

Plant regeneration through direct somatic embryogenesis from protoplasts of banana (*Musa* spp.)

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Summary. We report the isolation and regeneration of protoplasts from an embryogenic banana (*Musa* spp.) cell suspension culture initiated from *in vitro* proliferating meristems. A high yielding isolation method (up to 6×10^7 protoplasts.ml⁻¹ packed cells) is discussed. Optimal regeneration, with more than 50 % of the protoplasts showing initial cell division, occurred when high inoculation densities (10^6 protoplasts.ml⁻¹) or nurse cultures were applied. Under these conditions, the frequency of microcolony formation was 20-40%. These microcolonies developed directly, without an intervening callus phase, into somatic embryos which later germinated and formed plantlets.

Key words: Banana - Plant regeneration - Protoplast - Somatic embryogenesis - Suspension culture

Introduction

Bananas (*Musa* spp.) are the second largest fruit crop in the world (FAO 1991) and are produced in the tropical and subtropical regions of developing economies. It is an orphan crop since it has not received much research attention (Dale 1990). Many pests and diseases are threatening banana production among which are black sigatoka, a foliar fungal disease caused by *Mycosphaerella fijiensis*; Panama disease caused by *Fusarium oxysporum* f. sp. *cubense* which blocks the vessels; bunchy top, a virus disease causing plant distortion; nematodes and banana weevils. Because of high sterility levels and polyploidy of the edible varieties, classical *Musa* breeding remains a difficult endeavour (Swennen and Vuylsteke 1993). Biotechnology offers new prospects. For example, with advanced transformation techniques, foreign resistance genes can be introduced. These techniques largely depend on the successful regeneration of plants from cells or protoplasts. Recently, embryogenic banana suspension cultures were initiated from proliferating

meristems (Dhed'a *et al.* 1991), from corm tissue and leaf bases (Novak *et al.* 1989), from immature zygotic embryos (Escalant 1990), and from immature male inflorescences (Escalant *et al.* 1992). These suspension cultures can be regenerated into plantlets through somatic embryogenesis at high frequencies and grown in the field (Dhed'a *et al.* 1991; Novak *et al.* 1989).

There are only a few reports on protoplast culture in *Musa* spp. Bakry (1984) succeeded in isolating protoplasts from inflorescence-derived callus in Cavendish (*Musa* subgroup AAA). Regeneration was not mentioned. He reported that leaf material was unsuitable for protoplast isolation. Chen and Ku (1985) used the base tissue of the youngest leaf for the production of protoplasts. However, these protoplasts did not proceed with cell wall regeneration and cell division and survived only for 15 days. Matsumoto *et al.* (1988) obtained highest protoplast yields from bracts. They observed cell wall formation and cell division after 5 days but did not report any further development of the dividing protoplasts. Very recently, Megia *et al.* (1992) reported protoplast regeneration and subsequent callus formation from zygotic embryo-derived suspension cultures in the wild diploid AA banana *Musa acuminata* ssp. *burmannica* cv Long Tavoy. These reports reveal that *Musa* is very recalcitrant towards protoplast culture, a common characteristic for most monocotyledons, such as members of the *Gramineae* like rice and maize. Embryogenic suspensions have been the source of protoplasts in several of these reports (Rhodes *et al.* 1988). We report here the isolation of protoplasts and regeneration of plants by using regenerable meristem-derived *Musa* cell suspensions.

Materials and methods

Suspension cultures. Cell suspension cultures with proven regeneration capacity have been initiated from the cv. 'Bluggoe' (*Musa* spp., ABB subgroup), using explants from the upper part of proliferating shoot-tips as described by Dhed'a *et al.* (1991). The suspension has been

maintained in 60 ml of liquid MS medium (Murashige and Skoog 1962), but with the following modifications : half strength macro elements and iron, 0.4 mg.l⁻¹ thiamine, 10 mg.l⁻¹ ascorbic acid, 5 µM 2,4-D, 1 µM zeatin, and without myo-inositol, pH before autoclaving 5.8.

Initially, these cultures were very heterogeneous and contained large translucent cells as well as small dense cells (Fig. 1). When frequently subcultured at 2 week intervals, the suspension cultures became more uniform and consisted only of clusters of small tightly packed cells with a dense cytoplasm (Fig. 2). These cells are round and characterised by a relatively large nucleus, a very dense nucleolus, small multiple vacuoles and tiny starch and protein grains. These characteristics indicate morphogenic competence (Mc William *et al.* 1974) and are typical for the embryogenic cell suspension cultures of *Gramineae*.

Protoplast isolation. At different periods after their last subculture, embryogenic cell suspensions were concentrated to obtain a packed cell volume (PCV) between 15 and 20%. One millilitre of this suspension with known PCV was subsequently transferred to 6 ml of the enzyme solution containing cellulase 'Onozuka R-10' from *Trichoderma viride*, macerozyme 'R-10' from *Rhizopus* sp. and pectinase '5S' from *Aspergillus niger* (all from Serva, Heidelberg, Germany) at several concentrations and combinations (see Results). The solution also contained 7 mM CaCl₂.2H₂O, 0.7 mM NaH₂PO₄.2H₂O, 3 mM MES (2-N-morpholino ethane sulfonic acid) and 10% mannitol. The pH was adjusted to 5.8 and the solution is filter sterilised through 0.2 µm. This mixture was maintained on a rotary shaker (ca 40 rpm) in the dark for different time periods.

Protoplast purification. After the enzyme treatment, the suspension was washed by centrifugation at 66 x g for 5 min with 10 ml suspension culture medium devoid of plant growth regulators but enriched with 10% mannitol (washing solution). Protoplasts were purified either by flotation on 20, 22 or 25% (w/v) sucrose or sieving through a 100 µm and subsequently a 25 µm sieve.

Protoplasts were counted before and after purification to determine the purification effectiveness. Cell wall degradation and viability was visualised with an UV inverted microscope (Dialux 20 from Leitz, Wetzlar, Germany) using Calcofluor white (fluorescent brightener 28, Sigma, St. Louis, USA) and fluorescein diacetate (Sigma, St. Louis, USA) respectively.

Protoplast culture. The purified protoplasts were cultured according to the following methods:

1/ Culture in liquid media. Protoplasts were resuspended at densities ranging between 10⁵ and 10⁶ protoplasts per ml in the aforementioned 1/2 MS medium containing only 5 µM 2,4-D and 10% mannitol. Five millilitres of these protoplast suspensions were transferred to 100 ml Erlenmeyer flasks and maintained without or with shaking at 30 rpm.

2/ Culture in semi-solid medium. Protoplasts, at twice the desired concentration, suspended in the liquid protoplast culture medium, were mixed with an aliquot of the same medium but with twice the gellificant concentration. This mixture was then transferred to plastic petri dishes of 5.5 cm in diameter. The variable parameters were : final concentration of the gellificant (0.3 or 0.6% agar (Vel, Leuven, Belgium), 0.3 or 0.8% agarose (Sigma, St. Louis, USA)), mannitol concentration (5 and 10%), 2,4-D concentration (0, 1, 5 and 10 µM), protoplast density (10⁵, 2.5x10⁵ and 10⁶ protoplasts per ml) and the presence or absence of 100 mg.l⁻¹ myo-inositol.

3/ Culture on solid medium. Protoplasts were again diluted to a known density after which 1 ml was placed on the solid medium containing the 1/2 MS salt solution, 5% mannitol, 5 µM 2,4-D and 0.8% agarose or 0.2% Gelrite (Phytigel™, Sigma, St. Louis, USA), which was covered or not with a filter (black Millipore, n°AABG04700 ; black gridded, 0.8 µm, Ireland).

4/ Culture with feeder layers. Two types were applied : The first method was based on the principle developed by Rhodes *et al.* (1988). In a petri dish of 9 cm diameter 20 ml 1/2 MS medium supplemented with 5% mannitol, 2 g.l⁻¹ gelrite and 5 µM 2,4-D was poured. On this layer 2 ml of the nurse culture was placed. The nurse culture was obtained by mixing 1 ml of a one week old suspension culture concentrated to 25% PCV with 1 ml 1/2 MS medium supplemented with 10% mannitol, 200 mg.l⁻¹ myo-inositol, 5 µM 2,4-D and 1.6% agarose. On top of the feeder a filter (white Whatman filter N° 40, Maidstone, England or black Millipore filter, see above) was placed covered with 1 ml of protoplasts at a density of 10⁵, 2x10⁵, 5x10⁵ or

10⁶ protoplasts per ml. The second method was described by Hahne *et al.* (1990). The nurse culture had the same content as the previous one except that it was solidified with 0.4% agarose. This mixture was gently poured along the wall of a 55 mm petri dish, thus forming a ring that left a well in the centre where 0.5 ml of protoplast suspension at a density of 10⁵ protoplasts per ml was placed.

5/ Culture on preconditioned medium. This culture method resembles the first culture method with feeder layers. However, instead of 1 ml of nurse cells, 1 ml of a preconditioned medium was mixed together with 1/2 MS supplemented with 10% mannitol, 200 mg.l⁻¹ myo-inositol, 5 µM 2,4-D and 1.6% agarose. This preconditioned medium was prepared by separating the medium from a one week old culture by using a white Whatman filter n° 40, whereafter it was filter sterilised (0.2 µm ME Syringe Tip Filter, DynaGard, Microgon, Laguna Hills, USA).

Results

Protoplast isolation

The isolated protoplasts had a very dense cytoplasm and were very homogeneous in size (17 ± 3 µm) (Fig. 3). The cell wall was always completely digested and viability ranged between 90 and 100%. The influence of enzyme exposure times (4 and 24 h) and the enzyme solution composition is shown in Table 1. Since protoplast yields varied a lot, five independent experiments were carried out each showing the same tendency. A 4 h exposure time was not sufficient to release many protoplasts (especially when 2.5% cellulase was used as the sole cell wall degrading enzyme). A 24 h exposure time resulted in a 5 to 15 fold increase in protoplast yield for 2.5% (w/v) cellulase and the enzyme mixture (1% (w/v) cellulase, 1% (w/v) macerozyme and 1% (w/v) pectinase), respectively. Although suspensions of all five repetitions were taken two weeks after their last subculture, protoplast yields varied considerably.

Protoplast purification

Purification of the protoplasts through the flotation method proved to be inferior to the sieving method. The efficiency with flotation varied between 15 and 35% (mean is 21%) independent from the sucrose concentration used. The sieve method resulted in 74 to 100% purification efficiencies (mean is 85%). Therefore the latter method has been selected for further experiments.

Protoplast culture

Culture in liquid medium. After one day of liquid culture, protoplasts aggregate thus forming groups of hundreds of cells which made observations difficult. When shaken, viability of the protoplasts was totally lost after one week of culture. The cell wall regeneration was irregular and no cell division was observed. Without shaking, protoplasts formed already after 3 days a homogeneous cell wall, and remained alive for more than 3 weeks.

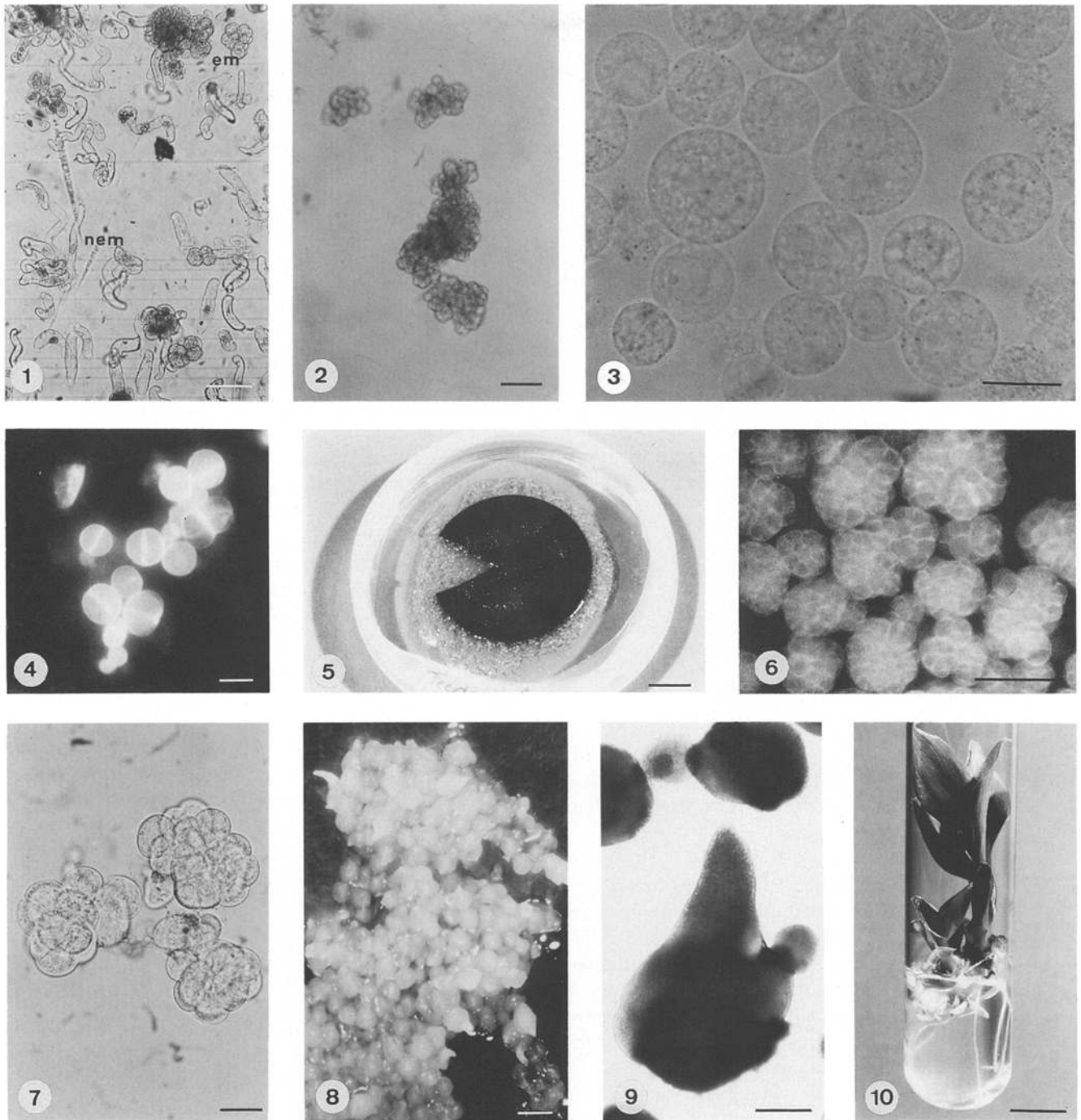


Fig.1. Heterogeneous cell suspension culture consisting of embryogenic clusters (em) and large non-embryogenic cells (nem) (bar = 100 μ m). **Fig.2.** Uniform embryogenic suspension culture (bar = 100 μ m). **Fig.3.** Freshly isolated protoplasts (bar = 10 μ m). **Fig.4.** Cell wall regeneration and first division of protoplasts, 8 days after inoculation on a feeder layer culture (stained with calcofluor white and observed under ultraviolet illumination) (bar = 20 μ m). **Fig.5.** Protoplast regeneration on a feeder layer culture, 1 month after inoculation (bar = 1 cm). **Fig.6.** Protoplast derived colonies, 5 weeks after inoculation on a feeder layer culture (stained with calcofluor white and observed under ultraviolet illumination) (bar = 100 μ m). **Fig.7.** Protoplast derived colonies, 5 weeks after inoculation on a feeder layer culture (observed with a light microscope) (bar = 20 μ m). **Fig.8.** Formation of somatic embryos, two months after protoplast isolation (bar = 1 mm). **Fig.9.** Emergence of the plumule from the somatic embryo (bar = 200 μ m). **Fig.10.** Protoplast derived plantlet 5 months after protoplast isolation (bar = 1 cm).

Table 1. Protoplast yields ($\times 10^5$ protoplasts per ml PCV)

enzyme	hours	repetition number					mean \pm s.d.
		I	II	III	IV	V	
cell ^a	4	0.2	0.3	1.0	3.6	0.2	1.1a ^c \pm 1.5
cell	24	8.9	21.2	28.3	12.3	8.1	15.8b \pm 8.7
mix ^b	4	43.1	47.6	270.8	28.0	15.1	80.9b \pm 106.9
mix	24	606.2	371.4	660.4	57.0	115.0	362.0b \pm 275.1

^a 2.5 % cellulase^b 1 % cellulase, 1 % macerozyme and 1 % pectinase^c Mean separation by Duncan's multiple range test at 5 % level of significance. Values followed by the same letter were not found to be significantly different.**Table 2.** Reaction of protoplasts to different culture methods^a

medium ^b	filter	density (protoplasts per ml)			
		10^5	2×10^5	5×10^5	10^6
0.2 % gelrite	NO	o/+	o/+	o/++	o/+++
0.8 % agarose	NO	*/-	*/-	*/+	o/+++
0.8 % agarose + PCM	NO	*/-	*/-	*/-	o/+++
0.2 % gelrite	YES	o/-	o/++	o/+	o/+++
0.8 % agarose	YES	*/-	*/-	*/-	o/+++
0.8 % agarose + PCM	YES	*/-	*/-	*/-	o/+++
0.8 % agarose + FL	YES	o/+++	o/+++	o/+++	o/+++

^a o : good uniform cell wall formation

* : irregular thin cell wall formation

- : lifetime protoplasts < 1 week, no division

+ : lifetime protoplasts < 2 weeks, no division

++ : lifetime protoplasts > 2 weeks, < 1 % division

+++ : lifetime protoplasts > 2 weeks, > 50 % sustained divisions

^b PCM : preconditioned medium, FL : feeder layer

Cell divisions were observed at very low frequencies (less than 0.5 %). This was independent of protoplast inoculum density.

Culture in semi-solid medium. The reaction towards this culture method was very heterogeneous. Indeed, some protoplasts formed rigid cell walls already after 3 days, others showed no sign of cell wall regeneration; some protoplasts died after 2 days, but others survived for 4 weeks. Bud formation on protoplasts was also frequently observed. Occasionally, divisions were seen but this could not be related to experimental parameters such as 2,4-D and mannitol concentration, protoplast inoculation density, gellificant concentration and the presence of myo-inositol.

Culture on solid media (including preconditioned medium and feeder layer culture). The results are shown in Table 2. Generally, cell wall regeneration began 24 h after protoplast inoculation. An inoculation density of 10^6 protoplasts per ml always resulted in a good cell wall formation. Very uniform cell walls were also formed on feeder layer cultures and on the gelrite medium irrespective of the filter presence. On 0.8 % agarose, the cell walls were irregular and very thin. Nor the filter nor the preconditioned medium had an influence.

Sustained cell divisions (with more than 50 % of the protoplasts showing division) were obtained with the feeder layer culture at all tested inoculum densities and with the highest inoculum density (10^6 protoplasts per ml) on all tested protoplast culture media. Consequently, a good correlation exists between cell wall regeneration and cell division capacity with exception of the gelrite media. The feeder layer culture method of Rhodes *et al.* (1988) resulted at day 8 in a division frequency of about 75 % with 10 % of the cells in their second division (Fig. 4). After five weeks of culture, similar sized groups of dividing cells containing hundreds of cells were distinguished (Fig. 5 and 6), indicating that each cluster originated from a different protoplast at the same time. These cell clumps (Fig. 7) resembled cells of normal cell cultures, with the embryogenic characteristics described above. The frequency of micro-calli formation from protoplasts cultured on this feeder layer varied between 20 and 40 %. The replacement of a Millipore filter by a white Whatman filter always resulted in protoplast death. The method of Hahne *et al.* (1990) which has no physical barrier between feeder layer cells and protoplasts, resulted in good cell wall formation but low cell division frequencies. Five to ten percent of the cells divided only once.

Plant regeneration

Cell clumps containing at least 100 or more cells, were transferred to a semi-solid culture medium without nurse cells and devoid of plant growth regulators and mannitol. Two weeks later all these cell groups formed epidermised globules after which they invaginated (Fig. 8), a process which was also observed during the normal somatic embryogenesis from embryogenic suspension cells (Dhed'a *et al.* 1991). At the site of invagination a small plumule emerged (Fig. 9), and later at the opposite side, a small root. The regeneration frequency of the invaginated globules into somatic embryos was estimated at 10-14 % (Dhed'a *et al.* 1991). They developed into rooted plantlets after three months. The *in vitro* plants regenerated from the protoplasts resembled normal *in vitro* plants obtained through meristem tip-culture and somatic embryogenesis (Fig. 10). About 100 plants were regenerated to the stage where they could be transferred to the greenhouse. All of them showed normal root and shoot development.

Discussion

A protoplast culture method for *Musa* is reported which results in high plantlet regeneration frequencies. Other advantages in respect to other methods are high protoplast yield, high plating efficiency and no intervening callus phase, thus reducing the risks for somaclonal variation. Our results confirm that a morphogenic embryogenic suspension culture is the material of choice for the protoplast culture of monocotyledons (He *et al.* 1992;

Horn *et al.* 1988; Vasil and Vasil 1980). The isolation method (enzyme mixture of 1% cellulase, 1% macerozyme and 1% pectinase, incubation for 24 h), yields up to 6.6×10^7 protoplasts per ml PCV (or 5×10^7 protoplasts per gram fresh weight). This yield is comparable with the highest yields obtained so far in monocots (He *et al.* 1992). The age of the suspension cultures to obtain high and comparable yields of regenerating protoplasts is of little importance as long as it ranges between 2 and 14 days. Relatively long enzyme incubation periods (24 h) are needed as compared with the 4 hours used in most isolation procedures. This is in combination with an enzyme mixture containing relatively low concentrations of cellulase (1%). The fact that purification of the digested suspension culture using flotation on sucrose results in poor efficiencies can be explained by the observation made by Millam *et al.* (1991) who found that highly meristematic (and thus dense) protoplasts sink in the sucrose solutions and sediment with the debris.

Liquid cultures are not suitable for *Musa* protoplasts since they tend to aggregate. In addition, it makes it difficult to determine plating efficiencies accurately (Vasil and Vasil 1987). If genetically transformed, these aggregates could lead to chimeras. This contrasts with protoplast culture on a semi-solid medium; protoplasts remain isolated and similar sized cell groups derive from each protoplast (Fig. 6).

Two main strategies can be followed for obtaining sustained division in *Musa* protoplasts, one using nurse cells, the other plating at very high densities. The use of feeder cells is essential for protoplast regeneration in many embryogenic monocot cultures like oat (Hahne *et al.* 1990), maize (Rhodes *et al.* 1988) and rice (Datta *et al.* 1992). In the latter report, however, feeder cells were successfully replaced by a preconditioned medium. No regeneration could be achieved in *Musa* for protoplast densities lower than 10^6 per ml. The fact that this is only possible in some species might be correlated with the observation of Jørgensen *et al.* (1992) who concluded that no universal conditioning factor is involved.

High plating densities (10^6 protoplasts per ml) can substitute for the feeder layers, protoplasts probably serving as their own feeder. However, this is only applicable when large amounts of protoplasts are made available and not, for example, after protoplast electroporation, where usually a high percentage of protoplasts are killed (Sagi *et al.* 1993). Nurse cells are then advisable.

In most reports the callus phase seems to be a necessary intermediate step in plantlet regeneration from protoplasts. In some cases the morphogenic capacity of donor cell suspensions can be partially or completely lost in protoplast derived calli (Jähne *et al.* 1991). We however, obtained protoplasts regeneration through direct embryogenesis, a process which was also described by Li *et al.* (1992) with protoplasts from wheat. Estimates about

the frequency of microcolony formation (20 to 40%) exceeds by far the values obtained in other plant species.

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