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

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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 peat moss and vermiculite (1:1).

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.
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The medium was gelled with 0.2% gellan (Dephyte, 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) after 3 weeks in culture. (A) Reversion of hyperhydric banana 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*                   | 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 ed      | 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 df           | 8.0 b                | 0.17 d            | 2.79 ab                           |                          |
| **Gellan**    |                         |             |                     |                  |                      |                   |                                   |                          |
| 0.00          | 50 b*                   | 9.3 bc      | 3.4 ed              | 5.7 ed           | 5.8 bc               | 0.12 e            | 1.28 c                            |                          |
| 0.25          | 100 a                   | 8.7 bc      | 5.6 bc              | 6.3 bcd          | 5.3 c                | 0.13 d            | 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 ** * *
Calcium nitrate (B) * ** * * NS
A × B NS * NS ** NS

*Values followed by the same letter in the same column are not significantly different at \( P ≤ 0.05 \) according to Duncan’s multiple range test.

**Microscopic observation of stoma.** For scanning electron microscopy, samples (4 mm²) were obtained from the leaves and fixed in glutaraldehyde (2.5%) for 4 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 (Hickelhoven 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 Chemilager 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.
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) × 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 ± 2 °C, 60% to 70% relative humidity, and 100 μmol-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 × 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 concentration. (Fig. 1B and C).

**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 FragariaSananassa (Barbosa et al., 2013), Thymus daenensis (Hassannejad et al., 2013), 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 O\(_2^-\) and H\(_2\)O\(_2\), 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 H\(_2\)O\(_2\) and O\(_2^-\) compared with those in hyperhydric tissues (Figs. 3 and 4). The excessive water accumulation in plant tissue,
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 a         | 8.8 ab            | 5.9 b            | 5.8 c                | 2.68 cd                   |
|                | 0.25                     | 92 b         | 13.5 ab           | 8.7 a            | 7.3 a                | 4.29 b                    |
|                | 0.50                     | 100 a        | 12.0 ab           | 12.6 a           | 7.2 b                | 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                     | 343 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  **  **  NS  *

*aValues followed by the same letter in the same column are not significantly different at P ≤ 0.05 according to Duncan’s multiple range test.

**Nonsignificant or significant at P ≥ 0.05 or 0.01, respectively.

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* (Balen 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; Komall et al., 1998). Hypolignification has been attributed to the decrease in enzyme activities, as reported for *Origanum vulgare* (Andarwulan and Shetty, 1999) and *Pruus avium* (Phan and Hegedus, 1986). Electrolyte leakage has been used to quantify damage to cell membranes in hyperhydric tissues (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–agar leaves the leaves of normal, hyperhydric, and reverted banana ‘Grand Naine’ shoots after 3 weeks in culture.
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.

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