Studies on Wheat Protoplasts - A Rapid and Large-Scale Isolation Method and Cell Wall Regeneration in Cultures

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One of the most significant developments in biology during the last decade has been the application of the technique of isolation, culture and fusion of protoplasts to studies on biochemical regulation of plant growth and development and to breed new species. Although during the last 20 years there have been extensive researches on protoplast cultures of oat^{4,8,25}, barley¹², rice^{5,6,27}, corn^{9,22,23}, and wheat^{8,19}, little success has been obtained so far because cereal tissue protoplasts are rather recalcitrant to sustain morphogenesis. Further success in isolation and culture of protoplasts of cereals in general and of wheat in particular has been rather limited. Hence, the importance of studies on wheat protoplasts cannot be overemphasized.

Our study reports a method for both rapid and large-scale isolation of protoplasts from mesophyll tissue of *Triticum aestivum* Linn. cv Norin 8. The incubation mixture standardized for this cultivar was used for isolation of mesophyll protoplasts from three other cultivars viz. Norin 61, Fukuho, and Chinese Spring.

Fluorescence microscopic studies were done to test protoplast viability, presence of lipids, and cell wall synthesis in both freshly isolated and cultured protoplasts. Sustained divisions leading to callus formation and plantlet regeneration are yet to

be achieved.

Materials and Methods

Grains of *Triticum aestivum* Linn. cultivars Norin 8, Norin 61, Fukuho and Chinese Spring were germinated aseptically on 0.7% agar medium under continuous light (ca 3000 lux) to obtain green leaves or in darkness to obtain etiolated leaves. To determine an osmoticum suitable for mesophyll protoplasts, mannitol solution (between 0.2 M and 0.8 M) at ranges of 0.05 M, sucrose (0.4 M) and sorbitol (0.7 M) were tested. Leaves from 7-day-old seedlings, and occasionally from 8-day-old seedlings, were used for protoplast isolation. The leaves were isolated aseptically, surface-sterilized with 50% ethanol for 30 s, and with 1% sodium hypochlorite for 10 min, and then washed thoroughly with sterile distilled water. The sterilized leaves were shredded longitudinally (5 mm long and 1 mm wide) using a sterilized scalpel. The shreds were immersed in the Millipore filter-sterilized incubation mixture, 5 ml/gm of shreds.

Table 1 gives details of isolation of protoplasts of the cultivar Norin 8. The incubation mixture, that was chiefly used in the present investigation was prepared in CPW medium²⁶ containing cellulase "Onozuka R 10" (Kinki Yakult, Nishinomiya, Japan), pectolyase Y-23 (Seishin Seiyaku, Tokyo, Japan), and 0.5 M mannitol at pH 5.8. The incubation was carried out in the dark at 25±1°C with mild shaking twice or thrice in 4 h.

After incubation, the incubation mixture together with the released protoplasts was sieved through two layers of stainless steel mesh (100 µm) to remove the undigested

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debris. The suspension was centrifuged at 100 g for 1 min to get a pellet of protoplasts; the pellet was washed twice with the same incubation mixture but without the enzymes and then suspended in 14% sucrose solution to obtain a pure band of protoplasts. The band containing protoplasts was finally washed with the culture medium. Table 2 lists the culture media tested. One of the following three media was used as the basal medium: (1) MS i.e. Murashige and Skoog's medium¹⁵, (2) MS 1/2, i.e. half the concentration of MS medium, and (3) MSH, i.e. half the concentration of only the mineral salts of MS. It was variously supplemented with organic salts such as citric acid, malic acid, and succinic acid; amino acids such as glutamic acid and proline; casein hydrolysate (CH) a source of reduced nitrogen; sugars such as dextrose, glucose, mannitol, sucrose and D-xylose; and growth substances such as 2,4-

dichlorophenoxyacetic acid (2,4-D), Kinetin (Kn), and benzylamino purine (BAP). The pH of every medium was adjusted to 5.8.

The protoplasts were cultured either in microdrops, or in semi-solid medium in Falcon petridishes (6 cm diameter) following the procedure of Nagata and Takebe¹⁸. The final density of the protoplasts determined by use of haemocytometer was either 10^5 or 2×10^5 /ml of the culture medium. The cultures were sealed with parafilm.

Owing to heavy bacterial contamination in protoplast cultures of cultivar Norin 8 (which was later found to be seed-borne) two antibiotics viz. cephaloridine (200 μ g, 250 μ g or 300 μ g/ml) and kanamycin sulphate (1 ppm, 2 ppm, 10 ppm, 20 ppm) were tested; prior to sowing for germination the grains were soaked for 5 h in an aqueous solution of one or the other antibiotic, or the antibiotic was added to the protoplast culture medium.

Table 1. Incubation mixtures tested for isolation efficiency of Norin 8 mesophyll protoplasts.

S.No.	Incubation mixture (pH 5.8)	Incubation period (h)	Yield/g fresh weight
1	CPW*+C** (4%)+pectinase (Serva) (1%)+0.5 M	...	4.11×10^5
2	CPW+C (4%)+pectinase (1%)+sucrose (0.4 M)	...	1.17×10^5
3	CPW+C (4%)+pectinase (1%)+Macerozyme (1%)†+0.5 M	17	2.4×10^5
4	CPW+C (4%)+pectinase (1%)+Macerozyme (1%)+driselase (1%)+0.5 M	...	3.5×10^4
5	CPW+C (4%)+pectinase (2%)+Macerozyme (1%)+0.5 M	17	4.0×10^5
6	CPW+C (4%)+Macerozyme (1%)+0.5 M	...	3.6×10^4
7	CPW+C (4%)+Macerozyme (1%)+Potassium dextran sulphate (1%)+0.5 M	...	4.5×10^4
8	CPW+C (4%)+pectolyase (0.1%)††+0.5 M	...	9.0×10^5
9	CaCl ₂ (1.2 mM)+C (4%)+pectinase (1%)+0.5 M	...	3.75×10^5
10	CaCl ₂ (1.2 mM)+C (4%)+pectinase (1%)+sucrose (0.4 M)	17	6.6×10^4
11	CaCl ₂ (1.2 mM)+C (4%)+pectolyase (0.05%)+0.5 M	...	9.3×10^5
12	CaCl ₂ (1.2 mM)+C (4%)+pectolyase (0.1%)+0.5 M	...	1.0×10^6
13	CaCl ₂ (1.2 mM)+C (4%)+pectolyase (0.2%)+0.5 M	...	8.1×10^5
14	CaCl ₂ (1.2 mM)+C (4%)+pectolyase (0.4%)+0.5 M	...	9.8×10^5
15	DW+C (4%)+Macerozyme (1%)+dextran sulphate (1%)+sulphur (17.3%)+sorbitol (0.7 M)	...	4.0×10^4
16	DW+C (2%)+pectolyase (0.1%)+0.5 M	...	2.73×10^5
17	DW+C (3%)+pectolyase (0.1%)+0.5 M	...	4.53×10^5
18	DW+C (4%)+pectolyase (0.1%)+0.5 M	...	7.65×10^5

*: Mixture of KH₂PO₄ 27.2, KNO₃ 101, CaCl₂ · 2H₂O 1480, MgSO₄ · 7H₂O 240, KI 0.16 and CuSO₄ · 5H₂O 0.25 mg/l.

** : Cellulase "Onozuka R 10". 0.5 M : Mannitol (in Molar concentration).

† : Macerozyme R 10. †† : Pectolyase Y-23. DW : Distilled water.

Table 2. Media used for culture of mesophyll protoplasts of wheat.

S.No.	Media*
1	MS**+sucrose (3%)+mannitol (0.35 M)+2, 4-D (5 ppm)+Kn (2 ppm)
2	MS+sucrose (3%)+mannitol (0.35 M)+2, 4-D (8 ppm)+Kn (2 ppm)
3	MS+sucrose (6%)+mannitol (0.35 M)+2, 4-D (8 ppm)+Kn (2 ppm)+dextrose (250 mg/l)
4	MS+sucrose (4%)+mannitol (0.35 M)+2, 4-D (8 ppm)+Kn (2 ppm)+dextrose (250 mg/l)
5	MS+sucrose (4%)+mannitol (0.35 M)+2, 4-D (8 ppm)+Kn (2 ppm)+dextrose (250 mg/l)+malic acid (10^{-6} M)+succinic acid (10^{-6} M)
6	No. 5+kanamycin sulphate (1 ppm)
7	MS+mannitol (0.5 M)+2, 4-D (8 ppm)+Kn (2 ppm)+dextrose (250 mg/l)+malic acid (10^{-6} M)
8	No. 7+kanamycin sulphate (2 ppm)
9	MS+sucrose (3%)+2, 4-D (2 ppm)
10	MS+sucrose (3%)+2, 4-D (2 ppm)+glucose (250 mg/l)
11	MS+sucrose (4%)+2, 4-D (2 ppm)+glucose (250 mg/l)
12	MS+2, 4-D (2 ppm)+glucose (5%)
13	MS 1/2†+sucrose (2.9%)+mannitol (0.3 M)+2, 4-D (4 ppm)+Kn (2 ppm)+kanamycin sulphate (10 ppm)
14	MS 1/2+sucrose (2.9%)+mannitol (0.3 M)+2, 4-D (4 ppm)+Kn (2 ppm)+dextrose (250 mg/l)+kanamycin sulphate (10 ppm)
15	MS 1/2+sucrose (14.53%)+2, 4-D (4 ppm)+Kn (2 ppm)+kanamycin sulphate (10 ppm)
16	MS 1/2+sucrose (14.53%)+2, 4-D (4 ppm)+Kn (2 ppm)+dextrose (250 mg/l)+kanamycin sulphate (10 ppm)
17	MSH††+sucrose (2.9%)+mannitol (0.3 M)
18	MSH+sucrose (2.9%)+mannitol (0.3 M)+kanamycin sulphate (10 ppm)
19	MSH+sucrose (2.9%)+mannitol (0.3 M)+2, 4-D (2 ppm)+kanamycin sulphate (10 ppm)
20	MSH+sucrose (2.9%)+mannitol (0.3 M)+2, 4-D (2 ppm)
21	MSH+sucrose (2.9%)+mannitol (0.3 M)+2, 4-D (2 ppm)+Kn (2 ppm)+CH (400 ppm)+malic acid (10 ppm)+citric acid (10 ppm)
22	MSH+sucrose (1%)+mannitol (0.3 M)+2, 4-D (2 ppm)+Kn (2 ppm)+CH (400 ppm)+malic acid (10 ppm)+citric acid (10 ppm)+glucose (4%)
23	MSH+sucrose (1%)+mannitol (0.3 M)+2, 4-D (2 ppm)+Kn (2 ppm)+CH (400 ppm)+malic acid (10 ppm)+citric acid (10 ppm)+glucose (4%)+D-xylose (250 mg/l)
24	MSH+mannitol (0.5 M)+2, 4-D (2 ppm)+Kn (2 ppm)+CH (400 ppm)+malic acid (10 ppm)+citric acid (10 ppm)
25	MSH (but vitamins and amino acid of WB)+sucrose (2.9%)+mannitol (0.3 M)+2, 4-D (2 ppm)+Kn (2 ppm)
26	MSH (but vitamins and amino acid of WB)+sucrose (2.9%)+mannitol (0.3 M)+2, 4-D (2 ppm)+Kn (2 ppm)+CH (400 ppm)+malic acid (10 ppm)
27	No. 26 (but with half the conc. of vitamins and amino acid of WB).
28	MS (with vitamins and amino acid of WB)+sucrose (3%)+BAP (2 ppm)+glutamine (37.5 mg/150 ml)+proline (37.5 mg/150 ml)
29	No. 28+2, 4-D (2 ppm)

* : Liquid as well as solid medium, pH 5.8.

** : Murashige and Skoog's medium (Murashige & Skoog 1962).

† : Half concentration of MS medium.

†† : MS containing half the concentrations of only the mineral salts.

WB : Modified White's medium (Rangaswamy 1961).

Fig. 1. Freshly isolated protoplasts of *Triticum aestivum* Linn. cv Norin 8. A. Protoplasts isolated from green leaves; note the peripheral distribution of chloroplasts ($\times 750$). B. As in A, but in lower magnification, to show clumping of cytoplasm (arrows) in some protoplasts ($\times 370$). C. Protoplasts stained with carbol fuchsin; nucleus is large and distinct ($\times 750$).

The cultures were maintained either in diffuse light (*ca* 2000 lux) or in a 16 h light regime for 35 days, at $25 \pm 1^\circ\text{C}$, by perio-

dically replenishing the medium. To observe the newly synthesized wall around the protoplasts, the cultured protoplasts were

Fig. 2. Percentage distribution of green and etiolated protoplasts of different sizes.

plasmolysed with 0.7 M mannitol.

For fluorescence microscopic studies, acridine orange (AO for short)²⁾, fluorescein diacetate (FDA for short)¹³⁾, 3,4-benzpyrene (Berg 1951, cited in Jensen 1962), and calcofluor White ST¹⁷⁾ were used. Cell wall regeneration in cultured protoplasts was tested with calcofluor every 6 h from culture up to 48 h.

For light microscopic studies, protoplasts were stained with carbol fuchsin¹¹⁾.

Results and Discussion

Unlike kanamycin sulphate, cephaloridine drastically lowered the germination percentage of grains from 90 to 20 and the resultant seedlings showed stunted growth. When incorporated in the protoplast culture medium cephaloridine decreased also drastically the protoplast viability to 14%. No such effects were seen when kanamycin sulphate was used. Hence kanamycin sulphate (10 ppm) was more effective than cephaloridine.

In order to find out a suitable osmoticum for wheat protoplasts, different sugars were tested and 0.5 M mannitol was found the most suitable. Sucrose and sorbitol were not so effective as mannitol (Table 1).

Of the 18 incubation mixtures tested, the best yields (9.0×10^5 to 1×10^6 /g fresh weight) of protoplasts were obtained in CPW or $\text{CaCl}_2 + 4\%$ cellulase + 0.1% pectolyase Y-23 + 0.5 M mannitol. Of the concentrations of cellulase tested, 4% proved the best (Table 1).

With pectinase (1%), although both the yield and the quality of protoplasts were satisfactory (nearly 85% of the protoplasts were viable), it required the longest incubation period of 17 h. On the other hand replacing pectinase with pectolyase Y-23 (0.1%) significantly shortened the incubation period to 4 h without affecting either the yield or the quality of protoplasts. With 0.05% pectolyase Y-23 also, both the yield (Table 1) and the quality of protoplasts were good (ca 83% protoplasts were viable), but to achieve it, the incubation period had to be increased from 4 to 6 h. Although higher concentrations of pectolyase Y-23 (0.2% and 0.4%) caused a higher yield of protoplasts (Table 1), the percentage viability, as tested by FDA and AO, decreased to nearly 55; also the protoplasts formed aggregates. Thus a one step treatment of the shredded leaf tissue with 0.1% pectolyase Y-23 in combination with 4% cellulase, proved the best method for isolation of wheat mesophyll protoplasts.

For isolation of rice and oat protoplasts also pectolyase Y-23 helped to considerably decrease the incubation period¹⁶⁾. Use of macerozyme as the first enzyme in sequential treatments for isolation of cereal protoplasts did not yield even free cells^{8,21)}. Similarly our preliminary experiments showed that pectolyase Y-23, alone did not yield a workable number of free cells.

Freshly isolated protoplasts (green as well as etiolated) were spherical and showed a

Fig. 3. Protoplast cultures of *Triticum aestivum* Linn. cv Norin 8. A. From 19-day-old culture; large droplets of lipid are obvious in two of the newly formed cells ($\times 700$). B. A plasmolysed entity from 16-day-old culture; the newly synthesized cell wall (arrow) is evident ($\times 700$). C, D. Budding in 16-day-old protoplast cultures ($\times 750$), ($\times 700$).

large nucleus (Fig. 1A–C). Chloroplasts usually presented a peripheral disposition (Fig. 1A), and seldom the cellular contents could be seen clumped in the centre of the protoplast or pushed against its plasma-membrane (Fig. 1B). However even in such clumped protoplasts the cytoplasm became evenly distributed within 2 h from culture.

Green protoplasts measured 10–50 μm ,

and etiolated protoplasts 20–75 μm in diameter (Fig. 2). However, in either type the maximal percentage of protoplasts were 40–45 μm in diameter. These protoplasts were larger than rice protoplasts, which were 10.3 μm in diameter¹⁴.

Fluorescence microscopic studies showed that nearly 85% of the freshly isolated protoplasts were viable as they fluoresced in-

Fig. 4. Representative preparations from 4 to 20-day-old protoplast cultures. A-C. *Triticum aestivum* Linn. cv Fukuho, 4-day-old protoplasts in liquid medium ($\times 350$), ($\times 400$), ($\times 350$). D. cv Norin 61, 20-day-old protoplasts in liquid medium ($\times 350$).

tensely bright yellow with FDA. Also, when tested with calcofluor, no remnants of original cell wall material could ever be seen on the surface of isolated protoplasts. Test for lipids with 3,4-benzpyrene showed rather tiny droplets of lipids in protoplasts of two of the four cultivars viz. Norin 8 and Fukuho.

Irrespective of the culture medium used, the protoplasts slightly increased in size, 1–3 days from culture in liquid medium, whereas in cultures in the semi-solid medium there was hardly any increase in protoplast size. Also, the etiolated protoplasts turned green within one hour of raising the protoplast culture during which the fluo-

rescent light was used. No increase in the number of chloroplasts in any of the protoplast cultures occurred. By 3 days from culture, large droplets of lipid could be seen in cultured protoplasts of cultivars Norin 8 and Fukuho (Fig. 3A). Lipid droplets have also been observed in protoplast cultures of *Vicia faba*³⁾.

Irrespective of the culture medium used, cell wall formation started nearly 6 h after protoplast culture, in 20% of the cultured protoplasts of Chinese Spring, and nearly 18 h after culture in 52–62% of the cultured protoplasts of the other three cultivars. Importantly, the newly synthesized wall material appeared as patches or as a

reticulum. Within two days from culture the percentage of cultured protoplasts which initiated cell wall regeneration increased from 20–62 to 90 in all the cultivars. Four to five days after culture a complete and uniform wall was recognizable around all those protoplasts which had started regenerating a cell wall. The walled protoplasts attained an oval shape confirming the synthesis of new wall material. Plasmolysis with 0.7 M mannitol of 4 to 5-day-old protoplast cultures gave a final confirmatory evidence for the presence of a newly regenerated wall around the protoplasts leading to the formation of cells (Fig. 3B). Distinct cell wall regeneration in wheat protoplast cultures has been reported previously also^{8,19}. Budding was a rare event in protoplast cultures of all the 4 cultivars of wheat (Fig. 3C,D).

Between 4 and 20 days from culture stage reminiscent of cell division were observed (Fig. 4A-D). However, such divisional stages could be observed in only 2.32% of the protoplasts cultured on media no. 5 and 14 only (Table 2).

Although we maintained to survive the protoplast cultures up to 35 days only *ca* 20% of the protoplasts remained viable, and sustained divisions leading to callus formation have never been observed, in spite of treatments with an auxin, 2,4-D, the most commonly used growth substance for initiation and maintenance of callus cultures of monocots¹⁹. Also from tissue culture studies on wheat^{1,7,20,28,29} it can be inferred that 2,4-D alone or in combination with kinetin serves as a cardinal factor for the morphogenesis *in vitro*.

In protoplast cultures of *T. monococcum* Németh and Dudits (1977) observed divisions on the 15th and the 20th day from culture in 6.54% and 11.41% of the protoplasts respectively; subsequently cell clusters giving rise to callus tissue were formed. There are only 3 other reports of callus formation in cereal protoplast cultures—*Hordeum vulgare*¹², *Oryza sativa*^{5,6}, and *Zea mays*^{22,23}. Indeed cereals are recalcitrant materials for the induction of cell division and the continued morphogenesis in protoplast cultures.

Summary

An improved method for the isolation of wheat mesophyll protoplast was standardized. The viability, presence of lipids and cell wall synthesis were also studied by aid of fluorescence microscopy.

Mesophyll protoplasts of 4 cultivars of *Triticum aestivum* Linn. were isolated from green as well as etiolated leaves of 7 and 8-day-old seedlings. Incubation mixture containing cellulase (4%), pectolyase Y-23 (0.1%) and mannitol (0.5 M) prepared in CaCl_2 (1.2 mM) or CPW medium (pH 5.8) proved the best not only in decreasing the incubation period to as short as 4 h from the usual 17 h⁸) but also in yielding as high as 1×10^6 protoplasts per gram fresh weight of the leaf material.

Fluorescence microscopic studies showed that nearly 85% of the freshly isolated protoplasts were viable and cell wall regeneration in cultured protoplasts started 6–18 h from culture. Cell division-like stages were observed in less than 3% protoplasts.

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〔和 文 摘 要〕

コムギのプロトプラストに関する研究——
迅速大量分離法と細胞壁の再生

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4品種のコムギを用いて、7日目および8日目の幼苗の、緑葉ならびに軟白葉の葉肉細胞から、プロトプラストを分離する方法を研究した。分離のためには、セルラーゼ4%, ペクトライエース Y-23 の0.1% およびマニトール 0.5 M を、1.2 mM の塩化カルシウム溶液または CPW 溶液 (pH 5.8) にて調整した処理液が、最も良かった。他の処理液で 17 時間も要した分離時間を、この処理液では、4 時間に短縮できた。また葉片生体重 1 g 当り、 1×10^6 個のプロトプラスト収量を、得ることができた。

蛍光顕微鏡観察で、分離直後のプロトプラストの約 85% が正常であり、また6時間から 18 時間培養したのちに、細胞壁の再生が開始されることがわかった。細胞分裂をおこしたと考えられる状態のものは、3%以下であった。