Journal of Agricultural Science; Vol. 7, No. 8; 2015
ISSN 1916-9752 E-ISSN 1916-9760
Published by Canadian Center of Science and Education

TDZ and 4-CPPU Induce Embryogenic Response on Scalps of Recalcitrant East African Highland Banana

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Received: December 1, 2014 Accepted: March 31, 2015 Online Published: July 15, 2015

doi:10.5539/jas.v7n8p215 URL: http://dx.doi.org/10.5539/jas.v7n8p215

Abstract

Breeding of banana conventionally is very difficult due to high levels of sterility arising from aneuploidy. Transformation of East African highland banana (EA-AAA banana) has been limited by difficulties in induction of embryogenic callus and/or embryogenic cell suspension (ECS), a requirement for efficient genetic transformation. In this study N-phenyl-N'-1,2,3-thidiazol-5-ylurea (TDZ) and N-(2-chloro-4-pyridyl)-N'-phenylurea (4-CPPU) that are highly active in regulating morphogenesis in tissue culture of many plant species were tested for enhancing embryogenic callus induction in recalcitrant EA-AAA banana through scalps. *In vitro* derived shoot-tips of banana cultivars Mbwazirume, Mpologoma, Nakabululu, Nakinyika and Nfuuka were subcultured on various combinations of TDZ and 4-CPPU till scalps were formed. In all the cultivars, ideal scalps for callus induction were achieved in over 50% of the shoot-tip cultures within four subculture cycles in the medium containing equal proportions of 9–13 µM of TDZ and 4-CPPU, and 26 µM of each. Embryogenic callus was formed on 2.5–20% of the scalp cultures depending on cultivar and, TDZ and 4-CPPU combination. Cultivars Nakinyika, Nakabululu, Mbwazirume and Nfuuka developed regenerable embryogenic callus. Of the plantlets regenerated 5 out of 12 and 4 out of 5 plantlets of cultivars Nakinyika and Mbwazirume, respectively, developed normal roots and were weaned onto soil. These results suggest that TDZ and 4-CPPU increases embryogenic response from scalps of EA-AAA banana which can enhance their genetic transformation.

Keywords: EA-AAA banana, forchlorfenuron, scalps, somatic embryogenesis, thidiazuron

1. Introduction

East African highland banana (EA-AAA banana) is an autopolyploid triploid (2n = 3x = 33) clone of *Musa acuminata* Colla, the parental donor of the A genome in banana (Pillay et al., 2006). It evolved through mutation and genetic restitution within the highlands of East Africa. Because of this, its genome is often denoted as EA-AAA to differentiate it from other banana with AAA genome composition such as Cavendish, Gros Michel and Yangambi Km5. It comprises over 120 cultivars grouped into five clone sets namely Musakala, Nakabulubu, Nakitembe, Nfuuka and Mbidde (Karamura & Pickersgill, 1999). The first four clone sets are usually cooked and served in different food recipes while the last one is processed into juice or beer. Being a major source of energy and having ability to produce fruit year-round, the banana is reckoned as a sustainable food security crop in the East African region.

Sustaining banana production in East Africa is increasingly becoming challenging. The predominant EA-AAA banana is susceptible to pests and diseases (Tushemereirwe et al., 2004). The most devastating pests are Banana weevil (*Cosmopolites sordidus* Germar) and plant parasitic nematode species *Radopholus similis*, *Pratylechus coffeae*, *P. goodeyi* and *Helicotylechus multicinctus*. The major diseases include Banana bacterial wilt (BBW) caused by *Xanthomonas campestris pv. musacearum* and Black Sigatoka caused by the fungus *Mycosphaerella fijiensis* Morelet (Tushemereirwe et al., 2004). The EA-AAA banana is also drastically affected by soil nutrient deficiency and drought (African Agricultural Technology Foundation [AATF], 2003). To address these production constraints sustainably requires deployment of improved varieties, but the improvement of EA-AAA banana through conventional breeding is very difficult due to its ploidy and limited genetic diversity. Though it has known female fertile cultivars, the EA-AAA banana is characterized by high female and male sterility

(Mukasa & Rubaihayo, 1993; Nyine & Pillay, 2007), and low seed viability (Ssebuliba et al., 2006). Besides, bananas in general have long generation time which also retards breeding efforts.

In vitro techniques have been widely applied to enhance genetic engineering as an alternative approach for crop improvement. However, application of genetic engineering for improvement of EA-AAA banana has been limited by difficulties in generating embryogenic callus (EC) and/or embryogenic cell suspension (ECS) as appropriate target material for transformation. Most of the EA-AAA banana cultivars are recalcitrant to regeneration protocols including through scalps. There has been limited success in developing regeneration protocols for EA-AAA banana through scalps (Sadik et al., 2007). However, scalps together with immature male flowers were reported to be the most responsive material for initiating embryogenic cultures (Meenakshi et al., 2007). Since some EA-AAA banana cultivars such as 'Endirira' do not produce male flowers (Schoofs et al., 1998), scalps remain the best material for callus induction. Furthermore, by using scalps, one can obtain explants from *in vitro* cultures or field plants at any growth stage and consequently avoid challenges of limited availability of mature flowers and/or the long time required to wait for banana plants to flower.

In vitro plant cell differentiation and morphogenesis is highly dependent on culture medium (Khaleda & Al-Forkan, 2006). Optimum proportion and/or concentration of growth regulators especially auxins is necessary for successful induction and maintenance of embryogenic callus or cell suspension cultures (Gomez et al., 2000). N-phenyl-N'-1,2,3-thidiazol-5-ylurea (thidiazuron) (TDZ) and N-(2-chloro-4-pyridyl)-N'-phenylurea (forchlorfenuron) (4-CPPU), both substituted phenylurea compounds, are reported to be highly active in regulating morphogenesis in many plant species (Fiore et al., 2004; Victor et al., 2004; Haruki et al., 2007). They are known to exhibit a unique property of mimicking both cytokinin and auxin effects on growth and differentiation of cultured explants (Murthy et al., 1998). At low concentrations TDZ and 4-CPPU stimulate shoot proliferation, simulating cytokinin activity while at high concentrations they induce callus, typical of an auxin activity (Vinayak et al., 2009). They are also reported to have distinct influence on crop varieties suggesting that they could be suitable candidates for determining variety specific regeneration protocols (Vinayak et al., 2009). This paper reports the efficacy of a combination of TDZ and 4-CPPU growth regulators in enhancing embryogenic callus induction in recalcitrant EA-AAA bananas through scalps.

2. Materials and Methods

2.1 Plant Material

Five EA-AAA banana cultivars namely Mbwazirume, Mpologoma, Nakabululu, Nakinyika and Nfuuka from different clone sets were selected for this study based on farmers' variety preference. Shoot-tip explants were extracted from young suckers of 40-100 cm height from a field at Makerere University Agricultural Research Institute, Kabanyolo (MUARIK). They were sterilized and cultured on the Murashige and Skoog (MS) medium modified for banana multiplication (Talengera et al., 1994) up to transfer 2 to produce adequate clean shoots for generating scalps.

2.2 Generation of Scalps

In vitro derived shoot-tips from transfer 2 on the MS derived banana multiplication medium described by Talengera et al. (1994) were put on the same medium modified with various concentrations of TDZ and 4-CPPU growth regulators. Equal concentrations of TDZ and CPPU of 5 µM, 7 µM, 9 µM, 11 µM and 13 µM and, single concentrations of TDZ and 4-CPPU at 26 µM, designated as media treatments M1, M2, M3, M4, M5, M6 and M7, respectively, were applied to generate scalps. Fifty shoot-tips were used in each hormone treatment and placed in a completely randomized design (CRD) in a dark growth room maintained at 26 ± 2 °C. The cultures were serially subcultured every four weeks until scalps were formed. The rate of scalp formation in the various media was determined by counting the number of shoots and buds in each subculture cycle until scalps were formed. Callus directly formed on the scalps being generated was also recorded. Other growth features or characteristics such as corm-like growth when observed were noted.

2.3 Callus Induction and Embryo Development

The scalps were excised under dissection microscope and cultured on callus induction medium derived by combining 5 µM 2,4-D (2,4-Dichlorophenoxyacetic acid) with TDZ and 4-CPPU singly and in combination at 5 µM, 10 µM and 15 µM as hormone treatment. Fifty scalps were used in each treatment. All the cultures were placed in a dark growth room maintained at 26±2 °C. They were observed every two weeks for four months to track embryogenic responses. Any embryogenic callus achieved was transferred to the MS banana multiplication medium supplemented with 5 µM benzyl amino purine (BAP). Embryo to plant development was observed for at least 28 days. The plantlets regenerated together with the number of normal roots (with hair roots) they

possessed were recorded.

2.4 Data Analysis

The data on rate of scalp formation was subjected to analysis of variance (ANOVA) using GenStat 13th edition (GenStat, 2010). The means were separated using least significant differences (LSD) at 5% level of significance. Comparisons were done on number of shoots and multiple buds across cultivars, subculture cycles and growth regulator combination. Callus directly formed on the scalp induction media was summarized as percentage of explants or cultures with callus on the scalps being formed. Induced callus was summarized as a percentage of scalps that generated callus to the total number of scalps initiated on callus induction medium.

3. Results

3.1 Scalp Formation

Scalp formation in all the cultivars varied across hormone treatment and subculture cycle. There were significant differences in mean shoot and bud proliferation on the different combinations of TDZ and 4-CPPU (Table 1). At subculture 1, medium M5 (13 µM TDZ + 13 µM 4-CPPU) induced significantly ($P < 0.05$) more multiple buds with fewer shoots in cultivars Nakinyika, Nakabululu and Nfuuka which favors scalp formation. Medium M1 (5 µM TDZ + 5 µM 4-CPPU) induced significantly ($P < 0.05$) higher number of multiple buds in cv. Mpologoma but it induced high shoot proliferation which does not favor scalp formation. However, at subculture 2, medium M1 (5 µM TDZ + 5 µM 4-CPPU) induced significantly ($P < 0.05$) higher number of multiple buds in cultivars Mbwazirume, Nakinyika and Nakabululu while cv. Nfuuka continued to produce significantly ($P < 0.05$) higher number of multiple buds in medium M5 (13 µM TDZ + 13 µM 4-CPPU).

There was significant increase in mean multiple bud proliferation from subculture 1 to subculture 3 in all the cultivars but at varying levels depending on hormone treatment (Table 1). In cycle 3, cv. Nakinyika had significantly ($P < 0.05$) the highest mean multiple bud proliferation on medium M6 (26 µM TDZ), followed by Nakabululu on M3 (9 µM TDZ + 9 µM 4-CPPU) and Mbwazirume on M5 (13 µM TDZ + 13 µM 4-CPPU). Cultivars Mpologoma and Nfuuka produced the same number of multiple buds but on medium M4 (11 µM TDZ + 11 µM 4-CPPU) and M7 (26 µM 4-CPPU), respectively.

Table 1. Cultivar shoot and multiple bud proliferation on different combinations of TDZ and 4-CPPU (M1, M2, M3, M4, M5, M6 and M7) at first, second and third subculture cycles

Subculture cycle	Cultivar	Organ	Mean number of shoots and multiple buds							$LSD_{(0.05)}$
			M1	M2	M3	M4	M5	M6	M7	
1	Mbwazirume	Shoots	1.50	1.00	1.30	1.40	1.50	1.60	1.10	0.244
		Multiple buds	3.90	1.60	2.80	2.90	3.20	2.70	2.40	0.626
	Mpologoma	Shoots	1.40	1.30	0.80	1.50	1.40	1.10	1.40	0.244
		Multiple buds	1.30	2.00	1.40	1.70	1.40	0.90	0.80	0.626
	Nakabululu	Shoots	1.30	1.70	1.10	1.30	1.20	2.40	1.50	0.244
		Multiple buds	3.00	3.60	5.30	4.20	6.50	2.10	4.50	0.626
	Nakinyika	Shoots	2.00	1.40	1.10	0.90	0.80	2.40	1.40	0.244
		Multiple buds	3.20	2.00	4.00	4.30	7.80	2.40	4.20	0.626
	Nfuuka	Shoots	1.90	1.40	0.90	0.70	0.30	1.10	2.20	0.244
		Multiple buds	1.50	3.20	3.10	4.00	4.30	2.90	1.90	0.626
2	Mbwazirume	Shoots	0.30	0.80	0.60	0.70	0.40	0.60	0.60	0.244
		Multiple buds	5.70	4.20	4.20	3.60	3.70	2.10	2.10	0.626
	Mpologoma	Shoots	0.70	0.60	0.50	0.60	0.00	0.60	1.40	0.244
		Multiple buds	2.30	3.10	2.70	2.10	2.60	2.00	1.00	0.626
	Nakabululu	Shoots	0.70	1.00	1.10	0.80	1.20	1.60	1.80	0.244
		Multiple buds	4.60	2.20	3.60	2.20	4.00	3.10	3.90	0.626
	Nakinyika	Shoots	0.30	0.30	0.30	0.50	0.40	1.80	1.30	0.244
		Multiple buds	5.30	2.00	3.50	2.90	4.50	2.20	3.70	0.626
	Nfuuka	Shoots	0.70	1.00	0.50	0.40	0.40	1.00	1.30	0.244
		Multiple buds	3.30	3.90	3.20	4.50	5.20	3.60	2.40	0.626
3	Mbwazirume	Shoots	1.70	2.30	0.70	1.00	0.60	0.90	2.00	0.244
		Multiple buds	5.80	6.20	4.40	8.80	9.10	8.60	6.90	0.626
	Mpologoma	Shoots	2.40	2.20	0.70	1.50	1.70	1.40	0.60	0.244
		Multiple buds	7.20	5.00	6.80	8.30	4.90	4.50	6.10	0.626
	Nakabululu	Shoots	1.40	0.80	0.70	1.60	1.40	0.80	1.40	0.244
		Multiple buds	8.60	6.60	9.30	8.50	8.60	8.50	8.40	0.626
	Nakinyika	Shoots	1.10	1.50	0.20	0.60	0.70	0.70	2.50	0.244
		Multiple buds	8.30	6.90	8.50	8.40	8.20	9.40	9.80	0.626
	Nfuuka	Shoots	1.40	1.30	1.40	1.10	0.30	1.00	1.00	0.244
		Multiple buds	8.20	7.80	7.60	7.70	7.30	7.20	8.30	0.626

Cycle $LSD_{(0.05)}$ = 0.160 for shoot and 0.410 for multiple buds

Cultivar $LSD_{(0.05)}$ = 0.206 for shoot and 0.529 for multiple buds

Scalps were achieved in all TDZ and 4-CPPU combinations (Table 2). With exception of cultivars Mpologoma and Mbwazirume, scalps were formed in over 50% of the cultures of the various cultivars by the fourth subculture cycle. Mpologoma produced fewer (≤ 50%) scalps in most of the media while Mbwazirume produced fewer scalps (37.5%) only in medium M7 (26 µM 4-CPPU). Cultivars Nakinyika and Nakabululu produced high number of scalps (≥ 70%) in all the media or treatments.

Table 2. Cultivar percentage ideal scalps on the different combinations of TDZ and 4-CPPU at the fourth subculture cycle

Cultivar	Percentage ideal scalps under different media treatment							Mean %
	M1	M2	M3	M4	M5	M6	M7	
Mbwazirume	75.0	60.0	60.0	70.0	70.0	66.7	37.5	62.74
Mpologoma	47.1	40.0	50.0	70.0	60.0	50.0	31.3	49.77
Nakabulu	70.6	75.0	82.4	72.2	71.4	75.0	87.5	76.30
Nakinyika	88.2	60.0	70.0	70.0	70.0	100.0	100.0	79.74
Nfuuka	76.5	82.4	73.3	94.1	69.2	50.0	50.0	70.79

3.2 Callus and Embryo Development

As the scalps formed on the scalp induction media containing various combinations of TDZ and 4-CPPU, compact yellowish, pinkish and cream callus tissue developed on them (Figure 1). The percentage of scalp cultures that had callus on them varied with hormone treatment and cultivar (Table 3). By cycle 4, all the cultivars produced callus on their scalp cultures even on the medium containing the lowest concentration of TDZ and 4-CPPU (5 µM TDZ + 5 µM 4-CPPU). Cultivar Nakinyika had the highest percentage of cultures with callus (20%) on medium M5 (13 µM TDZ + 13 µM 4-CPPU) and M6 (26 µM TDZ), Mpologoma (20%) on M6 (26 µM TDZ), Nfuuka (17.5%) on M1 (5 µM TDZ + 5 µM 4-CPPU), Nakabululu (17.5%) on M7 (26 µM 4-CPPU) and Mbwazirume (12.5%) on M6 (26 µM TDZ). All the cultivars performed well on MS supplemented with 26 µM TDZ with up to 12.5−20% of their scalp cultures having callus on them by cycle 4 (Table 3).

Table 3. Mean percentage callus formation on scalp cultures of EA-AAA banana cultivars on different combinations of TDZ and CPPU by the fourth subculture cycle

Cultivar	Mean percentage callus under different media treatment						
	M1	M2	M3	M4	M5	M6	M7
Mbwazirume	10.0	10.0	2.5	5.0	10.0	12.5	2.5
Mpologoma	2.5	5.0	2.5	10.0	17.5	20.0	2.5
Nakabululu	2.5	5.0	10.0	10.0	10.0	15.0	17.5
Nakinyika	7.5	5.0	7.5	7.5	20.0	20.0	17.5
Nfuuka	17.5	12.5	7.5	10.0	5.0	15.0	2.5
Treatment LSD $_{(0.05)}$ = 6.905; Cultivar LSD $_{(0.05)}$ = 5.836							

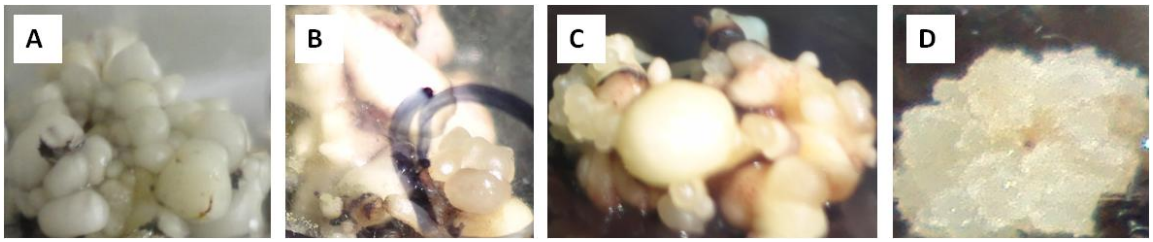

Figure 1. Embryogenic callus formation on scalps of cultivar Nfuuka on 9 µM TDZ + 9 µM 4-CPPU at the third subculture cycle: (A) Ideal scalp, (B) Callus initiation, (C-D) Embryogenic callus development

As the callus on scalps aged and became necrotic by cycle 4, some embryos developed on it and differentiated directly into shoot (Figure 2). This was observed in cv. Nakabululu on 13 µM TDZ + 13 µM 4-CPPU. When the callus from the different cultivars was removed and cultured on a hormone free medium, it differentiated into somatic embryos and shoots (Figure 3) regardless of initial hormone treatment implying that it was all embryogenic. When the callus from the different cultivars was removed and transferred to MS medium

containing 5 µM BAP, it only regenerated into plantlets (Figure 4) in a few cultures of cultivars Nakinyika, Nakabululu, Nfuuka and Mbwazirume (Table 4). Of the plantlets regenerated 5 out of 12 and 4 out of 5 plantlets of cultivars Nakinyika and Mbwazirume, respectively, developed normal roots (Table 4) and were weaned (Figure 5). But when the scalps were excised and cultured on a medium with 5 µM 2,4-D to generate callus they all died.

Table 4. Number of plantlets and their roots of various EA-AAA banana cultivars regenerated out of twenty (20) embryogenic callus cultures induced by TDZ and 4-CPPU growth regulators

Cultivar	Number of plantlets regenerated	Number of plantlets with roots	Mean root number
Mbwazirume	5	4	6.0
Mpologoma	0	0	0
Nakabulu	10	1	2.0
Nakinyika	12	5	5.4
Nfuuka	2	0	0

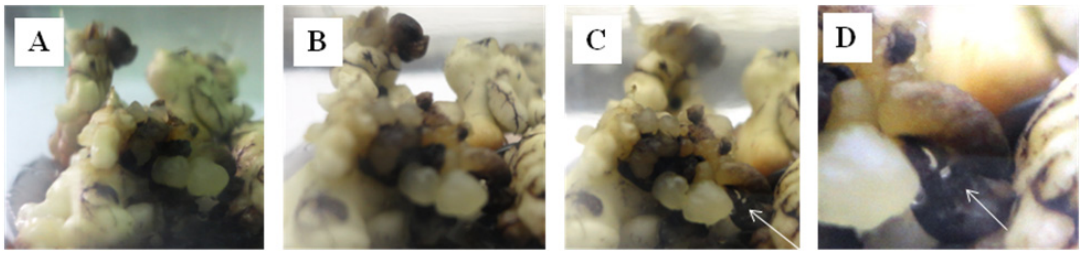

Figure 2. Direct embryogenesis in cv. Nakabululu on 13 µM TDZ+13 µM 4-CPPU: (A) Callus on scalp, (B) Embryo initiation on callus, (C–D) embryo to shoot development

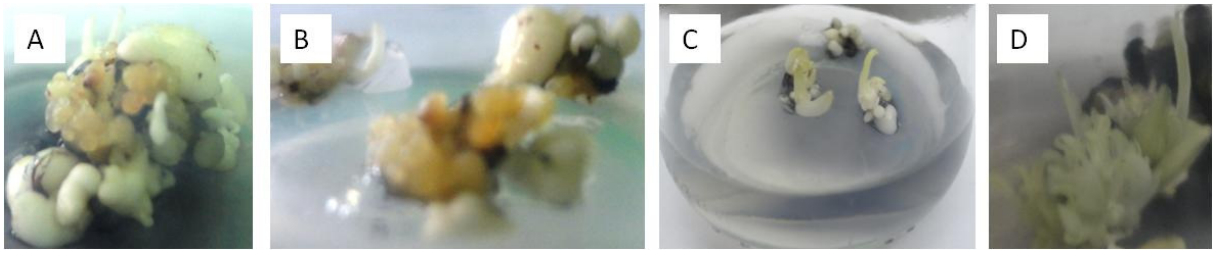

Figure 3. Differentiation of callus on scalps of cv. Nakinyika into somatic embryos and shoots on transfer to hormone free MS medium: (A) Callus on scalp (B) Embryo development, (C) Callus differentiation into shoot (E) Scalp tissue differentiation in into shoot

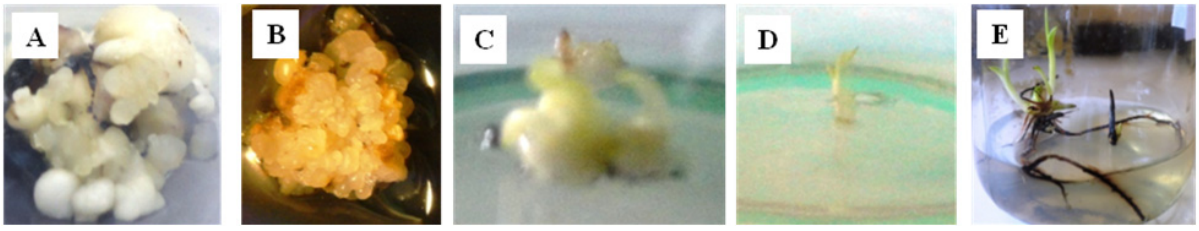

Figure 4. Different stages of plant regeneration through direct embryogenesis from scalps of cv. Mbwazirume after transfer of embryogenic callus to MS medium with 5 µM BAP: (A) Embryogenic callus development, (B) Somatic embryo development, (C–D) embryo to plant development, (E) Root development on the plantlets under the same medium

Figure 5. Plants of cvs Nakinyika and Mbwazirume regenerated through direct embryogenesis induced by TDZ and 4-CPPU after transfer of the embryogenic callus to MS medium with 5 µM BAP under acclimatization in soil

4. Discussion

Cultivars Nakinyika and Nakabululu produced more multiple buds on medium M5 (13 µM TDZ + 13 µM 4-CPPU) at subculture 1 but at subculture 2, they produced more multiple buds on M1 (5 µM TDZ + 5 µM 4-CPPU). This was probably due to changes in effect of the growth regulators with subculture cycle especially TDZ which was reported to have carry-over effect (Makara et al., 2010). Generally at subculture 1, multiple-bud proliferation increased as the concentration of TDZ and 4-CPPU increased. Concentrations of TDZ and 4-CPPU above 7 µM appear to suppress shoot apical dominance and favor multiple bud proliferation. However, in cv. Mbwazirume, mean bud proliferation on 5 µM TDZ + 5 µM 4-CPPU was significantly higher than on 7 µM TDZ + 7µM 4-CPPU suggesting that combined TDZ and 4-CPPU at low concentration permits vigorous proliferation of both shoots and buds depending on cultivar. TDZ is active in inducing adventitious and axillary shoot proliferation (Ashraf et al., 2004; Youmbi et al., 2006) which are precursors for scalp formation. Similarly, 4-CPPU induced multiple axillary shoot proliferation in *Impatiens walleriana* L. (Subotić et al., 2008).

Multiple bud proliferation was significantly ($P < 0.05$) higher on 13 µM TDZ + 13 µM 4-CPPU than on 26 µM TDZ and 4-CPPU when used individually. The interaction of TDZ and 4-CPPU to induce more multiple bud proliferation when used in combination at lower concentration (13 µM) compared to when used singly at concentration of 26 µM suggests both synergistic and additive effects and implies that high levels of each of them can be avoided. Achievement of plant regeneration at lower concentrations of combined TDZ and 4-CPPU could be advantageous in reducing somaclonal variation. In bananas, somaclonal variations of different types have occurred due to high concentrations of growth hormones such as 6-benzylaminopurine and dicamba (Leela et al., 2003).

Embryogenic callus was directly formed on the scalp induction medium containing TDZ and 4-CPPU. Commonly, embryogenesis is induced after inoculating the ideal scalps onto medium containing 2,4-D or other auxins (Schoofs et al., 1998; Sadik et al., 2007; Ramírez-Villalobos & De García, 2008). The formation of embryogenic callus under TDZ and 4-CPPU treatments as described in this study conforms to reports that at high concentrations, these urea-type cytokinins exhibit an auxin activity and thus could induce embryogenesis (Vinayak et al., 2009). Chhabra et al. (2008) suggested that at high concentrations, TDZ imposes stress on the explant and could redirect its development from shoot organogenesis to somatic embryogenesis. At concentrations higher than 2 µM, TDZ induced direct somatic embryogenesis on cotyledonary node explants of recalcitrant lentil (Chhabra et al., 2008). Earlier, TDZ was reported to have induced higher somatic embryogenesis in protocalli than even 2,4-D (Lehminger-Mertens & Jacobsen, 1989).

Although TDZ and 4-CPPU exhibit an auxin activity and induce somatic embryos in cultures grown on media devoid of auxins, the mechanism of these urea-type cytokinins to induce embryogenic callus in bananas are of interest as earlier reports suggested that somatic embryogenesis could be induced in dicot species on cytokinin-supplemented media (Raemakers et al., 1995) but auxins solely mediated the transition from somatic to embryogenic cells in angiosperm monocots (Víctor, 2001). TDZ induced somatic embryogenesis in rice but after a short treatment with 2,4-D (Aparna & Rashid, 2004). This implies that further research on whether auxins

solely mediate transition from somatic to embryogenic cells in angiosperm monocots is necessary.

Cultivar Nfuuka formed callus on medium M1 with low levels of TDZ and 4-CPPU. Relating to the name 'Nfuuka' ''meaning ever changing'' in one of the dialects in Central Uganda, the response of cv. Nfuka suggested that it is mutatic and may on all occasions respond to a wider range of treatments than other EA-AAA banana cultivars. However, the increase in callus formation on low levels of TDZ and 4-CPPU with subculture cycle was attributed to carry-over effect of urea-type cytokinins especially TDZ (Makara et al., 2010).

The differentiation of callus on scalps generated on TDZ and 4-CPPU into somatic embryos and shoots is in agreement with other reports. Razdan (1993) reported that a whole plant regeneration from callus tissue may occur either through organogenesis (shoot-bud differentiation) or somatic embryogenesis where the cell or group of cells initiate a development-pathway that leads to reproducible regeneration of non-zygotic embryos capable of germinating to form complete plants. There was more embryo development when the callus was removed and cultured on a hormone free medium probably due to reduction in auxin activity resulting from the high concentrations of TDZ and 4-CPPU. Though the process of somatic embryogenesis is often initiated in media containing high levels of auxins, later reduction in auxin concentration usually stimulates further embryo development since lower auxin concentrations induce pro-embryogenic cells to divide and develop into embryos (Sharp et al., 1980).

The scalps formed on media containing TDZ and 4-CPPU died when transferred on to medium containing 5μM 2,4-D possibly due to excessive cumulative auxin activity effect (George et al., 2008). Since urea-type cytokinins especially TDZ have carry-over effect (Makara et al., 2010), their auxin activity together with that of 2,4-D could have been too high for the stimulation of the cells in the scalp tissue to dedifferentiate and form callus but rather killed them. However, somatic embryos were produced from stem explants of Golden Pothos (*Epipremnum aureum*) on MS medium supplemented with 0.5 mgl-1 (2.26 μM) 2,4-D and 2 mgl-1 (8.07 μM) CPPU, and 0.5 mgl-1 (2.26 μM) 2,4-D and 2.0 mgl-1 (9.08 μM) TDZ (Zhang et al., 2004). This suggests that lower concentration of 2,4-D is required for scalps generated on media containing TDZ.

Among the explants that have been used in somatic embryogenesis in bananas such as immature zygotic embryos, immature male flowers, female flowers, scalps, leaf bases and corm slices, scalps were reported to be among the most responsive material for initiating regenerable embryogenic cell suspension cultures (Meenakshi et al., 2007). One impediment to using the scalp method to generate callus in EA-AAA bananas has been the long time required to achieve ideal scalps. Up to 9 subculture cycles on a medium containing 100 μM BAP (Schoofs et al., 1998) or 6 subculture cycles on a medium containing 10 μM TDZ (Sadik et al., 2007) were required.

In this study, scalps of EA-AAA bananas were achieved in over 50% of the scalp cultures within 4 subculture cycles and embryogenic callus was formed on 2.5–20% of the scalp cultures depending on cultivar and TDZ and 4-CPPU combination. Though the embryo to plant conversion rate was low, the embryogenic callus responses were within the range and in some instances higher than those reported in other fairly responsive genotypes using the scalp method (Xu et al., 2005; Ramírez-Villalobos & De García, 2008). The influence of TDZ on proliferation in EA-AAA bananas had been reported (Arinaitwe et al., 2000; Sadik et al., 2007; Makara et al., 2010). This is the first report on combined effect of TDZ and CPPU on scalps and regenerable embryogenic callus induction in EA-AAA bananas.

Application of TDZ and 4-CPPU growth regulators increased the rate and percentage of embryogenic callus induced from scalps of recalcitrant EA-AAA banana cultivars. Cultivars Nakinyika, Nakabululu, Mbwazirume and Nfuuka developed regenerable embryogenic callus and recorded plant regeneration through direct embryogenesis. Early plant regeneration at lower concentrations of combined TDZ and 4-CPPU could reduce occurrence of somaclonal variation and allow safe and enhanced genetic transformation of the EA-AAA bananas.

Acknowledgements

The study was funded by the World Bank and the Government of Uganda through the Millennium Science Initiative (MSI) Program of the Uganda National Council for Science and Technology (UNCST).

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