

Induction of Somatic Embryogenesis and Plant Regeneration in the Banana Cultivar 'Kluei Namwa' (*Musa sp. ABB*)

Beyene Demtsu^{1/}

Thunya Taychasinpitak^{2/}

Benya Manochai^{2/}

Shermarl Wongchaochant^{2/}

ABSTRACT

The study was conducted at the National Centre for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA). The objective of study was to investigate the efficiency of different auxins and auxin concentrations in inducing somatic embryogenesis and to identify suitable medium formulation for optimum germination of somatic embryos in the Thai banana cultivar 'Kluei Namwa' (*Musa sp. ABB* group). Complete plantlets were regenerated via somatic embryogenesis from shoot apex explants prepared from *in-vitro* propagated plants cultured on MS medium containing different concentrations of 2,4-D (2,4-dichlorophenoxy acetic acid) and picloram (4-amino-3,5,6-trichloropicolinic acid). Picloram at a concentration of 8 mg/l resulted the highest embryogenic calli while none of the explanted cultured on 2,4-D produced any callus. A suspension culture was successfully established by transferring embryogenic calli on to a multiplication medium (half strength MS medium, MS vitamins, 10 mg/l ascorbic acid, 0.22 mg/ zeatin, 30 g/l sucrose) supplemented with 8 mg/l picloram. Among different regeneration media formulations tested, a medium containing 5mg/l BA, 1 mg/l NAA 20 mg/l maltose 50 mg/l inositol, 0.25 g/l glucose and 0.25 g/l sucrose supplemented with 500 mg/l cassaminoacid was found to be the best formulation resulting healthy vigorous plantlets.

Key-words: somatic embryos, 'Kluei Namwa', picloram

^{1/} Tigrai Agricultural Research Institute (TARI), Tigrai, Ethiopia

^{2/} Department of Horticulture, Faculty of Agriculture, Kasetsart University, Chatuchak, Bangkok 10900

Introduction

Bananas and plantains are cultivated in over 100 countries in the tropical and subtropical regions of the world where they constitute a major staple food crop for millions of people, as well as providing a valued source of income through local and international trade (Frison and Sharrock, 1998). The world banana production averages 104 million tons per annum and almost 85 percent comes from relatively small plots and kitchen or backyard gardens (Anon, 2003). In many developing countries, the bulk of banana production is self-consumed or locally traded, thereby playing a crucial role in food security.

Somatic embryogenesis is used for mass clonal propagation (Schoofs, 1998, Kosky *et al.*, 2002), germplasm handling such as cryopreservation (Abdelnour-Esquivel *et al.*, 1992; Routefal, 2000) Agrawal *et al.*, 2004 and artificial seeds (Redenbaugh *et al.*, 1986). It is also an important tool for improving *Musa* sp. through nonconventional strategies, i.e. protoplast culture (Assani *et al.*, 2001; Haicour *et al.*, 2009), protoplast fusion (Assani *et al.*, 2005), mutagenesis (Jain, 2010), somaclonal variation (Mara and

Garca, 2000) and genetic transformation (Sagi *et al.*, 1995; Becker *et al.*, 2000). Somatic embryogenesis of banana cultivars of different groups has been successfully achieved (Cronauer-Mitra and Krikorian, 1988; Novak *et al.*, 1989; Dhed'a *et al.*, 1991; Escalant *et al.*, 1994; Cote *et al.*, 1996; Grapin *et al.*, 1998; Ganapathi *et al.*, 1999; Assani *et al.*, 2001); however, the conversion into plants is frequently low, thus limiting its use. Several factors are known to affect somatic embryogenesis such as explant type (Cronauer-Mitra and Krikorian, 1988; Novak *et al.*, 1989; Dhed'a *et al.*, 1991; Escalant *et al.*, 1994; Navarro *et al.*, 1997; Divakaran and Nair, 2011); genotype, growth regulators (type, amount, and combination), nitrogen source and others factors (Bhojwani and Razdan, 1996). In banana, different genotypes and different cultivars of the same genotype group react differently to the same culture condition while the same genotype react differently to varieties of plant growth regulators and their combinations (Fillipi *et al.*, 2001; Sidha *et al.*, 2007). Similar variation in response to culture environments was also reported in sweet potato (El Abidine Triqui *et al.*, 2008).

Moreover, irrespective of the methods used, the embryogenic frequency reported by different references so far remains very low (Xu *et al.*, 2005 and Strosse *et al.*, 2006). Establishment of embryogenic cell suspension (ECS) is an important step after induction of embryogenesis. Since ECSs frequently originate from a single cell, transformed plants circumvent the problem of chimeric plants encountered when using shoot tips as starting material hence are preferred material for genetic manipulation. ECSs are already used for genetic transformation (Sagi *et al.*, 1995; Becker *et al.*, 2000) and protoplast fusion (Xiao *et al.*, 2009) in banana plants.

Materials and Methods

Source and preparation of explant

In this study, *in vitro* plants of the Thai banana variety 'Kluei Namwa' (*Musa* sp. ABB) were provided by the Starch Biosynthesis Laboratory, National Centre for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency. Explants were prepared following the method used by Filippi *et al.* (2001). Plants of approximately similar height, diameter, and growth vigor were used for this purpose. Explants were

prepared by removing the roots and leaves and reduced to approximately 1.5 cm height and 0.5 cm diameter. These were cut into two by dissecting longitudinally in the middle to expose the meristem tissue thereby producing two explants from a single *in vitro* plant.

Induction of embryogenic callus

Explants were placed with the meristem (cut side) in contact to a medium containing MS salts and vitamins (Murashige and Skoog, 1962) supplemented with 8, 12, or 16 mg/l of either 2,4-(2,4-dichlorophenoxy acetic acid) and picloram (4-amino-3,5,6-trichloropicolinic acid) and 30 g/l sucrose and solidified with 2 g/l of gelrite. The media were adjusted to pH 5.8 with KOH (1N) and HCl (1N) prior to autoclaving and poured onto 9 cm diameter sterile plastic petridishes. Four explants were planted in each petridish and there were 20 petridishes per each hormone concentration. Plates were sealed with parafilm and maintained in culture room at 28 °C in darkness.

Initiation and maintenance of embryogenic cell suspensions

Cell suspension cultures were initiated by placing embryogenic callus in 150 ml Erlenmeyer flasks dispensed with 20 ml liquid multiplication medium (half strength MS medium, MS vitamins, 10 mg/l ascorbic acid, 0.22 mg/l zeatin, 30 g/l sucrose) supplemented with 8 mg/l picloram (the best auxin concentration for the induction of embryogenic calli) sealed with aluminium foil, and parafilm. suspensions were subsequently cultivated on a rotary shaker at 100 rpm under darkness and $26 \pm 1^\circ\text{C}$ for three months. The culture medium was refreshed every week for the first month and then every two weeks there after by keeping 10-20% of the old 'preconditioned' medium.

A batch of cell suspension cultures were prepared with an initial cell weight of 100 mg and added into 250 ml Erlenmeyer flasks containing 50 ml fresh multiplication medium to study cell growth by employing the fresh/dry weight method. Experiment was set up by measuring the fresh and dry weight by harvesting cells every 24 hours after inoculation over a time span of 17 days in three replications (three flasks per measurement). Cells were collected from each flask onto a preweighed whatman

filter paper using a vacuum filter and weighed for fresh weight and then oven dried for 48 hours or until the weight became constant by measuring at 2 hour intervals. Weight of the filter paper was then subtracted from both the readings of fresh and dry weight and plotted over the days in culture in the x axis.

Somatic embryos regeneration

Aliquots of 1 ml sedimented embryogenic cells were plated on petridishes containing maturation medium (multiplication medium without picloram) at pH 5.8 and incubated for two months under 16/8 hour light conditions at 27°C . After two months of culturing, matured embryos were individually transferred onto 150 ml test tubes containing different formulations of regeneration media (Table 1) containing 5 mg/l benzylamino purine (BA), 1 mg/l naphthalene acetic acid (NAA) and 50 mg/l inositol supplemented with different concentrations of carbon sources (maltose, glucose, sucrose) and casamino acid.

Results and Discussion

Induction of embryogenic callus

After 4 weeks in the culture media

Table 1. Formulations of different media tested for their efficiency in somatic embryos germination and conversion in the banana cultivar 'Kluei Namwa' (*Musa* sp. ABB)

Components added	Medium	Medium	Medium	Medium
to MS medium	1	2	3	4
BAP (benzyl amino purine)	5 mg/l	5 mg/l	5 mg/l	5 mg/l
NAA (naphthalene acetic acid)	1 mg/l	1 mg/l	1 mg/l	1 mg/l
Maltose	20 mg/l	50 mg/l	20 mg/l	20 mg/l
Inositol	50 mg/l	50 mg/l	50 mg/l	50 mg/l
Glucose	25 g/l	50 g/l	25 g/l	25 g/l
Sucrose	25 g/l	25 g/l	25 g/l	25 g/l
Casamino acid			200 mg/l	500 mg/l

containing picloram, explants started to change colour from gray to black followed by tissue development and growth which resulted in the bending of the explants in the 5th week (Figure 1). Yellow loose callus developed in the 6th week and globular somatic embryos started to develop starting the 7th week of culturing while steadily increasing in number and size into the 8th and 9th weeks. Explants cultured on the medium supplemented with 8 mg/l picloram gave rise to more number and faster development of embryogenic calli as compared to those on 12 and 16 mg/l. Explants cultured on the medium containing the highest picloram concentration (16 mg/l) turned black quickly; however the development of

yellow callus and somatic embryos was latest and smallest as compared to the lower concentrations.

As compared to picloram, explants cultured on the media supplemented 2,4-D did not result in the induction of embryogenic callus (Figure 2). Concentrations of 8 and 12 mg/l 2,4-D turned black and started to swell just like explants on picloram after 5th to 8th weeks in culture. However, all explants turned dark and eventually died with no callus development after the 9th week. Explants on the highest concentration of 2,4-D (16 mg/l) turned black and died in the 5th week. They did not show any sign of tissue development due to herbicidal effects of 2,4-D at high concentration. Tu *et al.*, (2001) explained that low concentrations of 2,4-D can

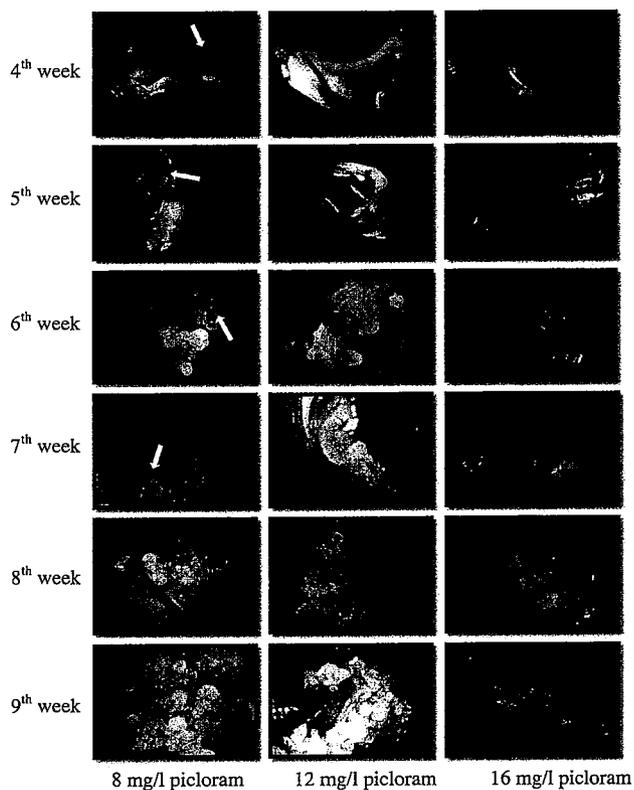


Figure 1. Response of banana cultivar 'Kluei Namwa' (*Musa sp. ABB*) to different concentrations of picloram through time in callus induction medium, arrows show tissue development and the bending of the explants (4th week), change of colour from gray to black (5th week), yellow loose callus (6th week) and globular somatic embryos (7th week) respectively while somatic embryos steady increase in number and size into the 8th and 9th weeks.

stimulate RNA, DNA, and protein synthesis leading to uncontrolled cell division and growth, and ultimately, vascular tissue destruction while on the other hand, high concentrations of 2,4-D

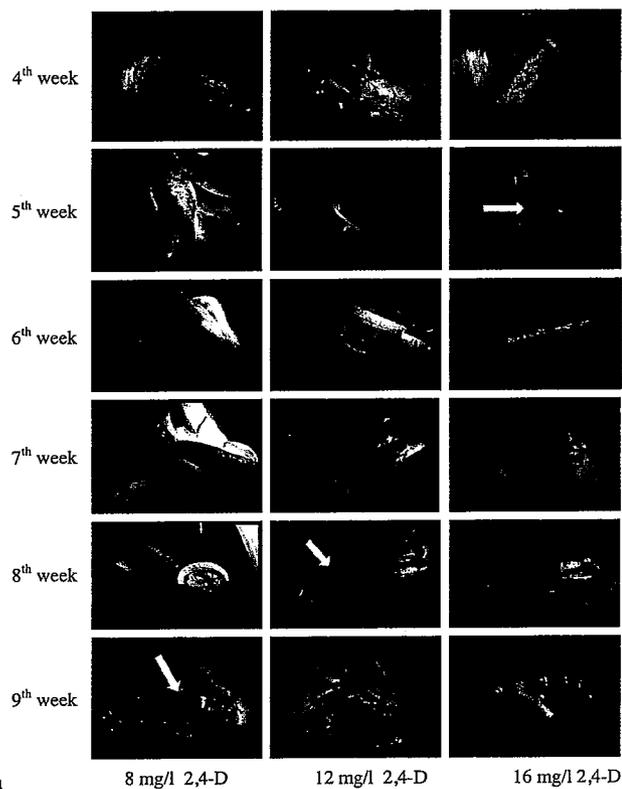


Figure 2. Response of banana cultivar 'Kluei Namwa' (*Musa sp. ABB*) to different concentrations of 2,4-D through time in callus induction medium, arrows show death of explants after the 5th, 8th and 9th weeks culture with 2,4-D concentrations of 16 mg/l, 12 mg/l and 8 mg/l respectively with no callus development.

can inhibit cell division and growth leading to plant death typically within three to five weeks following application.

Initiation and maintenance of embryogenic cell suspensions

Embryogenic suspensions were established by culturing calli on a liquid

multiplication medium containing half strength MS medium, MS vitamins, 10 mg/l ascorbic acid, 0.22 mg/l zeatin, 30 g/l sucrose and 8 mg/l picloram cultivated on a rotary shaker at 100 rpm under darkness and $26 \pm 1^\circ\text{C}$ for three months. Cell growth was studied using the fresh/dry weight method over a period of 17 days. The growth curves for both fresh and dry weight measurements were sigmoid (Figure 3) conforming to the theoretical cell suspension growth curves reported by Torres (1989), Razdan (1993). In the present study, the growth of embryogenic cells terms of cell proliferation (FW) indicated an increase in growth over a period of time until day 12. For example, the 4th day displayed an increase of 3 times, the 8th day had an increase of 6.75 times and the 12th day exhibited 10.48 times over the control (inoculation day).

Somatic embryos regeneration

In this study, embryo development was halted at the globular stage on media containing picloram. Further differentiation of somatic embryos beyond the globular stage and its subsequent maturation was possible upon their transfer to hormone free media. After two

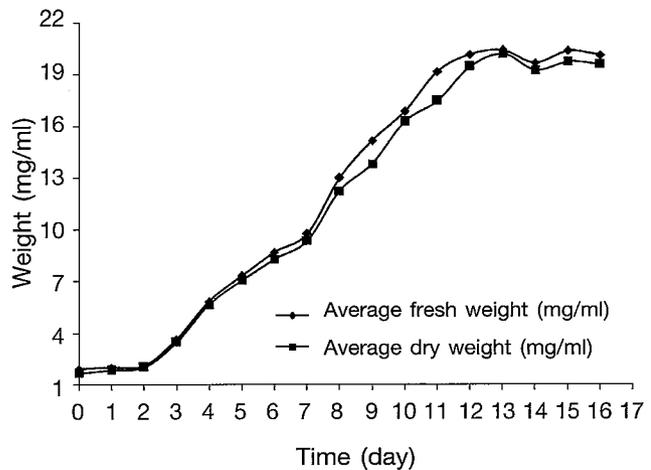


Figure 3. Growth curve of embryogenic cell suspension for the banana cultivar 'Kluei Namwa' (*Musa* sp. ABB) established by measurement of cell fresh and dry weight every 24 hours.

months of culturing, matured embryos were individually transferred onto 150 ml test tube containing different formulations of regeneration media. In this study, medium formulation 4 (Table 1) which included 5mg/l BA, 1 mg/l NAA, 20 mg/l maltose, 50 mg/l inositol, 0.25 g/l glucose, 0.25 g/l sucrose and supplemented with 500 mg/l casamino acid resulted in a high number of embryo germination of 92.7 % (139 embryos germinating out of 150 planted) followed by the medium containing similar components but 200 mg/l (Table 2). This result showed the importance of

Table 2. Effect of different media formulations on the regeneration of matured somatic embryos in the banana cultivar 'Kluei Namwa' (*Musa* sp. ABB group)

Medium	Number of mature embryos	Number of regenerated plantlets from embryos	Percentage of plant regeneration
1	150	85	56.7
2	150	78	52.0
3	150	111	74.0
4	150	139	92.7

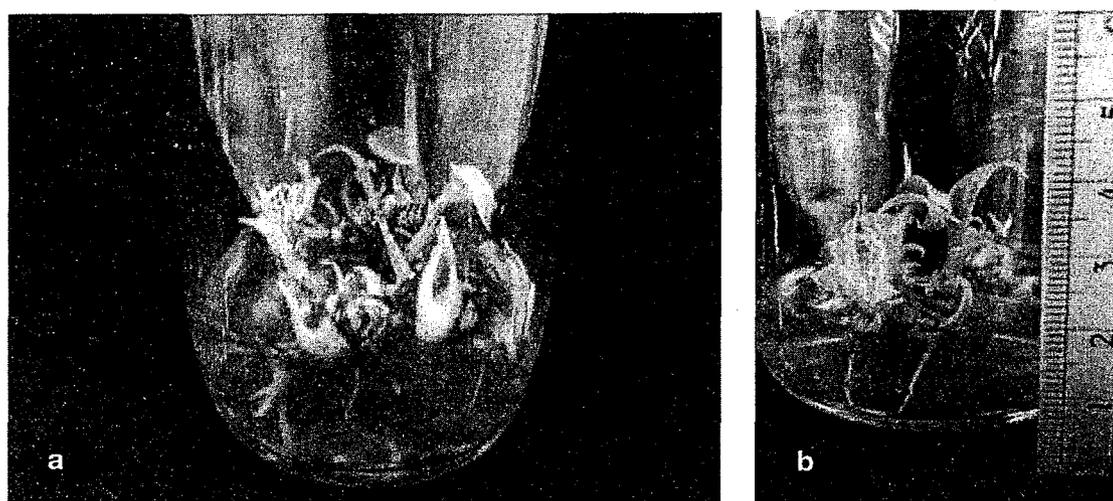


Figure 4. Germination of somatic embryos and plant regeneration of banana cultivar 'Kluei Namwa' (*Musa* sp. ABB): a) germination of an embryo into more than one plantlet and b) vigorous growth of regenerated plants

casamino acid in improving germination of somatic embryos in the banana cultivar 'Kluei Namwa'. Germinated embryos grew into healthy plantlets with some embryos yielding more than plantlets (Figure 4). Casamino acid is often used an important source of amino acids (Ramage and Williams, 2002; Davey and Anthony, 2010).

In another study, Sun and Hong (2010) reported that casamino acid improved germination of somatic embryos in *Leymus chinensis* (Trin.). Similarly, Kumar *et al.* (2008) reported that the addition of casamino acid into a regeneration medium increased the embryo regeneration in safflower.

Embryogenic response is known to be affected by genotype, type and concentration of growth regulator used, explants type and many other factors (Sidha *et al.*, 2007). Hence, although several commercial and elite clones have already been induced into embryogenesis, a large number of banana genotypes still need to be explored for embryogenic potential to assist propagation and genetic improvement. The auxin is the most frequently used auxin for the induction of embryogenic callus in banana and other plant species (Jimnez, 2001) however other auxins such as picloram and dicamba (3,6-dichloro-2-methoxybenzoic acid) are also used to induce embryogenic callus (Houllou-Kido *et al.*, 2005).

Filippi *et al.* (2001) reported that embryogenic response was obtained only from shoot apex explants of *Musa* spp. cv 'Nanico' cultured in picloram while none of the explants cultured on media containing 2,4-D showed no callus development in agreement with the results of this study. Similar results were also reported by Smitha and Nair (2011) from leaf sheath of *Musa acuminata* (diploid) cv 'Njalipoovan' (AB). In another

study conducted by Huang and Chi (1988), picloram also gave rise to the best induction of embryogenic callus from shoot tips of banana (*Musa sapientum* L.). However, in disagreement with our finding, they found out that 2,4-D also gave rise to callus development although only half of what was obtained with picloram. Picloram was also used to induce embryogenic callus from immature male flowers (IMF). In the study reported by Kulkarni *et al.*, (2002), highly embryogenic callus was observed on the seeded banana (*Ensete superbum* (Roxb.) Cheesman) using picloram while medium supplemented with only 2,4-D produced non-embryogenic callus. Similarly, Wei *et al.*, (2007) reported a high percentage of embryogenic calli from IMF of *Musa acuminata* cv 'Mas' (AA) from picloram although 2,4-D was also able to induce embryogenic calli. Houllou-Kido *et al.*, (2005) also reported a successful induction of embryogenic callus from IMF of the banana cv 'Ma' (AAB group). In general, the variations exhibited in all the studies are attributed to differential response of the genotypes to the auxins (Sidha *et al.*, (2007).

Conclusion

The auxin 2,4-D has been used to induce somatic embryogenesis both in banana and other species. However, in this study, it failed to induce any embryogenic callus from a shoot apex explant hence is not an option for somatic embryo induction for banana cultivar 'Kluei Namwa' (*Musa* sp. ABB). However, using the optimum concentration (8 mg/l), picloram was a better option for the induction of somatic embryogenesis in this cultivar. This study also found out that casamino acid enhanced the germination and conversion of somatic embryos obtained from embryogenic cell suspension into plantlets.

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