African Crop Science Journal, Vol. 12. No. 1, pp. 43 - 49, 2004
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ISSN 1021-9730/2004 $ 4.00

SOMATIC EMBRYOGENESIS FROM IMMATURE MALE INFLORESCENCES OF EAST AFRICAN HIGHLAND BANANA CV ' NAKYETENGU'

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ABSTRACT

The East African highland bananas (*Musa* spp. cv. EA-AAA), the most important staple food in Uganda, are susceptible to a range of pests and diseases. This has been attributed to a narrow genetic base of this banana group and thus the need to create a wide genetic base through development of resistant cultivars. The use of genetic transformation (engineering) approach has been identified as a potential option that could be utilised to facilitate and/or enhance the process of developing resistance cultivars. The objective is to establish an *In vitro* regeneration system for East African (EA-AAA) highland bananas. Cell suspensions are the material of choice for genetic transformation because of their regeneration capacity through somatic embryogenesis. In this study, immature male flowers of cultivar 'Nakyetengu' were isolated and initiated on callus induction media designated M1, M2, M4, M6, M7 on petri-dishes. Cultures were placed under controlled light and temperature conditions and monitored for embryogenic callus formation. Embryogenic callus composed of somatic embryos was obtained on medium M1 only, after a culture period of 3.5 to 4 months. Direct somatic embryo germination was achieved on embryo germination medium. Somatic embryo germination was recorded at a rate of 66.7%. Plant recovery was achieved on standard MS hormone free medium, all being normal plants with root and shoot at weaning. With regard to the pest and disease problems of the East African highland banana, these findings underscore the fact that somatic embryogenesis is essential in the development of an *In vitro* regeneration system and is a critical step for the development of resistant varieties through genetic transformation.

Key Words: Cell suspensions, embryogenic callus, medium, regeneration, somatic embryos

RÉSUMÉ

Les bananas de région de montagne de l'Afrique de l'est (*Musa* spp.cv. EA-AAA), le plus important aliment de base en Ouganda, sont susceptibles à une gamme des maladies et des pestes. Ceci a été attribué à une faible base génétique de ce groupe de bananes et ainsi le besoin de créer une large base génétique à travers le développement des variétés résistantes. L'usage de l'approche de transformation génétique (inginerie) a été identifié comme une option potentielle qui pourrait être utilisée pour faciliter et/ou améliorer le processus de développement des variétés résistantes. L'objectif est d'établir une regénération *In vitro* du système pour les bananas de région de montagne de l'Afrique de l'est (EA-AAA). Les suspensions des cellules sont les matériels de choix pour la transformation génétique à cause de leur capacité de regénération à travers l'embryogenèse somatique. Dans cette étude, les fleurs mâles immatures de variété 'Nakyetendu' étaient isolées et initiées sur le media induction durillon classées sur les boites de petri M1, M2, M4, M6, M7. Les cultures étaient placées sous les conditions contrôlées de température et lumière et surveillées pour la formation de durillon embryogenèse. Le durillon d'embryogenèse composé des embryons somatiques était obtenu sur seulement la moyenne M1, après une période de culture de 3,5 à 4 mois. La germination de l'embryon somatique direct était atteinte sur la germination moyenne de l'embryon. La germination de l'embryon somatique était enregistrée à un taux de 66,7%. Le recouvrement de plante était atteint sur l'hormone libre moyen standard MS, tout étant normal pour les plantes avec racines et rejeton au sevrage. Avec regard aux problèmes des maladies et pestes des bananas de région de montagne de

l'Afrique de l'est, ces résultats soulignent le fait que l'embryogenèse somatique est essentielle dans le développement d'un système de régénération *In vitro* et est une étape critique pour le développement des variétés résistantes à travers la transformation génétique.

Mots Clés: Suspensions des cellules, durillon embryogénique, moyen, regénération, embryons somatiques

INTRODUCTION

Bananas and plantains are a staple food for 400 million people of whom 7 million are Ugandans (Cammue *et al.*, 1993). In E. Africa, they are produced both for home consumption and local trade (Kashaija, 1996). The average national banana consumption of 400-600kg/person/year is the highest in the world (Tushemereirwe *et al.*, 2000). Pests and diseases are a threat to this banana group. This has been attributed to their narrow genetic base (Gold *et al.*, 1993; Tushemereirwe *et al.*, 1996). Lack of pests and diseases resistant varieties pose a major threat to food security. Development of resistant varieties through genetic improvement is a potential option that could be utilised to facilitate efforts geared towards increasing the genetic base.

Conventional breeding activities in East African highland bananas (EAHBs) has focused on pollinating triploid (Matooke) with resistant diploid male parents especially Calcutta 4 to produce hybrid cultivars. However, the hybrids produced so far have lower yield values and poor cooking qualities when compared with matooke. In addition, long cropping cycles, complex genetic constitution and low female fertility hampers development of disease resistant bananas by conventional breeding (Vuylsteke *et al.*, 1993; 1998; Panis *et al.*, 1995). This indicates that it is likely to take many years to develop an acceptable, pest and disease resistant EAHB hybrid using conventional breeding methods. Therefore, the potential role of genetic engineering is complementary to conventional breeding methods by a one step introduction of well-characterised genes conferring disease resistance without altering other valuable traits (Vuylsteke *et al.*, 1993) of matooke.

In order to carry out genetic transformation, an effective and highly performing *in vitro* regeneration system with a regenerable target tissue is required. Somatic embryogenesis and cell suspension cultures have made it possible to obtain banana and plantain plants developed *in vitro* (Escalant and Teisson, 1989; Novak *et al.*, 1989). Somatic embryogenesis has two objectives; the development of micropropagation techniques and cell regeneration systems. The main characteristic of such a system is that a normal true to type plant can be regenerated from the explant at high frequency (Sagi *et al.*, 1995).

Such explant regeneration has been achieved in cell or protoplast cultures derived from meristems (Novak *et al.*, 1989; Panis *et al.*, 1993; Schoofs, 1997; Schoofs, 1998) zygotic embryos (Escalant and Teison, 1989) and young male flower tissue (Escalant *et al.*, 1994; Cote *et al.*, 1996). However, the procedures remain genotype specific (Vuylsteke *et al.*, 1998). Embryogenic cell suspensions have also been reported for *Graminaea* spp. (Shatters *et al.*, 1994), highland Papaya (Jordan *et al.*, 1996), cassava *(Manihot esculentum)* (Sofiara *et al.*, 1997) and cocoa *(Theobroma cacao)*, (Alemanno *et al.*, 1996).

There are no reports on somatic embryogenesis of East African highland bananas and establishment of their embryogenic cell suspensions. The present study was therefore instituted to investigate somatic embryogenesis and plant regeneration of East African highland bananas using male flowers of cultivar 'Nakyetengu'.

MATERIALS AND METHODS

Ten male buds of cv 'Nakyetengu' were harvested from field growing plants after bunch formation was complete. Bracts were removed upto a final size of about 1.5cm width and 2.0cm length. Immature flowers (hands) were isolated from positions 8-15 under a binocular microscope and laminar flow hood and inoculated on semi-solid callus induction medium on 90mm x 15mm petridishes. The medium used designated here as M1, comprised of standard MS salts and vitamins (Murashige and Skoog, 1962), supplemented with 4.09µM biotin, 4.5µM 2,4-dichlorophenoxyacetic

acid (2,4-D), 5.37) µM indole acetic acid (IAA), 5.4µM naphthalene acetic acid (NAA), 20mg l^{-1} ascorbic acid, 3 sucrose, and 7.2mg l^{-1} agarose. In addition, various concentration of 2,4,D were also tested as follows: 2mgl'1; 4mgl'"; 6mgl"1; and 7mg^{-1} 2,4-D corresponding to M2, M4 (which is MAI according to Cote etal., 1996), M6, andM7, respectively. The pH was adjusted to 5.8 with IN NaOH and IN HCL before autoclaving. Culture dishes sealed with parafilm to avoid contamination, were placed in the dark at 28 ±1°C and 67 humidity and monitored for embryogenic callus formation for 5-6 months. The male buds used were obtained from Kawanda Agricultural Research Institute (KARI) in Kampala, Uganda, and in vitro experiments were conducted at the KARI tissue culture laboratory.

Embryo germination. Somatic embryos were initiated on embryo germination medium. This consisted of standard MS salts supplemented with Morel vitamins (Morel and Wetmore, 1951), 0.22µM benzylanimopurine, 1.14µM indole acetic acid, and 87µM sucrose, solidified with 7.2gl^{-1} agarose. The pH was adjusted to 5.8 with 1N NaOH and IN HCL before autoclaving. Plantlet regeneration was achieved on MS hormone free medium at 28 ±1 ^{0}C, 14:10 light: dark photo period.

Data collection. Embryogenic callus formation was recorded as percentage response. This was taken as number of hands (with embryogenic callus) out of the total number of buds used.
Somatic embryo germination was recorded as number of embryos with root and/or shoot out of total number of embryos cultured. Pictures were taken using a digital camera (Sony,Mavica, MVC-FD 75).

RESULTS AND DISCUSSION

Embryogenic callus production. Embryogenic callus was only obtained on M 1 medium after 3.5-4 months of culture, representing 20 embryogenic callus response for 10 buds (replicates) used, and 2.5 of the total 80 hands.The first sign of embryos was observed at 3.5 months of culture. This was characterised by somatic embryos growing from whitish compact tissue. By 4 months, the embryos had grown in number and size. No friable tissue developed at the base of embryos. The original tissue was maintained on the same media up to 6 months without sub-culturing. There was an increase in the number of somatic embryos obtained in the 5th to 6th month on the tissue where embryogenic callus was obtained. The increase in number of embryos results from maintaining the original explant on medium without subculturing (Escalant *et al.,* 1994). It also results from the fact that when tissues are subjected to extinctive stress, they respond by generation of several propagules as ameans to maintain their continuity and survival.No embryogenic response was obtained on media M2, M4, M6 and M7. Embryogenic callus was obtained by culturing'Nakyetengu' on medium M 1. No similar response was observed with other cultivars used i.e.,'Namwezi', 'Nakitembe', and 'Kisansa'. Varying levels of yellow non-embryogenic callus was observed. At Imgl-' and 2mgl^{-1} 2,4-D corresponding to medium Ml and M2, respectively, low levels of tissue necrosis were observed compared to medium M4, M6 and M7 where tissue necrosis was very high. This observation can be explained by the fact that production of embryogenic callus has a strongdependence on cultivar (Gamborg and Phillips,1995), genotype and on the ontogeneticdevelopmental stage of the tissue (Dixon and Gonzales, 1994) as reported in earlier studies.Medim Ml favoured cultivar 'Nakyetengu' and not the others. Embryogenic response was obtained

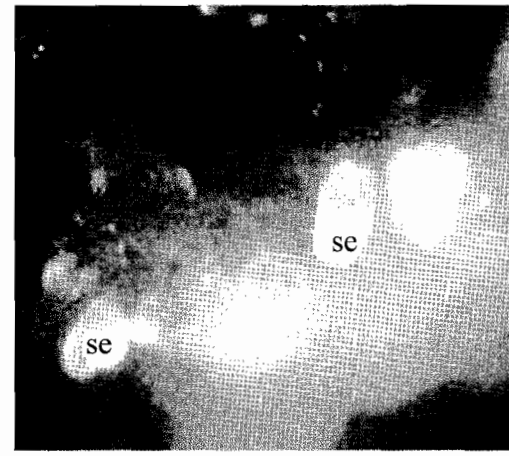

Plate 1. *se*, somatic embryos, without friable tissue

at a concentration that is four times lower than that previously used for *Musa* AAA cv Cavendish (Escalant et al., 1994; *Cote et al.,* 1996). Male flowers of 'Nakyetengu' cultured on MA1(Escalant *et al.,* 1994, *Cote et al.,* 1996) did notproduce somatic embryos under theseexperimental conditions. This probably implies that *Musa* AAA-EA cultivars require lower concentrations of auxin to produce embryogenic callus. This suggests a potential future working range that can be investigated with further experimentation to optimise conditions for cultivars belonging to different EAHB clone sets.

Different *Musa* AAA-EA cultivars may require variable medium alterations since in this case, only cultivar 'Nakyetengu' responded on one medium formulation. These results are however, preliminary, further investigation to optimise conditions in order to increase chances of obtaining embryogenic callus areon-going.

Embryo germination. Somatic embryos cultured on embryo germination media became swollen, vitrified and developed small root hairs in the first 7 days of culture (Plate 2a). Germination was complete after 14 days with a clear root and shoot structure ((Plate 2b). Plantlet development i.e., leaf and root elongation was achieved on standard MS hormone free media. Multiple roots started growing vigorously. However, there was some disorientation of root development observed, with some roots growing upwards. Within two months, the plantlets had at least 4 open leaves and well developed roots (Plate 2c).

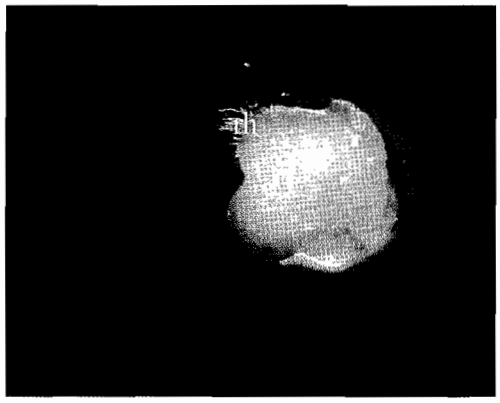

Plate 2a Embryo swollen, with root hairs, *(rh).*

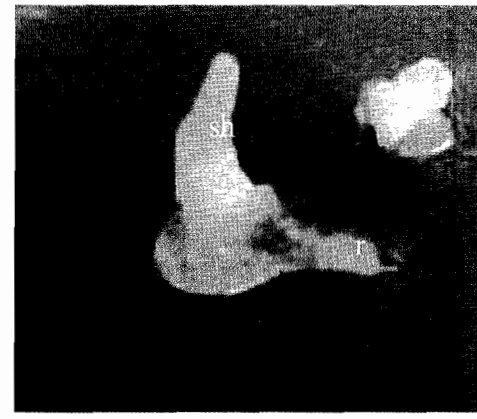

Plate 2b Germination with r, root and sh shoot .

Plate 2c Germination with shoot only

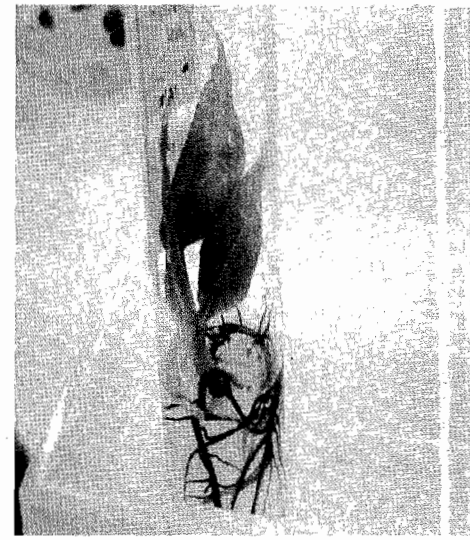

Plate 2d Plantlet with 3 leaves at 1.5 months

Somatic embryo germination exhibited a 66.7 germination success. This is comparable to what has been previously obtained from encapsulated somatic embryos of Musa AAB cv Rasthali (Ganapathi etal., 1999) somatic embryos of Musa AAA cv Cavendish i.e., ranging between 60 and 70 germination (Escalant et al., 1994). Subsequent embryos obtained from the same tissue after 5 months of culture showed reduced germination potential (Table 1). Two forms of germination were observed from these embryos. In the first one, they developed green shoots or pale green 'shoot- like' structures after 2-4 weeks (Plate 2d). These were transferred onto standard MS hormone free medium for root development, and this was complete in approximately 9-29days (Table 2). In the second, embryos germinated with both shoot and root together (Plate 2b). A few germinations were very prolific, giving more than one plant (Table 2). Normal weaned plantlets

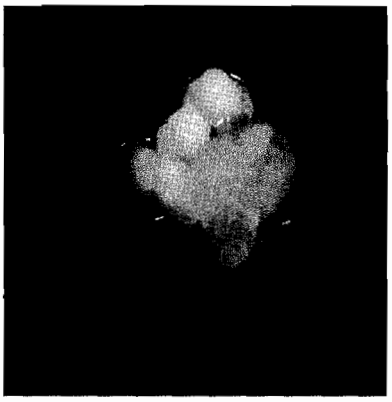

Plate 2e Vitrification and failed germination

Plate 2f Plantlet at nursery stage

TABLE 1. Direct somatic embryo germinations from cv 'Nakyetengu'

Observation	Number of embryos plated	Germination	Percentage germination
1	3	2	66.7
2*	19	8	42.1%

*Embryos were observed after 4.5 months of cultures

TABLE 2. Somatic embryo derived plants and their mode of germination

Observation 1	Germination code	Form of germination	No of days to rooting	No of plants derived
	*SED1	Shoot and root	NA**	1
	*SED2	Shoot and root	NA	25***
Observation 2	SED3	Shoot and root	NA	2
	SED4	Shoot	23	3
	SED5	Shoot	9	1
	SED6	Shoot	15	1
	SED7	Abn shoot		failed
	SED8	Abn shoot		failed
	SED9	Shoot	29	2
	SED10	leaf		failed

*SED(somatic embryo derived plant)
**NA (Not applicable)
***Plants were cultured on multiplication medium for 1 month

were established in the nursery for preliminary evaluation (Plate 2e). Some embryos turned into vitrified structures (Plate 2f) or produced only roots, and did not germinate into plants.

The results obtained with male flowers of EAHBs corroborate earlier findings on *Musa* spp. (Cote *et al.*, 1990). The formation of non-embryogenic, yellow nodular callus was also observed on Grand Naine (Escalant, 1994), French sombre (Grupin *et al.*, 1996), *Musa acuminata* (Navaro, 1997). However, development of embryogenic callus was achieved in this study through modification of a procedure previously used for these *Musa* spp. Embryogenic callus was obtained at a concentration of 2,4-D, 4 times less than that used for male flowers explants. This is an exception to the methodology described before believed to be applicable to all *Musa* genotypes without any changes (Grapin *et al.*, 1998). In regard to the difficulty in genetic improvement of bananas, the development of an *invitro* regeneration system for a range of EAHB cultivars remains essential. Cell suspensions are the material of choice for genetic transformation because of their regeneration capacity through somatic embryogenesis.

The practical implications of this observation is that somatic embryos obtained through this method can be used for genetic improvement of EAHBs by introducing desirable genes through particle bombardment. Relatively high levels of germination obtained i.e., more that 65%, show their regeneration capacity. Further more, the method has potential application to mass propagation of bananas. The successful application of this method will further depend on the trueness to type of the plants derived there from. Somatic embryo derived plants now in the nursery, are being prepared for field evaluation to ascertain their trueness to type.

ACKNOWLEDGMENT

The study was conducted in the framework of the Banana Biotechnology Project coordinated by INIBAP and funded by the Government of Uganda through the CGIAR.

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