Micropropagation of Banana: Reversion, Rooting, and Acclimatization of Hyperhydric Shoots

M.E. El-Mahrouk and A.R. El-Shereif

Department of Horticulture, Faculty of Agriculture, Kafrelsheikh University, Kafr El-Sheikh 33516, Egypt

Y.H. Dewir¹

Department of Horticulture, Faculty of Agriculture, Kafrelsheikh University, Kafr El-Sheikh 33516, Egypt; and Plant Production Department, College of Food and Agriculture Sciences, King Saud University, Riyadh 11451, Saudi Arabia

Y.M. Hafez and Kh. A. Abdelaal

EPCRS Excellence Center, Plant Pathology and Biotechnology Laboratory, Department of Agricultural Botany, Faculty of Agriculture, Kafrelsheikh University, Kafr El-Sheikh 33516, Egypt

S. El-Hendawy

Plant Production Department, College of Food and Agriculture Sciences, King Saud University, Riyadh 11451, Saudi Arabia; and Agronomy Department, Faculty of Agriculture, Suez Canal University, Ismailia 41522, Egypt

H. Migdadi and R.S. Al-Obeed

Plant Production Department, College of Food and Agriculture Sciences, King Saud University, Riyadh 11451, Saudi Arabia

Additional index words. abnormalities, calcium nitrate, electrolyte leakage, Musaceae, stomata

Abstract. Hyperhydricity is a physiological disorder impacting plant growth and multiplication and acclimatization of regenerated plantlets. We report the use of calcium nitrate for reversion and acclimatization of banana ‘Grand Naine’ hyperhydric shoots cultured on Murashige and Skoog medium containing agar or gellan. Although 100% rooting of hyperhydric shoots occurred at all concentrations of calcium nitrate, only 50% rooting was recorded in the absence of calcium nitrate. Electrolyte leakage decreased significantly in the reverted banana tissues compared with the hyperhydric tissues. Histochemical staining for reactive oxygen species indicated that reverted banana tissues possess lower levels of both hydrogen peroxide (H₂O₂) and superoxide (O₂⁻) than do hyperhydric tissues. Rooting, growth, and survival of the reverted banana plantlets were significantly influenced by calcium nitrate concentrations as well as the type of gelling agent. Reverted banana plantlets in medium containing calcium nitrate (0.5–1 g·L⁻¹) were acclimatized with 100% survival in a growing substrate of peatmoss and vermiculite (1:1).

Banana (*Musa* spp. AAA; Musaceae) is an economically important fruit crop in tropical and subtropical countries, and it ranks as the largest fruit crop produced worldwide (FAOSTAT, 2017). The plant is vegetatively propagated using corms and suckers, allowing for the spread of diseases. There has been a shift toward cyclic replacement with new plantation because the yield starts to decline after 3 to 5

years and then declines rapidly after 10 to 15 years (Singh et al., 2011). To overcome disease spread through vegetative propagation and to meet the commercial demand, plant tissue culture techniques have been routinely used for propagation. Several reports have reviewed the biotechnological improvements and progress in banana tissue culture, thereby highlighting clonal mass propagation through direct and indirect regeneration pathways (Deepika et al., 2018; Rout et al., 2000; Strosse et al., 2004). Developing efficient protocols for banana tissue culture is the foundation for producing high-quality and pathogen-free planting materials and reducing production costs.

Hyperhydricity has been described as a physiological disorder of tissue-cultured plants whereby the hyperhydric propagules become translucent due to excessive hydration of tissues and exhibit glassy morphology

(Dewir et al., 2014; Kevers et al., 2004). The limited aeration and ethylene accumulation and the high relative humidity inside the tissue culture container create an unsuitable environment for plant growth and induce physiological abnormalities such as hyperhydricity (Chakrabarty et al., 2006; Dewir et al., 2005, 2014; Rojas-Martinez et al., 2010). Moreover, cyclic subcultures and prolonged exposure to cytokinins, such as 6-benzylaminopurine (BAP) and thidiazuron, induce hyperhydricity (Dewir et al., 2018; Ivanova and Van Staden, 2011). As a consequence of the plant response to these in vitro stress conditions, the cell metabolism is altered and the production of reactive oxygen species (ROS) is increased (Balen et al., 2009; Franck et al., 1995; Tian et al., 2014) due to changes in the activity of antioxidant enzymes in hyperhydric tissues (Chakrabarty and Datta, 2008; Dewir et al., 2006; Gao et al., 2017a). Increasing evidence suggests a close link between oxidation stress and hyperhydricity (Chakrabarty et al., 2006; Dewir et al., 2006, 2014; Tian et al., 2014), ultimately resulting in plant malformation and malfunctioning. Several approaches, including modifications to the growth medium and improved aeration in the culture container, have been attempted to alleviate or eradicate hyperhydricity in plant tissue culture (Dewir et al., 2014; Hazarika, 2006). Although the majority of studies have focused on the prevention of hyperhydricity, few studies have investigated the reversion of hyperhydric propagules (Gao et al., 2017a, 2017b; Hassannejad et al., 2012; Reyes-Vera et al., 2008; Soundararajan et al., 2017).

Hyperhydricity is a common problem in plant tissue culture that hinders growth, multiplication, and acclimatization of regenerated plantlets (Debergh et al., 1992; Pospisilova et al., 2007). Losses of up to 60% in cultured shoots or explants have been reported due to hyperhydricity in commercial plant micropropagation (Piqueras et al., 2002; van den Dries et al., 2013). Consequently, hyperhydricity can limit the success and efficiency of micropropagation by decreasing the quantity and quality of the tissue-cultured plantlets and increasing the cost of production (Dewir et al., 2014; Gao et al., 2017a; Hazarika, 2006; Kozai et al., 1997). In this study, the effects of calcium nitrate on the reversion of hyperhydric banana ‘Grand Naine’, a commercially important dessert banana of the Cavendish subgroup, and the survival and acclimatization of the reverted plantlets under greenhouse conditions were investigated.

Materials and Methods

Plant material. Shoot tips of banana (*Musa xparadisiaca* L. ‘Grand Naine’) were cyclically subcultured four times (4 weeks per culture cycle) for multiplication on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 3% sucrose and supplemented with 3 mg·L⁻¹ 6-benzylaminopurine (BAP) and 1 mg·L⁻¹ Kinetin (Fig. 1A).

Received for publication 12 Mar. 2019. Accepted for publication 11 Apr. 2019.

The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding this work through research group NO (RGP-1438-012), and the Research Support & Services Unit (RSSU) for their technical support.

¹Corresponding author. E-mail: ydewir@hotmail.com or ydewir@ksu.edu.sa.

The medium was gelled with 0.2% gellan (Dephite, Hannover, Germany). The pH of the medium was adjusted to 5.8 before autoclaving at 121 °C and 118 kPa for 15 min. The cultures were incubated for 4 weeks at 25 ± 1 °C during a 16-h photoperiod at 25 µmol·m⁻²·s⁻¹ photosynthetic photon flux density (PPFD) provided by cool white fluorescent tubes. Ten percent of the proliferated shoots showed symptoms of hyperhydricity during the fifth re-culture. These hyperhydric shoots were used as the plant material for the reversion experiments (Fig. 1B).

Reversion of hyperhydric banana shoots. The hyperhydric shoots were cultured on MS medium supplemented with calcium nitrate [Ca (NO₃)₂] at different concentrations (0, 0.25, 0.50, 0.75, and 1 g·L⁻¹) and 3% (w/v) sucrose. The media were solidified using

0.8% (w/v) agar-agar (Dephite) or 0.2% (w/v) gellan. There were four replicates per treatment. Each replicate represented a culture of five individual shoots, resulting in a group of 20 shoots per treatment. All other culture conditions were as previously described. After 3 weeks of culture, the following parameters were recorded for each explant: rooting percentage, number of roots, root length (seedlings were washed and the longest root was measured), number of leaves, shoot length, and fresh weight.

Determination of chlorophyll content. Chlorophyll was extracted overnight at 5 °C with 5 mL of dimethyl-formamide and determined according to the methods of Moran and Porath (1982) using a double-beam ultraviolet/visible spectrophotometer (Libra S80PC; Biochrom, Cambridge, UK) at

663 nm and 647 nm. Chlorophyll concentration is expressed as mg·g⁻¹ fresh weight of leaves.

Microscopic observation of stoma. For scanning electron microscopy, samples (4 mm²) were obtained from the leaves and fixed in glutaraldehyde (2.5%) for 24 h at 4 °C. Then, they were postfixed in osmium tetroxide (1% OsO₄) for 1 h at room temperature (Harley and Ferguson, 1990). Samples were dehydrated by passing them through increasing concentrations of acetone (30% to 100%). Samples were air-dried until the critical point and sputter-coated with gold. Images were obtained using a JEOL JSM T330A scanning electron microscope (JEOL, Tokyo, Japan).

Histochemical analysis of ROS. Detection of superoxide (O₂⁻) and hydrogen peroxide (H₂O₂) were visualized as blue coloration of nitroblue tetrazolium (NBT) and red-brown coloration of 3, 3-diaminobenzidine (DAB). Cross and longitudinal leaf discs were vacuum-infiltrated with 10 mM of potassium phosphate buffer (pH 7.8) containing 0.1% (w/v) NBT (Sigma-Aldrich, Steinheim, Germany) according to the methods of Adam et al. (1989) or 0.1% (w/v) DAB (Fluka, Buchs, Switzerland). NBT-treated and DAB-treated samples were incubated in daylight for 20 min and 2 h, respectively, and subsequently cleared in 0.15% (w/v) trichloroacetic acid in ethanol: chloroform at 4:1 (v/v) for 1 d (Hückelhoven et al., 1999). Cleared samples were washed with water and placed in 50% glycerol before evaluation. Discoloration of stem discs resulting from NBT or DAB staining was quantified using a Chemilmager 4000 digital imaging system (Alpha Innotech Corp., San Leandro, CA).

Electrolyte leakage. Measurements were performed as described by Szalai et al. (1996) and Whitlow et al. (1992). Leaf discs of hyperhydric tissues and recovered tissues were placed individually in 25 mL of deionized water (Milli-Q 50; Millipore, Bedford, MA). Flasks were shaken for 20 h at ambient temperature to facilitate electrolyte leakage from injured tissues. Initial electrical conductivity (EC) measurements were recorded for each vial using an Acromet AR20 EC

Fig. 1. Reversion, rooting and acclimatization of hyperhydric banana 'Grand Naine' shoots. (A) Normal multiple shoots from the fourth subculture in Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine (3 mg·L⁻¹) and Kinetin (1 mg·L⁻¹). (B) Hyperhydric shoots obtained from the fourth subculture. (C) In vitro reversion and rooting of the hyperhydric shoots in agar (8 g·L⁻¹)-solidified MS medium supplemented with calcium nitrate (0.5 g·L⁻¹) after 3 weeks in culture. (D) Reverted plantlets after 4 weeks of acclimatization.

Table 1. Effects of calcium nitrate on rooting and growth of hyperhydric banana 'Grand Naine' shoots after 3 weeks in culture.

Gelling agent	Calcium nitrate (g·L ⁻¹)	Rooting (%)	Roots (no./plantlet)	Root length (cm)	Leaves (no./plantlet)	Shoot length (cm)	Total chlorophyll (mg·g ⁻¹ fresh wt)	Fresh wt per plantlet (g)
Agar	0.00	50 b ^c	2.7 d	1.1 d	4.3 f	4.4 c	0.15 de	1.09 c
	0.25	100 a	9.0 bc	9.1 ab	5.0 def	6.3 bc	0.26 c	2.86 ab
	0.50	100 a	6.0 cd	12.7 a	7.7 ab	7.7 b	0.38 a	2.67 ab
	0.75	100 a	8.3 bc	12.1 a	7.3 bc	7.6 b	0.29 bc	2.71 ab
	1.00	100 a	11.0 ab	9.8 ab	4.7 def	8.0 b	0.17 d	2.79 ab
Gellan	0.00	50 b	9.3 bc	3.4 cd	5.7 cdef	5.8 bc	0.12 e	1.28 c
	0.25	100 a	8.7 bc	5.6 bc	6.3 bcde	5.3 c	0.13 de	1.93 bc
	0.50	100 a	11.7 ab	9.8 ab	6.7 bcd	7.7 b	0.30 b	3.45 a
	0.75	100 a	13.7 a	11.4 a	8.3 a	10.3 a	0.35 a	2.90 ab
	1.00	100 a	10.3 ab	5.3 bcd	6.7 bcd	7.8 b	0.31 b	2.43 b
Significance								
Type of gelling agent (A)		NS	**	*	*	NS	NS	NS
Calcium nitrate (B)		*	**	**	**	**	**	**
A × B		NS	*	NS	**	NS	**	NS

^aValues followed by the same letter in the same column are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test.

NS, *, **Nonsignificant or significant at $P \leq 0.05$ or 0.01, respectively.

meter (Fisher Scientific, Chicago, IL). Flasks were then immersed in a hot water bath (Fisher Isotemp, Indiana, PA) at 80 °C for 1 h to induce cell rupture. The vials were again placed in an Innova 2100 platform shaker for 20 h at 21 °C. Final conductivity was measured for each flask. The electrolyte leakage percentage was calculated as follows: (initial conductivity/final conductivity) $\times$ 100.

Acclimatization. Banana plantlets at the five-leaf stage were transplanted to culture pots (diameter, 5 cm) filled with a mixture of sterilized peatmoss and perlite (1:2). Each treatment had three replicates, and each replicate was represented by 20 plantlets. The plantlets were covered with a clear plastic film during the first 15 d of culture in a shade-controlled greenhouse and watered with 1 g·L⁻¹ of solution containing 19N-8.3P-15.7K water-soluble fertilizer (Rosasol; Rosier, Moustier, Belgium). The environment of the greenhouse was adjusted to a temperature of 27 $\pm$ 2 °C, 60% to 70% relative humidity, and 100 μ mol·m⁻²·s⁻¹ PPFD. After 4 weeks of acclimatization, the following parameters were recorded for each plantlet: survival percentage, root length (seedlings were washed and the longest root was measured), shoot length, and fresh weight.

Experimental design and statistical analysis. All experiments had a completely randomized design. All data were subjected to an analysis of variance and Duncan's multiple range test using SAS statistical software (version 8.1; SAS Institute, Cary, NC).

Results and Discussion

Reversion and rooting of hyperhydric banana shoots. BAP is a commonly used cytokinin for micropropagation of *Musa* sp. (Bairu et al., 2008; Escalona et al., 2003; Hui et al., 2013; Vuylsteke, 1989). However, in this study, hyperhydricity (10%) was recorded during the fourth subculture (Fig. 1B) of 'Grand Naine' multiple shoots in MS medium fortified with BAP (3 mg·L⁻¹) and Kinetin (1 mg·L⁻¹). High BAP concentrations and/or cyclic subcultures on BAP-enriched media have been reported to induce hyperhydricity in *Musa* sp. (Buah et al., 1999; Jafari et al., 2011) and other plant species, including *Fragaria* $\times$ *ananassa* (Barbosa et al., 2013) and *Thymus daenensis* (Hassannejad et al., 2012). BAP has been associated with the rapid formation of N-glucosides, and its accumulation may enhance severe alterations in in vitro cultures (Bairu et al., 2007; Valero-Aracama et al., 2010).

Rooting and growth parameters (root length, number of leaves, shoot length, chlorophyll content, and fresh weight) of the hyperhydric shoots were significantly improved by the addition of calcium nitrate in the culture medium (Table 1; Fig. 1C). Although 100% rooting occurred at all concentrations of calcium nitrate, only 50% was recorded in the control experiments. The highest values of rooting and growth were obtained on gellan-solidified medium

supplemented with 0.75 g·L⁻¹ calcium nitrate. High rooting and growth were also observed in agar-solidified medium supplemented with 0.5 g·L⁻¹ calcium nitrate. The type of gelling agent also significantly affected the number of roots, root length, and

number of leaves; however, shoot length, chlorophyll content, and fresh weight were not significantly affected. The interaction effect for the type of gelling agent and calcium nitrate significantly influenced the number of roots and leaves and the chlorophyll

Fig. 2. Scanning electron microscopy of stomata in the leaves of banana 'Grand Naine' shoots after 3 weeks in culture. (A and B) Normal and hyperhydric shoots [fourth subculture in MS medium supplemented with 6-benzylaminopurine (3 mg·L⁻¹) and kinetin (1 mg·L⁻¹)]. (C) Reverted shoots [cultured in agar (8 g·L⁻¹)-solidified MS medium supplemented with calcium nitrate (0.5 g·L⁻¹)].

content (Table 1). A previous report by Buah et al. (1999) indicated that the type of gelling agent influenced the growth of banana shoots, mainly due to the physical properties of the medium (i.e., water potential). Moreover, the hardness of gellan-solidified medium decreases when calcium is reduced from 80 to 40 mg·L⁻¹, but it is unaffected in the agar-solidified medium (Cameron, 2001). De Klerk et al. (2017) proposed that chelating compounds excreted by plant tissues liquefy the gellan-solidified medium. Therefore, variations in the growth of banana shoots (Table 1) could be attributed to the enhanced water availability and nutrient uptake in gellan-solidified medium compared with that in agar-solidified medium.

Calcium is associated with several attributes, such as membrane structure and function, ion uptake, interactions with growth regulators, and enzymatic activation (via calmodulin) (Malavolta et al., 1997). The structural function of calcium is characterized by its use in the synthesis of new cell wall, particularly the middle lamellae that separate newly divided cells (Taiz and Zeiger, 2006). Calcium deficiency is well-known in the hyperhydric tissues of *Dianthus caryophyllus* (Kevers and Gaspar 1986) and *Petunia hybrida* (Zimmerman et al., 1988). Machado et al. (2014) demonstrated that the addition of calcium chloride (1.32 g·L⁻¹) to the MS culture medium reduced hyperhydricity in *Lavendula angustifolia* shoots from 23% and 30% to 6% and 1.3% in the first and second subcultures, respectively. Similar findings in *Cydonia oblonga* (Singha et al., 1990) and *Solanum tuberosum* (Sha et al., 1985) indicated that increases in calcium improve plant growth and reduce or eliminate deformities such as hyperhydricity and shoot tip necrosis in cultures.

Supplementation of growth media with calcium nitrate improved the chlorophyll content in the reverted banana shoots (Table 1). A decrease in the intensity of the chlorophyll pigment in the hyperhydric shoots of *Fragaria × ananassa* (Barbosa et al., 2013), *Thymus daenensis* (Hassannejad et al., 2012), and *Vanilla planifolia* (Sreedhar et al., 2009), compared with that in normal shoots has been reported. This decrease in chlorophyll concentration may be due to fewer chloroplasts in the hyperhydric leaves or the damaging effects of hyperhydricity on thylakoid membranes (Chakrabarty et al., 2006; Marschner and Possingham, 1975). The malformed non-functional stomata is a common abnormality in hyperhydric shoots (Apóstolo and Llorente, 2000; Barbosa et al., 2013; Gribble et al., 1996; Olmos and Hellin, 1998). Our results indicated the presence of widely open deformed stomata in the hyperhydric banana shoots (Fig. 2B), thus indicating abnormal functioning of stomata compared with that in normal and reverted shoots (Fig. 2A and C). Unlike the typical elliptical-shape cells found in normal and reverted banana shoots, the stomata in hyperhydric shoots are nearly round, with deformed guard cells (Fig. 2B). Guard cell deformity could be due to the

excessive water absorption leading to turgidity, consequently changing the cell wall structure and elasticity (Fontes et al., 1999).

Histochemical analysis of ROS. Histochemical staining for ROS, including O₂⁻ and H₂O₂, were visualized as blue and brown

colorations, respectively. NBT or DAB staining and quantification indicated that the recovered banana tissues possess lower levels of both H₂O₂ and O₂⁻ compared with those in hyperhydric tissues (Figs. 3 and 4). The excessive water accumulation in plant tissue,

Fig. 3. Histochemical analysis of reactive oxygen species in the leaves of normal, hyperhydric, and reverted banana 'Grand Naine' shoots after 3 weeks in culture. (A) Superoxide and (B) hydrogen peroxide.

Fig. 4. Quantification of reactive oxygen species in the leaves of normal, hyperhydric, and reverted banana 'Grand Naine' shoots after 3 weeks in culture. (A) Superoxide and (B) hydrogen peroxide.

which is the most characteristic symptom of hyperhydricity, generates aeration stress that depletes oxygen levels and limits its diffusion in cells. Therefore, it has been proposed that hyperhydric tissues can be under hypoxic stress (Franck et al., 1998, 2004; Gribble et al., 1996, 1998; Kevers and Gaspar, 1986; Kevers et al., 2004; Olmos et al., 1997). Increased levels of ROS involving the superoxide and hydroxyl free radicals as well as hydrogen peroxide have been observed in hyperhydric tissues of *Dianthus chinensis* (Gao et al., 2017a, 2017b), *Malus* sp. (Chakrabarty et al., 2006), and *Mammillaria gracilis* (Balén et al., 2009). Several reports suggested that oxidative stress, an important damaging factor in hyperhydricity induction, may be responsible for many metabolic changes in hyperhydric tissues such as lipid peroxidation and, consequently, membrane injury, protein degradation, enzyme inactivation, and DNA damage (Chen and Ziv, 2001; Dewir, 2005; Dewir et al., 2006; Franck et al., 1995, 1998; Olmos et al., 1997).

Electrolyte leakage. Electrolyte leakage was significantly decreased in the reverted banana tissues compared with that in hyperhydric tissues (Fig. 5). Cellular membrane

dysfunction due to stress increases permeability and the release of ions, which can be readily measured based on the efflux of electrolytes (Dewir et al., 2015a, 2015b). Cell wall properties and composition are considered some of the most important factors affecting the development of anomalous morphology in hyperhydric tissues (Dewir et al., 2014). Different researchers have shown modifications in the cell wall constituents of hyperhydric tissues, mainly cellulose, lignin, and pectins (Kevers et al., 1987; Majada et al., 2000; Olmos et al., 1997; Saher et al., 2005a, 2005b) and their mechanical properties (Kevers et al., 1987; Komali et al., 1998). Hypolignification has been attributed to the decrease in enzyme activities, as reported for *Origanum vulgare* (Andarwulan and Shetty, 1999) and *Prunus avium* (Phan and Hegedus, 1986). Electrolyte leakage has been used to quantify damage to cell membranes in hyperhydric *Saintpaulia ionantha* (Dewir et al., 2015b). Foyer et al. (1994) observed a higher rate of solute leakage in hyperhydric leaves compared with that in the control, indicating marked deterioration of the membrane. Our results indicated that banana shoots cultured on calcium nitrate–

perhydric shoots, indicating the protective role of calcium nitrate against oxidative stress.

Acclimatization and survival of the reverted banana plantlets. Survival and growth of the reverted banana plantlets were significantly influenced by calcium nitrate as well as gelling agents used during in vitro rooting (Table 2). Furthermore, 100% survival was recorded for plantlets reverted in medium containing 0.5 to 1 g·L⁻¹ calcium nitrate regardless of the solidifying agent. The reverted plantlets grown in medium lacking calcium nitrate and solidified with gellan or agar resulted in 43% and 83% survival, respectively. A low calcium nitrate concentration (0.25 g·L⁻¹) resulted in 85% and 92% survival of plantlets reverted on gellan and agar, respectively. Therefore, solidifying the MS medium with agar during the reversion of hyperhydric shoots was more efficient than using gellan for the survivability of plantlets. Dehydration and death of hyperhydric plants during the acclimatization stage were mainly due to water loss through epidermal discontinuities and nonfunctional stomata (Apóstolo and Llorente, 2000; Gribble et al., 1996; Olmos and Hellin, 1998). Hyperhydric *Simmondsia chinensis* shoots exhibiting malformed nonfunctional stomata fail to survive acclimatization (Apóstolo and Llorente, 2000). Nonfunctional stomata, hypolignification, and epidermal discontinuity resulted in the loss of protection needed for acclimatization. Calcium nitrate proved effective for reversion and acclimatization of ‘Grand Naine’ hyperhydric shoots. All plantlets cultured in a medium containing calcium nitrate (0.5–1 g·L⁻¹) were reverted and acclimatized (Fig. 1D). Previous studies reported varied percentages of reversion for hyperhydric shoots such as *Dianthus chinensis* (67% on medium containing 5 mg·L⁻¹ silver nitrate) (Gao et al., 2017b) and *Atriplex canescens* (39.7% on vented Magenta vessels; pore size, 0.22 µm) (Reyes-Vera et al., 2008), indicating that reversion to normal morphology is influenced by the culture conditions and plant genotype.

We concluded that 58% and 88% of the hyperhydric banana ‘Grand Naine’ shoots cultured in media lacking calcium nitrate

Fig. 5. Electrolyte leakage in the leaves of normal, hyperhydric, and reverted banana ‘Grand Naine’ shoots after 3 weeks in culture.

Table 2. Effects of calcium nitrate on survival and growth of the reverted banana ‘Grand Naine’ plantlets after 4 weeks of acclimatization in a greenhouse.

Gelling agent	Calcium nitrate (g·L ⁻¹)	Survival (%)	Shoot length (cm)	Root length (cm)	Leaves (no./plantlet)	Fresh wt per plantlet (g)
Agar	0.00	83 c ^z	8.8 ab	5.9 b	5.8 c	2.68 cd
	0.25	92 b	13.5 ab	8.7 ab	7.8 a	4.29 b
	0.50	100 a	12.0 ab	12.6 a	7.3 a	5.67 a
	0.75	100 a	12.0 ab	10.0 ab	6.5 b	4.34 b
	1.00	100 a	12.1 ab	7.2 b	6.5 b	3.49 bc
Gellan	0.00	43 d	7.5 b	6.0 b	5.2 c	2.11 d
	0.25	85 c	11.5 ab	6.0 b	5.8 c	3.65 bc
	0.50	100 a	10.0 ab	6.0 b	5.3 c	3.59 bc
	0.75	100 a	13.8 a	8.8 ab	6.1 b	4.26 b
	1.00	100 a	10.5 ab	8.5 ab	6.5 b	3.93 bc
Significance						
Type of gelling agent (A)		**	**	*	**	NS
Calcium nitrate (B)		**	**	**	NS	**
A × B		**	NS	**	**	*

^zValues followed by the same letter in the same column are not significantly different at $P \leq 0.05$ according to Duncan’s multiple range test.

NS, *, **Nonsignificant or significant at $P \leq 0.05$ or 0.01, respectively.

and solidified gellan or agar, respectively, were estimated as losses because these shoots failed to either root or survive past the acclimatization stage. Moreover, 100% rooting of hyperhydric shoots occurred at all concentrations of calcium nitrate. Growth and survival of the reverted banana plantlets were significantly influenced by calcium nitrate concentrations as well as the type of gelling agent used. Reverted banana plantlets in medium containing calcium nitrate (0.5–1 g·L⁻¹) were acclimatized with 100% survival.

Literature Cited

- Ádám, A., T. Farkas, G. Somlyai, M. Hevesi, and Z. Király. 1989. Consequence of O₂ generation during a bacterially induced hypersensitive reaction in tobacco: Deterioration of membrane lipids. *Physiol. Mol. Plant Pathol.* 34:13–26.
- Andarwulan, N. and K. Shetty. 1999. Influence of acetyl salicylic acid in combination with fish protein hydrolysates on hyperhydricity reduction and phenolic synthesis in oregano (*Origanum vulgare*) tissue cultures. *J. Food Biochem.* 23:619–635.
- Apóstolo, N.M. and B.E. Llorente. 2000. Anatomy of normal and hyperhydric leaves and shoots of *in vitro* grown *Simmondsia chinensis* (Link) Schn. *In Vitro Cell. Dev. Biol. Plant* 36:243–249.
- Bairu, M.W., W.A. Stirr, K. Dolezal, and J. Van Staden. 2007. Optimizing the micropropagation protocol for the endangered *Aloe polyphylla*: Can meta-topolin and its derivatives serve as replacement for benzyladenine and zeatin? *Plant Cell Tissue Organ Cult.* 90:15–23.
- Bairu, M.W., W.A. Stirr, K. Dolezal, and J. Van Staden. 2008. The role of topolins in micropropagation and somaclonal variation of banana cultivars ‘Williams’ and ‘Grand Naine’ (*Musa* spp. AAA). *Plant Cell Tissue Organ Cult.* 95:373–379.
- Balen, B., M. Tkalec, D. Pavokovic, B. Pevalek-Kozlina, and M. Krsnik-Rasol. 2009. Growth conditions in *in vitro* culture can induce oxidative stress in *Mammillaria gracilis* tissues. *J. Plant Growth Regul.* 28:36–45.
- Barbosa, L.M.P., V.B. de Paiva Neto, L.L.C. Dias, R.A. Festucci-Buselli, R.S. Alexandre, L. Iarema, F.L. Finger, and W.C. Otoni. 2013. Biochemical and morpho-anatomical analyses of strawberry plantlets hyperhydric tissues affected by BA and gelling agents. *Rev. Ceres* 60:152–160.
- Buah, J.N., Y. Kawamitsu, S. Sato, and S. Murayama. 1999. Effects of different types and concentrations of gelling agents on the physical and chemical properties of media and the growth of banana (*Musa* spp.) *in vitro*. *Plant Prod. Sci.* 2:138–145.
- Cameron, S.I. 2001. Use of a prototype gel hardness tester to demonstrate the effect of variable calcium concentration of gel rigidity. *In Vitro Cell. Dev. Biol. Plant* 37:419–424.
- Chakrabarty, D. and S.K. Datta. 2008. Micropropagation of gerbera: Lipid peroxidation and antioxidant enzymes activities during acclimatization process. *Acta Physiol. Plant.* 30:325–331.
- Chakrabarty, D., S.Y. Park, M.B. Ali, K.S. Shin, and K.Y. Paek. 2006. Hyperhydricity in apple: Ultrastructural and physiological aspects. *Tree Physiol.* 26:377–388.
- Chen, J. and M. Ziv. 2001. The effect of ancymidol on hyperhydricity, regeneration, starch and antioxidant enzymatic activities in liquid-cultured *Narcissus*. *Plant Cell Rep.* 20:22–27.
- De Klerk, G.-J.M., N. van den Dries, and F.A. Krens. 2017. Hyperhydricity: Underlying mechanisms. *Acta Hort.* 1155:269–276.
- Debergh, P., J. Aitkenchristie, D. Cohen, B. Grout, S. Von Arnold, R. Zimmerman, and M. Ziv. 1992. Reconsideration of the term vitrification as used in micropropagation. *Plant Cell Tissue Organ Cult.* 30:135–140.
- Deepika, C., B. Basanti, D.J. Singh, K. Subhash, and P. Anil. 2018. An insight into *in vitro* micropropagation studies for banana- Review. *Intl. J. Agr. Sci.* 10:5346–5349.
- Dewir, Y.H. 2005. Ornamental *Euphorbia* and *Spathiphyllum*: Application of bioreactor system and microponics for large-scale production, *in vitro* flowering and its physiology. Ph.D. dissertation, Chungbuk National University, South Korea.
- Dewir, Y.H., D. Chakrabarty, E.J. Hahn, and K.Y. Paek. 2005. Reversion of inflorescence in *Euphorbia milii* and its application to large scale micropropagation in an air-lift bioreactor. *J. Hort. Sci. Biotechnol.* 80:581–587.
- Dewir, Y.H., D. Chakrabarty, M.B. Ali, E.J. Hahn, and K.Y. Paek. 2006. Lipid peroxidation and antioxidant enzyme activities of *Euphorbia millii* hyperhydric shoots. *Environ. Exp. Bot.* 58:93–99.
- Dewir, Y.H., M.E. El-Mahrouk, Y.M. Hafez, H.Z. Rihan, C.A. Sáez, and M.P. Fuller. 2015a. Antioxidative capacity and electrolyte leakage in healthy versus phytoplasma infected tissues of *Euphorbia coerulescens* and *Orbea gigantea*. *J. Plant Physiol. Pathol.* 3:1, doi: 10.4172/2329-955X.1000139.
- Dewir, Y.H., M.E. El-Mahrouk, Y.M. Hafez, J.A. Teixeira da Silva, and Y. Naidoo. 2015b. Hyperhydricity in African violet (*Saintpaulia ionantha* H. Wendl)-biochemical aspects of normal versus hyperhydric shoots regenerated via direct adventitious shoots formation. *Propag. Ornament. Plants* 15:53–62.
- Dewir, Y.H., Nurmansyah, Y. Naidoo, and J.A. Teixeira da Silva. 2018. Thidiazuron-induced abnormalities in plant tissue cultures. *Plant Cell Rep* 37:1451–1470.
- Dewir, Y.H., Y. Indoliya, D. Chakrabarty, and K.Y. Paek. 2014. Biochemical and physiological aspects of hyperhydricity in liquid culture system, p. 693–703. In: K.Y. Paek, H.N. Murthy, and J.J. Zhong (eds.). *Production of biomass and bioactive compounds using bioreactor technology*. Springer, Berlin.
- Escalona, M., I. Cejas, J. Gonzalez-Olmedo, I. Capote, S. Roels, M.J. Canal, R. Rodriguez, J. Sandoval, and P. Debergh. 2003. The effect of meta-topolin on plantain propagation using a temporary immersion bioreactor. *InfoMusa* 12:28–30.
- FAOSTAT. 2017. <<http://www.fao.org/faostat/en/#data/QC>>.
- Fontes, M.A., W.C. Otoni, S.M.B. Carolino, S.H. Brommonschenkel, E.P.B. Fontes, M. Fari, and R.P. Louro. 1999. Hyperhydricity in pepper plants regenerative *in vitro*: Involvement of BiP (Binding Protein) and ultrastructural aspects. *Plant Cell Rep.* 19:81–87.
- Foyer, C.H., M. Lelandais, and K.J. Kunert. 1994. Photooxidative stress in shoots. *Physiol. Plant.* 92:696–717.
- Franck, T., C. Kevers, and T. Gaspar. 1995. Protective enzymatic systems against activated oxygen species compared in normal and vitrified shoots of *Prunus avium* L. raised *in vitro*. *Plant Growth Regulat.* 16:253–256.
- Franck, T., C. Kevers, C. Penel, H. Greppin, J.F. Hausman, and T. Gaspar. 1998. Reducing properties, and markers of lipid peroxidation in normal and hyperhydrating shoots of *Prunus avium* L. *J. Plant Physiol.* 153:339–346.
- Franck, T., C. Kevers, T. Gaspar, T. Dommès, C. Deby, R. Greimers, D. Serteyn, and G. Deby-Dupont. 2004. Hyperhydricity of *Prunus avium* shoots cultured on gelrite: A controlled stress response. *Plant Physiol. Biochem.* 42:519–527.
- Gao, H., P. Xu, J. Li, H. Ji, L. An, and X. Xia. 2017a. AgNO₃ prevents the occurrence of hyperhydricity in *Dianthus chinensis* L. by enhancing water loss and antioxidant capacity. *In Vitro Cell. Dev. Biol. Plant* 53:561–570.
- Gao, H., X. Xia, L. An, X. Xin, and Y. Liang. 2017b. Reversion of hyperhydricity in pink (*Dianthus chinensis* L.) plantlets by AgNO₃ and its associated mechanism during *in vitro* culture. *Plant Sci.* 254:1–11.
- Gribble, K., V. Sarafis, J. Nailon, P. Holford, and P. Uwins. 1996. Environmental scanning electron microscopy of the surface of normal and vitrified leaves of *Gypsophila paniculata* (Babies Breath) cultured *in vitro*. *Plant Cell Rep.* 15:771–776.
- Gribble, K., J. Tingle, V. Sarafis, A. Heaton, and P. Holford. 1998. Position of water in vitrified plants visualized by NMR imaging. *Protoplasma* 201:110–114.
- Harley, M.M. and I.K. Ferguson. 1990. The role of the SEM in pollen morphology and plant systematics, p. 45–68. In: D. Claugher (ed.). *Scanning electron microscopy in taxonomy and functional morphology*. Sys. Assoc. Spec., Clarendon Press, Oxford.
- Hassannejad, S., F. Bernard, F. Mirzajani, and M. Gholami. 2012. SA improvement of hyperhydricity reversion in *Thymus daenensis* shoots culture may be associated with polyamines changes. *Plant Physiol. Biochem.* 51:40–46.
- Hazarika, B.N. 2006. Morpho-physiological disorders in *in vitro* culture of plants. *Scientia Hort.* 108:105–120.
- Hückelhoven, R., J. Fodor, C. Preis, and K.H. Kogel. 1999. Hypersensitive cell death and papilla formation in barley attacked by the powdery mildew fungus are associated with hydrogen peroxide but not with salicylic acid accumulation. *Plant Physiol.* 119:1251–1260.
- Hui, A.V., A. Bhatt, S. Sreeramanan, and C.L. Keng. 2013. Establishment of a shoot proliferation protocol for banana (ABB Group) Cv. ‘Pisang Awak’ via temporary immersion system. *J. Plant Nutr.* 36:529–538.
- Ivanova, M. and J. Van Staden. 2011. Influence of gelling agent and cytokinins on the control of hyperhydricity in *Aloe polyphylla*. *Plant Cell Tissue Organ Cult.* 104:13–21.
- Jafari, N., R.Y. Othman, and N. Khalid. 2011. Effect of benzylaminopurine (BAP) pulsing on *in vitro* shoot multiplication of *Musa acuminata* (banana) cv. Berangan. *Afr. J. Biotechnol.* 10:2446–2450.
- Kevers, C. and T. Gaspar. 1986. Vitrification of carnation *in vitro*: Changes in water content, intracellular space, air volume and ion levels. *Physiol. Veg.* 24:647–653.
- Kevers, C., R. Prat, and T. Gaspar. 1987. Vitrification of carnation *in vitro*: Changes in cell wall mechanical properties, cellulose and lignin content. *Plant Growth Regulat.* 5:59–66.
- Kevers, C., T. Franck, R.J. Strasser, J. Dommès, and T. Gaspar. 2004. Hyperhydricity of micropropagated shoots: A typically stress-induced change of physiological state. *Plant Cell Tissue Organ Cult.* 77:181–191.
- Komali, A.S., M. Peleg, C. Gerhards, and K. Shetty. 1998. A study of the cell wall mechanical properties in unhyperhydrated shoots of oregano (*Origanum vulgare*) inoculated with *Pseudomonas* sp. by load deformation analysis. *Food Biotechnol.* 12:209–220.

- Kozai, T., C. Kubota, and B. Ryoung Jeong. 1997. Environmental control for the large-scale production of plants through *in vitro* techniques. *Plant Cell Tissue Organ Cult.* 51:49–56.
- Machado, M.P., A.L.L. da Silva, L.A. Biasi, C. Deschamps, J.C.B. Filho, and F. Zanette. 2014. Influence of calcium content of tissue on hyperhydricity and shoot-tip necrosis of *in vitro* regenerated shoots of *Lavandula angustifolia*. *Mill. Braz. Arch. Biol. Technol.* 57:636–643.
- Majada, J.P., F. Tadeo, M.A. Fal, and R. Sánchez Tamés. 2000. Impact of culture vessel ventilation on the anatomy and morphology of micro-propagated carnation. *Plant Cell Tissue Organ Cult.* 63:207–214.
- Malavolta, E., G.C. Vitti, and S.A. de Oliveira. 1997. Avaliação do estado nutricional das plantas: Princípios e aplicações. 2nd ed. Piracicaba: Associação Brasileira para Pesquisa da Potassa e do Fósforo.
- Marschner, H. and J.V. Possingham. 1975. Effects of K and Na on the growth of leaf discs of sugar beet and spinach. *Z. Pflanzenphysiol.* 75:6–16.
- Moran, R. and D. Porath. 1982. Chlorophyll determination in intact tissues using N,N-Dimethyl formamide. *Plant Physiol.* 69:1370–1381.
- Murashige, T. and F.A. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15:473–479.
- Olmos, E. and E. Hellin. 1998. Ultrastructural differences of hyperhydric and normal leaves from regenerated carnation plants. *Scientia Hort.* 75:91–101.
- Olmos, E., A. Piqueras, J.R. Martínez-Solano, and E. Hellin. 1997. The subcellular localization of peroxidase and the implication of oxidative stress in hyperhydrated leaves of regenerated carnation shoots. *Plant Sci.* 130:97–105.
- Phan, C.T. and P. Hegedus. 1986. Possible metabolic basis for the developmental anomaly observed in *in vitro* culture, called 'vitreous plants'. *Plant Cell Tissue Organ Cult.* 6:83–94.
- Piqueras, A., M. Cortina, M.D. Serna, and J.L. Casas. 2002. Polyamines and hyperhydricity in micropropagated carnation plants. *Plant Sci.* 162:671–678.
- Pospisilova, J., H. Synkova, D. Haisel, and S. Semoradova. 2007. Acclimation of plantlets to *ex vitro* conditions: Effects of air humidity, irradiance, CO₂ concentration and abscisic acid (a review). *Acta Hort.* 748:29–38.
- Reyes-Vera, I., C. Potenza, and J. Barrow. 2008. Hyperhydricity reversal and clonal propagation of four-wing saltbush (*Atriplex canescens*, Chenopodiaceae) cultivated *in vitro*. *Austral. J. Bot.* 56:358–362.
- Rojas-Martínez, L., R.G.F. Visser, and G. de Klerk. 2010. The hyperhydricity syndrome: Waterlogging of plant tissues as a major cause. *Propag. Ornament. Plants* 10:169–175.
- Rout, G.R., S. Samantaray, and P. Das. 2000. Biotechnology of the banana: A review of recent progress. *Plant Biol.* 2:512–524.
- Saher, S., A. Piqueras, E. Hellin, and E. Olmos. 2005a. Prevention of hyperhydricity in micro-propagated carnation shoots by bottom cooling: Implications of oxidative stress. *Plant Cell Tissue Organ Cult.* 81:149–158.
- Saher, S., N. Fernández-García, A. Piqueras, E. Hellin, and E. Olmos. 2005b. Reducing properties, energy efficiency and carbohydrate metabolism in hyperhydric and normal carnation shoots cultured *in vitro*: A hypoxia stress? *Plant Physiol. Biochem.* 43:573–582.
- Sha, L., B.H. McCown, and L.A. Peterson. 1985. Occurrence and cause of shoot-tip necrosis in shoot cultures. *J. Amer. Soc. Hort. Sci.* 110:631–634.
- Singh, H.P., S. Uma, R. Selvarajan, and J.L. Karihaloo. 2011. Micropropagation for production of quality banana planting material in Asia-Pacific. Asia-Pacific Consortium on Agricultural Biotechnology (APCoAB), New Delhi, India.
- Singha, S., E.C. Townsend, and G.E. Oberly. 1990. Relationship between calcium and agar on vitrification and shoot-tip necrosis of quince (*Cydonia oblonga* Mill.) shoots *in vitro*. *Plant Cell Tissue Organ Cult.* 23:135–142.
- Soundararajan, P., A. Manivannan, Y.S. Cho, and B.R. Jeong. 2017. Exogenous supplementation of silicon improved the recovery of hyperhydric shoots in *Dianthus caryophyllus* L. by stabilizing the physiology and protein expression. *Front. Plant Sci.* 8:738.
- Sreedhar, R.V., L. Venkatachalam, and B. Neelwarne. 2009. Hyperhydricity-related morphologic and biochemical changes in Vanilla (*Vanilla planifolia*). *J. Plant Growth Regul.* 28:46–57.
- Strosse, H., I. Houwe, B. Panis, S. Jain, and R. Swennen. 2004. Banana cell and tissue culture—Review. Banana improvement: Cellular, molecular biology, and induced mutations. Proc. Meeting, Leuven, 24–28 Sept. 2001, 1–12.
- Szalai, G., T. Janda, E. Paldi, and Z. Szigeti. 1996. Role of light in post-chilling symptoms in maize. *J. Plant Physiol.* 148:378–383.
- Taiz, L. and E. Zeiger. 2006. *Plant physiology*. 3rd ed. Sinauer Associates Inc., Sunderland, MA.
- Tian, J., F. Jiang, and Z. Wu. 2014. The apoplastic oxidative burst as a key factor of hyperhydricity in garlic plantlet *in vitro*. *Plant Cell Tissue Organ Cult.* 120:571–584.
- Valero-Aracama, C., M. Kane, S. Wilson, and N. Philman. 2010. Substitution of benzyladenine with meta-topolin during shoot multiplication increases acclimatization of difficult and easy-to-acclimatize sea oats (*Uniola paniculata* L.) genotypes. *Plant Growth Regulat.* 60:43–49.
- van den Dries, N., S. Giannì, A. Czerednik, F.A. Krens, and G.-J.M. De Klerk. 2013. Flooding of the apoplast is a key factor in the development of hyperhydricity. *J. Expt. Bot.* 64:5221–5230.
- Vuylsteke, D. 1989. Shoot-tip culture for the propagation, conservation and exchange of Musa germplasm. IBPGR, Rome.
- Whitlow, T.H., N.L. Bassuk, T.G. Ranney, and D.L. Reichert. 1992. An improved method for using electrolyte leakage to assess membrane competence in plant tissues. *Plant Physiol.* 98:198–205.
- Zimmerman, T.W., S.M.D. Rogers, and B.G. Cobb. 1988. Physiological differences between normal and vitreous petunias grown *in vitro*. ASHS/CSHS 1988 Ann Mtg, East Lansing, Mich, Prog & Abstr. HortScience 23:780 (abstr.).