Plant Cell Tiss Organ Cult (2006) 86:37–45
DOI 10.1007/s11240-006-9094-2

ORIGINAL PAPER

# Effect of primary culture medium composition on high frequency somatic embryogenesis in different *Coffea* species

**N. P. Samson · C. Campa · L. Le Gal · M. Noirot · G. Thomas · T. S. Lokeswari · A.de Kochko**

Received: 8 March 2005 / Accepted: 18 February 2006 / Published online: 21 April 2006


**Abstract** High frequency somatic embryogenesis can be obtained over a 7–8 month culture period when using current routing coffee tree micropropagation protocols. To reduce this response time and improve the embryo formation yield, eight different media were tested for primary culture. These media differed from the classically used ones by their mineral nitrogen and plant growth regulator concentrations. An increase from 0.66 to 0.75 in the $NO_3$/$NO_3$+$NH_4$ ratio and a 2-fold lower plant growth regulator concentration in the primary culture medium led to substantial improvements in terms of rapidity and embryo/plantlet regeneration frequencies. Embryo development time was reduced by up to 3 months with a 5-fold increase in the number of formed embryos. These results were obtained for the two cultivated coffee tree species, *Coffea canephora* and *C. arabica*, and for a wild one, *C. heterocalyx*, but not for a second wild species, *C.* sp. Moloundou showing a species-specific response. The new conditions described in this paper led to a substantial enhancement that should be particularly helpful for clonal propagation and genetic engineering of cultivated coffee plants.

N. P. Samson · C. Campa · L. L. Gal · M. Noirot · A. de Kochko (✉)
Génomique et qualité du café, UMR 1097 DGPC, Institut de Recherche pour le Développement, Centre de Montpellier, BP 64501, 34394, Montpellier, Cedex 5, France
E-mail: dekochko@mpl.ird.fr
Tel.: +33 (0)4 67 41 63 11
Fax: +33 (0)4 67 41 62 22

G. Thomas
Interfield Laboratories, 682005, Karuvelipady, Kochi, India

T. S. Lokeswari
Department of Biotechnology, Sri Ramachandra Medical College & Research Institute, College of Biomedical Science, Technology and Research, #24, Dr. Vasudev Nagar, 600 041, Thiruvanmiyur, Chennai, India

**Keywords** Ammonium–nitrate ratio · Clonal propagation · Coffee trees · Embryo development · Plant growth regulator

**Abbreviations**

| | |
|---|---|
| BAP | Benzylaminopurine |
| 2,4-D | 2,4-Dichlorophenoxyacetic acid |
| Kin | Kinetin |
| 2-iP | 2-Isopentenyladenine (6-dimethylaminopurine) |
| MS | Murashige and Skoog's salts |
| N | Nitrogen |
| $NO_3$ | Nitrate |
| $NH_4$ | Ammonium |
| PGR | Plant growth regulator |
| SE | Somatic embryogenesis |

## Introduction

Clonal propagation through somatic embryogenesis (SE) has become an essential method for the improvement of most economically important plants. Moreover, establishment of cultures yielding high frequency somatic embryogenesis (HFSE) would be useful for gene expression studies involving genetic transformation since a steady quantity of target tissue can be produced. Cell differentiation into somatic embryos is governed by environmental or cultural stimuli (Gaj 2004). Whatever the species or the plant organ considered, nitrogen is a major nutrient component that influences in vitro morphogenesis. The plant growth regulator concentration and especially the balance between auxins and cytokinins also appears to be a key factor in callus induction and SE initiation (Raimondi et al. 2001; Gaj, 2004). Numerous experiments have clearly shown that nature and balance of nitrogen sources can induce SE and regeneration in plants such as wild carrot (Wetherall and Dougall 1976), rice (Ozawa et al. 1996), white spruce (Barrett et al. 1997), sorghum (Elkonin and Pakhomova 2000), feijoa (Dal Vesco and Guerra 2001) or cotton (Ikram-ul-Haq and Zafar 2004).

For the *Coffea* genus, many schemes have been proposed to develop SE since the first observation of Staritsky (1970). Occurrence of SE in coffee plants has been reported from different explants for different species and genotypes such as hypocotyl (Ocampo and Manzanera 1991), stem (Nassuth et al. 1980), leaf (Söndahl and Sharp 1977; Dublin 1984; Yasuda et al. 1985; Berthouly and Michaux-Ferrière 1996), integument (Sreenath et al. 1995) and protoplasts (Spiral and Pétiard 1991; Tahara et al. 1994). Several investigations have focused on the effects of genotype, explant age (Molina et al. 2002), plant growth regulators (PGRs) and cultural conditions on the induction of SE in several *Coffea* species (Van Boxtel and Berthouly 1996; Berthouly and Etienne, 1999). Methods that yield HFSE from leaf explants were first described by Söndhal and Sharp (1977) through two steps, and later by Berthouly and Michaux-Ferrière (1996) in solid medium, as well as Van Boxtel and Berthouly (1996) and Ducos et al. (1999) in liquid medium. Large-scale production of somatic embryos using a temporary immersion bioreactor has also been recently developed (Etienne et al. 1997; Etienne-Barry et al., 1999). All of these techniques especially concern the two cultivated coffee tree species, *Coffea canephora* and *C. arabica* and require about 7–8 months to obtain embryos. In all of these cases, the first culture step appears to be the primary bottleneck for obtaining somatic embryos.

In this paper, we propose a new culture medium composition for the primary culture medium used in the two-step technique described by Berthouly and Michaux-Ferrière (1996), which improves SE induction in the cultivated species and extends the process to wild ones. We show that by lowering the nitrogen and PGR levels, but increasing the $NO_3/(NO_3+NH_4)$ and auxin/cytokinin ratios, HFSE from coffee leaf explants and plant regeneration can be significantly enhanced in some wild and cultivated species.

## Materials and methods

### Plant material

The most recently formed and fully expanded leaves were collected before flowering on trees grown in a tropical greenhouse at IRD Montpellier (France). This was described as being the best period for obtaining HFSE with *C. canephora* (Berthouly and Michaux-Ferrière 1996). Two trees were used for each of the four *Coffea* species studied: AR 38-05 and 26-06 for *C. arabica*, BD 69 and 71 for *C. canephora* Pierre, JC 62 and 63 for *C. heterocalyx* and OD 65 and 61 for *C*. sp. Moloundou. After five rinses in running water the leaves were surface-disinfected by immersion in 70% ethanol for 1 min. They were then immersed for 15 min in a solution containing 12° active chloride (commercial bleach of sodium hypochlorite diluted three times, Javel Oxena –SH pieriChime), ascorbic acid (4% w/v) and Tween-20 (1% v/v) as surfactant. Leaves were then washed with sterile water 3–5 times, and dissected into $\sim$1 $cm^2$ squares, devoid of midrib, to be placed on an appropriate medium for culturing.

### Culture media

For the first culture step, a new primary culture medium (NPCM), enriched with two different

combinations of growth regulators ($PGR_1$ and $PGR_2$), was assayed and compared to the classical salt solution of Murashige and Skoog (1962) at three different concentrations (full salt solution: MS; half salt solution: MS/2; quarter salt solution: MS/4) enriched with the same $PGR_1$ and $PGR_2$. The NPCM composition is given in Table 1. It differed from Yasuda medium (Yasuda et al. 1985) by some component concentration ($CaCl_2{\cdot}2H_2O$, $MnSO_4{\cdot}H_2O$, thiamine–HCl and Myo-inositol) and by the mineral nitrogen source, $NH_4NO_3$ was partially substituted by $Ca(NO_3)_2{\cdot}4\text{-}H_2O$. Its inorganic nitrogen content is also compared in Table 1 to the subsequent media for embryogenic calli induction, embryo regeneration and plantlet development (E, R and D, respectively). $PGR_1$ was composed of 2,4-D (2.25 μM) and 2-iP (4.93 μM) and $PGR_2$ of 2,4-D (2.25 μM), 2-iP (4.93 μM) and kinetin (4.65 μM). The eight media were adjusted to pH 5.6 using 1 N NaOH or 1 N HCl before adding 0.3% (w/v) Phytagel and autoclaving at 121°C for 25 min.

The compositions of culture media used for embryogenic calli induction and embryo regeneration (E and R, respectively) corresponded to the culture media used by Van Boxtel and Berthouly (1996). Plantlet development was obtained in D medium consisting of R medium deprived of PGR (Table 1). For all of these media, the mineral composition was

**Table 1** Composition of media used for the induction of high frequency somatic embryogenesis in *Coffea* species

| Components | NPCM | | E | R | D |
|---|---|---|---|---|---|
| | $PGR_1$ | $PGR_2$ | | | |
| $NH_4NO_3$ | 250 | 250 | 825 | 825 | 825 |
| $KNO_3$ | 400 | 400 | 950 | 950 | 950 |
| $Ca(NO_3)_2{\cdot}4{\cdot}H_2O$ | 300 | 300 | – | – | – |
| $MgSO_4{\cdot}7\ H_2O$ | 90 | 90 | 185 | 185 | 185 |
| $KH_2PO_4$ | 85 | 85 | 85 | 85 | 85 |
| $CaCl_2{\cdot}2\ H_2O$ | 160 | 160 | 220 | 220 | 220 |
| $H_3BO_3$ | 3.1 | 3.1 | 3.1 | 3.1 | 3.1 |
| $CuSO_4{\cdot}5\ H_2O$ | 0.0125 | 0.0125 | 0.0125 | 0.0125 | 0.0125 |
| $MnSO_4{\cdot}H_2O$ | 90.3 | 90.3 | 11.2 | 11.2 | 11.2 |
| $Na_2MoO_4{\cdot}2\ H_2O$ | 0.125 | 0.125 | 0.125 | 0.125 | 0.125 |
| $ZnSO_4{\cdot}7\ H_2O$ | 4.3 | 4.3 | 4.3 | 4.3 | 4.3 |
| $CoCl_2{\cdot}6H_2O$ | – | – | 0.025 | 0.025 | 0.025 |
| KI | – | – | 0.83 | 0.83 | 0.83 |
| Thiamine–HCl | 15 | 15 | 20 | 10 | 10 |
| Nicotinic acid | 1 | 1 | – | 1 | 1 |
| Pyridoxine–HCl | 1 | 1 | – | 1 | 1 |
| Myo-inositol | 130 | 130 | 200 | 200 | 200 |
| Glycine | – | – | 20 | 2 | 2 |
| Cysteine | – | – | 40 | – | – |
| Adenine sulfate | – | – | 60 | 40 | 40 |
| Malt extract | – | – | 800 | 400 | 400 |
| Caseine hydrolyzate | – | – | 200 | 400 | 400 |
| Fe–EDTA solution (100 × ) (Sigma) | 5 | 5 | 5 | 5 | 5 |
| 2,4-D | 2.25 | 2.25 | 4.50 | – | – |
| 2-iP | 4.93 | 4.93 | – | – | – |
| BAP | – | 4.65 | 17.75 | – | – |
| Kin | – | – | – | 9.30 | – |
| Sucrose | 30 | 30 | 30 | 35 | 30 |
| Total inorganic N (mM) | 12.74 | 12.74 | 30.00 | 30.00 | 30.00 |
| $NO_3$/Total N | 0.755 | 0.755 | 0.657 | 0.657 | 0.657 |

NPCM: Primary culture medium, E: culture medium for embryogenic callus induction, R: culture medium for embryo regeneration, D: culture medium for embryo development

The concentration in mineral and organic components (including sucrose) is expressed in mg $l^{-1}$; Plant growth regulator concentration is expressed in μM

2.4-D: 2,4-dichlorophenoxyacetic acid, 2-iP: 2-isopentenyladenine, BAP: 6-benzylaminopurine, Kin: kinetin

equivalent to MS/2 salts. As for primary culture media, the three media were adjusted to pH 5.6 using 1 N NaOH or 1 N HCl before adding 0.3% (w/v) Phytagel and autoclaving at 121°C for 25 min.

### Culture conditions

For the primary cultures, five leaf explants were inoculated in Petri dishes (120 × 10 mm) containing 20 ml of primary culture medium. Ten dishes were prepared for each of the eight media used. After sealing (Parafilm ''M'', American National Can™, Chicago), the dishes were maintained in the dark at 26 ± 1°C for callus induction. After 1 month, explants showing callus growth were transferred to Petri dishes (120 × 10 mm) containing E medium (Van Boxtel and Berthouly 1996). The cultures were kept in light (12 h $day^{-1}$ photoperiod) with a photon flux of 30 μmol $m^{-2}$ $s^{-1}$ provided by cool-white fluorescent lamps. When white globular somatic embryos appeared (between 1 and 3 months depending on the species), the embryogenic calli were isolated and separated in units of 100 mg each. For each primary culture medium, ten of these calli were spread thinly in tubes (20 × 150 mm) sealed with snap caps (Sigma-Aldrich Corporation, St. Louis, USA) containing R medium (Van Boxtel and Berthouly 1996) for embryo development under the same culture conditions. Green colored embryos appeared after 1 month and were then transferred to tubes containing D medium to promote the development of healthy plantlets. The well-developed plants were then transferred without modification (decapitation or reduction) into small pots containing sterilized vermiculite. All the transferred plants rooted and acclimated in the greenhouse.

### Morphological observations

All the experiments were performed according to a completely randomized design. The total number of compared treatments was 4 (culture medium composition) × 2 (PGRs combinations). Explants from two plants belonging to four species, *C. arabica, C. canephora, C. heterocalyx* and *C.* sp. Moloundou, were used to study the effect of the composition of primary culture medium on the development of embryogenic calli. As results were not significantly different between plants from the same species, only one plant per species was used to study the effect of this composition on the embryogenic competence and plantlet development. The number of somatic embryogenic calli (yellow friable calli) formed after 3 months of culture in E medium was taken into account to evaluate the percentage of explant response.

The number of somatic embryos was evaluated after 5 weeks of culture in R medium. The average of developed embryos and plantlets was calculated for ten calli (100 mg each) isolated from each primary culture medium and expressed per 100 mg of embryogenic callus. Variance analyses were performed using the Newman-Keuls test.

## Results

### Effect of primary culture medium on highly somatic embryogenesis

The calli emerged from the explants after 10 days of culture on NPCM medium for all the tested species. White when they appeared and becoming yellowish, they were observed on the edges of the explants. The well developed embryogenic calli were observed 2 months after transfer on medium E for explants from leaves of *C. arabica, C. canephora* and *C. heterocalyx*. These yellow friable calli were formed upon the explant surface. The first responses were observed with *C. arabica* and *C. canephora* explants in NPCM containing $PGR_1$ as plant growth regulator. In the case of *C.* sp. Moloundou, yellow embryogenic-like calli, with a more compact structure than that observed with the other species, appeared after 3 months.

As shown in Table 2, the best results were obtained for the four species when the primary culture was conducted on NPCM containing $PGR_1$ as plant growth regulator. For this culture medium (NPCM + $PGR_1$), depending on the tree tested, from 84 to 100% of the explants produced embryogenic calli. The two trees belonging to the same species responded similarly, as shown by the identical results obtained with *C. arabica* or slightly different with the other species (≤4%). For all species, cultures in NPCM with $PGR_2$, which consisted of $PGR_1$ plus kinetin (4.65 μM), gave lower rates of embryogenic calli formation than

**Table 2** Percentage of explants producing numerous embryogenic calli (or embryogenic-like calli for *C.* sp. Moloundou) after 4 weeks of culture in eight different primary culture media

| Species | Embryogenic callus frequency (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | MS | | MS/2 | | MS/4 | | NPCM | |
| | $PGR_1$ | $PGR_2$ | $PGR_1$ | $PGR_2$ | $PGR_1$ | $PGR_2$ | $PGR_1$ | $PGR_2$ |
| *C. arabica* | | | | | | | | |
| AR 38-05 | 17 $^{a}$ | 0$^{a}$ | 56$^{ab}$ | 56$^{b}$ | 84$^{b}$ | 84$^{b}$ | 100$^{b}$ | 84$^{b}$ |
| AR 26-06 | 22$^{a}$ | 8$^{a}$ | 54$^{ab}$ | 24$^{ab}$ | 84$^{b}$ | 26$^{ab}$ | 100$^{b}$ | 64$^{b}$ |
| *C. canephora* | | | | | | | | |
| BD 69 | 15$^{a}$ | 0$^{a}$ | 38$^{a}$ | 18$^{b}$ | 82$^{b}$ | 32$^{b}$ | 96$^{b}$ | 44$^{b}$ |
| BD 71 | 20$^{a}$ | 8$^{a}$ | 40$^{a}$ | 42$^{b}$ | 90$^{b}$ | 60$^{b}$ | 100$^{b}$ | 63$^{b}$ |
| *C. heterocalyx* | | | | | | | | |
| JC 62 | 16$^{a}$ | 24$^{a}$ | 46$^{ab}$ | 28$^{a}$ | 80$^{b}$ | 56$^{b}$ | 100$^{b}$ | 64$^{b}$ |
| JC 63 | 4$^{a}$ | 0$^{a}$ | 14$^{ab}$ | 22$^{bc}$ | 36$^{b}$ | 46$^{c}$ | 96$^{c}$ | 44$^{c}$ |
| *C.* sp. Moloundou | | | | | | | | |
| OD 65 | 18 $^{a}$ | 2 $^{a}$ | 33 $^{a}$ | 23 $^{ab}$ | 75 $^{b}$ | 69 $^{c}$ | 87 $^{b}$ | 67 $^{c}$ |
| OD 61 | 10 $^{a}$ | 0 $^{a}$ | 24 $^{a}$ | 0 $^{a}$ | 84 $^{b}$ | 56 $^{b}$ | 84 $^{b}$ | 64 $^{b}$ |

Two trees for each *Coffea* species were tested on MS salt solution (MS), half MS (MS/2), quarter MS (MS/4) and NPCM, and two solutions of plant growth regulators [$PGR_1$: 2,4-D (2.25 μM) and 2-iP (4.93 μM); $PGR_2$: 2,4-D (2.25 μM), 2-iP (4.93 μM) and kinetin (4.65 μM)]. Observations were performed after 3 months of culture on E medium. Fifty explants were used for each experiment. For a given genotype, means for $PGR_1$ on one hand and $PGR_2$ on another hand, followed by the same letter in superscript are not significantly different at the 5% level according to variance analysis and the Newman–Keuls test

NPCM containing PGR1 (44–84%). The effect of PGR was particularly marked with *C. canephora* C 69 and *C. heterocalyx* JC 63 explants on NPCM. Adding $PGR_2$ instead of $PGR_1$ to the other media (MS, MS/2 or MS/4) also resulted in a decrease in the embryogenic competence, except for *C. heterocalyx*. The lowest response was obtained when culturing explants on full MS salt solution (MS), especially when $PGR_2$ was used as plant growth regulator. The 2- or 4-fold dilution of the MS salt solution (MS/2 and MS/4) increased the development of embryogenic calli compared to MS, regardless of the PGR used. On average, the embryogenic callus development rate was 2.6- and 5.7-fold higher in MS/2 and MS/4, respectively, than in MS when the media were enriched with $PGR_1$. Apart from the wild species, particularly *C. heterocalyx*, the responses were quite uniform for the two trees belonging to the same species, except when cultures were performed with $PGR_2$.

## Effect of primary culture medium on embryogenic competence and plantlet development

Embryogenic calli developed on E medium and derived from primary cultures obtained on the eight primary culture media were spread thinly and, after weighing, plated on R medium (Table 1) to induce somatic embryo development. As the percentage of explants producing embryogenic calli in NPCM culture conditions was fairly the same for the two trees from the same species (Table 2), only one tree per species was kept to make this experiment (AR 38-05 for *C. arabica*, BD 69 for *C. canephora*, JC 62 for *C. heterocalyx* and OD 65 for *C.* sp. Moloundou). Numerous globular embryos (Fig. 1A,B) were produced by cultures initiated on NPCM and on diluted MS salts (MS/2 and MS/4) enriched with $PGR_1$ or $PGR_2$ after 5 and 7–8 weeks, respectively, of subculture on R medium. These embryos were issued from globular tissues that appeared on the calli surface and their formation took place continuously during the culture. When full MS had been used as primary culture medium, or in case of *C.* sp. Moloundou explants, no embryos were formed. For this recalcitrant species, irrespective of the mineral content and plant growth regulators used in the primary culture medium, embryogenic-like calli were unable to produce somatic embryos. For the three other species, the best results were generally obtained when primary cultures were conducted on NPCM containing $PGR_1$ as a source of plant growth regulators (Table 3). For *C. canephora*, the results

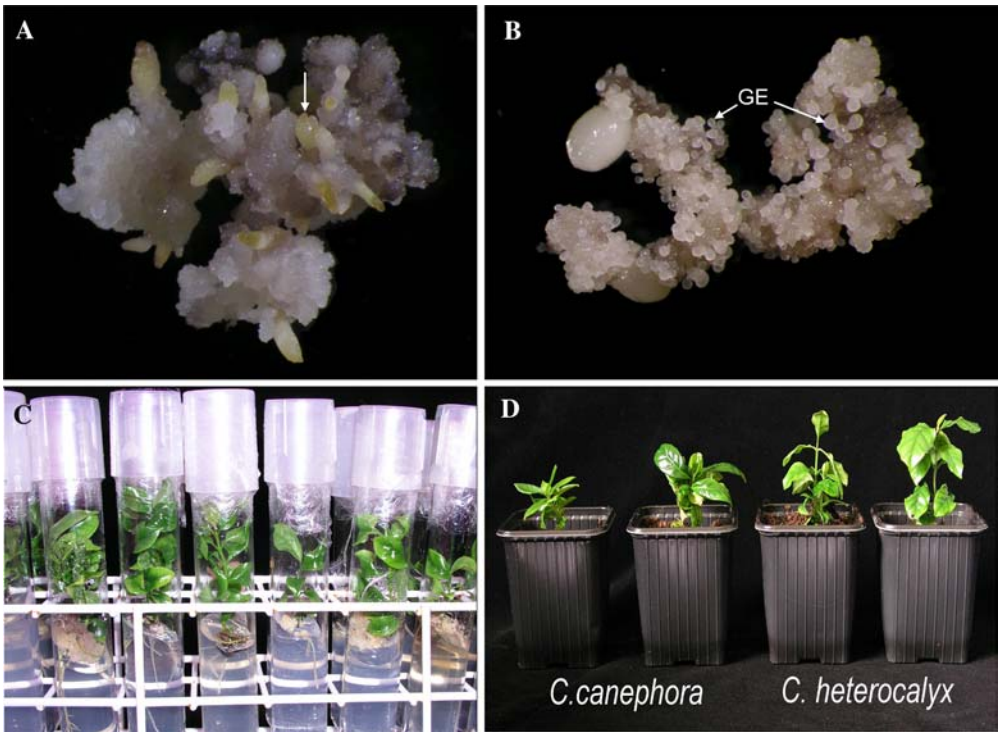


**Fig. 1** Developmental stages of coffee plant regeneration process through high frequency somatic embryogenesis. (**A**) Globular embryos from an embryogenic callus of *C. heterocalyx*. Arrows indicate early stage of globular embryos (GE). (**B**) Developing torpedo-stage embryos from an embryogenic callus of *C. heterocalyx*. (**C**) Developed plantlets of *C. canephora*. (**D**) Acclimatized plants of *C. canephora* and *C. heterocalyx* Bar in (**A**) and (**B**) = 2 mm

**Table 3** Somatic embryo (SE) induction and plantlet development in different *Coffea* species from calli initially cultured on eight different media: MS salt solution (MS), half MS (MS/2), quarter MS (MS/4), or NPCM solution and two concentrations of plant growth regulators [$PGR_1$: 2,4-D (2.25 μM) and 2-iP (4.93 μM); $PGR_2$: 2,4-D (2.25 μM), 2-iP (4.93 μM) and kinetin (4.65 μM)]

| Species | Plant growth regulators | Number of SE | | | | Number of plantlets | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | MS | MS/2 | MS/4 | NPCM | MS | MS/2 | MS/4 | NPCM |
| *C. arabica* AR 38-05 | $PGR_1$ | 0 | $62 \pm 10^a$ | $198 \pm 22^b$ | $306 \pm 22^c$ | 0 | $31 \pm 8^a$ | $56 \pm 7^b$ | $157 \pm 10^c$ |
| | $PGR_2$ | 0 | $35 \pm 4^a$ | $151 \pm 25^b$ | $239 \pm 21^c$ | 0 | $17 \pm 4^a$ | $41 \pm 7^b$ | $117 \pm 22^c$ |
| C. *canephora* BD 69 | $PGR_1$ | 0 | $16 \pm 12^a$ | $98 \pm 11^b$ | $124 \pm 30^b$ | 0 | $8 \pm 6^a$ | $33 \pm 14^b$ | $55 \pm 7^b$ |
| | $PGR_2$ | 0 | $23 \pm 8^a$ | $75 \pm 10^b$ | $127 \pm 47^c$ | 0 | $12 \pm 4^a$ | $21 \pm 9^a$ | $60 \pm 26^b$ |
| *C. heterocalyx* JC 62 | $PGR_1$ | 0 | $50 \pm 10^a$ | $131 \pm 20^b$ | $285 \pm 53^c$ | 0 | $25 \pm 4^a$ | $61 \pm 27^b$ | $145 \pm 23^c$ |
| | $PGR_2$ | 0 | $18 \pm 7^a$ | $113 \pm 37^b$ | $179 \pm 52^c$ | 0 | $10 \pm 3^a$ | $60 \pm 21^b$ | $90 \pm 26^b$ |
| *C.* sp Moloundou OD 65 | $PGR_1$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | $PGR_2$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Observations were performed after 10 weeks of culture on R medium for somatic embryo induction and 3 months on R medium for plantlet development. The results are expressed as number of SE obtained per 100 mg embryogenic calli. Ten embryogenic calli were used for each treatment. Means in the same line followed by the same letter in superscript are not significantly different at the 5% level according to variance analysis and the Newman–Keuls test

were not significantly different between explants cultured on NPCM containing kinetin or not ($PGR_1$ or $PGR_2$). Lower results were obtained when cultures were performed in MS salts. For these culture media, the response increased with the dilution of the salt solution. The highest number of somatic embryos was obtained in the set of *C. arabica* cultures without kinetin ($PGR_1$), with 306 embryos formed per

100 mg of embryogenic calli on average. A similar response pattern was obtained with *C. heterocalyx*.

The higher mean number of plantlets developed on D medium was also observed with *C. arabica* and *C. heterocalyx* explants grown in NPCM supplemented with $PGR_1$ (Table 3). *C. canephora* also generated more plantlets when using this medium for primary culture. For the three species, 3- and 5-fold lower results were obtained using quarter and half strength MS salts, respectively. Interestingly, the percentage of embryos forming viable plantlets was around 50%, except for *C. arabica* and *C. canephora* explants that were initially cultured on MS/4 salts.

After 2 months, plants regenerated from embryogenic calli of *C. arabica, C. canephora* and *C. heterocalyx* were isolated for subsequent culture on D medium. When they reached 3–4 cm high, they were transferred into sterilized pots, where all of them successfully rooted (Fig. 1C–E).

## Discussion

Culture of coffee leaf explants on NPCM enriched with $PGR_1$ as plant growth regulator led to the development of a high percentage of embryogenic calli (near 100%) which formed embryos that could readily develop into plantlets with a high success rate (close to 50%). This was noted with the two cultivated species *C. arabica* and *C. canephora*, but also with a wild species, *C. heterocalyx*. Moreover, compared to previous results, variability in the embryogenic callus frequency observed in this medium within the cultivated species appeared to be low (under 4%). This could be attributed to the NPCM medium, as it has been shown that variability in the *C. canephora* response may be reduced by 50% depending on the medium used (Berthouly and Michaux-Ferrière 1996).

For three of the four species cultured in this medium, the first somatic embryos began to appear after 12 weeks (4 weeks of culture on NPCM and 8 weeks on E medium), when calli were transferred to R medium. Maximal regeneration was observed after 1 month on this medium. For all the media containing MS salts at different concentrations, the best results were similarly obtained after 1 month and with $PGR_1$ as plant growth regulator. In $PGR_1$, the auxin/cytokinin ratio was 1/2 and 2-fold higher than in $PGR_2$. This balance ratio was described by Berthouly and Michaux-Ferrière (1996) as being the best one to obtain HFSE in *C. canephora*. However, the final concentration of both auxin and cytokinin in $PGR_1$ was 2-fold less than in the hormonal solution proposed by these authors. In their culture conditions, which was equivalent to our MS/2 medium enriched with $2 \times PGR_1$, 14 weeks were needed to obtain friable calli able to form embryos after 3–4 months. Compared to these results, the response time for embryo development was thus shortened by at least 2–3 months using NPCM medium. A reduced response time was previously obtained with *C. arabica* but under very special conditions, i.e. in vitro pre-conditioning of seedling leaves for 2 months with growth regulators (Quiroz-Figueroa et al. 2002). The present results, combined with those obtained previously by the cited authors, reinforce the hypothesis that lowering the PGR concentration triggers embryogenic callus initiation. Accordingly to results obtained by Van Boxtel and Berthouly (1996) showing that the endogenous hormone concentration in non-embryogenic calli of a recalcitrant genotype was 3 fold higher than in calli able to produce somatic embryos, the delay in embryogenic callus initiation and the lack of embryo formation observed for *C.* sp. Moloundou could be attributable to a high endogenous hormone level in this species. But the endogenous hormone level and the aptitude of somatic embryo production should be compared in different *Coffea* species in order to confirm this hypothesis.

NPCM allowed us to obtain a high number of embryos and plantlets as compared to the other media (Table 3). On average, for the three embryo-forming species, the number of developing plantlets was 5-fold higher in this medium than in MS/2 enriched with $PGR_1$ (equivalent to Berthouly and Michaux-Ferrière's conditions). The mineral composition of NPCM consisted of slightly modified MS/4 salts (Yasuda et al. 1985) in which the mineral composition, and particularly the inorganic nitrogen source, has been modified. It mainly differed by the ammonium concentration, with the nitrate content being almost equivalent (Table 1). Then the $NO_3$ and $NH_4$ concentrations were 2- and 3-fold lower, respectively, in NPCM than in MS/2 medium. The $NO_3$/inorganic N ratio was increased by 15% in NPCM (0.75 instead of 0.66) compared to all the other media. Enhanced embryo production was observed in *Mangifera indica*

when the total nitrogen concentration in the medium, particularly ammonium, was low (Laxmi et al. 1999). Similarly, a low nitrate level (11.6 mM) was found to induce higher embryogenesis in *Santalum album* (Das et al. 2001) and sorghum (Elkonin and Pakhomova 2000). In *Medicago sativa*, the increase in the $NO_3/NO_3 + NH_4$ ratio to 0.8 in the primary culture medium stimulated SE induction (Meijer and Brown 1987). The decrease in the mineral nitrogen concentration, and especially the increase of the $NO_3$/total N ratio could be, along with the PGR concentration, another factor that could enhance the embryogenic competence of coffee leaf explants.

These results underline that the first medium composition was a key factor in obtaining successful induction of high frequency embryogenic calli and embryogenesis. This culture step seemed to be crucial for conditioning tissues to subsequently produce somatic embryos. The new primary culture medium described here can be used to produce a higher yield of developed embryos in a significantly shorter time, and to extend the embryogenesis procedure to some wild coffee tree species. In addition, reducing the culturing time might decrease the somaclonal variation frequency; another major commonly encountered difficulty (Barry-Etienne et al. 2002a,b), and it will probably ensure the formation of more homogenous plantlets. The fact that this new medium improves the yield and reduces the culture time required makes it more time and cost effective. It will be particularly interesting and helpful for clonal mass propagation and/or genetic engineering in coffee plants after testing its efficiency on large samples representative of intraspecific genetic variability. It could also be interesting to test this medium with other woody plants such as poplar or vines.

**Acknowledgements** This work was financed by the Indo-French Center for the Promotion of Advanced Research (IFCPAR) and IRD. NPS was supported by IFCPAR.The authors are grateful to Dr. H. Etienne for his critical reading of this manuscript.

## References

Barrett JD, Park YS, Bonga JM (1997) The effectiveness of various nitrogen sources in white spruce [*Picea glauca* (Moench) Voss] somatic embryogenesis. Plant Cell Rep 16:411–415

Barry-Etienne D, Bertrand B, Schlonvoigt A, Etienne H (2002a) Comparison of somatic embryogenesis-derived coffee (*Coffea arabica* L.) plantlets regenerated in vitro or ex vitro: morphological, mineral and water characteristics. Plant Cell Tiss Org Cult 68:153–162

Barry-Etienne D, Bertrand B, Vasquez N, Etienne H (2002b) Morphological variability within a population of coffee somatic embryos produced in a bioreactor affects the regeneration and the development of plants in the nursery. Ann Bot 90:77–85

Berthouly M, Etienne H (1999) Somatic embryogenesis in coffee. In: Jain SM, Gupta PK & Newton RJ (eds) Somatic embryogenesis in woody Plants, Vol 5 (pp. 259–287). Kluwer Academic Publishers, Dordrecht

Berthouly M, Michaux-Ferrière NM (1996) High frequency somatic embryogenesis in *Coffea canephora* – Induction conditions and histological evolution. Plant Cell Tiss Org Cult 44:169–176

Dal Vesco LL, Guerra MP (2001) The effectiveness of nitrogen sources in Feijoa somatic embryogenesis. Plant Cell Tiss Org Cult 64:19–25

Das S, Ray S, Dey S, Dasgupta S (2001) Optimisation of sucrose, inorganic nitrogen and abscisic acid levels for *Santalum album* L. somatic embryo production in suspension culture. Process Biochem 37:51–56

Dublin JP (1984) Techniques de reproduction végétative in vitro et amélioration génétique chez les caféiers cultivés. Café Cacao Thé 28:231–244

Ducos JP, Gianforcaro M, Florin B, Pétiard V & Deshayes A (1999) Technically and economicaly attractive way to propagate elite *Coffea canephora* (Robusta) clones: in vitro somatic embryogenesis. In: ASIC Publishers (eds) 18th International Scientific Colloquium on Coffee, Helsinki, Finlande, Paris

Elkonin AL, Pakhomova NV (2000) Influence of nitrogen and phosphorus on induction embryogenic callus of sorghum. Plant Cell Tiss Org Cult 61:115–123

Etienne H, Bertrand B, Anthony F, Côte F & Berthouly M (1997) Somatic embryogenesis, a tool for coffee genetic improvement. In: ASIC Publishers (eds) 17th International Scientific Colloquium on Coffee, Nairobi, Kenya, Vevey, Switzerland, pp 457–465

Etienne-Barry D, Bertrand B, Vasquez N, Etienne H (1999) Direct sowing of *Coffea arabica* somatic embryos mass-produced in a bioreactor and regeneration of plants. Plant Cell Rep 19:111–117

Gaj MD (2004) Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.) Heynh. Plant Growth Reg 43:27–47

Ikram-ul-Haq, Zafar Y (2004) Effect of nitrates on embryo induction efficiency in cotton (*Gossypium hirsutum* L.) Afr. J Biotechnol 3(6):319–323

Laxmi DV, Sharma HC, Kirti PB, Mohan ML (1999) Somatic embryogenesis in mango (*Mangifera indica* L.) cv. Amrapali Curr. Sci 77:1355–1358

Meijer EGM, Brown CWB (1987) Role of exogenous reduced nitrogen and sucrose in rapid high frequency somatic embryogenesis in *Medicago sativa*. Plant Cell Tiss Org Cult 10(1):11–19

Molina D, Aponte M, Cortina H, Moreno G (2002) The effect of genotype and expant age on somatic embryogenesis of coffee. Plant Cell Tiss Org Cult 71:117–123

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

Nassuth A, Wormer TM, Bouman F, Staritsky G (1980) The histogenesis of callus in *Coffea canephora* stem explants and the discovery of early embryoid initiation. Acta Bot Neerl 29:49–54

Ocampo C, Manzanera LM (1991) Advances in genetic manipulation of coffee plant. In: Quatorzième Colloque Scientifique International sur le Café, ASIC, Montpellier, pp 73–81

Ozawa K, Ling D-H, Komamine A (1996) High-frequency somatic embryogenesis from small suspension-cultured clusters of cells of an interspecific hybrid of *Oryza*. Plant Cell Tiss Org Cult 46:157–159

Quiroz-Figueroa FR, Fuentes-Cerda CFJ, Rojas-Herrera R, Loyola-Vargas VM (2002) Histological studies on the developmental stages and differentiation of two different somatic embryogenesis systems of *Coffea arabica*. Plant Cell Rep 20:1141–1149

Raimondi JP, Camadro EL, Babinec FJ (2001) Somatic embryogenesis in *Asparagus officinalis* L. cv. Argenteuil: interactions between genotype, explant type and growth regulators on induction, growth and embryogenic differentiation. Biocell 25(2):147–154

Söndahl MR, Sharp WR (1977) High frequency induction of somatic embryos in cultured leaf explants of *Coffea arabica* L. Z Pflanzenphysiol 81:395–408

Spiral J & Pétiard V (1991) Protoplast culture and regeneration in Coffea species. In: Quatorzième Colloque Scientifique International sur le Café, ASIC, Montpellier, pp 383–391

Sreenath HL, Shanta HM, Babu KH, Naidu MM (1995) Somatic embryogenesis from integument (perisperm) cultures of coffee. Plant Cell Rep 14:670–673

Staritsky G (1970) Embryoid formation in callus tissues of coffee. Acta Bot Neerl 19:509–514

Tahara M, Yasuda T, Uchida N, Yamaguchi T (1994) Formation of somatic embryos from protoplasts of *Coffea arabica* L. Hort Science 29:172–174

Van Boxtel J, Berthouly M (1996) High frequency somatic embryogenesis from coffee leaves. Factors influencing embryogenesis, and subsequent proliferation and regeneration in liquid medium. Plant Cell Tiss. Org Cult 44:7–17

Wetherall DF, Dougall DK (1976) Sources of nitrogen supporting growth and embryogenesis in cultured wild carrot tissue. Plant Physiol 37:97–103

Yasuda T, Fujii Y, Yamaguchi T (1985) Embryogenic callus induction from *Coffea arabica* leaf explants by benzyladenine. Plant Cell Physiol 26:595–597