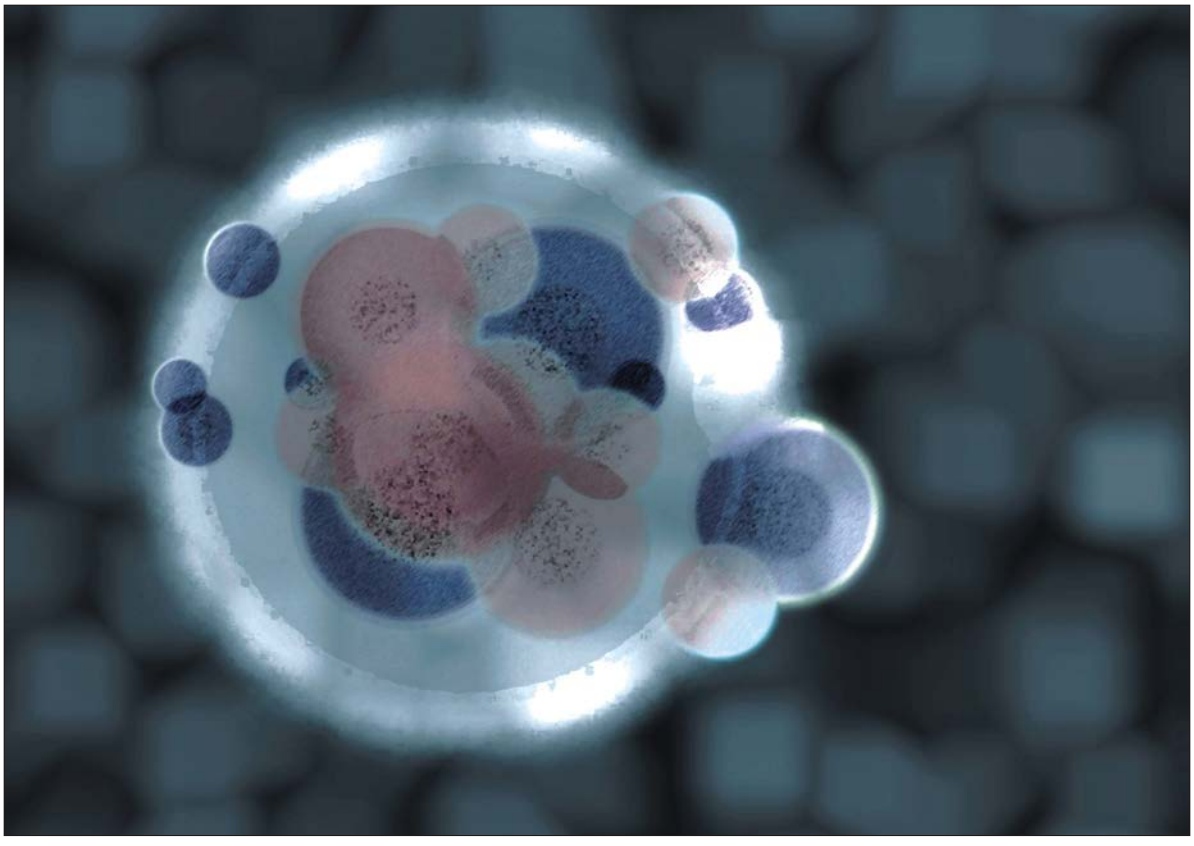

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Bio-Chemical Contents and Micro-Propagation Efficiency in Banana

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Abstract: The rate of banana micro-propagation depends on the balanced bio-chemical contents within the cultured explants, which are maintained by specific hormone/s in the medium. A normal mode of organogenesis and than shoot induction, are the hormonal derived steps for *in vitro* banana micro-propagation. To know about what a situation exist, under specific hormonal conditions, an experiment was designed. The meristematic stem tips were cultured for organogenesis on a number of media supplemented with NAA, BA and IAA separately and/ in a combination. After 2 weeks, the organogenised explants were sub-cultured on shoot induction medium, maximum plantlets (6.20±2.50) were observed on the medium supplemented with BA. Such efficient plant regeneration was occurred, when organogenesis was carried out on the medium supplemented with BA and IAA. Among these explants maximum Na^+, Cl•, while decrease in Ca^{2+}, K^+, reducing sugar, total sugars and total protein contents were observed. All of these phenomena were reversed in the presence of BA only (shooting culture). These bio-chemical contents in the cultured tissue are specified due to the presence of s balanced form of specific hormone/s in the medium. A specific combination of hormones may be involved to direct organogenesis or shoot induction in the cultured explants.

Key words: *In vitro*, *Musa* sp., Basrai, micro-propagation, nutrient contents, L-cystein, immersion system and meristematic shoot tip culture

INTRODUCTION

Banana is one of the most important table fruit crop. In Pakistan, its annual production is ~102 million tons per year (FAO, 2002). It is rich with various carbohydrates and vitamins (Vuylsteke and Ortiz, 1996). However, the banana production is limited by the shortage of pathogen free plant material as the transmission of harmful insects, nematodes and viral disease progressing in the banana grown fields among its suckers. Such biotic constraints have prompted the interest for the use of aseptic culture techniques. Through which such disorders may be reduced and/eliminated from the desired crops.

The growth medium acts as a tool, to induce rapid shoot multiplication (Hamill *et al*., 1993; Hwang *et al*., 1984; Schenk and Hildebrandt, 1972; Boxus *et al*., 1991) because it 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; Ma and Shii, 1978; Krikorian and Croncauer, 1984). Among the organics, hormones have a key role for developing a specific mode of growth in the cultured tissue as by changing its amounts and types in the medium, the cells are stimulated to develop into shoots and/or roots or even their death may occurs. 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. The auxin and/or cytokinin develop a specific balanced ratio of the organic and inorganic contents in the cell. The balanced chemical contents in the cells lead to trigger, a polar cell division in the meristmatic regions, which results into a specific structural development (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 huge numbers but normal and fertile plantlets. It is possible only when the concentration, type, time and combination of specific auxins and/or ctokinins 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 (medium solidification) of the growth culture (Escalona *et al*., 1999; Alvard *et al*., 1993). Each step of the micro-propagating plantlets is varied in a capability to absorb nutrients from the medium.

The present study was conducted to know about what a type of biochemical and micro-propagation efficiency come to be existed under different combinations of auxins and/or cytokinin and which extent of the medium solidification is suitable for banana micro-propagation.

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MATERIALS AND METHODS

Four suckers of banana (*Musa* sp.) variety Basrai were selected from the greenhouse; Vice Chancellor's house, University of Sindh, Jomshoro, Pakistan, to use as experimental material (Hirimburegama and Gamage, 1997) or explant.

Meristematic stem tips were excised and surface disinfected from micro-organisms by washing with ethanol (90%) for 1 min, than shacked 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 for organogenesis on MS [Murashige and Skoog (1962) basal medium supplemented with vitamins B5 (Gamborg *et al*., 1968); 3% sucrose; semi-solidified with 3.60 g $L^{\bullet 1}$ phytagel] medium (initial culture) supplemented with various hormone/s for organogenesis. After 2-weeks, the explants were shifted to the shooting

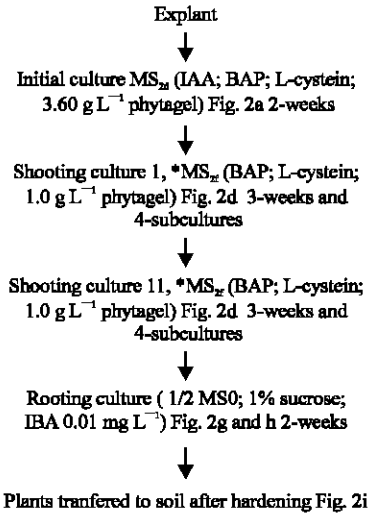

Fig. 1: A schematic representation of the optimized protocol for the micro-propagation of banana cv. Basrai (*Musa* sp.)

Table 1: Effect of different hormonal combinations on the bio-chemical contents and micro-propagation efficiency of banana (*Musa* sp.) cv. Basrai

	MS with different hormonal combinations							
Parameters/Characters	Hormones	MS_o	MS_{2a}	MS_{2b}	MS_{2c}	MS_{2d}	MS_{2e}	*MS_{2f}	**MS_{2f}
Treatments	BA	0.00	8.00	8.00	8.00	8.00	8.00	10.00	10.00
	NAA	0.00	0.00	4.00	4.00	0.00	0.00	0.00	0.00
	IAA	0.00	0.00	0.00	10.00	10.00	0.00	0.00	0.00
a. Organogenesis (2-weeks culture).									
Explant proliferation (g)		-0.212±2.25	0.421±1.05	0.553±2.50	0.458±1.50	0.350±1.25	0.930±3.25	0.403±3.60	0.360±4.01
Dry wt. (g)		0.25±3.02	0.85±1.25	0.96±1.50	0.89±1.80	1.75±0.75	1.92±0.65	2.35±0.75	2.01±2.25
b. Shoot induction (4-weeks culture), explants (after organogenesis) from MS_{2d} were sub-cultured on each medium.									
Plant height (cm)		-	-	-	-	0.40±1.75	2.95±1.05	3.41±0.12	2.80±1.50
No. of plantlets/explant		-	-	-	-	0.04±2.25	2.25±0.75	6.20±0.50	2.75±1.25
c. Biochemical analysis (explant were taken after 4-weeks of culturing on each respective medium during organogenesis).									
Total protein (mg $g^{\bullet 1}$)		10.25±3.30	32.06±0.75	14.16±1.20	42.56±1.75	25.90±2.25	28.82±2.25	38.42±1.20	25.28±1.80
Reducing sugar (mg $g^{\bullet 1}$)		9.25±3.20	13.40±1.23	13.67±0.80	18.60±1.26	14.23±2.25	14.56±2.01	16.85±0.65	15.68±2.20
Total sugar (mg $g^{\bullet 1}$)		11.35±2.20	18.02±2.20	22.46±1.15	36.96±2.25	22.38±1.75	16.52±1.65	27.05±1.85	20.41±2.80
Na^+ (g $kg^{\bullet 1}$)		3.41±3.65	3.96±1.26	4.01±2.00	5.01±1.12	4.68±2.20	4.01±1.70	3.50±1.25	3.60±1.24
K^+ (g $kg^{\bullet 1}$)		12.21±4.01	18.25±2.42	19.02±2.40	18.53±1.90	17.64±3.25	20.26±2.20	24.20±2.24	23.65±1.60
$Cl^{\bullet}$ (g $kg^{\bullet 1}$)		6.32±3.92	8.25±2.30	7.60±2.26	7.95±2.62	5.35±2.80	5.06±1.66	4.30±1.60	4.68±0.85
Ca^{++} (g $kg^{\bullet 1}$)		5.21±4.25	7.62±2.90	8.50±1.90	5.60±2.54	6.35±2.22	8.66±1.62	9.70±2.25	9.59±2.75

Note: MS_{2d}; Initial culture; *MS_{2f} and **MS_{2f}: Used as a shooting culture by culturing explants after organogenesis on MS_{2d} (2-weeks) as in Fig. 1. *MS_{2f} and **MS_{2f}: With and without L-cystein (Both of the media were used for the explants proliferation/organogenesis; also for shoot induction and their multiplication in the explants which were organogenised on MS_{2d} medium for 2-weeks); -Each experiment comprised on 4 replicates; Explant proliferation was calculated in grams (g) after 4-weeks by formula (final wt of explant-initial wt of the explant); Shoots weight were measured, which plants were of 5 mm or more in height; Box observations: The # of the regenerated plantlets on *MS_{2f} and **MS_{2f} media, here sub-cultured explants were organogenised on MS_{2d}

Table 2: Effect of different culturing conditions on micro-propagation efficiency in banana (*Musa* sp.) variety Basrai

Parameters	Culture a	Culture b	Culture c	Optimized protocol (Fig. 1)
a. Organogenesis	3.60 g $L^{\bullet 1}$ phytagel	2.0 g $L^{\bullet 1}$ phytagel	1.0 g $L^{\bullet 1}$ phytagel	3.60 g $L^{\bullet 1}$ phytagel
(IAA and BA)	2-weeks culture	2-weeks culture	2-weeks culture	2-weeks culture
b. Shoot	3.60 g $L^{\bullet 1}$ phytagel	2.0 g $L^{\bullet 1}$ phytagel	1.0 g $L^{\bullet 1}$ phytagel	1.0 g $L^{\bullet 1}$ phytagel
Induction (BA)	2-weeks culture	2-weeks culture	2-weeks culture	2-weeks culture
c. Shoot	3.60 g $L^{\bullet 1}$ phytagel	2.0 g $L^{\bullet 1}$ phytagel	1.0 g $L^{\bullet 1}$ phytagel	2.0 g $L^{\bullet 1}$ phytagel
multiplication (BA)	3-weeks culture	3-weeks culture	3-weeks culture	3-weeks culture
a. No. of shoots/explant	2.35	3.70	1.67	11.02
b. Shoot height (cm)	5.32	4.80	3.30	3.23
c. Pseudostem diameter (cm)	0.50	0.43	0.36	0.53
d. Root induction (%)	65.15	38.20	-	85.00

Fig. 2: Different steps in micro-propagation of banana cv. Basrai (*Musa* sp.). a) Explants on MS_{2i} medium for organogenesis (2 weeks); b) Shoot induction on shooting culture I (after 4 weeks), best one stage for shifting to shooting culture II, for the purpose to minimize plant abnormality; c) Shoot induction on the shooting culture I with 3.60 g $L^{\bullet 1}$ phytagel (after 4 weeks); d) Shoot multiplication on shooting culture II (after 4 weeks); e) Meristematic micro-stem cuttings from shooting culture II for sub-culturing on the same medium; f) Shoot multiplication on shooting culture II (after 3 weeks; repeat this culture for 3 times, each for 4 weeks before going to root induction); g) Micro-propagated stem cuttings (with plantlets) from shooting culture II used for the root induction; h) A micro-propagated plantlet on root induction medium for 2 weeks and i) A plant (after 1 week of its hardening), growing on soil in wire-house

culture I for shoot induction (2-weeks) and its multiplication (3-weeks) was carried out on the shooting culture II (Fig. 1, Table 1 and 2).

The established cultures on shoot induction medium were routinely transferred after every 3 weeks by subdividing the clustered mass of plantlets (Fig. 2d) into a number of micro cuttings, according to that, the number of plantlets per explant with a sterile scalpel. These micro-cuttings (Fig. 2g) of the micro-propagated plantlets were sub-cultured for 4 times on the same medium. The root induction was carried out by culturing onto MS basal medium supplemented with 0.01 mg $L^{\bullet 1}$ IBA. After 4th sub-culturing on the shooting culture II, the cultures were refreshed by taking new young sucker from field.

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

After 4 weeks, the explants from each culture medium (Initial culture; Fig. 1; Table 1c) were removed, washed with water 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 for ion concentration (Na^+, K^+ and Ca^{2+}) as by Malavolta *et al*. (1989) methods. Chloride contents were measured by Chloro-counter following the instruction in the instrument operating manual (Marius Instrumenten, Utrecht, The Netherlands). Total sugars and reducing sugar contents were analyzed according to Montgomery (1961) and Miller (1959) respectively, while total protein contents were measured according to Lowery *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 different cytokinin and auxin for organogenesis. Explants proliferation was measured after 4 weeks, in all the cultured explants, maximum was observed on MS_{2e} and minimum on MS_{2d} (Table 1).

During organogenesis; after two weeks, the organogenised explants (Fig. 2a) from MS_{2d} were cultured on all media. After 4 weeks on shooting culture (Table 1b), a numbers of plantlets were observed on $*MS_{2f}$ (Table 1; Fig. 2b and c) after 6 weeks, the abnormal plantlets were also developed on MS_{2d}. Normal plantlets were also observed on $**MS_{2f}$ but can not survive for long terms (not able to grow after 2nd sub-culturing), because of the appearance of culture blackening, which was due to, the phenolics oxidation.

To know, what an effect of the solidification of culture medium (physical conditions) on the rate of shoot induction and its multiplication rate. The maximum numbers of plantlets per explant were observed on the shooting culture II ($*MS_{2f}$; Table 2), while numerous, very small and abnormal plantlets were developed on the medium solidified with 1.0 g $L^{\bullet 1}$ phytagel. Rooting was failed in such plantlets (Fig. 2f). Maximum plant heights were measured on the medium solidified with 3.60 g $L^{\bullet 1}$ phytagel. During the experiment, a co-relation was observed between the shoot multiplication rate and plant heights, with the increase in shoot height the number of plantlets decreased and vice versa ($*MS_{2f}$; $**MS_{2f}$; Table 1 and 2). The maximum number of shoots with reasonable height was observed (Table 2 and Fig. 1), when the shooting culture I (1.0 g $L^{\bullet 1}$ phytagel) and II (2.0 g $L^{\bullet 1}$ phytagel) were inserted in the MS_{2f} culture system.

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 0.01 mg $L^{\bullet 1}$ IBA, about 90% of the shoots, rooted within 2 weeks (Fig. 2h). 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 weeks, were established under greenhouse conditions.

Under the influence of different hormonal combinations and/or separately leads to a change in the organic and inorganic contents in the proliferating tissues. Each medium with a specific hormonal condition develops a specific combination of the physio-chemical contents in each medium. Both Na^+ and $Cl^{\bullet}$ contents were increased under the influence of BA, NAA and IAA (MS_{2c}) than others (Table 1), while the K^+ and Ca^{2+} contents were decreased. However, in the presence of BA (MS_{2f}), the situation was reversed (Table 1).

Similar characters were also observed for the organics like as total protein, reducing sugars and total sugar contents. Maximum reducing sugar contents were observed in MS_{2c} as this is a specific character for the indication of stressed condition on the explants. While total protein and sugar contents were observed maximum in the presence of BA ($*MS_{2f}$), which is a cause for the best shoot induction and with exponential multiplication within the organogenised explants (from MS_{2e}). So the presence of a specific hormone/s in the culture medium develops a specific inorganic and organic combination in the growing tissue, which reflects the rate and efficiency of the shoot induction and its multiplication in the micro-propagating plantlets.

DISCUSSION

Today, tissue culture is the basic need, to develop rapid and surface growing pathogen free plants. However, raising plants from seeds (or spores) is the traditional means of production, which 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 may also be effective for banana; which is an vegetatively propagating crop for the purpose to develop its disease free clones (Arinaitwe *et al*., 2000; Vuylsteke, 1998; Mendes *et al*., 1999; Wojtania and Gabryszweska, 2001; Ortiz and Vuylsteke, 1994).

The phytohormones regulate and integrate the overall growth, development and reproductions in plants. The morphogenesis devolves basically around the processes of cell division, cell elongation and differentiation. Hormones are factors which involved in regulating all of them. Auxins are typically associated with cell elongation Reinhardt *et al*. (2003, 2000); Jouve *et al*. (1999), Jenson *et al*. (1998) and Rayle and Cleland (1992), 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 (Hirimburegama and Gamage, 1997; Hutchison *et al*., 1999; Kadota and Nimii, 2003); the mode of roots and shoots within a cell and/or tissue are specified, while alteration results into altered signals. Such alterations develop specific balanced ratios of the specific organics and inorganics in the

growing tissues (Table 1). The balanced cell's chemical contents lead to trigger a phenomena cell polarity in the meristematic regions of the cultured tissues.

During organogenesis, the optimal combination of auxins and cytokinins in the medium both IAA and BA are perhaps critical phenomena among others i.e., BA, IAA and NAA while the enhanced shoot multiplication rate in a particular explant is the reflection of IAA and BA which is appeared in the presence of BA only (Fig. 2d). In general, higher levels of BA in the medium increases the number of shoots per explants, but the shoot height decreased or even abnormal shoot buds developed, which laterally not enable itself to develop into shoot (Van den *et al*., 1998; Victor *et al*., 1999).

While, the progress in a specific mode of development during banana micro-propagation is also dependent on the physical conditions of the medium either semi-solidification and/or liquefaction. The capability of the micro-propagating plantlets for nutrients absorption is different at different stage. Liquid media induction during micro-propagation is considered to be the ideal solution for automation and reducing production costs. However, it causes many problems such as asphyxia, hyper-hydricity (Etienne and Berthouly, 2002) which causes to induce abnormalities in the developing plantlets. All of these problems are avoidable by using different solidification conditions (Borroto, 1999; Damells, 1997) of the medium at different stages of plant micro-propagation (Fig. 1) Such abnormalities are not easy to un-install (Vuylsteke, 1998; Matsumoto and Brandao, 2002; Daquinta *et al*., 2000; Murch *et al*., 2004; Noh *et al*., 2003; Cosgrove *et al*., 2002; Arinozue and Maloof, 2006; Etienne and Berthouly, 2002). The abnormal buds are not suitable for banana micropropagation 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.

One step of liquification induction during micro-propagation has a positive effect on *in vitro* shoot proliferation (Alvard *et al*., 1993; Escalona *et al*., 1999; Murch *et al*., 2004). Presently three different media solidification conditions are optimized (timing and concentration of phytagel) for banana variety Basrai. The presence of a specific hormone/s in the medium has a key role in switching the banana micro-propagation character (Roels *et al*., 2005). IAA (Auxin) and BA (cytokinin) are appeared to be the better choice for the purpose to develop a mode (organogenesis) or initials development for shoot induction (BA) laterally. Both IAA and BA in the medium develop somewhat stress on the cultured tissue which is hinted because of the appearance of the high level of reducing sugars. While at the shooting stage it was lowered down and others i.e., total protein, total sugars and other mineral (K^+ Ca^{2+}) were increased. It gives an indication that the cultured tissue is metabolically active in the presence of BA only, as same situation also presented by MS_{2e} medium where highest proliferated mass of the explant was observed. Both physical conditions of the medium (phytagel) and specific hormones behaves in an interconnected form, which are involved to trigger a specific character otherwise, it causes to develop abnormalities in the developing plantlets.

By using, the optimized scheme (Fig. 1) any desirable genotypes of the banana (*Musa* sp.) can be micro-propagated into a large numbers; within a short time period by making somewhat change in the respective media composition or the culture timing.

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