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# Plant regeneration from embryogenic cell suspensions derived from anther cultures of barley (*Hordeum vulgare* L.)

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**Summary.** We have established embryogenic cell suspension cultures of barley (*Hordeum vulgare* L. cultivars Igri, Gimpel, Princesse, and Baronesse) from anther-derived embryogenic callus. Suspension cultures of cultivars Igri and Gimpel were regenerable. The most successful cultivar was Igri, from which a number of independent cell lines producing plantlets were established. Plants could be transferred to soil; up to now, 50% of more than 200 regenerated plants were morphologically normal and fertile. The relative frequency of sterile plants increased as suspensions aged. Suspensions older than 1 year produced embryogenic callus but only albino plantlets could be regenerated.

**Key words:** Barley anther culture – Suspension establishment – Plant regeneration

## Introduction

Immature embryos are the most frequently used explants for producing callus lines suitable for the establishment of suspension cultures of barley. In the last years there has been considerable progress in establishing morphogenic suspensions from this donor material; Lührs and Lörz (1988) were able to regenerate albino plantlets and Lazzeri and Lörz (1990) transferred green plants regenerated from suspensions to soil, although these plants were sterile. Recently, there has been success in establishing barley suspension cultures directly from immature embryos and from these suspensions regenerable protoplasts could be isolated (Yan et al. 1990). The efficiency of suspension establishment from embryo-derived cultures is low, however. As an alternative, it has been shown that microspore-derived tissues are a suitable source for the establishment of morphogenic suspensions in wheat (Harris et al. 1988), maize (Mitchell and Petolino 1990), rice (Datta et al. 1990), and also barley (R. Lührs and K. Nielsen, personal communication).

Anther cultures are an attractive source for the initiation of morphogenic suspension cultures, as they are highly embryogenic and may be transferred to liquid medium relatively soon after initiation. We present here our data on the use of anther cultures for the initiation of regenerable suspensions.

## Materials and methods

### *Plant material and growth conditions*

Experiments were carried out with three spring-type cultivars (Princesse, Baronesse, and Gimpel) and one winter-type cultivar (Igri) of barley (*Hordeum vulgare* L.). The plants were grown in a greenhouse under controlled conditions. Seeds were germinated in a peat/soil mix at approximately 20 °C with 16–18 h light for 8 days. Plants of winter-type barley, about 5 cm in length, were vernalized at 4 °C with 10 h light (4,000 lx) for 6–8 weeks. For further cultivation, the seedlings were transplanted to 9 × 9 cm pots with fresh soil. They were grown at 10–12 °C with 16 h light (10,000 lx) for about 7 weeks. Two weeks before the spikes were harvested, the temperature was increased to 18 °C. Liquid fertilizer (Wuxal, Schering; N : P : K = 12 : 4 : 6) was applied once per week. The plants were grown without application of fungicides or insecticides.

### *Culture media*

The media used for anther culture were modified MS (MSm) media (Murashige and Skoog 1962) and modified L media (Lazzeri et al. 1991) (see Table 1). The L medium was modified by decreasing the $NH_4NO_3$ concentration (L3). For anther culture we used 6-benzylaminopurine (6-BAP) at a concentration of 1 mg/l (MSmB1, L3B1). We also tested a combination of 1 mg/l 2,4 dichlorophenoxyacetic acid (2,4-D) and 1 mg/l 6-BAP (MSmD1B1, L3D1B1). For initiation and maintenance of cell suspension cultures, we used the L1 medium with an auxin concentration of 2 mg/l 2,4-D (L1D2, Lazzeri et al. 1991).

**Table 1.** Composition of media

| | MSm | L1 | L2 | L3 |
|---|---|---|---|---|
| **Macroelements (mg/l)** | | | | |
| $NH_4NO_3$ | 165 | 700 | 1,500 | 200 |
| $KNO_3$ | 1,900 | 1,750 | 1,750 | 1,750 |
| $CaCl_2 \times 2 H_2O$ | 440 | 450 | 450 | 450 |
| $MgSO_4 \times 7 H_2O$ | 370 | 350 | 350 | 350 |
| $KH_2PO_4$ | 170 | 200 | 200 | 200 |
| $Na_2EDTA$ | 37 | 37 | 37 | 37 |
| $FeSO_4 \times 7 H_2O$ | 28 | 28 | 28 | 28 |
| **Microelements (mg/l)** | | | | |
| $H_3BO_3$ | 6.20 | 5.00 | 5.00 | 5.00 |
| $MnSO_4 \times 4 H_2O$ | 22.30 | 25.00 | 25.00 | 25.00 |
| $ZnSO_4 \times 4 H_2O$ | 8.60 | 7.50 | 7.50 | 7.50 |
| KI | 0.83 | 0.75 | 0.75 | 0.75 |
| $Na_2MoO_4 \times 2 H_2O$ | 0.25 | 0.25 | 0.25 | 0.25 |
| $CuSO_4 \times 5 H_2O$ | 0.025 | 0.025 | 0.025 | 0.025 |
| $CoCl_2 \times 6 H_2O$ | – | 0.025 | 0.025 | 0.025 |
| **Vitamins (mg/l)** | | | | |
| Ascorbic acid | – | 1.00 | – | 1.00 |
| Biotin | – | 0.005 | – | 0.005 |
| Ca-Pantothenate | – | 0.50 | – | 0.50 |
| Choline chloride | – | 0.50 | – | 0.50 |
| Folic acid | – | 0.20 | – | 0.20 |
| myo-Inositol | 100.00 | 100.00 | 100.00 | 100.00 |
| Nicotinic acid | 1.00 | 1.00 | 1.00 | 1.00 |
| p-Aminobenzoic acid | – | 1.00 | – | 1.00 |
| Pyridoxine HCL | 1.00 | 1.00 | 1.00 | 1.00 |
| Riboflavin | – | 0.10 | – | 0.10 |
| Thiamine HCL | 10.00 | 10.00 | 10.00 | 10.00 |
| **Amino acids (mg/l)** | | | | |
| Glutamine | 750 | 750 | 750 | 750 |
| Proline | 150 | 150 | 150 | 150 |
| Asparagine | 100 | 100 | 100 | 100 |
| **Organic acids (mg/l)** | | | | |
| Citric acid | – | 10 | – | 10 |
| Fumaric acid | – | 10 | – | 10 |
| Malic acid | – | 10 | – | 10 |
| Sodium pyruvate | – | 5 | – | 5 |
| **Sugars (g/l)** | | | | |
| Cellobiose | – | 0.125 | – | 0.125 |
| Fructose | – | 0.125 | – | 0.125 |
| Mannose | – | 0.125 | – | 0.125 |
| Rhamnose | – | 0.125 | – | 0.125 |
| Ribose | – | 0.125 | – | 0.125 |
| Xylose | – | 0.125 | – | 0.125 |
| Maltose | 60.000 | 50.000 | 30.000 | 30.000 |
| **Hormones** | | | | |

were added in various concentrations and combinations as mentioned in the text

To test the morphogenic capacity of cell suspension cultures, various media were used (MSm, L1, L2, and L3), supplemented with differing concentrations and combinations of auxins (2,4-D: 0.5–2.5 mg/l; picloram: 2 mg/l; phenolic acetic acid (PAA): 100 mg/l) and cytokinin (6-BAP: 0.5–1 mg/l). The regeneration medium for both anther and suspension cultures was the modified MS medium with 30 g/l maltose and 0.5 mg/l 6-BAP.

All media were adjusted to pH 5.6 with KOH and filter sterilized. For solid media, the solutions were made double-concentrated and mixed with an equal volume of double-concentrated, autoclaved (0.8%) agarose (Sigma, Type I-A) solution.

*Anther culture*

Anther culture was performed according to a modified protocol of Olsen (1987). Spikes containing anthers in the miduninucleate stage were cold pretreated at 4 °C for 14 days in a two-compartment petri dish, as described by Huang and Sunderland (1982). Anthers surviving the cold pretreatment were removed using fine-tipped forceps (Aesculap BD 321) and transferred directly onto solid medium. Plates were sealed with Nescofilm and incubated at 25 °C in the dark. After 1 month, the anther response was determined by counting anthers that gave rise to callus or embryoids. At this stage, about 10% of the dishes were used to test the regeneration capacity of the anther-derived tissues. The remaining dishes were used for the initiation of suspension cultures.

*Initiation of cell suspension cultures*

For the initiation of cell suspensions, 4-week-old anther-derived, embryogenic callus was used. Callus from one spike was taken to initiate one suspension line. All germinating embryos were removed, and the finer aggregates were selected for culture in macroplates (Greiner) and incubated in 1 ml L1D2 medium. After 1 week, all germinated embryos were again removed and 1 ml fresh medium was added. The suspensions were then subcultured weekly by replacing the old medium by an equal volume of fresh medium. With each subculture, the suspensions were selected for finer aggregates. After 2 months, suspensions were transferred to larger plastic vessels (Greiner, volume ca. 190 ml) and then maintained by culturing approximately 5 g (fresh weight) of cells with 10 ml medium. The cultures were kept on a rotary shaker at 100 rpm at 25 °C under low-light conditions (ca. 200 lx).

*Regeneration of morphogenic cell suspensions*

The morphogenic capacity of cell suspension cultures was tested monthly by transferring aggregates from liquid onto different solid media. In the first 4 months of culture, aggregates from suspensions were transferred directly to regeneration medium and incubated at 24 °C with 16 h light. Aggregates of older suspensions were transferred to different solid media (see 'Culture media' above) for induction at 26 °C in darkness. Embryogenic calli that appeared after 1 month of culture were transferred to regeneration medium and incubated at 24 °C with 16 h light. After 4–6 weeks of culture, plantlets were transferred to pots containing a peat/soil mix and grown at 20 °C with 16–18 h light for about 2 months. Regenerants from the winter-type cultivar Igri were vernalized for 6–8 weeks at 4 °C with 10 h light (4,000 lx). After vernalization, plants were grown to maturity in the glasshouse.

## Results

*Anther culture*

Anther response was assessed after 4 weeks (Table 2). For all genotypes, the MSmB1 medium supported the highest anther response. Genotypes Igri and Gimpel also gave good anther response when the L3 medium was used. The addition of 1 mg/l 2,4-D did not influence the

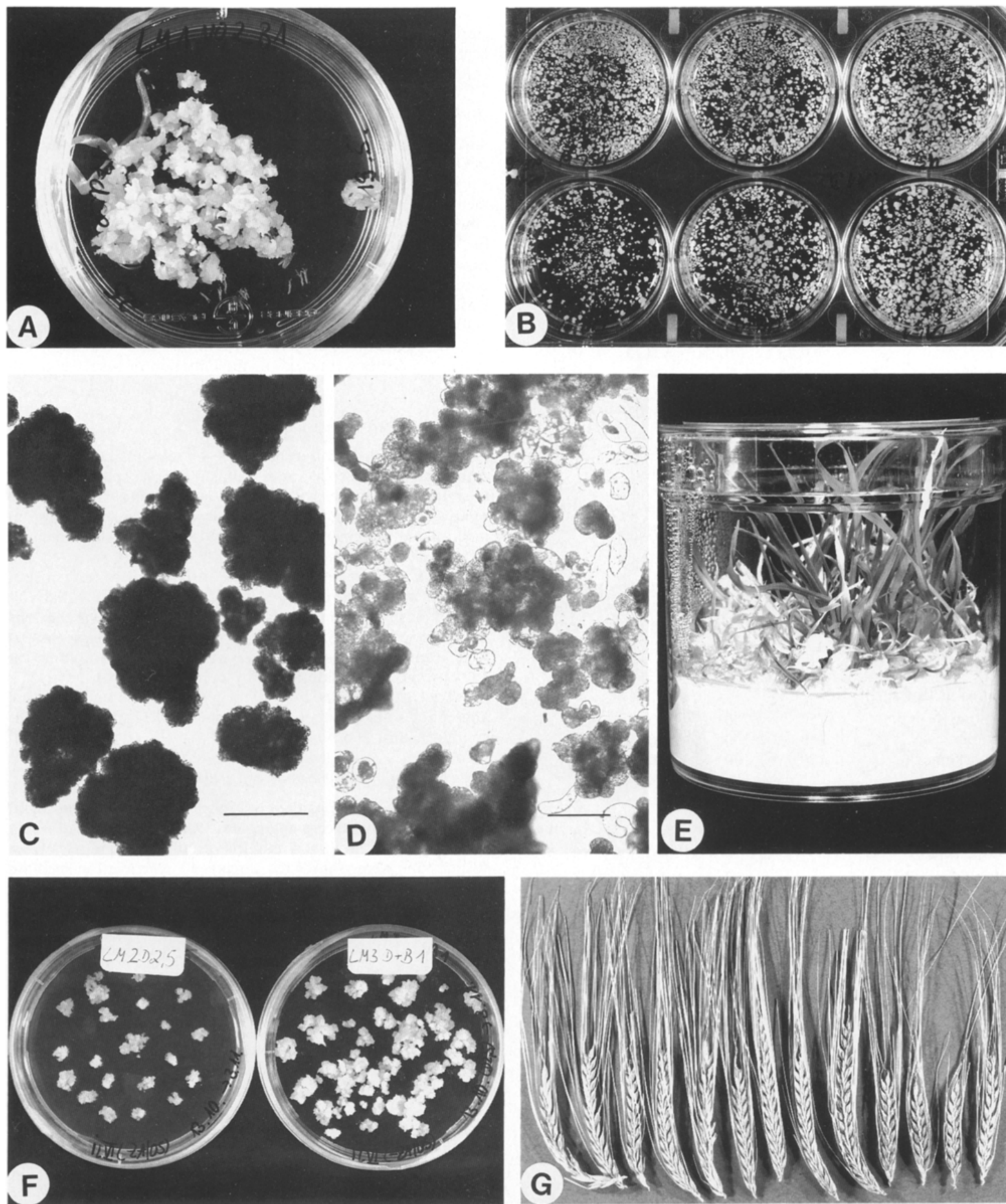


**Fig. 1 A–G.** Suspension establishment and regeneration of barley. **A** Anther-derived embryogenic callus after 1 month on induction medium; **B** initiation of anther-derived suspensions in macroplates; **C** suspensions after 8 weeks of culture consisting of large and compact aggregates (scale bar = 200 μm); **D** suspensions after 12 weeks of culture (scale bar = 100 μm); **E** regenerative capacity of a 6-week-old suspension; **F** aggregates from a suspension plated on different media; **G** fertile ears of plants regenerated from suspension cultures

**Table 2.** Influence of culture media on the androgenetic response and regeneration capacity of four barley cultivars

| Cultivar | Medium | Plated anthers | Responding anthers | Plantlets per responding anther | |
|---|---|---|---|---|---|
| | | | | green | albino |
| Igri | MSmB1 | 2,160 | 92% | 7.2 | 0.01 |
| | L3B1 | 2,490 | 74% | 3.4 | 0.01 |
| | L3D1B1 | 3,030 | 85% | 5.8 | 0.01 |
| Princesse | MSmB1 | 1,110 | 50% | 0.8 | 1.3 |
| | L3B1 | 1,110 | 36% | 0.2 | 0.1 |
| Baronesse | MSmB1 | 990 | 72% | 0.9 | 1.6 |
| | L3B1 | 1,070 | 23% | 0.1 | 0.1 |
| Gimpel | MSmB1 | 1,040 | 98% | 8.1 | 0.1 |
| | L3B1 | 1,040 | 87% | 6.7 | 0.2 |
| | L3D1B1 | 1,070 | 90% | 7.4 | 0.1 |

**Table 3.** Suspension initiation and regeneration of spring- and winter-type cultivars of barley

| | 1989 | | | 1990 | |
|---|---|---|---|---|---|
| | Igri | Princesse | Baronesse | Igri | Gimpel |
| No. of lines initiated | 42 | 16 | 19 | 65 | 18 |
| No. of lines selected after 1 month | 27 | 9 | 7 | 45 | 6 |
| No. of lines producing embryos or shoots on solid medium | 25 | 9 | 4 | 38 | 2 |
| No. of lines producing plantlets | 9 | 4 | 4 | 36 | 2 |
| Average no. of plantlets produced per 2 g cells | | | | | |
| (a) green | 15 | – | – | 18 | 3 |
| albino | 2 | 18 | 12 | 1 | 8 |
| (b) green | 4 | – | – | not yet known | |
| albino | 8 | – | – | not yet known | |

(a) up to 4 months, (b) after 4 months of culture

anther response significantly. The frequency of embryo formation was higher on the MSm medium than on the L3 media; anther culture using L3 media resulted in more callus than embryos. In the case of cultivars Princesse and Baronesse, anther response was significantly reduced on the L3B1 medium. These spring-type cultivars produced a higher proportion of albino plantlets. The highest frequency of green plantlet production was observed for cultivars Igri and Gimpel, with Gimpel producing more green plantlets than Igri.

*Initiation of cell suspension cultures*

For the establishment of cell suspensions, anther-derived embryogenic calli (Fig. 1 A) were used as inoculation material. The selection of callus suitable for the initiation of suspensions was very important. It was necessary to remove all fully-developed somatic embryos and to select fine aggregates and proembryos before transferring callus to liquid medium. Incubation of somatic embryos in advanced stages resulted in germination of the embryos during the 1st week of culture. Callus initiated on all the different anther culture media dispersed easily and started to grow immediately in liquid culture, although the aggregates remained quite hard and compact (Fig. 1 B and 1 C). However, after 8 weeks of culture it could be seen that lines initiated from embryogenic callus from L3 media continued to grow quickly, whereas lines derived from MSm medium gradually ceased growth. At this stage, the better suspensions with finer aggregates were selected for culture in plastic vessels. After the 3rd month of culture, suspensions became rather heterogeneous (Fig. 1 D). They consisted of a few elongated empty cells, cell debris, and many highly-cytoplasmic cells and cell aggregates. With regular subculture, the suspensions became more homogeneous until they were composed of cytoplasm-rich aggregates. Single cells were seen only rarely; these established suspensions consisted mainly of larger aggregates, with ca. 20–80 cells. Cells of suspension aggregates always contained at lot of starch, which did not disappear even when suspensions were subcultured with a reduced sugar concentration (10–20 g/l maltose).

The frequency of suspension initiation was strongly genotype-dependent. The best genotype was Igri, from which a total of 107 lines was initiated. After 1 month of culture, some of the lines became brownish and some ceased growth, but nevertheless, we could select a total of 72 independent lines for further cultivation. In the case of spring-type cultivars, it was much more difficult to select callus suitable for suspension initiation (Table 3). Their somatic embryos had a higher tendency to germinate on the anther culture medium and there was often not enough callus remaining to establish a cell line, as suspension initiation was unsuccessful when smaller amounts of callus were used.

*Plant regeneration from cell suspensions*

For up to 4 months of culture, suspension aggregates were plated directly on the regeneration medium, which was also used to regenerate plantlets from anther culture. Plants could be directly regenerated from these young suspensions without plating cells on an induction

**Table 4.** Comparison of media for regeneration of suspensions of barley cv Igri

| Medium | Culture development | Morphogenic capacity | Average no. of embryos per 2 g suspension cells |
|---|---|---|---|
| L1D2 | Soft callus | No | – |
| L2D2.5 | Compact callus | No | – |
| L3D2 | Compact callus | No | – |
| L3D2B1 | Embryogenic callus | Embryos | 11 |
| L3D1B1 | Embryogenic callus | Embryos and shoots | 18 |
| L3D1B0.5 | Embryogenic callus | Embryos and shoots | 15 |
| L3D0.5B0.5 | Embryogenic callus | Embryos and shoots | 20 |
| L3D0.5B1 | Embryogenic callus | Embryos and shoots | 21 |
| L3B1 | Soft callus | No | – |
| MSmD1B1 | Embryogenic callus | Embryos and shoots | 37 |
| MSmD1B0.5 | Embryogenic callus | Embryos and shoots | 32 |
| MSmD0.5B1 | Embryogenic callus | Embryos and shoots | 35 |
| MSmD0.5B0.5 | Embryogenic callus | Embryos and shoots | 29 |
| L3P2 | Soft callus | No | – |
| L3A100 | Soft callus | No | – |

D = 2,4-D; B = 5-BAP; P = picloram; A = PAA; concentration in mg/l

medium (Fig. 1 E). However, the formation of distinct somatic embryos could not be observed in liquid culture, although cell aggregates were compact and regular in shape. With advancing age of suspensions, regeneration capacity declined and the proportion of albino plantlets increased.

For the genotype Igri, 45 lines were selected, from which on average 16.5 green plants per 2 g suspension cells could be regenerated during the first 4 months of culture. These plants were transferred to soil and most of them survived this transfer. In the case of cultivars Princesse and Baronesse, we never succeeded in establishing a suspension line from which green plantlets could be regenerated; these lines had embryogenic capability, but it was only possible to regenerate albino plantlets. The genotype Gimpel showed very good anther culture response with high yields of green plants, but for suspension cultures this line was inferior to Igri (Table 3).

When cells from suspensions older than 4 months were plated directly on regeneration medium, plantlet production was only rarely observed. It was to be found necessary to plate the cells on an induction medium before transferring them to regeneration medium. We compared various solid media for the induction of embryogenesis of suspensions from the genotype Igri (Table 4). A solidified suspension medium (L1D2) only gave rise to soft callus. On a medium with an increased $NH_4NO_3$ concentration (L2D2.5), compact callus developed, but the formation of distinct embryos could not be observed. On media with decreased $NH_4NO_3$ concentrations (L3), embryos and shoots developed when the cytokinin 6-BAP was added (Fig. 1 F). When the medium was supplemented with 6-BAP only and 2,4-D was omitted, soft callus developed. The culture of suspension cells on solid L3 media supplemented with the auxins picloram or PAA always resulted in the production of soft callus. The induction of embryogenesis was most efficient when MSm media with combinations of 2,4-D and 6-BAP were used. It was still possible to induce embryogenesis from more than 15-month-old cell suspensions on these media.

When embryos or shoots appeared, the callus was transferred (approximately 1 month after plating of suspension cells) from induction medium to regeneration medium. The regeneration of plants was still possible, but the number of albino plantlets was twice as high as the proportion of green plantlets. These regenerants were also transferred to soil. In total, from the experiments in 1989, more than 150 regenerants of cultivar Igri from 2- to 6-month-old suspensions was grown in soil; 50% of the regenerants was morphologically normal and fertile (Fig. 1 G). Some plants from suspensions older than 6 months were also fertile, but the percentage of sterile plants increased as suspensions aged. From the experiments in 1990 to date, 85 plants from genotype Igri and 8 plants from Gimpel have been transferred to soil. These plants appear to be morphologically normal, but their fertility status is not yet known.

## Discussion

The data reported here show that the use of anther-derived tissues is an efficient approach for the establishment of embryogenic barley suspension cultures. It is now reproducibly possible to initiate embryogenic suspensions from which fertile plants can be regenerated during at least 6 months of culture. Barley now belongs to the list of major cereals for which the regeneration of fertile plants from suspension cultures has been reported (wheat: Redway et al. 1990; maize: Prioli and Söndahl

1989; Shillito et al. 1989; rice: Fujimura et al. 1985 and Datta et al. 1990).

Two factors important for the production of regenerable suspensions are the use of highly embryogenic source material and the time taken for suspension establishment. To date, immature embryos have been mostly used for the initiation of barley suspension cultures (Lührs and Lörz 1988; Lazzeri and Lörz 1990). In these explants, the establishment of suspensions took several months (for first callus culture and then suspension initiation), and only albino plantlets or sterile green plants could be regenerated. By comparison, suspension production from microspore cultures is faster and more efficient (R. Lührs and K. Nielsen, personal communication). Faster suspension establishment should minimize the loss of regeneration capacity and the accumulation of somaclonal variation (Lührs and Lörz 1988; Gaponenko et al. 1988).

In order to test the embryogenic capacity of our anther cultures, we determined the number of plantlets per responding anther. The results obtained are comparable to other reports (Olsen 1987; Hunter 1987; Datta and Potrykus 1989). Obviously, the anther response as well as the regenerative capacity are not only genotype dependent, but are also influenced by the composition of the culture media. The MSmB1 medium with a very low amount of nutrients supported the best anther response and, as regards regeneration capacity, was superior to L3 media that are supplemented with complex vitamins and organic nutrients. For the initiation of suspension cultures, calli from L3 media were better. Calli induced on MSmB1 media gradually ceased growth during the 1st month of suspension initiation, whereas those from L3 media maintained growth. It appears important to use induction media that are similar to the suspension media in order to make the adaptation phase easier. In anther cultures, the addition of 2,4-D slightly increased the anther response and the regenerative capacity for cv Igri as well as for cv Gimpel, in contrast to previous reports (Kuhlmann and Foroughi-Wehr 1989). The very high responsiveness of cv Igri has previously made it the model genotype for barley anther and microspore culture; our results show that the spring-type cultivar Gimpel is even superior to Igri.

The assumption that genotypes that are very responsive in anther culture are also suitable for the establishment of suspensions could not be proven. For the genotype Igri, we were able to establish regenerable suspensions with high frequency, whereas it was very difficult to initiate regenerable suspensions of cv Gimpel. For the spring-type cultivars Princesse and Baronesse, it was not possible to establish suspensions with regenerative capacity, although they responded well in anther culture.

The regeneration capacity of the suspension cultures declined gradually with time, as is generally observed for cereal suspensions. In order to improve the regeneration frequency we tested different media. Our experiments show that modified MS media support the highest morphogenic capacity. It seems that complex organic nutrients present in L media are not necessary for embryogenesis, and may even be inhibitory.

The ability to produce suspensions that give rise to dividing protoplasts while still being regenerable is probably the most important factor for the regeneration of fertile plants from protoplasts. We have used our suspension cultures for the isolation of protoplasts from which fertile plants have been regenerated (Jähne et al. 1991).

Such regenerable protoplast cultures provide the potential for the production of transgenic barley plants, if stable transformation of protoplasts, which we have recently reported (Lazzeri et al. 1991) and efficient plant regeneration can be combined.

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