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Culture of and fertile plant regeneration from regenerable embryogenic suspension cell-derived protoplasts of wheat (*Triticum aestivum* L.)

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Summary. Regenerable embryogenic cell suspensions initiated from immature embryo-derived friable, fast growing, embryogenic calli of GK Ságvári winter wheat (*Triticum aestivum* L.) served as sources of protoplasts, which were cultured in different liquid or agarose-solidified media. Protocallus formation was best on KM_{8p} (Kao and Michayluk 1975) and GM (Li and Murai 1990) media, and protocallus growth on MS (Murashige and Skoog 1962) callus growing medium. Green shoot/plant regeneration occurred on MS regenerating medium, and rooting on MS or N6M (Mórocz et al. 1990) hormone-free media. Protocalli maintained their morphogenic capacity over 4 months, and with multiple subcultures on half-strength MS regenerating medium, the total number of regenerants could be increased. Approximately 1000 shoots/plants were regenerated and over 500 plants were transplanted in the greenhouse. The majority of them had an abnormal chromosome number and low viability, however, one plant grew to maturity and set seed.

Key words: Cell suspension - Protoplast culture - Plant regeneration - Wheat (*Triticum aestivum* L.)

Abbreviations: BAP = 6-benzylaminopurine; 2,4-D = 2,4-dichlorophenoxyacetic acid; ECS = embryogenic cell suspension; GA_3 = gibberellic acid; GM = General medium; IAA = indole-3-acetic acid; IBA = indole-3-butyric acid; MS = Murashige and Skoog medium; NAA = 1-naphthaleneacetic acid; RECS = regenerable embryogenic cell suspension.

Introduction

Reproducible regeneration of plants from protoplasts is indispensable for successful application of genetic manipulation techniques in plants, especially in cereals (Lazzeri and Lörz 1988; Lörz et al. 1988; Vasil 1988; Mórocz et al. 1990; Potrykus 1989; 1991). Cereal plants have already been regenerated from cultured protoplasts (Lazzeri and Lörz 1988; Lörz et al. 1988; Vasil 1988; Roest and Gilissen 1989) of embryogenic cell suspension-origin (Jones 1985; Morrish et al. 1987; Lazzeri and Lörz 1988; Lörz et al. 1988; Vasil 1988; Potrykus 1989). However, from protoplast cultures of wheat regeneration often fails; its frequency is too low or the regenerated plants have low viability and cannot set seed. From wheat protoplasts derived from cell suspensions, Maddock (1987) could regenerate only roots, Hayashi and Shimamoto (1988) roots and albino shoots, Lee et al. (1988) shoot meristems and leaves, Djardemaliev et al. (1992) roots and embryogenic structures.

Nevertheless, other authors were successful in regenerating green plantlets and plants from protoplasts of wheat (Harris et al. 1988; Wang et al. 1988; 1990; Vasil et al. 1990; Chang et al. 1991; Guo et al. 1991; He et al. 1992; Li et al. 1992a, b; Qiao et al. 1992). Vasil et al. (1990) reported that protoplasts from non-regenerable embryogenic suspension cells of wheat produced protocolonies only, while the regenerable embryogenic counterparts developed into plantlets. Independently of the original callus type as source of suspension cell culture, regenerative capacity of the suspension cells seems to be a prerequisite of plant regeneration from protoplasts of wheat, similarly to other cereals and grasses (maize: Prioli and Söndahl 1989; Shillito et al. 1989; Mitchell and Petolino 1991; barley: Jähne et al. 1991; rice: Fujimura et al. 1985; Jenes and Pauk 1989; Datta et al. 1990; napier grass: Vasil et al. 1983; ryegrass: Dalton 1988).

In this paper, we describe a protocol for protoplast isolation from highly embryogenic suspension cell lines of immature embryo- and friable embryogenic callus-origin in wheat (*Triticum aestivum* L.), for protoplast culture on different media, where the embryogenic capacity of protocalli could be maintained over 4 months by multiple subcultures, and for the subsequent plant regeneration resulting in a fertile plant. None of the authors cited above mentioned regeneration of mature, fertile plants from wheat protoplast cultures.

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Materials and methods

Cell suspension culture. Fine regenerable embryogenic cell suspension cultures (RECS), suitable for protoplast isolation were initiated from friable, non-mucilagineous, rapidly growing and embryogenic calli of winter wheat cv. GK Ságvári (Ahmed and Sági, unpublished; Fig. 1). Weekly subcultures were made in liquid MS medium supplemented with 2 mg/l 2,4-D, agitated on a rotary shaker (130 rpm) at 26°C under 16 h/8 h light/dark cycle.

Protoplast isolation and purification. Protoplasts were isolated from 4-12 month-old RECS (KSV-90A and KSV-91A cell lines). Two to six days after subculture, a fine suspension of cells (1 gram fresh weight) was incubated with 10 ml filter-sterilized enzyme solution (pH 5.6) in 100x15 mm Petri dishes at 26°C in the dark without shaking for 3 to 4 hours. The modified enzyme solution (Djardemaliev et al. 1992) consisted of 2% (w/v) cellulase "Onozuka" RS (Yakult, Japan), 0.5% (w/v) Driselase (Fluka, Switzerland), 0.1% (w/v) Pectolyase Y-23 (Seishin, Japan) dissolved in 6.5% (v/v) glycerol, 1.0% (w/v) $CaCl_2$. $2H_2O$, 0.1% (w/v) $MgSO_4$. $7H_2O$ and 0.05% (w/v) KH_2PO_4. The enzyme solutions described by Vasil et al. (1990), and Wang et al. (1990) were also used. The protoplast-enzyme mixture was filtered through a series of nylon mesh sieves (pore sizes: 80, 60, and 40 μm) with W5 washing solution (Menczel et al. 1981). Then, the protoplasts were collected by centrifugation at 800 rpm for 5 min. The sediment was resuspended in 5 ml sucrose solution (0.50-0.56 M), overlayered with 2 ml W5 solution, and centrifuged at 800 rpm for 5 min. The floating protoplasts were collected, resuspended in W5 solution, and counted using a Buerker chamber before the last and 3rd centrifugation.

Protoplast culture. Purified protoplasts were cultured in 1 to 2 ml liquid or low-gelling-temperature agarose (Sigma) medium in 35x10 mm plastic Petri dishes (Corning, N.Y, USA), at a density of $5x10^5$/ml. Four basal media were used: KM8p (Kao and Michayluk 1975), GM (Li and Murai 1990), L_1 (Lazzeri et al. 1991), and MS (Murashige and Skoog 1962). All media were supplemented with 0.6 M glucose, 1 or 2 mg/l 2,4-D, and 600 mg/l MES (2 [N-morpholino]-ethanesulfonic acid) buffer. Prior to filter sterilization, the pH of the media was adjusted to 5.8 with diluted HCl or NaOH, and osmolarity to 700±50 mOsm/kg H_2O with mannitol. For the agarose-embedded culture, the protoplasts were suspended in a double-strength liquid medium and mixed with the melted agarose solution at a ratio of 1:1 (final agarose concentration: 1.2%). Protoplast cultures were incubated at 26°C in the dark for 4 to 6 weeks. Division frequency (dividing protoplasts in per cent of total protoplasts plated) was determined after 2 weeks in liquid culture and plating efficiency (number of protocalli about 1 mm Ø, as a per cent of the actual number of cultured protoplasts) was estimated after 5 weeks. Five to six week-old protocalli were transferred onto callus growing MS medium (supplemented by 0.5 mg/l each of nicotinic acid, pyridoxine.HCl, thiamine.HCl, 2 mg/l glycine, 150 mg/l asparagine, 100 mg/l myo-inositol, 1 mg/l each of IAA and zeatin, 3% sucrose, 0.2% Gelrite). Cultures grew under 26°C in the dark or in 16 h photoperiods for 4 to 5 weeks.

Plant regeneration. Protocalli were transferred to MS regeneration medium (similar to MS callus growing medium, but containing 0.5 mg/l each of IAA and BAP, 2% sucrose, 10 mg/l $AgNO_3$) in 16 h light. Shoots/plants were transferred for rooting on full- or half-strength hormone-free N6M (Mórocz et al. 1990) or MS medium. After removal of the regenerants, remnant highly embryogenic callus pieces were subcultured on fresh MS regeneration medium (half-strength, with 0.5 mg/l each of IAA and BAP). Subcultures were repeated every 4-5 weeks. The at least 6 cm tall plants were transplanted to clay pots filled with garden earth, covered with plastic bag and watered with Hoagland nutrient solution (Gamborg and Wetter 1975) for 1 to 2 weeks. The 4 week-old established plants were vernalized at 5°C for 7 weeks, then grown in the greenhouse under a light and temperature regime of 16/8 h and 20/15°C, respectively.

Determination of chromosome number. Metaphase chromosome number of several regenerated plants was determined in root tip squashes. Root tips were immersed in 0.1% (w/v) colchicine solution for 2 h at room temperature, fixed overnight with acetic acid : ethanol (1:3), stored in 70% ethanol, rinsed with water and hydrolyzed for 12 min in 1N HCl at 60°C. Staining was then performed with 0.5% acetocarmine and the chromosomes were counted under a light microscope.

Results

Protoplast isolation and purification

From RECS of KSV-90A and KSV-91A cell lines, 2 to 6 days after subculture, $8x10^6$ purified protoplasts per one gram initial fresh weight could be obtained in average at the end of a 4 h incubation period, using the modified enzyme solution of Djardemaliev et al. (1992). Effectiveness of this enzyme solution decreased when glycerol was replaced by mannitol (Table 1). Two other enzyme solutions (Vasil et al. 1990, Wang et al. 1990) released fewer protoplasts, even after a prolonged incubation (Table 1). Gentle shaking (2-3 times in hourly intervals) completed cell wall digestion. Spontaneous protoplast fusions were rare. The purified protoplasts were spherical, rich in cytoplasm and free from undigested cells (Fig. 2).

Table 1. Activity of various enzyme solutions on the yield of wheat protoplasts released from RECS, KSV-90A cell line. Data are averages of three experiments

Enzyme solution		Incubation time (h)	No. of purified protoplasts ($x10^6$/gram)
Vasil et al. (1990)		16	3
Wang et al. (1990)		8	5
Djardemaliev et al. (1992)	with glycerol	4	8
	with mannitol	5	4

Protoplast culture

After 48-72 h in culture, the protoplasts increased in size, regenerated new cell walls and became oval. The first cell divisions occurred after 4 d of culture, followed by microcolony formation (Figs. 3,4) and colony formation after 3 weeks (Fig. 5). Division frequency and plating efficiency were found to be dependent on the protoplast culture media. Protoplast division frequency varied from 8% to 40%. Plating efficiency was highest in agarose and liquid KM8p (0.11%, 0.10%) and in liquid and agarose GM media (0.09%, 0.08%), respectively. Protoplast-derived cells divided in MS and L_1 medium at low frequency, and calli were formed in MS medium only (Table 2).

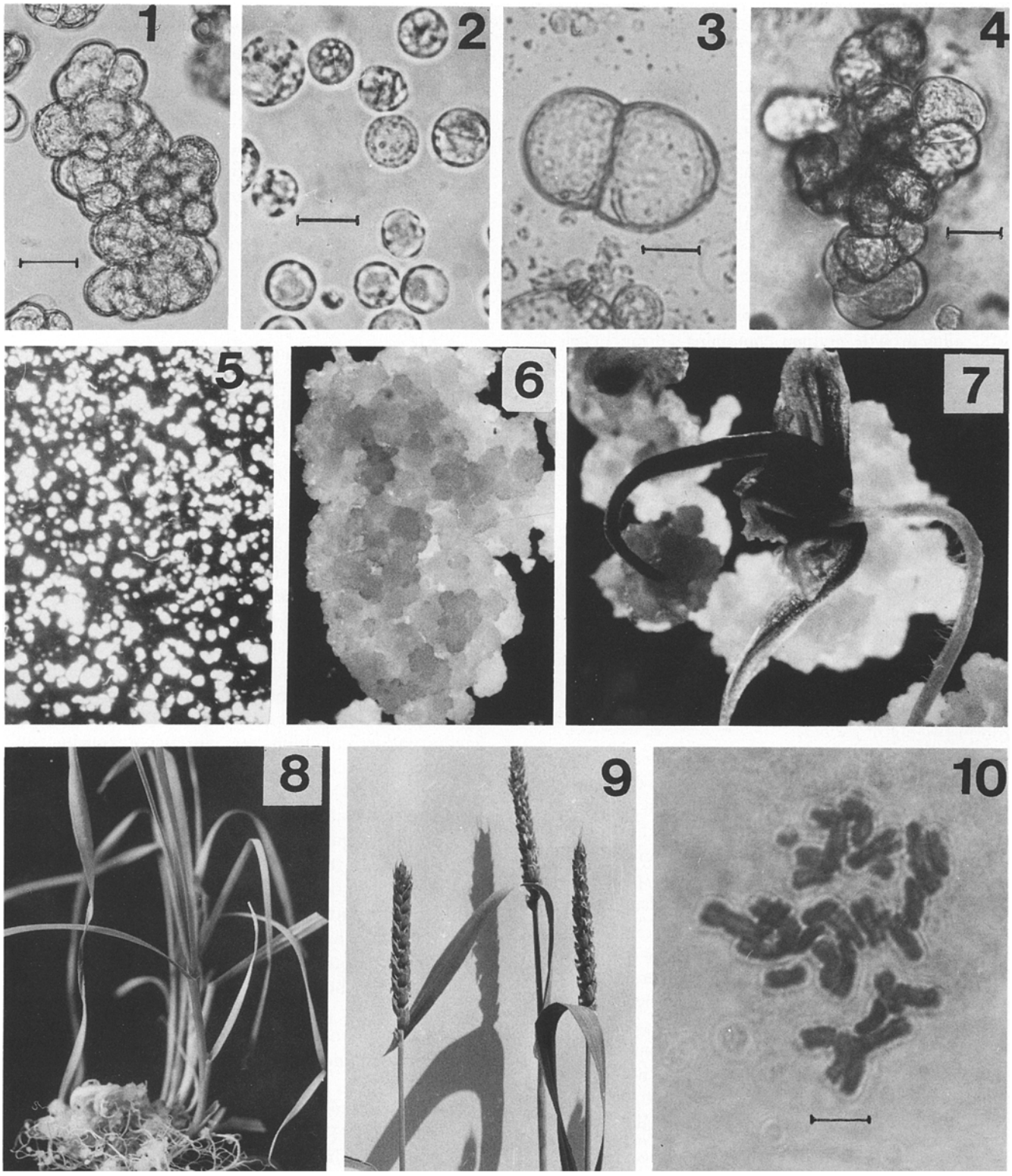

Fig. (1-10). Culture of and regeneration from RECS-derived GK Ságvári wheat protoplasts. 1) Clusters of densely cytoplasmic cells, bar = 100 μm; 2) freshly isolated, RECS-derived protoplasts, bar = 100 μm; 3,4) first cell division and cell colony formation from RECS-derived protoplasts, 4 and 10 days after plating in solid $KM_{8}p$ medium, respectively, bar = 25 μm and 100 μm; 5) protocalli (approx. 1 mm) formed from protoplasts cultured in liquid GM medium, 5 weeks post-isolation (2x); 6) embryogenic calli developed from protocalli on MS growing medium (7.5x); 7) regenerating plantlet on MS regenerating medium (7.5x); 8) complete plant ready for transplanting; 9) fertile regenerant at anthesis; 10) Aneuploid chromosome number (2n = 31) of a regenerated plant, bar = 10 μm.

Table 2. Division frequency and plating efficiency of cultured RECS-derived wheat protoplasts in different liquid or agarose-solidified media as a per cent of actually cultured protoplasts. Data are averages of three experiments

Culture media	Division frequency (%) [a]	Plating efficiency (%) [b]	
		Liquid	Agarose 1.2%
KM8p	40	0.10	0.11
GM	37	0.09	0.08
MS	20	0.03	0.02
L_1	8	0.00	0.00

[a] at two weeks in liquid culture
[b] at five weeks in liquid or agarose culture

Protocalli of 1 mm diameter were transferred to solid MS callus growth medium containing 1.0 mg/l each of IAA and zeatin (Ahmed and Sági, unpublished). In this medium, the protocalli grew rapidly (about 10-fold mass multiplication in every 4-5 weeks) at 26°C in the dark or in 16 h photoperiods. They were friable, nodular, highly embryogenic, and similar to the calli used to initiate RECS (Fig. 6).

Plant regeneration

Protocalli, divided to small pieces (3-4 mm Ø) and plated on MS regeneration medium supplemented with IAA+BAP, 0.5 mg/l each, grew and formed embryogenic structures which developed into shoots/plants after 11 to 12 weeks of protoplast isolation (Fig. 7). The primary regenerants were transferred either to fresh MS regeneration medium or to rooting media.

Protocalli maintained their morphogenic capacity over 4 months. After removal of regenerants, callus pieces were transferred to fresh half-strength MS regeneration medium containing IAA+BAP, 0.5 mg/l each. Multiple subcultures resulted in an increased total number of regenerants, e.g. 51.5% shoots/plants at the 2nd subculture (16 weeks after protoplast isolation, Table 3).

Root formation was induced on hormone-free MS or N6M medium (Fig. 8), but the regenerants rooted poorly. Rooting was not supported by 0.5 mg/l IAA+0.02 mg/l kinetin or other hormones (BAP, IBA, GA3, NAA, 2,4-D) used singly or in combination at 0.05-5.0 mg/l. Putrescin (2.4 mg/l) did not promote root formation significantly. However, to date more than 1000 shoots/plant have been recovered.

Table 3. Number of regenerated shoots/plants from wheat protocalli by multiple subculturing on MS regeneration medium (2nd to 4th subculture on half-strength MS medium)

No. and (%) of protocalli transferred to MS regeneration medium	No. and (%) of regenerated shoots/plants from different subcultures				
	1st	2nd	3rd	4th	total
476 (100.0)	17 (4.7)	185 (51.5)	105 (29.2)	52 (14.5)	359 (75.4)

Over 500 plants regenerated from different protocalli and subcultures were transplanted into pots, but only 50% of them survived 2 to 16 weeks after transplantation in the greenhouse. However, one tiller of a regenerated plant grew further and produced six secondary fertile tillers. All these tillers headed and set normal, germinable seeds (Fig. 9).

The mitotic chromosome number of the regenerants ranged from 14 to 42 (Fig. 10). Only 7% of the root tip cells had the normal bread wheat chromosome complement (2n = 6x = 42).

Discussion

According to the literature, cereal and grass plants can successfully be obtained from protoplasts of RECS (Vasil et al. 1983; Fujimura et al. 1985; Dalton 1988; Jenes and Pauk 1989; Prioli and Söndahl 1989; Shillito et al. 1989; Datta et al. 1990; Vasil et al. 1990; Chang et al. 1991; Jähne et al. 1991; Mitchell and Petolino 1991; He et al. 1992; Qiao et al. 1992). RECS of GK Ságvári wheat variety, which served as sources for regenerable protoplasts, maintained their morphogenic capacity over 2 years and the protocalli derived from their protoplasts also remained regenerable in long-term culture. Therefore, before starting protoplast isolation from embryogenic cell suspension (ECS) of cereals and grasses, it is advisable to test the regenerating ability of the donor ECS. Frequency of plant regeneration from ECS can provide useful information about the effect of media, culture conditions etc., also when studying cell-protoplast-plant systems in monocots (Vasil et al. 1990, Jähne et al. 1991).

In agreement with Djardemaliev et al. (1992), it was found that for preparing wheat protoplasts from suspension cells, glycerol was superior to mannitol as osmoticum. Thus, besides the composition of the enzyme solution, the nature of the osmoticum also plays an important role in the cell-wall digestive activity (Table 1). However, protoplasts obtained with a glycerol-containing enzyme solution needed careful washing and purification. We could increase the yield of purified protoplasts up to 18×10^6/g f. w. using a longer centrifugation (15 min) and a larger volume of W5 washing solution.

Among the tested protoplast culture media, KM_8p and GM proved best (Table 2). Vasil et al. (1990), Wang et al. (1990) and Chang et al. (1991) also found the KM medium to be most suitable for protocallus formation in wheat. On GM medium low in ammonium, rice protoplasts could be plated with high efficiency (Li and Murai 1990). As stated by Datta et al. (1990) and Vasil et al. (1990), application of nurse cells (Hayashi and Shimamoto 1988) was not necessary.

Division frequency (37-40%, Table 2) of the protoplasts was comparable with those of Wang et al. (1990)

Djardemaliev et al. (1992) and Qiao et al. (1992) but considerably higher than that described by Maddock (1987), and Li et al. (1992b). Frequency of protocallus formation (0.11% at best, Table 2) did not reach the frequencies (0.2% and 0.1-5%) reported by Maddock (1987) and He et al. (1992), respectively.

The protocalli developed on MS callus growth medium into friable, nodular and embryogenic calli, resembling to those used for initiation of the suspension cultures, as observed by Maddock (1987) and Müller et al. (1989).

In our hands the protocalli regenerated shoots or plants with much higher frequency (4.7% to 51.5%, depending on the subcultures, Table 3) than in the experiments of He et al. (1992, 0.5% to 17%). Calculated on the average number of plated wheat protoplasts ($0.5x10^6$), this corresponds to 0.075% for the total four subcultures. Li et al. (1992a,b) reported a regeneration frequency of 0.0015-0.0018% and 0.00001%, respectively. Regeneration results (68 shoots/plantlet from $1.8x10^7$ protoplasts) of Chang et al. (1991) were even lower.

In our wheat protoplast system, plant regeneration occurred indirectly, via callus formation and somatic embryogenesis, as described by Harris et al. (1988), Hayashi and Shimamoto (1988), Wang et al. (1988), He et al. (1992) and Li et al. (1992b). Regeneration through direct embryogenesis excluding somaclonal variation and producing plants from protoplasts in a shorter time was achieved by Vasil et al. (1990), Guo et al. (1991) and Li et al. (1992a). However, direct somatic embryogenesis is not a guarantee for a prolonged regenerating ability (Li et al. 1992a).

It is known that in wheat suspension cells, as well as in protoplasts and in plants recovered from them, chromosomal aberrations can occur (Karp et al. 1987; Wang and Nguyen 1990; Chang et al. 1991; Shang and Wang 1991). In contrast to the results of He et al. (1992), the mitotic chromosome number of our regenerants was normal (2n = 6x = 42) only in exceptional cases. Thus, restricted rooting, formation of functionally defective roots or low viability as observed by Chang et al. (1991) and Li et al. (1992a) can be due to aneuploidy or other chromosomal anomalies. The chromosomal defects have probably been repaired in the tillers, which grew normally, produced fertile ears, set normal, germinable seeds and reached maturity.

To the best of our knowledge, regeneration of fertile plants from protoplast culture of wheat has not yet been reported. The wheat has never been selected for regeneration from cell and protoplast culture, therefore, it is plausible that the output of fertile plants from such an *in vitro* culture is occasional. However, further research certainly will solve this particular problem, as happened formerly in the case of somatic callus and anther culture of wheat and other cereals.

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