ORIGINAL ARTICLE

Thidiazuron induced plant regeneration in callus culture of triploid banana (*Musa* sp.) 'Gros Michel', AAA group

Amornwat Srangsam[1] and Kamnoon Kanchanapoom[2]

Abstract

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Thidiazuron induced plant regeneration in callus culture of triploid banana (*Musa* sp.) 'Gros Michel', AAA group
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Yellow friable calluses of banana (*Musa* sp.) 'Gros Michel', AAA group were initiated from *in vitro* shoot buds cultured on Murashige and Skoog (MS) solid medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and coconut water (CW). Small spherical, compact calluses were formed after friable calluses were transferred to half-MS liquid media supplemented with 1.5 mg/l 2, 4-D or 1.5 mg/l 2, 4-D in combination with 5% CW or without 2,4-D and CW. However, no shoots were produced from these media. Embryogenic calluses were induced followed by subculture the spherical, compact calluses to half-MS solid medium in the presence of thidiazuron (TDZ). These embryogenic calluses gave rise to shoots on MS germination medium containing 2.0 mg/l α-naphthaleneacetic acid (NAA) and 1.0 mg/l 6-benzyladenine (BA). Complete banana plants were recovered on MS maturation medium containing 0.5 mg/l BA and 0.2% activated charcoal. Morphological observation of plantlets did not show any abnormality.

Key words : banana, callus culture, 'Gros Michel' AAA group, organogenesis, thidiazuron

[1]M.Sc.(Biological Sciences), [2]Ph.D.(Botany), Assoc. Prof., Department of Biology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand.
Corresponding e-mail: kkamnoon@ratree.psu.ac.th
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บทคัดย่อ

อมรวรรธน์ สร้างสาม และ คำนูณ กาญจนภูมิ
Thidiazuron ชักนำการเกิดพืชจากการเพาะเลี้ยงแคลลัสกล้วยทริพลอยด์ (*Musa* sp.) 'Gros Michel', AAA group
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ชักนำแคลลัสสีเหลืองฟรายเอเบิลของกล้วยหอมทอง (*Musa* sp.) 'Gros Michel, AAA group' จากตายอดของต้นที่เลี้ยงไว้ในสภาพปลอดเชื้อ โดยใช้อาหารแข็งสูตร Murashige and Skoog (MS) ที่เติม 2,4-dichlorophenoxy-acetic acid (2,4-D) และน้ำมะพร้าว เมื่อย้ายเลี้ยงในอาหารเหลวสูตร 1/2 MS ที่มี 2,4-D 1.5 มก/ล, 2,4-D 1.5 มก/ล ร่วมกับน้ำมะพร้าว 5% หรือไม่มีทั้ง 2,4-D และน้ำมะพร้าวจะเกิดแคลลัสรูปร่างกลมแข็งสีเหลืองแต่ไม่เกิดยอด เมื่อย้ายแคลลัสรูปร่างกลมแข็งสีเหลืองนี้ลงเลี้ยงในอาหารแข็งสูตร 1/2 MS ที่มี thidiazuron (TDZ) จะเกิด embryogenic callus ขึ้น และ embryogenic callus จะพัฒนาเป็นยอดเมื่อเลี้ยงบนอาหารแข็งสูตร MS ที่มี NAA 2.0 มก/ล และ BA 1.0 มก/ล เมื่อย้ายยอดที่เกิดขึ้นไปเลี้ยงบนอาหารแข็งสูตร MS ที่มี BA 0.5 มก/ล และผงถ่าน 0.2% จะเกิดรากได้เป็นต้นกล้วยที่สมบูรณ์ โดยมีลักษณะปกติเหมือนต้นกล้วยหอมทองในธรรมชาติ

ภาควิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยสงขลานครินทร์ อำเภอหาดใหญ่ จังหวัดสงขลา 90112

Triploid banana, 'Gros Michel', AAA group, plays a role as a major cash export commodity and a staple food crop in tropical and subtropical countries. Primarily viral and fungal diseases as well as insect and nematode pest problems limit the production. The application of classical methods of breeding for both disease and pest resistance has resulted in only limited success due to the long generation times and the high sterility of most cultivated bananas (Sasson, 1997). The integration of genetic engineering into breeding programs may provide powerful tools to overcome these limitations by introducing specific genes that can be utilized for banana improvement within a short period of time. However, these applications require reliable plant regeneration protocols for banana. Much progress has been made in application of tissue culture methods for bananas and plantains (Chen and Ku, 1985; Cronauer and Krikorian, 1985; Wong, 1986; Balakrishnamurthy and Sree Rangaswamy, 1988; Doreswamy and Sahijram, 1989; Eckstein and Robinson, 1995; Okole and Schulz, 1996; Kanchanapoom and Chanadang, 2001). These methods were successfully performed on a wide range of banana cultivars. However, explant sources differed from one another and this caused several problems such as disease infection. Therefore *in vitro* plantlets are good sources of explants in case of unlimitation and disease-free explants.

In the present work we demonstrated plant regeneration from *in vitro* shoot buds-derived calluses of triploid banana, 'Gros Michel', AAA group. The feasibility of recovering and converting to plantlets is also reported.

Materials and Methods

Plant materials

Aseptic clean cultures of the *Musa* sp. 'Gros Michel', AAA group were used in this study. They were multiplied by using *in vitro* shoot bud cultured on MS (Murashige and Skoog, 1962) medium containing 5.0 mg/l BA and 15% CW as described by Kanchanapoom and Chanadang (2001).

Culture media and conditions

The culture media used in these experiments were those of MS constituents. The pH of all media was adjusted to 5.7 with either 1 N NaOH or 1 N HCl prior to autoclaving for 20 min at 120ºC and

1.05 kg/cm^2. The cultures were maintained at 26ºC with a photoperiod of 16/8 h under an illumination of 20 µmol $m^{-2}s^{-1}$ provided by cool white fluorescence lamps. All plant materials were cultured in 115 ml screw-topped jars each containing 20 ml of medium. One explant was implanted per culture and 10 cultures were raised for each treatment unless otherwise stated.

Callus induction

The shoot buds with 3-5 cm in length were selected and dissected aseptically down to 4 mm before inoculated onto the induction medium (designated MS1, Table 1) to induce callus formation. The MS1 medium contained MS basal salts supplemented with 3% sucrose, 5% coconut water (CW), 1.5 mg/l 2,4-D and 0.72% agar.

Embryogenic callus induction and plant regeneration

After 5 weeks of culture, calluses proliferated from the explants and the obtained calluses were subcultured to the new medium at a 3-week interval. For embryogenic cell production, yellow friable calluses from MS1 medium were transferred to MS liquid media which contained half-MS salts, MS-vitamins, with (mg/l) glutamine 100, proline 50, cysteine 50, biotin 1, myo-inositol 100 and with different combinations of CW and 2,4-D (designated MS2-8, Table 1).

For shoot bud induction, solid MS media were employed. The constituents of these media were the same as the liquid medium but TDZ at the concentrations of 1.0, 2.0 and 3.0 mg/l were incorporated instead of 2,4-D and CW (designated MS9-11, Table 1). For shoot bud differentiation, MS medium composed of 20g/l sucrose, 2.0 mg/l NAA and 1.0 mg/l BA were employed (designated MS12, Table 1). These shoot buds were subcultured onto maturation medium supplemented with 0.5 mg/l BA, 0.2% activated charcoal and 2% sucrose (designated MS13, Table 1) for 3 weeks. Complete plantlets were obtained and then transferred to pots.

Table 1. Culture media used for plant regeneration through somatic embryogenesis from shoot bud-derived callus of banana cv. 'Gros Michel' (*Musa* sp., AAA group).

Culture media	MS salts	Half-MS salts	Sucrose (g/l)	2,4-D (mg/)	Coconut water (%)	TDZ (mg/)	NAA (mg/l)	BA (mg/l)	Biotin (mg/l)	Glutamine (mg/l)	Proline (mg/l)	Activated charcoal (%)	Agar (%)
MS1		-	30	1.5	5	-	-	-	-	-	-	-	0.72
MS2	-		30	1.5	5	-	-	-	1.0	100	50	-	-
MS3	-		30	1.5	-	-	-	-	1.0	100	50	-	-
MS4	-		30	0.5	5	-	-	-	1.0	100	50	-	-
MS5	-		30	0.5	-	-	-	-	1.0	100	50	-	-
MS6	-		30	-	5	-	-	-	1.0	100	50	-	-
MS7	-		30	-	10	-	-	-	1.0	100	50	-	-
MS8	-		30	-	-	-	-	-	1.0	100	50	-	-
MS9	-		30	-	-	1.0	-	-	1.0	100	50	-	0.72
MS10	-		30	-	-	2.0	-	-	1.0	100	50	-	0.72
MS11	-		30	-	-	3.0	-	-	1.0	100	50	-	0.72
MS12		-	20	-	-	-	2.0	1.0	-	-	-	-	0.72
MS13		-	20	-	-	-	-	0.5	-	-	-	0.2	0.72

Results

Callus induction

During the first phase of culture, yellow friable callus was formed at the apical shoot buds of explants after 4 weeks of culture on MS1 medium (Figure. 1A). The frequency of callus formation was up to 100% of the cultured explants but the proliferation was low. However, long term culture induced phenolic compound in the medium and later caused necrosis and sudden death of the callus. Frequent subculture at a 3-week interval could minimize the accumulation of phenolic compound and increase the rate of proliferation. Hence these calluses were suitable for somatic embryo induction in further experiments.

Embryogenic callus induction and plant regeneration

After completion of the callusing phase, primary calluses were transferred to liquid media either with 0.5, 1.5 mg/l 2, 4-D and 5, 10% CW (MS2-7) or without 2, 4-D and CW (MS8). Small spherical and hard compact calluses were formed and disaggregated into fine suspension upon agitation in the culture flask (Figure 1B). No morphogenetic response occurred in MS2, 3 and 8 media while differentiating rhizogenesis were evidenced in MS 4, 5, 6 and 7 media (Table 2). However, none of these displayed all the characteristics that would make them typical shoots at the end of treatments.

These small spherical calluses obtained from MS2 were transferred to solid MS9-11 media containing TDZ and embryogenic calluses were evidenced on the surface of these calluses (Figure 1C). Macroscopic observations revealed that only embryogenic calluses incubated on MS10 medium supplemented with 2 mg/l TDZ underwent shoot development after a total of 30 days. The shoot-like structures could be distinguished by the presence of green, opaque, and compact nodules. A distinct shoot and scutellum were observed as shown in Figure 1D, indicating a monopolar structure characteristic of shoots. In contrast, cultures on MS9 and 11 showed only large cell aggregates with white and green color and no differentiation were observed. At the end of the experiments these aggregates became brownish and no persistent growth or further differentiation took place.

Differentiation of shoots on MS10 was evidenced when they were transferred to MS12 medium supplemented with 2 mg/l NAA combined with 1 mg/l BA for 3 weeks. These plantlets continued to maturity and root formation was seen

Table 2. Effect of 2, 4-D and coconut water on responses of callus in MS liquid medium after 4 weeks in culture.

Culture Media	2,4-D (mg/l)	CW (%)	Browning (%)	Compact callus formation (%)	Root formation (%)	Plantlet formation (%)
MS2	1.5	5	100	100	0	0
MS3	1.5	-	100	100	0	0
MS4	0.5	5	100	0	100	0
MS5	0.5	-	100	0	100	0
MS6	-	5	100	0	100	0
MS7	-	10	100	0	100	0
MS8	-	-	100	100	0	0

Values represent the mean number of ten replicates per treatment of two independent experiments.

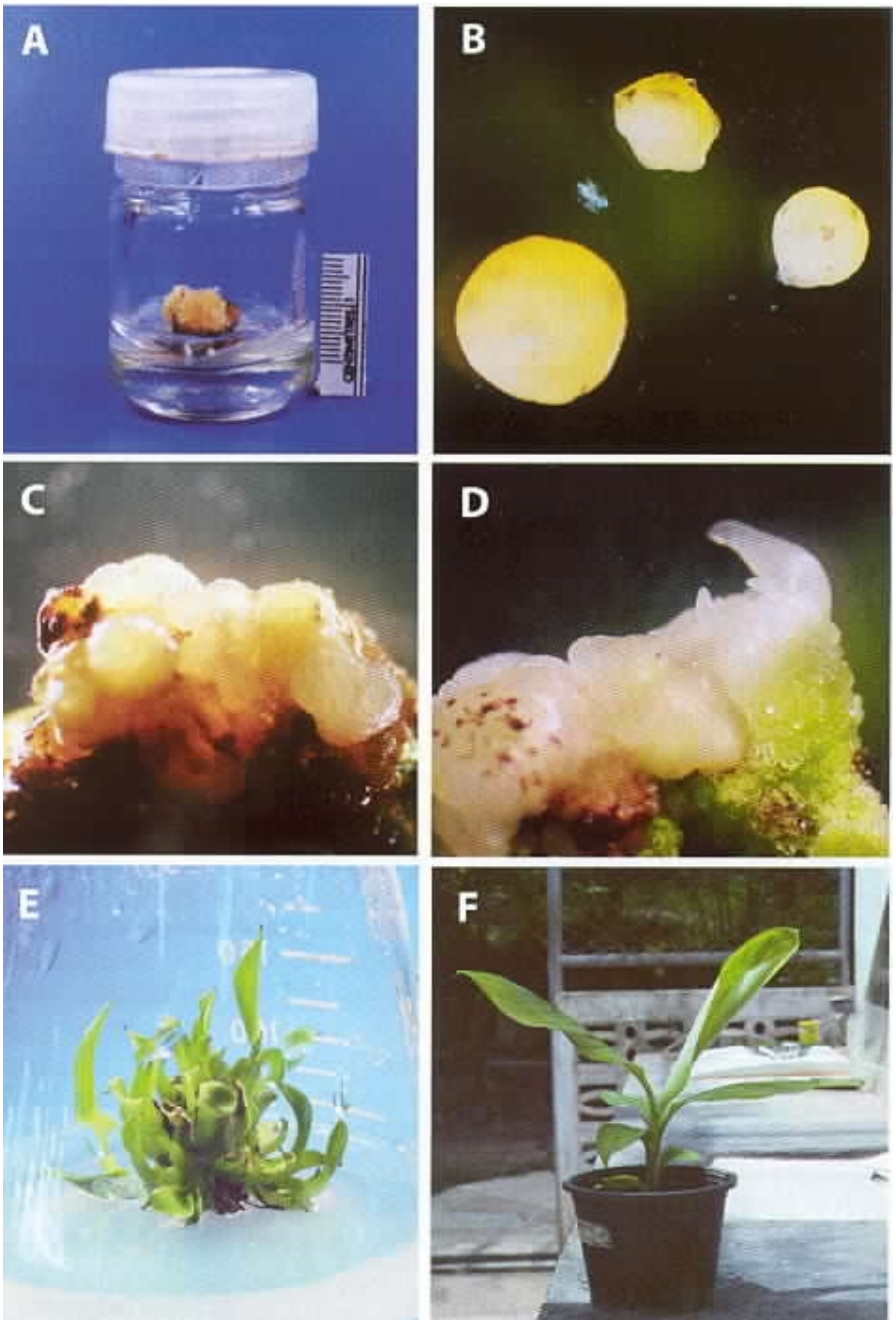

Figure 1. Plant regeneration from callus culture 'Gros Michel', AAA group. (A) Callus formation from shoot bud segment on MS medium containing 1.5 mg/l 2, 4-D and 5% CW. (B) Spherical, compact calluses in half MS liquid medium. (C) Embryogenic callus formation on a solid medium supplemented with 2.0 mg/l TDZ. (D) Shoot primodium emerged from embryogenic mass. (E) Development of plantlets on MS solid medium containing 0.5 mg/l BA. (F) A normal looking banana plant in potted soil.

after being transferred to MS13 medium (Figure 1E). Since roots could be formed easily with 0.5 mg/l BA and 0.2% activated charcoal incorporated in the medium, rooted shoots were transplanted to pots and normal looking banana plants with 100% survival rate were obtained (Figure 1F).

Disussion

One major problem was encountered during *in vitro* culture of banana, viz. exudation of phenolic compound. The inclusion of activated charcoal in the nutrient medium seems to alleviate this problem in several plant species (Wang and Huang, 1976). The most important aspects of activated charcoal are adsorption of toxic brown/black pigments and other unknown colorless toxic compounds. In our experiments, we did not utilize activated charcoal but frequent subculture to new fresh medium instead. Banerjee and Langhe (1985) could overcome the problem of blackening of the culture of seven *Musa* cultivars by a combined effect of the addition of ascorbic acid and frequent transfer of the tissue to fresh medium. Novak *et al*., (1989) reported that combination of 30 µM Dicamba with 5 µM TDZ improved callus formation and overcame the problem of browning.

In this study, we have shown that compact calluses derived from shoot-apices can be used to initiate embryogenic calluses and shoot-bud induction with subsequent regeneration of banana plants. Our result is in agreement with Novak *et al*., (1989) that a morphogenic embryogenic calluses was the material of choice for banana regeneration through somatic embryogenesis. Plant regeneration in *Musa* sp. was obtained from embryogenic cultures induced by several plant growth regulators such as zeatin (Novak *et al*., 1989); BA and IAA (Ganapathi *et al*., 1999); IAA, 2, 4-D and NAA (Grapin *et al*., 2000); 2, 4-D (Gomez, 2001). However, TDZ was never employed in the induction of *Musa* sp. TDZ has been used to induce shoot organogenesis in several woody species (Huetteman and Preece, 1993). High activities of TDZ in plant regeneration were also reported in other ornamentals (Tsung *et al*., 2001; Sunpui and Kanchanapoom, 2002) and vegetables (Compton and Gray, 1993; Gray *et al*., 1993).

In our experiments the possible role of TDZ in shoot induction of the *Musa* sp. Gros Michel', AAA group through callus growth is characterized by the presence of TDZ. Embryogenic calluses were initiated from MS solid medium containing 2.0 mg/l TDZ. A two-stage process was developed, the first stage involved initiation of embryogenic callus on TDZ containing media. For the second stage of the process, these calluses were transferred to NAA and BA containing medium. On this medium, leaf-like structure was formed and developed further until plantlets occurred. The NAA/BA balance is one of the factors determining patterns of morphogenesis in order to increase the number of vegetative shoots. These combinations caused interactions that resulted in altered morphogenetic responses. Hence prolonged culture on this medium allowed the differentiation of shoot-like growth structures and subsequent complete plantlets.

In conclusion, this research demonstrates that plant regeneration via callus culture of shoot bud of the *Musa* sp. Gros Michel', AAA group corresponds to an organogenesis through the development of induced embryogenically determined cells (Ammirato, 1987). It seems that 2, 4-D plays an important role in the induction medium; TDZ is necessary for maturation and NAA in combination with BA are used for development. The procedure described here could be applied to other bananas with the AAA genome.

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