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Influences of phytohormones in plant nutrient cultures of *in-vitro* multiplying banana plantlets alters the cellular biocontents

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Abstract

Banana is an important fruit as well as staple food crop. Its production is decreasing by the infection of a number of inter and intra-cellular pathogens. Banana micropropagation under *in-vitro* is being a possible way for development of pathogen free plants. In this experiment, banana (cv., Basrai) multiplication was optimized and certain biochemical analysis was performed among the organogenesis and plant multiplication cultures of banana. Maximum plantlets were propagated when meristematic micro-stem cuttings were cultured for organogenesis on MS_2 medium supplemented with 10µM 6-Benzyleaminopurine (BAP), 15µM Indole aceticacid (IAA) and solidified with 0.36% phytagel. After 3rd week of incubation, cultures were sub-cultured on MS_3 (10 µM BAP, 0.12% phytagel) for shoot induction (for 2- weeks) than for plant or shoot multiplication was carried on MS_4 (8µM BAP, 0.2% phytagel) medium (for 4-weeks). Organogenesis process was induced under a specific combination of hormonal (IAA and BAP) stress, while plant multiplication (without IAA) occurs with the elevation of IAA stress due to the adjustment of specific biocontents among the cultured tissues. Likely to that plant biomass, *Chl* a/*Chl* b and K^+/Cl^- ratios were increase (p 0.05) while *Chl* ab/carotenoids and Na^+ and K^+ ratios (p 0.05) were decreased among organogenesis cultures than plant shoot multiplication cultures. Meanwhile, developed plantlets through this protocol has shown normal plant growth under *ex-vitro* environment.

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Introduction

Edible banana (*Musa* sp.) is one among major staple food and cash fruit crops with annual revenue production ~102MT year^{-1} (WHO/FAO, 2002). From time to time, its production is decreasing with systematic increasing levels of biotic and abiotic stresses. Limited banana availability due to shortage of pathogen free banana plant material has initiated promptly the farmer's increasing interest for cultivation of *in-vitro* micro-propagated plantlets. Now it is possible to reduce or removal of plant growth retarding surface growing disorders from crop of interest. Under *in-vitro*, plant tissue shows plasticity and enable to initiated single or multiple shoots from the explant than roots or even go to tissue death (Hirimburegama and Gamage, 1997; Jouve *et al.*, 1999; Georget *et al.*, 2000; Reinhardt *et al.*, 2003) in the plant cultures. On the basis of increasing demand of farmers, a number of efficient *in-vitro* plant propagation protocols have been established by different institutes for various crops.

An optimized plant micropropagation system means to propagate normal and fertile plantlets. Under aseptic culture conditions, normal plant propagation depends on specific concentrations, types, time interval and combination of phytohormones i.e. auxins and/or cytokinins. Incubation of these cultures under specific environmental conditions also being beneficial for proper functioning of the phytohormones.

Such spatial and temporal conditions trigger to induce a specific and systematic mode of development. Inspite of above, concentration of solidifying agent (i.e. agar or phytagel) of medium also impact on the rate of rate of progress in specific growth mode. Every plant species shows specific nutritional absorption capacity for specific nutrients from the medium, which also further dependent on plant growth stage either at explant or tissue proliferation or organogenesis or shoot multiplication on the plant culture medium. In actual, all steps in plant micro-propagation is demands for specific medium solidification state (Alvard *et al.*, 1993; Escalona *et al.*, 1999; Scherer *et al.*, 2013).

During plant micropropagation, both hormones and medium solidification levels play very important role for the initiation of specific mode of plant growth among the cultured tissues for organogenesis or shoot multiplications even their survival under artificial aseptic culture conditions. Variations in the environmental conditions determine the rate of plant growth, which reflects the rate of agricultural production. Both vegetative as well as reproductive growth rates even senescence of plant organs can be altered with modulation of nutrient as well as hormonal remobilization efficiencies (Paltridge *et al.*, 1984; Jing *et al.*, 2005; Iqbal *et al.*, 2012).

Over supplementation of phytohormones increases growth rate of plant biomass either under *in-vivo* or *in-vitro* conditions as it controls plant growth and senescence (Gaspar *et al.*, 1996; Pierik *et al.*, 2006; Nazar *et al.*, 2014; Voesenek *et al.*, 2015; Bielach *et al.*, 2017). Such promotions or inhibitions of plat growth processes are depending on hormones concentrations and their application timing on which plant species (Konings and Jackson, 1979; Khan, 2005; Pierik *et al.*, 2006; Ahmad and Khan, 2008). The influences of hormones and physical medium solidification also causes alterations in the concentration of certain stress related metallic ions as well as total carotenoids and chloroplasts within the cultured tissues. Present study, appears somewhat quite interesting to analysis the biochemical and phonotypical alterations in the cultured plant tissue at different growth stages under different hormone influences in plant growth cultures.

Materials and methods

Collection of plant material and preparation of explant

Around 2-3 months aged young and phenotypically healthy banana (*Musa* spp.) suckers of Basrai cultivar selected in open fields of banana and used as an initial plant material for this experiment. The inner-most 4-5 whorls of meristematic leaflets with sucker were excised carefully and used as an explant. It was washed with ethanol (90%) for surface disinfection. After 1 min, explants were stirred in 30% robin bleach (5.25% NaOCl) on continuous electric magnet stirrer for exact 30min.

They were washed with magnet stirrer for 3 times with sterile distilled water under aseptic condition and each stir was performed for 3 minutes.

Plant micropropagation cultures

After sterilization, explants were kept in sterile petri-dishes and again outermost leaf-whorl was peeled-off. These rest sterile shoot tips (almost 3 to 4mm thick) were cultured on different MS_2 (Murashige and Skoog, 1962) medium for organogenesis (Table 1). The composition of MS_2 medium was MS_0 [MS basal salts, 3% sucrose, 5 ml L^{-1} B5 vitamins (Gamborg *et al.*, 1968)], 15µM Indole aceticacid (IAA) and 10µM 6-Benzyleaminopurine (BAP) and solidified with 0.36% phytagel. These organogenesing cultures were incubated in dark at 37°C for 3-weeks. These bulky explants transferred to fresh plant or shoot induction MS_3 (MS_0, 10µM BAP, 0.12% phytagel) medium for 2-weeks. When the initiation of shoot development was started, these cultures were sub-cultured on shoot multiplication MS_4 (MS_0, 8µM BAP, 0.2% phytagel) medium for 4-weeks. The regenerated plantlets were sub-culture 3-times on same but fresh MS_4 medium for increasing the number of plantlets. At 4th subculture, plantlets were cultured on root induction MS_1 (½MS_0, 0.5mg L^{-1}, 0.36% phytagel) medium.

In-vitro culture conditions

All medium as presented in Table 1 were contained MS basal salts in according to object of the study, while they all had supplemented with L-cysteine (30.0mg L^{-1}), sucrose (3.0%). The pH of these medium were adjusted to 5.7-5.8. Medium was autoclaved for 15min at 121°C with 20 lbs pressure. The cultures were maintained at 25±2°C in dark as well as under 18/6h light (light intensity ~2000 lux) conditions.

Morphological data collection

At the stage of 3rd week of organogenesis and 4th week of plant or shoot micropropagation, plantlets were plant tissue and plantlets (respected cultures) were harvested and washed with tap water. Plant fresh biomass was taken and chlorophyll contents and carotenoids were determined (Dere *et al.*, 1998; Bojović and Stojanović, 2005).

Biochemical analysis

Plant tissue and shoots were also dried in electric oven at 72°C for 2-days. Plant material was weighed and its dry weight was measured. This dried plant material was agitated in H_2O_2 for overnight and subjected for analysis of ionic concentrations (Na^+ & K^+) as by (Körner, 1989) and Cl^- as reported by (Glass *et al.*, 1996; Lassmann and Nosske, 2013).

Statistical analysis

In final, for comparative significance analysis of the collected data, it was computed by *COSTAT* (*CoHort* software, Berkeley, USA) computer based package.

Results and discussion

Banana micropropagation and phytohormones

Specific concentrations of phytohormones regulate and integrate a specialized mode of growth either plant regeneration from tissues or vegetative and reproductive developments in plants. Morphogenesis of developing plantlets basically devolves through systematic biological processes of cell division, cell elongation (cells or tissues growth) and differentiation that are regulated under the presence of specific types and concentrations of hormones within or outside the tissue. Among the plant tissue culture, it has been confirmed that cell elongations and its divisions are associated with the concentration of auxins typically (Rayle and Cleland, 1992; Jensen *et al.*, 1998; Jouve *et al.*, 1999; Reinhardt *et al.*, 2003). Infect, its combination with cytokinins act synergistically to add up differentiation with the regulation of cell division process in cultured tissues (Hwang and Sheen, 2001; Hwang *et al.*, 2012).

The specific ratios of auxins and cytokinins in the nutrient medium induces organogenesis (mode/initiation of plant shoots) in cultured single somatic cell (callus) and/or meristematic tissues. It triggers cell's polarity in meristematic tissues. Even alterations in ratios of phytohormones concentrations results into induction of abnormal characters or growth retardation and death of tissues due to imbalance ratios of specific organic as well as inorganic compounds in growing tissues is possible.

Maintenance of micropropagation cultures and plant morphogenesis

For the establishment and optimization of banana (*Musa* sp.) cv., Basrai micropropagation, the fresh micro-stem cuttings were cultured on six plant nutrient cultures. These cultures were supplemented with basic plant nutrient salts with influence of different combinations of phytohormones like as auxins and cytokinins (IAA and BA) for organogenesis (Table 1). After 3rd-week of cultures, the organogensised explants from MS_2 medium (Fig 1a) were sub-cultured on all the six medium for shoot initiation. After 3-weeks, plant cultures from MS_3 medium (Fig 1b) were shifted again on all on six medium for shoot multiplication (Fig 1c). At the stage of 4th sub-culture, a large number of plantlets were observed on MS_3 medium, while abnormal plantlets were also developed among normal ones.

However, maximum of normal plantlets were developed on MS_4 cultures. Well-developing normal plantlets were rooted on half-strength MS_1 medium. Almost 90% propagated plantlets were rooted on MS_1 medium within 2-weeks and transferred to pots containing soil for plant hardening.

Phytohormones and plant regeneration efficiency

In actual, among the cultures on MS_2 medium supplemented with the specific concentrations of cytokinins (BAP) and auxins (IAA) in combination induces organogenesis with cell proliferation, while except IAA, influence of BAP only (as in MS_3) initiate the development of shoots (Hwang and Chen, 1984; Daniells, 1997; Hirimburegama and Gamage, 1997; Jambhale *et al.*, 2001; Roels *et al.*, 2005; Vasudevan and van Staden, 2011). Number of plantlets per explant as well as plant heights of the propagated plants varied dramatically from cultures to culture.

It could be resulted due to the influence of different concentrations of cytokinins and auxins either alone or in a combination supplemented in Plant MS medium (Kaur & Bhutani, 2012). It is cleared that high number shoot induction depends on the hormonal combination used for organogenesis step (Table 1, Fig 1).

Micropropagation efficiency and medium to pant biochemical contents

Regeneration of plantlets from the meristematic plant tissue after its processing through a series of plant nutrient cultures especially influence of phytohormones and level of medium solidification. It is important to know about the bio-chemical nature of developed plantlets under above mentioned environmental distinct composition of nutrient cultures. Among the cultures high rate of explant proliferation was observed in MS_2 cultures that enable the cultured tissues to develop shoots in same MS_2 medium as well as in MS_3 and MS_4 also but maximum abnormalities among the developed plantlets were observed in MS_2 (Table 1, Fig 4).

At shoot multiplication stage both plant fresh and dry weight were higher in MS_4 cultures ($p>0.05$). Level of total proteins and sugar contents were lower in all culture than MS_4, while reducing sugar contents showed diverse results (lesser in MS_4, Fig 2). Ratios of chlorophyll contents (chla/chlb) also noted lower in all cultures than MS_4. Similarly among the metallic ions concentrations, Na^+/K^+ ratios were measured higher in same MS_4 cultures (Fig 3, 4).

Table 1. Composition of plant nutrient cultures with specific combinations of auxins and cytokinins used for banana micropropagation under different solidification conditions.

#s.	Medium	Composition of medium	Solidification
01.	MS_0	MS basal salts + 3% sucrose + 30 mg L^{-1} L-cystein	0.36 % Phytagel
02.	MS_1	½MS_0, 0.5 mg L^{-1} IBA	0.36% Phytagel
03.	MS_2	MS_0 + 10.0 µM BAP + 15.0 µM IAA	0.36 % Phytagel
04.	MS_3	MS_0 + 10.0 µM BAP	0.15 Phytagel
05.	MS_4	MS_0 + 8.0 µM BAP	0.2 % Phytagel
06.	MS_5	MS_0 + 15.0 µM IAA	0.2 % Phytagel

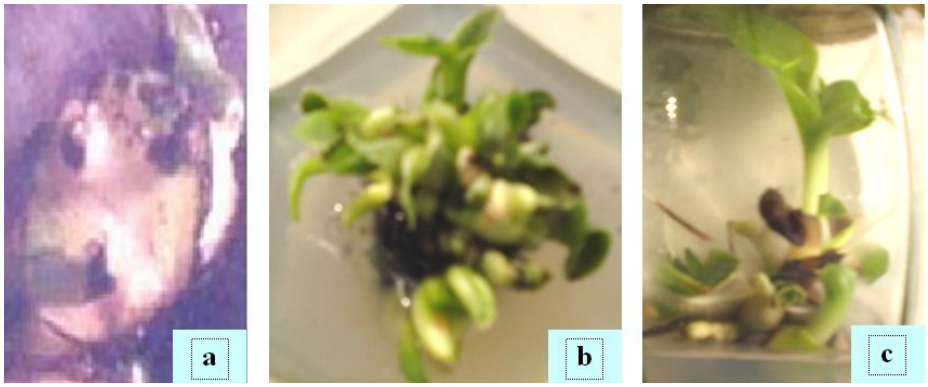

Fig. 1. Comparative morphogenesis at different plant micro-propagation stages in banana (*Musa* spp.) var., Basrai a: Micro-stem cutting proliferated after 3-weeks of culture on organogenesis (MS_2) medium, b: Developing plantlets or shoots on shoot induction (MS_3) medium, c: Development and micropropagation of plants on shoot multiplication (MS_4) medium.

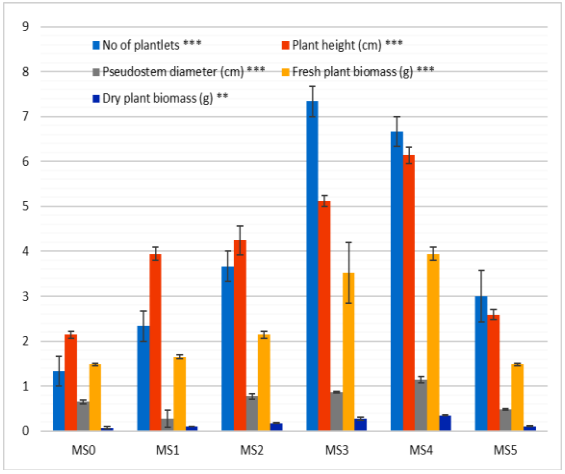

Fig. 2. Different morphological attributes of micro-propagating banana (*Musa* spp.) var., Basrai at the stage of 4th week of cultures (Cultures were maintained from MS_4 medium).

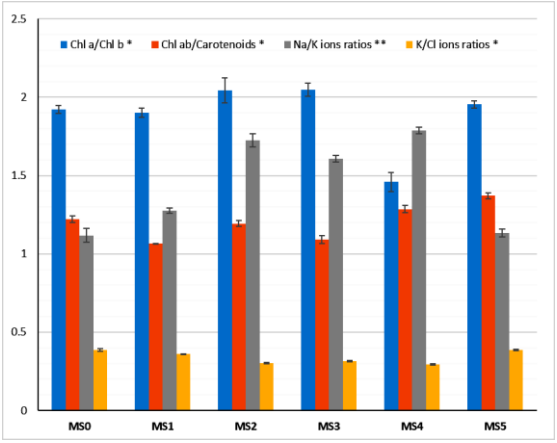

Fig. 3. Different ratios of chloroplast contents and metals among the micro-propagated banana (*Musa* spp.) var., Basrai at the stage of 4th week of cultures (Cultures were maintained from MS_4 medium).

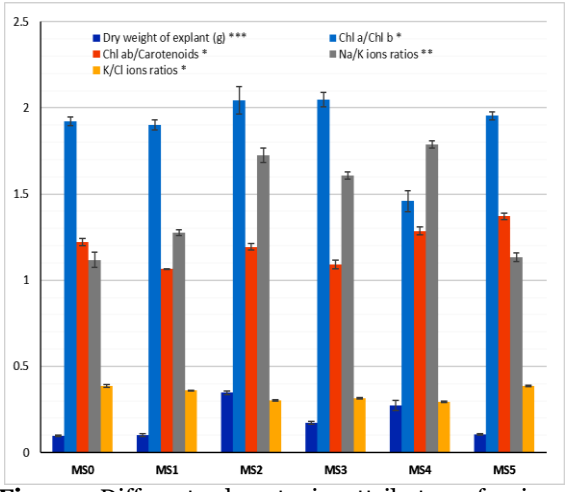

Fig. 4. Different phenotypic attributes of micro-propagating banana (*Musa* spp.) cv., Basrai at organogenesis stage of 3rd week of cultures (initial micro-stem cuttings on MS_2 medium).

Plant biocontents and abnormality in developed plantlets

According to the existed situation of biochemical contents and morphological attributed of regenerated normal or abnormal plantlets on all six culture shows specific impacts of hormones as well as medium solidification. Especially among the culture where no shoot multiplication or abnormal plantlets are regenerated (in MS_2, MS_3 and MS_5). Even tissue habituation is observed among the abnormal plantlets, which is not breakable or not possible to un-install (Vuylsteke *et al.*, 1988; Cosgrove *et al.*, 2002; Etienne and Berthouly, 2002; Noh *et al.*, 2003; Gübbük and Pekmezci, 2004; Murch *et al.*, 2004; Roels *et al.*, 2005; Nozue and Maloof, 2006; Georgiev *et al.*, 2014; Cosgrove, 2015).

Such developed abnormal plantlets regenerated among the normal plantlets, which are rarely suitable for banana micro-propagation purpose and even not for field production. Objection on these abnormal plantlets is that are these may be fertile or sterile under field conditions.

Conclusions

Phytohormones are major regulators of plant physiological as well as developmental mechanisms, which resist against the impacts of nutritional stresses in their habitats. When plant meristematic tissues are cultured on a series of nutrient cultures under *in-vitro*. The complex networks of cell signaling lead to prevent or attenuate to adopt new imposed habitat. The crosstalk mechanisms between bio-components of cultured tissues and supplied plant hormones increases pleiotropy in intra- and inter-cellular as well as spatial and temporal distribution of medium solidification. Influence of specific combinations of cytokinins and auxins and their specific concentrations in plant nutrient medium is playing a key role in switching a specific mode of banana micro-propagation like as organogenesis, shoot induction and shoot multiplication. These specialized talks adjust the accumulation of specific intra-cellular biocontents, which triggers the development of specific mode of plant development.

Inspite of phytohormones, medium solidification is also playing important role in enhancing efficiency of banana micropropagation rate. Like as low concentration of phytagel increases shoot multiplication rate, while optimization of both phytohormones and medium solidification encourages to develop normal plantlets.

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