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TDZ AND 4-CPPU IN GAMBORG B5 SALTS WITH MS VITAMINS DOUBLES EMBRYOGENIC RESPONSE FROM MALE FLOWERS OF EA-AAA BANANA

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ABSTRACT

Conventionally, auxins have been used in MS medium in combination or without purine-based cytokinins for induction of embryogenesis in EA-AAA banana (*Musa* spp.). Besides, low embryogenic response, it has been rare for more than two cultivars to respond similarly to a single treatment. This study investigated the efficacy of urea-type cytokinins, N-phenyl-N'-1,2,3-thidiazol-5-ylurea (TDZ) and N-(2-chloro-4-pyridyl)-N'-phenylurea (4-CPPU); and salt formulations, Chu (N_6), Eriksson, Gamborg B5, MS, Nitsch, NLN, SH and White for embryogenic callus induction in different EA-AAA banana cultivars. Immature male flowers of cultivars Mpologoma, Mbwazirume, Nakabululu, Nakinyika and Nfuuka were cultured on callus induction medium, supplemented with different TDZ and 4-CPPU combinations. Most of the cultivars had embryogenic response to the medium with 10µM TDZ+10µM CPPU. Cultivar Nakabululu recorded 22.2% embryogenic response, followed by Mwazirume (5.7%), Nakinyika (5.3%) and Mpologoma (4.6%). Cultivar Nfuuka had 9.1% embryogenic response on 15µM TDZ+15µM CPPU. When cultivars Mpologoma and Nakinyika were cultured on the same medium containing 10µM TDZ+10µM CPPU, but the MS salts substituted with the other salt formulations, their cultures recorded 11.4 and 8.3% embryogenic response, respectively to Gamborg B5 salts; which was almost twice their response to MS medium. The results suggested that TDZ and 4-CPPU, particularly in Gamborg B5 salt formulation, could increase percentage of embryogenic callus induced from male flowers of EA-AAA banana cultivars, and would improve plant regeneration and consequently help in the process of genetic improvement of EA-AAA banana.

Key Words: Cytokinins, embryogenic response, *Musa* spp., Thidiazuron

RÉSUMÉ

Conventionnellement, les auxines ont été utilisees dans le medium MS en combinaison avec ou sans cytokinines à base de purine pour induction de l'embryogenèse dans la banane EA-AAA (*Musa* spp.). En plus d'une faible réponse embryogénique, il a été rare pour plus de deux cultivars de répondre de façon similaire à un seul traitement. Cette étude a été conduite pour évaluer l'efficacité des cytokinines de type urea, N-phenyl-N'-1,2,3-thidiazol-5-ylurea (TDZ) et N-(2-chloro-4-pyridyl)-N'-phenylurea (4-CPPU) ; et les formulations du sel, Chu (N6), Eriksson, Gamborg B5, MS, Nitsch, NLN, SH et blanc pour l' induction du callus embryogénique dans différents cultivars de banane EA-AAA. Des cultivars Mpologoma des fleurs males immatures Mbwazirume, Nakabululu, Nakinyika et Nfuuka étaient cultivés sur le medium d'induction du callus, supplémentée avec différentes combinaisons de TDZ et 4-CPPU. La plupart des cultivars avaient une réponse embryogénique au medium avec 10µM TDZ+10µM CPPU. Le cultivar Nakabululu a réalisé 22.2% de réponse embryogénique, suivi de Mbwazirume (5.7%), Nakinyika (5.3%) et Mpologoma (4.6%). Le cultivar Nfuuka avait 9.1% de réponse embryogénique sur 15µM TDZ+15µM CPPU. Lorsque les cultivars Mpologoma et Nakinyika étaient cultivés sur le même medium contenant 10µM TDZ+10µM CPPU, mais les sels MS substitués par d'autres

formulations de sels, leurs cultures ont enregistré 11.4 et 8.3% de réponses embryogéniques, respectivement, aux sels Gamborg B5; qui faisait presque le double de leur réponse au medium MS. Les résultats ont suggèrent que TDZ et 4-CPPU, particulièrement dans la formulation du sel Gamborg B5, pourrait augmenter le pourcentage induit du callus embryogénique des fleurs males des cultivars de banane EA-AAA et pourrait améliorer la régénération des plants et en conséquence aider dans le processus de l'amélioration génétique de la banane EA-AAA.

Mots Clés: Cytokinines, réponse embryogénique, *Musa* spp., Thidiazuron

INTRODUCTION

East African Highland banana (EA-AAA banana) is a distinct triploid banana group grown in the Great Lakes region of Eastern Africa, covering parts of Uganda, Rwanda, Burundi, Tanzania, Kenya and Democratic Republic of Congo (DRC) (Smale and De Groote, 2003). It constitutes the largest proportion of banana produced in the region, particularly in Uganda, Africa's first and world's second leading producer of banana after India (AATF, 2009). Being triploid in nature, the improvement of the EA-AAA banana through conventional methods is very difficult. Tissue culture techniques have been applied to enhance banana breeding efficiency (Vuylsteke *et al*., 1997).

Somatic embryogenic techniques have been used to facilitate genetic transformation as an avenue to bypass challenges of polyploidy, low fertility, limited genetic variability and long generation time faced during banana breeding. Embryogenic calli or embryogenic cell suspensions (ECS) - the standard material for genetic engineering have been generated using mainly immature male flower (Cote *et al*., 1996; Grapin *et al*., 1996; Ghosh *et al.*, 2009; Mohandas *et al.,* 2011) and scalp methods (Schoofs *et al.,* 1998; Ramírez-Villalobos and De García, 2008) in different banana genome groups and cultivars including the EA-AAA banana (Sadik *et al*., 2007). However, there have been difficulties in callus induction and ECS development in the EA-AAA banana, hindering routine genetic transformation.

The frequency of embryogenic callus encountered on scalps, under standard *in vitro* callus induction procedure, has been zero while that on immature male flowers ranges from 0.01 to 0.02% in most of the EA-AAA banana cultivars (Strosse *et al*., 2003). It has also been rare for two or more cultivars to show similar embryogenic response on the same protocol (Tripathi *et al*., 2008a)

Several authors suggested protocols for transformation of banana through shoot tips (May *et al*., 1995; Tripathi *et al*., 2003b, 2008a) to avoid the challenges of generating callus and establishing cell suspension cultures. Shoot tips are amenable to transformation regardless of ploidy or the genotype of the banana (Tripathi *et al*., 2003b, 2008a), but somatic embryogenic techniques offer unparallel means for genetic improvement of banana. Plants regenerated from ECS frequently originate from a single cell and thus in case of transformation, it reduces occurrence of chimeric plants, a problem commonly encountered when using shoot tips as the target material (Strosse *et al*., 2003).

Somatic embryogenic techniques also offer opportunities for protoplast culture and spontaneous or induced mutagenesis to be applied. Besides facilitating genetic engineering through micro-injection, electroporation or Agrobacterium techniques, protoplast culture enables transfer of organelles such as mitochondria and/or chloroplasts between sexually incompatible parents and thus creation of novel hybrids.

Culture medium nutrient components and growth regulator combinations greatly influence plant cell differentiation and morphogenesis *in vitro* (Kantharajah, 2001). Auxins, particularly 2,4-dichlorophenoxyacetic acid (2,4-D), have been widely used in combination with cytokinins such as Benzyl adenine (BA) (Sannasgala *et al*., 1992), Zeatin (Schoofs *et al.,* 1998; Ramírez-Villalobos and De García, 2008) and Benzyl amino purine (BAP) (Sadik *et al*., 2007) for initiation and maintenance of embryogenic cell suspension (ECS) in banana.

Whereas urea-type cytokinins, N-phenyl-N'-1,2,3-thidiazol-5-ylurea (thidiazuron) (TDZ) and N-(2-chloro-4-pyridyl)-N'-phenylurea (forchlorfenuron) (4-CPPU) have desirable attributes for embryogenic callus induction from explants (Murthy *et al*., 1998; Haruki *et al.*, 2007), they have not been widely tried for induction of somatic embryogenesis in EA-AAA banana, especially through immature male flower buds. On the other hand, a number of tissue culture salt formulations were described by different authors including MS (Murashige and Skoog, 1962), White (White, 1963), Eriksson (Eriksson, 1965), Gamborg B5 (Gamborg *et al*., 1968), Nitsch (Nitsch and Nitsch, 1969), SH (Schenk and Hilderbrandt, 1972), Chu N6 (Chu, 1975) and NLN (Lichter, 1982).

During the establishment of *in vitro* micro-propagation procedure for the EA-AAA banana, only a few salt formulations were evaluated (Talengera *et al*., 1994). Although MS medium was adopted for routine micropropagation of the EA-AAA banana through shoot tip culture, there has been no justification that its salt mixture is also the best for callus induction or ECS development. The aim of this study was to test efficacy of TDZ and 4-CPPU, and different salt formulations for embryogenic callus induction in EA-AAA banana cultivars through immature male flowers.

MATERIALS AND METHODS

Plant material. Five EA-AAA banana cultivars, namely Mbwazirume, Mpologoma, Nakabululu, Nakinyika and Nfuuka from different clone sets were used in this study. Sukali Ndizi (AAB), which is responsive to different regeneration protocols, was included as a check. Male flowers were collected 1 to 3 weeks after flowering, from the field banana germplasm at Makerere University Agricultural Research Institute, Kabanyolo (MUARIK) in Uganda and the banana fields of the National Banana Program at National Agricultural Research Laboratories (NARL), Kawanda, both sites in Uganda. They were reduced to 0.8 - 2 cm within 6 hours after collection and the reduced immature male flowers were surface sterilised in 70% ethyl alcohol for 2 minutes before they were initiated within two hours.

Callus induction on medium supplemented with TDZ and 4-CPPU. Using a scalpel with fine blades, immature flower bud clusters were excised under dissection microscope from the 18 to 8th position and cultured on a callus induction medium containing 5 µM 2,4-D, supplemented with different combinations of TDZ and CPPU alone or in presence of IAA and NAA: 5µM TDZ+5µM 4-CPPU, 10µM TDZ+10µM 4-CPPU, 15µM TDZ+15µM 4-CPPU, 5µM TDZ+5µM 4-CPPU+1mgl^{-1} IAA+1mgl^{-1} NAA and 10µM TDZ+10µM 4-CPPU+1 mgl^{-1} IAA+1mgl^{-1} NAA designated as H1, H2, H3, H4 and H5, respectively. In each treatment, fifty male flowers of each cultivar were initiated on separate petridishes and placed in a completely randomised design (CRD) in a dark growth room maintained at 26 ± 2°C. The cultures were observed every two weeks for 8 months. Yellow callus (YC) and embryogenic callus (EC) developing from the flower buds was recorded. Organ-like structures developing on the callus or alongside embryos were noted.

Callus induction on basal salt mixtures substituted on callus induction medium. By substituting only MS inorganic salts (MS vitamins and other medium components retained) from the callus induction medium supplemented with 10µM TDZ+10µM 4-CPPU (the most efficacious combination in pure MS medium), salt formulations of Chu (N_6), Eriksson, Gamborg B5, Nitsch, NLN, SH and White were tested for embryogenic callus induction in EA-AAA banana cultivars. Immature male flower buds of two cultivars, Mpologoma and Nakinyika, were cultured on semisolid callus induction media containing the various salt formulations, full strength MS vitamins, 30 gl^{-1} sugar, 5µM 2,4-D and 10µM TDZ+10µM 4-CPPU at pH 5.7. Fifty male flowers of each cultivar were used in each salt mixture treatment and placed in a CRD in a dark growth room maintained at 26 ± 2°C. The cultures were observed every two weeks for at least 8 months.

The number of flower buds on each petridish that responded (grew or differentiated into callus)

was recorded. Yellow callus, embryogenic callus and organ-like structures developing on the callus or alongside embryos were recorded. Any callus with embryos achieved from both experiments was transferred onto hormone free MS banana multiplication medium described by Talengera *et al.* (1994) and embryo to plant development observed for two months.

Data analysis. The data on callus formation were summarised in percentages using Excel Computer Program. Yellow callus response was presented as percentage of male flower explants that produced yellow callus to the total number of male flowers initiated on callus induction medium. Embryogenic callus response was expressed as proportion of callus cultures with embryos. The data on number of immature male flower bud clusters that responded on the various basal salt mixtures were subjected to ANOVA using GenStat 13th Edition (GenStat, 2010). The means were separated using the Least Significant differences (LSD) at 5% level of significance. Comparisons were done on number of flower bud clusters that responded across cultivars and salt formulations.

RESULTS

Effect of TDZ and 4-CPPU on callus induction. The response of the immature male flower buds of the different cultivars on the callus induction medium containing various combinations of TDZ and 4-CPPU did not vary during the first two months except some cultures of cv. Mpologoma on medium H1 (5µM TDZ+5 µM 4-CPPU) dried without forming yellow callus. From 3-8 months, there were differences in embryogenic response and drying of yellow callus among the cultivars on the different growth regulator combinations (Table 1). Between 3-4 months the cultures of Mpologoma and Mbwazirume showed embryogenic response on H2 (10µM TDZ+10µM 4-CPPU) (formed a white translucent callus with somatic embryos on its surface) of up to 4.6 and 5.7%, respectively (Table 1).

The cultures of other cultivars had not yet formed embryogenic callus. Between 5-6 months, Nakabululu and Nakinyika showed embryogenic response of 4.8% on H3 (15µM TDZ+15µM 4-CPPU) and 5.3% on H5 (10µM TDZ+10µM 4-CPPU+10 mgl^{1-} IAA+10mgl^{1-} NAA), respectively. From 7-8 months, Nakabululu showed 22.2% embryogenic response on H2 (10µM TDZ+10µM 4-CPPU); while Mpologoma and Nfuuka had 2.4 and 9.1% embryogenic response, respectively on H3 (15µM TDZ+15µM 4-CPPU). Most of the callus was white and translucent and formed on primary yellow callus (Fig. 1). About 5% of the embryogenic calli had friable tissue. Most of the flower buds of Sukali Ndizi did not differentiate into callus instead proliferated extensively as floral tissue. A few flower buds of EA-AAA banana cultivars also proliferated as floral tissue without differentiating into callus particularly on low TDZ and 4-CPPU concentrations.

Callus induction on different basal salt mixture substituted on callus induction medium. In the first two months there were no differences in response of the cultivars on the various salt mixtures except 5.9% of the cultures of cultivar Mpologoma formed callus with somatic embryos on Gamborg B5 basal salt mixture which increased in 3-4th months to 11.43% (Table 2). Between 3-4 months, 8.33% and 3.33% of cultures of cultivar Nakinyika formed embryos on Gamborg B5 and White basal salt mixtures respectively.

The cultures of both cultivars on Nitsch, NLN and SH salt mixtures dried within 4 months without forming somatic embryos (Fig. 3c). Those on Chu N6 and Eriksson salt mixtures dried in 6 months; while those on Gamborg B5 and White salt mixtures dried after 8 months. On pure MS medium, cultures of cultivar Mpologoma had 4.6% embryogenic response between 3-4 months and dried in 6 months; while cultures of cv. Nakinyika dried after the same period without showing any embryogenic response.

There was significantly ($P < 0.05$) higher survival and differentiation of the flower buds into callus on Chu N6 and White basal salt mixtures than on other salt mixtures except flower bud cultures of cultivar Nakinyika which had also high survival in MS medium (Table 3). The survival and differentiation of the flower buds of both cultivars on Nitsch was significantly ($P < 0.05$) low. The response of the cultures of both cultivars to Eriksson (ER) and SH was moderate.

TABLE 1. Yellow callus (%YC) and embryogenic callus (%EC) formed on callus induction medium supplemented with various combinations of TDZ and 4-CPPU

Cultivar	Hormone treatment	Months taken to induce embryos					
		3 - 4		5 - 6		7 - 8	
		%YC	%EC	%YC	%EC	%YC	%EC
Mpologoma	H1	66.7	0	23.3	0	3.3	0
	H2	100	4.6	76.5	0	0	0
	H3	100	0	42.9	0	23.8	2.4
	H4	100	0	0	0	0	0
	H5	96.8	3.2	32.3	0	0	0
Mbwazirume	H1	67.8	1.7	65.5	1.7	0	0
	H2	97.1	5.7	97.1	5.7	61.3	0
	H3	100	0	100	0	4.4	0
	H4	100	0	0	0	0	0
	H5	100	0	0	0	0	0
Nfuuka	H1	100	0	100	0	100	0
	H2	100	0	92.9	0	42.9	0
	H3	72.7	0	72.7	0	54.6	9.1
	H4	0	0	0	0	0	0
	H5	100	0	0	0	0	0
Nakabululu	H1	100	0	70	0	70	0
	H2	80	0	77.8	0	44.4	22.2
	H3	100	0	95.2	4.8	90.5	9.5
	H4	100	0	100	0	18.2	0
	H5	0	0	0	0	0	0
Nakinyika	H1	100	0	100	0	0	0
	H2	100	0	69.2	0	0	0
	H3	100	0	100	0	67.7	0
	H4	34.2	0	0	0	0	0
	H5	79	0	63.2	5.3	63.2	5.3
Sukali Ndzi	H1	100	0	83.3	0	0	0
	H2	100	0	0	0	0	0
	H3	100	0	100	0	66.7	0
	H4	100	0	90.9	0	0	0
	H5	100	0	100	0	0	0

Cultures of cultivar Nakinyika had significantly ($P < 0.05$) lower survival than those of Mpologoma on Gamborg B5 (Gamb.), Nitsch and NLN basal salt mixtures.

The morphology and growth characteristics of the cultures also varied among the basal salt mixtures. The flower buds of both cultivars grew normally on Gamborg B5 and White salt mixtures and differentiated into callus, some of which had visible embryos (Fig. 2). The embryos survived for over 6 months, though some of the callus cultures had blackening at the base. Undefined organ-like structures also grew on some of the calli.

The cultures on Chu N6 and Eriksson grew vigorous, but most of them had water cells especially in cv. Nakinyika (Fig. 3). A few flower buds differentiated into yellow callus and grew

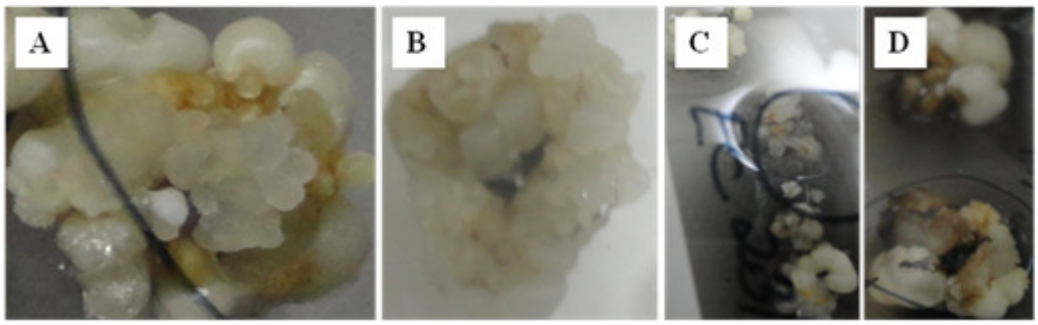

Figure 1. Different stages of callus development from immature male flower buds of cv. Nfuuka on callus induction medium supplemented with 15µM TDZ+15µM 4-CPPU: (A) white translucent callus on primary yellow callus, (B) white callus, (C) Callus with primary embryos, and (D) Embryogenic callus with friable tissue.

TABLE 2. Yellow callus (%YC) and embryogenic callus (%EC) of cv.s Mpologoma and Nakinyika on various basal salt mixtures substituted on callus induction medium containing MS vitamins supplemented with 10µM TDZ+10µM 4-CPPU

Cultivar	Basal salt mixture	Months taken to induce embryos					
		3 - 4		5 - 6		7 - 8	
		% YC	% EC	% YC	% EC	%YC	% EC
Mpologoma	Chu N6	100	0	83.9	0	0	0
Nakinyika	Chu N6	82.6	0	82.6	0	0	0
Mpologoma	Eriksson	90	0	90	0	0	0
Nakinyika	Eriksson	100	0	84.4	0	0	0
Mpologoma	Gamborg B5	100	11.4	97.1	11.4	97.1	11.4
Nakinyika	Gamborg B5	86.2	8.3	86.2	8.3	86.2	8.3
Mpologoma	Nitsch	100	0	0	0	0	0
Nakinyika	Nitsch	97.1	0	0	0	0	0
Mpologoma	NLN	94.3	0	0	0	0	0
Nakinyika	NLN	91.4	0	0	0	0	0
Mpologoma	SH	97.5	0	0	0	0	0
Nakinyika	SH	53.9	0	0	0	0	0
Mpologoma	White	87.2	0	87.2	0	87.2	0
Nakinyika	White	100	3.3	100	3.3	100	3.3
Mpologoma	MS	100	4.6	76.5	0	0	0
Nakinyika	MS	100	0	69.2	0	0	0

TABLE 3. Immature male flower buds of cultivars Mpologoma and Nakinyika that responded or differentiated into callus on various basal salt mixtures substituted on callus induction medium supplemented with 10 µM TDZ+10 µM 4-CPPU

Cultivar	Basal salt mixture							LSD (0.05)
	Chu N6	ER	Gamb.	Nitsch	NLN	SH	White	MS	
Mpologoma	7.26	5.53	6.28	3.35	6.54	5.84	7.94	5.91	1.219
Nakinyika	7.26	5.09	4.59	3.39	4.75	6.41	7.94	7.94	
LSD (0.05)	0.61								

ER = Eriksson, Gamb. = Gamborg B5, SH = Schenk and Hilderbrandt, MS = Murashige & Skoog

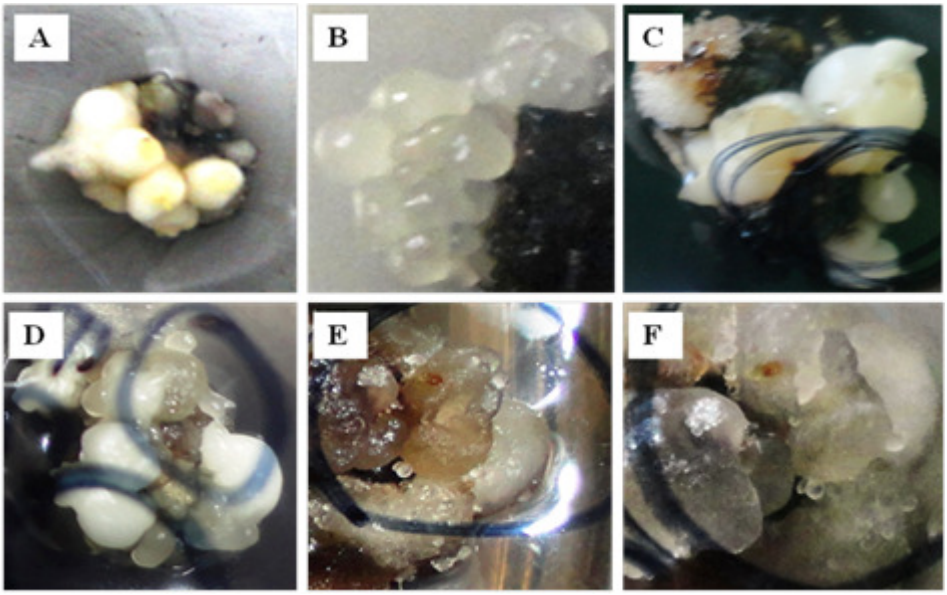

Figure 2. Different stages of callus development from immature male flower buds of cv. Mpologoma on Gamborg B5 basal salt mixture substituted on callus induction medium supplemented with 10 µM TDZ+10 µM 4-CPPU: (A) flower bud differentiation into callus, (B) white callus, (C) development of embryos together with organ-like structures, (D) development of secondary embryos, (E) Embryogenic callus with friable tissue, and (F) Callus with somatic embryos at different stages.

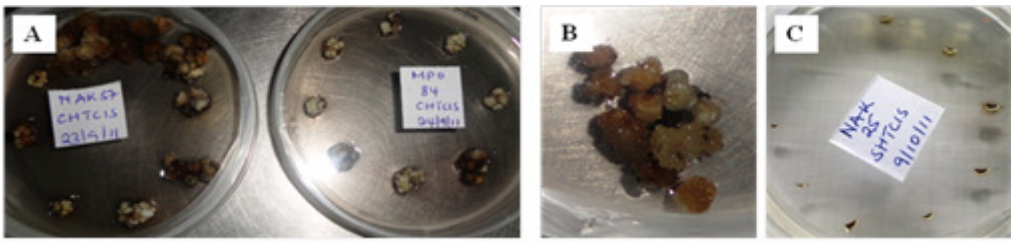

Figure 3. Growth characteristics of immature male flower buds of cv.s Mplogoma and Nakinyika on Chu N6 and SH basal salt mixtures substituted on callus induction medium supplemented with 10 µM TDZ+10 µM 4-CPPU: (A) flower bud differentiation into water cells, (B) Proliferation of water cells, and (C) Early dried flower buds on SH salt mixture.

normally until they dried due to age. The cultures on Nitsch, NLN and SH salt mixtures had slow growth, characterised with blackening at the base of the initiated flower buds. Most of the flower buds on these three salt formulations dried at an early stage without showing any sign of embryogenic response (Fig. 3). When the callus of the various cultivars on the different salt formulations substituted on the callus induction medium supplemented with TDZ and 4-CPPU was removed and cultured on a hormone free MS medium, it regenerated into shoots through both organogenesis and somatic embryogenesis (Fig. 4) regardless of salt formulation confirming embryogenic competence. The plantlets developed adequate roots, which enabled them to survive when transferred into soil (Fig. 5).

DISCUSSION

Callus induction on medium supplemented with TDZ and 4-CPPU. Embryogenic callus formation among the cultivars on the callus induction medium supplemented with various combinations of TDZ and 4-CPPU varied widely. However, all the EA-AAA banana cultivars showed embryogenic response, implying that a combination of TDZ and 4-CPPU with 2,4-D is

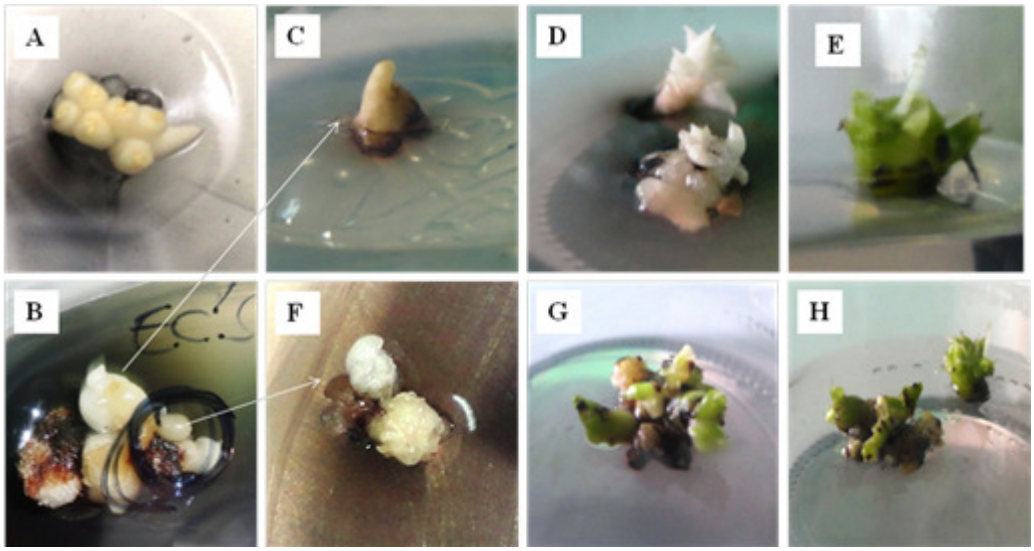

Figure 4. Organogenesis and somatic embryogenesis from immature male flower buds of cv. Mplogoma on Gamborg B5 basal salt mixture, substituted on callus induction medium supplemented with 10 µM TDZ+10 µM 4-CPPU: (A) flower bud cluster, (B) Differentiation into embryos and shoot-like structure, (C-E) Direct organogenesis on hormone free medium, and (F-H) plant development through embryogenesis on hormone free medium.

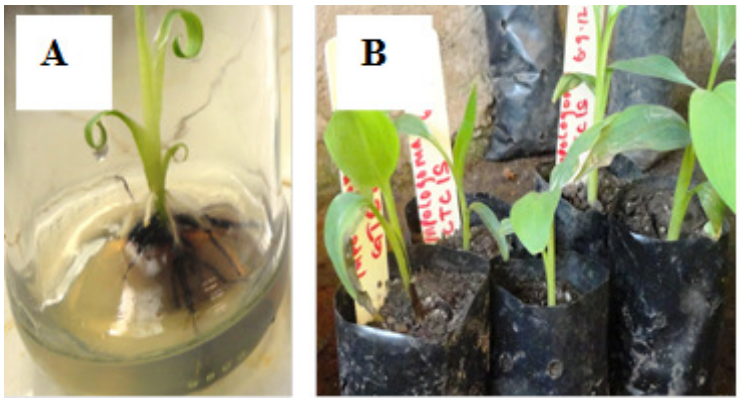

Figure 5. Plants regenerated through somatic embryogenesis from immature male flower buds of cv. Mplogoma on Gamborg B5 basal salt mixture substituted on callus induction medium supplemented with 10 µM TDZ+10 µM 4-CPPU: (A) after transfer on hormone free medium, and (B) under acclimatization in soil.

efficacious for callus induction from male flower buds of EA-AAA banana cultivars. Favourability of combinations of urea-type cytokinins with auxins for induction of embryogenesis was reported in other banana genotypes (Divakaran *et al*., 2011), where embryogenic callus was generated from bract explants of diploid ($2n = 22$) banana cultivars on MS medium supplemented with 0.045–9.00 µM TDZ. When Divakaran *et al.* (2011) transferred the embryogenic callus on MS basal medium supplemented with biotin and or glutamine, it produced somatic embryos and consequently regenerated normal plants with little somaclonal variation and survival rate in soil of 90%.

In other crops, the combined effect of TDZ and 2,4-D induced somatic embryogenesis in non-responsive caryopses of rice (Aparna and Rashid, 2004). Tuong Huan *et al.* (2004) achieved more embryogenic calli induction from protocorm

of *Cymbidium, a* hybrid orchid, on medium containing a combination of NAA and TDZ than on NAA alone. A combination of 2,4-D and TDZ also changed granular callus into a favourable friable form in *Oncidium* (Fang *et al.,* 2006). Fiore *et al.* (2002) reported higher percentage of embryo formation in both sweet orange (*C. sinensis* L.) and lemon (*Citrus limon* L.) on 4-CPPU when they used media supplemented with different combinations of 2,4-D and 4-CPPU to induce callus and somatic embryogenesis.

All the cultivars, except Nfuuka, formed more somatic embryos on the medium supplemented with 10 µM of each of TDZ and 4-CPPU than on the medium supplemented with 5 µM or 15 µM of each of them. The low response of the cultures on the callus induction medium supplemented with 15 µM of each of TDZ and 4-CPPU was attributed to excessive auxin effect. At high concentrations, urea-type cytokinins behave typical like auxins and, thus the auxin activity from them added to that of 2,4-D could have been too high to induce embryogenic response. This implies that cv. Nfuuka, which responded best on the medium supplemented with 15 µM of each of TDZ and CPPU require higher auxin activity to stimulate embryogenesis in it.

Instead of differentiating into callus on the medium supplemented with 5 µM of each of TDZ and 4-CPPU, some of the flower buds proliferated into new floral tissue. Perez-Hernandez and Rosell-Garcia (2008) reported inflorescence proliferation in dwarf Cavendish male flower on medium supplemented with 5 µM TDZ. At low concentrations, urea-type cytokinins exhibit cytokinin activity, which favours tissue proliferation suggesting that at 5 µM, TDZ and 4-CPPU added none or minimal auxin activity to that of 2,4-D, which was not sufficient to induce embryogenic response. It further implies that cv. Sukali Ndizi (AAB) whose cultures had excessive proliferation on all the treatments require much higher auxin supply and thus needed higher TDZ and 4-CPPU to stimulate embryogenesis. Bananas with AAB genome appear to require higher auxin concentration to induce embryogenesis (Sukhada *et al*., 2010). Embryogenic callus and somatic embryos were generated from male flower buds of banana cv. Rasthali (AAB) on MS medium supplemented with 18.10 µM 2,4-D, 5.37 µM NAA and 5.71 µM IAA (Sukhada *et al*., 2010).

Callus induction on different basal salt mixture supplemented with TDZ and CPPU. Under MS vitamins and, TDZ and CPPU hormone treatment, embryogenic response was observed on Gamborg B5, MS and Whites basal salt mixtures, but none on Chu N6, NLN, Nitsch and SH salt formulations. Other workers reported on callus induction and ECS development from immature male buds of different banana genotypes on some of these salt formulations. Cote *et al.* (1996) achieved embryo development in *Musa* AAA cv. Grande Naine (AAA), by plating its cell suspension on a medium of SH with MS vitamins. Gomez *et al.* (2000) achieved embryo formation in hybrid cultivar FHIA–18 (AAAB) using a medium of SH with MS vitamins. When Ganapathi *et al.* (1999) initiated young banana male flowers on full strength MS medium and White's medium, they found MS medium to be significantly better than the White's medium in terms of embryogenic callus induction. This study suggested that SH was not effective in inducing embryogenic callus, while cultivar Nakinyika showed embryogenic response on white basal salt mixture but not on MS medium.

Despite the cultivar differences, the performance of the various basal salt mixtures was influenced by their interaction with TDZ and CPPU in the media. Urea-type cytokinins, especially TDZ in synergy with Ca^{2+} stimulate ethylene production (Wing-Kin and Shang, 1986), which caused the cultures on the basal salt mixtures with high calcium content especially MS and NLN to die and dry early (Table 2). Gamborg B5 basal salt mixture has low calcium content compared to the other salt mixtures; that was why both its callus and embryos survived for longer time than cultures on the other salt mixtures (Table 2).

Both cultivars, Mpologoma and Nakinyika, formed more embryos on Gamborg B5 basal salt mixture than on other salt mixtures, including on MS medium one of the most commonly used media for plant tissue culture. Compared to the other salt formulations, Gamborg B-5 basal salt mixture has also the lowest total nitrogen content, of which 93% is in nitrate form and only 7% in

ammonical form. Since banana cultures have a high demand for nitrogen (Stover and Simmond, 1987) and mostly prefer ammonical nitrogen (Marchal, 1990), the limited amount of nitrogen in it created a stress which probably favoured embryo formation. It was because of the same reason that the White basal salt mixture, which has lower total nitrogen induced embryogenic response in cv. Nakinyika (Table 2).

The trend in survival and response of the flower buds on each petridish was not clear. However, the survival of flower buds in the culture medium was linked with their embryogenic response. The cultures of both Mpologoma and Nakinyika on Gamborg B5 salt mixture survived for the same period, but cultivar Mpologoma whose flower buds had significantly ($P < 0.05$) higher survival rate showed higher embryogenic response. The higher survival of the flower buds of cv. Mpologoma on the callus induction medium resulted in increased chances for embryogenic response, implying that individual flower bud survival and duration of survival of the cultures on the various salt formulations determined embryogenic response.

When the callus of the various cultivars from the different salt formulations on callus induction medium supplemented with TDZ and CPPU was transferred into hormone free MS derived medium, it developed into shoot through both organogenesis and somatic embryogenesis (Fig. 4). Victor (2001) reported that both organogenesis and somatic embryogenesis can occur in the same explant, but originating from particular tissue layers or cells within the explant. Besides differences in physiological state of the flower buds or tissues and cells within them, the occurrence of organogenesis and somatic embryogenesis together in this study was attributed to the presence of TDZ and CPPU in the media and their interaction with the various salt mixtures. Murthy *et al*. (1998) reported that urea-based growth regulators, especially TDZ induced a diverse array of culture responses including induction of callus, formation of somatic embryos and other structures. Similarly, Chhabra *et al.* (2008) reported that TDZ induced both direct shoot organogenesis and somatic embryogenesis on cotyledonary node explants of lentil (*Lens culinaris* Medik.) This is possible due to its unique property of mimicking both auxin and cytokinin effects on growth and differentiation of cultured explants (Murthy *et al*., 1998).

Previously, auxins particularly 2,4-D were used in combination with purine-based cytokinins such as BA, BAP and Zeatin for induction of embryogenesis in EA-AAA banana. Besides low embryogenic responses, it was rare for more than two cultivars to show embryogenic response on a single treatment, though embryogenic response in banana is genotype dependent (Youssef *et al.*, 2010). In this study, where the callus induction medium was supplemented with urea-type cytokinins TDZ and CPPU, all the studied EA-AAA banana cultivars produced regenerable embryogenic callus, especially on the callus induction medium supplemented with 10 µM TDZ+10 µM CPPU.

When cultivars Mpologoma and Nakinyika were cultured on the same medium containing 10 µM TDZ+10 µM CPPU with the MS salts substituted with other salt formulations, 11.4 and 8.3% of their cultures, respectively had embryos on Gamborg B5 salt formulation almost twice their response on pure MS medium. Since the male flowers were collected randomly from the same fields and there were no instances of differences in embryogenic response due to differences in developmental stage of the flower buds (Youssef *et al*., 2010), these responses were rated to those reported in other genotypes such as Cavendish cultivar Robusta (AAA) by Ghosh *et al*. (2009). This implies that application of TDZ and 4-CPPU overcomes recalcitrance in EA-AAA banana cultivars and makes them responsive to somatic embryogenesis to levels comparable to those on non EA-AAA banana genotypes.

Some of the regenerable embryogenic calli had friable tissue which is desirable for establishment of ECS, the best material for genetic engineering. Though some cultures such as those of Sukali Ndizi proliferated instead of forming regenerable embryogenic callus, given the limited availability of immature flowers and/or long time required to wait for banana plants to flower, the induced floral tissue could be used instead of flower buds for more callus induction. Perez-Hernandez and

Rosell-Garcia (2008) found newly induced floral tissues from banana inflorescence cultured on 5 µM TDZ to be embryogenically competent.

CONCLUSION

The callus induction medium supplemented with a combination of TDZ and CPPU induces various levels of embryogenic response from immature male flower buds of EA-AAA banana cultivars Mbwazirume, Mpologoma, Nakabululu, Nakinyika and Nfuuka. The callus induction medium, supplemented with 10 µM TDZ+10 µM CPPU, induces efficient embryogenic response, although Gamborg B5 salt mixture substituted on the callus induction medium, supplemented with 10 µM TDZ+10 µM CPPU is the best. These results show that TDZ and 4-CPPU particularly in Gamborg B5 salt formulation increases percentage of embryogenic callus induced from male flowers of EA-AAA banana cultivars and improves plant regeneration and consequently is useful in genetic improvement of EA-AAA banana.

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