used. However, major differences in the density-dependent distribution profiles and average buoyant densities were observed in protoplasts from different tissues. Differences in average buoyant densities of up to 23 mg/cm^3 were found between maize protoplasts from mesophyll and stem internode parenchyma and also between tobacco mesophyll and epidermis protoplasts. Etiolation of leaves also leads to an alteration of the density-dependent distribution profiles and average buoyant densities. Protoplasts from green mesophyll have average buoyant densities 8-11 mg/cm^3 higher than those from etiolated primary leaves. Differentiation and the number of plastids seem to strongly affect the buoyant density of protoplasts. Different ploidy levels of tobacco mesophyll or epidermis protoplasts did not affect the respective density-dependent distribution profiles (Table 1) and no significant effects of protoplast size on buoyant density were observed. When the mean protoplast diameter for each of the protoplast fractions was determined, smaller protoplasts always tended towards higher densities (Fig. 4). However, protoplasts from the same density fraction may differ considerably in diameter, as can be seen when comparing amphidiploid and amphihaploid protoplasts in Figure 4. To test the practicability of the KMC/S-density gradient system under sterile conditions, tobacco mesophyll protoplasts were fractionated and cultured. These experiments were also designed to reveal any negative or toxic effects of the gradient media. However, despite the relatively high salt concentrations of the gradient media, the plating efficiency, colony formation and, finally, plant regeneration were unaffected. No abnormalities in cytokinesis of the regenerated cells have been observed of the type described after prolonged culture in high salt concentrations (Meyer 1974; Meyer and Abel 1975). Protoplasts have been incubated in KMC solution for several hours without reducing their potential to form colonies. The multiple effects on protoplasts of more extended incubation in high concentration mineral media will be reported elsewhere (Harms and Potrykus, in preparation).

The obvious differences in the density-dependent fractionation profiles of protoplasts from varying sources have been used in experiments on the enrichment of fusion products by fractionation in iso-osmotic KMC/S-density gradients (Harms and Potrykus, 1978).

The experiments reported here provide evidence that the fractionation of mixed populations of protoplasts is achieved by use of a simple gradient system. The over-all iso-osmolality and the physiological compatibility of this type of gradient should prove useful in studies with purified fractions of protoplasts as obtained after fractionation of mixed populations isolated from different tissues and organs.

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