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Wheat protoplast culture: embryogenic colony formation from protoplasts

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ABSTRACT

Wheat (Triticum aestivum L. cv Chinese Spring) protoplasts were isolated from immature embryos or embryogenic calli (3-4 weeks of culture on MS medium with 32 mg/1 dicamba) and cultured in R2 medium containing 2 mg/1 2,4-D by the nurse culture methods originally developed for rice protoplasts (Kyozuka et al. 1987). Protoplasts isolated from embryogenic calli started to divide within 3-5 days and formed colonies at frequencies up to 2% after 3-4 weeks of culture, while protoplasts isolated from immature embryos formed colonies at much lower frequency (less than 0.1%). Some of these colonies were "embryogenic", and they appeared at a frequency of approximately 0.5% of colonies formed when callus-derived protoplasts were used. From two of those "embryogenic" colonies, calli were regenerated and albino shoots and roots were obtained.

INTRODUCTION

Recent progress in genetic manipulation of plant cells has opened new possibilities in crop improvement. In particular, protoplast culture plays a key role in developing such new techniques as somatic hybridization and gene transfer. However, application of these techniques in major cereals has been hampered by lack of reproducible methods for plant regeneration from protoplasts (Ozias-Akins and Lörz 1984; Vasil 1987).

Recently, reproducible methods for plant regeneration from rice protoplasts were established (Fujimura et al. 1985; Yamada et al. 1986; Toriyama et al. 1986; Abdullah et al. 1986; Kyozuka et al. 1987), and somatic hybrids of rice and barnyard grass (Terada et al. 1987), rice and wild Oryza species (Hayashi et al. 1988) have been obtained. In maize, successful plant regeneration from protoplasts was reported (Rhodes et al. 1988), though all the protoplast-derived plants were sterile. Also, albino plantlets were obtained from barely protoplasts (Lührs and Lörz 1988). These successes were accomplished by using embryogenic or morphogenic suspension cultures as the protoplast source (Vasil and Vasil 1980).

In wheat, plant regeneration from protoplasts has not been reported. Potrykus and Petruska (1983) reported that fast growing callus cultures or suspension cultures could not be developed and that protoplasts isolated from early shoot-meristem and root-meristem suspension cultures did not divide. Maddock (1987) reported the establishment of rapidly-growing suspension cultures and reproducible callus formation from protoplasts. No shoots, however, were obtained. One possible reason is that the suspension cultures she used for protoplast isolation were established after culture and selection for more than 4 years. Therefore, the culture was no longer embryogenic at the time of protoplast isolation, as she mentioned that plants were never regenerated from the suspension cultures used for protoplast isolation. These studies show that in wheat it is difficult to develop embryogenic suspension cultures and that dividing protoplasts can only be isolated from non-morphogenic, long-established cell suspensions. Therefore, to investigate an alternative approach, we used immature embryos or freshly induced embryogenic calli as protoplast sources instead of suspension cultures. This approach proved useful in rice in combination with nurse culture methods. In this paper, reproducible formation of embryogenic colonies and plantlet regeneration from protoplast-derived callus are described.

MATERIALS AND METHODS

Induction of Embryogenic Callus From Immature Embryo

Seven cultivars of spring wheat (Triticum aestivum L. cv. Chinese Spring, Ciano, Oxley, Zenith, Yang mai 1, Beijing 8, Norin 61) and one cultivar of semi-winter wheat (T. aestivum L. cv. Pitic 62) were used as sources of immature embryos. Seeds of Chinese Spring were provided by Dr. T. Shimada at Ishikawa College of Agriculture (Nonoichi, Ishikawa). The rest of the seeds were obtained from National Institute of Agribiological Resources, Tsukuba. Vernalization of semi-winter wheat was performed by growing plants (3 weeks after germination) at 10°C for 2 weeks. All plants were grown in a greenhouse.

Immature dehusked seeds (10-14 days after pollination) were surface-sterilized in 70% ethanol for 5-10 sec, in 3% sodium hypochlorite for 15 min and washed 3 times in sterile distilled water. Immature embryos (0.5-2.5 mm in diameter) were aseptically excised under a stereo-microscope with fine forceps.

Embryos were either used for protoplast insolation or inoculated with the scutellum upwards on MS (Murashige and Skoog 1962) or N6 (Chu et al. 1975) agar medium (0.7% Agarose Type 1, Sigma) supplemented with various concentrations of auxins and organic supplements to induce calluses. As auxins, 2,4-dichlorophenoxyacetic acid (2,4-D) and 3,5-dichloro-2-methoxybenzoic acid (dicamba, Serva, FRG) were used at 0.5, 1, 2 mg/1 and 2, 4, 8, 32 mg/1, respectively.

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To promote induction of embryogenic calluses, 10 mM proline and 100 mM tryptophan were added.

Protoplast Isolation

Immature embryos or embryogenic calli derived from immature embryos were cut into small pieces with a razor and suspended in an enzyme mixture consisting of 4 or 6% Cellulase RS (Kinki Yakult, Japan), 1% Macerozyme R-10 (Kinki Yakult, Japan), 0.5% calcium chloride, 0.5% potassium dextran sulfate and 0.4M mannitol. In some experiments, 0.01 or 0.1% Pectolyase Y-23 (Seishin Pharmaceutical, Japan) was added. After 5-6 hr of incubation at 27°C without shaking, 3 x volume of KMC solution (Harms and Potrykus 1978) was added and passed through 20 µm nylon mesh. Protoplasts were collected by centrifugation (800 rpm for 8 min) and washed twice in KMC solution by centrifugation (400 rpm for 5 min). Calcofluor white (0.1%) was used to determine the presence of undigested cells.

Protoplast Culture and Plant Regeneration

Protoplasts were suspended in various media consisting of basal components, auxin, organic supplements, and 0.4 M sucrose. To this suspension, equal volume of molten protoplast medium containing 2% agarose (Sea plaque, FMC) was added. Protoplast density was 1 x 10^6 protoplasts/ml. Solidified agarose containing protoplasts was cut into blocks and floated in 6 ml of protoplast medium. Two different methods of nurse culture, the mixed nurse method and the Millicell method, were used (Kyozuka et al. 1987). In the former method, nurse cells (approximately 100 mg/plate) were added in the liquid part of the culture. In the latter method, nurse cells were suspended outside a culture plate insert (Millicell HA, 30 mm diameter, 0.45 µm pore size, Millipore USA). The latter method was used only for subculture of colonies. As nurse cells, three different types of cultures were used. Calli of *Triticum aestivum* L. cv Chinese Spring were induced by plating immature embryos on MS medium with 32 mg/1 dicamba. The Oc suspension culture of rice is a gift from Dr. K. Shono at Tokyo University. It has been maintained for more than 7 years and is no longer competent for plant regeneration. A cell line of *Aegilops squarrosa* was provided by Dr. C. Ishii at Saitama University. The Oc suspension grew rapidly and was subcultured every week, while the suspension of *Aegilops squarrosa* grew more slowly and was subcultured every three weeks.

Basal media used were MS, KM (Kao and Michayluk 1975), P2 (Chuang et al. 1978), and R2 (Ohira et al. 1973) supplemented with 1, 2 mg/1 2,4-D or 1, 4, 10 mg/1 dicamba. Other organic supplements used were 100 mg/1 m-inositol, 100 mg/1 carbenicillin (Sigma), 100 mg/1 cefotaxime (Calbiochem), and 50 mg/1 $AgNO_3$. They were all reported to promote growth of wheat calli (Ozias-Akins and Vasil 1983; Mathias and Boyd 1986; Purnhauser et al. 1987). Frequencies of colonies consisting of more than 10 cells were scored after 3 weeks of culture. At the same time, frequencies of embryogenic type colonies were scored. Subculture of embryogenic colonies regenerated from protoplasts was performed as follows; (1) renewal of half or total volume of liquid protoplast medium, (2) renewal of half or all of nurse cells, (3) transfer of agarose blocks to soft agar medium with or without feeder cells, (4) transfer of the "embryogenic" colonies to fresh medium and culture by the Millicell method. At two different periods of culture, 3 or 7 weeks of culture, the above treatments were done. When colonies became ca. 2-3 mm in diameter, they were transferred to N6 agar medium containing 1% agarose (Type 1, Sigma) and 6% sucrose without growth hormones. Regenerated plants were transferred to 1/2 MS medium with 0.5% agarose and 1.5% sucrose.

RESULTS AND DISCUSSION

1. Induction of Embryogenic Calli From Immature Embryos

In wheat, genotypic effect on culture response has been reported (Mathias and Simpson 1986; Felsenburg et al. 1987). For this reason, eight cultivars were examined for induction of embryogenic calli, and Chinese Spring induced embryogenic calli with the highest frequency (88% of inoculated immature embryos). Therefore, all the following experiments were performed with cv. Chinese Spring.

Immature embryos of 10 days after pollination (1-2 mm in diameter) were found to be the best explant for induction of embryogenic calli. They were inoculated on MS basal medium supplemented with various concentrations of auxins. Embryogenic calli (Fig. 1-A) were most frequently induced on MS basal medium supplemented with 32 mg/1 dicamba (Table 1). In contrast, secondary embryos (Fig. 1-B) were formed on MS medium supplemented with 0.5-1 mg/1 2.4-D. An attempt to maintain embryogenic calli by transferring to fresh medium was not successful, because they lost embryogenic properties soon after the transfer. Therefore, freshly induced calli (4 weeks after initial plating of embryos) were used for protoplast isolation.

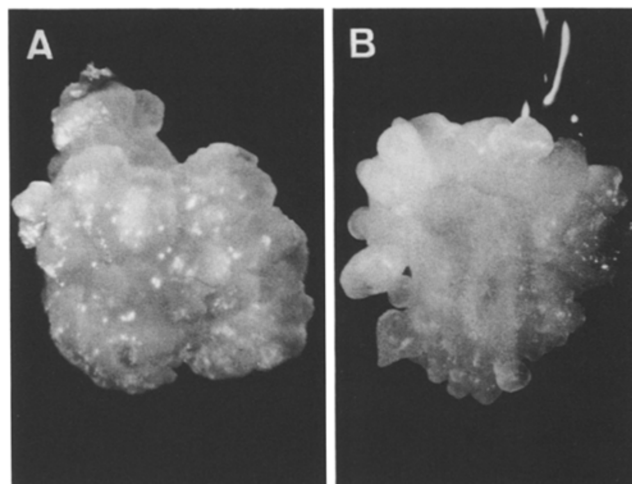

Fig.1. Embryogenic callus and secondary embryos formed on the immatue embryo of cv. Chinese Spring: **A.** Embryogenic callus induced on MS medium with 32 mg/1 dicamba; **B.** Secondary embryos induced on MS meduim with 2 mg/1 2,4-D.

Table 1. Effect of auxin on frequencies of embryogenic callus and secondary embryo formation from immature wheat embryos

Auxin	Conc.(mg/1)	Embryogenic callus	Secondary embryo (%)
2,4-D	0.5	10	82
	1.0	28	44
	2.0	39	33
Dicamba	2.0	25	19
	4.0	38	61
	8.0	50	36
	32.0	88	0

For each experiment 20-50 embryos (10 days after pollination) were used.

Embryogenic calli were also suspended in various liquid media such as R2, P2, KM in order to establish embryogenic suspension cultures. However, they became loose rapidly and did not continue to grow embryogenically in any of the liquid media.

2. Protoplast Isolation

Immature embryos and embryogenic calli induced on MS medium with 32 mg/1 dicamba were examined as protoplast sources. Dissection with a razor of these embryos or calli prior to the enzyme treatment was required for efficient protoplast isolation. Incubation (5-6 hr at 27°C) in the enzyme mixture consisting of 4% Cellulase RS, 1% Macerozyme R-10, and 0.4 M mannitol without shaking gave the highest yield of viable protoplasts. The average yield was 10^5 protoplasts/100 embryos or $5x10^5$ protoplasts/g fresh weight callus. Higher concentration (6%) of Cellulase RS or addition of 0.01 or 0.1% Pectolyase Y-23 did not increase the yield of viable protoplasts.

Two types of protoplasts were noticed among protoplasts isolated from immature embryos or embryogenic calli (Fig.2-A). The first type was highly vacuolated (Fig.2-B, left) and the second was cytoplasmically dense with many starch grains (Fig.2-B, right). The latter tended to fuse during isolation. Contamination of cell debris and undigested cells could not be detected microscopically. Moreover, calcofluor white staining revealed the absence of undigested cells in the protoplast preparations. Therefore, we concluded that the procedure for protoplast isolation adopted in this study was effective for obtaining virtually pure populations of protoplasts.

3. Protoplast Culture

Requirement of Nurse Cells to Induce Division of Protoplasts

The presence of nurse cells was indispensable for induction of division of protoplasts. Protoplasts isolated from immature embryos or embryogenic calli divided after 3-5 days of culture (Fig.2-C). Cytoplasmically-dense protoplasts divided predominantly, and formed colonies. After 3 weeks of culture, some of these colonies became visible (up to 400 µm in diameter) (Fig.2-D).

With regard to the nurse cells, the Oc cultures of rice were the most effective. Mixing the Oc cultures with two types of wheat cells, suspension cultures of *Aegilops squarrosa* or calli of *Triticum aestivum* cv. Chinese Spring, did not increase the frequency of protoplast division, although these two types of wheat cells could independently induced low frequency of protoplast division when used alone. This suggests that the ability of nurse cells to induce protoplast division depends more on the cell type than on species used.

Colony Formation From Protoplasts

Frequencies of colonies were examined after 3 weeks of culture with various combinations of basal media and auxins. R2 medium with 2 mg/1 2.4-D gave the highest frequency (ca. 2% of protoplasts), when the embryogenic calli were used for protoplast isolation (Fig.2-E). Protoplasts directly isolated from immature embryos showed much lower plating efficiency (less than 0.1%).

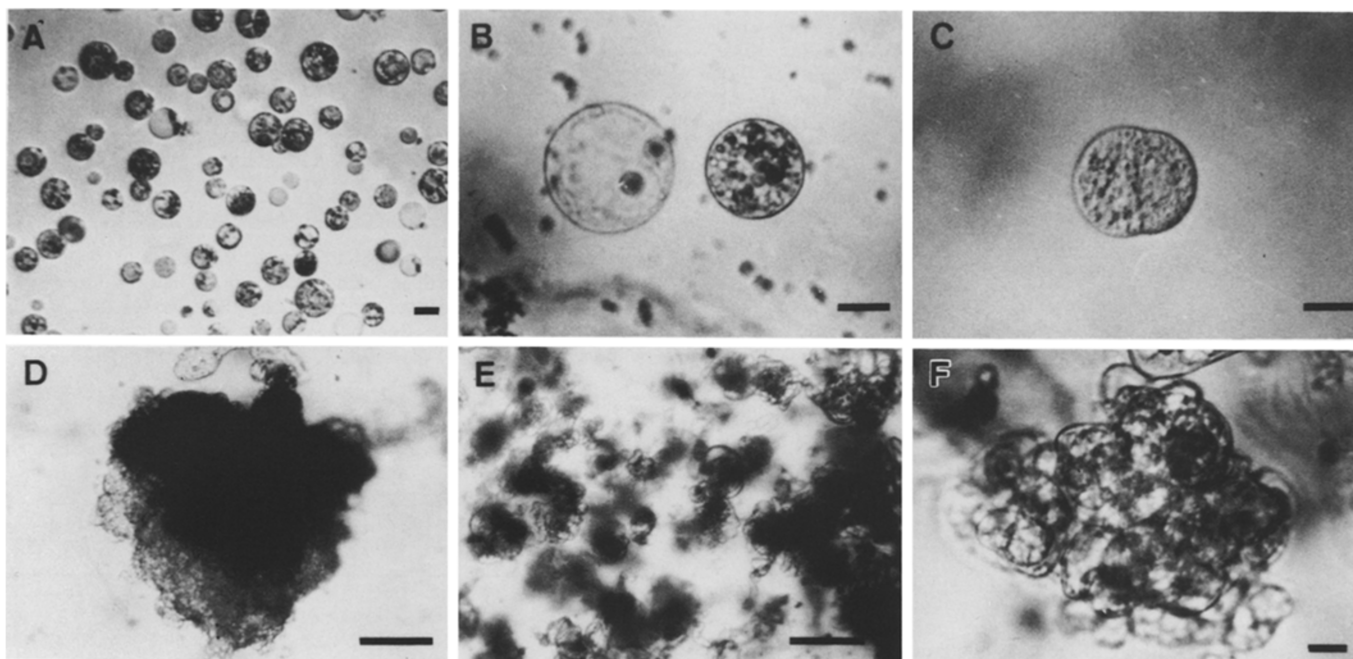

Fig.2. Colony formation from protoplasts isolated from embryogenic callus: A. Protoplasts isolated from embryogenic callus; B. Highly vacuolated protoplast (left), cytoplasmically dense protoplast (right); C. First division of protoplast; D. "Embryogenic type" colony; E. A microscopic view of protoplast-derived colonies in an agarose bead; F. "Non-embryogenic type" colony. Scale bar in A,B,C,F is 10 µm; in D,E scale bar is 100 µm.

Colonies formed from protoplasts could be morphologically classified into two types, "embryogenic type" and "non-embryogenic type". While "embryogenic type" colonies consisted of cytoplasmically dense cells (Fig.2-D), "non-embryogenic type" colonies consisted of highly vacuolated and elongated cells (Fig.2-F). The latter constitutes the majority of colonies and the former appeared at frequencies not higher than 0.01%. Protoplasts isolated from embryogenic calli formed "embryogenic type" colonies at higher frequency than those isolated from immature embryos. When embryogenic calli induced with 2 mg/1 2.4-D were used, no increase of that frequency was observed.

4. Callus Formation and Morphogenesis

During culture of the "embryogenic type" colonies, vacuolation of cells at their periphery was often noticed after 4-5 weeks of culture and colony proliferation stopped ca. 3 weeks later. In order to inhibit this vacuolation and to stimulate colony proliferation, various treatments, such as renewal of nurser cells and/or medium, transfer of the agarose beads to the soft agar medium, and addition of m-inositol or $AgNO_3$ or antibiotics were examined at various stages of culture. However, none of these treatments was effective.

Two calli were regenerated when colonies from embryogenic callus-derived protoplasts were picked up and cultured with the Millicell method as reported previously (Fig.3-A). Albino shoots and roots appeared from them on N6 agar medium without hormones (Fig.3-B,C).

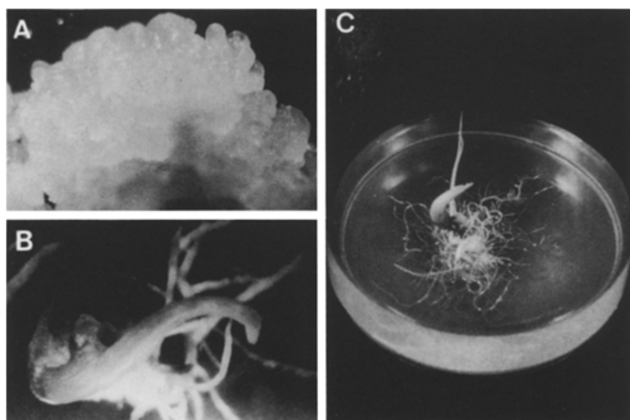

Fig.3. Plant regeneration from protoplast-derived callus: **A.** Protoplast-derived callus; **B.** Shoot and root formation; **C.** Developing albino plantlet.

Our studies suggest that formation of "embryogenic type" colonies is possible by the nurse culture method when protoplasts are isolated from freshly induced embryogenic calli. Although our culture system gave a low frequency of colony formation, this could be a unique approach in wheat protoplast culture circumventing difficulties of establishing embryogenic suspensions. The "embryogenic type" colonies appear to be competent for plant regeneration although plants were not reproducibly obtained. A possibility of contamination of meristems in the protoplast population was not unequivocally excluded although undigested cells were not observed by calcofluor staining.

The "embryogenic type" colonies rarely formed calli, just as embryogenic calli did not proliferate continuously. Similar cases have been reported in maize protoplast culture (Imbrie-Milligan and Hodges 1986) and wheat pollen culture (Wei 1982). Inability to maintain the competence both for morphogenesis and proliferation remains a problem. For reproducible plant regeneration from protoplasts, further investigations on factors influencing these competences are required.

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