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## Micro-Propagation Efficiency in Banana (*Musa sp.*) under Different Immersion Systems

Ikram-ul-Haq and Muhammad Umar Dahot  
Institute of Biotechnology and Genetic Engineering (IBGE),  
University of Sindh, Jamshoro, Pakistan

**Abstract:** The establishment of a micro-propagation protocol for banana (*Musa sp.*) cv Basrai, was carried out by using meristematic stem cuttings, as an explant. Upto 60% micro-propagation efficiency was increased, when organogenesis (MS; 10.0  $\mu\text{M}$  BA; 15.0  $\mu\text{M}$  IAA) was carried out on medium solidified with 3.60  $\text{g L}^{-1}$  phytagel for 3 weeks, while shoot induction and its multiplication (MS; 10.0  $\mu\text{M}$  BA) were obtained on the medium with 1.0  $\text{g L}^{-1}$  phytagel for 10 days and 2.0  $\text{g L}^{-1}$  phytagel for 20 days, respectively. One culture (20.0  $\mu\text{M}$  BA and 4.0  $\mu\text{M}$  NAA or/and 6.0  $\mu\text{M}$  TDZ) somewhat callus formation was observed but later on was proceeded to death, instead of multiplication. The developed plantlets were cultured on MS basal medium supplemented with IBA (0.50  $\text{mg L}^{-1}$ ) for root induction. Through this method, complete micro-propagated plantlets were obtained within 3 months.

**Key words:** *In vitro*, *Musa sp.*, basrai, micro-propagation, l-cystein, medium solidification, meristematic tip culture

### INTRODUCTION

In Pakistan, Banana is one of the most important cash crops with annual production ~102 million tons per year (FAO, 2002). It is rich with 25% of carbohydrate (Vuylsteke and Ortiz, 1996). Cultivated banana (3n) derived from two diploid species of the genus *Musa*, *M. acuminata* (Malaysia) and *M. balbisiana* (India) diploid (2n) parent genomes (Stover and Simmonds, 1987; Simmonds, 1962; Georget *et al.*, 2000). However, expansion of banana production is limited by shortage of plant material. The transmission of harmful insects, nematodes and viral disease by field-grown suckers has prompted interest in the use of aseptic culture techniques.

High production costs generally limit the commercial use of *in vitro* micro-propagation because of its low efficiency. However, by using liquid medium is considered to be the ideal solution for automation and reducing production costs. Meanwhile, the use of liquid media can be responsible for other problems such as asphyxia, hyper-hydricity and the need for more complex equipment (Etienne and Berthouly, 2002).

The growth medium is designed to induce rapid shoot multiplication (Hamill *et al.*, 1993; Hwang *et al.*, 1984; Schenk and Hildebrandt, 1972; Boxus *et al.*, 1991; Vuylsteke and De Langhe, 1985). This results in several

clones of the original plant being produced over a short period of time. The growth medium provides all the requirements for growth: inorganic and organic salts (macronutrients, micronutrients and vitamins); moisture; a support matrix and sugar (Jambhale *et al.*, 2001; Arias, 1992). By changing the amounts and types of growth regulators in the medium, the cells can be stimulated to develop into shoots and/or roots or even may die, if we change the medium solidification, the micro-propagation efficiency also altered (Escalona *et al.*, 1999; Alvard *et al.*, 1993).

Through banana micro-propagation we may be able to get plantlets free from insects, bacteria and other microorganisms (Krikorian and Cronauer, 1984; Ma and Shii, 1972; Vuylsteke, 1998). With the correct diet of nutrients the tissues grow into plantlets that multiply indefinitely. Propagation is highly efficient, allowing a large turnover of plants in a very short time within a very little space. The process also produces genetically uniform plants. It is the only way to produce pathogen free clones from the infected plants.

The present research, suggests a rapid banana (cv. Basrai) multiplication protocol from meristematic stem tips, by using a medium with optimized concentration of the auxins/cytokinins either through temporary immersion system or permanent immersion system. Present findings

may be helpful for the establishment of banana micro propagation laboratory to produce its clean clones. It may be of great valuable for the future research studies.

### MATERIALS AND METHODS

Four newly young banana (*Musa* sp.) cv. Basrai plants were selected to use as an experimental material, in the open-field conditions from Vice Chancellor's house, University of Sindh, Jomshoro, Pakistan.

Meristematic stem tips of banana (*Musa* sp.) cv., Basrai were excised from young suckers, which were used as an explant, surface disinfected by washing with ethanol (90%) for 1 min and then stirred in 10% commercial Robin bleach (5.25% NaOCl) for 30 min afterwards, they were rinsed 3 times with sterile distilled water.

Shoot tips (3-4 mm) were isolated aseptically and cultured for organogenesis on MS [Murashige and Skoog, (1962) basal medium; B5 vitamins (Gamborg *et al.*, 1968); 3% sucrose; 3.60 g L<sup>-1</sup> phytigel] medium supplemented with 10.0 μM BA; 15.0 μM IAA; 30.0 mg L<sup>-1</sup> L-cystein for 3 weeks. After organogenesis, the explants were subcultured on the medium supplemented with only 10.0 μM BA and 30.0 mg L<sup>-1</sup> L-cystein (Table 1 and 2) for shoot induction (1.0 g L<sup>-1</sup> phytigel) and its multiplication (2.0 g L<sup>-1</sup> phytigel).

In these experiments, three different media with different solidification conditions i.e., 3.60 g L<sup>-1</sup> phytigel;

2.0 g L<sup>-1</sup> phytigel and 1.0 g L<sup>-1</sup> phytigel were used during organogenesis, shoot induction and than their multiplication (Table 2). All of these cultures were similar in minerals composition, the difference is only, solidification (Roels *et al.*, 2005) of the medium.

All cultures were supplemented with 30.0 mg L<sup>-1</sup> L-cystein, 30% sucrose and their pH was adjusted to 5.7-5.8 before autoclaving at 121°C for 15 min. Each culture was maintained at 25±2°C under the light conditions with intensity of ~2000 lx provided by growth chamber with 18/6 h photoperiod.

The established cultures on shoot induction medium were routinely transferred after every 3 weeks by subdividing bulky mass of suckers with a number of plantlets, into a number of micro cuttings, according to that, the number of plantlets per micro-propagated explant with a scalpel. These micro cuttings of the micropropagated clusters were sub-cultured 4 times on the same medium, than the cultures were refreshed by taking new explants.

Before each sub-culturing, at the micro-propagation stage, the number of shoots per explant (determined by counting the number of shoots/explant) and average shoot length (6 shoots were randomly chosen of ~5 mm in size from each micropropagated cluster by measuring the area between the starting point of the pseudostem and the point from where the last leaf emerged) were measured. The diameter of the pseudostem was also measured from

Table 1: Optimization of the micro-propagation efficiency in banana (*Musa* sp.) cv. Basrai under different hormonal combinations

| Medium abbreviations       | Hormonal combinations           |                           |                           |                           |                          |
|----------------------------|---------------------------------|---------------------------|---------------------------|---------------------------|--------------------------|
|                            | L-cystein (mg L <sup>-1</sup> ) | TDZ (μM L <sup>-1</sup> ) | IAA (μM L <sup>-1</sup> ) | NAA (μM L <sup>-1</sup> ) | BA (μM L <sup>-1</sup> ) |
| Control (MS <sub>0</sub> ) | 30.0                            | -                         | -                         | -                         | -                        |
| B                          | 30.0                            | -                         | -                         | -                         | 10.0                     |
| BN                         | 30.0                            | -                         | -                         | 6.0                       | 10.0                     |
| BNI                        | 30.0                            | -                         | 15.0                      | 6.0                       | 10.0                     |
| BNIT                       | 30.0                            | 6.0                       | 15.0                      | 6.0                       | 10.0                     |
| NIT                        | 30.0                            | 6.0                       | 15.0                      | 6.0                       | -                        |
| IT                         | 30.0                            | 6.0                       | 15.0                      | -                         | -                        |
| T                          | 30.0                            | 6.0                       | -                         | -                         | -                        |
| NI                         | 30.0                            | -                         | 15.0                      | 6.0                       | -                        |
| BI                         | 30.0                            | -                         | 15.0                      | -                         | 10.0                     |
| BT                         | 30.0                            | 6.0                       | -                         | -                         | 10.0                     |
| Shifting A                 | 30.0                            | -                         | -                         | -                         | 10.0                     |
| Shifting B                 | -                               | -                         | -                         | -                         | 10.0                     |

Table 2: Different culturing conditions, used during the optimization of micro-propagation efficiency in banana (*Musa* sp.) cv. Basrai

| Culturing systems              | Organogenesis                   | Shoot induction                 | Shoot multiplication            | Possible shifting |
|--------------------------------|---------------------------------|---------------------------------|---------------------------------|-------------------|
| Routinely used cultures (S)    | IAA (15.0 μM)                   | BA (10.0 μM)                    | BA (10.0 μM)                    | S to S            |
|                                | BA (10.0 μM)                    | 3.60 g L <sup>-1</sup> Phytigel | 3.60 g L <sup>-1</sup> Phytigel | S to T            |
|                                | 3.60 g L <sup>-1</sup> Phytigel | 10 days culture                 | 20 days culture                 | S to T            |
| Temporary immersion system (T) | 2.0 g L <sup>-1</sup> Phytigel  | 2.0 g L <sup>-1</sup> Phytigel  | 2.0 g L <sup>-1</sup> Phytigel  | T to T            |
|                                | Hormones as in i                | Hormones as in i                | Hormones as in i                | T to S; T to P    |
| Permanent immersion system (P) | 1.0 g L <sup>-1</sup> Phytigel  | 1.0 g L <sup>-1</sup> Phytigel  | 1.0 g L <sup>-1</sup> Phytigel  | P to P            |
|                                | Hormones as in i                | Hormones as in i                | Hormones as in i                | P to S; P to T    |
| Optimized protocol (Fig. 6)    | 3.60 g L <sup>-1</sup> Phytigel | 1.0 g L <sup>-1</sup> Phytigel  | 2.0 g L <sup>-1</sup> Phytigel  | (As in Fig. 6)    |
|                                | Hormones as in i                | Hormones as in i                | Hormones as in i                |                   |

its starting point after transversely cutting with scalpel. The root induction in the shoot cuttings was carried out by culturing onto MS basal medium supplemented with 0.50 mg L<sup>-1</sup> IBA.

**RESULTS**

In order to establish an efficient *in vitro* micro-propagation system for banana (*Musa sp.*) cv Basrai, fresh meristematic stem tip-cuttings were cultured on MS basal media supplemented with a number of combination of different cytokinins and auxins (Table 1) for organogenesis. Explants proliferation was measured after 3 weeks, in all the cultured explants. The maximum proliferated explant-mass was observed on Shifting A medium and minimum on T and IT (Table 1), while not any detectable proliferation was seen on MS control and BI media (Fig. 1a). The reason is that Shifting A explants were initially cultured on BI medium for week.

After 3 weeks of culture for organogenesis; all cultures were refreshed on the same media for shoot induction. Except Shift A, not any measurable change was observed on other cultures. After waiting for 4 weeks, all cultures were refreshed but the explants in which organogenesis was carried out through Shift A were cultured on each medium with an additional medium, which is without L-cystein (Shift B medium; Table 1).

The numbers of shoots were counted after 4 weeks before its sub-culturing on the same shoot induction media. Maximum numbers of shoots/plantlets were observed on Shifting A and B (Fig. 1b), however abnormal shoots were developed on B, BN, BNI On BI normal shoots were developed but can not survive for long terms. The shoots developed on Shifting B were less in number than Shifting A, there somewhat, culture blackening was observed, which is because of the phenolics oxidation.

After 5 weeks of culture on shoot induction medium, shoot height and pseudostem diameter of the developing plantlets were also measured (Fig. 1c and d). Both were observed maximum in BI medium. It showed a correlation among the media as which explants have developed high numbers of shoots both of them were decreased. The pseudostem diameter was observed maximum in the culture where plant height is high. With the decrease in plant height the pseudostem diameter was also decreased (Fig. 1c and d), but the numbers of shoots per explant were increased.

Well developed shoots of about 3 weeks old after their 4th sub-culturing were excised and cultured on half-strength MS basal medium supplemented with 0.50 mg L<sup>-1</sup> IBA. More than 90% of plantlets were rooted within

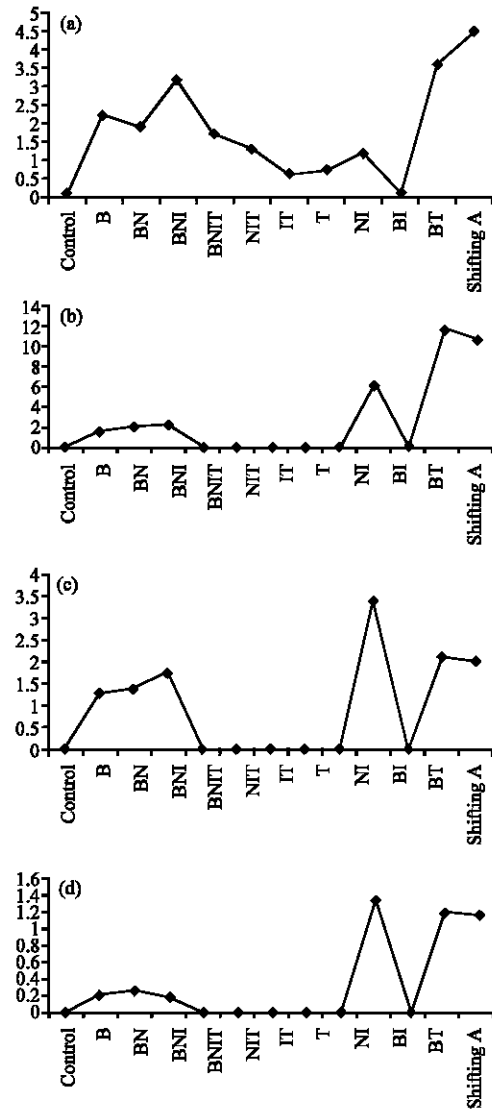


Fig. 1: Effect of different hormones and their combinations on different vegetative growth characteristics during micro-propagating banana (*Musa sp.*) cv. Basrai. a): The meristematic explants proliferation (g) 2 week culture, b): # of shoots per explant, 4 weeks culture, c): Shoot heights (cm), 4 weeks culture and d): Pseudostem diameter (cm) 4 weeks culture

4 weeks (Fig. 2d and e), then rooted plantlets were transferred to earthen pots (covered with a polythene bags for a few days to prevent wilting) containing soil, for plant hardening. After 2 weeks, all of them were established under greenhouse conditions.

To know, what an effect of the culture medium solidification (physical conditions) has on the micro-propagation efficiency in banana (Fig. 3 and 4), a clear cut

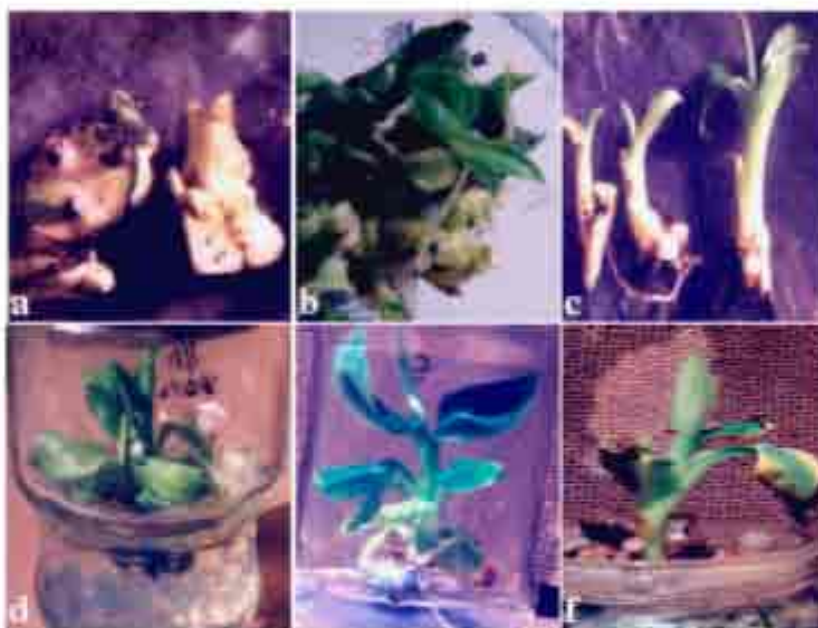


Fig. 2: Different steps during micro-propagation of banana (*Musa sp.*) cv. Basrai, a): Explant proliferation/organgogenesis on MS medium (BI), b): Shoot induction on Shifting A (Table 1), c): Microstem-cuttings, after 4 weeks of culturing on the shoot induction medium (shifting A), d): Micro-propagating, microstem cutting on the shoot induction medium (shifting A), e): Root induction in the micro-propagated plantlets on 1/2 MS basal medium supplemented with IBA ( $0.50 \text{ mg L}^{-1}$ ) and f): Establishment of banana plantlets in the soil, after plant hardening



Fig. 3: Effect of the medium-solidifying (phytagel) on the rate of growth and morphological behavior of the micro-propagating banana (*Musa sp.*) cv. Basrai plantlets. A): Shoot induction in microstem-cuttings on the medium supplemented with  $2.0 \text{ g L}^{-1}$  phytagel (temporary immersion system), b): Shoot induction on the medium solidified with  $3.60 \text{ g L}^{-1}$  phytagel (Routinely used tissue culture system), c): Micro-propagating plantlets on the routinely used tissue culture system and d): Micro-propagating plantlets on the temporary immersion system supplemented with phytagel  $2.0 \text{ g L}^{-1}$

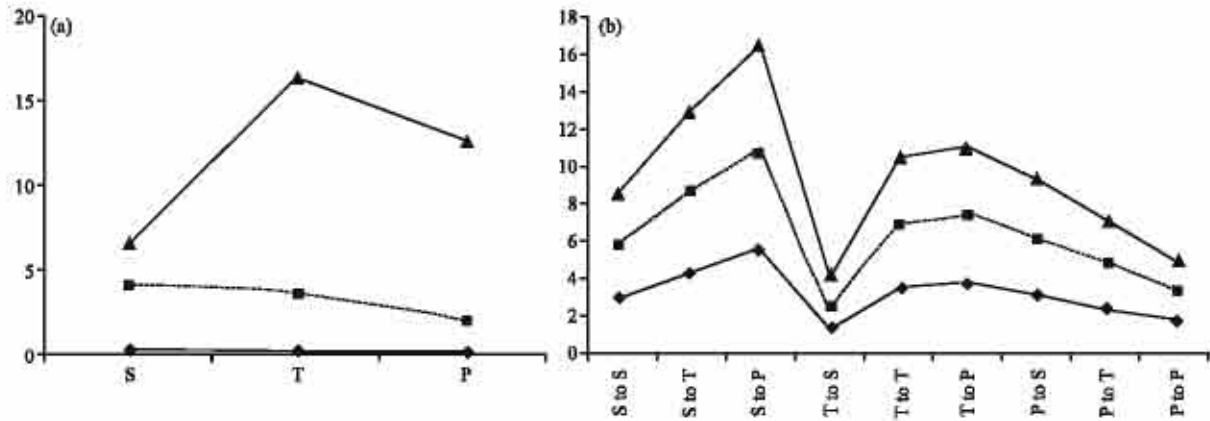


Fig. 4: Effect of different physical conditions (different amount of phytigel used for medium solidification) on different vegetative growth parameters of the banana (*Musa* AAA sp.) cv Basrai (4 weeks culture). Different banana growth parameters like # of shoots/explant (—▲—), plant height (cm) (—■—), pseudosem diameter (cm) (—◆—) were measured. a): The organogenesis/explant proliferation was carried out by culturing explants on Shift A medium (Fig. 1a), here difference is only the amount of phytigel used for medium solidification was different i.e., S: Solidified medium/routinely used tissue culture system ( $3.60 \text{ g L}^{-1}$ ); T: Temporary immersion system ( $2.0 \text{ g L}^{-1}$ ); P: Permanent immersion system ( $1.0 \text{ g L}^{-1}$ ). After 2 weeks from B.I medium the explants were cultured on the shoot induction medium B with different immersion systems and b): the developing plantlets were shifted from STP (Table 2) to each of their respective medium after 4 weeks culture



Fig. 5: Different modes of the shoot induction in micro-propagating banana (*Musa* sp.) cv. Basrai plantlets by culturing under different immersion systems. a): Organogenesis and shoot induction was carried out on the medium with  $3.60 \text{ g L}^{-1}$  phytigel, b): Shoot induction ( $2.0 \text{ g L}^{-1}$  phytigel) in the explant; in which the organogenesis was carried out on the routinely used tissue culture medium, c): Shoot induction ( $1.0 \text{ g L}^{-1}$  phytigel) in the explant, in which the organogenesis was also carried out on the same medium and d): Micro-propagated plantlets (after 4 weeks) on the temporary immersion system ( $2.0 \text{ g L}^{-1}$  phytigel) in which the organogenesis was carried out on routinely used tissue culture system ( $3.60 \text{ g L}^{-1}$  phytigel) for 2 weeks and than to the permanent immersion system ( $1.0 \text{ g L}^{-1}$  phytigel) for 10 days

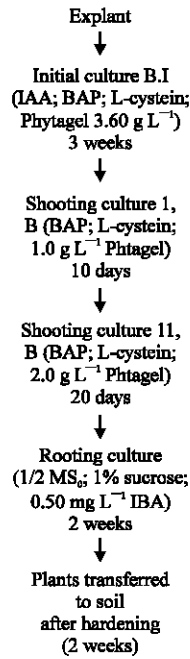


Fig. 6: A schematic representation of the optimized protocol for the micro-propagation of the banana (*Musa sp.*) cv. Basrai

difference was observed in both plantlets growth rate and their physical appearance. An experiment was conducted to develop a system with maximum micro-propagation rate. Initially 3 culture systems were established, by taking meristematic shoot cuttings as explants from the Shifting A (Fig. 2b and 1a). After 4 weeks, each explant was fresh weighed, number of shoots, shoot height and pseudostem diameter of the developing plantlets were measured (Fig. 4). Then they were sub-cultured on the same medium but difference is that the developing micro-stems on the routinely used tissue culture system (S) were also cultured on temporary immersion system (T) and the permanent immersion system (P). In the case of temporary and permanent immersion system were also cultured same as for routinely used tissue culture system, the maximum numbers of shoots with moderate plant height were observed on the cultures which were sub-cultured from routinely used tissue culture system to permanent immersion system and than to temporary immersion system (Fig. 4a and b). However, the maximum shoot height was observed on the medium subcultured from the solid tissue culture system to permanent immersion system with maximum pseudostem diameter (Fig. 4b).

The cultures that were developed from solidified to solidified and permanent to permanent immersion system showed many drawbacks, such as producing high levels of verifications, meristematic rhizome growth and many

abnormal buds that are not suitable for banana micro-propagation purpose (Fig. 5a and c) not such but somewhat low abnormalities were seen on the culture developed from solidified to the temporary immersion system (Fig. 1, 6 and Table 2).

In banana tissue culture, the goal is to produce a maximum number of shoots with long enough for the rooting under *in vitro* conditions. In this respect, the schematic represented protocol with different solidification and/or immersion system gave the overall best results among the different cultures and the system which, we have conducted in our lab. The findings are much consistent with the previous studies that have been conducted in various labs.

## DISCUSSION

Today, tissue culture is the basic need, for quite some time to develop rapid and surface growing pathogen free plants. However, raising plants from seeds (or spores) is the traditional means of production and reflects the sexual reproductive approaches, while vegetative propagation is the process of using a part of an existing plant (stem cutting, suckers, or other tissues) to grow into a new one under *in vitro* conditions. This method is effective for some plants, relatively easy, inexpensive and widely used. In banana, the most widespread used technique for vegetative propagation is reproduction by taking actively growing pieces of stems, called as micro-cuttings for *in vitro* micro-propagation under varying concentrations of different cytokinins and auxins (Arinaitwe *et al.*, 2000; Vuylsteke, 1998; Mendes *et al.*, 1999; Wojtania and Gabryszweska, 2001; Vuylsteke and Ortiz, 1996).

In order to establish, an efficient *in vitro* micro-propagation system for banana cv Basrai, fresh meristematic stem cuttings were considered as a best explant. An efficient micro-propagation was observed on B medium (Table 1 and Fig. 1) when organogenesis was carried on BI medium, where a non-prolific mass was observed (Daniells, 1997; Jambhale *et al.*, 2001; Kadota and Niimi, 2003; Hirimburegama and Gamage, 1997). The optimal combinations of auxins and cytokinins in the medium are perhaps the most critical factor for enhancing shoot multiplication rate in a particular explant. In the present study, the numbers of shoots per explant and shoot lengths also varied dramatically with the change/alteration in the cytokinin and auxin combinations (i.e., BA, IAA, NAA and TDZ) in the MS basal medium (Fig. 1). In the presence of BA (10.0  $\mu$ M), a moderate rate of shoot multiplication was obtained (Fig. 5d). In general, high BA levels enhance the number of shoots per

explants, but the shoot height decreased or even abnormal shoot buds were developed, which laterally not enable itself to develop into shoot.

Both shoot numbers and their heights are the results of the presence of specific combinations of the auxins and cytokinins (BA, IAA, NAA and TDZ) in the media while the addition of the NAA and TDZ among BA or separately lead to trigger the callus induction, not shoots multiplication which ultimately go to death. However, the shoot multiplication is stimulated by BA only, not through any other (Van den *et al.*, 1998; Victor *et al.*, 1999; Vuylsteke and De Langhe, 1985). However, the instance of the occurrence of normal plantlets with changed morphology (abnormal plant development among normal plantlets), which could be due to a continuous *in vitro* sub culturing on the same medium, such lakes can be eliminated either by refreshing the cultures from organogenesis to shoot induction (Fig. 6) or by taking new explants after every 3-5 sub-culturing.

The culture media series with different solidification and/or its liquefaction conditions are also effective to enhance the rate of micro-propagation and also physical appearance of the developing plantlets (Fig. 3 and 4). The temporary immersion system produced the tallest shoots than the other two systems, while the permanent immersion system is also similar when explants were culture from solidification and/or temporary systems. The abnormal characters are also possible, when the explants were sub-cultured on the same medium [S to S and P to P (Fig. 4b)] during and after organogenesis. Such abnormalities are not easy to install into normal (Vuylsteke, 1998; Matsumoto and Brandao, 2002; Daquinta *et al.*, 2000; Murch *et al.*, 2004). The abnormal buds are not suitable for banana micro-propagation purpose. From them, the normal tissues can be developed either by changing its medium composition or culturing times but objection is that such developed plantlets may be sterile in the field.

In banana tissue culture, the goal is to produce a maximum number of shoots with long enough for the rooting under *in vitro* conditions. In this respect, the schematic represented protocol with the solidification to permanent and/or temporary immersion systems gave the overall best results among other different cultures.

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#### ABBREVIATIONS

MS: Murashige and Skoog medium, TDZ: Thidiazuran, NAA:  $\alpha$ -Naphthalene acetic acid, IAA: Indole acetic acid, IBA: Indole butyric acid, BA: Benzyle aminopurine, L-cyst: L-cystein.

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