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Histology of Callogenesis in Diploid Bananas (*Musa acuminata*, AA Group) 'Kluai Sa' and 'Kluai Leb Mu Nang'

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Abstract

Yellow compact calluses were induced from *in vitro*-grown shoot tips of diploid bananas (*Musa acuminata*, AA group) 'Kluai Sa' and 'Kluai Leb Mu Nang' on a modified Murashige and Skoog (MS) medium containing 100 mg/L malt extract, 50 mg/L proline, 50 mg/L cysteine, 100 mg/L glutamine, 1 mg/L biotin, 7 mg/L Dicamba and 2 mg/L TDZ. Green shoot buds were induced after transfer of the yellow compact calluses to the same MS medium but supplemented with 1 mg/L NAA and 3 mg/L BA and plant regeneration was achieved through organogenesis in callus cultures. Regenerated shoots were rooted on MS medium containing 0.2% activated charcoal but without plant growth regulators. Histological analysis revealed that calluses originated from small dense cells with well stained cytoplasm and nucleus typical of meristematic cells.

Keywords: callus culture, dessert bananas, micropropagation, organogenesis, shoot tip culture

Introduction

Banana (*Musa* sp.) frequently plays role as major cash export commodity and a complementary food in local diets of tropical and subtropical countries. Traditionally, most of the edible clones are seedless, sterile and propagated by suckers. For these reasons, classical breeding is difficult. The multiplication of a clone is slow, laborious, and time-consuming as far as to obtain a large number of homogeneous plants (Sasson, 1997). Recent advances in biotechnology for crop improvement have had a great impact on banana cultivation. Plant regeneration via *in vitro* culture has been described by a number of authors, from various sources of explants including male floral apices (Murali and Duncan, 1995), zygotic embryo (Navarro *et al*., 1997), meristem (Bhagyalakshmi and Singh, 1995), shoot tip (Israeli *et al*., 1996), leaf (Okole and Schulz, 1996), rhizome (Lee *et al*., 1997), cell suspension (Cote *et al*., 1996), and protoplast (Panis *et al*., 1993).

In vitro regeneration via callus and cell suspension cultures is not only important for an integral part of genetic transformation but also a selection of useful somaclonal variants. Embryogenic callus cultures of two important Thai banana cultivars, *Musa acuminata* 'Kluai Sa' and 'Kluai Leb Mu Nang', initiated from shoot tips, have been reported (Koarapatchaikul and Kanchanapoom, 2010). Both banana cultivars are native and well known as local economic bananas in southern Thailand. They are seedless, tasty, odorous and highly priced. Histological knowledge of callus induction can provide important information for improving the somatic embryogenesis for this crop. However, to date no precise histological study in two Thai banana cultivars has been performed.

Therefore, the present investigation deals with a histological analysis from the initial callogenesis followed by differentiation in the callus culture of *M. acuminata* 'Kluai Sa' and 'Kluai Leb Mu Nang'.

Materials and methods

Callus induction and culture conditions

Shoot tips (3-5 mm long) from sword suckers of two Thai banana cultivars, 'Kluai Leb Mu Nang' and 'Kluai Sa', were dissected and propagated aseptically on a solid MS (Murashige and Skoog, 1962) medium as previously described by Koarapatchaikul and Kanchanapoom (2010). Briefly, *in vitro*-grown shoot tips of each cultivar were cultured on MS medium with macro elements reduced to half and five organic addenda with (mg/L) malt extract 100; glutamine 100; proline 50; cysteine 50; and biotin 1 served as callus induction medium. TDZ (Fluka™) at concentration of 2 mg/L and Dicamba (Sigma-Aldrich) at concentration of 7 mg/L were added to the callus induction medium. All media were solidified by 0.17% Gelrite™ (Merck and Co., Kelco Div., NJ, USA). The pH of all media was adjusted to 5.7 with 1 N NaOH or 1 N HCl prior to autoclaving at 121°C, 1.1 kg cm^{-2} pressure for 20 min. The cultures were maintained at 25±1°C with a 16 h photoperiod and 20 µmol m^{-2} s^{-1} photosynthetic photon flux density provided by cool-white fluorescent lamps. All calluses were transferred to a fresh medium and subcultured at 3-week intervals. One explant was planted per culture and 20 cultures were raised for each treatment. All experiments were conducted on 2 different days.

Plant regeneration and acclimatization

Six-week-old yellow compact calluses with 1cm in diameter were cultured on MS medium supplemented with 1 mg/L NAA and 3 mg/L BA for plant regeneration. A mass of growing buds was formed on these calluses. To fos-

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ter shoot elongation, these buds were cultivated for three weeks on MS medium supplemented with 3 mg/L BA, 3% sucrose and solidified by 0.75% agar. The regenerated shoots, that developed, were transferred for germination in individual glass-capped test tubes each containing 20 ml of MS plant growth regulator-free medium but containing 0.2% activated charcoal for rooting. Gelrite™ was carefully washed from the well-rooted plantlets and they were then transferred to black plastic pots filled with potting soil and watered twice daily for a period of 2 months in greenhouse conditions. Normal looking banana plants were obtained before being planted in the field.

Histological analyses

Morphological characteristics of callus were examined using an Olympus stereo microscope SZH10. The paraffin method was used for light microscopy following the method described by Johansen (1940). The samples of calluses were randomly taken at day 21 and day 40 after culture initiation and fixed in FAA II solution containing 95% ethyl alcohol: glacial acetic acid: formaldehyde: water (10:1:2:7 v/v). Fixed specimens were dehydrated through a tertiary-butyl alcohol series, embedded in Paraplast plus (Melting point 56°C) using Histo-embedder (Jung, Leica, Germany). Sections were cut using a rotary microtome and mounted in Permount (Fisher Scientific International Inc., USA). Serial sections were observed under a light microscope. At least 5 samples for each developmental stage were observed. Scanning electron microscopy (SEM) was used to provide details of shoot bud formation. The samples were fixed in FAA II, dehydrated through a graded ethanol series, dried in a critical point dryer and coated with gold. These samples were imaged, using a scanning electron microscope JEOL 5800LV operated at 10-15 KV, followed by the visual examination of the hard copies.

Results and discussion

M. acuminata 'Kluai Sa' and 'Kluai Leb Mu Nang' are native bananas in southern Thailand and represent a major source of income and employment. Both banana cultivars are propagated by suckers but this method is laborious and time consuming. Furthermore, traditional propagation methods are unable to cope with the demand for huge planting materials. Under these circumstances, the use of tissue culture technique has had a great impact on banana cultivation. There are few reports on successful regeneration from callus cultures of diploid banana cultivars; most of the work comes from triploid cultivars. In this context plant regeneration through callus culture of two important Thai bananas is described (Koarapatchaikul and Kanchanapoom, 2010). Yellow compact calluses were initiated from shoot tip explants of *M. acuminata* 'Kluai Sa' and 'Kluai Leb Mu Nang' after 3 weeks of culture on MS medium fortified with 7 mg/L Dicamba in combination with 2 mg/L TDZ (Fig. 1a). By successive subculture, masses of proliferating callus have been established. After completion of the callusing phase, the yellow compact calluses were subcultured on MS medium containing 1 mg/L NAA and 3 mg/L BA and they formed green shoot buds.

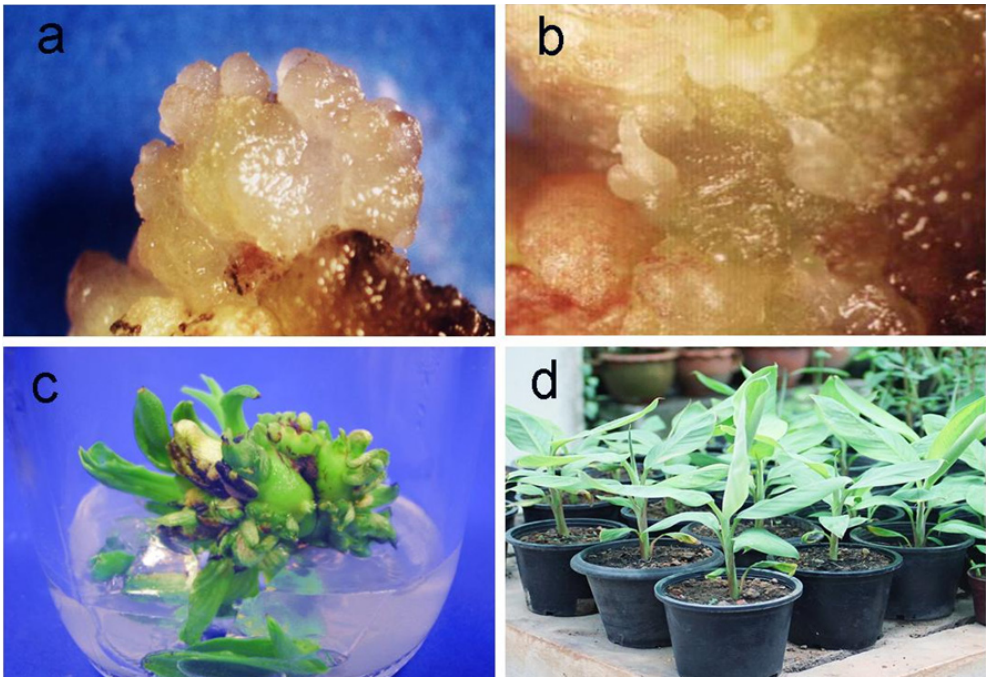

Fig. 1. Plant regeneration from callus cultures in 'Kluai Sa' and 'Kluai Leb Mue Nang'. (a) Callus formation from shoot tip culture on MS medium supplemented with 7 mg/L Dicamba and 2 mg/L TDZ. (b) Small shoots emerged from compact calluses. (c) Development of multiple shoots on MS medium supplemented with 1 mg/L NAA and 3 mg/L BA. (d) Normal looking banana plants in potting soil

Regenerated shoots could be distinguished from the callus by the presence of opaque, and compact nodules of varying sizes linked to one another (Fig. 1b). Prolonged culture on this medium allowed the differentiation and subsequent complete plantlet (Fig. 1c). To establish a rooting protocol, the healthy green shoots were excised from each other and transferred to MS plant growth regulator-free medium for 3 weeks for rooting. Two weeks of rooting incubation was adequate before transplanting to polystyrene pots containing soil mixture (1 sand: 1 manure: 1 decayed leaves). *In vitro*-derived plants did not display any phenotypic variation during subsequent vegetative development (Fig. 1d).

Histological monitoring of the callogenesis revealed many spherical globules (Fig. 2a). Microscopic examination of these globules indicated that they were made up of meristematic cells that had a dense and well stained cytoplasm attached to each other (Fig. 2b). The determination of histology and origin of callus tissue is considered important in the isolation of mutants and nonchimeric plants. The findings of callus formation may prove useful in the improvement of this crop plant through genetic modification at the cellular level since there have been several reports on genetic transformation in banana (Acereto-Escoffié *et al*., 2005; Khanna *et al*., 2004; Sreeramanan *et al*., 2006; Tripathi *et al*., 2007). However, it cannot be determined from the present study whether callus proliferation was of a single or multicellular origin. In other monocots, the single origin is widely encountered e.g. date palm (Tisserat and DeMason, 1980), cork oak (El Maataoui *et al*., 1990) while the multicellular origin is reported at oil palm (Schwendiman *et al*., 1988). These globules developed into a compact nodular structure and emerged through the surface of the callus tissue (Fig. 2c). Shortly after several cell divisions within adjacent cells resulting in the formation of shoot apex-like structures or meristemoids (Fig. 2d).

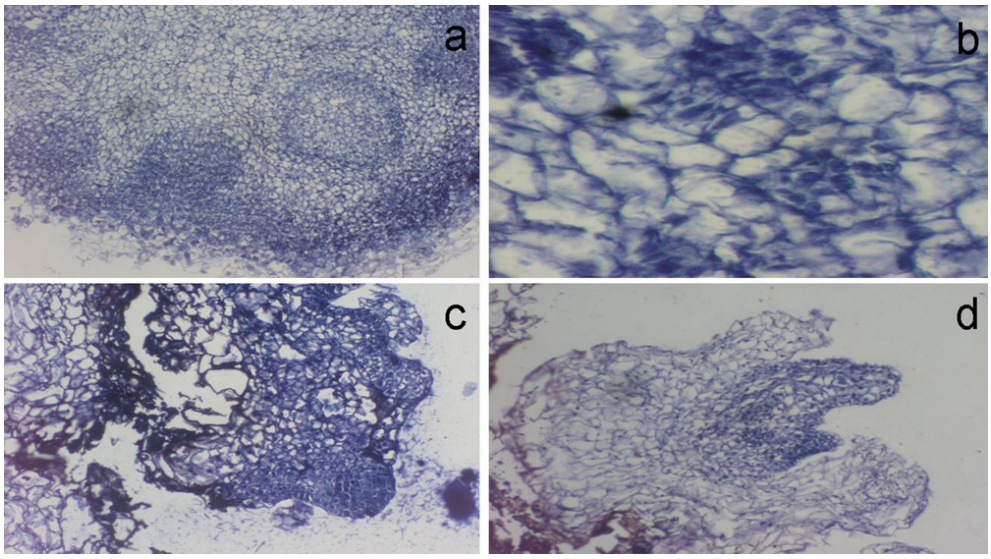

Fig. 2. Histology of callus showing (a) scattered spherical globules embedded in callus tissue; (b) Meristematic cells with densely stained cytoplasm and nucleus in the globules; (c) Nodular compact structure or meristemoids protruded from callus tissue; (d) Formation of shoot apex and leaf primordium

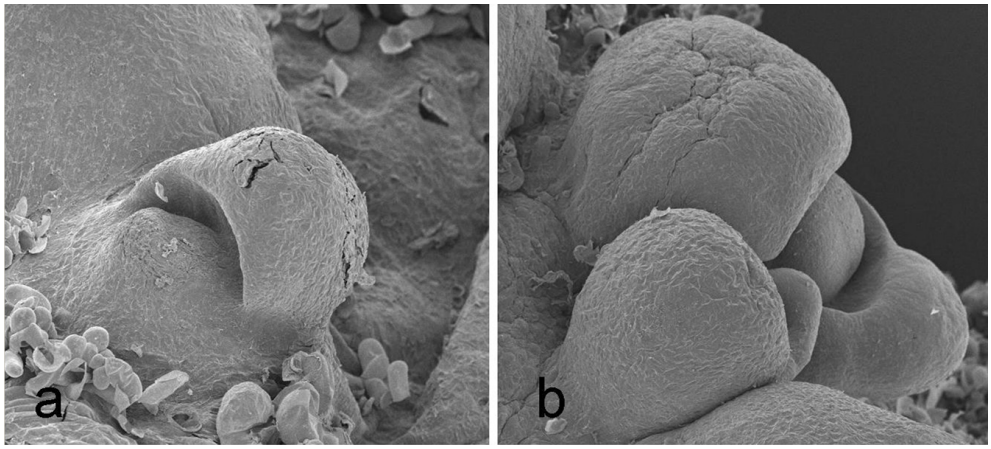

Fig. 3. Scanning electron micrographs of (a) single regenerated shoots and (b) multiple shoots

Typical characteristics of meristemoids were small cell size with dense cytoplasm, minimal vacuoles and large well-stained nuclei. Scanning electron microscopic images of meristemoids also revealed the similarity in the structure of shoot apical meristem and the leaf primordium in a single shoot (Fig. 3a) or multiple shoots (Fig. 3b).

Conclusions

In conclusion, plantlets were regenerated from callus derived from shoot tip culture of *M. acuminata* 'Kluai Sa' and 'Kluai Leb Mu Nang' via organogenesis. The histological observation contributes to understanding the formation of callus and subsequent plantlets regeneration.

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