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Full Length Research Paper

Effect of immersion systems on chlorophyll contents in micro-propagating banana

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Banana is a nutritionally as well as economically important plant for which (Basrai variety) an efficient micro-propagation protocol was developed by using micro-stem cutting, as an explant. The maximum numbers of plantlets with higher chlorophyll and lower carotenoid contents were observed, which developed through routinely used tissue culture system (10.0 µM BA; 8.0 µM IAA; 3.0 g/L phytagel) for organogenesis, permanent immersion system (10.0 µM BA; 1.0 g/L phytagel) for shoot induction and temporary immersion system (10.0 µM BA; 2.0 g/L phytagel) for shoot multiplication. The developed plantlets were cultured on 0.5 MS medium with IBA (0.01 mg/L). Rooted plantlets were successfully transferred to field after initial acclimatization.

Key words: *In-vitro*, *Musa* spp, Basrai; micro-propagation, IAA, L-cystein, immersion system and meristematic shoot tip culture, chlorophyll contents.

INTRODUCTION

Bananas (*Musa* spp.) are a giant perennial herb, cultivated in the tropical and sub-tropical regions of world and Pakistan has annual production about 40 tons per year (FAO, 2005) where they serve as staple and cash crops. A major problem of plantain and banana landraces is their susceptibility to various fungal, viral, nematodal and insect attacks. If unchecked, all of these causes to leaf decay thereby reducing the photosynthetic area, and causing a reduction in yield (Mobambo et al., 1993).

The expansion of banana production is limited, because of the shortage of healthy plant material availability to the farmers. The transmission of harmful insects, nematodes, and viral disease by field-grown suckers has prompted interest in the use of aseptic culture techniques. Through banana micro-propagation, it is possible to get plantlets free from insects, bacteria and other microorganisms (Krikorian and Cronauer, 1984; Ma and Shii, 1972; Vuylsteke, 1998; Ortiz and Vuylsteke, 1996; Tenkouano et al., 1998a). 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 growth medium is a means 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), which results into several clones of the original plant, 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; Georget et. al., 2000; Haq and Dahot 2007a). 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.

The nutrient uptake power from the culture medium is a variant property of the specific plant species so with the change of the medium solidification, plant micro-propagation efficiency is also altered (Escalona et al., 1999; Alvard et al., 1993; Haq and Dahot, 2007b). High production costs generally limit the commercial use of *in-vitro* micro-propagation of banana because of its low efficiency. However, using liquid medium (a step with temporary

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Abbreviations: MS, Murashige and Skoog medium; IAA, indole acetic acid; IBA, indole butyric acid; BA, benzyle aminopurine; Chl, chlorophyll.

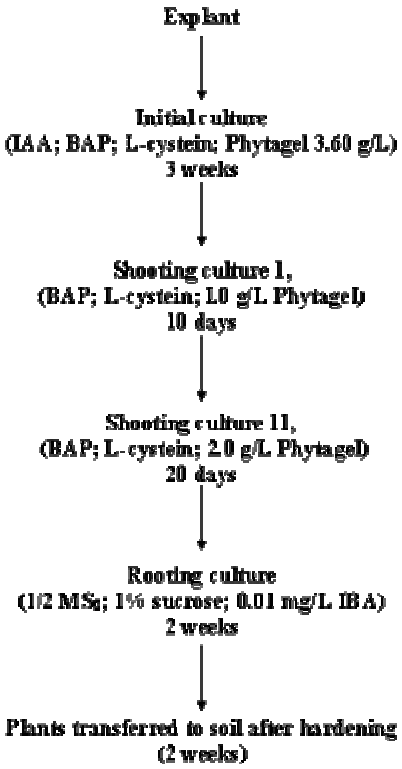

Figure 1. A schematic representation of the optimized protocol (Table 1) for banana micro-propagation.

and/or permanent immersion system) during routinely used banana micro-propagation system is considered to be ideal for automation and production cost reduction.

The present work introduces a rapid *in vitro* multiplication protocol for banana with optimized concentration of the auxins/cytokinins either through temporary immersion system and/or permanent immersion system.

MATERIALS AND METHODS

Four newly young banana (*Musa spp*) cv Basrai plants were selected. Meristematic stem tips were excised and used as an explant. They were surface sterilized by washing with ethanol (90%) for 1 min and then stirred in 20% commercially available robin bleach (5.25% NaOCl) for 30 min. Afterwards, they were rinsed with sterile distilled water 3 times in the laminar air flow cabinet.

Shoot tips (3 - 4 mm) were isolated aseptically and divided into two parts, then they were cultured for organogenesis on MS (Murashige and Skoog, 1962) basal medium, B5 vitamins (Gamborg et al., 1968), 3% sucrose, 3.60 g/L phytagel] medium supplemented with 10.0 µM BA, 15.0 µM IAA, and 30.0 mg/L L-cystein for 3 weeks (Figure 1). After organogenesis, the explants were sub-cultured on the medium supplemented with only 10.0 µM BA and 30.0 mg/L L-cystein (Table 1) for shoot induction (1.0 g/L phytagel) and its multiplication (2.0 g/L phytagel). The root induction in the shoot cuttings was carried out by culturing onto 0.5 MS basal medium supplemented with 0.01 mg/L IBA.

During these experiment three different media with different solidification conditions i.e. 3.60 g/L phytagel, 2.0 g/L phytagel and 1.0 g/L phytagel were used during organogenesis, shoot induction and their multiplication, respectively (Table 1). 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 L-cysteine, 3% sucrose, and their pH was adjusted to 5.7 - 5.8 before autoclaving at 121°C for 15 min. Each culture was maintained with seven replicates at 25 ± 2°C under 18/6 h day and night photoperiod in growth chamber with light intensity ~2000 lx. The established cultures on shoot induction medium were routinely transferred after every 3 weeks by subdividing bulky mass of plantlets into a number of micro cuttings with a scalpel. These micro cuttings of the micro-propagated clusters were sub-cultured 4 times on the same medium. The cultures were refreshed by taking new explants from the open field grown plants.

For chlorophyll determination, fresh leaves were enclosed in black plastic bags, placed on ice in a bucket, brought to laboratory, extracted (0.5 g sample) in 80% acetone (10 ml) using a blender, and vacuum filtered. The filtrate was immediately determined for chlorophyll contents at 645, 652 and 663 nm (Witham et al., 1971) using a spectrophotometer *U-2000* (Hitachi Instruments, Tokyo, Japan).

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 micro propagated 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 its starting point after transversely cutting with scalpel.

RESULTS

In order to establish an efficient *in vitro* micro-propagation system for banana (*Musa* spp.) cv Basrai, fresh and sterilized meristematic stem tips were cultured on MS basal medium (initial culture) supplemented with BA and IAA for the purpose of inducing shoot/organogenesis. After 2-weeks, a measurable explant proliferation was observed (Table 1). Then cultures were transferred onto shoot induction medium supplemented with BA only, and a measurable change or a pattern of shooting was observed in them. Plantlets regeneration was observed (Figure 2b). The numbers of plantlets/explant were counted after 4th week, before their sub-culturing on the same shoot induction media (repeated for 4-times).

After 5th weeks of culturing on shoot induction medium, the shoot height and the pseudostem diameter of the developing shoots were also measured. Such very low micro-propagation system for banana, limits its commercial uses. Thus there is need to develop an efficient plant regeneration protocol with high numbers of plantlets by determining the effect of medium solidification (physical conditions) on the micro-propagation efficiency in banana. Initially the explants were organogenised, through three different culture systems (Table 1). Then they were sub-cultured not only on the same medium i.e. routinely used tissue culture system (S) but also cultured on temporary immersion system (T) and permanent immersion system (P). Two additional plant culture systems (ja and Jb) were also maintained by adding two different medium solidification conditions during shoot induction

Table 1. Different culturing conditions, used during the optimization of the micro-propagation efficiency in banana (*Musa spp.*) cv Basrai

#s Culturing systems	Organo genesis	Shoot Induction	Shoot multiplication	Explant proliferation (g)	# of shoots/ explant	Shoot height (cm)	Pseudostem Diameter (cm)	Root induction (%)	Chl amgg^{-1}	Chl b mg g^{-1}	Ch lab mg g^{-1}	Carotenoinds mg g^{-1}
a. Routinely used tissue cultures system (S)	IAA (15.0 µM) BA (10.0 µM) 3.0 g/L Phytagel 3-weeks culture	BA (10.0 µM) 3.0 g/L Phytagel 10 days culture	BA (10.0 µM) 3.0 g/L Phytagel 20 days culture	0.192	2.92	5.01	0.55	74.15	0.439	0.227	0.666	541.05
b. S to P (After organogenesis)	-	1.0 g/L Phytagel	1.0 g/L Phytagel	0.285	4.98	2.15	0.4 2	12.29	0.424	0.210	0.634	564.05
c. S to T (After organogenesis)	-	2.0 g/L Phytagel	2.0 g/L Phytagel	0.206	4.39	3.70	0.46	19.94	0.430	0.219	0.649	558.89
d. Temporary immersion system (T)	2.0 g/L Phytagel Hormones as in i	2.0 g/L Phytagel Hormones as in i	2.0 g/L Phytagel Hormones as in i	1.259	3.95	4.92	0.49	32.21	0.402	0.202	0.604	561.62
e. T to S (After organogenesis)	-	3.0 g/L Phytagel	3.0 g/L Phytagel	1.025	1.50	5.39	0.57	42.74	0.385	0.184	0.569	575.78
f. T to P (After organogenesis)	-	1.0 g/L Phytagel	1.0 g/L Phytagel	2.212	3.72	3.18	0.42	12.00	0.411	0.209	0.620	585.23
g. Permanent immersion system (P)	1.0 g/L Phytagel Hormones as in i	1.0 g/L Phytagel Hormones as in i	1.0 g/L Phytagel Hormones as in i	2.125	1.73	3.35	0.34	-	0.392	0.196	0.588	584.54
h. P to S (After organogenesis)	-	3.0 g/L Phytagel	3.0 g/L Phytagel	1.511	2.92	3.98	0.47	22.29	0.379	0.180	0.559	590.28

Table 1. Contd.

i. P to T (After organogenesis)	-	2.0 g/L Phytagel	2.0 g/L Phytagel	2.002	2.32	3.29	0.35	5.92	0.404	0.220	0.624	582.22
ja. Optimized protocol (Fig 1)	3.0 g/L Phytagel Hormones as in i (Initial culture)	1.0 g/L Phytagel Hormones as in i (Shooting culture I)	2.0 g/L Phytagel Hormones as in i (Shooting culture II)	3.212	13.29	3.45	0.56	92.95	0.512	0.321	0.833	534.35
jb. Optimized protocol	3.0 g/L Phytagel Hormones as in i (Initial culture)	2.0 g/L Phytagel Hormones as in i (Shooting culture II)	1.0 g/L Phytagel Hormones as in i (Shooting culture I)	3.492	10.23	2.31	0.42	68.75	0.435	0.314	0.749	552.70

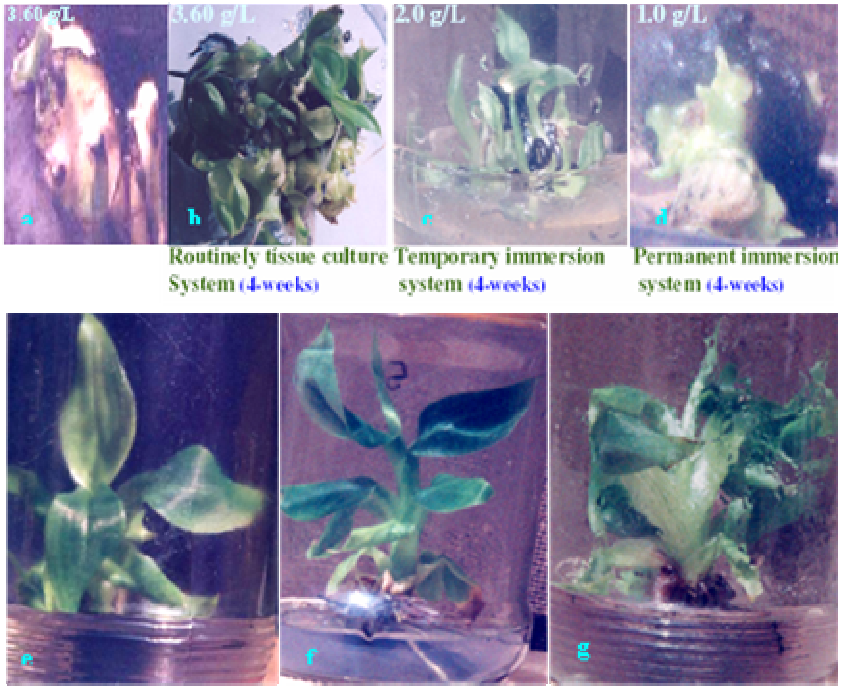

Figure 2. Different steps for banana micro-propagation under various immersion systems. **a.** Micro-stem cutting (explant) proliferating on initial culture (3.60 g/L phytagel) for organogenesis. **b.** Micro-propagating plantlets on shooting culture II (3.60 g/L phytagel). **c.** Plantlets on shooting culture II, supplemented with 2.0 g/L phytagel. **d.** Plantlets growing on shooting culture II, solidified with 1.0 g/L phytagel. **e.** Micro-propagated plantlets growing on rooting medium, which was developed through routinely used tissue culture system. **f.** Plantlets growing on rooting medium, developed through temporary immersion system. **g.** Plantlets growing on rooting medium, developed through optimized protocol.

and its multiplication (shooting culture I and shooting culture II). Each explant's fresh weight was measured (□0.20 g) before culturing on each system. During their growth, a clear cut difference in both plantlets growth rate and their physical appearance was observed. After four weeks, the number of shoots, shoot height and pseudostem diameter of the developing plantlets were measured (Table 1).

After the 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 then to temporary immersion system (Table 1; Figure 2c). 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 (Figure 2f). Somewhat 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 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 (Figure 2c). The maximum numbers of shoots with moderate shoot height were observed on optimized protocol culture i.e. ja (Table 1) which were developed from the solidified (organogenesis) to permanent immersion system and then to the temporary immersion system (Figure 1), while similar results were also noted on culture jb (Table 1) but rooting efficiency was very low in them.

The chlorophyll contents and carotenoids were measured, after 4-weeks of culture on the shoot induction II medium (Figure 1; Table 1). The chlorophyll contents were appeared to be dependent on medium solidification. The chlorophyll contents (chl a, b and ab) were observed as minimum in the permanent immersion system (Table 1) while increased with the increase in with medium solidification. Chlorophyll contents were maximum in the plantlets which were developed through routinely used tissue culture system but carotenoids were appeared as in a reversed form (Table 1). However, the alteration of the medium solidification during different stages of the micro-propagating plantlets leads to increase in the number of plantlets development. In all of these plantlets, higher chlorophyll contents were observed while catrotenoids

were minimum (Table 1).

After 4th sub-culturing on the shooting medium, well developing plantlets (Figure 2g) of about 3 weeks old were excised and cultured on 0.5 MS basal medium supplemented with 0.01 mg/L IBA for 2-weeks. 89.90% plantlets were rooted. Rooted plantlets were then transferred to pots (covered with a polythene bags for a few days to prevent wilting) for plant hardening. After 2-weeks, all of them were established under greenhouse conditions (Figure 2f).

DISCUSSION

Today, tissue culture is the basic need, to develop rapid and surface growing pathogen free plants. In banana, the most widespread used technique for vegetative propagation is *in vitro* micro-propagation by culturing actively growing pieces of plants, under varying concentrations of different cytokinins and auxins (Arinaitwe et al., 2000; Vuylsteke, 1998; Mendes et al., 1999; Wojtania and Gabryszweska, 2001; Ortiz and Vuylsteke, 1994). For regeneration in a well differentiated tissue, IAA and BA are best for banana cv Basrai, while only BA triggers/induces mass proliferation (Figure 4b) within 2 - 3 weeks (Daniells, 1997; Jambhale et al., 2001; Kadota and Niimi, 2003; Hirimburegama and Gamage, 1997).

The optimal combinations of auxins and cytokinins in the medium (IAA and BA) are perhaps a critical phenomena during organogenesis. The enhanced shoot multiplication rate in a particular explant is the reflection of IAA and BA (Figure 2c). In general, higher levels of BA in the medium increases the number of shoots per explants, but the shoot height decrease or even abnormal shoot buds develop, which laterally will not enable itself to develop into shoot (Van den et al., 1998; Victor et al., 1999; Haq and Dahot 2007c).

The physical conditions of the medium i.e. solidification and/or its liquefaction are also effective on the rate of micro-propagation in banana which may also causes to develop abnormality in the multiplying plantlets (Etienne and Berthouly 2002). Such developed plantlets are not suitable for further banana micro-propagation purpose (Vuylsteke, 1998; Matsumoto and Brandao, 2002; Daquinta et al., 2000; Murch et al., 2004). However, the normal plantlets can be obtained from them by repeating the organogenesis to shoot induction steps but such developed plants may be sterile in the field.

During *in vitro* development, the plant survives and multiplies on already supplied nutrients in the medium. When they are abolished, the plant photosynthetic apparatus (chlorophyll contents) become active and start to prepare its carbon complexes. However, if the nutrients are present in the medium, but plant is again unable to absorb nutrients properly due to the physical conditions (solidification rate) of the medium, this can stimulate the photosynthetic machinery. When a plant is fixing CO_2, the rate of shoot multiplication decreases as compared to that growing on proper *in vitro* conditions. The carotenoid contents present in minimum amount in the tissues which are photosynthtically active where reversed when it is inactive. So the alternate steps during banana micro-propagation is to activate and inactivate its assimilatory organelles during organogenesis/shoot induction and its multiplication/maturation, respectively (Pospisilova, et al., 1999; Te chato, et al., 2005; Arigita, et al., 2002).

In banana tissue culture, the goal is to produce a maximum number of shoots with rooting under *in vitro* conditions. In this respect, the physical conditions of the medium like as the routinely used tissue culture system, temporary immersion system and permanent immersion system should be used as in interconnected form. Through this optimized protocol (Figure 1), pathogen free plantlets can be developed within 2 - 3 months. By using the optimized scheme, any desirable genotypes of the banana (*Musa* spp.) can be micro-propagated within a short time period by making some changes in respective medium composition or its culture timing. Our findings may be of great value during the establishment of banana micro-propagation laboratory to produce in future.

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