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Production of carrot somatic embryos in a bioreactor

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Abstract. Somatic carrot embryos were grown as batch cultures in a stirred 10-l bioreactor. Embryo production in the bioreactor was comparable to that obtained in shake-flasks. A production of about $50 \cdot 10^3$ embryos/l per day was commonly achieved with an inoculum density of 0.1% volume of tissues/volume of medium. Regularly changing of the medium increased embryo viability. A filtering unit coupled to the bioreactor was developed in order to calibrate embryos. The characteristics of the population of harvested embryos are described.

Introduction

It is well established that somatic embryo production in liquid medium can be used for mass production in plant propagation systems such as vitroplants or artificials seeds (Redenbaugh et al. 1988). Somatic embryos can easily be obtained in suspension cultures of several species including carrot, alfalfa, coffee and celery. Nevertheless, from an economical point of view, large production volumes and process optimization are necessary to develop an efficient production system of vitroplants or artificial seeds. Production in a bioreactor allows scale-up and operations can be more easily controlled and automated than in a small individual culture vessel, including transferring cultures and collecting embryos, medium renewal, monitoring different parameters such as dissolved oxygen concentration (DO), pH, CO_2 concentration, and sampling. Drew (1979), Kessel and Carr (1972), Ammirato and Styer (1985) and Petiard et al. (1987) were able to grow carrot somatic embryos in bioreactors. Recently, the feasibility of embryo culture in a bioreactor has been reported for alfalfa (Chen et al. 1987; Stuart et al. 1987), sandalwood (Bapat et al. 1990), *Digitalis lanata* (Greidziac et al. 1990) and poinsettia (Preil and Beck 1991). Nevertheless, embryo-to-plant conversion frequencies were not reported in these works. More information is needed to monitor and optimize embryogenesis in liquid medium.

The aims of the present work were to determine whether a classical stirred bioreactor could be used or not for carrot somatic embryo production, to describe the kinetics of carrot somatic embryo development, to test a filtering system, added at the bioreactor outlet in order to calibrate embryos that are developed asynchronously and to specify the quality of the embryo harvest (size, viability, embryo-to-plant conversion rate).

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Materials and methods

Plant material and culture conditions. Carrot strains were initiated from aseptic seedlings of a cytoplasmic male sterile cultivar kindly provided by I.R.T. (Institut de Recherche Tezier, Valence, France). Hypocotyl segments were inoculated onto a semi-solid basal medium (MS+) comprising the inorganic salts of Murashige and Skoog (1962), the mixture of organic substances of Halperin (1964), 0.1 mg/l of 2,4-dichlorophenoxyacetic acid (2,4 D), 20 g/l of sucrose and 8.0 g/l of Bacto-Difco agar. The pH was adjusted to 5.8 prior to autoclaving for 20 min at 115° C. The developed calli were transferred into liquid medium of the same composition (MS+) in erlenmeyer flasks and placed on a gyratory shaker (100 rpm) at 23° C under white light ($3\ \mu E \cdot m^{-2} \cdot s^{-1}$) with a 16 h/8 h photoperiod. In MS+ medium a fine cell suspension was obtained and maintained by repeated subcultures every 12 days at an inoculation density of 1 g fresh weight/100 ml. For the induction of embryogenesis, a 10- to 14-day-old culture was filtered through two nylon meshes, 100- then 50-μm pore sizes (except in some experiments where 180- then 50-μm pore sizes were used). The cell clumps remaining on the latter filter were washed three times in fresh basal medium lacking 2,4-D (MSO) and then inoculated into the same medium with an inoculum density estimated on a packed cell volume per millilitre basis.

Cultures in bioreactors. The bioreactors used in this work were stirred fermentors of 10-l working volume (Magnaferm and Microferm model, New-Brunswick Edison, USA). These fermentors are equipped with three four-blade Rushton impellers. The agitation was 100 rpm and the temperature was regulated at 26° C. The bioreactor were autoclaved with 10 l of MSO medium for 45 min at 120° C. DO was measured with New Brunswick or Ingold electrodes. In some experiments, DO was controlled by direct feedback adjustment of air input using a Setric controller. In some

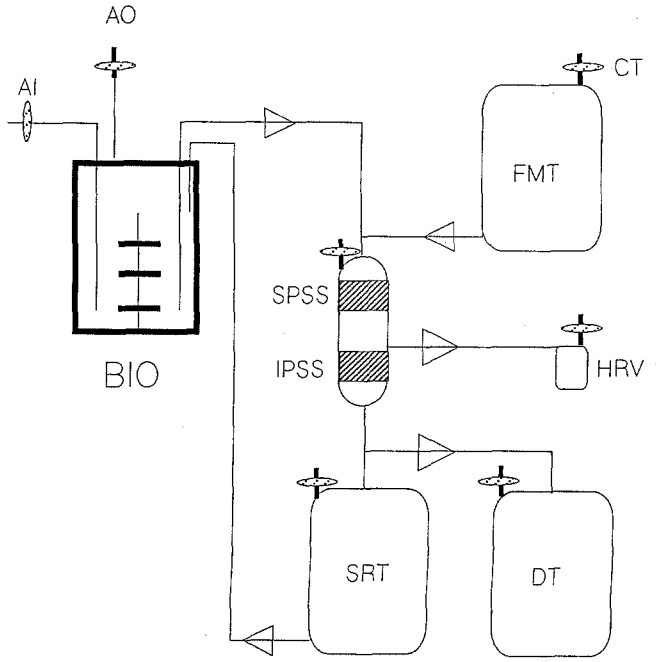

Fig. 1. Filtering apparatus: AI, air inlet; AO, air outlet; SPSS, superior pore size sieve; IPPSS, inferior pore size sieve; SRT, suspension receiving tank; DT, draining tank; HRV, harvest receiving tank; FMT, fresh medium tank; CT, cotton filter; Bio, bioreactor

trials, spent medium was regularly removed by air pressure through a 25-µm sieve and replaced by fresh medium.

Filtering apparatus. During each harvest the following operations were made with the apparatus shown in Fig. 1. By air pressure the embryo suspension was allowed to fill the filtering unit and to flow through 280-µm then 180-µm sieves towards the suspension receptor tank (SRT). In some experiments, a 380-µm then a 180-µm sieve were used. The resulting suspension was flushed back to the fermentor, then 2–5 l of fresh medium was air-propelled from the fresh medium tank (FMT) to the sieves, in order to rinse them, and then to the draining tank (DT). The embryos on the surface of the second sieve (IPSS) were harvested and propelled by fresh medium to the harvest receptor vessel (HRV). Before and after each harvest, samples were kept to count the number of embryos eliminated from the suspension. For some experiments cumulative data are presented.

Measurements of cell dry weight, fresh weight, embryo number and carbohydrate concentration. In order to establish the kinetics, a measured volume of tissue suspension was collected every 3 or 4 days. The fresh weight (FW) was recorded and the sample dried at 110° C for 24 h for measuring the dry weight (DW). For embryo counts 1 ml was added in a Petri dish (8.0 cm diameter) and mixed with 4 ml of 2% agar (w/v). Then the embryos were counted using a microscope. Four counts were made for each sample. Embryos were counted according to their shape and size, which are 100–200 µm, 200–400 µm, 400–1000 µm and more than 1000 µm for globular-, heart-, torpedo- and cotyledonary-plantlet stages, respectively. The carbohydrate concentration in the medium was estimated by the enzymatic method using hexokinase and glucose-6-phosphate dehydrogenase (Boeringher kit).

Measurements of size, viability and embryo-to-plant conversion rate. Each size value represents the mean + standard error of at least 50 embryos. For viability tests, embryos were placed in petri dishes containing 10 ml MSO medium at a concentration lower than 5 embryos/ml (three petri dishes for each estimate). After 7 days, survival expressed as the percentage of embryos exhibiting a bipolar development (root growth and differentiation of chlorophyllous cotyledons) was recorded. To estimate the embryo-to-plant conversion rate, 100 embryos were transferred at the plantlet stage (about 1000 µm) from liquid medium to the same basal medium solidified with Bacto-Difco agar (0.8%, w/v). Conversion rates were determined as the percentage of viable embryos producing normal plantlets after 4 weeks of culture on semi-solid medium.

Results

Somatic embryo production in shake-flasks

In 0.5-l shake-flasks containing 0.25 l MSO medium inoculated with 0.1%, v/v (volume of tissue/volume of medium), the maximum total embryo concentration was 1.9 to $11.0 \cdot 10^5$-embryos/l (Table 1). These maximal concentrations were reached during the third week of culture after transfer of tissue into a hormone-free medium. The mean production varied between 1.6 and $7.5 \cdot 10^4$ embryos/l per day (values derived by dividing the maximum yield by the time taken to achieve that yield). After 2 weeks of culture, 20–50% of the embryos had reached the torpedo stage. The number of plantlets increased and their roots became very long (more than 5 cm); consequently, it was impossible to take out samples after 3 weeks of culture.

Preliminary studies have shown that an inoculum density of 0.1% (v/v) can be considered the optimal density. In fact, if the inoculum size was increased tenfold, production was multiplied by about ten, but the percentage of torpedo and plantlet-stage embryos decreased: it never exceeded 12% and the size of plantlet roots never exceeded 1 cm (Table 1).

Somatic embryo production in a bioreactor

Embryo yields in a 10-l bioreactor were nearly comparable to those obtained in shake flasks; production of 1.4 to $5.3 \cdot 10^4$ and $49.6 \cdot 10^4$ embryos/l per day could be obtained for inocula of 0.1% (v/v) and 1.0% (v/v), respectively (Table 2). An example of embryo development kinetics with an inoculum size of 0.1% (v/v) is reported in Fig. 2A (see also Table 2, trial 1). In this experiment the whole suspension had been regularly filtered on 280-µm then 180-µm sieves. After each filtration the total embryo concentration dropped. In this way, the concentration never exceeded $5 \cdot 10^5$ embryos/l. As the largest embryos were eliminated from the suspension, plantlets did not appear in the suspension, so sampling was possible even after 3 weeks. The cumulative growth curves show that the maximum total embryo yield, reached after 2 weeks of culture, was about $8 \cdot 10^5$ embryos/l (Fig. 2B). During these 2 weeks, the production remained constant with a mean value of about $5.3 \cdot 10^4$ embryos/l per day. It is necessary to emphasize that the biomass concentration at the time of the inoculation was very low (0.4 g FW/l) and that, due to regular filtration, it remained

Table 1. Somatic carrot embryo production in shake-flasks (0.25 l)

Strain	Age (subculture number)	Inoculation density (%, v/v)	Maximum total embryo yield ($\times 10^5$ embryos l)	Time (days)	Production ($\times 10^4$ embryos per day)	Maximum torpedo and plantlets (%)
C1	15	0.1	8.9± 2.4	17	5.2	23
	15	1.0	75.9±12.7	17	44.6	5
	40	0.1	5.4± 0.4	22	2.5	29
C3	10	0.1	6.6± 1.6	15	4.4	21
	17	0.1	9.5± 3.3	14	6.8	30
	17	1.0	89.9±16.4	18	49.8	12
	19[a]	0.1	10.4± 1.8	14	7.4	32
	19[a]	1.0	82.0±10.0	17	48.2	7
	23	0.1	6.7± 1.4	16	4.2	28
C4	4	0.1	9.9± 1.2	18	5.5	45
	4	1.0	70.4± 3.3	15	46.9	12
	9	0.1	7.9± 2.2	19	4.2	17
	17[a]	0.1	7.0± 0.9	14	5.0	20
	20	0.1	5.2± 0.5	14	3.7	21
C5	4	0.1	8.4± 2.6	15	5.6	30
C6	3	0.1	8.4± 2.0	14	6.0	38
	7	0.1	1.9± 0.7	12	1.6	40

[a] Tissues (50/180 μm) used to initiate embryogenesis

Table 2. Somatic embryo production in a 10-l bioreactor

Trial	Strain	Age (subculture number)	Inoculation density (%, v/v)	Aeration (vvm)	Initial $K_L a$ (h^{-1})	Dissolved oxygen evolution (% of saturation)	Harvest[b]	Change of medium	Maximum total embryo yield ($\times 10^5$ embryos l)	Time (days)	Production ($\times 10^4$ embryos per day)
1	C1	15	0.1	0.05	0.7	100–80	+	–	7.9± 0.5	15	5.3
2		20	0.1	0.05	0.7	100–60	+	+	10.9± 0.6	22	4.9
3	C3	19	1.0	0.1–0.2	2.7	100–10	–	–	87.2±19.7	16	49.6
4	C4	9	0.1	0	0	20 Controlled	+	–	8.3± 0.8	27	3.1
5[c]	C5	0.1	0.05	0.7	100–40	–	–		5.9± 1.6	11	5.3
6	C6	3	0.1	0.3	6.2	100–75	+	–	6.8± 2.1	14	4.9
7		7	0.1	0.3	6.2	100–51	+	+	1.6± 0.5	12	1.4

$K_L a$, volumetric mass transfer coefficient
[a] Tissues (50/180 μm) use to initiate embryogenesis
[b] Harvested by filtration on 280-μm then 180-μm sieves except for trial 7 (380/280 μm)
[c] Sampling impossible after day 11

at a low value throughout the run, never more than 7.0 g/l for the FW and 0.40 g/l for the DW.

The sugar uptake curve shows that sucrose was hydrolysed during these 2 weeks (Fig. 2C). Nevertheless, when embryo production stopped, the sugar concentration was as high as 20 g/l (10 g/l for glucose and 10 g/l for fructose). So, embryo production arrest did not result from sugar depletion; it seems that production stopped because there was a depletion of proembryos in the medium. This observation is consistent with the idea that in the absence of 2,4-D there is no proliferation of proembryos.

Figure 3A shows the kinetics of embryo development with an inoculum size of 1.0% (v/v) (see also Table 2, trial 3). In this experiment no harvest was made during the culture. Because of the inhibition of embryo development there were no plantlets in the suspension. So, sampling was possible after 3 weeks even if the suspension had not been filtered. The first heart-shaped embryos appeared after 3 days, torpedo-shaped embryos after 6 days. The total embryo concentration increased until 16 days with an average value of about $49 \cdot 10^4$ embryos/l per day, peaked at this time ($87.2 \cdot 10^5$ embryos/l), then the total embryo concentration tended to decrease. As the appearance rate of the torpedo-shaped embryo is never identical to the one of heart-shaped, it can be assumed that the inhibition of development took place as early as day 6. Although sucrose totally disap-

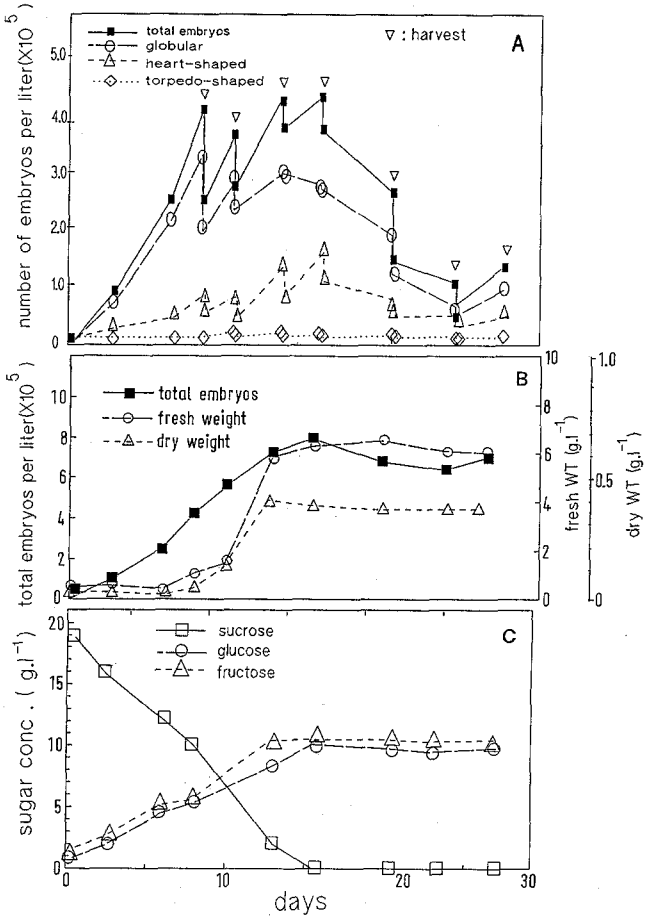

Fig. 2A–C. Production of carrot somatic embryos in a 10-l bioreactor with an inoculum density of 0.1% (v/v) (trial 1). **A** Kinetic development of embryos. **B** Cumulative growth curve in terms of total embryos, fresh and dry weight (WT). **C** Concentration of carbohydrates

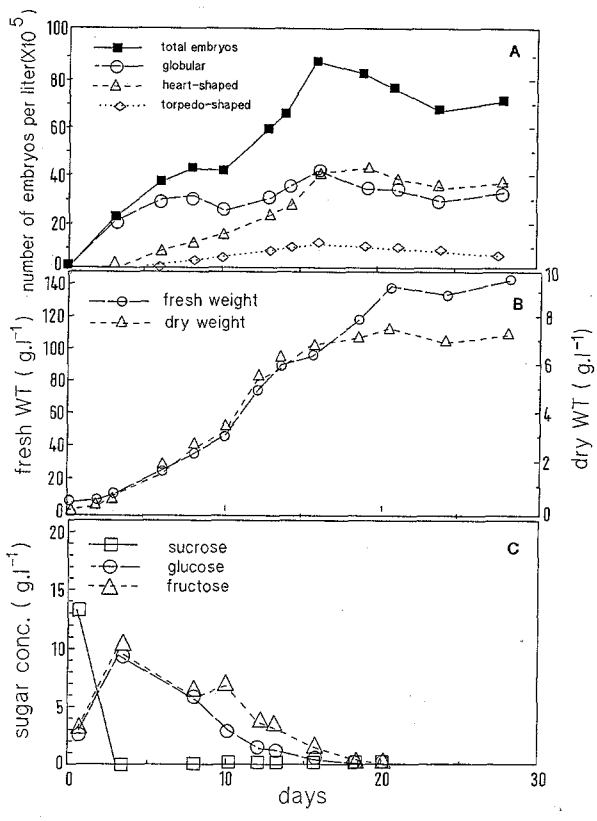

Fig. 3A–C. Production of carrot somatic embryos in a 10-l bioreactor with an inoculum density of 1.0% (v/v) (trial 3). **A** Kinetic development of embryos. **B** Growth curve in terms of fresh and dry weight. **C** Concentration of carbohydrates

peared after 3 days, glucose and fructose were completely consumed only after 18 days (Fig. 3C). Moreover, data presented in Fig. 3B show that the DW and FW continued to increase after day 6, until 21 days (7.7 g DW/l and 138 g FW/l, respectively). Thus, it can be concluded that cessation of embryo development is related to self-inhibition (accumulation of toxic compounds in the medium) rather than depletion of carbohydrate or nutrients.

Filtering system

So far, filtration through a nylon or steel screen provides the only method for the purification of embryos at different developmental stages from large volumes of embryo suspensions (Rodriguez et al. 1990). Data presented in Table 3 show the quantity and the quality of embryo populations obtained after each harvest during the experiment shown in Fig. 2 (trial 1). In total, $9 \cdot 10^5$ embryos were collected after 7 filtrations (280/180 µm). The global filtration yield (i.e. number of embryos harvested/total number of embryos in the bioreactor) was about 11%. The average size of these populations was about 260 ± 100 µm. A second experiment was monitored with the same strain using the same culture conditions as in trial 1 except that the spent medium was removed twice a week and replaced by fresh medium (see Table 2, trial 2). Production was the same as in trial $1:5 \cdot 10^4$ embryos/l per day during 2 weeks of culture. The total number of embryos collected was about $2.9 \cdot 10^6$; the global filtration yield reached in this case was 27%.

Viability of the harvested embryos

During trial 1, viability of the embryos decreasing during embryogenesis: in the last harvest at day 27 only 7% of the embryos were viable (Table 3A). In fact brown-coloured embryos appeared in the collections and their percentage increased with the age of the suspension. The existence of these brown-coloured embryos is not due to a high DO level because they also appeared if DO was regulated at 20% of saturation (see Table 2, trial 4). Moreover, in erlenmeyer flasks the DO remained as high as 60% for 10 days (data not shown) but we never observed necrotic embryos. They also appeared in the bioreactor even if no harvests were made (see Table 2, trial 5).

When the spent medium was removed twice a week, as in trial 2, embryo viability remained as high as 68%

Table 3a, b. Embryo populations harvested during somatic embryo production in a 10-l bioreactor. **a** Without changing the medium (trial 1). **b** With medium changed twice a week (trial 2)

	Date of harvest (days)	Number of embryos harvested ($\times 10^6$)	Size (μm)	Viability (%)
a	8	0.07	276±112	44
	10	0.12	287±112	42
	13	0.14	240± 98	24
	15	0.24	—	10
	20	0.22	—	0
	23	0.09	—	10
	27	0.05	—	7
b	8	0.29	289±121	86
	11	0.25	276±110	—
	15	0.48	254±107	68
	18	0.67	245±117	76
	22	0.99	232±130	77
	25	0.26	279±124	77

—, not measured
[a] Mean of three replicates

throughout embryogenesis (Table 3b) and brown-coloured embryos were not observed in the collections. Another experiment including regular medium replacement confirmed this result (see Table 2, trial 7): viability was as high as 77% at the end of this run (Table 4).

Synchronization of a few successive embryo harvests

Three harvests on 380/280-μm sieves achieved during trial 7 were placed at 4° C in a 1.0-l erlenmeyer flask without agitation (0.6 l MSO medium, 100 embryos/ml, in the dark). The efficacy of this conservation procedure was tested for 36 days after the beginning of the trial (culture + conservation time) according to three parameters: conservation of the mean size, viability, and embryo-to-plant conversion (Table 4). The embryo mean size and potential to develop into a plant were maintained for at least 24 days. Surprisingly, the embryo-to-plant conversion of the viable embryos seemed to increase in the case of stored embryos. For collections 1, 2 and 3, viability dropped by 8, 28 and 11%, respectively.

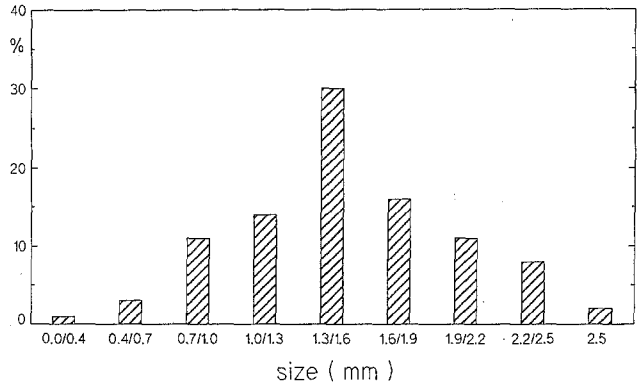

Fig. 4. Distribution according to the size of a population of embryos obtained by 1.0/0.7 mm filtration after a two-step maturation

Nevertheless, this procedure seems to be a good approach for both conserving all embryo harvests achieved during a first development step, and maintaining their synchronization throughout a second maturation step.

At the end of the trial 7 the three harvests were grouped together in a 1-l shake-flask (concentration: 50 embryos/ml, 110 rpm, 0.5 l MSO medium). After 3 days, the suspension was filtered on a 1000-μm then a 700-μm pore size nylon fltler. The embryo population obtained (about 10000 embryos) was 100% viable and the embryo-to-plant conversion was 77%. The mean size was 1547±536 μm (90% of the embryos were at the torpedo-shaped stage, between 400 and 2200 μm). The distribution of these embryos according to their size is shown in Fig. 4.

Discussion

Embryo production depends on the age of the cell lines. Like other authors (Smith and Street 1974), we observed a decline of the embryogenic potential of carrot tissue suspensions with the number of subcultures. In practice, we utilized carrot strains aged less than 6 months (i.e. about 15 subcultures). In this way a mean production of about $50 \cdot 10^3$ embryos/l per day is commonly achieved for an inoculum size of 0.1% (v/v) (i.e. 0.2–0.4 g FW/l). The self-inhibition of the heart-shaped embryo devel-

Table 4. Synchronisation of the 380/280-μm harvests obtained during somatic embryo production in a 10-l bioreactor (trial 7) by chilling

Before conservation phase					After conservation phase		
Harvest date (days)	Size (μm)	Viability[a] (%)	Embryo-to-plant conversion ($n=100$) (%)	Conservation duration (days)	Size (μm)	Viability[a] (%)	Embryo-to-plant conversion ($n=100$) (%)
12	427±207	95	56	24	498±207	88	72
15	482±182	90	66	21	479±223	62	74
19	363±181	77	54	17	445±200	66	81

[a] Mean of three replicates

opment linked to the size of the inoculum has been previously reported for carrot (Wetherell 1984), rice (Ozawa and Komamine 1989) and the coffee tree (Zamarripa et al. 1991). Moreover, we observed that this inhibition is reversible: it could be suppressed by diluting the culture with fresh medium. In a 10-l bioreactor we obtained a production of embryos of the same magnitude as in shake-flasks. Thus it can be considered that culture conditions in a stirred bioreactor do not affect embryo production. The reason for the reduction in embryo viability remains unclear. It appears that the DO concentration is not implicated, but we must consider the possibility that embryo viability may be affected by the aeration rate of the medium, and therefore due to changes in the concentration of other gases, such as CO_2 or ethylene. As no physical damage can be seen on the surface of these necrotic embryos it can be assumed that neither regular suspension filtering nor direct mechanical effect of the impellers on the embryos are involved. The fact that medium renewal suppresses this phenomenon suggests this hypothesis: large vacuolated and distorted cells present in the inoculum are probably more fragile than small, densely granular proembryo cells, and more susceptible to mechanical agitation. In fact, more cell fragments are observed in bioreactors than in sake-flasks. It can be assumed that these large cells, after their destruction, release toxic compounds in the medium, which can affect embryo viability if they are not diluted by fresh medium. Further studies are needed to specify the origin of this necrosis phenomenon.

According to our culture conditions the filtering system utilized in this work allows the production embryo populations free of undifferentiated tissues with more than 95% of single embryos. Rinsing the sieves is a very important step: the more the filters are rinsed, the better the calibration of the population collected, but the greater the number of embryos lost. The global filtration yield is about 10–30%. Embryos were lost during each filtration on the surface of the first sieve (in the case of embryos that were too long) and during the rinsing step (in the case of embryos that were too small). The quantity of embryos collected on the second filter can be increased in two ways, firstly by increasing the frequency of collecting operations, consequently the number of embryos that are too long at each collection will decrease, and secondly by using filtered medium on a 25-μm sieve already contained in the bioreactor for the rinsing step. In this way small embryos can be recovered and flushed back into the suspension. By using sieves of different pore sizes, by rinsing the filtering unit with increasing medium volumes during collections, and by increasing filtration frequency, it would be possible to control embryo population characteristics such as mean size and size distribution.

Such a powerful process of vegetative multiplication provides the preliminary step for encapsulating embryos to obtain what are called "artificial seeds". It will be necessary to determine embryo population characteristics consistent with the chosen somatic embryo distribution and coating method. In carrot breeding, such a multiplication process could be of interest for maintaining and multiplying male sterile plants as parents of hybrids. Therefore it will be necessary to test the flowering and fertility abilities of embryo-derived plants.

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