Improvement of shoot-tip culture proliferation in banana using PEG6000

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Abstract

In order to improve the shoot proliferation rate of ‘Dwarf Cavendish’ banana shoot tip cultures, polyethylene glycol (PEG_{6000}) was employed at concentrations of 2, 4, and 6% (w/v). The medium without PEG was considered as negative control. An increasing of shoot proliferation was achieved in MS medium containing 2% PEG. Furthermore, application of 2% PEG significantly increased the shoot dry weight (DW) as well as root/shoot DW ratio of the regenerated explants. The root/shoot DW ratio, chlorophyll a and chlorophyll b contents of leaves were decreased by the increase in PEG percentage. Application of PEG at high concentrations increased the leaf tissue chlorosis and necrosis. The proline and potassium contents of leaf tissue were increased in regenerated shoots from the media containing 2 and 4% PEG. Application of 2% and 4% PEG increased ex vitro plantlets survival rate up to 34% and 40%, respectively compared to the control. In conclusion, the mild osmotic stress caused by the application of PEG 2% improved the in vitro shoot proliferation rate accompanied by an increase in the ex vitro survival rate of regenerated plantlets. Seemingly, the promising results using PEG_{6000} at the resultant level (2%) are in the main part due to the stress simulation effects and later plantlets’ acclimatization through physio-biochemical mechanisms.

Keywords: Musa spp; osmotic stress; tissue culture; proline

Abbreviations: Chl: Chlorophyll; DW: Dry Weight; FW: Fresh Weight; PEG: Polyethylene Glycol; RWC : Relative Water Content ; TW :Turgor weight.


Introduction

Banana is growing worldwide in more than 100 countries throughout the tropic and sub-tropic regions of the world. Nowadays, the global markets are dominated by ‘Dwarf Cavendish’ cultivar due to its public favorite, transport feasibility, and unripe picking up (INIBAP, 2013). This cultivar is triploid and cannot produce fertile gametes. Therefore, the plants are generally propagated vegetatively via the asexual procedures. But, the limited number of propagules from a maternal donor, the long period of propagation cycle, serious damages of

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Received: January, 2016
Accepted: May, 2017
individual plant by the diseases and the non-
homogenous growth of propagated plants are the 
main disadvantages of the traditional asexual 
propagation of banana. Currently, tissue culture 
techniques are routinely used in banana 
propagation. Continuous efforts have been made 
to improve the efficiency of the propagation 
through improvements in the culture media 
compositions. Ngomuo et al. (2014) reviewed the 
*in vitro* propagation techniques for producing 
banana using shoot tip cultures. Hussein (2012) 
studied the effects of nutrient media constituents 
on growth and development of banana (Musa 
spp.) shoot tips cultured *in vitro*. He found that BA 
concentration at 7.0 mg/l significantly promoted 
bud proliferation. In the same manner, Kacar et al. 
(2010) investigated the effects of different culture 
vessels and gelling agents on the multiplication of 
banana explants. However, debate continues 
about the best strategies for improving the 
efficiency of the tissue culture propagation 
methods for this highly valued crop.

Polyethylene glycol has been commonly 
used in plant tissue culture to induce controlled 
water stress because it modifies the 
osmotic potential of the medium and induces 
plant water deficit in a relatively controlled 
manner (Bidabadi et al., 2012 a; Putnik-Delic et al., 
2013). In an attempt to develop tolerant lines of 
banana for water stress, shoot tips were subjected 
to a stepwise selection procedure contained 
different levels (10, 20 and 30 g L⁻¹) of PEG-induced 
water stress (Bidabadi et al., 2012 b). They showed 
that water stress adversely affected fresh weight 
increase, shoot vigor and multiplication rate. 
However, a banana line L2-5 on the medium 
fortified with 30 g L⁻¹ PEG, had significantly the 
greatest fresh weight increase. The culture of 
tomato callus under conditions of water stress, 
which was induced by addition of PEG6000 in the 
medium, decreased relative growth rate and increased dry matter content in all treatments 
compared with the control (Aazami et al., 2010). 
Also, the study showed that in all studied cultivars, 
proline levels increased in response to water 
stress. Callus induction, proliferation and plantlet 
regeneration from the unexpanded spindle leaf 
sheath explants were declined with increasing the 
PEG level (Begum et al., 2011). Therefore, in this 
study we investigated the effects of PEG6000-
induced osmotic stress on shoot tip proliferation 
of *Musa spp.* cv. Dwarf Cavendish. We also 
examined PEG-induced acclimatization effects on 
*ex vitro* survival rate of regenerated plantlets.

**Materials and Methods**

Plantlets of banana, *Musa spp.* cv. Dwarf 
Cavendish, under *in vitro* culture condition were 
provided by Hessami Institute of Tissue Culture 
(Karaj, Iran) and transferred to the Horticultural 
Biotechnology Lab in the University of Zanjan. 
Twelve shoot tips (about 14 mm in length) were 
re-cultured in solid MS medium (Murashige and 
Skog, 1962) aseptically. Micropropagation 
medium consisted of the MS basal medium containing 7.4 g L⁻¹ Na₂EDTA (Merck), 5.57 g L⁻¹ 
FeSO₄ (Merck), 30 g L⁻¹ sucrose (Merck), and 6.5 
g L⁻¹ agar (Sigma). The medium was supplemented 
with 5 mg L⁻¹ BA, 1 mg L⁻¹ IAA, 20 mg L⁻¹ 
glycin, 5 mg L⁻¹ nicotinic acid, 5 mg L⁻¹ pyrodoxal 
hydrochloride, 1 mg L⁻¹ thiamin, and 100 mg L⁻¹ myo-inositol. The final pH of the media was adjusted to 5.7 with 0.1 
N NaOH, and then autoclaved at 121 °C for 20 min. 
Osmotic stress in the reference medium was 
induced by adding 2, 4 and 6% (w/v) of PEG6000 
resulting in 0.04, -0.08 and -0.12 MPa osmotic 
potential in the media, respectively. The medium 
without PEG was considered as negative control. 
Three explants were transferred to each 
experimental unit. The cultures were maintained 
at 24 °C under 16 h illuminations. After eight 
weeks of explants growth, the proliferation rate 
(No. of shoots per explants), root and shoot dry 
weight (DW) and shoot DW/FW (fresh weight) 
ratio, No. of leaf abscission and shoots length (cm) 
were measured. Also, different physiological 
parameters including RWC, free proline, 
chlorophyll a (chlₐ), chlorophyll b (chlₐ) and 
potassium content in the leaf tissue were 
analyzed. Leaf chlorosis and necrosis were visually 
assessed. Measurement of the RWC was 
performed according to Bidabadi et al., (2012 a), 
and was calculated using Equation 1.

Equation (1):

\[
\text{RWC(\%) = } \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DT}} \times 100
\]

where, FW, DW and TW represented fresh, dry, and turgor weights of 
leaf tissue, respectively.
Proline concentration of leaves was spectrophotometrically measured as described by Bates (1973) at 520nm using spectrometer (Milton ROY-21D). Free proline concentration was calculated in µmol/gr FW using proline standard solution according to equation 2.

Equation (2):

\[
\text{Proline} = \frac{\text{Proline (µ mol/ml) x Toluene (ml)} \times 155.5}{\text{Sample FW (gr)}}
\]

Fresh leaf samples were removed from in vitro regenerating shoot tips for chlorophyll measurement. The chlorophyll pigments were extracted with 90% acetone and later were assayed for their content spectrophotometrically (spectrometer model Milton ROY-21D), and calculated using Equation 3 and 4 given by Porra (2002).

Equation (3):

\[
\text{Chl, mg}^{-1}\text{FW} = [12.7(A_{645}) - 2.69(A_{663})] \times (V/1000) \times W
\]

Equation (4):

\[
\text{Chl, mg}^{-1}\text{FW} = [22.9(A_{645}) - 4.68(A_{663})] \times (V/1000) \times W
\]

in which, V is extract volume (ml), W; leaf sample weight (g), FW; leaf fresh weight (g); A_{663}; light absorbance at 663 nm and A_{645}; light absorbance at 645 nm. Leaf potassium content was measured based on standard potassium solution using flame photometer instrument (Jeneway PFP7-England).

To measure the survival rate of regenerated explants, the plantlets were transplanted into plastic pots containing a mixture of perlite and sand (1:2 V/V) in the greenhouse. Explants regenerated from the shoot tips cultures of 6% PEG were not transplanted successfully because of the severity of root and shoot necrosis. The transplant rate was evaluated by the number of survived plants of each treatment after four weeks growing in 75% relative humidity and 25±1 °C. The experiment was conducted as completely randomized design with four replications. Analysis of variance was carried out by SAS.9.0 and means were compared using Duncan’s multiple range test at 1% probability level.

Results

Application of PEG in high concentrations, especially 6% PEG caused severe leaf tissue chlorosis and necrosis (Fig. I). The proliferation rate was suppressed up to 90% using 6% PEG compared with control. Although explants showed good appearance in control medium however, high number of explants per shoot tip culture was achieved in 2% PEG and the proliferation was increased by increasing of PEG towards (Fig. II). Based on the results, mild osmotic stress induced by 2% PEG had promotive effect on shoot proliferation rate. Using 2% PEG in shoot tip culture medium increased the shoot DW/FW as well as root/shoot DW ratios of regenerated plantlets significantly. However, these ratios were declined by any increase in the PEG concentration (Table 1). The length and RWC of regenerated plantlets were decreased by increasing the PEG concentration. Two percent PEG didn’t affect the content of chla in explants compared with control. However, the chlb content was increased by 2% PEG (Table 1).

The proline content in leaf tissue of regenerated shoots was increased by PEG treatments and the maximum amount was achieved using 4% PEG (Fig. 3 A). In this study, the highest K⁺ content was achieved by 2% PEG (Fig. III B).

Discussion

Bidabadi et al. (2012a), reported the decreasing of shoot tip proliferation rate induced by the increasing of PEG in Musa acuminata cv. Berangan. Interestingly, our results showed contradictory findings about proliferation rate in medium containing 2% of PEG which were higher, even those than control medium. These contradictory finding could be mainly in part due to the genetically differences among banana cultivars. It is encouraging to compare these results with that were found by Benderradji, et al. (2012). They showed that moderate heat stress (30°C) increased the embryonic capacity of wheat callus in cv. Mahon-Demias. However, the heat stress with its all levels decreased the embryonic callus efficiency in cv. Hidhab. To screen the Musa biodiversity for drought tolerant varieties,
Vanhove et al. (2012), developed a screening test for in vitro plants. The 'ABB' variety showed the smallest stress induced growth reduction in comparison to other varieties.

Extended root development and raised root/shoot dry weight ratio are the at-hand mechanisms of plants to cope with the drought stress (Mohammadzade, Z. and Soltani, 2015). Our finding is in agreement with Putink-Delic, et al. (2013) findings whom showed the reduction of fresh weight, tissue water content and axillary bud formation in sugar beet explants because of drought stress imposed by increasing of PEG to 5% (w/v). Both chl_a and chl_b contents were decreased by increasing the PEG concentration. These results are consistent with those of Bidabadi et al. (2012 a), who reported the decrease in chl_a and chl_b contents in regenerated plantlets of banana by increasing the PEG concentration. The chl_b hold a pivotal role in plant photosynthesis system. In

![Image of plant samples](Fig. 1. Shoot proliferation, chlorosis and necrosis of ‘Dwarf Cavendish’ banana leaves growing at MS medium containing A (0%), B (2%), C (4%) and D (6%) of PEG_6000.)
Applying PEG6000 for banana shoot-tip culture

In the current work, the regenerated shoots from 2% PEG treatment were retained their common photosynthesis activity by the maintenance of appropriate chl$_a$ level as well as by the increasing chl$_b$ levels in the leaves.

The proline content in leaf tissue of regenerated shoots was increased by PEG treatments. These results corroborated those of Putink-Delic, et al. (2013), who reported the increasing of leaf proline content of sugar beet explants by PEG. Bidabadi et al. (2012 a), reported the increase in proline content of banana explants resulted by the application of 3% PEG in culture medium. The level of proline which accumulates varies greatly and is highly dependent on the plant species (Burritt, 2012). Proline is considered to act as an osmolyte, a ROS scavenger, and a molecular chaperone stabilizing the structure of proteins, thereby protecting cells from damage caused by stress (Krasensky and Jonak, 2012).

Potassium has an important role in controlling transpiration levels and also it causes the continuity of water and other nutrient absorption through root system (Wang et al., 2013). The potassium content of banana explant at 4% PEG was less than those of growing at 2% PEG. This result may be explained by the fact that 2% PEG resulted in a mild osmotic stress in comparison with its higher levels. Based on this, the plantlets were able to adjust their tissue osmotic potential to maintain the optimum physiological activities by increasing the potassium absorption.

The 2 and 4% PEG in banana shoot tip culture medium were increased the ex vitro plantlets survival rate up to 34% and 40%, respectively. However, 4% PEG had no positive effect on the majority of plantlet growth and physiological traits particularly shoot proliferation rate. In sugarcane tissue culture for as an example, callus induction, proliferation and plantlet regeneration was reduced with the increased levels of PEG. However, the ex vitro survival rate of regenerated plantlets was increased due to their more likely drought tolerance (Begum et al., 2011). The shoot and root systems of plants produced in vitro are fragile and vulnerable. Therefore, the transfer of plantlets from the in vitro to the ambient environment can be a challenge that may result in death or damage to a large percentage of micropropagated plants (Preece, 2010). Huang et al. (2011), studied the effects of acclimatization on Aechmea fasciata plantlet growth. They showed that those plantlets grown under a middle light intensity (50 μmol.m$^{-2}$s$^{-1}$) showed a dramatic increase in survival percentage and the maximum number of newly developing roots. In the case of banana, in vitro plantlets acclimatization caused by mild osmotic stress using 2% PEG, which affected the ex vitro plantlets survival by increasing their

**Table 1**

<table>
<thead>
<tr>
<th>PEG 6000 (%)</th>
<th>No. of leaf abscission</th>
<th>Shoot length (cm)</th>
<th>RWC (%)</th>
<th>Shoot DW/FW ratio</th>
<th>Root/Shoot DW ratio</th>
<th>Chl$_a$ (mg.g$^{-1}$ FW)</th>
<th>Chl$_b$ (mg.g$^{-1}$ FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.41±0.23</td>
<td>0.19±0.04</td>
<td>1.47±0.2</td>
<td>0.43±0.07</td>
<td>0.49±0.02</td>
<td>88±2.3</td>
<td>7.53±0.75</td>
</tr>
<tr>
<td>2</td>
<td>0.66±0.12</td>
<td>0.25±0.02</td>
<td>1.35±0.3</td>
<td>0.54±0.04</td>
<td>0.53±0.08</td>
<td>67±2.8</td>
<td>6.22±0.32</td>
</tr>
<tr>
<td>4</td>
<td>2.16±0.41</td>
<td>0.11±0.03</td>
<td>1.25±0.1</td>
<td>0.35±0.02</td>
<td>0.39±0.04</td>
<td>55±1.8</td>
<td>5.82±0.22</td>
</tr>
<tr>
<td>6</td>
<td>4.25±0.54</td>
<td>0.09±0.01</td>
<td>0.9±0.07</td>
<td>0.33±0.04</td>
<td>0.33±0.02</td>
<td>47±0.9</td>
<td>5.56±0.6</td>
</tr>
</tbody>
</table>

†Means with the same letter in each column had not significantly different at 1% probability

**Fig. III.** Mean comparison of leaf Proline (A) and Potassium (B) contents of Dwarf Cavendish’ banana plantlets growing at different concentrations of PEG6000 in MS culture medium

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**Fig. II.** Trend monitoring of PEG6000 effect on proliferation No. of ‘Dwarf Cavendish’ banana shoot tip culture
root/shoot dry weight ratio as well as high proline and potassium contents of leaves.

As a conclusion, the mild osmotic stress caused by the application of 2% PEG in ‘Dwarf Cavendish’ banana tissue culture medium showed stimulatory effect on the adventitious shoot tip proliferation. It also increased the ex vitro survival rate of regenerated plants by influencing on their acclimatization process through growth and physiological mechanisms.

References


Preece, J.E. 2010. 'Acclimatization of plantlets from in vitro to the ambient environment'. In 'Encyclopedia of Industrial Biotechnology' (ed.). Wiley online library, DOI: 10.1002/9780470054581.eib593


'Screening the banana biodiversity for drought tolerance: can an in vitro growth model and proteomics be used as a tool to discover tolerant varieties and understand homeostasis'. Front. Plant Sci. 3: 1-10.
