In Vitro Cell. Dev. Biol. 30P:181–186, October 1994

1054-5476/94 $02.50+0.00

AMPLIFIED SOMATIC EMBRYOGENESIS FROM MALE FLOWERS OF TRIPLOID BANANA AND PLANTAIN CULTIVARS (*MUSA* SPP.)

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(Received 6 December 1993; accepted 26 February 1994; editor R. H. Smith)

SUMMARY

Somatic embryogenesis and plant regeneration of banana and plantain cultivars (*Musa* spp.) were obtained by culturing young male flowers. Multiplication and maintenance of embryogenic cultures were achieved by culturing somatic embryos in a temporary immersion system (SIT). A multiplication rate of 40 allowed us to obtain more than 6000 somatic embryos after 6 mo. of subculture. Plant recovery frequencies were 60 to 70%. This method was expanded to different banana and plantain genomic groups.

Key words: banana; plantain; triploids; somatic embryogenesis; in vitro; temporary immersion system; plant regeneration.

INTRODUCTION

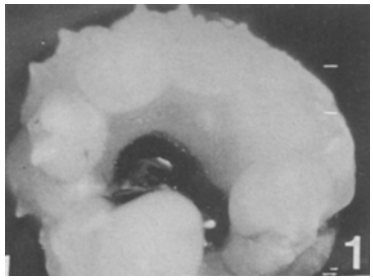
Internationally, banana and plantain are among the most important cultivated species, with a production of approximately 70 million tons annually. The incidence of numerous diseases such as black Sigatoka (*Mycosphaerella fijiensis*), *Fusarium* wilt (*Fusarium oxysporum* var. *cubense*), viruses (bunchy top and CMV), and nematodes (*Radopholus similis*) affecting production and its cost make the development of new varieties increasingly urgent. For more than 60 yr, different programs of genetic improvement have been developed around the world (Rowe, 1984; Bakry et al., 1990). However, because of sterility and polyploidy of banana and plantain the development of new varieties through conventional breeding programs remains difficult. Due to these problems, new strategies consisting of tissue culture and molecular biology techniques to complement breeding programs have been undertaken (Murfett and Clarke, 1987). Among these techniques, genetic transformation permits the introduction of foreign genes for insect and virus resistances. However, the major drawback is that these methods usually depend on an in vitro culture system that allows regeneration of mature plants (Weising et al., 1988). For several years, different techniques such as somatic embryogenesis and cellular suspension cultures have made it possible to obtain banana and plantain plants developed in vitro (Escalant and Teisson, 1989; Novak et al., 1989; Dhed'a et al., 1991; Chou Tou Shii et al., 1992). The complete mastery of the somatic embryogenesis system should make it of value not only to current genetic improvement programs, but also for industrial production of tissue-culture-derived plants for use as planting material. Here we describe a new method for enhanced somatic embryogenesis in sweet and cooking triploid cultivars. This method now permits continuous recovery of banana plants from a new and original system.

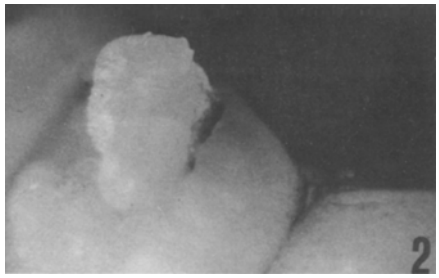
MATERIALS AND METHODS

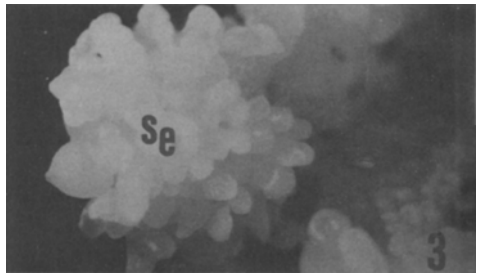
Somatic embryogenesis. Embryogenic cultures were obtained using the method described by Chou Tou Shii et al. (1992). Immature male flowers from positions 0 to 15 (0 being the floral meristem) were isolated from male buds (tip portion of inflorescence) using a dissecting microscope. The following genotypes were tested: *Musa* AAA cvs. "Grande Naine" and "Yangambi", *Musa* AAB cvs. "French Plantain", "Mysore" and "Silk", and *Musa* ABB cv. "Pelipita". Young flowers were cultured on an MI semisolid medium which consisted of MS salts and vitamins (Murashige and Skoog, 1962) supplemented with 4.09 μM biotin, 5.7 μM indole-3-acetic acid (IAA), 18.1 μM 2,4-dichlorophenoxyacetic acid, 5.37 μM naphthaleneacetic acid, 0.87 M sucrose, and solidified with 7 $g \cdot \text{liter}^{-1}$ agarose (Fig. 1). The pH was adjusted before sterilization to 5.7 with KOH (1 N) and HCl (1 N). Cultures were kept at 27° ± 2° C under fluorescent light (10 $w \cdot m^2$) on a 16:8 light:dark cycle and were maintained without subculturing. After 3 to 5 mo., depending on the genotype, somatic embryos obtained were isolated and transferred to MG germination medium, which consisted of MS salts and Morel's vitamins (1948) supplemented with 0.22 μM BAP, 1.14 μM IAA and 0.87 M sucrose, and solidified with 2 $g \cdot \text{liter}^{-1}$ gelrite (Marroquin et al., 1993). After germination of somatic embryos, plantlet development was achieved on an MS hormone-free, semisolid medium supplemented with Morel's vitamins, 0.87 M sucrose, and solidified with 2 $g \cdot \text{liter}^{-1}$ gelrite.

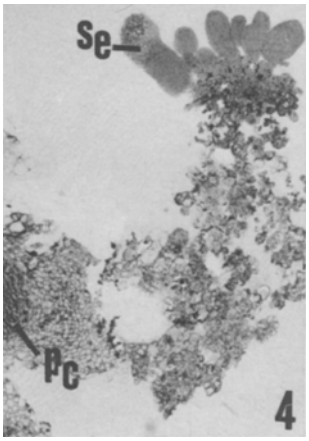
Somatic embryogenesis through temporary immersion. With the goal of finding a simple, rapid, and effective method, which could be used in a genetic transformation program as well as for mass propagation, we tested the association of two methods used extensively by our team. The first concerns the efficiency of picloram to induce somatic embryogenesis and embryogenic suspension from zygotic embryos (Escalant and Teisson, 1989; Marroquin et al., 1993). In this way, we used the semisolid medium and the method developed on zygotic embryos. The second method concerns the use of the temporary immersion system (Alvard et al., 1993). We have thus compared the development of somatic embryos placed in the presence of picloram in such a system.

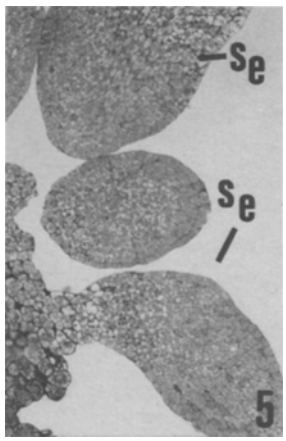
Proliferation of somatic embryos from the cultivar "Grande Naine" was achieved by using embryogenic cultures established as mentioned above. The young embryogenic cultures obtained from the male flowers were iso-

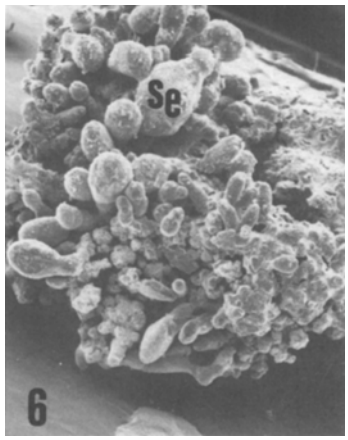












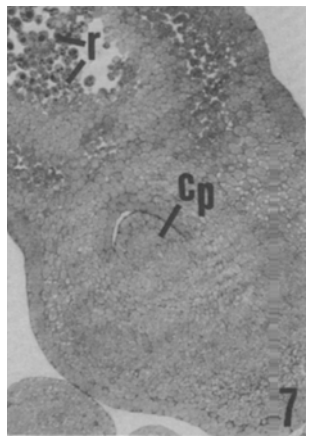


FIG. 1. Young cluster of male flowers after 15 days of culture. ×10.

FIG. 2. Small yellow callus derived from male flower after 2 mo. of culture. ×30.

FIG. 3. White and translucent callus with somatic embryos (*se* = somatic embryos). ×20.

FIG. 4. Cross section of callus with compact tissue in the middle part; *note* the pseudocambial zone (*pc*) and loose tissue carrying the embryos on the surface (*se*). ×50.

FIG. 5. Cross section of embryogenic tissue carrying the somatic embryos (*se*); *note* the embryogenic cells with reserves (*r*) and the groups of two to several cells (*g*). ×230.

FIG. 6. Somatic embryos (*se*) derived of translucent and friable embryogenic tissue (scanning electron micrograph); *note* the great number of somatic embryos at various stage of development. ×25.

FIG. 7. Transverse section of somatic embryos; *note* the cauline pole (*cp*) and the haustorium like structure with proteical reserve (*r*). ×230.

lated, weighed, and evaluated regarding the number of somatic embryos. Cultures were initiated with approximately 0.250 *g* of translucent embryogenic culture bearing an average of 150 somatic embryos. The MP culture medium, used during temporary immersion, consisted of MS mineral salts supplemented with Morel's vitamins, 2.2 μM picloram [4-amino-3,5,6-trichloropicolinic acid] (Escalant and Teisson, 1989), and 0.87 *M* sucrose, with the pH adjusted to 5.8. Culture conditions were the same as described above. Proliferation on semisolid medium ($2\ g \cdot \text{liter}^{-1}$) was compared with that occurring in liquid medium with temporary immersion of the embryogenic cultures for 1 min every 6 h. This new culture system has been previously described (Alvard et al., 1993) and is referred to as temporary immersion system (TIS). Specially modified autoclavable filter units (Nalgene, Nalge Co.) were used as culture vessels. Embryogenic cultures were placed in the upper compartment and immersed only when liquid medium in the lower compartment was pushed up by air pressure. When the pressure was released, the liquid medium flowed back by gravity, keeping the explant in a condition of high relative humidity. Effects of treatments on the rate of proliferation and the fresh matter weight were evaluated monthly from the second month of culture. The medium was refreshed each month. Germination and development of somatic embryos were achieved transferring somatic embryos on, respectively, MG germination medium and MS hormone-free, semisolid medium supplemented with Morel's vitamins,

TABLE 1

RESULTS OBTAINED AFTER 4 MO. OF CULTURE OF DIFFERENT GENOTYPES[a]

	"Yangambi" AAA	"Plantain" AAB	"Mysore" AAB	"Silk" AAB	"Pelipita" ABB
Callogenic clusters, %	15	20	15	18	17
Embryogenic clusters, %	5	2	3	7	0

[a] They were obtained by the culture of five male buds per cultivar.

0.87 *M* sucrose, and solidified with 2 $g \cdot liter^{-1}$ gelrite. After development, plantlets were transferred to a greenhouse for acclimatization and somaclonal variation evaluation.

Histologic studies. Specimens selected for histologic studies were fixed with a glutaraldehyde solution (2%) in a phosphate buffer (0.2 *M*; pH 7.2), to which was added acroline (2%) and caffeine (1%). They were next dehydrated and embedded in a water-soluble resin (Kulzer 7100). Sections 3-μm thick were cut, and prepared sections were stained with a double staining procedure which used periodic acid Schiff (PAS) to reveal the polysaccharides and naphthol blue-black to show proteins of a specific nature.

Results

Somatic embryogenesis. Young flowers responded after 1 to 2 mo. of culture by forming a small yellow callus (Fig. 2) which appeared on the upper surface of the flower. During the following 3 to 5 mo., a white and translucent callus formed on the yellow callus, and somatic embryos appeared on its surface. When the number of somatic embryos was greater than 20, it was possible to observe that they were formed from a translucent and very friable tissue (Fig. 3).

In cross-section, the three parts of the previously described callus are distinguishable (Fig. 4). The base of the callus is composed of highly differentiated cells corresponding to the necrotic portion of the explant; the middle part is composed of compact tissue corresponding to the yellow, nodular callus; and the surface portion is composed of loose tissues carrying the embryos and corresponding to the previously described translucid and friable callus. The transition between the nodular callus and the friable tissue is characterized by cellular isolation, with hydrolysis of starch and production of a polysaccharide mucus. The cells with embryogenic potential, loaded with protein reserves, are thus observable (Fig. 5). These cells evolve into embryos. The presence of developing embryos consisting of groups of two to several cells suggests that they are probably of unicellular origin (Fig. 5). The formation of an epidermis and the beginning of polarization, as demonstrated by a localized accumulation of starch, are observable in the embryos (Fig. 5).

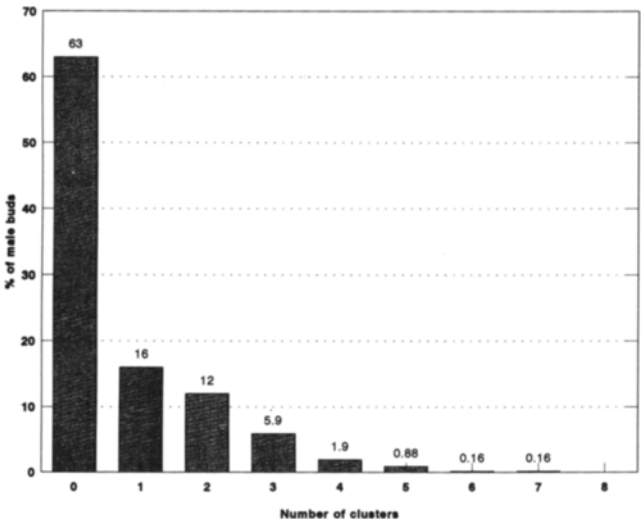


Fig. 8. Distribution of the male buds, depending of the number of embryogenic clusters. *Musa* AAA cv. "Grande Naine".

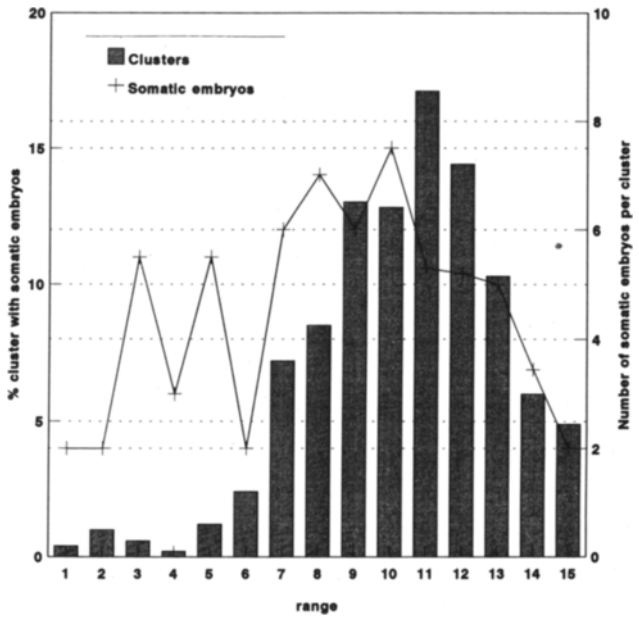


Fig. 9. Embryogenic potential of male flower clusters of *Musa* AAA cv. "Grande Naine", according to their position.

When the translucent and friable embryogenic tissue is highly developed, maintenance of the explant for 2 to 3 mo. more without subculturing can result in a considerable increase in the number of somatic embryos (rate 1 to 20) (Fig. 6). It is important to emphasize that the monthly subculture of embryogenic cultures onto fresh MI medium does not permit their proliferation. Apparently, the main point required for the multiplication of the embryogenic cultures is

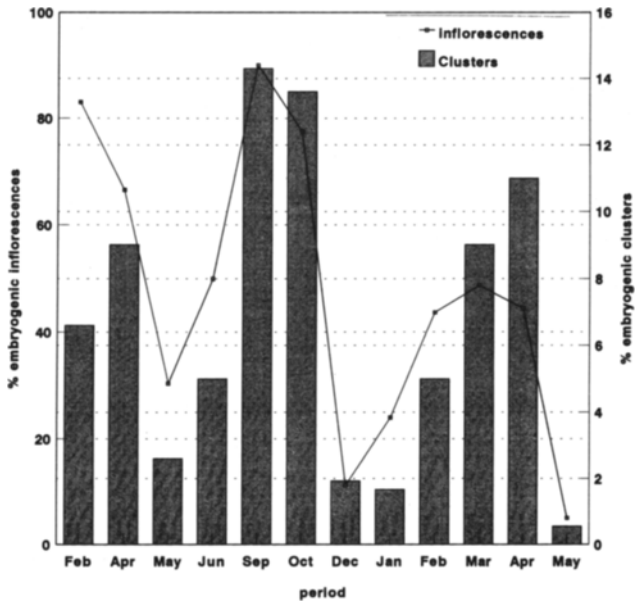
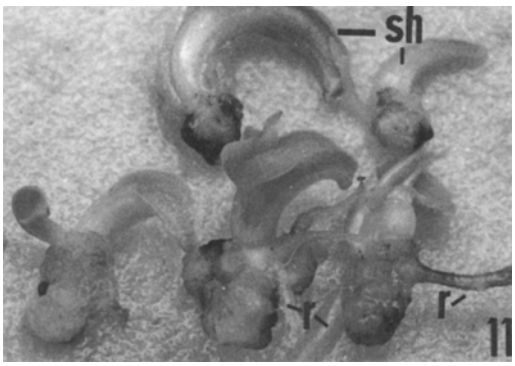
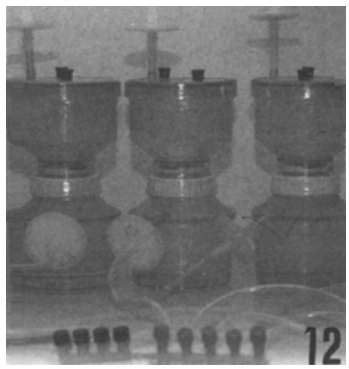


Fig. 10. Seasonal effect on the embryogenic potential of the inflorescences and clusters.





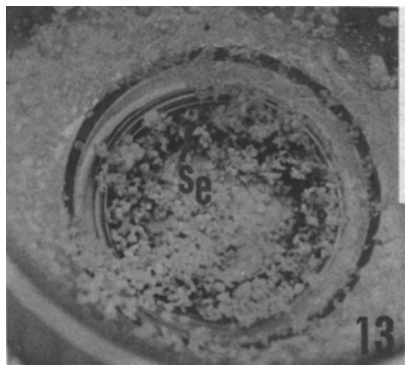


FIG. 11. Germinating somatic embryo; *note* the young shoot growth (*sh*) and root development (*r*). ×0.6.
FIG. 12. System of temporary immersion. ×0.07.
FIG. 13. Somatic embryos (*se*) obtained through the temporary immersion system after 3 mo. of culture. ×0.37.

either the permanence of the primary explant or the age of the medium or both. Somatic embryos appear as small (0.5 mm), translucent structures with a smooth surface and cotyledonary slit, as the histologic section shows (Fig. 7).

Although somatic embryogenesis has been obtained with the majority of the cultivars tested, we used the *Musa* AAA cv. "Grande Naine" in detailed studies because of the availability of vegetative material. The culture of 635 male buds (9525 clusters of flowers) permitted us to obtain the following results: only 37% of the male buds were embryogenic, among those 28% represent one or two embryogenic flower clusters per bud (Fig. 8). However, some of them (0.16%) had up to five embryogenic clusters per bud. We should also mention that 74% of the embryogenic clusters were distributed between positions 7 and 13 (Fig. 9). The frequency and intensity of embryogenesis demonstrates again a part of the uncertainty in which a seasonal factor can have some influence (Fig. 10). This observation must be taken into account in the comparative study involving five male buds per variety conducted on the principal genomic groups in which the cultivars are found (Table 1).

The embryogenic response is general, with the exception of the cultivar "Pelipita" of the ABB group, but occurs at a lower level than for "Grande Naine". Experiments repeated during the course of a year would eventually be able to improve response.

The transfer of somatic embryos onto MG medium resulted in two germination processes. In the first process (type I), after 1 wk of culture, the somatic embryos germinate with the emergence of a chlorophyllious plumule, quickly followed by the development of roots (Fig. 11). In the second germination process (type II), the somatic embryo turns into a vitrified structure, from which after 3 to 4 wk a complete plant emerges. To reduce the phenomena of vitrification and delayed germination, a piece of filter paper was inserted between the semisolid medium and the somatic embryos. The results presented in Table 2 show that the rate of type I germination goes from 26% on the MG medium to 59.5% on the MG + filter paper medium. Also *note* that the germination percentages are increased to 80 and 88%, respectively. The development of somatic embryos is obtained after transfer of young plantlets onto a growth medium free of hormones.

TABLE 2

PERCENTAGE OF GERMINATION OBTAINED FOLLOWING TRANSFER OF SOMATIC EMBRYOS TO MG AND MG + FILTER PAPER MEDIA[a]

	Germination Type I, %	Germination Type II, %	Germination Total, %
MG medium	26	54	80
MG medium + filter paper	59.5	28.5	88

[a] Average obtained by four replications with 100 embryos in each treatment).

Somatic embryogenesis through temporary immersion. After 2 mo. the temporary immersion system showed its superiority over the semisolid medium: the coefficient of multiplication of the em-

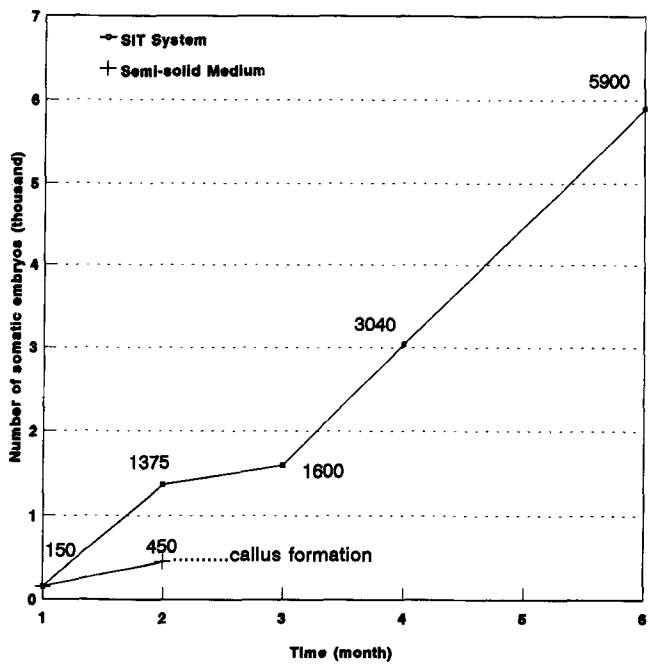
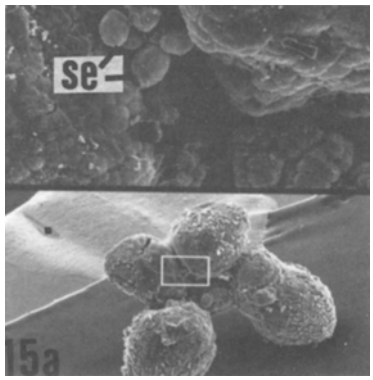


FIG. 14. Mean number of *Musa* AAA cv. "Cavendish" somatic embryos produced after 6 mo; comparison between SIT system and semisolid medium culture.



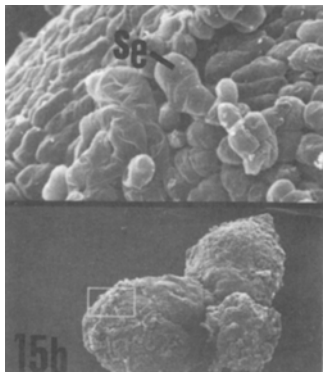


FIG. 15. Multiplication of somatic embryos in the temporary immersion system. *a*, Young somatic embryos forming at the base of primary embryos; *note* the group of several cells (*g*) as future embryos. ×22. *b*, Young somatic embryos (*se*) forming from epiderm cell (scanning electron micrograph). ×30.

FIG. 16. Plants obtained from somatic embryos of *Musa* cv. AAA "Grande Naine".

bryos was 9 for the former and 3 for the latter. After 6 mo. of culture, the SIT permitted us to multiply the initial population by 40 (Fig. 14). Macroscopic and microscopic observations of the SIT cultures allowed us to verify that multiplication occurs by adventitious somatic embryogenesis. The somatic embryos were multiplied in cascades, each one forming 4 to 5 new embryos which then separated and continued the phenomenon. Somatic embryos can be formed at the base or from some epidermal cells of the primary embryos (Fig. 15 *a,b*). Furthermore, after 2 mo. of incubation we observed that the somatic embryos maintained on semi-solid medium had become transformed into a compact, white, callus-like structure, while after 6 mo. of subculture, those in the SIT system retained a more satisfactory appearance and maintained embryogenic proliferation. The SIT system permits one to easily divide up the population at any time by transferring a portion of the embryos to other (SIT) vessels. Germination was carried out by transferring the embryos onto semisolid MG medium. In both cases, the rates of germination varied between 60 and 70%. Germination could also be achieved without having to transfer somatic embryos to another SIT container, by simply exchanging the MP + picloram medium for the MG medium. Due to the high number of embryos per SIT vessel and the resulting competition for space, light, and nutrients, it is preferable to regularly remove the embryos that germinate and to transfer them to the medium for plantlet development. By this method we were able to obtain more than 2000 plantlets from the approximately 3000 somatic embryos produced after 4 mo. This process was repeated over a period of several months, always with equal success.

DISCUSSION AND CONCLUSION

In regard to the multitude of problems to be solved for the genetic improvement of banana and plantain, the development of new in vitro regeneration systems that cover a larger range of genotypes remains essential.

Somatic embryogenesis was obtained by culturing young male flowers of different genomic groups of sweet and cooking bananas. The originality of this method is characterized by its application to a large range of genotypes and by the introduction of the temporary immersion system as a very efficient propagation technique. Although this is not the first report of regeneration in the *Musa* genus, the method of regeneration is original and different from any previously reported (Dhed'a et al., 1991; Novak et al., 1989). The efficiency of this method will allow its application in plant transformation through bombardment with particles (Weising et al., 1988). In addition, due to the high proliferation rate obtained in the SIT system, additional research should be conducted to determine its commercial applicability for mass propagation. For the same reason, it would be interesting to consider this method in a germplasm conservation program by developing additional studies for producing synthetic seeds (Villalobos et al., 1991). Moreover, the temporary immersion system could also be used with other species, perhaps permitting a considerable improvement in the results obtained by somatic embryogenesis or mass propagation. Nevertheless, all of these applications would require more information on the eventual presence of somaclonal variants among the plants obtained (Fig. 16). At present, 1500 plants are in the process of field evaluation.

ACKNOWLEDGMENTS

We thank Prof. Ma and his team from Taiwan University for their help and collaboration. We also acknowledge Dr. Nicole Michaux-Ferrières and Mrs. A. Grapin from the CIRAD's Histological Laboratory for their collaboration with the histologic studies and MSc Nelly Vasquéz for her collaboration with the scanning microscopic analyses. This work was supported by a grant from the European Economic Commission (EEC-STD3). We also acknowledge support from the "Centro Agronomico de Investigacion y Enseñanza (CATIE)" and from the "Centre International de Recherche en Agronomie pour le Développement (CIRAD)".

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