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EFFECT OF SALINITY ON THE MICRO-PROPAGATION EFFICIENCY IN BANANA (*Musa* spp.)

Ikram-ul-Haq*, Faheeda Sumroo and Muhammad Umar Dahot
Institute of Biotechnology and Genetic Engineering (IBGE), University of Sindh, Jamshoro
*Email: ikramnibge@yahoo.com

Abstract

To know what a physio-chemical situation in the micro-propagating banana variety Basrai under saline conditions, an experiment was conducted. *In vitro* plantlets were developed by culturing meristematic stem tips (explant) for 3 weeks on organogenesis (8.0 µM BA; 10.0 µM IAA; 3.0 g l^{-1} phytagel), 3 weeks on shoot induction (15.0 µM BA; 1.0 g l^{-1} phytagel) and 4 weeks on shoot multiplication (10.0 µM BA; 2.0 g l^{-1} phytagel) media. The micro-propagating plantlets were cultured on 3 different saline conditions (0 mM, 50 mM and 100 mM NaCl), variable morpho-biochemical characters were observed. With the increase in NaCl stress; Ca^{2+}, K^{+}, total protein, explant proliferation, fresh weight and dry weight were decreased while Na^{+}, Cl^{-} and reducing sugar contents were increased, however reversed situation was noted in plantlets cultured on normal micro-propagating medium. 80% > plantlets were rooted in the presence of 0.5 mg l^{-1} IBA while reduced to >10% and >1% at 50 mM and 100 mM NaCl, respectively.

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

Abbreviations: MS: Murashige and Skoog medium, IAA: Indole acetic acid. IBA: Indole butyric acid. BA: Benzyle amino-purine, NaCl: Sodium chloride.

INTRODUCTION

Banana is one of the most important cash crops with annual production ~102 million tonnes per year (FAO, 2002). Its increasing demand is because, rich with various carbohydrates and minerals equally beneficial for both child and adults (Vuylsteke and Ortiz, 1996). However, the banana production is going to be decreasing due to a number of biotic and a-biotic constraints. Because of limited availability of pathogen free plant material has prompted the interest for the use of aseptic culture techniques. Through which such disorders may be reduced/eliminated from the desired crops.

An efficient plant micro-propagation is possible under *in-vitro*, not *in-vivo*. 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 cytokines 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 (light, temperature, pH, salinity and/or drought) of the growth medium. Among the physical conditions, salinity is one,

which has a serious effect on plant micro-propagation rate. It is not effective only under natural but also under *in-vitro* conditions. While a temporary immersion system during shoot induction causes to increase the shoot multiplication efficiency (Alvard et al., 1993; Escalona et al 1999). Both hormones and physical conditions are playing very important role in developing a specific mode of growth and other for its progress during plant micro-propagation.

MATERIALS AND METHOD

Meristematic stem tips of banana (*Musa* spp.) cv. Basrai 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 (3x5 min) with sterile distilled water.

The micro-propagation cultures were establish by culturing micro-cuttings of stem for 3-weeks on MS [Murashige and Skoog, (1962) basal medium supplemented with vitamins B_5 (Gamborg et al 1968); 3% sucrose] medium, supplemented with 10 µM IAA and 8 µM BA and solidified with 3.60 g l^{-1} phytagel for organogenesis while shoot induction was carried on MS with 15 µM BA and 1.0 g l^{-1} phytagel. After 2-weeks, organogenised explants were shifted to the shoot multiplication medium (MS with 15 µM BA and 1.0 g l^{-1} phytagel) for 28-days (Figure 1). The established cultures on shoot multiplication cultures were sub-cultured after 4-weeks by subdividing the clustered mass of plantlets (Figure 2d) into micro cuttings, according to the number of plantlets per explant with a sterile scalpel. The number of plantlets per explant, pseudostem diameter, plant height, fresh and dry wt of the plantlets were measured on each culture.

The root induction was carried out by culturing onto $\frac{1}{2}$ MS basal medium supplemented with 0.10 mg l^{-1} IBA.

The micropropagating plantlets were sub-cultured under different saline stresses. Three levels of salinity i.e. 0 mM, 50 mM, 100 mM and 150 mM NaCl were maintained in micro-propagation medium by keeping one control (plant multiplication medium with out salinity).

All cultures were supplemented with 20.0 µM L-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-weeks, the plantlets 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^+ $Mg^{2+}$and Ca^{2+}) as by Malavolta et al., (1989) methods. 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 measured according to Lowery et al. (1951) method. The praline (Magne and Larker, 1992), glycinebetaine (Bessieres et al., 1999) and phenol (Ozyigit et al., 2007) were also measured.

For nitrate determinations, plantlets were dried at 70ºC in an air-forced oven 48 h, and ground to pass 40 meshes. Nitrate was determined by the method of Cataldo et al. (1975).

RESULTS AND DISCUSSION

The shoot multiplication medium used for the experiment was non-saline with favourable properties for the plant micro-propagation (Table 1). The salinity levels maintained by single salt (NaCl) environments while under natural saline conditions there is always a mixture of different ions. It was observed that the numbers of plantlets were decreased significantly less than 150 mM NaCl during 28 days of growth. The plant height was also decreased under saline conditions during the whole growth period, while non-significant effects of salinity were measured in fresh weight of plantlets. However the pseudostem diameter and dry weight of the plantlets were decreased with the increase in saline stress. The carbohydrate contents were measured higher in the cultures which were maintained on the normal shoot multiplication medium. Consequently, it

provided extra energy to overcome salt stress experienced by the plant for a certain extent but decreased with the increase in salinity levels.

Protein contents were decreased significantly at level of 150 mM NaCl, it was also noted that both praline and glycinebetain contents were increased significantly, where protein and carbohydrates were decreased significantly. However the reducing sugars contents were increased with the increase in saline severity.

Data on proline accumulation pattern – its retention or leaching in tissue was subjected to NaCl stress-indicated that along with the inorganic ions, this organic osmolyte was also a osmotic modulator responsible for protecting the tissue from stress injury. Inorganic ions are known to act as the first line of defence to combat the NaCl-stress before the organic components become activated (Jain and Selvaraj, 1997). Apparently, low proline content under control and a higher praline level under saline conditions indicated that a high level of proline along with high Na^+ and K^+ concentrations were responsible for imparting salt tolerance to the tissue of the tolerant variety. That proline is a potent osmoregulator in plant tissues has been shown by Basu et al. (1995; 1999), contrary to the finding that proline accumulation seemed to be a symptom of injury rather than an indicator of salinity resistance (Lutts et al., 1999). Like the K^+, praline was also leached out. But, NaCl shock resulted in almost a six-fold increase of total proline content in the cultured plantlets. Decline to incline of total proline from low NaCl stress to and its sudden increase during shock treatment could be explained by the fact that the critical point of proline accumulation in the salt stressed plantlets might be high (Jain et al., 1991) and proline was overproduced only when the degree of the stress was above that critical point.

Potassium ions are known to be a major component of osmotic adjustment during stress (Wu et al., 1996); but the data of the present experiment for K^+, Na^+ in stressed apparently was not consistent with the earlier finding of Dvorak and Gorham (1992) and Watad et al. (1991). However, plantlets on normal micro-propagation cultures were inherently rich in K^+ in comparison to that of saline cultures (Table 2), so, these ions were released in an environment rich in NaCl. The presence of a high concentration of K^+ in control one, which was supposed to act as the natural inorganic osmoregulator perhaps allowing Na^+ to enter in the callus which explains the situation of lower K^+ content after shock treatment as the physiological 'window' of optimum K^+ concentrations narrows in the presence of increasing amounts of Na^+ (Marschner, 1995; Maathius and Amtmann, 1999). A higher level of endogenous K^+ content was also reported in Nona Bokra, another salt-resistant cultivar (Lutts et al., 1999).

Statistical analyses of the data derived from the contents of Na^+, K^+ and proline in different tissues – either untreated or shock treated, and correlating especially the contents those retained within the tissues after NaCl-shock with the data of tissue growth (the visible indicator of tolerance) – lead us to deduce that: – Accumulation of either inorganic (K^+ and Na^+) and/or organic solute (proline) in tissues was correlatable and the relative presence of these components in tissues after the shock treatment were important to support differential regrowth capacity of the shock treated tissues; – presence or retention of K^+ was a key factor for the indication of the non saline stress. It was observed to be positively correlated with the growth of the plantlets. However, the correlation between growth and Na^+ was negative, when it increases the growth of the plantlets decreases significantly. While a positive correlation praline, which was negative for plant growth.

These results probably indicate that accumulation of K^+ is the first option to counteract the negative water potential of the external medium, while accumulation of proline is probably the last metabolic device that the plant tissues chemical marker for when those are being challenged by osmotic stress.

The nitrate concentration in the micro-propagating plantlets was not significantly influenced by 50 mM NaCl. At 150 mM NaCl, nitrate concentration was reduced highly significantly in comparison with the control (Table 2). Salinity interferes with N acquisition and utilization. Saline conditions can influence the different steps of N metabolism, such as uptake; reduce ion and protein synthesis that may be responsible, at least in part, for the observed

reduction in plant growth rate (Frechill et al., 2001). Salinity affects nitrate uptake at two levels: by direct competition of chloride with nitrate and at the membrane level and/or the membrane proteins by changing plasmalemma integrity (Cramer et al., 1985).

Maximum phenol contents were measured on control while decreased with the increase in saline stress. A negative correlation between phenolics and saline stress was observed.

The phenol exudation, medium discoloration and rapid explant browning of the culture. The synthesis of phenol is dependent on phenol oxidase (POD) and polyphenol oxidase (PPO) activity (Cox, 1996; Laukkanen, 1999; Thomas and Ravindra, 1999). Literature indicates that the role of a phenolic depends on its chemical structure, plant species, the biological process in study (organogenesis or somatic embryogenesis) and its developmental step. We believe that metabolized phenols affect tissue culture systems positively with auxin metabolism (rapid cell division and synthesis of the cell wall and other related components). But oxidized phenols turn into highly toxic quinones and polymerized material causing discoloration of the medium and death of the explants. It will be very useful to find out the least phenol synthesizing germination age for some problematic species (in tissue culture studies) like cotton, mango, coconut, banana etc. This study will help in the establishment of tissue culture and a criterion for the soil selection for the cultivation of banana. The banana is a much sensitive crop for saline factor, which is the main reason for the lower regeneration efficiency in it under *in vivo* growth.

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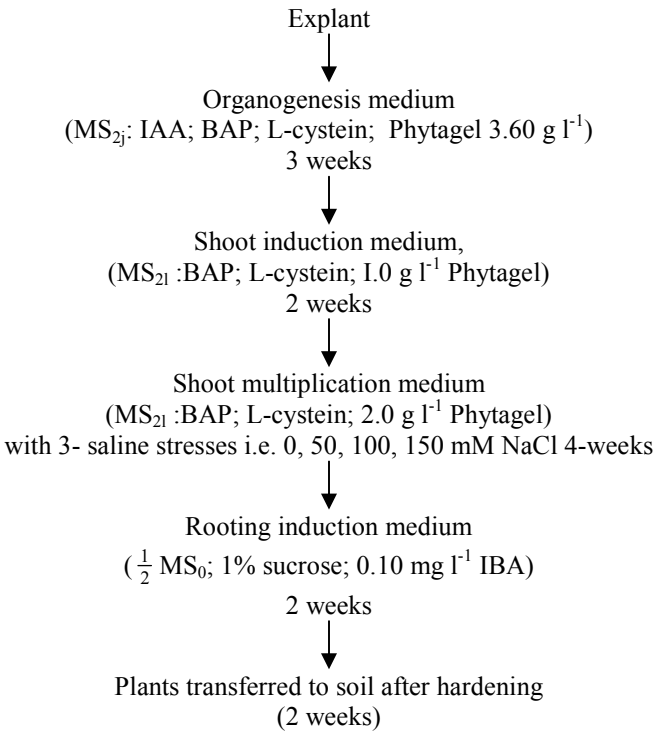


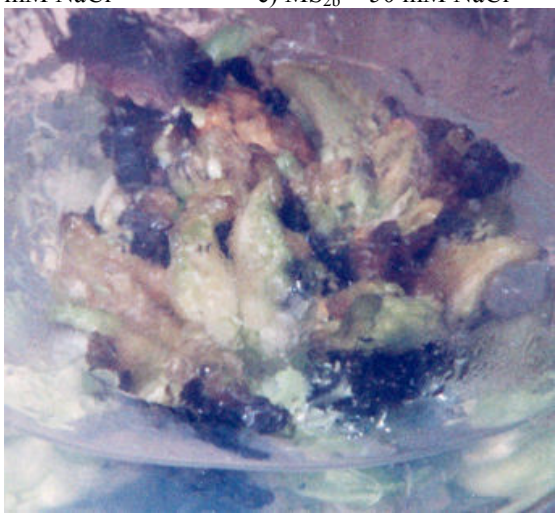
Figure 1: Schematic representation of the optimized protocol for the micro-propagation of the banana (*Musa* spp.) cv. Basrai

a) MS_0 + 0 mM NaCl

b) MS_{2b} + 0 mM NaCl

c) MS_{2b} + 50 mM NaCl

d) MS_{2b} + 100 mM NaCl



e) MS_{2b} + 150 mM NaCl

Figure 2: The plantlets micro-propagation on shoot multiplication medium supplemented with different levels of NaCl

Table 1: Effect of NaCl under *in-vitro* conditions for 28-days culture on some morphological characteristics of banana (*Musa* spp.) cv. Basrai

	Parameters	MS_0	MS_2	MS_2+50 mM NaCl	MS_2+100 mM NaCl	MS_2+150 mM NaCl	Mean values (ANOVA)
a)	Number of plantlets	5.40±0.219	6.01±0.837	2.42±0.219	4.23±0.769	2.76±0.820	19.9581***
b)	Plant height (cm)	2.14±0.078	2.94±0.586	2.86±0.654	1.44±0.320	1.58±0.338	2.1964*
c)	P diameter (cm)	0.321±0.023	0.262±0.036	0.258±0.054	0.282±0.052	0.165±0.036	0.0011*
d)	Fresh weight (g)	0.262±0.115	0.671±0.242	0.428±0.067	0.333±0.076	0.198±0.038	0.1646^{ns}
e)	Dry weight (g)	0.046±0.006	0.032±0.009	0.022±0.007	0.015±0.009	0.009±0.003	0.0221*

* P diameter: Pseudostem diameter

Table 2: Effect of NaCl under *in-vitro* conditions for 28-days culture on some biochemical contents of banana (*Musa* spp.) cv. Basrai

	Parameters	MS_0	MS_2	MS_2+50 mM NaCl	MS_2+100 mM NaCl	MS_2+150 mM NaCl	Mean values (ANOVA)
A. Organics (mg/g)							
a)	Total protein	1.165±0.02	1.14±0.01	1.203±0.030	1.991±0.029	0.574±0.032	1.2767***
b)	Reducing sugars	0.460±0.018	0.367±0.01	0.406±0.019	0.403±0.01	0.510±0.023	0.0168***
c)	Total sugars	8.45±0.142	7.90±0.11	6.35±0.161	8.301±0.145	3.152±0.128	24.6768***
d)	Proline (µmol/g)	6.28±0.50	5.92±1.00	6.83±1.18	7.65±0.08	8.06±1.10	2.4347***
e)	Glycinebetain	7.12±0.72	6.84±0.80	7.46±1.00	8.82±0.07	9.05±0.86	3.0628***
f)	Phenol	0.232±0.65	0.212±0.25	0.256±0.05	0.27±0.16	0.292±0.35	0.0030***
B. Inorganics(mg/g)							
a)	a Na^+	3.92±0.90	3.52±1.50	4.18±0.95	7.06±1.21	9.58±1.30	20.3428***
b)	b K^+	7.97±1.50	10.21±1.20	8.43±0.80	6.29±0.95	5.25±1.81	41.0807***
c)	c Ca^{2+}	7.89±0.75	8.92±0.81	7.07±1.02	6.92±0.80	6.05±1.75	3.5401**
d)	d Cl^-	4.50±1.20	4.39±1.50	5.17±1.48	8.68±1.10	11.52±1.20	29.6657***
e)	e NO_3^-	1.488±0.01	1.54±0.02	1.517±0.01	1.488±0.01	1.546±0.003	0.0037***