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Morpho-physiological Aspects of Micro-propagating Banana under Different Hormonal Conditions

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Abstract: An experiment was designed, to know what a physio-chemical situation developed in the micro-propagating banana variety Basrai on either organogenesis or shooting stage under specific hormonal conditions. *In vitro* plantlets were developed by culturing meristematic stem tips (explant) on organogenesis medium (8.0 μM BA; 10.0 μM IAA; 3.0 g L^{-1} phytigel) for 3 weeks, shoot induction medium (15.0 μM BA; 1.0 g L^{-1} phytigel) for 3 weeks and shoot multiplication medium (10.0 μM BA; 2.0 g L^{-1} phytigel) for 4 weeks. The micro-propagating plantlets and freshly excised explant from young banana plant from field were cultured on each of these 3 cultures separately. Variable morpho-biochemical contents were observed in each cultured tissue. On the organogenesis medium, reduction in Ca^{2+} , K^{+} , total protein and explant proliferation, fresh and dry weight, rate of micro-propagation but increase in Na^{+} , Cl^{-} and reducing sugar contents were measured, in comparison to both shooting cultures, where all of these were observed in a reversed form. So organogenesis resulted under stressed condition which is developed due to IAA and BA, later on relaxation leads into shoot induction, when only BA is present in the medium. Both steps i.e., organogenesis and shooting are directed due to the presence of specific hormones in the medium.

Key words: *In vitro*, *Musa* spp., basrai, micro-propagation, ionic contents, total proteins, reducing sugars, meristematic shoot tip culture

INTRODUCTION

In Pakistan, Banana is one of the most important cash crops with annual production ~ 102 million tons per year (FAO, 2002). However, the banana production is limited by a number of biotic and a-biotic constraints. Because of the shortage of normal and healthy plant material has prompted an interest for the use of aseptic culture techniques. Through which environmental disorders may be reduced and/eliminated from the desired crops.

Among the organics in the culture medium, hormones play a key role for developing a specific mode of growth in the cultured tissue which may be due to the accumulation of specific biochemical contents in them. The single or the combination of different hormones in the medium causes to maintain, specific and balanced inorganic and organic contents in the growing tissue. Under such specified situations, cells or tissues may be stimulated to develop either into shoots and/or roots or even their death may occur. Both organogenesis and shoot induction in the explant depends on the presence or absence of specific hormone individually or in a combination in the respective medium

(Reinhardt *et al.*, 2000; Dowries and Crowell, 1998; Jouve *et al.*, 1999; Georget *et al.*, 2000) in the plants.

An efficient micro-propagation means to develop a large numbers but normal and fertile plantlets, it is possible only when the concentration, type, time and combination of specific auxins and/or cytokinins are maintained, which are involved to trigger a specific mode of development. However, the rate of progress in a specific mode also depends on the physical conditions (concentration of the solidifying agent i.e., phytigel) of the growth medium. Each plant has a specific ability to absorb nutrients from the medium either when a tissue is developing on the culture medium either on organogenesis or shoot induction/multiplying stage. So each step of the micro-propagating plants is dependent on the nature of medium solidification (Alvard *et al.*, 1993; Escalona *et al.*, 1999). Both hormones and physical conditions of the medium are very important either for developing a specific mode of growth in cultured explant and others for shooting or its survival, during micro-propagation of the plantlets.

The present study, illustrate, what an effect of hormones on the accumulation of bio-chemical contents

in the micro-propagating banana either on organogenesis or shooting stage. At which medium solidification state, proper plant regeneration with normal and fertile plantlets is possible. The optimization of timing and amount of both of these factors has been carried out for the purpose to get an efficient protocol for plant multiplication. Such findings may be useful for the further improvement in the banana tissue culture in near future.

MATERIALS AND METHODS

An experiment was conducted during June, 2005-April, 2006 for the purpose to optimize micro-propagation efficiency in banana. So four young banana (*Musa* sp.) suckers of variety Basrai were selected from the greenhouse; Vice Chancellor's house, University of Sindh, Jomshoro, Pakistan, to use as experimental material.

Meristematic stem tips were excised and surface disinfected from micro-organisms by washing with ethanol (90%) for 1 min, than stirred with electric magnet stirrer in 30% commercial bleach (5.25% NaOCl) for 30 min. Afterwards, washed for 3 times (3×5 min) with sterile distilled water.

Aseptically isolated shoot tips (3-4 mm) were cultured on organogenesis medium on MS_{2a} (Murashige and Skoog, 1962) basal medium supplemented with B5 vitamins (Gamborg *et al.*, 1968) 3% sucrose; 3.60 g L⁻¹ phytigel], supplemented with 10 μM IAA and 8 μM BA (under dark conditions) for organogenesis. After 3 week, the explants were shifted to the shoot induction medium MS_{2b} (15 μM BA; 1.0 g L⁻¹ phytigel) for 3 weeks and its multiplication was carried out on the shoot multiplication medium MS_{2c} (10 μM BA; 2.0 g L⁻¹ phytigel; Table 1) for 4 weeks. The established cultures on shoot induction medium were routinely refreshed after every 4 week by subdividing the clustered mass of plantlets into a number of micro cuttings, according to that, the number of plantlets per explant with a sterile scalpel. These micro-cuttings of the micro-propagated plantlets were sub-

cultured for 4 times on the shooting culture II. The root induction was carried out by culturing onto ½ MS basal medium supplemented with 0.5 mg L⁻¹ IBA.

In these experiments three different media with different solidification conditions i.e., 3.60 g L⁻¹ phytigel; 2.0 g L⁻¹ phytigel and 1.0 g L⁻¹ phytigel were used during organogenesis, shoot induction and than their multiplication (Table 1). All of these cultures were similar in minerals composition, the difference is only, solidification of the medium.

All cultures were supplemented with 30.0 mg L⁻¹ cystein, 3.0% sucrose and their pH was adjusted to 5.7-5.8 before autoclaving (121°C and 20 lbs for 15 min). Each culture was maintained at 25±2°C with 18/6 h photoperiod (light intensity ~2000 lux).

After 4 week, the explants from each culture medium (Initial culture; Table 1 and Fig 1a) and micro-propagating plantlets (shooting culture II; Fig. 1c) were removed, washed with water, fresh weighed and dried in electric Owen at 72°C for 2-days till a constant dry weight was measured. Dried plant material was subjected to chemical analysis. Ion concentration (Na⁺, K⁺ and Ca²⁺) were measured as by Malavolta *et al.* (1989) and chloride contents were measured by Chloro-counter by following the instruction in the instrument operating manual (Marius Instrumenten, Utrecht and The Netherlands). Reducing sugar contents were analyzed according to Miller (1959) while total protein contents were also measured according to Lowry *et al.* (1951) method.

RESULTS

To establish an efficient *in vitro* micro-propagation system for banana (*Musa* sp.) variety Basrai, fresh meristematic shoot cuttings were cultured on MS basal medium supplemented with a combination of auxin and cytokinin (IAA and BA) for organogenesis (Fig. 1a). After 4 week, the organogenised explants from initial culture were cultured on shooting culture I (Fig. 1b), after 3 week

Table 1: Effect of phytigel on the micro-propagation efficiency in banana (*Musa*) sp. variety Basrai

Parameters	Routinely used cultures (S)	Temporary immersion system (T)	Permanent immersion system (P)	Optimized protocol
a. Organogenesis	10 μM IAA; 8.0 μM BA 3.60 g L ⁻¹ Phytigel 3 weeks culture	10 μM IAA; 8.0 μM BA 2.0 g L ⁻¹ Phytigel 3 weeks culture	10 μM IAA; 8.0 μM BA 1.0 g L ⁻¹ Phytigel 3 weeks culture	MS _{2a} :10 μM IAA; 8.0 μM BA 3.60 g L ⁻¹ Phytigel 3 weeks culture
b. Shoot induction	15 μM BA 3.60 g L ⁻¹ Phytigel 3 weeks culture	15 μM BA 2.0 g L ⁻¹ Phytigel 3 weeks culture	15 μM BA 1.0 g L ⁻¹ Phytigel 3 weeks culture	MS _{2b} :15 μM BA 1.0 g L ⁻¹ Phytigel 3 weeks culture
c. Shoot multiplication	10 μM BA 3.60 g L ⁻¹ Phytigel 4 weeks culture	10 μM BA 2.0 g L ⁻¹ Phytigel 4 weeks culture	10 μM BA 1.0 g L ⁻¹ Phytigel 4 weeks culture	MS _{2c} :10 μM BA 2.0 g L ⁻¹ Phytigel 4 weeks culture
a. # of shoots/explant	2.87	3.51	1.62	17.65
b. Shoot height (cm)	5.21	4.85	3.31	3.42
c. <i>Pseudostem</i> diameter (cm) 0.52	0.46	0.34	0.52	
d. Root induction (%)	70.25	30.82	-	89.90

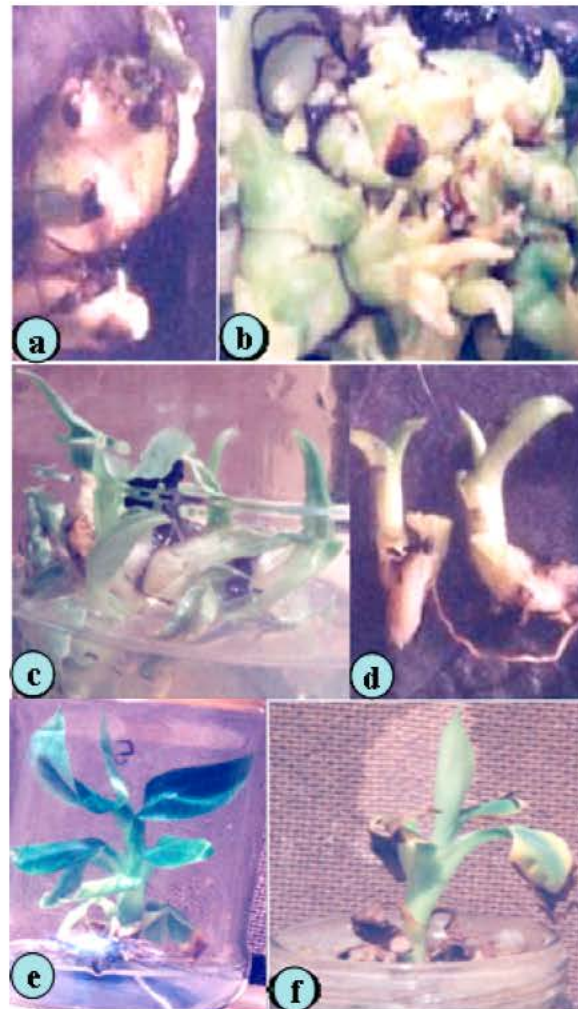


Fig. 1: Different steps involved in micro-propagation of banana (*Musa* sp.) variety Basrai. (a): explants on MS_{2a} medium for organogenesis (3 weeks); (b): Shoot induction on shoot induction (MS_{2b}) medium (after 3 weeks); (c): Multiplying shoots on shoot multiplication medium (MS_{2c}), after 4 weeks; (d): Meristematic micro-stem cuttings from shoot multiplication culture for its sub-culturing on the same medium and also used for the root induction; (e): A micro-propagated plantlet on root induction medium for 2 weeks; (f): A plant (after 1-week of its hardening), growing on soil in wire-house

were shifted to shooting culture II for 4 week, a numbers of normal plantlets were observed (Fig. 1c), however the abnormal plantlets were also developed when continued for sub-culturing after 4th sub-culturing on the shoot multiplication medium.

To know, what is the effect of medium solidification (physical conditions) state on the micro-propagation efficiency in banana. Meristematic stem tips (explants) were cultured for organogenesis (3 week), shoot induction (3 week) and shoot multiplication (4 week) on 4 different culture systems i.e., a, b, c and d (Table 1). After 4th week, the maximum numbers of shoots were observed on the

culture which was sub-cultured from routinely used culture system (S) to permanent immersion system and than to temporary immersion system (Table 1 and Fig. 1c). However, the maximum shoot height was observed on the medium which was sub-cultured from the temporary culture system (T) to routinely used culture system (S), where highest pseudostem diameter was also measured. Similar characters were also measured on routinely used culture, where the difference is only that the numbers of shoots per explant were very low.

The cultures that were developed from solidified to solidified and permanent to permanent immersion system

Table 2: Effect of different hormonal conditions on the morpho-chemical aspects of the micro-propagating banana (*Musa sp.*) variety Basrai

Parameters/characters	Organogenesis medium (MS _{2a}) (10 µM IAA; 8 µM BA; 3.60 g L ⁻¹ phytigel)	Shoot induction medium (MS _{2b}) (15 µM BA; 1.0 g L ⁻¹ phytigel)	Shoot multiplication medium (MS _{2c}) (10 µM BA; 2.0 g L ⁻¹ phytigel)	Cultured material
A				
a. Explant proliferation (g)	4.35±0.15	7.24±1.21	9.40±0.17	Explant excised from young banana suckers after 4 weeks
b. Explant dry wt (g)	1.75±0.25	3.35±1.50	3.35±0.15	
c. Plant height (cm)	-	-	-	
d. Fresh wt. of plantlets (g)	-	-	-	
e. Dry wt. of plantlets (g)	-	-	-	
f. # of plantlets/explant	-	-	-	
g. Total protein (mg g ⁻¹)	25.90±1.25	30.42±0.75	38.42±1.20	
h. Reducing sugars (mg g ⁻¹)	16.23±1.15	15.20±1.25	14.85±1.65	
i. Na ⁺ (g kg ⁻¹)	4.68±1.20	4.02±0.80	3.50±0.25	
j. K ⁺ (g kg ⁻¹)	4.35±0.80	4.76±1.28	5.30±0.60	
k. Cl ⁻ (g kg ⁻¹)	24.64±1.50	20.14±1.75	17.20±0.24	
l. Ca ²⁺ (g kg ⁻¹)	6.35±1.22	7.75±1.80	9.70±0.25	
B				
a. Plant height (cm)	5.21±1.16	3.05±2.20	3.41±1.20	Micro-propagating plantlets taken from shooting MS _{2c} cultured for 4 weeks
b. Fresh wt. of plantlets (g)	5.26±0.75	3.25±1.65	4.39±1.62	
c. Dry wt. of plantlets (g)	2.15±0.35	0.79±1.60	0.85±0.45	
d. # of plantlets/explant	0.30±1.06	6.35±1.75	7.20±1.14	
e. Total protein (mg g ⁻¹)	32.50±1.54	38.05±2.05	42.32±1.24	
f. Reducing sugars (mg g ⁻¹)	15.13±1.35	14.00±1.90	12.56±1.16	
g. Na ⁺ (g kg ⁻¹)	4.25±1.26	3.96±0.80	3.15±0.35	
h. K ⁺ (g kg ⁻¹)	4.37±0.65	5.37±1.26	6.65±0.75	
i. Cl ⁻ (g kg ⁻¹)	26.14±1.35	22.35±1.05	19.55±1.24	
j. Ca ⁺⁺ (g kg ⁻¹)	6.96±1.65	8.28±0.95	10.15±1.25	

showed many drawbacks, such as, producing high levels of verifications, meristematic rhizome growth and many abnormal buds that were not suitable for banana micro-propagation purpose. The maximum numbers of shoots with moderate shoot height were observed on optimized protocol culture i.e., d (Table 1) which were developed from the solidified (organogenesis) to permanent immersion system and then to the temporary immersion system (Table 1).

Well developing plantlets of about 3 weeks old after 4th sub-culturing on the shooting culture II were excised and cultured on half-strength MS basal medium supplemented with 0.5 mg L⁻¹ IBA, more than 90% of the plantlets, rooted within 2 week. Rooted plantlets were then transferred to pots containing soil (covered with a polythene bags for a few days to prevent wilting) for plant hardening, after 2 week, were established under greenhouse conditions.

For the purpose to know about the existing biochemical situation in the micro-propagating plantlets either at organogenesis medium and/or shooting media, for which two experiments, were conducted. A- he fresh explants, excised from young banana suckers (Fig. 1a and Table 2A) B-Micro-stem cutting (Fig. 1d; Table 2B) were cultured on organogenesis medium (MS_{2a}), shooting media (MS_{2b} and MS_{2b}) for 4 weeks. During organogenesis (MS_{2a}; Table 2A), explant proliferation, fresh weigh, dry weight and total protein contents were decreased while Na⁺, Cl⁻ and reducing sugars were increased in comparison to K⁺, Ca²⁺ contents.

All of these characters were appeared to be in a reversed in both shooting cultures (Table 2A).

Variant micro-propagation efficiency was observed, when micro-propagating plantlets were cultured on MS_{2a}, MS_{2b} and MS_{2c} media. Maximum numbers of plantlets per explant were observed on MS_{2c} medium while maximum plant heights were observed on MS_{2a} medium. During the experiment, a significant co-relation was observed between the shoot multiplication rate and plant heights, with the decrease in shoot height the number of plantlets/explant were increased and vice viscera (Table 2B).

Under the influence of different hormonal combinations and/or separately leads to a change in the organic and inorganic contents even in the micro-propagating plantlets, when cultured on the medium supplemented with various combinations of the hormones. Each medium with a specific hormonal condition develops a specific combination of the physio-chemical contents in cultured plantlets. So again both Na⁺ and Cl⁻ contents were increased under the influence of BA and IAA (MS_{2a}) than others (Table 2), while the K⁺ and Ca²⁺ contents were decreased. However in the presence of BA (MS_{2b} and MS_{2c}), each of which was observed as in a reversed form.

Similar phenomena were also observed for the organics like as total protein, reducing sugars and total sugar contents (Table 2A, B). Maximum reducing sugar contents were observed in the organogenesis medium, as this is a specific indicator of the stressed condition on the

explants. While total protein and sugar contents were observed maximum in the presence of BA (shooting media), which may be a cause for the shoot induction and its exponential multiplication in the organogenised explants. So a specific inorganic and organic combinations in the growing tissue, reflects the efficiency of the shoot induction and its multiplication in the micro-propagating plantlets.

DISCUSSION

The phytohormones regulate and integrate the overall growth, development and reproductions in plants. The morphogenesis devolves basically around the process of cell division, cell elongation and differentiation; hormones are involved to regulate all of them. Auxins are typically associated with cell elongation (Jensen *et al.*, 1998; Jouve *et al.*, 1999; Rayle and Cleland, 1992; Reinhardt *et al.*, 2000 and 2003) while auxin and cytokinin act synergistically to regulate the process of cell division (Dowries and Crowell, 1998; Hwang and Sheen, 2001). Depending upon, the ratio of auxins and cytokinins, the organogenesis; the mode of root and/or shoot induction in a cell and/or tissue are specified, while alteration results into altered signals. Such alterations in hormonal conditions develop a specific balanced ratio of the specific organics and inorganics in the growing tissues (Table 2). The balanced cell's chemical contents lead to trigger a phenomena 'cell polarity' in the meristematic regions of the cultured tissues. So fresh meristematic stem cuttings (explants) were cultured on MS basal medium supplemented with a combination of cytokinin (BA) and auxin (IAA) which causes to induce organogenesis (Initial culture) and no mass proliferation of the tissue, while only BA, due to which both mass proliferation and shooting occurs (Hwang *et al.*, 1984; Daniells, 1997; Hirimburegama and Gamage, 1997; Hutchison *et al.*, 1999; Jambhale *et al.*, 2001; Kadota and Niimi, 2003; Roels *et al.*, 2005). The numbers of shoots per explant and their heights varied dramatically in the presence of different cytokinin and auxin combinations in the MS basal medium (Van-den *et al.*, 1998; Victor *et al.*, 1999), so the induction of a specific mode of banana development is a hormonal specified step (Table 2).

The medium solidification state also have a key role in plant development, so the progress in a specific mode of development during banana micro-propagation is dependent on the physical conditions of the medium either semi-solidification and/or liquefaction. The explants on shoot induction (MS_{2b}) and shoot multiplication (MS_{2c}), during shooting stage in organogenised explant, the normal and an efficient multiplication was observed.

However, if both shoot induction and their multiplication was carried out on shooting induction medium only, the abnormal plantlets were observed. Such abnormalities are not easy to un-install (Vuylsteke, 1998; Cosgrove *et al.*, 2002; Daquinta *et al.*, 2002; Etienne and Berthouly, 2002; Matsumoto and Brandao, 2002; Noh *et al.*, 2003; Murch *et al.*, 2004; Arinozue and Maloof, 2006). 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 plants may be sterile in the field (Haq and Dahot, 2007a and b).

In conclusion, the presence of a specific hormone/s in the medium has a key role in switching the banana micro-propagation character while, the physical conditions of the medium (3.60, 2.0, 1.0 g L⁻¹ phytigel) are interconnected with the developmental stages of the micro-propagating plantlets. For normal growth, each stage require a proper medium solidification.

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