INIBAP Technical Guidelines 8

Banana and plantain embryogenic cell suspensions

Hannelore Strosse, Régis Domergue,
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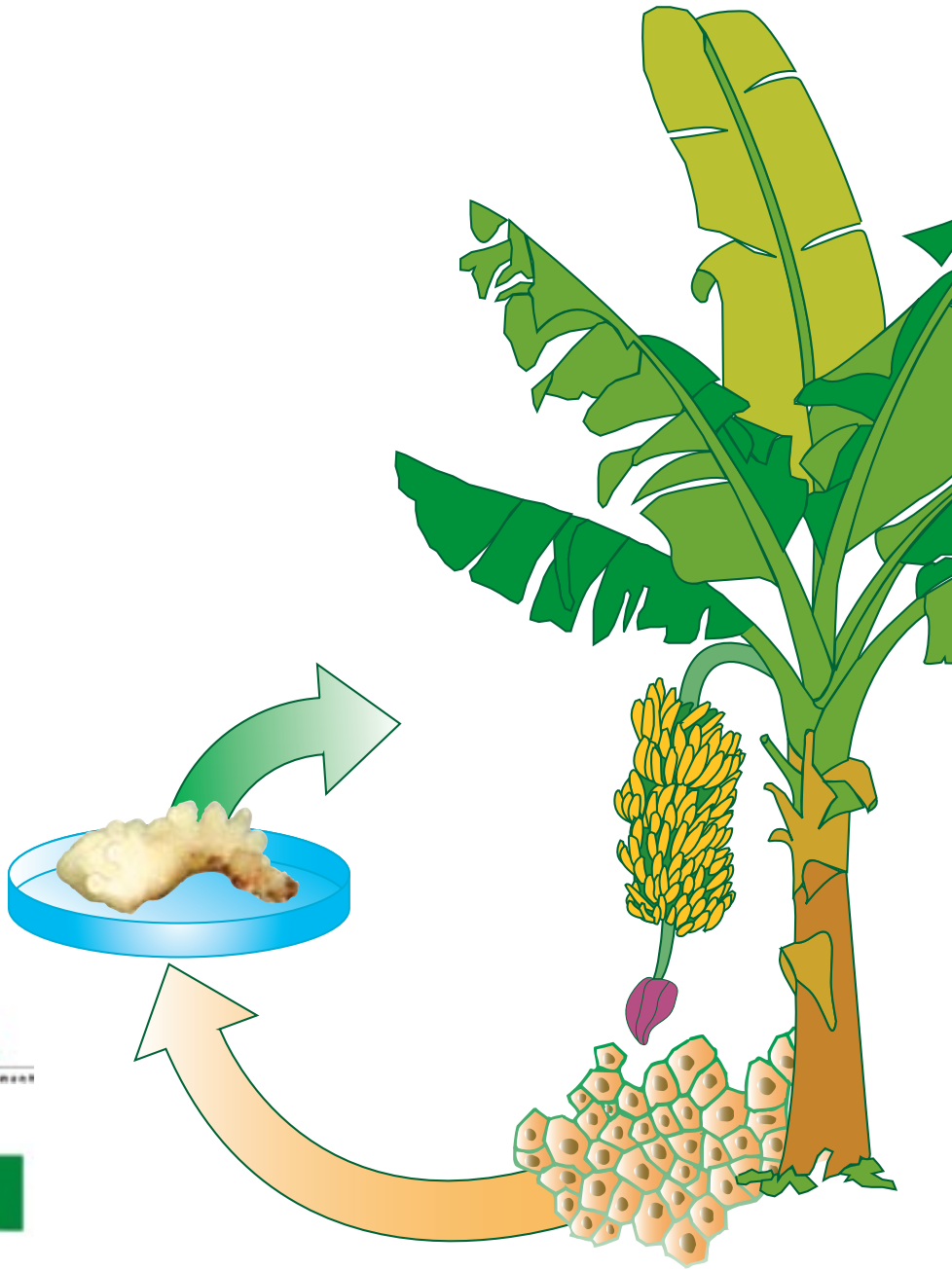

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To promote and strengthen collaboration and partnerships in banana-related research activities at the national, regional and global levels

To strengthen the ability of NARS to conduct research and development activities on bananas and plantains

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Foreword

The protocols were prepared by the Laboratory of Tropical Crop Improvement of KULeuven and the cellular biology laboratory of the *Centre de coopération internationale en recherche agronomique pour le développement* (Cirad, Biotrop laboratory).

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Introduction

Somatic embryogenic techniques were originally developed to meet two main goals: mass micropropagation and the development of cellular tools for genetic improvement (e.g. genetic transformation and protoplast fusion). These techniques rely on the use of synthetic growth regulators (auxins) to induce the dedifferentiation of tissues and the formation of embryogenic tissue (callus). The callus provides the starting material for the development of embryogenic cell suspensions (ECS). From these suspensions, embryos are produced and plants are regenerated.

Despite its high regeneration potential, the technique to produce ECSs is not operational for mass micropropagation. The main reason is the increased somaclonal variation compared to the level observed with the classical technique of shoot tip culture. However ECS are already used for genetic transformation and protoplast fusion in banana plants. Plants regenerated from an ECS frequently originate from a single cell. In the case of transformed plants, this circumvents the problem of chimeric plants (plants containing genetically transformed cells alongside untransformed cells) encountered when using shoot tips as starting material.

Four procedures have been tested with banana plants. Each relies on different types of explants: zygotic embryos (Cronauer and Krikorian 1988, Escalant and Teisson 1989), rhizome slices and leaf sheaths (Novak *et al.* 1989), immature male/female flowers (Ma 1991, Escalant *et al.* 1994, Grapin *et al.* 1996, Grapin *et al.* 1998) and proliferating meristem cultures (scalps) (Dhed'a *et al.* 1991, Schoofs 1997). ECSs are most commonly initiated from immature male flowers or scalps.

The development by Professor Ma at the University of Taiwan of a culture medium and a methodology to obtain ECSs from male flowers (Ma 1991) was a major breakthrough in the development of a somatic embryogenesis system for bananas and inspired numerous studies. The first steps, up to the formation of the embryogenic callus, are described in Escalant *et al.* (1994). Descriptions of the initiation, maintenance and regeneration phases of cellular suspensions are available in Grapin *et al*. (1996) and Côte *et al*. (1996). Ma's method has also been used with immature female flowers for those cultivars that do not produce male flowers (Grapin *et al*. 2000).

The scalp method used at *Katholieke Universiteit Leuven* (KULeuven) relies on highly proliferating cultures initiated from shoot tips. This method was first described by Dhed'a (1992) and optimized by Schoofs (1997).

In these guidelines, only the methods based on immature male flowers and scalps are described, as they are supported by numerous publications and have been replicated in several laboratories. The protocols are followed by chapters on how to evaluate the quality of ECSs and on the limitations of this technique.

1. Somatic embryogenesis

Somatic embryogenesis of bananas, via male flowers and scalps, is illustrated in Figure 1 and detailed below. "Initial explant" refers to the explant that develops into an embryogenic callus when placed in a callus induction medium. Depending on the method, the initial explant is an immature flower or a scalp derived from a shoot tip. But whereas immature flowers are collected directly from the male bud, an *in vitro* culture phase is needed to obtain an initial explant when using the scalp method.

Given that these methods differ only in the first steps leading to the embryogenic callus, the protocols are presented in parallel up to that point. Thereafter, a common protocol, in which the particularities for each method are noted, is presented.

Callus induction

Preparation of initial explant

Scalp method	*Immature flower method*
Starting material	
Virus-indexed shoot tips (Diekmann and Putter 1996) are used as starting material. They can come from rooted *in vitro* plants (Figure 2), or greenhouse or field plants that have been surface sterilized (Hamill *et al*. 1993).	Use male buds (Figure 8) collected 1 to 10 weeks after flowering. The buds can be kept for 24 hours before inoculation of the immature flowers. For cultivars that do not produce male flowers, such as the Horn plantains, female flowers can be used. In this case, the bud is taken from inside the pseudostem before flowering. The method is described in Grapin *et al*. 2000.
Remove the roots and leaves and cut 0.5 cm above the apical meristem (Figure 3). Making longitudinal incisions can enhance the multiplication of individual shoots.	In non-sterile conditions, reduce the size of the male bud until the explant is 0.8 cm x 2 cm (Figure 9). The reduced bud is kept in non-dehydrating conditions until sterilization, e.g. in a container with a few drops of water and sealed with a plastic film.

Scalp method	*Immature flower method*
Sterilization	
Screen for the presence of slow-growing bacteria. Although they do not generally interfere with *in vitro* shoot multiplication, bacteria might be problematic in later stages of cell suspension initiation. Cut a slice at the base of the corm and rub it on the bacteriological medium. Incubate at 28°C and 4 to 6 weeks later examine the culture medium for the presence of bacterial colonies (Van den Houwe *et al*. 1998).	The reduced buds are surface-sterilized in 70% ethyl alcohol for 1 minute (Figure 10). It is not necessary to rinse them with distilled water. The time lapse between disinfection and inoculation should be less than 1 hour.
Inoculation	
Inoculate explants on 25 ml of P5 medium (Table 1 in Appendix 2) in 150 ml test tubes (1 explant/test tube) or baby food jars (3 to 5 explants/jar). The explants are cultured under **standard conditions**: 27°C under a continuous light intensity of 50 µE m^{-2} s^{-1} or a 12 h photoperiod and a relative humidity over 70%. One month later, evaluate the starting material and select the proliferation medium that will be used for subculturing, depending on the proliferation capacity of the clone (for a detailed description of the different proliferation classes, see Schoofs 1997). For cultivars with a high proliferating capacity (most cultivars belonging to the ABB group), clusters of small shoots and groups of up to 20 closely packed meristems should be observed at the base of the explant	Go to "Induction of embryogenesis" section.

Scalp method	*Immature flower method*
one month after inoculation on P5 medium (Figure 4). These cultivars should be kept on P5 medium until the production of 'cauliflower-like' meristem cultures.	
For cultivars with moderate to low proliferating capacity (most bananas and plantains), clumps of shoots and rooted plantlets, without meristem formation at the base of the leaf, will form 1 to 3 months after inoculation on P5 medium (Figure 5). These cultivars need to be transferred to P4 medium (Table 1 in Appendix 2) to obtain cauliflower-like meristem cultures (Figure 6).	

Subculturing

Once a month, select small shoots with groups of meristems at the base of the leaf until the groups are about 0.5 cm in diameter and can be cultured separately. The culture conditions, hereafter referred to as **culture conditions 2**, are the same as the standard conditions mentioned above, except that the cultures are kept in darkness.

Meristem cultures should be subcultured until they consist of clusters of small, white meristems surrounded by only a few, very small leaves (Figure 6). The required number of subcultures varies from clone to clone, but is usually between 2 and 10. The required and maximum numbers of cycles are listed by Schoofs (1997) for a broad range of cultivars. When proliferating capacity decreases fol-

Go to "Induction of embryogenesis" section.

Scalp method	*Immature flower method*
lowing repeated subculturing on P4 medium, it is recommended to do 1 or 2 subcultures on P5 medium before resuming subcultures on P4 medium.	

Induction of embryogenesis

Starting material

Using a binocular microscope, excise good quality scalps (3 × 3 × 3 mm to 3 × 3 × 5 mm) from the meristem cultures (Figure 7). These scalps should have a high ratio of meristematic domes/corm and leaf tissue. The presence of too much corm and leaf tissues should be avoided, as corm tissue tends to swell and leaf tissue gives rise to too many and watery calli.	Under a sterile hood, immature male flowers from reduced and surface-sterilized buds are isolated using a binocular (Figure 11). The immature flowers should be taken from position 16 to 8 (1 being the immature flower closest to the meristematic dome) (Figure 12). Use a scalpel with fine blades (e.g. Feather No. 11). The flowers in the 8th to 16th position are the most responsive ones in terms of embryogenesis. The ones below tend to become necrotic on MA1 medium, whereas the ones above tend to produce non-embryogenic callus.

Inoculation

Scalps are inoculated on semi-solid ZZ medium (ZZss, Table 1 in Appendix 2) in test tubes (1 explant/test tube), baby food jars or Petri dishes (3 to 5 explants/9 cm Petri dish), under culture conditions 2. The cut surface of the scalp is pushed into the culture medium, ensuring that the meristematic domes are not in direct contact with the culture medium.	Flowers from the 8th to 16th position are cultured on MA1 medium (Table 2 in Appendix 2) in baby food jars or 9 cm Petri dishes (Figure 13). The cultures are kept in total darkness under high humidity conditions (>70% RH) at 27°C. The cut surface is in contact with the medium. Containers are sealed with Parafilm® or household foil to avoid

Scalp method	*Immature flower method*
The containers are sealed with Parafilm® or household foil to avoid evaporation and to reduce contamination. This is of the outmost importance as scalps are kept on the same culture medium during the entire embryogenesis induction phase (3 to 8 months).	evaporation (a low relative humidity reduces the chances of success) and to reduce contamination. This is of the outmost importance as male flowers are left on the same culture medium during the entire embryogenesis induction phase (4 to 7 months).

The following sections are valid for both the scalp and the immature flower methods.

Callus formation

The cultures need to be checked monthly during the first 3 months and every two weeks thereafter. The process of embryogenesis induction depends on the cultivar and method used but in general the following phases can be distinguished.

• 0 to 4 weeks:

– dedifferentiation of leaf tissue into watery callus (*scalp method*);

– appearance of wound callus (*scalp and male flower methods*);

– the male flower explant curls up and becomes almost circular.

• Around 4 weeks:

– appearance of meristematic globules on the explant (most of the surface is turning black, a positive sign indicating that not too much leaf tissue has been inoculated).

• From 6 weeks on:

– swelling of corm tissue (lower part of the scalp);

– development of secondary, non-embryogenic yellow nodular calli (Figure 14) not suitable for the initiation of a suspension;

– formation of heterogeneous globules;

– formation of compact (non-embryogenic) calli not suitable for the initiation of a suspension (Figure 15);

– development of embryogenic calli (EC) consisting of individual embryos (Figure 16) or of compact calli (CC) (Figure 17) not suitable for the initiation of a suspension. The highest frequency of EC is usually observed after 3 months of culture (*immature flower method*);

– formation of a friable embryogenic callus (called IC for "ideal callus") bearing many translucent proembryos (generally more than 10) (Figures 18 and 19). The callus is suitable for transfer to liquid medium. The highest frequency of IC are usually observed after 4 to 5 months of culture (*immature flower method*). See Escalant *et al*. 1994 and Grapin *et al*. 1996 for information on the histology of an IC.

• From 6 to 8 months on:

– globules and secondary calli turn brown.

• At the end of the culture period, three main developmental patterns are distinguished:

– no embryogenic response (50 to 100% of explants depending on the cultivar and method);

– embryogenic calli consisting of individual embryos (on average, 15% of inoculated male flowers);

– friable "ideal" embryogenic calli (on average, 0.8% of inoculated male flowers or 8% of male buds if 10 immature male flowers per male bud are inoculated).

See section 2, "Evaluation criteria", for more information on how to calculate the success rate of embryogenic callus formation.

Initiation of cell suspension

Selection of embryogenic callus

The success rate of initiating a good quality embryogenic cell suspension (ECS) depends on the quality and volume of the selected embryogenic calli, as determined by the presence of only a few developed embryos. Careful and regular observation of the IC callus is necessary to select embryogenic calli that are at equilibrium between the "right size" and the "right developmental phase".

Select tissue containing highly embryogenic calli and early stage, transparent proembryos. It is important to remove embryos at the cotyledonary stage, meristematic globules and compact calli.

Transfer the IC to liquid culture medium: liquid ZZ (ZZl, in Table 1 in Appendix 2) in the case of IC derived from scalps, or liquid MA2 (Table 2 in Appendix 2) in the case of IC derived from male flowers.

Different types of containers can be used as long as a minimum inoculum density is respected.

- Erlenmeyer flask: 1 embryogenic complex per flask, 3 to 6 ml of liquid ZZl culture medium per flask. Two-thirds of the bottom of the flask should be covered by the embryogenic complex.
- Multi-well plate (1 embryogenic callus per well, each well 3 cm in diameter, 6 to 8 ml of liquid MA2 per well).

Cover the container with aluminium foil, seal with Parafilm® and put on a rotary shaker (70 to 100 rpm) under standard conditions 2.

IC are friable and immediately fall apart in liquid medium.

Subculture and quality improvement

The goal is to improve the quality of the suspensions in order to obtain homogeneous ECSs. An inverted microscope is used for observation. When enough material is available, a sample can be taken for observation under a light microscope. All non-desired components are removed by using a pipette.

0 to 3 months after initiation

Newly established ECSs (Figure 20) are composed of:

- embryogenic cell aggregates;
- heterogeneous globules releasing embryogenic cell clusters at their surface;
- distinct small, transparent embryos producing embryogenic cells near the base (Figure 21);
- empty yellowish meristematic globules and/or dense cells rich in starch (Figure 22);
- whitish embryos at the cotyledonary stage dedifferentiating into meristematic globules and/or releasing phenolic compounds that oxidize, causing blackening of the cell suspension.

Refresh part of the culture medium every 7 to 10 days. Depending on the growth rate of the ECS, keep 10 to 20% of the old preconditioned medium.

Remove the yellowish meristematic globules, the whitish embryos at the cotyledonary stage, the necrotic tissue and the highly vacuolated cells.

Every month, transfer a sample to a bacteriological medium (Van den Houwe 1998).

Transfer the ECS to a bigger container depending on the growth rate of the settled cell volume of the suspension.

3 months after initiation until the establishment of the cell suspension (6^{th} to 9^{th} month)

A 3-month-old ECS is composed of:

- proliferating embryogenic cell aggregates;

- whitish somatic proembryos resulting from the differentiation of embryogenic cells;
- yellowish meristematic globules resulting from the conversion of heterogeneous globules;
- whitish isolated dense cells released by meristematic globules;
- highly vacuolated cells released by meristematic globules.

To test the viability of the ECS, add a few drops of fluorescein diacetate (FDA) stock (-20°C, dissolved in acetone-water) to distilled water until a blue shine is observed. Add 1 to 2 drops of this diluted stock to a suspension sample. Viable tissues fluorescence brightly green when observed under ultra-violet light.

When the volume is sufficient, spreading a very small quantity of cells on the regeneration medium is a quick and simple way of determining the embryogenic character of the suspension.

Refresh part of the culture medium every 10 to 14 days. Depending on the growth rate of the ECS, keep 10 to 20% of the old preconditioned medium.

The settled cell volume at the onset of a subculture should be 1.5 to 3%. Use a larger Erlenmeyer flask or divide the contents between Erlenmeyer flasks if necessary.

Remove meristematic globules and proembryos when they are present in high amounts. Remove large cell clusters by sieving, retaining the fraction between 250 to 500 µm.

Every month, transfer a sample to a bacteriological medium.

Maintenance of cell suspension

Although it is relatively easy to obtain the multiplication of banana cells in liquid medium, the resulting suspensions can have varying regenerating capacities. A detailed histological description of the various parts of an ECS, of their development over time and their embryogenic potential can be found in Georget *et al*. 2000.

A good quality ECS is characterized by:

- the presence of a high proportion (>80%) of proliferating embryogenic cell aggregates (Figure 23);
- a colour generally ranging from bright to light yellow (pale white suspensions are not desirable as this is often an indication of the presence of a high proportion of nonregenerable cells rich in starch);
- a quick (within 1 minute) precipitation of cells when the suspension is removed from the orbital shaker, indicating a high density of cellular contents;

- a viability of embryogenic cell clusters that is over 80% according to the FDA test;
- a multiplication ratio between 1.5 to 2 per two-week subculture period;
- a high regeneration capacity, i.e. 100 to over 300 000 embryos per ml of settled cells.

Regularly screen for contamination and check the regeneration capacity (see section on plant regeneration below) and growth rate. For the latter, the following parameters can be considered:

- settled cell volume (SCV) (precipitation by gravity forces);
- packed cell volume (PCV) (precipitation by centrifugation);
- fresh and dry weight.

The quality of an ECS decreases with the number of subcultures. This results in an increased probability of contamination and a decreased growth rate and regeneration capacity, due for example to fast-growing dense cells rich in starch taking over (Georget *et al*. 2000). A direct relationship between time spent in culture and somaclonal variation is also expected. To reduce the problems related to subculturing, a cryopreservation protocol was developed, making it possible to store ECSs for unlimited periods (Panis and Thinh 2001).

Plant regeneration

A good quality ECS easily regenerates into somatic embryos and, subsequently, into plants.

Development of embryos

At the onset of a subculture period:

- transfer a representative sample of ECS to a graduated tube and adjust the SCV to 3% by adding liquid maintenance medium: ZZl (*scalp method*) or MA2 (*immature flower method*) (Figure 24);
- transfer 1 ml of this solution onto a Whatman filter paper in a 90 mm Petri dish containing 25 ml of regeneration medium: RD1 (*scalp method*) or MA3 (*immature flower method*);
- incubate under standard conditions 2.

One to 3 months after initiation, depending on the ECS, the developing embryos should look as in Figure 25.

Germination of embryos

Transfer a sample of mature embryos (3 to 4 months after plating) to a Petri dish containing 25 ml of germination medium: RD2 (*scalp method*) or M4

(*immature flower method*). Incubate under standard conditions for 1.5 months to obtain germinated embryos (Figure 26).

Regeneration into plantlets

Transfer the germinated embryos to P6 medium. Incubate under standard conditions for 1 to 1.5 month. At this stage, the plantlets look like those obtained using the classical *in vitro* method (Figure 27).

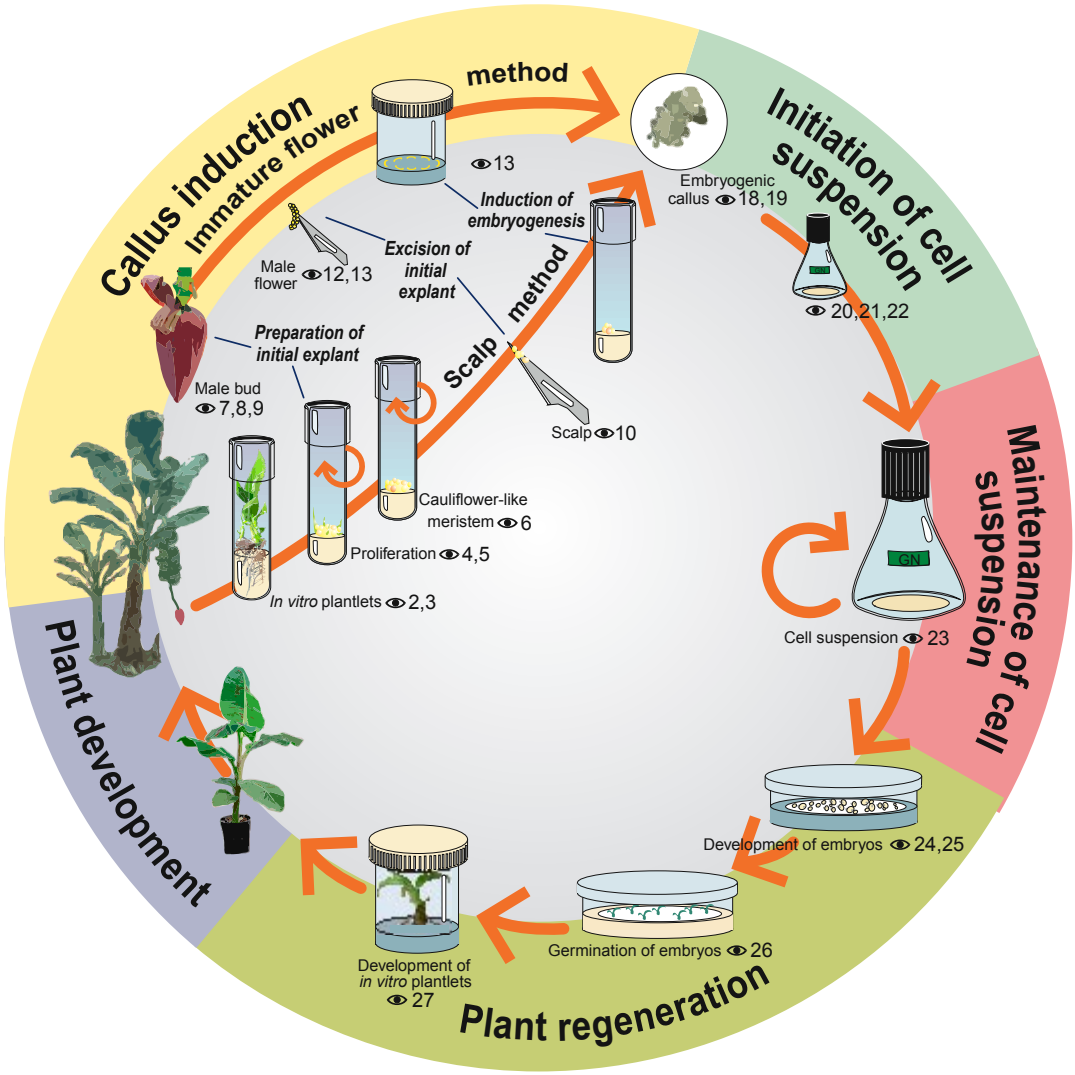

Figure 1. Steps of the two main methods used to produce banana and plantain embryogenic cell suspensions.

Refers to the figures.

The scalp method

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Figure 2. Rooted *in vitro* plantlet.

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Figure 3. Initial explant.

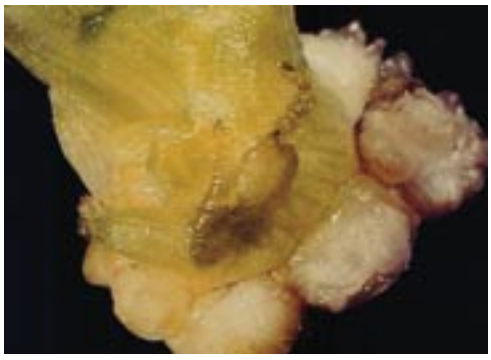

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Figure 4. Cultivar with a high proliferating capacity (one month after inoculation).

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Figure 5. Cultivar with a moderate to low proliferating capacity (1-3 months after inoculation).

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Figure 6. Meristem culture.

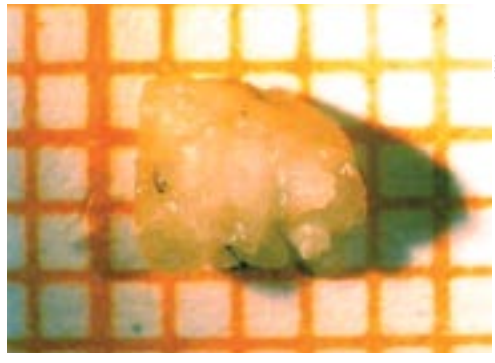

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Figure 7. Scalp excised from meristem culture.

The male flower method


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Figure 8. Male buds.


Régis Domergue, Cirad

Figure 9. Preparation of initial explant.


Régis Domergue, Cirad

Figure 10. Surface sterilization.

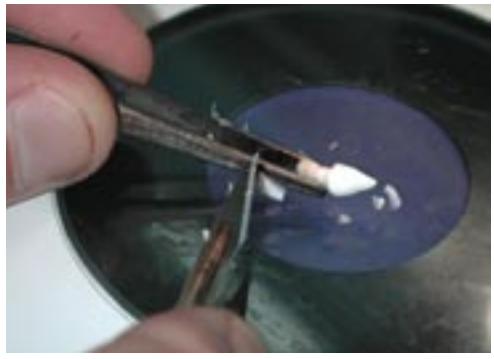
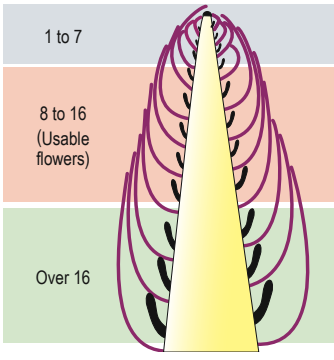

Régis Domergue, Cirad

Figure 11. Isolation of initial explant.



Régis Domergue, Cirad

Figure 12. Disposition of flowers in the male bud.


Régis Domergue, Cirad

Figure 13. Inoculation of male flowers.

Callus

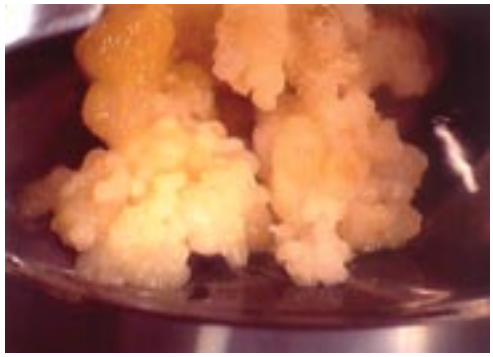

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Figure 14. Non-embryogenic yellow nodular callus.


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Figure 15. Compact (non-embryogenic) callus.

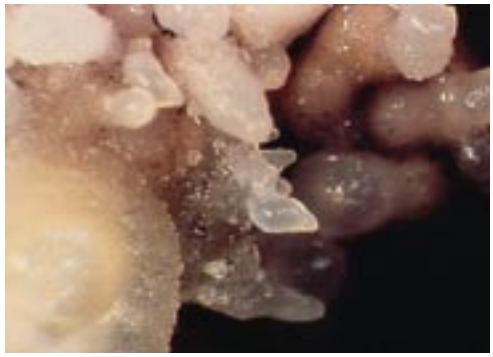
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Figure 16. Individual embryos.

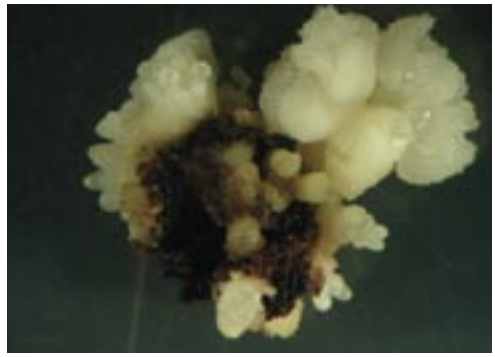
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Figure 17. Embryos and compact callus.

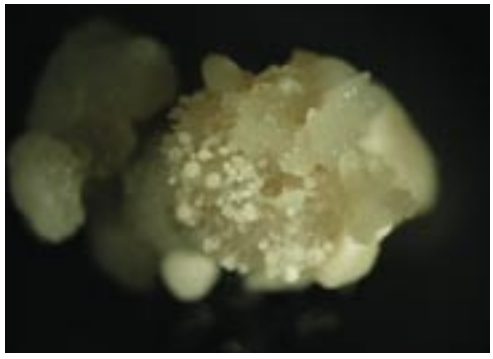
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Figure 18. Ideal callus.


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Figure 19. Ideal callus with translucent proembryos.

Cell suspension and regeneration

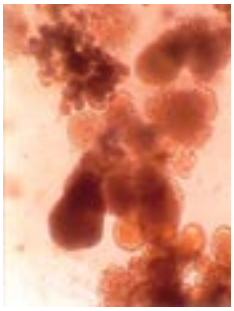
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Figure 20. Newly established ECS.

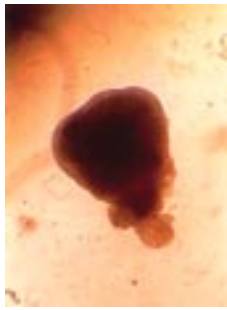
KULeuven

Figure 21. Transparent embryo.

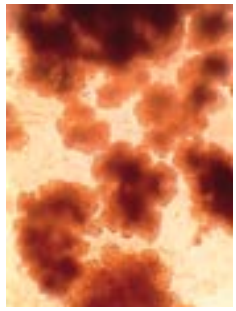
KULeuven

Figure 22. Yellowish meristametic globules.

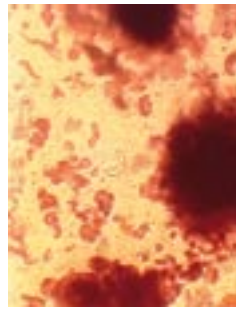
KULeuven

Figure 23. Proliferating embryogenic cell aggregates.

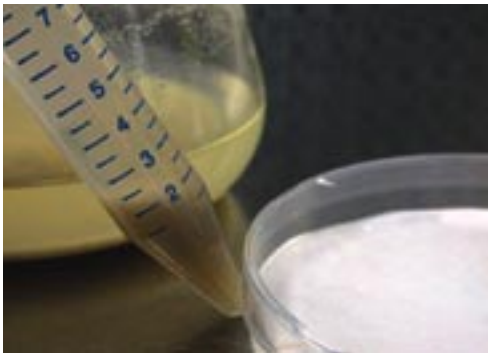
Régis Domergue, Cirad

Figure 24. Subculture of ECS.

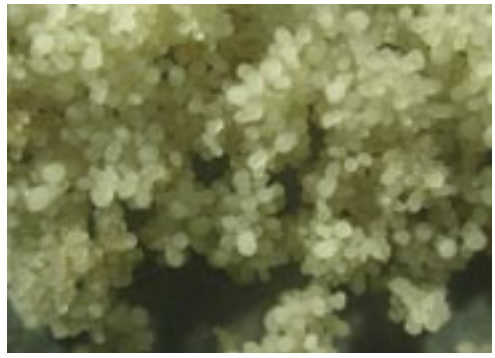
Régis Domergue, Cirad

Figure 25. Developing embryos.


Régis Domergue, Cirad

Figure 26. Germinating embryos.


Régis Domergue, Cirad

Figure 27. Regenerated plantlets.

2. Evaluation criteria

Common criteria are needed to evaluate the performance of the technique used and to facilitate the exchange of information between teams working on banana somatic embryogenesis. The following are a selection of qualitative and quantitative indicators that cover the main steps.

Ideal callus formation

Scalp method

% of IC = number of IC/number of inoculated scalps

Immature flower method

% of IC = number of IC/number of inoculated male buds

The value obtained for 'Grande naine' is generally between 3 to 10%, using the scalp method, and 8% on average, using the immature flower method. However the values for the two methods are not comparable since it is the immature flowers in the male bud that are inoculated, not the male bud itself.

Embryogenic cell suspension establishment

% of ECS initiated = number of ECSs/number of IC placed in liquid medium

This percentage is between 10 and 30% with 'Grande naine' (Domergue and colleagues unpublished results).

A good quality ECS is formed of homogenous cellular aggregates. This homogeneity is observed after many months of culture (generally 6 to 9 months). An ECS can be characterized by the monthly increase in the settled cell volume, which is usually between 1.5 and 4. Once established, an ECS can keep on multiplying for months, even years.

Embryo formation

The number of embryos obtained per volume of plated cells is the key criteria by which to evaluate the quality of a suspension. For example, 1 ml of settled cells can yield between 100 to 300 000 embryos (Grapin *et al*. 1996, Côte *et al*. 1996, Strosse *et al*. in press).

Success rate of embryo formation = number of embryos/ml of plated cells

Regeneration capacity

The success of germination is often used to describe the regeneration process. Values up to 80% have been reported by different teams. Beyond the variability that is explained by the use of different methodologies, the range of results

observed probably also reflects the difficulty of estimating the number of embryos. We suggest the following criteria.

% of germination = number of plantlets obtained/number of embryos put on germination medium

Another way of evaluating the performance of the regeneration process is to calculate the regeneration capacity.

Regeneration capacity = number of vitroplants produced/ml of plated cells

With 'Grande naine' values in the order of 35 000 plantlets/ml of plated cells have been observed (Georget and colleagues unpublished data, Strosse *et al*. in press).

In order to determine the regeneration capacity of a suspension, a quantitative assay is necessary. The following method is based on the weight and number of germinating embryos and plants from representative samples.

- Determine the total weight of the regenerated cell culture, i.e. the weight of embryos (be careful to conserve only the embryos).
- Pick three representative samples of the cell culture, weigh them and count the number of embryos in each one.
- Take another set of three representative samples, weigh them and transfer each one to a test tube (*scalp method*) or a 90 mm Petri dish (*immature flower method*) containing germination medium: 12 ml of RD2 (*scalp method*) or 25 ml of M4 (*immature flower method*).
- Incubate under standard conditions.
- 1.5 months after initiation of germination:

– determine the average number of green shoots;

– transfer the green shoots to MS rooting medium (approximately 3 ml per plant);

– incubate under standard conditions.

- 1.5 to 2 months after initiation of rooting on MS:

– determine average amount of rooted shoots.

These data are used to determine the total number of embryos or rooted shoots obtained from a specific quantity of cell suspension. For example, the number of plants regenerated per ml of settled cells of a Cavendish ECS can be more than 10 000. A mean of 35 000 rooted plantlets/ml of plated cells has been observed in large scale experiments (Georget and colleagues unpublished data).

A list of cultivars on which the scalp method or the immature flower method yielded positive results is given in Appendix 3. The results come from the authors' labs.

3. Limitations of somatic embryogenesis

Although somatic embryogenesis in banana is well established and standard techniques are available, the initiation of an ECS cannot still be considered a routine procedure (Schoofs *et al.* 1999). This is mainly because of the low embryogenic response of banana tissues, the long time needed to obtain an embryogenic cell suspension, the risk of somaclonal variation and the occurrence of contamination.

The main problem in using edible (and thus seedless) bananas is that the embryogenic callus needs to be initiated from differentiated tissues and not, as is the case with most monocotyledons, from generative tissues such as zygotic embryos. As a consequence, hundreds of explants (flowers or scalps) need to be initiated to obtain one good quality embryogenic callus. Moreover, the time needed between the inoculation of the explant on the induction medium and the establishment of a good quality suspension is relatively long: between 7 and 14 months. In the case of the scalp method, a scalp preparation period of 3 to 14 months, depending on the cultivar, needs to be added.

Culture conditions

One important consequence of this low embryogenic response is that optimization of the culture medium is very difficult. An elaborate and labour-intensive experimental set-up is needed. This is probably the main reason why the ZZ and MA1 culture media used for the induction of somatic embryogenesis have not changed over the last decade, despite the need to optimize the media for certain cultivars.

The following are some suggestions to optimize somatic embryogenesis in bananas.

- Optimizing the starting material (size, osmotic state…).
- Changing the osmotic condition of the culture medium.
- Changing the concentration of the auxins that are responsible for the unorganised growth of the embryogenic cells.
- Adding embryogenesis-inducing compounds such as amino acids and polyamines.
- Changing the physiological conditions (pH, temperature…).
- Applying nurse (feeder) cultures.
- Setting up a procedure to obtain embryogenic calli based on the successive use of two culture medium (auxin choc).

Somatic embryogenesis, like most *in vitro* techniques, relies on experimental results. Fundamental research using molecular biology techniques may lead to advances in the methodology.

Somaclonal variation

Few studies have been published on the incidence of off-types among banana plants produced through somatic embryogenesis. In 'Grande naine', a number of plants derived from 4-month-old embryogenic cell suspensions were observed to be true-to-type and to have agronomic characteristics comparable to *in vitro* plantlets (Côte *et al*. 2000a). Similar findings were obtained with 'IRFA 903' plants derived from 7-month-old cell suspensions (Côte *et al*. 2000b). These data suggest that somatic embryogenesis can be used for genetic transformation given that a proportion of variants can be tolerated for that purpose.

It is not yet clear, however, whether somatic embryogenesis can be used to mass propagate banana plants. The available results were obtained in fairly young suspensions (4 to 7 months), but since only a small number of plants can be regenerated at present such suspensions, this technique cannot be used for multiplication on a large scale. Moreover, unpublished results indicate an increase in somaclonal variation as culture time is increased. Proportions varying between 15 and 100% of somaclonal variants have been observed in 'Grande naine' after 15 months of culture (Côte and colleagues, unpublished data).

Many teams are looking for molecular markers of somaclonal variation. Such markers would help understand how somaclonal variation is generated and identify the factors that influence its development. Data using flow cytometry would also help better understand how somatic embryogenesis techniques generate somaclonal variants (Roux *et al*. in press).

The concern for somaclonal variation also extends to cryopreservation, which is used to conserve embryogenic cell suspensions. The true-to-typeness of plants recovered from cryopreserved embryogenic cell suspensions of banana was screened by Côte *et al.* (2000b). They observed no difference between the agronomic performance of plants regenerated from cryopreserved ECS and control plants. A cryopreserved ECS also needs to retain its characteristics (like its transformation capacity). Recently, comparable levels of transient expression and stable transformation were observed using ECS of bananas that had been cryopreserved and ECS that had not been cryopreserved (Panis *et al*. in press).

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Appendix 1. Acronyms

2,4-D: 2,4-dichlorophenoxyacetic acid

BA, BAP: 6-benzylaminopurine

CC: compact callus

EC: embryogenic callus bearing only few embryos

ECS: embryogenic cell suspension

FDA: fluorescein diacetate

IAA: indoleacetic acid

IC: ideal callus suitable for ECS initiation

MA1: embryogenic callus induction medium for immature flower method

MA2: cell suspension culture medium

MA3: embryo development medium

MA4: embryo germination medium

PCV: packed cell volume

P6: MS medium supplemented with 1 µM IAA and 1 µM BAP

P5: MS medium supplemented with 1 µM IAA and 10 µM BAP

P4: MS medium supplemented with 1 µM IAA and 100 µM BAP

RD1: ZZss supplemented with 100 µg/L myo-inositol and devoid of plant growth regulators

RD2: RD1 supplemented with 1 µM BAP

RH: relative humidity

SCV: settled cell volume

ZZl: liquid half-strength MS medium supplemented with 5 µM 2,4-D and 1 µM zeatine

ZZss: ZZl solidified with 3 g/L of Gelrite

Appendix 2. Culture media

Table 1. Composition of culture media used in somatic embryogenesis of *Musa* spp. (*scalp method*)

	P6	P5	P4	ZZss	ZZI	RD1	RD2
Macro-elements	MS	MS	MS	½ MS	½ MS	½ MS	½ MS
Micro-elements	MS	MS	MS	MS	MS	MS	MS
Vitamins	MS	MS	MS	MS	MS	MS	MS
Ascorbic acid (mg/L)	10	10	10	10	10	10	10
Myo-inositol (mg/L)						100	100
IAA (mg/L)	0.175	0.175	0.175				
BAP (mg/L)	0.227	2.273	22.73				0.227
2,4-D (mg/L)				1	1		
Zeatin (mg/L)				0.219	0.219		
Sucrose (g/L)	30	30	30	30	30	30	30
Gelling agent (Gelrite) (g/L)	3	3	3	3		3	3
pH	5.8	5.8	5.8	5.8	5.8	5.8	5.8

Table 2. Composition of the MA culture media (in reference to Dr Ma's research) that are used in somatic embryogenesis of *Musa* spp. (*immature flower method*)

	MA1 Callogenesis	MA2 Multiplication	MA3 Regeneration	MA4 Germination
Macro-elements	MS	MS	SH	MS
Micro-elements (except iron)	MS	MS	SH	MS
FeEDTA	+	+	+	+
Vitamins*	MA	MA	MA	Morel
IAA (mg/L)	1			2
2,4-D (mg/L)	4	1		
NAA (mg/L)	1		0.2	
Zeatin (mg/L)**			0.05	
2iP (mg/L)			0.2	
Kinetin (mg/L)			0.1	
BAP (mg/L)				0.5
Saccharose (mg/L)	30	45	45	30
Lactose (g/L)			10	
Amino acids (mg/L)	Glutamine 100 g/L	Glutamine 100 g/L	Glutamine 100 g/L Proline 230 g/L	
Malt extract (mg/L)	100	100	100	
Gelling agent (g/L)	Agar Type II 7 g/L		Phytagel 4 g/L	Phytagel 3 g/L
pH	5.7	5.3	5.8	5.8

* See tables 3 and 4

** Filter (0.2 µm), autoclave and add when the temperature of the medium has come down to 50˚C

Table 3. Composition of Ma vitamins (Ma 1991)

	Concentration (mg/L)
Biotine	1
Glycine	2
Myo-inositol	100
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1

Table 4. Composition of Morel vitamins (Morel and Wetmore 1951)

	Concentration (mg/L)
Biotine	0.01
Calcium panthotenate	1
Myo-inositol	100
Nicotinic Acid	1
Pyridoxine HCl	1
Thiamine HCl	1

Appendix 3. Lists of cultivars for which embryogenic calli or embryogenic cell suspensions have been obtained

Table 5. Cultivars for which embryogenic calli (IC) or embryogenic cell suspensions (ECS) have been obtained using the immature flower method

Cultivar	Genetic group	IC	ECS	Reference
Col. 49	AA	X	X	Grapin *et al.* 1998
SF 265	AA	X	X	Grapin *et al.* 1998
IRFA 903	AA	X	X	Côte *et al.* 2000b
Grande naine	AAA	X	X	Escalant *et al.* 1996, Côte *et al.* 1996
Gros Michel	AAA	X	X	Grapin *et al.* 1998
Yangambi km 5	AAA	X	Not tested	Grapin *et al.* 1998
French sombre	AAB	X	X	Grapin *et al.* 1996
Dominico	AAB	X	X	Grapin *et al.* 1998
Mysore	AAB	X	Not tested	Grapin *et al.* 1998
Silk	AAB	X	Not tested	Grapin *et al.* 1998
Curare	AAB	X*	X	Grapin *et al.* 2000
Curare enano	AAB	X*	X	Grapin *et al.* 2000
FHIA-01	AAAB	X		Grapin *et al.* 1998
FHIA-02	AAAB	X		Grapin *et al.* 1998

* Female flower

Table 6. Cultivars for which embryogenic calli (EC/IC) or embryogenic cell suspensions (ECS) have been obtained using the scalp method

Cultivar	Genetic group	EC	IC	ECS	Reference
Calcutta 4	AA	X			Not published
Kamaramasenge	AB	X	X	X	Schoofs 1997
Kisubi	AB	X			Schoofs 1997
Musa balbisiana 'tani'	BB	X			Schoofs 1997
Grande naine	AAA	X	X	X	Schoofs 1997, Strosse *et al.* in press
Highgate	AAA	X			Schoofs 1997
Williams	AAA	X	X	X	Schoofs 1997, Strosse *et al.* in press
Igitsiri	AAAh	X			Schoofs 1997
Agbagba	AAB	X		X	Strosse *et al.* in press
Bise egome	AAB	X	X	X	Schoofs 1997
Lady finger	AAB	X			Schoofs 1997
Prata	AAB	X			Schoofs 1997
Orishele	AAB	X	X	X	Strosse *et al.* in press
Three hand planty	AAB	X	X	X	Schoofs 1997
Bluggoe	ABB	X	X	X	Dhed'a 1991
Cardaba	ABB	X			Dhed'a 1992
Saba	ABB	X		X	Dhed'a 1992
Obino l'ewai	AAB	X		X	INIBAP 2000

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