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Induction of microspore embryogenesis in *Camellia japonica* cv. Elegans

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Abstract

Three methods of microspore culture were tested for the induction of microspore embryogenesis in *Camellia japonica* L. cv. Elegans. Culture was performed on 17 different media consisting of Murashige and Skoog (MS) and N6 basal media with different combinations of carbon, growth regulators, serine and glutamine. Microspore suspensions plated over solid MS medium containing 4.5 μM 2,4-dichlorophenoxyacetic acid and 0.5 μM kinetin, with sucrose (MS6) or glucose (MS9) were seen as the best culture conditions for induction of embryogenesis. The development of microspore derived proembryos was obtained in MS medium supplemented with 2.2 μM N^6-benzyladenine (MS10) and reached the highest level when the microspores were cultured in MS6 inducing medium. The development of microspore-derived embryos ceased at the maturation stage.

Abbreviations: BA – N^6-benzyladenine, 2,4-D – 2,4-dichlorophenoxyacetic acid

Introduction

Camellia japonica L. (*Theaceae*) is an ornamental woody species. Some in vitro culture systems for direct embryogenesis have been established for this species (Pedroso & Pais 1993; Pedroso-Ubach 1991). These studies showed the potential for *C. japonica* to be used for studying somatic embryogenesis at cytological, biochemical and molecular levels (Pedroso & Pais 1992).

Although in the majority of species pollen-derived plants are obtained by anther culture, plant regeneration from isolated microspores has been successfully achieved in a few species (Bajaj 1990). Microspore culture presents a number of potential advantages over anther culture, mainly in relation to in vitro selection strategies, to genetic studies and genetic transformation. The culture of isolated microspores has become a valuable system for studies of embryogenesis in vitro (Taylor et al. 1990). Direct microspore embryogenesis, if achieved for *C. japonica*, would be potentially valuable for studying gene activity during microspore embryogenesis and the factors that divert microspore development into an embryogenic pathway.

In the present study, we report the results obtained on the induction of microspore embryogenesis in *Camellia japonica* cv. Elegans.

Materials and methods

Plant material

Unopened floral buds were harvested at the begining of flowering period from a single adult plant (> 50 years old) of *Camellia japonica* cv. Elegans (private garden in S. Paio, Gouveia, Portugal). Outdoors growth temperature for donor plant was 13°/1°C day/night.

Disinfestation

Floral buds were surface disinfested for 20 min in full-strength domestic bleach (5% active chlorine), washed 4 times in sterile distilled water and immediately dissected under aseptic conditions for anther isolation.

Culture conditions

Using the media composed of inorganic salts and vitamins of Murashige and Skoog (MS)(1962) Chu (N_6) (1978), 17 different combinations of carbon source, growth regulators, serine and glutamine were tested (Table 1). Dithiothreitol (DTT) at 5 mg l^{-1} was added to all media. The pH was adjusted to 5.5 before autoclaving. Agarose (Type VII, Sigma, USA) at 6 g l^{-1} was added to solid media. Autoclaving was performed at 120°C, 10^5 Pa, for 20 min. Serine (Ser) and glutamine (Gln) solutions were sterilized by filtration (pore size 0.22μm). The cultures, performed in 50 mm glass petri dishes, with 3 ml of culture medium per dish, and sealed with parafilm, were incubated in growth chambers at 24 ± 1°C, in darkness or under 16-h photoperiod (26 μmol m^{-2} s^{-1}, Philips white TLD 18W/84 tubes). Cultures established in liquid media, in 100 ml Erlenmeyer flasks closed with aluminium foil, were kept on shakers at 50 r.p.m. agitation.

Microspore isolation and culture

Three methods were tested: microspore culture immediately after anther isolation (method A and B) and after anther incubation (method C).

Method A, spontaneous release. The microspores were spontaneously released through natural dehiscence of anthers cultured in 20 ml of liquid media (Table 1) in 100 ml Erlenmeyer flasks at a density of 4 anthers per ml. After microspore release, anthers were removed from the medium and the microspores kept in suspension culture.

Method B, isolation through homogenisation and filtration. The anthers (8 to 10) were carefully crushed in 5 ml of MS or N_6 basal medium without growth regulators (washing solution, WS). The microspore suspension was filtered through a mesh of 200 μm and centrifuged at 80 *g* for 5 min. The pellet was resuspended in WS and centrifuged twice (washing step). After washing, the microspores were resuspended in a variable volume of liquid culture medium (Table 1) in order to achieve a determined inoculum density. The microspore suspensions were then plated over solid medium and cultured under light. Twenty different inoculum densities were tested ranging from 2.0×10^3 to 1.1×10^6 microspores per ml. Microspore density was determined by direct counting using a hemocytometer. The effect of inoculum and uninucleate microspore densities on embryo and callus formation was recorded 6 weeks after culture initiation, for microspore suspensions plated over solid MS9 medium and cultured under light. Microspore development in vitro was followed by periodic observation of microspores cultured on 0.4% agarose medium (immobilized). Values are the mean of at least three experiments with six replicates each, a total of 1.3×10^6 microspores (120 anthers).

Method C, microspore isolation after anther incubation. The anthers (6 anthers per ml) were incubated at 24 ± 1°C for 1 week on 0.4% agarose-solidified MS6, MS9 and MS10 media under light. After anther incubation, microspores were isolated following method B and plated over solid MS6, MS9 and MS10 media and cultured under light. Cultures initiated on the same day without previous anther incubation (method B) were used as controls. Values are the mean of six experiments with 10 replicates each.

Samples were taken at the time of inoculation at 0, 2, and 6 weeks after microspore culture initiation. Cultures and samples were observed using an inverted epifluorescence microscope (Nikon, Narashige, Japan). Microspore culture over solid media was performed by adding, every 4 weeks, 2 ml of fresh medium per petri dish. Twelve weeks after culture initiation, microspore suspension cultures and microspore cultures plated over solid medium were transferred to MS10 medium (maturation stage). We considered as 'sporophytic induction' (as opposed to gametophytic) the pathway of development leading to embryo or callus formation. Embryo and callus production efficiency (percentage of embryos and calli produced by induced microspores) of the three methods was determined for uninucleate microspores plated at a density of 1.6×10^5 microspores per ml over solid MS6, MS9, and MS10 media, under light conditions. Results were recorded at the time of inoculation, 2, 6, 8, and 12 weeks after culture initiation.

Optical microscopy

Microsporogenesis stage (at the time of inoculation) and gametophytic and embryogenic response (following microspore culture) were determined by staining with 4% acetocarmine (McClintock 1929) and DAPI (4',6-diamidino-2-indolephenyl) (Pedroso-Ubach 1991). Microspore viability was determined by staining with fluoresceine diacetate (FD) (Widholm 1972). Samples were observed 5 to 10 min after staining under a Leitz-Wetzlar SM-lux microscope (excitation at 340-380 nm and emission at 430 mm for DAPI; excitation at 450-490 nm and emission at 512 nm

Table 1. Culture media tested for microscope culture of *Camellia japonica* cv. Elegans

Media	Suc. (gl^{-1})	Gluc. (gl^{-1})	m-Inos (mgl^{-1})	Gln (mgl^{-1})	Ser (mgl^{-1})	2,4-D (μM)	Kinetin (μM)	IBA (μM)	BA (μM)	AC (gl^{-1})
MS1	30	-	1000	-	-	4.5	0.5	-	-	-
MS2	30	-	1000	-	-	-	-	19.1	8.9	-
MS3	30	-	1000	-	-	-	-	-	-	-
MS4	20	-	100	-	-	4.5	0.5	-	-	-
MS5	20	-	100	-	-	4.5	0.5	-	-	2.0
MS6	20	-	100	800	200	4.5	0.5	-	-	-
MS7	-	25	100	-	-	4.5	0.5	-	-	-
MS8	-	25	100	-	-	4.5	0.5	-	-	2.0
MS9	-	25	100	800	200	4.5	0.5	-	-	-
MS10	20	-	100	800	200	-	-	-	2.2	-
N1	100	-	100	-	-	4.5	0.5	-	-	-
N2	20	-	100	-	-	4.5	0.5	-	-	-
N3	20	-	100	800	200	4.5	0.5	-	-	-
N4	100	-	500	800	200	6.8	2.3	-	-	-
N5	100	-	100	-	-	-	-	-	-	-
N6	100	-	100	-	-	6.8	2.3	-	-	-
N7	-	25	100	-	-	4.5	0.5	-	-	-

MS = inorganic salts and vitamins of modified Murashige & Skoog (1962) medium.N = inorganic salts and vitamins of modified Chu (1978) medium.Suc. = sucrose; Gluc. = glucose; m-Inos. = meso-inositol; Gln = L-glutamine; Ser = L-serine; 2,4D = 2,4-dichlorophenoxyacetic acid; IBA = indol-3-butyric acid; BA = N^6 -benzyladenine; AC = activated charcoal.

for FD). Photomicrographs were taken using Kodak Ektachrome 400 ASA film.

Results

Microspores were extremely sensitive to variations of chemical and physical culture conditions. The cell volume increase occurred during the first 2 weeks of culture and was used as a microspore-response indicator. Three groups of microspores were considered. Group 1 comprised microspores showing 0 to 90% volume increase, negative or reduced acetocarmine staining, and a break of gametophytic development after culture initiation. These microspores were at tetrad, early uninucleate and late binucleate stages upon isolation. Group 2 comprised microspores showing 100 to 150% volume increase and a normal gametophytic development until pollen grain germination. Group 3 comprised microspores showing 200 to 400% volume increase and following a sporophytic pathway of development. Groups 2 and 3 were composed of microspores isolated at the uni- and binucleated stages, 0.98% (±3.6) of them (uninucleate microspores with peripheral nucleus and a dense cytoplasm) highly reactive to acetocarmine. The microspores from group 3 presented two types of sporophytic response: embryo and callus formation. DAPI staining showed that embryo and callus were originated from uninucleated microspores. A first symmetric mitotic division gave rise to proembryos while assymmetric divisions originated callus. After transfer to MS10 medium (embryo maturation medium), proembryos slowly turned into milky-white small spherical embryos. Three embryos reached a torpedo-like shape but they underwent severe browning. Microscopic observation of hand-made sections showed that the embryos obtained had two poles but cells from protoderm presented an uncommon form compared to those from protoderm of somatic embryos.

Method A

Anther dehiscence in suspension culture occurred during the first 3 weeks of culture. The mean of microspores released was 8.9×10^2 to 2.3×10^3

microspores per anther. The microspores were at the uninucleate stage with peripheral nucleus or at the binucleate stage and demonstrated a viability of 94.3% (±4.2). Microspore suspension culture enabled the induction of embryogenesis. However, proembryo development was only obtained by plating microspore suspension cultures over solid MS10 medium.

Method B

Isolation by homogeneization and filtration allowed the immediate release of 2.3×10^3 to 2.4×10^4 microspores per anther with a viability of 99.4% (±0.6). The isolated microspores were initialy at the uninucleate stage. Purification of microspore suspensions did not significantly affect microspore viability.

The microspores cultured in MS4, MS6 to MS10, N5, and N6 media underwent sporophytic divisions. However, a successful induction of embryo and/or callus formation was only achieved in MS6, MS9, and MS10 media. The highest value (1.3 microspores undergoing division per 1000 microspores) was recorded for microspores cultured in MS9 medium (Fig. 1a). First sporophytic divisions were observed at week 2 of culture for 0.56% of the cultured microspores and increased until week 4 (Fig. 1a). Microspore culture over solid medium enabled the induction of embryogenesis, callogenesis and proembryo development. Only culture under light conditions enabled proembryo formation. At 6 weeks after culture initiation, the number of microspores undergoing division per 100 anthers for cultures under light was 3.6-fold that of cultures in darkness (380 versus 105). For microspores cultured in darkness embryo and callus induction ceased at the week 6 of culture, after the first or second mitotic division.

The optimal values of inoculum density varied from 1.5 to 3.7×10^5 microspores per ml (Fig. 2, assays 5, 6). Lower microspore densities (3.0×10^4 to 1.0×10^5) stopped sporophytic development after 6 weeks in culture (Fig. 2, assays 3, 4). Inoculum densities lower than 3.0×10^4 and higher than 5.6×10^5 inhibited sporophytic induction (Fig. 2, assays, 1, 2, 8). A high inoculum density containing only 9.5 to 49.7% of uninucleated microspores did not enable either sporophytic induction (Fig. 2, assay 2) or efficient embryogenic development (Fig. 2, assays 3, 4). Identical results were observed when a low inoculum density (8×10^3) consisting of 100% of uninucleated microspores was used (Fig. 2, assay 1). Only microspores at the uninucleate stage inoculated at optimal densities (>60% of total population) enabled embryo formation (Fig. 2, assays 5, 6).

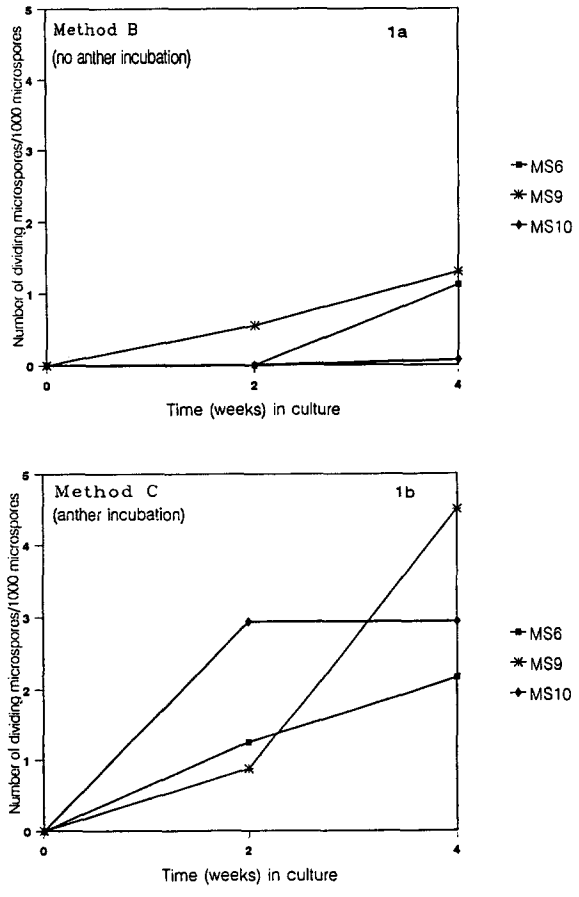

Fig. 1. Sporophytic induction (number of dividing microspores per 1000 microspores) in microspore cultures of *Camellia japonica* cv. Elegans. *(a)* Microspores isolated by homogenization and filtration (method B). *(b)* Microspores isolated after anther incubation (method C). Isolated microspores were plated over solid MS6, MS9, and MS10 media (Table 1), under 16-h photoperiod. Values are the mean of six experiments with 10 replicates each.

Method C

Anther incubation prior to microspore isolation and culture enhanced sporophytic induction (Fig. 1b) when compared with direct microspore culture using method B (Fig. 1a). Microspores isolated from anthers previously incubated underwent the first sporophytic division 2 weeks after culture initiation (Fig. 1b). Method C allowed earlier sporophytic divisions in MS10 medium in which all the induced microspores presented a first division at week 2 of culture. In MS6 and MS9 media, sporophytic induction was not synchronous,

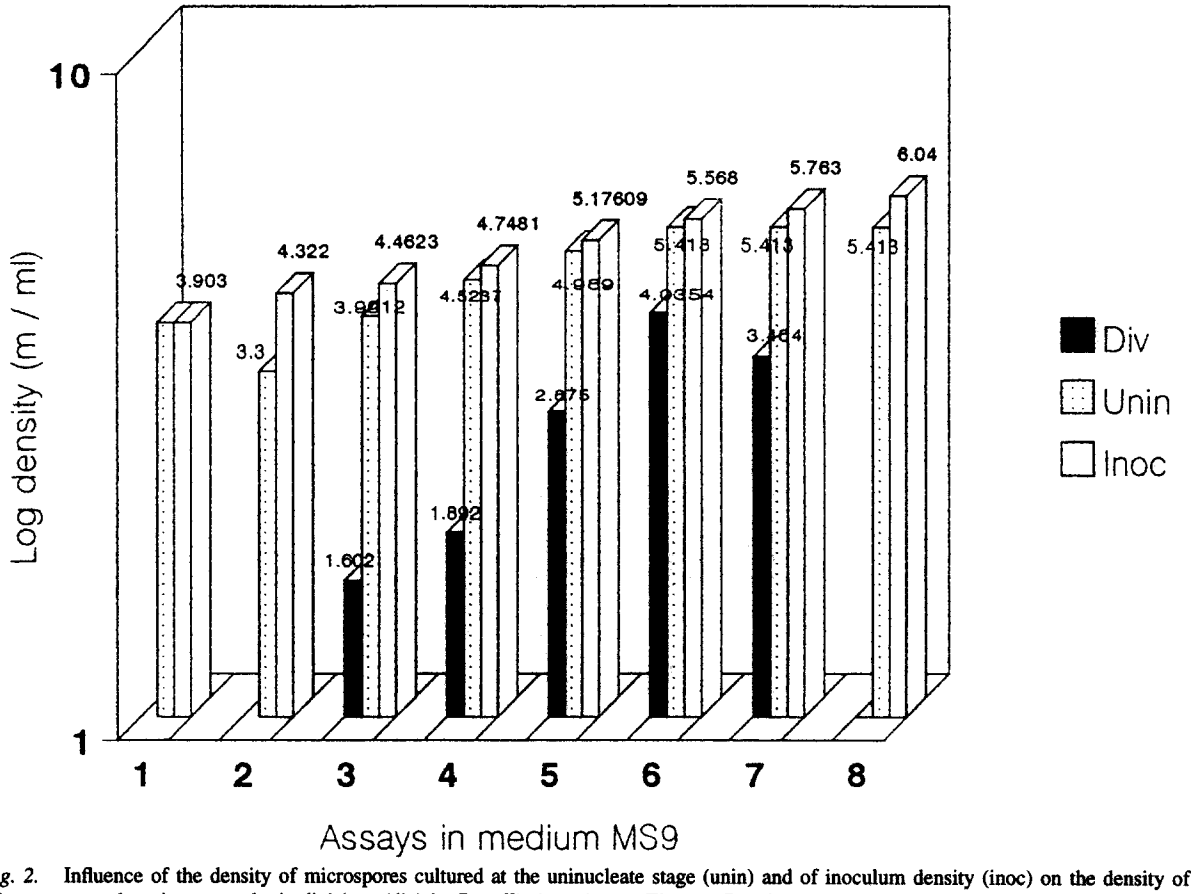

Fig. 2. Influence of the density of microspores cultured at the uninucleate stage (unin) and of inoculum density (inoc) on the density of microspores undergoing sporophytic divisions (div) in *Camellia japonica* cv. Elegans. Results are expressed in log of the density (number of microspores per ml) (m ml^{-1}). Microspores were isolated following method B and cultured over solid MS9 medium (Table 1) under 16-h photoperiod. Values are the mean of three experiments with six replicates each and were recorded 6 weeks after culture initiation.

being proembryo development concomitant with first sporophytic divisions until 6 weeks after culture initiation. The MS9 medium allowed, at week 4, the highest sporophytic induction, with 4.5 microspores undergoing division per 1000 microspores (Fig. 1b).

Efficiency of the assayed methods

Embryo formation occurred only on microspore cultures induced in MS6 and MS9 media (Table 2). From the three methods assayed, method C was the most efficient for sporophytic induction (proembryos and callus), but not for embryo maturation. Embryo and callus formation (maturation stage) were higher using method B and MS6 medium for the induction stage (Table 2). Induction of microspores in MS10 medium inhibited proembryo development during the maturation stage. Embryo production efficiency (Table 3) was higher in MS6 medium using method B and callus production efficiency was highest when MS10 medium and method B were used.

Discussion

The best culture conditions for microspore-derived embryo production were

- isolation by method B, and
- culture of 1.5 to 3.7 × 10^5 uninucleated microspores per ml, under light, in MS6 medium for the induction stage, and in MS10 medium for the maturation stage.

Anther incubation prior to microspore isolation (method C) was advantageous for the induction of sporophytic divisions, but less efficient for embryo maturation than method B. Microspore isolation from

Table 2. Embryo and callus induction (First divisions) and maturation in Camellia japonica cv. Elegans, expressed in dividing microspores, embryos or callus per 100 anthers (anth) and per 1000 microspores (mic) for the three methods of isolation and culture of microspores tested, A, B, and C.

Method	Induction			Maturation				
	Media	First divisions		Medium	Embryos		Callus	
		/100 anth	/1000 mic		/100 anth	/1000 mic	/100 anth	/1000 mic
A	MS6	603	0.620	MS10	4.92	(0.00050)	1.96	0.006
	MS9	200	0.186	MS10	0.86	(0.00008)	0	0
	MS10	0	0	MS10	0	0	0	0
B	MS6	380	1.430	MS10	14.28	0.053	2.33	0.013
	MS9	629	1.300	MS10	6.45	0.013	6.45	0.013
	MS10	63.4	0.090	MS10	0	0	2.86	0.004
C	MS6	2484	2.170	MS10	2.86	0.003	0	0
	MS9	1250	4.500	MS10	0.93	0.007	1.85	0.006
	MS10	1666	2.940	MS10	0	0	6.67	0.001

Composition of the media used is given in Table 1. Values were recorded after 6 weeks in induction medium and after 12 weeks in maturation medium.

Table 3. Efficiency (%) of embryo and callus production in *Camellia japonica* cv. Elegans, for the three methods of isolation and culture of microspores tested, A, B and C, in MS6, MS9, and MS10 media (see Table 1).

Method	Efficiency (%)					
	Embryos			Callus		
	(MS6)	(MS9)	(MS10)	(MS6)	(MS9)	(MS10)
A	0.08	0.04	0	0.96	0	0
B	3.71	1.00	0	0.91	1.00	4.44
C	0.14	0.14	0	0	0.13	0.03

incubated anthers seems more traumatic than isolation from freshly collected anthers, probably due to the deficiencies of the homogenisation medium.

Embryo and callus induction from microspores at the uninucleate stage was highest in MS6, MS9, and MS10 media. These three culture media were already successfully used for *C. japonica* anther culture (Pedroso-Ubach 1991), However, the presence of BA in MS10 medium, the best for embryo formation from anthers (Pedroso Ubach 1991), only enabled the formation of microspore-derived callus when used for the induction and maturation stages. According to Raghavan & Nagmani (1989), the presence of cytokinins in the culture medium can negatively affect pollen embryogenesis, inducing only callus formation and stopping the development of callus-derived embryos. Microspore embryogenesis occurred only when microspores were cultured in the presence of 2,4-D and kinetin (MS6 and MS9 media) and were transferred to MS10 medium for further development. The occurrence of both symmetric and assymmetric divisions originating, respectively, embryos and callus seems to indicate that sporophytic response of microspores in *C. japonica* cv. Elegans follows at least two pathways of development identical to those reported for *Hevea brasiliensis* Muell.-Arg. (Chen 1990), and corresponding to pathways B and A described by Sunderland & Dunwell (1974). According to Chen [1990] the majority of the microspores follows pathway B of development when in the presence of 2,4-D and kinetin, which agrees with the results obtained for *C. japonica*. The death

of microspore-derived embryos has also been reported for other woody species (Milewska-Pawhiczuk & Kubicki 1977; Milewska-Pawliczuk 1990; Zhang et al, 1990) and is frequently associated with an inadequate selection of the culture medium. It could have been the case, however, that abnormal protoderm formation was probably the cause of embryo death. The results obtained on anther-derived embryo formation (Pedroso- Ubach 1991) suggested that successful embryo development was related to the presence of a deposition layer on the embryogenic regions and globular embryos.

The quantification of embryo and callus induction and maturation, per 100 crushed anthers and per 1000 microspores, does not give concordant results. It was probably due to the differences in densities of microspores isolated from the same number of crushed anthers. We opted to present both forms of quantification for comparison purposes with results reported for other woody species. In *C. japonica*, embryo and callus induction per 1000 microspores and embryo maturation per 100 anthers were, respectively, 3-and 357-fold higher than those reported for *Malus domestica* Borkh (Milewska-Pawliczuk 1990). The highest value obtained for callus formation in *C. japonica* was 6.7 calli per 100 anthers, while for *Populus* spp. 2.25 to 9.92 calli per 100 anthers (Wang et al 1975) and for *Litchi chinensis* Sonn. 10 calli per 100 anthers (Lianfang 1990) were reported. The optimization of anther culture in *Hevea brasiliensis* Muell.-Arg. (Chen et al 1978) led to the production of 44 embryos per 100 anthers and 34 embryos per 1000 microspores, respectively, ca. 3- and 7.5-fold higher than those recorded for *C. japonica*.

Our results show that microspore-derived embryos can be produced in *C. japonica* through direct microspore culture without previous anther incubation or floral bud cold-pretreatment. This method also enables the inoculation of microspores with a precise control of the optimal stage of microsporogenesis and direct observation of cultures and the stages of microspore embryogenesis. This culture system, when optimized, can be used as an experimental system to study, at cellular and molecular levels, the induction of microspore embryogenesis in this woody angiosperm plant.

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