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Full Length Research Paper

Generation of cell suspensions of East African highland bananas through scalps

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The improvement of East African highland bananas (EAHBs) using conventional breeding methods is difficult due to their biology and therefore focus on improving them has shifted to exploring methods for establishment of embryogenic cell suspensions, which can then be targeted, for genetic transformation. Shoots of four cultivars namely 'Musakala', 'Kibuzi', 'Mbwazirume' and 'Lwadungu' were transferred to a multiplication media modified by adding a combination of N6-benzylaminopurine (BAP) and thidiazuron (TDZ) at concentrations of 24.8/0.45, 16.2/1.14, 14.4/3.50, 12.4/4.55, 10/5.68 µM for scalp generation. These media are referred to as M1, M2, M3, M4 and M5, respectively. Two other treatments designated as M6 and M7 with concentrations of 10 µM TDZ and 100 µM BAP, respectively were included for comparison purposes. The scalps developed were excised and inoculated into liquid induction medium supplemented with either BAP or Zeatin to generate cell suspensions. Scalp formation was achieved earlier and at much lower concentrations of combined BAP and TDZ than when singly applied. Combinations of 12.4/4.55 and 10/5.68 µM BAP/TDZ produced the best scalps. The structure of the cell suspension and the rate of cell growth were found to be dependent on the cultivar regardless of the hormone treatment in the induction medium. Cultivars 'Musakala', 'Kibuzi' and 'Mbwazime' produced cell culture of clustered and aggregated cells and high cell numbers, which are a prerequisite for embryo cells development.

Key words: Banana, N6-benzylaminopurine**,** thidiazuron, scalps, embryogenic cell suspensions.

INTRODUCTION

Banana and plantain (*Musa* sp.) is a crop of tremendous economic and social importance in the humid and subhumid tropical regions of the world (Robinson, 1996). Its perennial nature and ability to produce fruit throughout the year provides food and income security for millions of people in Africa and other countries. Banana and plantain also has high industrial potential for the production of juice, wine and a variety of post-harvest products (Ssebuliba et al., 2000). In Uganda, the East African Highland banana (EAHB) is a major staple food crop and is cultivated extensively. Uganda's annual production of banana is estimated at 8.45 million tons, accounting for 15% of the world's banana/plantain output. The per capita consumption of banana in Uganda estimated at 220–240 kg is the highest in the world (Anon, 2000). There has been a decline in banana production per unit land area especially in Central Uganda over the last 20 years (Tushemereirwe, 1996). This decline has been attributed to pests, diseases and declining soil fertility (Davies, 1995). The most important diseases are black Sigatoka leaf spot (*Mycosphaerella fijiensis*) and fusarium wilt (*Fusarium oxysporium* f.sp. *cubense*). The latter mainly affects the exotic banana such as cultivars 'Pisang awak', 'Sukali ndizi' and 'Gros Michel'. The major pests are a complex of nematodes (*Radopholus similis, Pratylenchus goodeyi, Helicotylenchus multicinctus and Meloidogyne* sp.) and the banana weevil, *Cosmopolites sordidus*.

Conventional breeding methods for the improvement of EAHBs are complicated by high sterility, different polyploidy levels and long generation times. Genetic engineering provides an alternative method for crop improvement. Most transformation protocols for banana and plan-

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Figure 1. Mature scalps of 'Musakala' ready for inoculation into liquid induction medium.

tain rely on the production of cell suspensions (Sagi et al al., 1995; Côte et al., 1997; Becker et al., 2000; Ganapathi et al., 2001). However, a transformation protocol applicable to a wide range of banana genotypes using shoot tips has also been developed (May et al., 1995; Tripathi et al., 2002, 2003). The cell suspension phase offers an opportunity for various selection, mutage-nesis or genetic transformation schemes to be superim-posed on the system prior to regeneration. Transforma-tion at the cellular level is considered to reduce the occurrence of chimerism that is associated with the use of meristems (Schoofs et al., 1999).

Regenerable embryogenic cell suspensions for most banana genome groups have been obtained from immature zygotic embryos, immature male or female flowers, somatic tissues such as *in vitro* leaf bases, corm slices and proliferating meristems (Schoofs et al., 1999). Namanya et al. (2002) generated embryogenic cell suspendsions from immature male flowers of the EAHB cultivar 'Nakyetengu' but none has been generated from scalps (proliferating meristems). The establishment of embryogenic cell suspensions from scalps offers a better alternative for plant regeneration, since some edible bananas including the EAHBs (Endirira) do not produce male flowers on which the male bud method depends (Schoofs et al., 1998). In addition, the use of scalps to generate cell suspensions allows one to obtain explants from *in vitro* cultures or plants at any growth stage and thus saves time, as most banana genotypes take up to twelve months or more to flower.

Schoofs et al. (1998) developed a widely applicable methodology for the initiation of embryogenic cell suspensions using scalps. However, this method that uses a high dose (100 µM) of N6-benzylaminopurine (BAP) produces scalps in the EAHBs only after 9 subculture cycles and is cultivar dependent. The prolonged use of a high dose of BAP has the disadvantage of producing somaclonal variation and/or an undesirable decrease in somatic embryogenesis (INIBAP, 2000).

Nahamya (2000) achieved relatively rapid production of scalps in EAHBs by using 10 µM of thidiazuron (TDZ), a urea-based compound that shows cytokinin type activity instead of 100 µM BAP but the competence of these scalps for somatic embryogenesis was not well established. Sudarsono and Goldy (1991) observed that the addi-tion of low (<1 µM) concentrations of TDZ to BAP con-taining medium enhanced axillary shoot proliferation in *Vitis rotundifolia*. However, when higher concentrations (>1 µM) of TDZ were added to BAP containing medium both axillary and adventitious shoots were produced in *Cercis canadensis* var. alba L. (Yusnita et al., 1990).

The objective of this study was to determine the most effective combination of BAP and TDZ for scalp production in EAHB and the potential of these scalps for generation of embryogenic cell suspensions.

MATERIALS AND METHODS

The study was conducted in the plant tissue culture laboratory at Makerere University Agricultural Research Institute Kabanyolo (MUARIK) in Uganda. Four EAHB cultivars 'Musakala', 'Kibuzi', 'Mbwazirume' and 'Lwandugu' were used in this study. The explants were obtained from *in vitro* cultures of these plants.

Scalp induction

Shoot tips from the *in vitro* plants were transferred to a multiplication medium described by Talengera et al. (1994). The media was modified by adding a combination of BAP and TDZ at concentrations of 24.8/0.45, 16.2/1.14, 14.4/3.50, 12.4/4.55, 10/5.68 µM for scalp formation. These media are referred to as M1, M2, M3, M4 and M5, respectively. Two other treatments designated as M6 and M7 with concentrations of 10 µM TDZ and 100 µM BAP, respectively, were included for comparison purposes. Ten shoot tips (explants) were used for each treatment. Sub-culturing was done every four weeks until scalps were formed.

Generating cell suspensions

Thin sections of the scalps were carefully excised avoiding any corm and green leaves (Figure 1) and transferred to 125 ml Erlenmeyer flasks containing 15 ml of liquid induction medium composed of: half strength MS (Murashige and Skoog, 1962) mineral salt mixture, 30 g/l sugar, 100 mg/l myo-inositol, 2.0 mg/l glycine, 0.5 mg/l thiamine HCl, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine and 20 mg/l ascorbic acid, adjusted to pH 5.8. Two combinations of hormone treatments were used: 5 µM 2, 4-dichlorophenoxy acetic acid (2,4-D) and 1 µM zeatin (Schoofs et al., 1998), and 5 µM 2,4-D and 5 µM BAP (Cronauer and Krikorian, 1983). Ten scalps were used from each cultivar for each media. The cultures were placed on an orbital shaker at 70 rpm and maintained in the dark at 27 ± 2°C according to Gomez et al. (2000).

The cultures from the liquid medium were refreshed by removing the meristematic layers and all blackened and dead tissue cut off before they were re-inoculated into 15 ml of fresh medium of the same composition in a 125 ml Erlenmeyer flask. Any cells that had sloughed off into the culture medium were allowed to settle. About 3 - 5 ml of the cell were transferred with a pipette to 15 ml of fresh liquid medium to allow adequate gas exchange and prevent the cells from drowning due to too much liquid medium (Kenneth and

Table 1. Means for cultivar proliferation on the various combinations of BAP and TDZ at fifth and sixth subculture cycles.

S. cycle	Cultivar	Organ	Mean number of shoots and multiple buds							
			M1	M2	M3	M4	M5	M6	M7	LSD(P=0.05)
5	Musakala	Shoots	1.0	1.0	0.6	0.4	0.5	1.2	3.6	1.240
		Multiple buds	14.2	12.7	13.0	13.7	10.9	10.2	8.5	5.549
	Kibuzi	Shoots	3.0	1.6	1.2	1.1	0.4	1.7	2.9	0.917
		Multiple buds	11.1	15.7	17.9	15.4	14.3	9.7	8.9	4.897
	Mbwazirume	Shoots	0.8	1.0	0.5	0.4	0.3	1.1	1.7	0.898
		Multiple buds	15.2	15.4	14.4	18.8	18.2	14.7	8.0	6.073
	Lwadungu	Shoots	1.0	1.0	0.3	0.9	0.9	0.7	2.5	0.904
		Multiple buds	8.1	8.9	11.4	9.8	10.1	7.9	5.4	4.231
6	Musakala	Shoots	3.3	0.6	0.1	0.3	0.9	1.3	1.2	1.104
		Multiple buds	13.2	15.5	17.4	21.4	18.0	16.7	18.4	4.466
	Kibuzi	Shoots	3.8	0.9	1.7	0.5	0.6	1.7	1.3	1.179
		Multiple buds	9.1	15.8	13.4	18.2	19.8	14.9	15.9	4.022
	Mbwazirume	Shoots	1.6	1.0	1.2	0.7	0.2	1.0	1.6	1.149
		Multiple buds	17.0	18.9	17.4	17.1	18.1	15.7	13.6	4.366
	Lwadungu	Shoots	3.1	0.1	0.2	0.1	0.2	0.1	1.3	0.907
		Multiple buds	13.8	16.9	17.6	20.5	20.4	19.8	16.4	4.149

S. cycle= Subculture cycle

Torres, 1989). Initially the cultures were refreshed weekly to avoid scalp blackening and death due to oxidation of phenolic compounds, but after 3 weeks a 2 - 3 week interval was used as described by Schoofs et al. (1998). Cell growth was determined by counting the number of cells in 1 ml of the cell suspension with a haemocytometer and growth curves for each cultivar established.

RESULTS AND DISCUSSION

Scalp induction

The shoot tips in the modified media (M1-M7) produced a mixture of shoots with multiple buds in varying numbers that depended on the proportion of BAP to TDZ in the media. Bud formation is a pre-requisite for scalp formation in banana (Schoofs et al., 1999). At the fifth cycle, bud proliferation was greater in the treatments that included TDZ (M1, M2, M3, M4, M5, and M6). Bud proliferation was lowest in M7 that did not contain TDZ in the fifth cycle. However, at the sixth cycle there were no significant differences in bud proliferation rates between TDZ and BAP containing media (Table 1). All the cultivars produced more buds with further sub-culturing. At the sixth cycle, most cultivars produced more than 10 multiple buds per meristem (Table 1). The cultivar 'Lwadungu' which had the least proliferation in the fifth cycle showed a marked increase in bud formation in the 6^{th} cycle.

The different cultivars responded differently to the various media used in this study. For example, 'Kibuzi' produced most buds in M5 while the other cultivars performed best in M4.

The mean multiple bud proliferation increased signifycantly from 13.2 in M1 to 21.4 in M4 for 'Musakala', from 9.1 in M1 to 19.8 in M5 for 'Kibuzi' and from 13.8 in M1 to 20.5 in M4 for 'Lwadungu' demonstrating that combinations M4 and M5 were the best for multiple bud formation in these cultivars.

The formation of scalps from the multiple buds was cultivar and medium dependent began in the fifth cycle and became fully developed in the sixth to ninth cycle. In the case of' 'Musakala', 'Kibuzi' and 'Lwadungu', most scalps were formed in the sixth cycle on both M4 and M5. The multiple buds in cultures on M2, M3 and M6 were not fused and did not form uniform scalps (Figure 2a) as compared to cultures in M4 (Figure 2b). All the cultivars on M1 and M7 showed no sign of scalp formation till the eighth and ninth cycle (data not shown).

The results (Table 1) showed higher multiple bud proliferation, which indicates better scalp formation in the treatments with TDZ than BAP. The mean multiple bud proliferation increased as the concentration of TDZ increased in combination with BAP. Nahamya (2000) reported higher multiple bud proliferation and earlier scalp formation in EAHBs with TDZ than with BAP when the two cytokinins were used at concentrations of 10 µM and 100 µM, respectively. Arinaitwe (1999) also reported an increase in bud proliferation rate as the concentration of TDZ was increased from 0.045 to 6.81 µM. The higher cytokinin activity of TDZ was attributed to its ability to accumulate in cultured tissues to act as endogenous cytokinins (Huetteman and Preece, 1993). Similar reports were made in woody plants (Thomas and Katterman, 1986), in

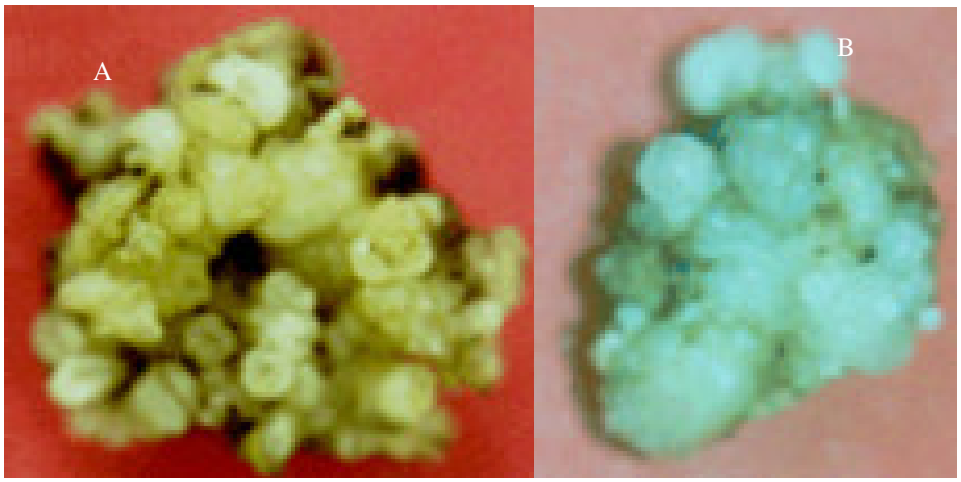

Figure 2. 'Kibuzi' Scalps formed at the sixth cycle of subculture on (A) M2 (B) M4.

Table 2. Percentage of scalps of the different cultivars that released cells in the induction medium with different cytokinin supplements.

	Percentage of scalps that released cells	
Cultivars	**2,4-D/Zeatin**	**2,4-D/BAP**
Musakala	55%	60%
Kabuki	85%	90%
Mbwazirume	40%	30%
Lwadungu	60%	55%

evergreen azalea (Korban and Chi-Ni, 1997), and in banana (Arinaitwe et al., 2000). TDZ is also less susceptible to degradation by enzymes within the tissues and, therefore, is stable and biologically more active than adenine-type cytokinins (Mok et al., 1987).

The results of scalp proliferation showed that in a mixture of BAP and TDZ (Table 1), low concentrations of 12.4 and 4.55 µM BAP and TDZ, respectively, were able to induce scalp formation. When used separately, BAP and TDZ have been reported to require concentrations of ≥100 µM (Schoofs et al., 1998) and ≥10 µM (Nahamya, 2000), respectively, to induce scalp formation. The combined effect of the two cytokinins in the culture medium appears to have influenced both their uptake and metabolism in the plant tissues (Korban and Chi-Ni, 1997), which likely caused a synergistic stimulation of scalp proliferation. Jeanette et al. (1995) similarly repor-ted more axillary shoot formation when shoots of *Miscanthus* X *ogiformis* Honda 'Giganteus' were transfer-red from medium with benzyladenine (BA) to a combina-tion of BA and TDZ than shoots grown continuously on either medium and subsequently suggested synergism between the two cytokinins in axillary shoot formation depending on the sequence of their application in that crop.

Generation of cell suspensions

The percentage of scalps that released cells in the liquid induction medium was cultivar specific. The response of the scalps in producing cells in the liquid induction media did not differ in the two hormone treatments (Table 2). More than 80 % of the scalps from 'Kibuzi' produced cells in the induction media while only 40% of scalps from 'Mbwazirume' were of cell producing type. Sixty percent of the scalps of 'Musakala' and 'Lwadungu' produced cells. Schoofs et al. (1998) reported a similar variation in cultivar response when the percentage of scalps producing embryogenic complexes on semi-solid induction medium was studied. A higher proportion of scalps that releases cells is favorable for the production of good cell suspensions.

In the liquid induction medium, the scalps became swollen as they absorbed much of the medium and produced callus-like substances on their surfaces. Cells were observed in the liquid media in 3 - 4 weeks after induction (Figure 3). Nahamya (2000) reported similar results within the same time.

Upon transfer of the initial cell suspension to fresh induction medium, the cell suspension of all the cultivars showed a similar growth pattern (although at different rates) regardless of hormone treatment in the induction medium (Figure 1). The growth curves were nearly sigmoid conforming to the theoretical cell suspension growth curves reported by Kenneth and Torres (1989), Razdan (1993) and Kantharajah (2001). In general 'Kibuzi' had the highest cell growth (5.1 x 10^4 cells/ml) after 5 weeks with a more sigmoid type growth curve. Such a growth curve is conducive for formation of somatic embryos as described by Nahamya (2000). 'Mbwazirume' consistently had the least cell growth and attained peak cell number in the 4th week (Figure 1). The cultivars 'Musakala' and 'Lwadungu' attained moderate cell num rers after 5 - 6weeks (Figure 4).

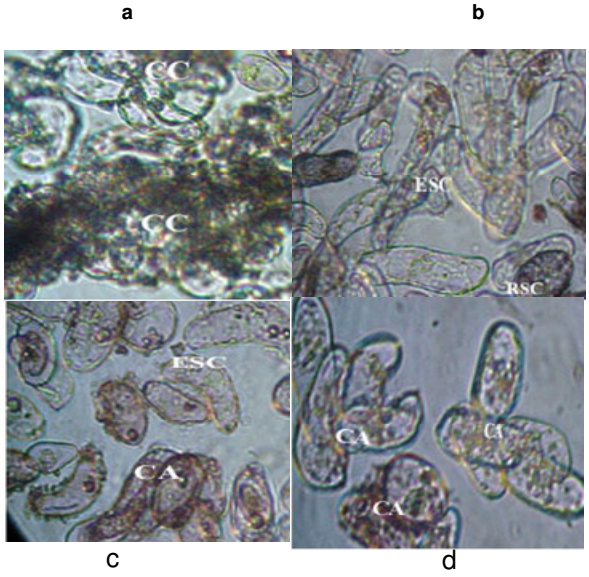

Figure 3. Cell suspension derived from scalps of various cultivars (a) 'Kibuzi' (b) 'Lwadungu' (c) 'Musakala' (d) 'Mbwazirume' (CA= cell aggregate, CC= cell cluster, ESC=elongated single cells).

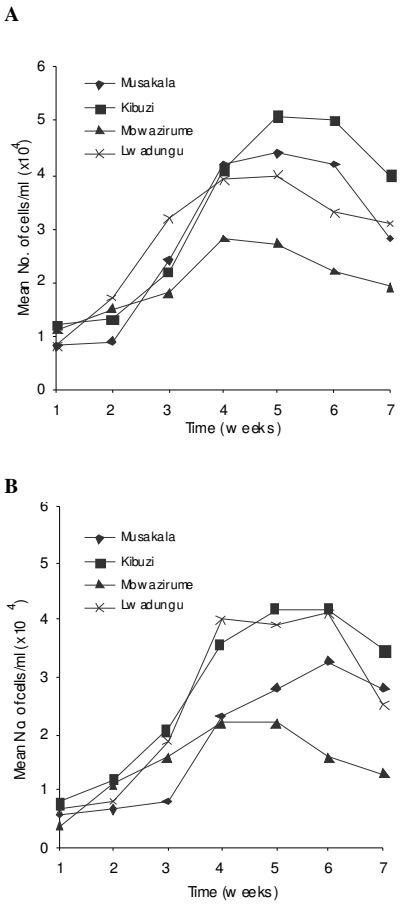

Figure 4. Growth pattern of cell suspension in induction medium: (A) supplemented with 2,4-D/Zeatin (B) supplemented with 2,4-D/BAP.

The different response of cultivars to produce cells was attributed to genotypic differences related to variations in endogenous hormone levels as reported by (Norstog, 1970). The fewer cells produced by 'Mbwazirume' was possibly due to the phenolic compounds released by the cells into the induction medium and which is known to inhibit cell division (Kenneth and Torres, 1989). Nevertheless, by the 4^{th} to 6^{th} week all the cultivars produced fairly good cell numbers (> 2 x 10^4 ml^{-1}). Beyond the 6^{th} week, there was a decline in cell number perhaps due to death of cells and the lack of cell division, as a result of exhaustion of some nutrients and/or accumulation of toxic substances in the medium (Kenneth and Torres, 1989) suggesting that cultures should be maintained by sub culturing every 4 - 6 weeks.

The morphology and composition of the cells varied according to the cultivar regardless of the cytokinin used in the induction medium when observed at 3 weeks. The cell suspensions of 'Kibuzi' (Figure 3a) consisted mainly of cell clusters with dense cytoplasm, while those of 'Lwadungu' (Figure 3b) were composed of elongated single cells with thin cell walls and no distinct nucleus. The suspensions of 'Musakala' (Figure 3c) and 'Mbwazirume' (Figure 3d) consisted of small cell aggregates and/or single cells. A unique feature of the 'Musakala' cell suspensions was the possession of more prominent nuclei suggesting higher activity (Georget et al., 2000).

Cell suspensions with composition/structure comparable to those observed in 'Lwadungu' were reported in *Saba* and *Pelipita* (*Musa* ABB) by Cronauer and Krikorian (1983) and Georget et al. (2000) described cell aggregates similar to those of 'Musakala', 'Kibuzi' and 'Mbwazirume' cell suspensions in a suspension generated from cv. *Grandenaine (Musa* AAA). Only cell suspensions with composition/structure comparable to those of 'Musakala', 'Kibuzi' and 'Mbwazirume' have been reported to be highly embryogenic (Georget et al., 2000). This is probably because cell suspensions with aggregates/clusters typically displayed by 'Musakala', 'Kibuzi' and 'Mbwazirume' are less exposed to 2, 4-D which although induces onset of somatic embryogenesis, is reported to retard their further development to somatic embryos (Georget et al., 2000).

Conclusion

In this study scalp formation was achieved at much lower concentrations of combined BAP and TDZ than when singly applied, suggesting synergism of the two cytokinins for scalp production in banana. Earlier and better scalps among cultivars were obtained from the cultures on BAP/TDZ. Combinations 12.4/4.55 and 10/5.68µM BAP/TDZ produced the best scalps. Reduced levels of these cytokinins and the smaller time interval required to produce scalps could be advantageous in reducing som-

aclonal variation. The structure of the cell suspension and the rate of cell growth were found to be dependent on the cultivar regardless of the hormone treatment in the induction medium. Cultivars 'Musakala', 'Kibuzi' and 'Mbwazime' produced cell culture of clustered and aggregated cells and high cell numbers (> 2 x 10^4/ ml of cell suspension), which are a prerequisite for embryo, cells development.

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