FERTILE WHEAT (*TRITICUM AESTIVUM* L.) REGENERANTS FROM PROTOPLASTS OF EMBRYOGENIC SUSPENSION CULTURE

János PAUK[1], Zoltán KERTÉSZ[1], Barnabás JENES[2], László PURNHAUSER[1], Outi MANNINEN[3], Seppo PULLI[3], Zoltán BARABÁS[1] and Dénes DUDITS[4]

[1] Cereal Research Institute, Wheat Genetics and Breeding Department, H-6701 Szeged, P.O. Box 391, Hungary

[2] Agricultural Biotechnology Center, Institute for Plant Science, H-2101 Gödöllő, P.O. Box 170, Hungary

[3] Agricultural Research Centre, Institute of Plant Breeding, SF-31600 Jokioinen, Finland

[4] Biological Research Center, Institute of Plant Physiology, H-6701 Szeged, P.O.Box 521, Hungary

Author for correspondence to

János PAUK[1]

Telephone: 36-62-435-235

FAX: 36-62-434-163

We report the regeneration of fertile, green plants from wheat (*Triticum aestivum* L. cv. Aura) protoplasts isolated from an embryogenic suspension initiated from somatic early-embryogenic callus. The present approach combines the optimization of protoplast culture conditions with screening for responsive genotypes. In addition to the dominant effect of the culture media, the increase in fresh mass and the embryogenic potential of somatic callus cultures varied considerably between the various genotypes tested. Establishment of suspension cultures with the required characters for protoplast isolation was improved by reduction of the ratio between cells and medium and by less frequent (monthly) transfer into fresh medium. These suspension cultures with different enzyme solutions yielded a sufficient number of protoplasts and a new washing solution was introduced to avoid the aggregation of protoplasts. However, the influence of the culture medium on cell division was variable in the different genotypes. We could identify cultures from cultivar Aura that showed a near by 9% cell division frequency and morphogenic response. The protoplast-derived microcolonies formed both early and late-embryogenic callus on regeneration medium and green plants were obtained through somatic embryogenesis. Regenerants were transferred to soil and a few fertile plants were recovered under greenhouse conditions. Their self and cross-pollinated progenies were 92% viable.

Key words: protoplast culture, somatic embryogenesis, plant regeneration, fertile plant, *Triticum aestivum* L.

Introduction

Genetic manipulation of higher plants has been successfully achieved by the use of protoplast technology for somatic hybridization and efficient production of transformants in a wide range of plant species, including cereal crops [1]. It is evident that regeneration of fertile plants from cultured protoplasts is an essential prerequisite for the application of these methodologies. In contrast with several important cereals such as rice [2], maize [3,4] and barley [5], progress in the development of a culture system for regeneration of plants from wheat protoplasts has been limited mainly by the low efficiency and the lack of seed production on protoplast-derived regenerants.

Despite the fact that cell division and colony development from wheat protoplast cultures were reported [6,7,8] long time ago, the hexaploid (2n=6x=42) *Triticum aestivum* L. has been found to be one of the most recalcitrant cereal species as far as the establishment of a suspension culture of totipotent cells [9] and a protoplast-plant system [10,11] are concerned.

Considerable efforts have been made to isolate protoplasts from different inoculates in wheat [7,9,12], but the most successful way to sustain divisions in protoplast culture is via the use of a suspension culture as the source of protoplasts [9,13], similarly as for other monocot species [4,14,15]. Protoplasts isolated from suspension cultures could readily be induced to divide in culture after resynthesis of the cell wall [9,16,17]. In attempts to culture wheat protoplasts, the first green protoplast-derived plantlet was regenerated from an anther-derived suspension culture [18]. This result was confirmed by others [19,20]. The approach used by Hayashi and Shimamoto [19] was based on the isolation of protoplasts from immature embryos or young embryogenic calli. These experiments resulted only in the low regeneration of green or albino plantlets from wheat protoplast cultures. Regeneration of fully developed plants was not achieved in these culture systems.

An essential increase in the number of protoplast-derived plants was achieved by Vasil et al. [10] through the initiation of suspensions from an immature embryo-

derived 'aged' compact callus. This tissue material exhibited structural characters similar to Type II [21] or early-embryogenic [4] callus tissues in maize. Protoplasts isolated from these suspensions continued to form microcalli, and a relatively high number of green plants were obtained through somatic embryogenesis. Morphologically healthy plants were transferred to soil, but fertile seeds were not obtained in these experiments [13]. Furthermore, a few reports recently described plant regeneration from cultured wheat protoplasts [22,23,24,25]. These protoplast-derived plantlets were successfully transferred to soil [22,24,25], but fertile plants could not be obtained [22,25] or the production of seeds was not reported [23,24,25].

The present publication describes the regeneration of morphologically normal and fertile plants from suspension-derived protoplasts of the Finnish cultivated winter wheat cv. Aura, and an improvement of the protoplast technology of hexaploid wheat.

Materials and methods

Terminology

In *in vitro* cultures of various organs, two types of callus tissues can be clearly distinguished: (1) homogeneous tissues with fully dedifferentiated cells and a relatively high water content; (2) fast-growing friable or compact tissues with embryogenic structures. The embryogenic potential is a great importance for plant regeneration and the differentiated morphogenic centers can show different morphological features during ontogeny of the culture. The friable embryogenic tissues consist cytoplasm-rich cells, and show similarities to cultures called Type II [21], aged friable embryogenic [22,26] or early-embryogenic [4] cultures. The other tissue type is a more compact, whitish callus which exhibits embryo-like structures on its surface and is named Type I [21], compact embryogenic [26] or late-embryogenic [4]. We will adopt the terminology of Mórocz et al. [4] in order to eliminate the ambiguity often found in the literature.

Plant material and callus initiation

Suspension cultures were initiated from haploid and somatic callus cultures of Hungarian and Finnish spring and winter wheat varieties and breeding lines. Somatic callus cultures were developed from immature inflorescences and embryos of field-grown plants. Caryopses and internodes containing the suitable inoculum were treated as described previously [27]. The haploid callus was obtained from anther culture [28] and maintained on $MSOu_1$ medium (see below). Callus cultures were initiated, selected and propagated in a dark thermostat at 28 ^{o}C and subcultured every 4 weeks. For induction of regeneration, the callus cultures were incubated at 28 ^{o}C in a growth room with a 16 h day/8 h night cycle, under fluorescent light of about 1500 lux intensity.

Media of callus, suspension and protoplast cultures

The primary callus cultures were generally initiated on $MSOu_2$ medium, which was an agar-solidified MS medium [29] supplemented with 150 mg/l asparagine [30] and 2 mg/l 2,4-D. $MSOu_1$ medium was the same as MSOu2, but the amount of 2,4-D was reduced to 1 mg/l. In addition, G_1 [31], N_6M_1 [4] and D_1 basic [32] media were prepared after recipe from the authors and supplemented with 1 mg/l 2,4-D and 3% sucrose. The culture media were solidified with 2.5 g/l Gelrite and the pH was adjusted to 5.8 with 1 M and 0.1 M KOH before autoclaving. Suspensions were initiated and maintained in a liquid medium, $MSOu_1$.

Using the agarose bead culture technique [33], the following three protoplast media were tested: **R-2PWpp**: major and minor elements, iron complex, vitamins and glycine after Kyozuka et al. [2]; **MSWpp**: M 5519 powder medium (Sigma) in the normal dose; **KaoWpp**: double strength KM8p without hormones [34]. Each medium was supplemented with 1 mg/l 2,4-D and $\approx$0.6 M glucose and adjusted to 700 mOsm. The pH was adjusted to 5.6 before sterile filtration. An agarose component was prepared from 1.2% low-melting Sigma type VII agarose in R-

2PWpp and MSWpp medium under sterile conditions and heated in a pressure cooker for 5 minutes. In the case of KaoWpp, the 1.2% agarose component was prepared in doubly distilled H_2O and autoclaved for 18 min. Agarose components were preserved at 42 ^{o}C till use and mixed with liquid culture medium component (1:1 ratio) including protoplasts in Greiner plastic Petri dishes.

Isolation of protoplasts

Protoplasts were isolated after 4 days of subculturing. Approximately 1.5 g settled cell and tissue material from the suspension culture was treated in 10 ml enzyme solution prepared as follows: $\mathbf{E_4K}$ modified from Jenes and Pauk [35]: Onozuka RS cellulase 3% (Yakult Honsha Co., Tokyo), pectinase 1%, Driselase 2% (Fluka), MES 585 mg/l, NaH_2PO_4 100 mg/l, $CaCl_2x2H_2O$ 1.0 g/l, mannitol of 0.3 M, D-sorbit of 0.3 M, pH 5.6; $\mathbf{E_D}$ (D. Djardemaliev pers. comm.): Onozuka RS cellulase 2%, Driselase 0.5%, pectolyase Y-23 0.1% (Seishin Pharmac. Co., Tokyo), glycerine 11%, $CaCl_2x2H_2O$ 1%, $MgSO_4x7H_2O$ 0.1%, KH_2PO_4 0.05%, pH 5.6; $\mathbf{E_{LL}}$ [14]: Onozuka RS cellulase 1%, Macerozyme R-10 0.5% (Yakult Honsha Co.), pectolyase Y-23 O.1%, casein hydrolysate 0.1%, mannitol 0.6 M, pH 5.8, 700 $\pm$ 5 mOsm.

The mixture of undigested cells and protoplasts was filtered through a nylon mesh series (120-80-40 μm) and sedimented for 5 min. at 90 g. If necessary, protoplasts were further purified by banding over 0.5 or 0.55 M sucrose, overlayered in washing solution. Since the W_5 [36] washing solution caused the aggregation of protoplasts during the washing procedure, we used W_{MS} (M 5519 powder medium 4.4 g/l, Sigma) as washing solution with mannitol at 700 mOsm. Protoplasts were washed and sedimented twice in washing solution. The protoplast viability was determined by fluorescein diacetate staining and the protoplast density was measured with a haemacytometer. Protoplasts were suspended at a density of $0.8x10^6$/ml in liquid culture medium for 15 min. and mixed with the same quantity of 1.2% agarose component.

Plant regeneration in vitro and growth in greenhouse

After 6-8 weeks in culture, the protoplast-derived callus colonies 1 mm in diameter were plated onto MSR_1 regeneration medium, previously described by Fekete and Pauk [27]. This medium contained of 4.4 g/l Sigma MS 5519 powder medium, 3% sucrose, 0.5 mg/l 2,4-D, 0.5 mg/l kinetin and 2 g/l Gelrite. The pH was adjusted to 5.6 before autoclaving. The plates were incubated in dim light for 2 weeks, then at 1500 lux intensity with a 16 h day/8 h night cycle. The microcalli showed the first organized structures in about 4 weeks. When the late-embryogenic callus started to produce shoots, moderate feeding with hormone-free liquid medium was applied weekly. The components of the liquid medium were the same as in MSR_1, but without hormones and Gelrite. We found it very important to avoid transfer of the developed organized structures onto a new medium during the initiation phase of regeneration. After reaching a height of 2 cm, the plants were transplanted for rooting into MSR_2 medium: 4.4 g/l Sigma MS 5519, 1.5% sucrose, 2 mg/l IAA and 7.5 g/l Sigma agar.

From the tubes, well-rooted and tillered plants were transplanted into pots filled with a mixture of 3:1 common and peat soil without autoclaving. Transplanted plants were covered with plastic bags for about 1 week, depending on the individuals, and then kept under greenhouse conditions. The growth conditions corresponded to the phenophase of the plants during the whole growing season. Vernalization was carried out in a cold chamber for 6 weeks at 2-4 ^{o}C under continuous dim light.

Results

Establishment of callus and suspension cultures

The basic principle in the present work was to combine the optimization of the culture media with screening for responsive genotypes. The effects of three different agar media supplemented with the same auxin and sucrose concentrations were

tested on callus growth and embryogenic character. Fig. 1 depicts the increase in fresh mass and the percentage of embryogenic callus of two spring and six winter wheat genotypes on the three media. As shown by the average fresh mass of callus tissues ($MSOu_1$ x=546%, N_6M_1 x=368%, G_1 x=292%), $MSOu_1$ medium was the most effective in supporting callus growth, but considerable differences were seen between the various genotypes. On the basis of the LSD values, on each medium different varieties exhibited a high, a medium and a low responsiveness (Fig. 1). In addition to analysis of the increase in fresh weight of the callus tissues, the percentage of cultures with embryogenic structures was also scored. Two media ($MSOu_1$ and N_6M_1) can be considered appropriate for the support of embryogenic callus development. The spring breeding line 3908, GK Örzse and GK Barna showed a high embryogenic response on both media. Callus tissues of doubled haploid 812 and the winter variety Aura produced a considerable number of embryogenic calli (early and late) only on $MSOu_1$ medium.

After optimization of the conditions for suspension culture initiation, the $MSOu_1$ medium and its slightly modified form (D_1) were tested with calli from four different genotypes (Fig. 2 A). When the suspensions were initiated in $MSOu_1$ liquid medium, the average increase in fresh mass (x=540%) during 4 weeks was higher than in D_1 medium. In subsequent experiments, $MSOu_1$ liquid medium was used, in which the Aura callus tissues displayed a high growth rate. We also analyzed the influence the of frequency of medium change and the mass of inoculated tissue material on the growth of tissues in suspension (Fig. 2 B). While a suspension initiated from 1 g early-embryogenic callus (Fig. 3 a) increased its fresh mass 6.5-fold without additional passage during 4 weeks, the suspension maintained by weekly passage increased the tissue weight 2-fold. These experiments suggested the use of a reduced inoculum mass and the less frequent transfer of tissues into fresh medium.

Isolation, purification and culturing of wheat protoplasts

Three different enzyme solutions were tested during the optimization of procedures for the isolation of protoplasts from multicellular colonies grown in suspension cultures (Fig. 4 A). The number of protoplasts as a function of the duration of digestion was monitored by using three enzyme combinations. Two enzyme mixtures produced 0.8-0.9 x 10^6/ml protoplasts during the first period (up to 8 h) of the digestion (Fig. 4 A). The prolonged enzyme treatment was less harmful in the case of E_{LL} enzyme. Protoplasts isolated after 14 h were easily damaged during isolation and purification.

Initially, W_5 washing solution was used for the washing and sieving of protoplasts, but aggregation of protoplasts in this solution caused a significant protoplasts loss. The aggregated protoplasts could usually not be dispersed by agitation or other manipulation. Subsequently, a new MS-based washing solution (W_{MS}) was prepared and compared with W_5 solution as concerns the number of single protoplasts during washing and storage (Fig. 4 B). The numbers of protoplasts after two washings (2nd cond.) and 1-hour storage (3rd cond.) in W_5 were significantly lower than in W_{MS}.

The freshly isolated protoplasts were heterogeneous in size and the prepared protoplast population had to be purified. The use of sucrose density gradient centrifugation effectively yielded relatively uniform protoplasts. The damaged, elongated cells did not float on a sucrose cushion after mechanical separation. First, each cell line was tested for the behaviour of their protoplasts during sucrose gradient separation. The optimal sucrose gradient region was determined, and later only one sucrose concentration was used for purification. 0.5-0.55 M sucrose was generally found to be best for density gradient centrifugation (data not shown). Protoplast populations isolated from suspension cultures of different genotypes contained a high number of protoplasts with dense cytoplasm after flotation on a sucrose cushion (Fig. 3 b).

Since the potential of protoplast-derived cells for division is one of the key components in the culture system, we compared the frequencies of divided cells from a variety of suspension cultures, including morphogenic and non-morphogenic lines. The protoplasts were cultured in agarose-solidified media, and the numbers of divided cells were counted 14 days after initiation of the cultures (Table I). Of 7 different cell lines, the oldest non-morphogenic one (H-1132 white) displayed the highest plating efficiency in each medium. Cell lines with a shorter period of *in vitro* culturing gave a division frequency under 10%. We could not detect a correlation between morphogenic potential and division capacity. The influence of the culture medium was variable, but the simple media (MSWpp and R-2PWpp) seemed superior to the more complex (mainly in organic components) KaoWpp (Table I.).

The first protoplast divisions occurred 4-6 days after the initiation of protoplast culturing (Fig. 2 c, d) and the second and third divisions were observed within 10 days (Fig. 2 e). After the fourth week of subculturing, the development of microcolonies was clearly visible. In 8-week-old cultures, the colonies reached 1 mm in diameter. At this stage, the colonies growing intensively in agarose blocks were transferred to regeneration medium.

Recovery of fertile plant and progeny test

For regeneration experiments, we used culture medium previously tested for embryogenic callus formation. Since the medium with 1:1 auxin/cytokinin (0.5-2 mg/l) ratio supported the development of early and late- embryogenic callus in wheat somatic tissue cultures, this medium was also used for the regeneration of protoplast-derived calli. After 4-6 weeks of incubation, the formation of late-embryogenic structures (Fig. 3 f) could be observed. In the first attempts, the late-embryogenic structures were transferred to regeneration medium supported with kinetin as proposed by Fekete and Pauk (1989). In the case of protoplast-derived callus, this step hindered shoot and root development and the transferred late-

embryogenic calli turned brown. Therefore, instead of transferring to a new medium, we fed the cultures with hormone-free MS liquid medium (1.5% sucrose) on the surface of the regeneration medium. This feeding supported the germination of embryos developed *in vitro*. As shown in Table II, we found a genotype-dependent morphogenic response. Despite the fact that calli from protoplasts of both Aura and 1132 genotype showed an organized structure, somatic embryos developed only from cultures of Aura. The majority of regenerated plants originated from these embryogenic cultures (Table II). Nine well-rooted plants were transplanted into soil in the greenhouse, vernalized and grown (Fig. 2 h). In spite of ideal greenhouse conditions, 3 plants did not survive the acclimatization and another 2 died during vernalization (Table II). We determined the chromosome number of the protoplast-derived plants. 67% of the tested root tips exhibited 42 chromosomes, demonstrating their hexaploid nature (Fig. 5 A). 33% showed mostly nulli- and monosomes (Fig. 5 B) or in a few cases we detected trisomes. Out 4 plantlets which survived vernalization, 3 proved to be fertile and 1 sterile. Each plant produced 5-8 heads, which were isolated before blossoming. 1-2 heads/plant were artificially pollinated and these heads showed 95% fertility, while only 42-70% of inbred heads produced seeds. The collected seeds were sown for further tests and propagation. 92% of protoplast-derived inbred R_0 seeds germinated. Among the root tips of R_0 seeds (50% of the seed set was tested), we did not find an abnormal chromosome number. R_1 plants will be grown and used for further tissue culture experiments.

Discussion

This paper demonstrates that the recovery of fertile plants via protoplast technology in wheat (*Triticum aestivum* L.) can be achieved by a complex approach based on the search for responsive genotypes and optimization of the conditions for the callus and suspension culture media, the enzyme and washing solutions, and culture for regeneration of protoplast-derived tissues.

Of three basically different media, MS-based nutrient medium was found to be the most effective in supporting both the growth of the callus fresh mass and the development of an embryogenic callus type, similarly to previously published results on wheat [22,23,26]. Asparagine-supplemented [30] $MSOu_1$ medium supported the induction and maintenance of embryogenic callus in wheat, as in maize [37]. In our experiments, the G basic medium which was successfully used in rice [31], and N_6M [4] applied for maize, both supplemented with 1 mg/l 2,4-D for wheat, provided less optimal conditions for increasing the callus fresh mass. In order to decrease the effect of stresses induced by the *in vitro* culture system we propose the use of a liquid form of $MSOu_1$ medium - with the same medium condition, as in callus culture - for suspension initiation.

Our experiments to develop suspension cultures showed that a weekly change of medium - frequently suggested in the literature for rice [38], maize [3,33] and wheat [10,17] - hindered growth of the fresh cell mass of suspension. A relatively long (one-month) incubation period for the commencement of suspension initiation increased the fresh mass of suspension about 6-fold in one month, in contrast with a weekly passage, which merely doubled the fresh mass during the same period. The beneficial effects of a long passage time in the initiation of suspension cultures for protoplast isolation were suggested by Mórocz et al. [4]. The presented data also confirmed the importance of a low inoculum/medium ratio in the establishment of an appropriate culture, in accordance with the similar results of Mórocz et al. [4] on maize, but in contrast with previous findings on wheat [26], barley [14] and rice [39], which suggested a high inoculate/medium ratio and weekly passage during initiation of the suspension culture.

For the isolation of suspension-derived protoplasts, three different enzyme solutions were tested. In the present work, the advantage of E_{LL} was shown by the isolation of healthy protoplasts that could tolerate various manipulation procedures. The basic components of this solution were adapted from the barley protoplast isolation recipe [14] and modified for wheat. In wheat, E_{LL} was found to be

superior to the E_4K [35] and E_D (Djardemaliev, pers. comm.) solutions used with good results in rice and wheat, respectively.

W_5 washing solution is widely applied in protoplast purification [36,40]. We found that a longer incubation of wheat protoplasts in W_5 solution caused a disagreeable clumping of protoplasts, with an essential loss ($\approx$50%) of protoplasts. W_{MS} includes similar ions as in the suspension and the protoplast culture medium and the protoplasts preserved their form and quantity for a longer period (1 h). The washing solution for barley protoplasts was prepared with a similar considerations [5], while others have used a simple washing solution containing mannitol with optimal molarity [10].

A relatively uniform fraction enriched in cytoplasmic dense protoplasts were obtained by the use of 0.5 M sucrose density gradient centrifugation. The present results proved that this procedure was effective for wheat protoplast purification, similarly as for other monocot species, such as rice [41] and maize [3,33], and dicots [42].

In our experiments, MSW_{pp} medium was found to support a higher rate of cell division and colony formation than G [31] or KM8p [34] based media. The results presented here are in agreement with data of Vasil et al. [10], Chang et al. [22] and Qiao et al. [25], who based their protoplast cultures on MS basic medium and obtained good responses in culturing wheat protoplasts.

On the basis of our experiments, we propose that the similarities in the conditions for various steps in the culturing procedures, e.g. the use of MS basic media for callus induction, propagation and selection, the establishment of suspension cultures and the cultivation of protoplasts can reduce stresess presumably caused by change of the culture media.

In regeneration experiments, a 1:1 auxin/cytokinin ratio in the medium supported the development of an early and late-embryogenic callus similarly as found in previous experiments with wheat somatic callus cultures [27]. When the late-embryogenic callus type was not removed from the original medium and fed

only with hormone-free medium (1.5% sucrose), scutellar-like structures were induced and germinating embryos could be observed in protoplast-derived cultures. In the previously used protoplast culture system, presumably because of deleterious chromosome changes [10,22], fertile plants could not be obtained from protoplast-derived callus cultures. According to the present cytological analysis, 67% of the root tip cells of the regenerated plants exhibited a hexaploid chromosome set and about 33% aneuploid cells were detected. This chromosomal constitution allowed the regeneration of fertile plants, but partial male sterility was also observed. The artificially pollinated heads matured nearly 100% seed sets, while inbred heads (isolated with cellophane bags) produced only 40-70% fertility. 92% of the collected and tested R_0 seeds germinated, and the seedlings showed appropriate viability.

In summary, the described culture conditions permit the production of fertile regenerants from protoplast-derived embryogenic callus cultures. We consider that the observed tissue culture responses can be attributed largely to the special features of cultivar Aura. Further experiments are in progress to increase the efficiency of the described protoplast culture system and to use wheat protoplasts for the production of transformants.

Acknowledgements. We are very grateful to I. Bartók, Á. Fejérvári and I. Pusztai for excellent assistance, and to M. Csősz and B. Dusha for preparing the illustrations. This research was supported by OTKA grant T 5221 and bilateral cooperation between CRI, Szeged, Hungary, and MTTK, Jokioinen, Finland.

References

1 A. Fehér and D.Dudits, Plant protoplasts for cell fusion and direct DNA uptake: culture and regeneration system. In I.K. Vasil (ed.), Plant Tissue Culture: Methods and Applications (1993) in press.

2 J. Kyozuka, K. Shimamoto and H. Ogura, Regeneration of plants from rice protoplasts. In Bajaj (ed.), Plant Prot. Gen. Eng., Vol. 8, Springer-Verlag, 1982, pp. 109-123.

3 L.M. Prioli and M.R. Söndahl, Plant regeneration and recovery of fertile plants from protoplasts of maize (Zea mays L.). Bio/technology, 7 (1989) 589-594.

4 S. Mórocz, G. Donn, J. Németh and D. Dudits (1990) An improved system to obtain fertile regenerants via maize protoplasts isolated from a highly embryogenic suspension culture. Theor. Appl. Genet., 80 (1990) 721-726.

5 A. Jähne, P.A. Lazzeri, M. Jäger-Gussen and H. Lörz, Plant regeneration from embryogenic cell suspensions derived from anther cultures of barley (*Hordeum vulgare* L.). Theor. Appl. Genet., 82 (1991) 74-80.

6 D. Dudits and G. Németh, Methods of somatic plant genetics in cereal research. Faculte des Sciences Agronomiques de L'état. Gembloux, (1976) 127-139.

7 J.C. Chin and K.J. Scott, A large-scale isolation procedure for cereal mesophyll protoplasts. Ann. Bot., 43 (1979) 23-32.

8 I. Potrykus and J. Petruska, In: Potrykus I (ed), Proc. of 6th Int. Prot. Symp., Birkhauser Verlag, 1983, pp 12-14.

9 S.E. Maddock, Suspension and protoplast culture of hexaploid wheat (*Triticum aestivum* L.). Plant Cell Rep., 6 (1987) 23-26.

10 V. Vasil, F. Redway and I.K. Vasil, Regeneration of plants from embryogenic suspension culture protoplasts of wheat (*Triticum aestivum* L.). Bio/technology, 8 (1990) 429-434.

11 H. Bialy, Recalcitrant Cereal. Transgenic wheat finally produced. Bio/technology, 10 (1992) 630.

12 F. Sarhan and D. Cesar, High yield isolation of mesophyll protoplasts from wheat, barley and rye. Physiol. Plant., 72 (1988) 337-342.

13 I.K. Vasil and V. Vasil, Advances in cereal protopast research. Physiol. Plantarum, 85 (1992) 279-283.

14 R. Lührs and H. Lörz, Initiation of morphogenic cell-suspension and protoplast cultures of barley. Planta, 175 (1988) 71-81.

15 S.K. Datta, K. Datta and I. Potrikus, Fertile indica rice plants regenerated from protoplasts isolated from microspore derived cell suspensions. Plant Cell Rep., 9 (1990) 253-256.

16 I.K. Vasil, Isolation and culture of protoplasts of grasses. Int. Rev. Cytol. Suppl., 16 (1983) 79-88.

17 W.C. Wang and H.T. Nguyen, A novel approach for efficient plant regeneration from long-term suspension culture of wheat. Plant Cell Rep., 8 (1990) 639-642.

18 R. Harris, M. Wright, M. Byrne, J. Varnum, B. Brightwell, K. Schubert, Callus formation and plantlet regeneration from protoplasts derived from suspension cultures of wheat (*Triticum aestivum* L.). Plant Cell Rep., 7 (1988) 337-340.

19 Y. Hayashi, K. Shimamoto, Wheat protoplast culture: embryogenic colony formation from protoplasts. Plant Cell Rep., 7 (1988) 414-417.

20 H.B. Wang, X.H. Li, Y.R. Sun, J. Chen, Z. Zhu, R. Fang, P. Wang and J.K. Wei, Culture of wheat protoplast - High frequency microcolony formation and plant regeneration. Science in China, 33 (1990) 294-302.

21 C.E. Green, Somatic embryogenesis and plant regeneration from the friable callus of Zea mays. In: Proc. 5th Int. Congr. Plant Tiss. Cell Cult. Fujiwara, Tokyo, 1982, pp. 107-108.

22 Y.F. Chang, W.C. Wang, C.Y. Warfield, H.T. Nguyen and J.R. Wong, Plant regeneration from protoplasts isolated from long-term cell cultures of wheat (*Triticum aestivum* L.). Plant Cell Rep., 9 (1991) 611-614.

23 Y.M. Yang, D.G. He and K.J. Scott, Establishment of embryogenic suspension cultures of wheat by continuous callus selection. Aust. J. Plant Physiol., 18 (1991) 445-452.

24 D.G. He, Y.M. Yang and K.J. Scott, Plant regeneration from protoplasts of wheat (*Triticum aestivum* cv. Hartog). Plant Cell Rep., 11 (1992) 16-19.

25 Y.M. Qiao, M. Cattaneo, F. Locatelli and E. Lupotto, Plant regeneration from long term suspension culture-derived protoplasts of hexaploid wheat (*Triticum aestivum* L.). Plant Cell Rep., 11 (1992) 262-265.

26 F.A. Redway, V. Vasil and I.K. Vasil, Characterization and regeneration of wheat (*Triticum aestivum* L.) embryogenic cell suspension cultures. Plant Cell Rep., 8 (1990) 714-717.

27 S. Fekete and J. Pauk, Study of the effect of 2,4-D and kinetin on plant regeneration in wheat: two-step efficient plant regeneration. Cer. Res. Comm., 17 (1989) 237-244.

28 J. Pauk, O. Manninen, I. Mattila, Y. Salo and S. Pulli (1991) Androgenesis in hexaploid spring wheat F2 populations and their parents using a multiple-step regeneration system. Plant Breeding, 107 (1991) 18-27.

29 T. Murashige and F. Skoog, A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., 15 (1962) 473-497.

30 G. Ou, W.C. Wang and H.T. Nguyen, Inheritance of somatic embryogenesis and organ regeneration from immature embryo cultures of winter wheat. Theor. Appl. Genet., 78 (1989) 137-142.

31 Y. Chen, Anther and pollen culture of rice. In: H. Hu, H. Yang (eds): Haploids of higher plants *in vitro*. Springer-Verlag, 1986, pp 3-25.

32 L. Purnhauser, P. Medgyesy, M. Czakó, P.F. Dix and L. Márton, Stimulation of shoot regeneration in *Triticum aestivum* and *Nicotiana plumbaginifolia* Viv. tissue culture using the ethylen inhibitor $AgNO_3$. Plant Cell Rep., 6 (1987) 1-4 .

33 R.D. Shillito, G.K. Carswell, C.M. Johnson, J.J. DiMaio and C.T. Harms, Regeneration of fertile plants from protoplasts of elite inbred maize. Bio/technology, 7 (1989) 581-587.

34 K.N. Kao and M.R. Michayluk, Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at very low population density in liquid media. Planta, 126 (1975) 105-110.

35 B. Jenes and J. Pauk, Plant regeneration from protoplast derived calli in rice (*Oryza sativa* L.) using Dicamba. Plant Sci., 63 (1989) 187-198.

36 Menczel L, Nagy I, Kiss Zs and P. Maliga, Streptomycin-resistant and sensitive somatic hybrids of *Nicotiana tabacum* + *Nicotiana knightiana*: correlation of resistance to *N. tabacum* plastids. Theor. Appl. Genet., 59 (1981) 191-195.

37 E.C. Green and R.L. Philips, Plant regeneration from tissue cultures of maize. Crop Sci. 15 (1975) 417421.

38 J.A. Thompson, R. Abdullah and E.C. Cocking, Protoplast culture of rice using media solidified with agarose. Plant Sci., 47 (1986) 123-133.

39 R. Abdullah, E.C. Cocking and J.A. Thompson, Efficient plant regeneration from rice protoplasts through somatic embryogenesis. Bio/technology, 4 (1986) 1087-1090.

40 K. Glimelius, High growth rate and regeneration capacity of hypocotyl protoplast in some Brassicaceae. Physiol. Plant., 61 (1985) 38-44.

41 K. Masuda, A. Kodo-Shiratori and M. Inoue, Callus formation and plant regeneration from rice protoplasts purified by density gradient centrifugation. Plant Sci., 62 (1989) 237-246.

42 C.T. Harms and I. Potrykus, Fractionation of plant protoplast types by iso-osmotic density gradient centrifugation. Theor. Appl. Genet., 53 (1978) 57-63.

Legends of Figs 1-5. and Table I., II.

Figures:

Fig. 1 Genotype-dependent *in vitro* responses in wheat callus cultures. Increase in fresh weight and frequency of embryogenic callus of 8 different wheat genotypes on 3 different agar media. Genotypes: 1. 3908 (spring=s), 2. Siete Cerros (s), 3. Aura (winter=w), 4. M.808 (w), 5. DH 812 (w), 6. GK Örzse (w), 7. GK Barna (w) 8. GK Zombor (w)

Fig. 2 Influence of culture conditions: (A) increase in embryogenic callus fresh weight in callus culture on two media, genotypes: 1. 3908, 2. Siete Cerros, 3. Aura, 4. M. 808; and (B) fresh weight of Aura suspension cultures initiated with weekly and monthly passage.

Fig. 3 a-h. Protoplast-plant system in wheat (cv. Aura) via protoplast culture and plant regeneration. (a) Established early-embryogenic callus for initiation of suspension culture, (b) freshly isolated and purified protoplasts with dense cytoplasm after centrifugation in W_{MS} washing solution, (c) protoplast, with initiation of cell wall formation (arrows) after 4 days of culturing, (d) the first division, (e) actively dividing cells and not-dividing large, vacuolated protoplast (arrow) after 6-7 days of culturing, (f) late-embryogenic callus section (arrows) surrounded by an early-embryogenic tissue developed on MS_R medium, (g) development of shoot on MS_R medium after feeding with hormone-free MS liquid medium, (h) fertile protoplast-derived plant in greenhouse before ripening.

Fig. 4 Optimization of protoplast isolation and washing conditions. Isolation and washing of wheat suspension-derived protoplasts (genotype: 1132 white): (A) isolation of protoplasts in three different enzyme mixtures (E_{LL}, E_D and E_4K) as a

function of time, (B) effects of washing solutions (W_5 and W_{MS}) on percentage of single protoplasts; conditions: (1) protoplasts in enzyme mixture, (2) protoplasts after two washings and centrifugations, (3) protoplasts in washing solution after 1-h incubation.

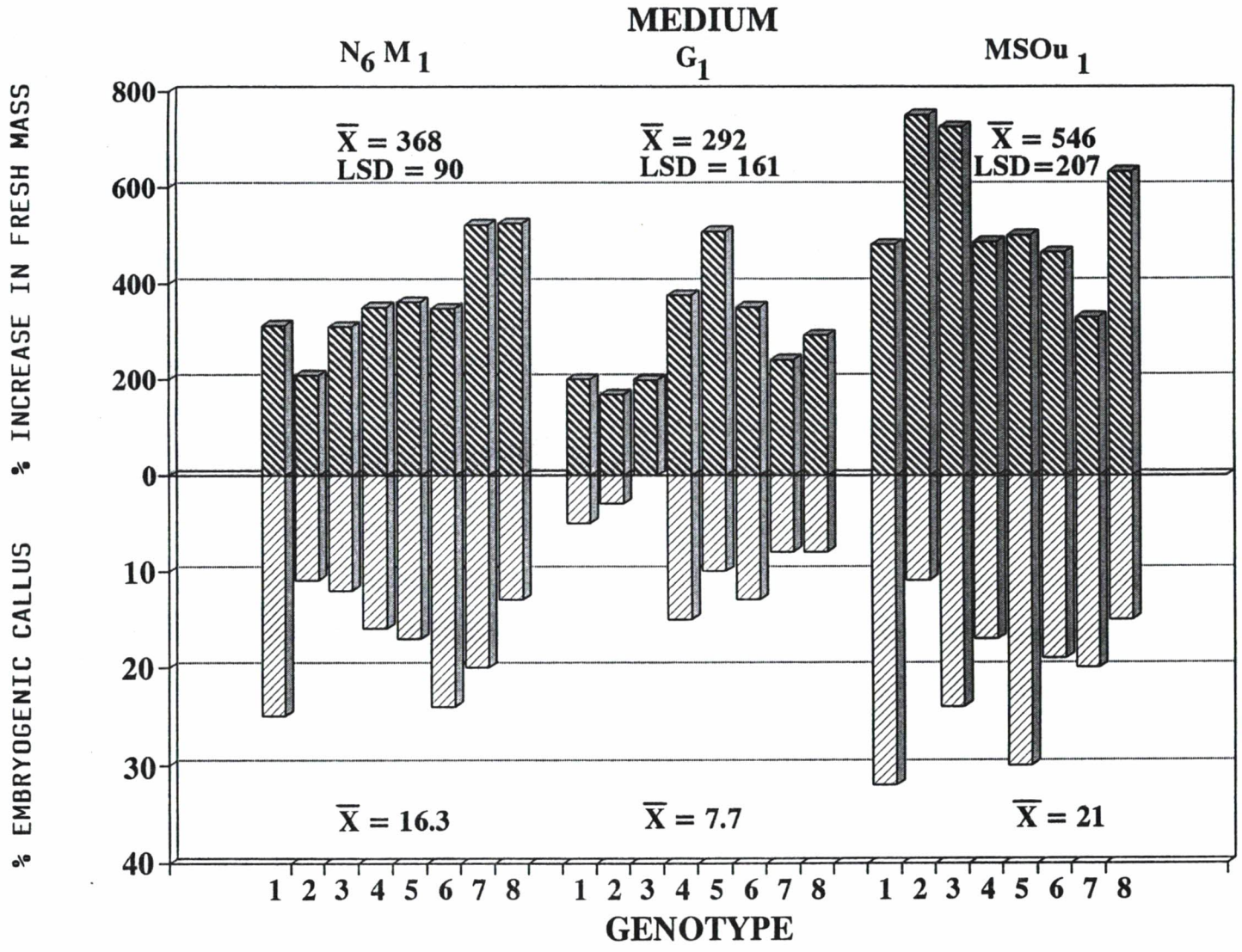
Fig. 5 A-B. Chromosome sets of 'Aura' protoplast-derived plants: (A) normal chromosome number (2n=6x=42) and (B) aneuploid, monosome chromosome set (2n-1=6x-1=41) from cells of root tip of R_0 plant.

Tables:

Table I. Comparison of cell division efficiencies from different cell lines of different ages cultured in three different agarose media on 14th day of culture.

Table II. Regeneration of protoplast-derived callus cultures of five different wheat genotypes.

Fig. 1.


MEDIUM
N6 M 1
G1
MSOu 1
X̄ = 368
LSD = 90
X̄ = 292
LSD = 161
X̄ = 546
LSD=207
X̄ = 16.3
X̄ = 7.7
X̄ = 21
800
600
400
200
0
10
20
30
40
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
GENOTYPE
% EMBRYOGENIC CALLUS % INCREASE IN FRESH MASS

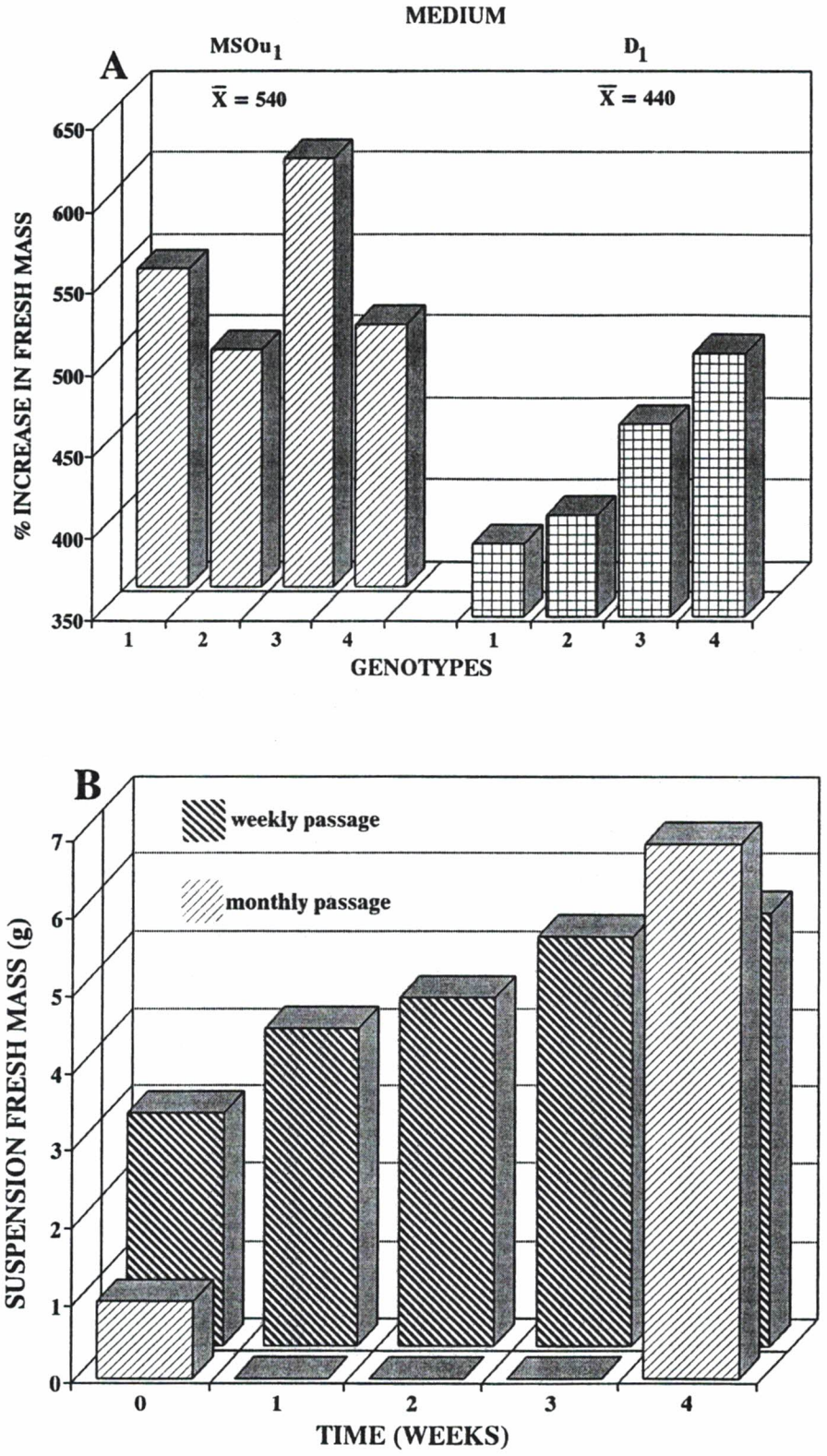

A
MEDIUM
MSOu1
D1
X̄ = 540
X̄ = 440
% INCREASE IN FRESH MASS
650
600
550
500
450
400
350
1
2
3
4
1
2
3
4
GENOTYPES
B
weekly passage
monthly passage
SUSPENSION FRESH MASS (g)
7
6
5
4
3
2
1
0
0
1
2
3
4
TIME (WEEKS)

Fig. 2.

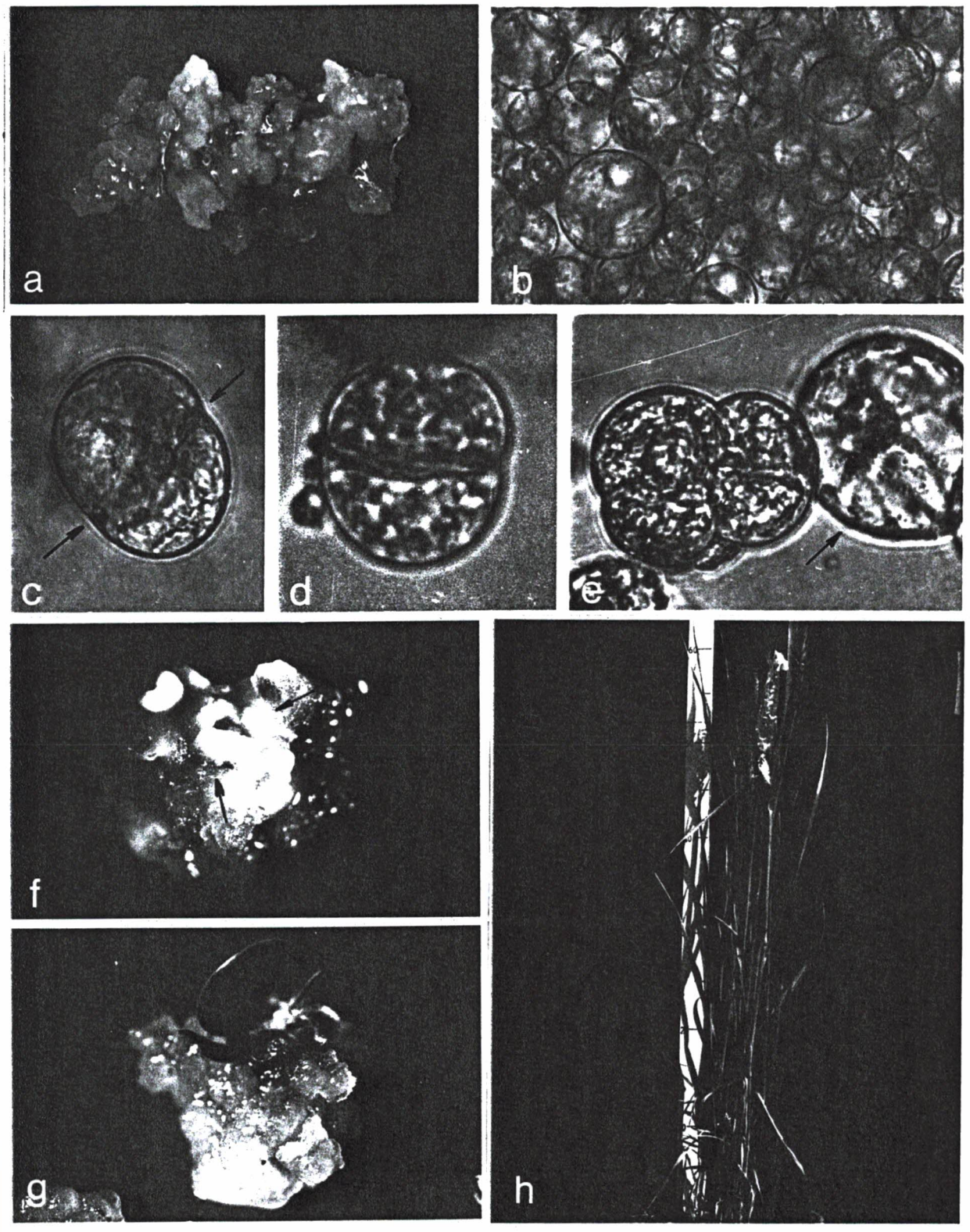
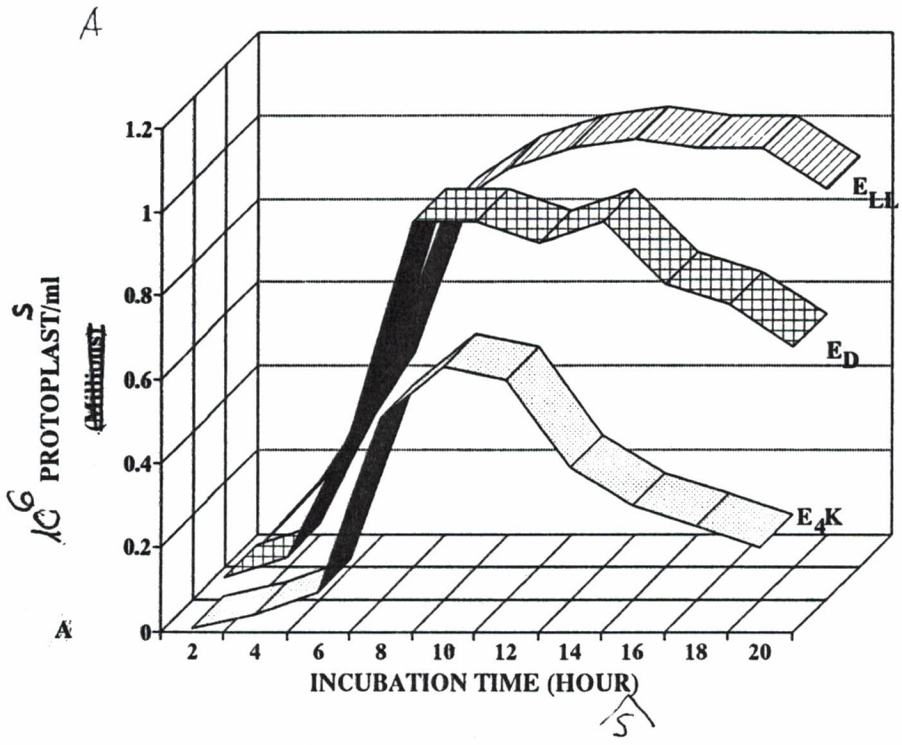


Fig. 3


A
1.2
1
0.8
0.6
0.4
0.2
0
E_{LL}
E_D
E_4K
2
4
6
8
10
12
14
16
18
20
INCUBATION TIME (HOUR)

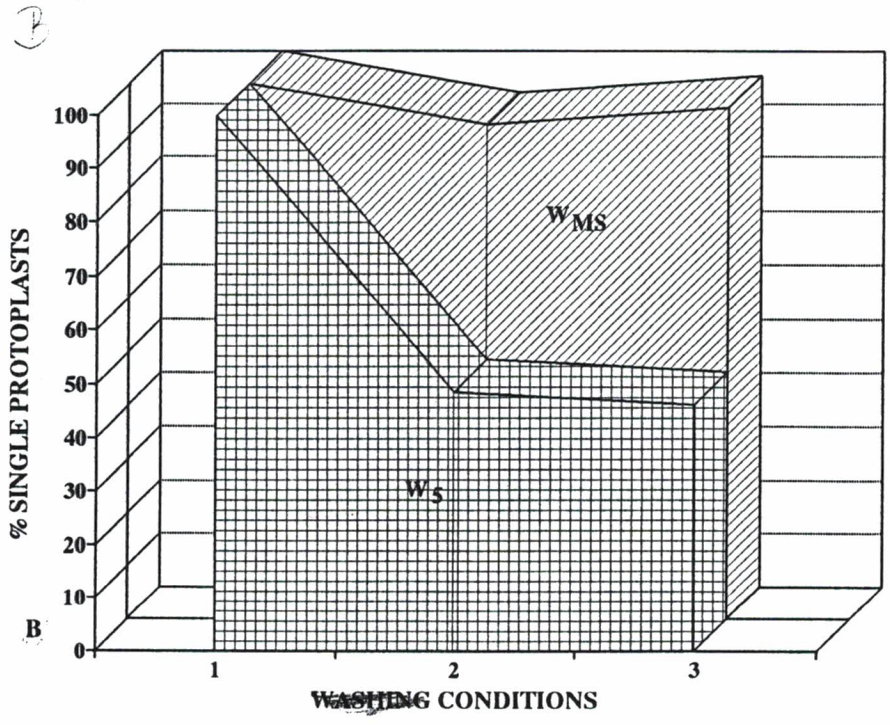

B
100
90
80
70
60
50
40
30
20
10
0
% SINGLE PROTOPLASTS
W_{MS}
W_S
1
2
3
WASHING CONDITIONS

Fig. 4 A-B

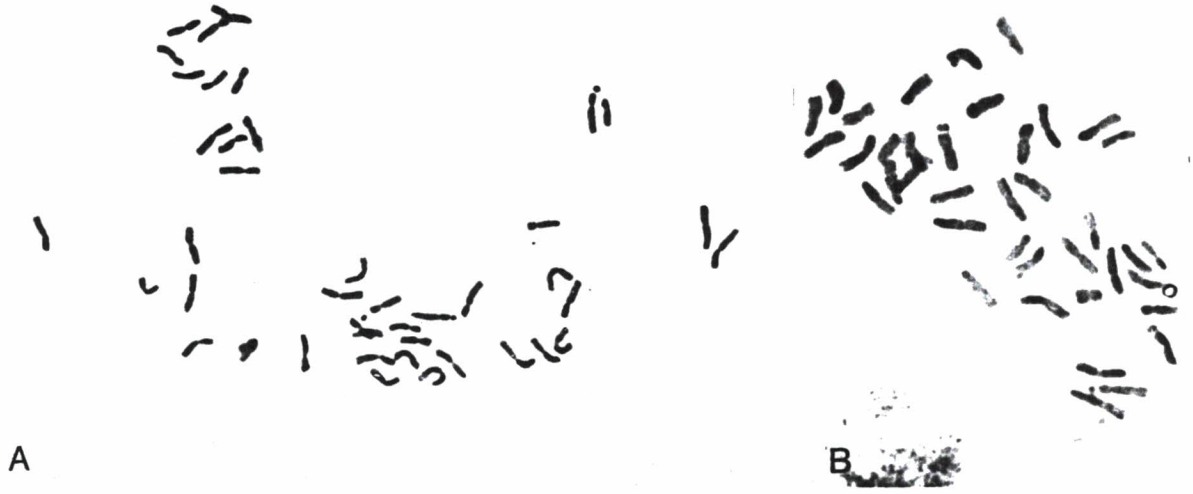


Fig. 5.

Table I.

Cell line	Age of suspension (months)	Culture medium	Divided at least once
H-1132 nm* (white)	37	R-2PWpp MSWpp KaoWpp	13.14 11.04 10.08
H-1132 m** (green)	25	R-2PWpp MSWpp KaoWpp	- 9.61 4.70
H-104 nm	23	R-2PWpp MSWpp KaoWpp	- 3.76 3.58
DH-104 nm	19	R-2WPpp MSWpp KaoWpp	7.40 - -
DH-2260 m	11	R-2PWpp MSWpp KaoWpp	1.8 - -
Aura m	6	R-2PWpp MSWpp KaoWpp	9.13 9.25 7.25
Jo.8351 m	13	R-2PWpp MSWpp KaoWpp	4.19 7.11 3.57

* non-morphogenic
** morphogenic

Table II.

Genotype	Age of suspension at isolation (months)	Transferred calli for regeneration	Morphogenic calli	Embryo-genic calli	Regenerated and transplan-ted plantlets	Adult plants	Fertile plants
Aura	4	382	82	27	8	4	3
1132 green	25	860	610	-	1	-	-
1132 white	37	510	-	-	-	-	-
HD 2260	11	87	14	-	-	-	-
Jo. 8351	9	194	13	-	-	-	-