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S.M. Khalil · K.T. Cheah · E.A. Perez · D.A. Gaskill
J.S. Hu

Regeneration of banana (*Musa* spp. AAB cv. Dwarf Brazilian) via secondary somatic embryogenesis

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Abstract A method has been developed for the regeneration of the banana cultivar Dwarf Brazilian (*Musa* spp. AAB group). Primary somatic embryos were produced when explants of immature male flower buds were cultured on Murashige and Skoog (MS) medium plus 1 mg/l biotin, 100 mg/l malt extract, 100 mg/l glutamine, 4 mg/l 2,4-dichlorophenoxyacetic acid, 1 mg/l indole-3-acetic acid (IAA), 1 mg/l α-naphthaleneacetic acid, 30 g/l sucrose and 2.6 g/l Phytagel, pH 5.8 (M1 medium) and then transferred to M1 medium plus 200 mg/l casein hydrolysate and 2 mg/l proline. Subsequent transfer to MS supplemented with 10% coconut water produced rapidly proliferating embryogenic callus that developed into secondary somatic embryos (SE_2); these were subcultured on half-strength MS supplemented with 5 mg/l 6-benzylaminopurine (BA). Differentiated embryos were transferred to MS medium supplemented with 5 mg/l BA for development, and mature SE_2 were isolated and cultured on hormone-free MS for germination and development into plantlets. Approximately 90% of the SE_2 germinated and developed into plantlets, and these were subcultured onto MS medium plus 0.1% activated charcoal, 1 mg/l BA and 1 mg/l IAA where complete plantlets developed. Morphologically normal banana plants developed from all of the regenerated plantlets, the first of which were produced within 6 months of culture initiation.

Keywords In vitro · Plant tissue culture · Monocot

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S.M. Khalil
Agricultural Genetic Engineering Research Institute (AGERI), ARC, 12619, Giza, Egypt

K.T. Cheah · E.A. Perez · D.A. Gaskill · J.S. Hu (✉)
Department of Plant and Environmental Protection Sciences, University of Hawaii, Honolulu, HI 96822, USA
e-mail: johnhu@hawaii.edu
Tel.: +1-808-956-7281, Fax: +1-808-956-2428

Abbreviations *MS*: Murashige and Skoog (1962) medium · *BA*: 6-benzylaminopurine · *2,4-D*: 2,4-dichlorophenoxgace acid · *IAA*: indole-3-acetic acid · *NAA*: α-naphtale acetic acid · SE_2: secondary somatic embryos/embryogenesis

Introduction

Banana (*Musa* spp.) is one of the most important of the cultivated tropical fruits, with world production in 2000 estimated at 64.6 million metric tons (Anonymous 2001). It is a staple food for nearly 400 million people and is an essential source of income for many national economies with world imports in 1995 valued at $USA 5.3 billion (Sasson 1997). Production is limited primarily by viral and fungal diseases as well as insect and nematode pest problems. The application of classical methods of breeding for both disease and pest resistance has resulted in only limited success due to the long generation times for banana and the high sterility and triploidy of most cultivated bananas (Sasson 1997). The integration of genetic engineering into breeding programs may provide powerful tools to overcome these limitations by introducing specific genetic changes that can be utilized for banana improvement within a short period of time. However, these applications require reliable plant regeneration protocols for banana.

Although in vitro culture of banana has been extensively used to quickly propagate vegetative clones of many genotypes (Vuylsteke and De Langhe 1985; Das et al. 1998), many obstacles remain to be overcome before an efficient banana regeneration protocol suitable for genetic transformation is developed. Plants regenerated through organogenesis are not appropriate for genetic transformation since many chimeric plants are produced. Current protocols for somatic embryogenesis using embryogenic cell suspension cultures have the potential to produce non-chimeric plants, but they are limited by low embryo germination [1.5–12%, Novak et al. (1989); 10–23%, Dhed'a et al. (1991); 3–20%, Côte et al. (1996)] and plant regeneration rates [13–25%,

Navarro et al. (1997)] and long culture times from culture initiation to plant regeneration [16 months, Côte et al. (1996); 18 months, Becker et al. (2000); 10–11 months, Ganapathi et al. (2001)]. Kosky et al. (2002) recently reported high (89.3%) germination rates for banana cv. FHIA-18 (AAAB) using a system of cell suspension, liquid culture in a bioreactor and temporary immersion. However, not all laboratories have access to the bioreactor or temporary immersion equipment required for this system.

Immature male inflorescences have been used to initiate cultures of several banana and plantain cultivars (Shii et al. 1992; Escalant et al. 1994; Côte et al. 1996; Navarro et al. 1997; Sagi et al. 1998; Becker et al. 2000). We have used immature male inflorescences to initiate cultures with the objective of developing a protocol for the regeneration of plants via secondary somatic embryogenesis (SE_2). SE_2 is the process of induction of new somatic embryos from pre-existing embryos (Raemakers et al. 1995). Since new embryos are continually formed from existing embryos, SE_2 has the potential to produce many plants and, once initiated, may continue to produce embryos over a long period of time. The use of SE_2 could provide an efficient solution to the problems limiting plant regeneration in other banana cultivars. Escalant et al. (1994) suggested that somatic embryos are probably of unicellular origin, making them an excellent candidate for genetic transformation since the potential for production of chimeric plants is low. Although the regeneration of banana through SE_2 has been previously reported (Escalant et al. 1994; Kosky et al. 2002), these protocols rely upon equipment, such as a temporary immersion system, which may not be available to all laboratories. The objectives of the investigation reported here were to establish a regeneration system through SE_2 that is suitable for genetic transformation and that has the potential to regenerate a large number of plants without passing through a temporary immersion system or cell suspension.

Materials and methods

Plant material and culture initiation

Male flower buds were excised from 1-month old male flower clusters (approximately 30 cm in length) and cultured using a procedure modified from Escalant et al. (1994). Flowers were collected in October 1999 from field-grown banana [*Musa* spp. AAB cv. Dwarf Brazilian (syn. Santa Catarina Prata)] on the island of Hawaii. This cultivar is referred to as 'Apple' or 'Hawaiian Apple' in the local markets in Hawaii. Male flowers were washed with 1% (v/v) liquid detergent solution for 5 min. The outer bracts were removed until the inflorescence was 5 cm long, then the inflorescence was surface-sterilized in 5.25% sodium hypochlorite for 15 min, followed by three rinses with sterile water. The inner layers of bracts were peeled off with forceps until the flower axis was 1.5 cm in length. The remaining flower bud was cut into four segments longitudinally through the shoot tip and then transversely into 1-mm segments.

Production of primary and secondary somatic embryos

Sixteen explants from each of five immature male flowers were cultured on solid M1 medium (Table 1) and incubated in the dark at 28°C with monthly transfers to fresh media. All cultures were

Table 1 Media used for plant regeneration from immature male flower buds of banana cv. Dwarf Brazilian (*Musa* spp., AAB group) through secondary somatic embryogenesis (*BA* 6-benylaminopurine, *2,4-D* 2,4-dichlorophenoxyacetic acid, *IAA* indole-3-acetic acid, *NAA* α-naphthaleneacetic acid)

Culture media[a]	Sucrose (g l^{-1})	Malt extract (mg l^{-1})	2,4-D (mg l^{-1})	IAA (mg l^{-1})	Biotin (mg l^{-1})	Glut-amine (mg l^{-1})	NAA (mg l^{-1})	Casein hydro-lysate (mg l^{-1})	Proline (mg l^{-1})	Coconut water (%)	Zeatin[b] (mg l^{-1})	6-BA (mg l^{-1})	Activated Charcoal
M1	30	100	4	1	1	100	1	–	–	–	–	–	–
MM1	30	100	4	1	1	100	1	200	2	–	–	–	–
SK1	45	–	–	–	–	–	–	–	–	5	–	–	–
SK2	45	–	–	–	–	–	–	–	–	–	0.2	–	–
SK3	45	–	–	1	–	–	–	–	–	–	–	0.2	–
SK4	30	–	–	–	–	–	–	–	–	10	–	–	–
SK5	30	–	1	–	–	–	–	–	–	10	–	–	–
SK6	60	–	–	0.2	–	–	–	–	–	–	–	2	–
SK7[c]	40	–	–	–	–	–	–	–	–	–	0.5	–	–
SK8[c]	30	–	–	–	–	–	–	–	–	–	–	5	–
GTR[c,d]	30	500	–	–	–	–	–	–	–	–	–	–	0.1
SKAW	30	–	–	–	–	–	–	–	–	–	–	5	–
SK10	30	–	–	–	–	–	–	–	–	–	–	–	–
SK11	30	–	–	1	–	–	–	–	–	–	–	1	0.1
M2[e]	45	100	1	–	1	100	–	–	–	–	–	–	–

[a] All media used in this study contained MS salts, myo-inositol (100 mg l^{-1}), nicotinic acid (1 mg l^{-1}), thiamine HCl (1 mg l^{-1}) and pyridoxine HCl (10 mg l^{-1}), solidified with Phytagel (2.6 g l^{-1}) and adjusted to pH 5.8 before autoclaving at 15 psi, 121°C for 20 min, except where noted. Cultures were incubated at 28°C under a photoperiod of 16/8-h (light/dark) except for M1 and MM1, which were incubated in the dark. Tissue was cultured on M1 for 2 months, MM1 for 2 weeks, SK4 for 4 weeks, SK8 for 3 weeks, SKAW for 2 weeks, SK10 for 2–3 weeks and SK11 for 2–3 weeks

[b] Zeatin was added cold filter-sterilized

[c] Media contained half-strength MS salts

[d] Ganapathi et al. (1999)

[e] Liquid medium M2 did not contain Phytagel and was adjusted to pH 5.3 before autoclaving

grown in petri dishes (100×15 mm) sealed with Parafilm until the plantlets became too large, at which point plantlets were grown in magenta boxes. After 2 months, white non-friable calli were produced. These calli were separated from the explant, transferred to MM1 (Table 1) and kept in the dark. Compact, friable, embryogenic tissues with globular structures containing primary somatic embryos were formed within 2 weeks and subsequently used to initiate SE_2.

The compact white calli were transferred to proliferation media SK1, SK2, SK3, SK4, SK5, SK6, or SK7 (Table 1), where new calli producing SE_2 were obtained after 4 weeks. These cultures, and all subsequent transfers, were incubated at 28°C under a 16/8 h (light/dark) photoperiod with light supplied by 40 W cool-white fluorescent lamps. At this stage, SE_2 were maintained on either SK4 or M2 media (Table 1) or cultured on either SK8 or GTR media (Table 1) for embryo development. The response of SE_2 to several different concentrations of the cytokinin 6-benzylaminopurine (BA) in the differentiation media SK8 was investigated. Differentiation media were prepared with 0.0, 2.5, 5.0, 7.5 or 10.0 mg/l BA, and the production of mature embryos and plantlets from each medium was recorded. After 2 weeks, cultures were transferred from SK8 or GTR to SKAW medium (Table 1) for further differentiation into mature embryos with leaf primordia.

Embryo germination, plantlet formation and acclimatization of plants

SE_2 germinated after a 2-week culture on SK10 medium (Table 1). The plantlets were then transferred for elongation to SK11 medium (Table 1). Mature plantlets 5- to 6-cm-long were obtained after 2–3 weeks. Magenta boxes containing plants suitable for acclimatization were placed in a greenhouse maintained at 28°C for 3–5 days. Medium was rinsed from the plantlets, and the latter were transplanted to 5-cm-diameter pots containing Sunshine Mix no. 4 (Sun Gro Horticulture, Bellevue, Wash.) and vermiculite (2:1; v:v). The plantlets were acclimated for 3 weeks in a greenhouse at 28°C, after which they were transferred to a greenhouse without temperature control.

Results and discussion

Initiation of primary somatic embryos

Immature male flowers isolated and cultured in the dark on M1 medium became swollen 2 weeks after the cultures were initiated. These cultures were transferred to fresh M1 medium after 1 month, and white tissue protruded from the inflorescence tissues within 2 months after culture initiation. The white tissues were excised from the mother tissue and transferred to MM1 medium where compact white callus, non-friable embryogenic tissues and early stages of embryos were formed within 2 weeks of the transfer (Fig. 1A), with 47 out of 80 explants producing embryogenic callus (58.75%). Escalant et al. (1994) reported 0–7% embryogenic clusters in five banana cultivars but also reported differences in embryogenic potential based on seasonal effects. Additional experiments are required to determine if the high percentage of embryogenic callus obtained through our method is due to cultivar or seasonal effects. The inclusion of proline and casein hydrolysate in the MM1 medium led to increased callus proliferation and production of primary somatic embryos and may increase the embryogenic potential in other banana cultivars.

Establishment of SE_2

Callus proliferated and differentiated into SE_2 from primary somatic embryos using different SK media (Table 1). Primary embryos responded best on SK4 medium containing 10% coconut milk. A relatively high percentage (57.1%) of explants produced only SE_2, and an additional 39.8% of the explants produced non-embryonic callus in addition to SE_2 (Table 2). In several monocot species, including *Hemerocallis* sp., *Dactylis glomerata*, *Oryza sativa*, *Panicum maximum*, *Pennisetum americanum*, *Triticum aestivum* and *Zea mays*, the induction of SE_2 has been found to require continuous exposure to growth regulators (reviewed in Raemakers et al. 1995). The transfer of the primary somatic embryos to other media (SK1, -2, -3, -5, -6 and -7) resulted predominantly in non-embryogenic callus production only (73.4–94.5% of explants), with a low percentage of explants (5.5–26.6%) producing some SE_2 in addition to non-embryonic callus, and no explants producing only SE_2 (Table 2). Cultures of SE_2 were multiplied and maintained for 4 months on SK4 medium, although some embryos reverted to callus after two subcultures.

SE_2 typically entered the globular stage directly from primary somatic embryos and occurred in clusters (Fig. 1b), initially appearing as small, hyaline protuberances on the surface of the clusters. The embryos passed through a recognizable heart-shaped stage, a primary torpedo stage (Fig. 1b), a later torpedo stage (Fig. 1c), and finally green plumules emerged from these embryos (Fig. 1d). Concomitant with morphological development there was a change in opacity from hyaline to translucent to opaque with a simultaneous change in color from pale white to yellow to green.

Effect of BA concentration on the development of embryos

The highest percentages of mature embryos and regenerated plantlets were obtained when SE_2 were cultured onto SK8 medium [half-strength Murashige and Skoog (l962); MS] supplemented with 5 mg/l BA (Fig. 2). BA concentrations above or below 5 mg/l produced lower percentages of mature embryos and plantlets, while SE_2 cultured on cytokinin-free medium produced 9% and 3% mature embryos and plantlets, respectively (Fig. 2). Vuylsteke (1989), Lee (1992) and Okole and Schulz (1996) reported that high BA concentrations induced

Fig. 1A–F SE_2 and the production of plantlets from immature male flower bud explants of *Musa* spp. cv. Dwarf Brazilian (AAB). **A** Primary somatic embryos on MM1 medium (*bar*: 1 cm), **B** cluster of globular-stage secondary somatic embryos on SK4 medium with *arrow* showing early torpedo-stage SE_2 (*bar*: 1 mm), **C** late torpedo-stage SE_2 on SK4 medium (*bar*: 1 mm), **D** green plumules (*arrow*) emerging from a SE_2 on SK4 medium (*bar*: 1 mm), **E** mature embryo with leaf primordia (*LP*), root initial (*RI*) and crown region (*CR*) on SKAW medium (*bar*: 1 mm), **F** germination of SE_2 with shoot development (*SD*) and root development (*RD*) on SK10 medium (*bar*: 1 mm) ▶

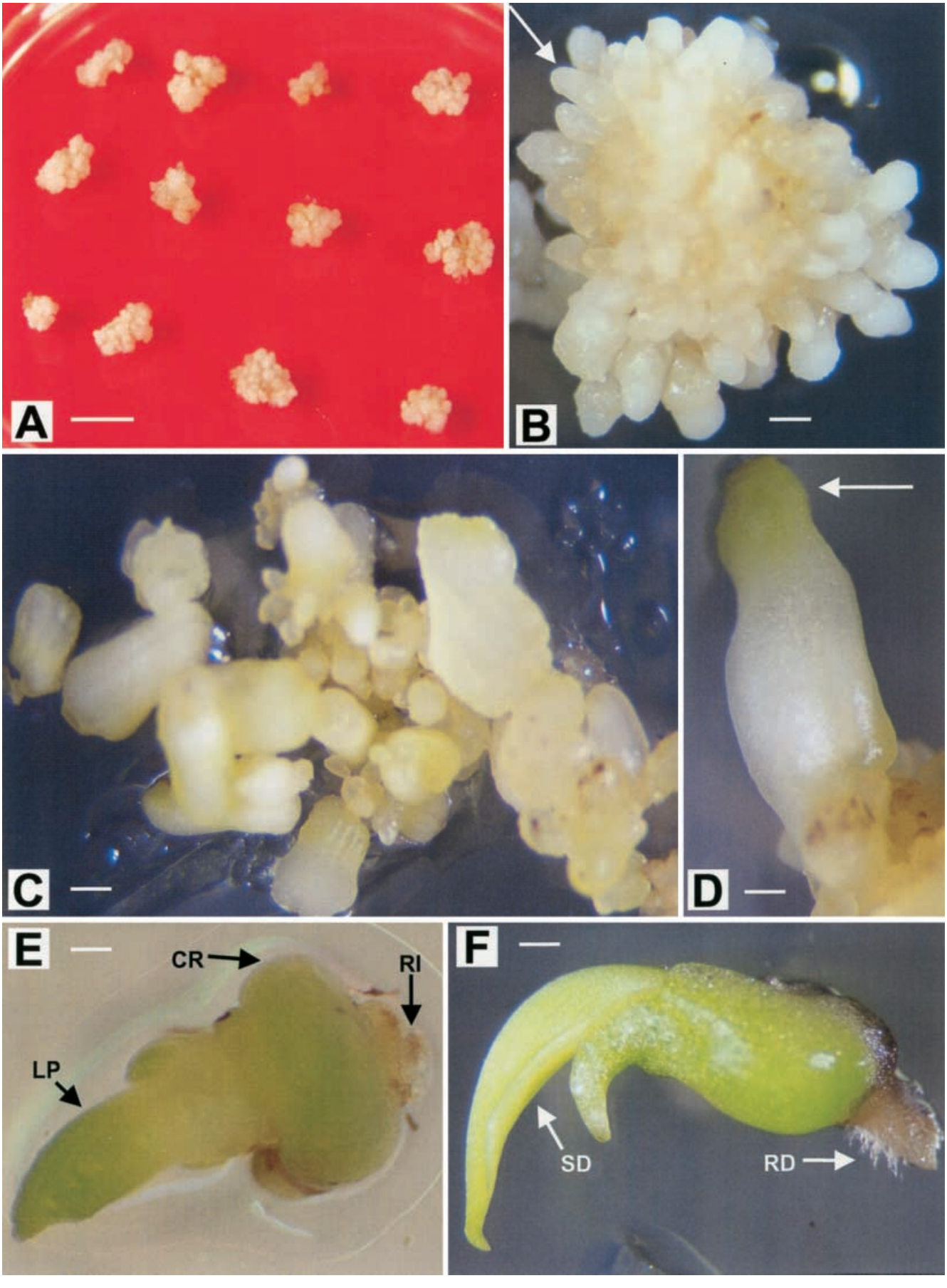
A
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Table 2 Response of explants containing primary somatic embryos transferred to several proliferation media for the production of secondary embryos. Approximately 40 explants composed of embryogenic callus containing primary somatic embryos were used per treatment in a single trial. Data were taken 4 weeks after transfer to proliferation media

Medium	Non-embryogenic calli only (%)	Non-embryogenic calli and some secondary embryos (%)	Secondary embryos only (%)
SK1	73.4	26.6	0.0
SK2	88.9	11.1	0.0
SK3	94.5	5.5	0.0
SK4	3.1	39.8	57.1
SK5	75.0	25.0	0.0
SK6	73.4	26.6	0.0
SK7	86.7	13.3	0.0

Table 3 Effect of differentiation medium on the development of secondary embryos, embryo germination and production of banana plantlets

Medium	Number of explants	Number of mature secondary embryos	Number of embryos germinated on SK10	Number of plantlets produced on SK11
SK8	40	388	10	9
GTR[a]	40	80	0	0

[a] Differentiation media of Ganapathi et al. (1999)

Table 4 Effect of SKAW medium on the development of secondary embryos, embryo germination and production of banana plantlets

Medium[a]	Number of explants	Number of mature secondary embryos	Number of embryos germinated on SK10	Number of plantlets produced on SK11
SK8 to SKAW	40	998	950	893
GTR[b] to SKAW	40	250	29	19

[a] After 3 weeks on differentiation medium (SK8 or GTR), secondary embryos were subcultured onto SKAW medium for the development of mature embryos
[b] Differentiation media of Ganapathi et al. (1999)

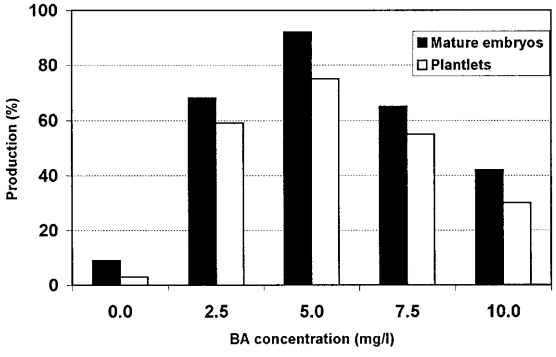

Fig. 2 Production of mature SE_2 and plantlets of *Musa* sp. AAB cv. Dwarf Brazilian from early-stage (globular, heart or torpedo) secondary embryos. *Bars* represent the percentage of 100 early-stage secondary embryos in a single experiment producing mature embryos (*black*) or plantlets (*white*) on media containing variable concentrations of BA in differentiation media SK8

shoot formation; however, our results showed a lower formation of mature embryos and plantlets at high (>5 mg/l) BA concentrations.

Effect of two media on the development of SE_2 and the regeneration of plantlets

The effects of SK8 medium from this present study and a differentiation medium (GTR) from Ganapathi et al. (1999) on the maturation of embryos and development of plantlets was compared (Table 3). Cultures on SK8 medium produced a higher number of mature SE_2 than cultures on GTR medium (Table 3). Additionally, a few SE_2 germinated and a few plantlets were produced when SE_2 were cultured on SK8 medium, while none were produced on GTR medium (Table 3). The transfer of explants to SKAW resulted in a threefold increase in the number of SE_2 that developed, irrespective of whether they were originally cultured on SK8 or GTR media (Table 4 versus Table 3). However, explants originally cultured on SK8 produced four times more mature SE_2 when transferred to SKAW than explants originally cultured on GTR (Table 4). Furthermore, transfer from SK8 to SKAW dramatically increased the number of germinated embryos and plantlets when compared to those that formed on SK8 medium only (Table 4 versus Table 3).

Germination of SE_2 and development of regenerated banana plants

Mature SE_2 possessed a well-defined late torpedo-stage morphology with a cylindrical, distinct apical dome with leaf primordia and crown region from which shoot initials and root initials developed (Fig. 1E). Embryos that germinated with shoot initials and root initials produced plantlets when placed directly on SKAW medium or

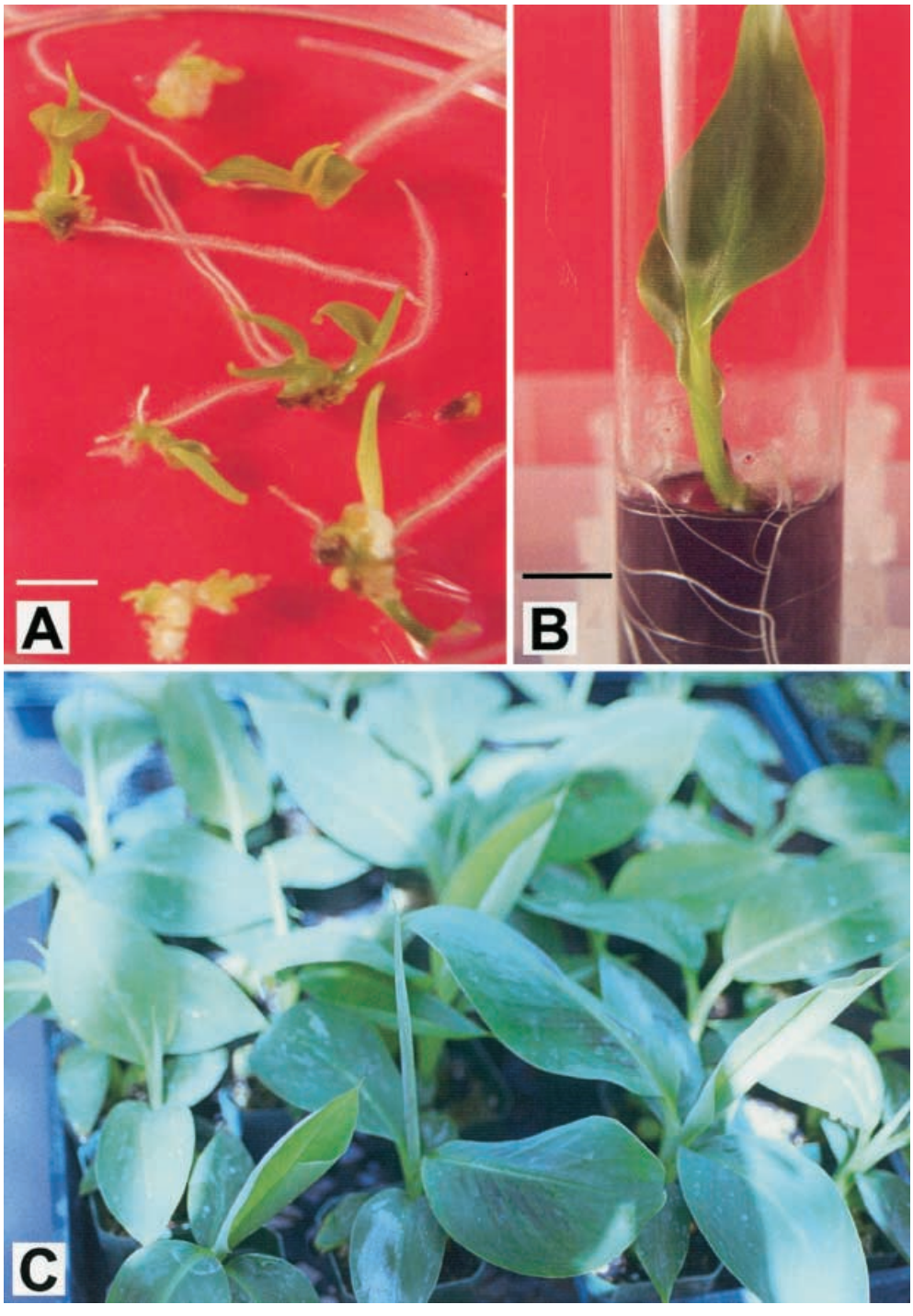

Fig. 3 **A** Germination of secondary embryos and production of small plantlets on SK10 medium (*bar*: 1 cm). **B** Elongation and development of mature regenerated plantlet of banana on SK11 medium (*bar*: 1 cm). **C** Regenerated banana plants, 4 weeks after transplanting

when separated from the culture mass and placed directly on SK10 (Fig. 1F). On SK10 medium, which contained no plant growth regulators, SE_2 gave rise to small plantlets within 10–15 days (Fig. 3A). After 2–3 weeks of culture on SK10 medium, plantlets were subcultured onto SK11 medium for elongation and the development of new leaves (Fig. 3B). After 2–3 weeks, the plantlets were removed from the SK11 medium and acclimated to greenhouse conditions. All of the plants regenerated through SE_2 and transplanted into the greenhouse survived (Fig. 3C). To date, over 1,000 plants have been maintained in the shade house for more than 6 months. No abnormal morphological variations have been observed, and these plants will be transplanted to the field for further observation of growth and fruit production.

Starting from 40 explants containing SE_2, the number of plantlets was increased at every subculture, resulting in 890 regenerated banana plants after 15 weeks (Fig. 4). The percentage of secondary embryos that germinated and developed into completely regenerated banana plants

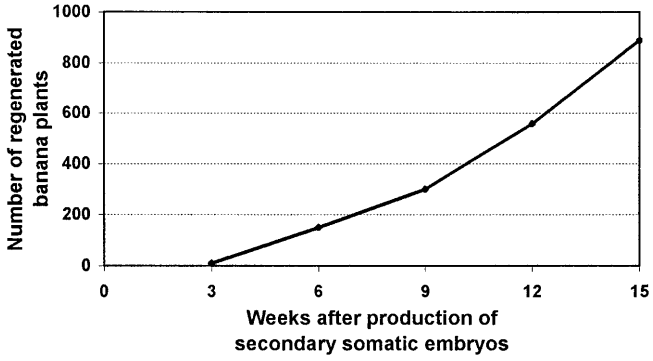

Fig. 4 Increase in the number of regenerated banana plants following the production of SE_2

was 89.5%, which is much higher than the figure reported by Novak et al. (1989) – 1.5–12% embryo germination – or Côte et al. (1996) – 3–20% of the germinated embryos developed into plantlets. Our results are similar to those of Escalant et al. (1994), who reported 60–70% germination using a temporary immersion system (TIS), and Kosky et al. (2002), who recently reported an 89.3% regeneration using TIS and cell suspension using a bioreactor. However, our method does not require TIS, cell suspension or a bioreactor, and can be adopted by any tissue culture laboratory, even those without specialized equipment.

Maintenance and proliferation of SE_2 for long-term culture

We have used two media to preserve SE_2: (1) solid SK4 medium and (2) liquid M2 medium (Table 1). The liquid M2 was used to maintain and proliferate cultures for 16 months. During this time, the cultures proliferated through SE_2. On solid SK4 medium, some of the embryos returned to callus after two subcultures. We have successfully regenerated plants using the system described in this paper from cultures maintained in liquid M2 medium on several occasions, both for plant propagation and for current experiments on genetic transformation, indicating that this protocol is reproducible. This is the first report of tissue culture of the banana cultivar Dwarf Brazilian.

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