Journal of Applied Sciences Research, 9(6): 3781-3790, 2013
ISSN 1819-544X
This is a refereed journal and all articles are professionally screened and reviewed

ORIGINAL ARTICLES

Callus Induction, Regeneration and Molecular Characterization of Cassava (*Manihot esculenta* Crantz)

[1]Neama A. Abd Alla, [2]Mohamed E. Ragab, [2]Salah El-Deen M. El-Miniawy, [1]Hussein S. Taha

[1]Plant Biotechnology Dept., National Research Center, Dokki, Cairo, Egypt.
[2]Horticulture Dept., Fac. of Agriculture, Ain Shams University, Shoubra El-Kheima, Cairo, Egypt.

ABSTRACT

Cassava (*Manihot esculenta* Crantz) is considered as an important energy plant due to the fact that its tuber roots contain high starch content. In this study, explants leaf, stem and root of one month old *in vitro* growing seedlings of cassava were utilized for callus induction on MS medium supplied with different concentrations (0, 5, 10, 15 and 20 mg L^{-1}) of 2,4-D. The results demonstrated that leaf explants were superior in callus formation and MS medium supplemented with 15 mg L^{-1} 2, 4- D was the best medium for callus induction and growth development of cassava plant. For enhancement of plantlets regeneration, induced calli were transferred to MS medium modified with different concentrations of BA, 2ip and TDZ (0, 0.1, 0.5 and 1.0 mg L^{-1} from each one alone). The data showed that MS medium containing 1 mg L^{-1} TDZ was propitious to plantlets regeneration compared with other regeneration media. Moreover, the molecular characterization of the *in vivo* growing plants comparing with the *in vitro* derived plantlets (*in vitro* propagated and regenerated plantlets) was performed using RAPD-PCR and SDS-PAGE techniques. The results demonstrated that there were not observed variations between them. These results confirmed that tissue culture technique was providing safety for *in vitro* propagation of cassava plant.

***Key words*:** Cassava, callus induction, regeneration and molecular characterization.

Introduction

Among all the tuber crops grown in Africa, cassava (*Manihot esculenta* Crantz) is the most largely grown and therefore, serves as the major tuber food crop for human consumption and also as animal feed. Cassava is considered as 'life-blood' because the starchy tuber is consumed as a staple and also supports the starch, textile, paint and pharmaceutical industries. Modern biotechnological applications for economically-important crops like cassava begins with successful establishment of stable and active callus cultures which can be genetically manipulated and induced into planting materials. Nowadays focus is being placed on cassava callus induction and plantlets regeneration (Fletcher *et al.,* 2011).

Callus is a relativity undifferentiated tissue consisting primarily of parenchymatous cells. Callus tissue can serve an experimental system to investigate and solve a broad range of basic research problems in plant cytology, physiology, morphology, anatomy, biochemistry, pathology, and genetics. It can also be used to resolve applied research problems in organogenesis and embryogenesis related to the propagation of horticultural and agronomic plants (Caponetti, 2000). Furthermore, used to study protoplast isolation, cell type, cellular selection and secondary metabolite production (Kondamudi *et al.*, 2009). Earlier studies on callus induction from different explants of cassava have been reported by several workers (El-Zeiny *et al.*, 2001; Joseph *et al.*, 2001; Ogburia, 2002; Fletcher *et al*. 2011).

Callus induction and plant regeneration are one of the key tools in plant biotechnology that exploits the totipotent nature of plant cells (Mukherjee *et al.*, 2011). Tissue culture serves as an indispensable tool for transgenic plant production. For nearly any transformation system, an efficient regeneration protocol is imperative (Stewart and Cardoza, 2008). Previous investigations on adventitious shoots formation from callus cultures of family euphorbiaceae were conducted (Sujatha and Mukta, 1996; Mussio *et al.*, 1998; Matand *et al*., 2004; Ahn *et al.* 2007; Kumari *et al.*, 2008; Li *et al*., 2012).

Biochemical and molecular approaches including RAPD techniques have been shown to be potentially useful in fingerprinting cassava germplasm collections. However; they will be even more useful if markers for linamarin content/cyanogenic potential in cassava could be identified (Wong *et al.*, 1997). DNA fingerprinting is increasingly being used for identification of varieties, quantification of similarity between varieties and study of genetic diversity available in the population. DNA fingerprinting using RAPD method has been standardized for cassava (Pillai and Sundaresan, 2007).

Corresponding Author: Dr. Hussein S. Taha, Plant Biotechnology Dept., National Research Center, Dokki, Cairo, Egypt.
E-mail: hussein.taha2@yahoo.com

The protein population of cassava root layers was characterized by SDS-PAGE technique. Although the storage root of cassava is a primary source of food, few studies on gene expression in this organ have been reported. However a survey was initiated on cassava root formation and protein profiles of roots at various stages of development (De Souza *et al.*, 2002 and 2003).

The aim of this study was establishment applicable protocols for callus induction and plantlets regeneration from cassava plant. In addition carrying out biochemical fingerprints for *in vitro*, regenerated plantlets through RAPD-PCR and SDS-PAGE techniques compared with mother plants.

Material And Methods

This investigation was carried out in Plant Biotechnology Department, Genetic Engineering and Biotechnology Division, National Research Center, Cairo, Egypt, in cooperation with Horticulture Department, Faculty of Agriculture, Ain Shams University, from 2009 to 2011, to establish an applicable protocol for callus induction and plantlets regeneration of cassava plant. Also, carrying out of RAPD-PCR and SDS-BAGE for distinguish the differences between *in vitro* derived plantlets compared with mother plant.

Callus induction and growth development:

- *Plant materials:*

Immature leaf, stem and root segments were taken from *in vitro* growing plantlets (one month old), and cut into pieces (explants 3-4 mm), then cultured on solidified MS-medium, 50 mg L^{-1} casein hydrolysate, 3% (w/v) sucrose and 0.7% agar and sortifed with different concentrations of 2,4-D as follow:

C_1 = MS Basal (free growth regulators)
C_2 = MS + 5 mg L^{-1} 2,4-D
C_3 = MS + 10 mg L^{-1} 2,4-D
C_4 = MS + 15 mg L^{-1} 2,4-D
C_5 = MS + 20 mg L^{-1} 2,4-D

The pH of all tested media was adjusted to 5.8 with 1 N KOH or HCl. The tested media were distributed into 100 ml glass jars containing 25 ml and sterilized by autoclaving for 15 min at 121ºC and 1.2 Kg Cm^{-2}. Each treatment consisted of 5 replicates (jars) and each replicate contained 4 explants. Cultures were incubated under light for 16/8 h of white cooling fluorescent lamps at 26±1ºC for four weeks. Callus formation (%) and calli fresh, dry weights (g/ jar) were recorded after four weeks.

- *Frequency of callus formation (%):*

Frequencies of callus formation were calculated by the following equation:
Frequencies of callus formation (%) = (Explants produced callus / Total cultured explants) × 100

Shootlets regeneration:

An equal weight of friable leaf calli (~ 250 mg) was cultured on MS medium supplemented with different concentration of cytokinins (BA, 2iP or TDZ) as follow:

T_1 = MS Basal (free growth regulators)
T_2 = MS + 0.1 mg L^{-1} BA
T_3 = MS + 0.5 mg L^{-1} BA
T_4 = MS + 1.0 mg L^{-1} BA
T_5 = MS + 0.1 mg L^{-1} 2iP
T_6 = MS + 0.5 mg L^{-1} 2iP
T_7 = MS + 1.0 mg L^{-1} 2iP
T_8 = MS + 0.1 mg L^{-1} TDZ
T_9 = MS + 0.5 mg L^{-1} TDZ
T_{10} = MS + 1.0 mg L^{-1}TDZ

After four weeks, regeneration percentage and No. of regenerated shootlets were recorded.

J. Appl. Sci. Res., 9(6): 3781-3790, 2013

Molecular analysis:

RAPD-PCR:

- *Isolation of genomic DNA:*

DNA was isolated from *in vitro* derived shootlets compared with leaves of mother plant as described method by Doyle and Doyle (1990).

- *Polymerase Chain Reaction (PCR)*

PCR amplification was performed as described method by Williams *et al.* (1990). The AM7, AM8 and AM9 operon primers were used to distinguish the differences molecular weights between *in vitro* derived shootlets compared with the mother plant as shown in Table (1).

Table 1: The random primer names and sequences used for RAPD analysis

No.	RAPD primers	
	Name	Sequences 5'---------- 3'
1	AM7	CTTCGGCAGCATCTCTTCAT
2	AM8	CAGTGTGGAAGCCGATTATG
3	AM9	ATGTGTTGTCTGGCTTGGTA

- *Electrophoresis:*

The amplification products were analyzed by electrophoresis according to described method by Sambrook *et al.* (1989). Nucleic acids bands were photographed and detected under short wave of UV light.

SDS-PAGE:

SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970).

Statistical analysis:

Data were statistically analyzed using Duncun's multiple range test at 5 % level (CoHort, 2004) according to Snedecor and Corchan (1982) to verify the differences between means of treatments. All experiments were designed in a completely randomized design.

Results And Discussion

Callus induction and growth development:

Effect of MS medium supplemented with different concentrations of 2,4- D on callus induction from leaf, stem and root explants of cassava was investigated as shown in Tables 2, 3, 4 and 5 and Fig. (1).

- *Frequency of callus formation (%):*

Frequency of callus formation showed significant differences between treatments. The best medium for callus induction was C_4 (MS medium supplemented with 15 mg L^{-1} 2, 4- D) which recorded 74.9 % and the best explant was leaf which recorded 79.1 % (Table 2). The maximum frequency (100%) of callus formation was obtained when leaf explant cultured on C_4, while root explant produced the lowest value (16.6%) when cultured on C_2 (MS medium supplemented with 5 mg L^{-1} 2, 4- D). Moreover, there was no response for callus induction on C_1 (MS basal medium) for all explants. So, the results demonstrated that culturing of leaf explant on MS supplemented with 15 mg L^{-1} 2, 4- D gave the best result of callus induction of cassava plant.

Table 2: Effect of MS medium supplemented with different concentrations of 2, 4- D on callus formation (%) from leaf, stem and root explants of cassava plant after four weeks of culturing and incubated under light condition for 16/8 h at 26±1ºC

Treatments	Explants			Mean
	Leaf	Stem	Root	
C_1 = MS Basal medium	0.00 i	0.00 i	0.00 i	0.0
C_2 = MS +5 mg L^{-1} 2,4-D	58.33 c-f	50.00 d-g	16.66 hi	41.6
C_3 = MS+10 mg L^{-1} 2,4-D	66.66 b-e	58.33 c-f	25.00 g-i	49.9
C_4 = MS+15 mg L^{-1} 2,4-D	100.00 a	83.33 a-c	41.66 e-h	74.9
C_5 = MS +20 mg L^{-1} 2,4-D	91.66 ab	75.00 a-d	33.33 f-h	66.6
Mean	79.1	66.6	29.1	

Each treatment was the average of 5 replicates and each Jar contains four explants
Values followed by the same letters were not significantly different by Duncan's test at 0.05 level

- *Callus fresh weight (g/jar):*

Data tabulated in Table (3) clearly showed that there were significant differences between treatments. The highest fresh weights of callus cultures derived from leaf, stem and root explants of cassava plantlets recorded 6.30, 5.30 and 4.54 (g/jar), respectively after four weeks of culturing on C_4 (MS medium supplemented with 15 mg L^{-1} 2,4- D). However, C_2 (MS medium supplemented with 5 mg L^{-1} 2, 4- D) showed the lowest values of fresh weights (4.37, 3.67 and 2.87 (g/jar), respectively). While MS basal medium recorded negative results for all used explants. So, the obtained results indicated that leaf explant produced the maximum fresh weight when cultured on C_4 medium while fresh weight of callus derived from root explants recorded the minimum value when cultured on C_2 medium.

Table 3: Effect of MS medium supplemented with different concentrations of 2,4- D on callus fresh weights (g/jar) from leaf, stem and root explants of cassava plant after four weeks of culturing and incubated at 26± 1ºC and 16/8 h day light

Treatments	Explants		
	Leaf	Stem	Root
C_1 = MS Basal medium	0.00 j	0.00 j	0.00 j
C_2 = MS + 5 mg L^{-1} 2,4-D	4.37 f	3.67 h	2.87 i
C_3 = MS + 10 mg L^{-1} 2,4-D	4.88 de	4.52 ef	3.92 gh
C_4 = MS + 15 mg L^{-1} 2,4-D	6.30 a	5.30 c	4.54 d-f
C_5 = MS + 20 mg L^{-1} 2,4-D	5.77 b	4.92 cd	4.23 fg

Each treatment was the average of 5 replicates and each Jar contains four explants
Values followed by the same letters were not significantly different by Duncan's test at 0.05 level

- *Callus dry weight (g/jar):*

As shown in Table **(**4) data clearly indicated that there were significant differences between treatments. The highest dry weights of callus cultures derived from leaf, stem and root explants of cassava plantlets recorded 0.581, 0.458 and 0.381 (g/jar), respectively after four weeks of culturing on C_4 (MS medium supplemented with 15 mg L^{-1} 2,4- D). However, C_2 (MS medium supplemented with 5 mg L^{-1} 2, 4- D) showed the lowest values of dry weights 0.374, 0.302 and 0.231 (g/jar), respectively. Whereas MS basal medium showed negative results for all subjected explants. So, the obtained results indicated that leaf explant produced the maximum dry weight when cultured on C_4 medium while callus derived from root explant recorded the minimum value when cultured on C_2 medium.

Table 4: Effect of MS medium supplemented with different concentrations of 2,4- D on callus dry weights (g/jar) from leaf, stem and root explants of cassava plant after four weeks of culturing and incubated at 26± 1ºC and 16/8 h day light

Treatments	Explants		
	Leaf	Stem	Root
C_1 = MS Basal medium	0.000 h	0.000 h	0.000 h
C_2 = MS + 5 mg L^{-1} 2,4-D	0.374 d	0.302 f	0.231 g
C_3 = MS + 10 mg L^{-1} 2,4-D	0.428 c	0.381 d	0.325 ef
C_4 = MS + 15 mg L^{-1} 2,4-D	0.581 a	0.458 c	0.381 d
C_5 = MS + 20 mg L^{-1} 2,4-D	0.512 b	0.422 c	0.352 de

Each treatment was the average of 5 replicates and each Jar contains four explants
Values followed by the same letters were not significantly different by Duncan's test at 0.05 level

In general, it could be concluded that maximum value of callus induction was recorded for leaf followed by stem and root explants. MS medium supplemented with 15 mg L^{-1} 2, 4- D compared with other supplementations was the best for cassava callus production.

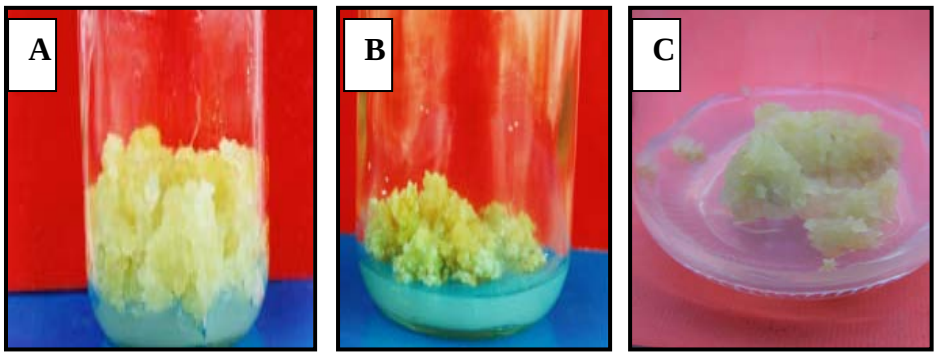

Fig. 1: Callus produced from leaf (A), stem (B) and root (C) explants after four weeks of culturing on MS medium supplemented with 15 mg L^{-1} 2, 4- D

The power of plant hormones to promote development in tissue culture has been amazed and baffled plant scientists for many years (Che *et al.*, 2002). Plant growth regulators, as one kind of signal molecule, have been shown to play an important role during the callus formation (Sugiyama and Imamura, 2006). An auxin is generally required for the induction of callus from explants. Applied auxins seem to be capable of fundamentally altering the genetically programmed physiology of whole plant tissues, which had previously determined their differentiated state. Cells, which respond to auxin, revert to a dedifferentiated state and begin to divide. The auxin most frequently employed to initiate callus cultures is 2, 4- D. However, since cultures maintained on 2, 4-D may become genetically variable, some investigators prefer NAA or IAA, or a transfer of callus to a medium containing one of these alternative compounds once it has been initiated by 2, 4-D (Machakova *et al*., 2008).

The obtained results from the present study were in agreement with the results which reported by Joseph *et al.* (2001); Fletcher *et al.* (2011) on cassava and Yang *et al.* (2009) on sun spurge (*Euphorbia helioscopia*).

There was different than what reported by El-Zeiny *et al.* (2001) on cassava; Catapan *et al*. (2000) on carolina leaf-flower (*Phyllanthus caroliniensis*); Sujatha *et al.* (2008) on castor and Kumar *et al.* (2008) on jatropha. In general, these different results may be due to the different plant.

Shootlets regeneration:

The effect of MS medium supplemented with different concentrations of BA, 2iP and TDZ on plantlets regeneration *via* callus culture of cassava was investigated. Regeneration percentage (%) and number of regenerated shootlets were recorded after 4 weeks of subculturing on regeneration media. Data listed in Table (6) clearly showed that there were significant differences between treatments. MS free growth regulators have no response to regeneration. TDZ was the most effective to plantlets regeneration *via* callus cultures of cassava comparing with BA and 2iP. MS medium supplemented with 1.0 mg L^{-1} TDZ was the best for plantlets regeneration (Fig. 2), where it recorded 73.3 % and 2.2 for regeneration percentage and number of regenerated shootlets, respectively. Whereas, there were insignificant differences between TDZ treatments. These results were in agreement with the results obtained by Kumari *et al.* (2008) on castor and Li *et al*. (2012) on jatropha.

Moreover Kumar and Reddy (2010) showed that TDZ is one of the several substituted ureas that have been investigated recently for their cytokinin-like activity, which is known to be more active than zeatin for stimulating the growth, differentiation and organogenesis when added to a tissue culture medium at a low concentration. These results suggest that TDZ plays a very important role in the formation of adventitious shoot buds from callus. These effects may be involved in stimulating *de novo* synthesis of auxins by increasing the levels of IAA and its precursor, tryptophan, as well as increase in contents of endogenous cytokinin and ethylene. The other possibilities include the modification in cell membranes, energy levels, nutrient uptake, or nutrient assimilation (Murthy and Saxena, 1998).

Table 6: Effect of MS medium supplemented with different concentrations of BA, 2iP and TDZ on regeneration percentage (%) and No. of regenerated shootlets from callus of cassava plant after four weeks of subculturing and incubation in light for 16/8 h at 26±1ºC

Treatments	Regeneration (%)	No. of regenerated shootlets
T_1 = MS Basal medium	0.00 g	0.0 g
T_2 = MS + 0.1 mg L^{-1} BA	13.32 fg	0.4 fg
T_3 = MS + 0.5 mg L^{-1} BA	19.98 e-g	0.6 e-g
T_4 = MS + 1.0 mg L^{-1} BA	26.60 d-f	0.8 d-f
T_5 = MS + 0.1 mg L^{-1} 2iP	33.30 c-f	1.0 c-f
T_6 = MS + 0.5 mg L^{-1} 2iP	39.90 b-e	1.2 b-e
T_7 = MS + 1.0 mg L^{-1} 2iP	46.60 b-d	1.4 b-d
T_8 = MS + 0.1 mg L^{-1} TDZ	53.30 a-c	1.6 abc
T_9 = MS + 0.5 mg L^{-1} TDZ	59.9 ab	1.8 ab
T_{10} = MS +1.0 mg L^{-1} TDZ	73.30 a	2.2 a

Each treatment was the average of 5 replicates and each Jar contains 1 g callus divided to four parts
Values followed by the same letters were not significantly different by Duncan's test at 0.05 level

Fig. 2: *In vitro* regenerated shootlets produced from callus cultures of cassava plant after 4 weeks of subculturing on MS medium supplemented with 1.0 mg L^{-1} TDZ

Molecular analysis:

RAPD-PCR:

Three random primers (AM7, AM8 and AM9) were screened in RAPD analysis for their ability to produce sufficient amplification products. The results of DNA fingerprints generated by PCR amplification using the three primers are presented in Tables (7, 8) and Fig. (3). The number of fragments generated per primer varied between 1-3, 1-6 and 1-5. The primer AM8 gave the highest number of bands (6) and the percentage of polymorphism were 88.8, while, the primer AM7 gave the highest percentage of polymorphism (100) and the number of bands was (3).

RAPD marker generated with primer (AM7) showed that there are no differences between all treatments and the mother plant. RAPD marker generated with primer (AM8) showed that the differences with two fragments, the first at 966 bp with mother plant and *in vitro* plants while didn't showed with regenerated plants, the second at 100 bp with mother plant and regenerated plants while didn't observed with *in vitro* plants. In addition, data showed that there are four common bands in all tested samples at 791, 700, 500 and 187 bp.

RAPD marker generated with primer (AM9) showed minimal DNA amplification differences between mother plant and *in vitro* plants, regenerated plants and the mother plant. Three bands are common with all at 368, 267 and 187 bp, while two bands showed with mother plant and regenerated plants but didn't showed with *in vitro* plants.

However, these polymorphisms are considered to be primarily due to the variation in the primer annealing sites. RAPDs have been used for many purposes, ranging from studies at the individual levels (e.g. genetic identity) to studies involving closely related species. Due to their high genomic abundance, RAPDs have also been applied in gene mapping studies (Williams *et al.,* 1990).

In this respect, RAPD markers have been able to assess the genetic stability of *in vitro* propagated plants of flora plena (*Deutzia scabra*) (Sayed and Gabr, 2009) almonds (Martins *et al*., 2004), cassava (Angel *et al*., 1996), ginger (Rout *et al*., 1998), air potato (*Dioscorea bulbifera* L.) (Dixit *et al*., 2003), turmeric (Tyagi *et al*., 2007), and yams (Ahuja *et al*., 2002). As RAPD markers amplify different regions of the genome, their simultaneous analyses give a better interpretation of the genetic stability of the *in vitro* regenerants (Martins *et al*., 2004).While, De Masi *et al.* (2003) reported that RAPD analysis used to distinguish between two more

largely widespread fig cultivars in Italy (Bianco del Cilento and Dottato) and their clones. RAPD- PCR produced amplification patterns that did not significantly vary between the two cultivars. However, RAPD analysis allowed clone differentiate using a single primer.

Table 7: RAPD pattern with primers (AM7, AM8 and AM9) (M). DNA marker (100 – 1500 bp) (1) mother plant (2) plantlets through *in vitro* propagation (3) regenerated plantlets of cassava

Band No.	M	Primer (1) AM7			Primer (2) AM8			Primer (3) AM9		
		1	2	3	4	5	6	7	8	9
1	1500	-	-	-						
2	966	-	-	-	+		+			
3	860	-	-	-						
4	791	-	-	-	+	+	+			
5	700	-	-	-	+	+	+			
6	600	-	-	-						
7	500	+	+	+	+	+	+	+		+
8	400	+	+	+				+		+
9	368	+	+	+				+	+	+
10	267	-	-	-				+	+	+
11	187	-	-	-	+	+	+	+	+	+
12	100	-	-	-	+	+				

Table 8: Polymorphism percentage of treatments and mother plant based on RAPD product of AM7, AM8 and AM9 primers

Primers	No. of polymorphism	Total No. of polymorphism	Polymorphism %
AM7	3	3	100
AM8	6	7.8	88.8
AM9	5	4.3	86

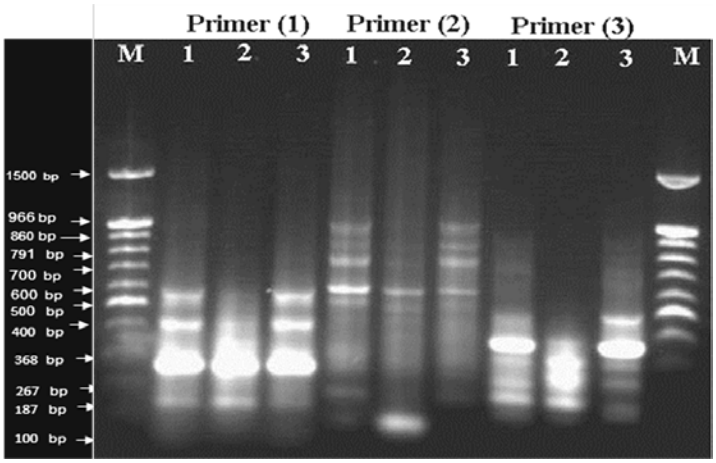

Fig. 3: RAPD pattern with primers (AM7, AM8 and AM9). (M) DNA marker (100 – 1500 bp) (1) *in vivo* plant (2) *In vitro* plantlet (3) regenerated plantlet of cassava.

SDS-PAGE:

SDS-PAGE analysis was performed to detect biochemical changes between *in vivo* plants and *in vitro* regenerated plantlets to identify any variation in protein banding patterns in response to plant growth regulators used in tissue cultures.

Comparison of protein banding patterns of *in vivo* plant (lane 1) with that of *in vitro* regenerated plantlet (lane 2) indicated that polypeptide band of 36 kDa was over expression in *in vitro* regenerated plantlet and not detected in *in vivo* plant (as shown in Fig. 4). So, the differences between in *vivo* plant and *in vitro* regenerated plantlet were not clear. The results demonstrated that there no observed variations between them were detected. These results were in agreement with Rady and Ali (1999) on sugar beet, Saker *et al.* (1999) on faba bean, El-Kazzaz and El-Bahr (2003) on date palm (cv. Samany) and De Souza *et al.*, 2002 and 2003 on cassava.

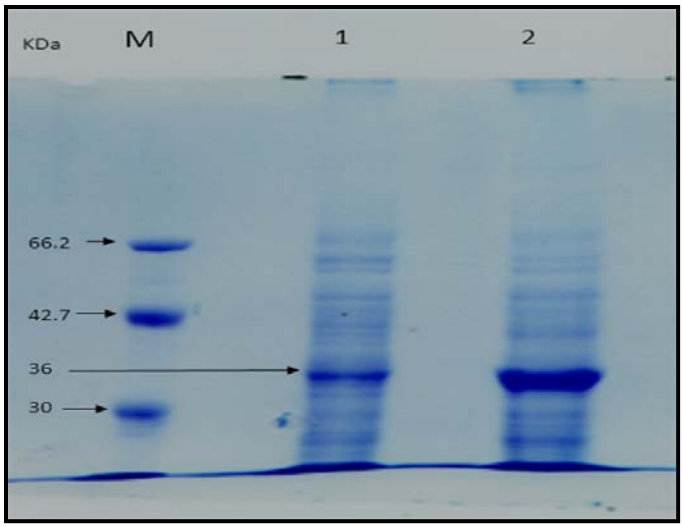

Fig. 4: SDS-PAGE analysis of total protein bands extracted from leaves of mother plant (1), indirect regenerated plantlet (2) of cassava and marker protein (M)

Conclusion:

It could be concluded that an active and stable protocol for callus cultures production from different explants of cassava plant have been successfully established; callus cultures can be genetically manipulated and induced into planting materials. It was clearly shown that the system of excellent induction and growth of callus achieved when leaf explant cultured on MS medium supplemented with 15 mg L^{-1} 2, 4- D.

Moreover, an applicable protocol for indirect plantlets regeneration from callus cultures of cassava plant was established. The results demonstrated that MS medium containing 1 mg L^{-1} TDZ was the best for plantlets regeneration. Finally, molecular characterization of the *in vivo* growing plants and the *in vitro* derived plantlets through RAPD-PCR and SDS-PAGE techniques was performed to identify the differences between them. The results indicated that there were no observed differences between *in vitro* derived plantlets and the mother plant.

Acknowledgement

I am grateful to Dr. Ahmed Magdy Gabr, Researcher of Plant Biotechnology, Department of Plant Biotechnology, National Research Center; for his help to perform analysis of DNA

Abbreviations:

MS, Murashige-Skoog; 2, 4-D, 2, 4-dichlorophenoxyacetic acid; BA, benzyladenine; 2ip, 6-(*γ*, *γ* -dimethylallylamino)-purine; TDZ, thidiazuron; RAPD-PCR, Random Amplified Polymorphic DNA-Polymerase Chain Reaction; SDS-PAGE, Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis.

References

Ahn, Y.J., L. Vang, A. Thomas, Mc Keon and G.Q. Chen, 2007. High-frequency plant regeneration through adventitious shoot formation in castor (*Ricinus communis* L.). *In Vitro* Cell. Developmental Biology-Plant, 43: 9-15.

Ahuja, S., B.B. Mandal, S. Dixit and P.S. Srivastava, 2002. Molecular phenotypic and biosynthetic stability in *Dioscorea floribunda* plants derived from cryopreserved shoot tips. Plant Sci., 163: 971-97.

Angel, F., V.E. Barney, J. Tohme and W.M. Roca, 1996. Stability of cassava plants at the DNA level after retrieval from 10 years of *in-vitro* storage. Euphytica, 90: 307-313.

Caponetti, J.D., 2000. Nutrition of callus cultures. *In*: Trigiano, R. N. and D. T. Gray. "Plant tissue culture concepts and laboratory Exercises". 2nd Ed. CRC press LLC, USA.

Catapan, E., M.F. Otuki and A.M. Viana, 2000. *In vitro* culture of *Phyllanthus caroliniensis* (Euphorbiaceae). Plant Cell, Tissue and Organ Culture, 62: 195-202.

Che, P., D.J. Gingerich, S. Lall and S.H. Howell, 2002. Global and hormone induced gene expression changes during shoot development in Arabidopsis. Plant Cell, 14: 2771-2785.

CoHort, 2004. CoHort Software Version 6.3. Monterey, CA, 93940, USA
De Souza, C.R.B., L.J.C.B. Carvalho, E.R.P. de Almeida and E.S. Gander, 2002. Towards the identification of cassava storage root protein genes. Plant Foods Hum. Nutr., 57: 353-363.
De Souza, C.R.B., E.R.P. de Almeida, L.J.C.B. Carvalho and E.S. Gander, 2003. Studies toward the identification of transcription factors in cassava storage root. Braz. J. Plant Physiol., 15(3): 167-170.
De-Masi, L., M. Cipolloro, G. Bernardo, di. U. Galderisi, G. Galano, A. Cascino, G. Grassi, E. Pavone and A. Simeone, 2003. Clonal selection and molecular characterization by RAPD analysis of the fig (*Ficus carica* L.). "Dottato and Bianco del cilento" cultivars in Italy. Acta Hort., 605: 65-68.
Dixit, S., B.B. Mandal, S. Ahuja and P.S. Srivastava, 2003. Genetic stability assessment of plants regenerated from cryopreserved embryogenic tissues of *Dioscorea bulbifera* L. using RAPD, biochemical and morphological analysis. Cryo Letters, 24 (2):77-84. (c.f. computer search).
Doyle, J.J. and J.L. Doyle, 1990. Isolation of DNA from fresh tissue. Foucs, 12: 13-15.
El-Kazzaz, A.A. and M.K. El-Bahr, 2003. Some factors affecting *in vitro* development of date palm (Samany) plantlets and molecular characterization. Bull. NRC, 28(1): 101-108.
El-Zeiny, O.A.H., S.A. Mansour, M.H. Edress and A.F. Abo-Haded, 2001. Towards commercial production of cassava *in vitro*. Egypt. J. Horti., 28(3): 387-394.
Fletcher, E.K.A., T.N.E. Amoako and P. Twumasi, 2011. Effect of 2, 4-D, explants type and cultivar on the callogenesis expression of cassava (*Manihot esculenta* Crantz) in Ghana. African Journal of Biotechnology, 10 (46): 9396-9401.
Joseph, T., H.H. Yeoh and C.S. Loh, 2001. Linamarin content and genetic stability of cassava plants derived by somatic embryogenesis. Euphytica, 120: 7-13.
Kondamudi, R., K.S. Murthy and R.T. Pullaiah, 2009. Euphorbiaceae – A critical review on plant tissue culture. Tropical and Subtropical Agroecosystems, 10: 313-335.
Kumar, N. and M.P. Reddy, 2010. Plant regeneration through the direct induction of shoot buds from petiole explants of *Jatropha curcas*: a biofuel plant. Ann. Appl. Biol., 156: 367-375.
Kumar, N., S.D.V.N. Pamidimarri, M. Kaur, G. Boricha and M.P. Reddy, 2008. Effects of NaCl on growth, ion accumulation, protein, proline contents and antioxidant enzymes activity in callus culture of *Jatropha curcas.* Biologia, 63(3): 378-382.
Kumari, K.G., M. Ganesan and N. Jayabalan, 2008. Somatic organogenesis and plant regeneration in *Ricinus communis*. Biologia Plantarum, 52: 17-25.
Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London), 227: 680-685.
Li, Z.G., M. Gong, S.Z. Yang and W.B. Long, 2012. Efficient callus induction and indirect plant regeneration from various tissues of *Jatropha curcas*. African Journal of Biotechnology, 11(31): 7843-7849.
Machakova, I., E. Zazimalova and E.F. George, 2008. Plant Growth Regulators I: Introduction; Auxins, their Analogues and Inhibitors. *In*: Plant Propagation by Tissue Culture - The Background Vol 1. The Background. E. F. George, M. A. Hall, G.-J. De Klerk (Eds.). 3rd Edition, Springer, Dordrecht, The Netherlands.
Martins, M., D. Sarmento, and M. Oliveira, 2004. Genetic stability of micropropagated almond plantlets, as assessed by RAPD and ISSR markers. Plant Cell Reports, 23: 492-496.
Matand, K., G. Acquaah and M. Burns, 2004. A one-step *in vitro* procedure for induction of callus and shoots in cassava (*Manihot esculenta* Crantz). Plant Growth Regulation Society of America (PGRSA) Quarterly, 32: 4, 124.
Mukherjee, P., A. Varshney, T.S. Johnson and T.B. Jha, 2011. *Jatropha curcas*: a review on biotechnological status and challenges. Plant Biotechnol. Rep., 5: 197-215.
Murthy, B.N.S. and P.K. Saxena, 1998. Somatic embryogenesis and plant regeneration of Neem (*Azadirachta indica* A. Juss). Plant Cell Rep., 17: 469-475.
Mussio, I., M.H. Chaput, I. Serraf, G. Ducreux and D. Sihachakr, 1998. Adventitious shoot regeneration from leaf explants of an African clone of cassava (*Manihot esculenta* Crantz) and analysis of the conformity of regenerated plants. Plant Cell, Tissue and Organ Culture, 53: 205-211.
Ogburia, M.N., 2002. An *in-vitro* study of callogenesis in recalcitrant plant organs of cassava (*Manihot esculenta* Crantz). Niger Delta Biologia, 4(1): 1-5.
Pillai, S.V. and S. Sundaresan, 2007. DNA fingerprinting in tapioca (*Manihot esculenta* Crantz.). Recent trends in horticultural biotechnology, Vol. II. ICAR national symposium on biotechnological interventions for improvement of horticultural crops: issues and strategies, Vellanikkara, Kerala, India, 10 – 12 January, 2005: 525-529.
Rady, M.R. and Z.A. Ali, 1999. Comparison of physiology and anatomy of seedlings and regenerates of sugar beet. Biologia Plantarum, 42(1): 39-48.
Rout, G.R., P. Das, S. Goel and S.N. Raina, 1998. Determination of genetic stability of micropropagated plants of ginger using Random Amplified Polymorphic DNA (RAPD) markers. Bot. Bull. Acad. Sin., 39: 23 -27.

Saker, M.M., A.E.M. Reda, M.K. El-Bahr and A.A. El-Kazza, 1999. Induction of embryogenic callus and plant regeneration in faba bean. Egypt. J. Agron., 21: 87-97.

Sambrook, J., E.F. Fritsch and T. Maniatis, 1989. Molecular Cloning: A laboratory Manual. Second edition Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA, pp: 1659.

Sayed, S.S. and A.M.M. Gabr, 2009. *In vitro* culture and RAPD analysis of *Deutzia scabra* Thunb. The 2nd International Conference of Natural Resources in Africa on "Sustainable Development of Natural Resources in the Nile Basin Countries" Cairo, EGYPT.

Snedecor, G.W. and W.G. Cochran, 1982. Statistical Methods. 7th Ed. The Iowa State Univ. Press. Ames. Iowa, USA.

Stewart, C.N. Jr. and V. Cardoza, 2008. Tissue Culture: The Manipulation of Plant Development. *In*: Plant Biotechnology and Genetics: Principles, Techniques, and Applications (Stewart, C. N. Jr. Ed.). John Wiley & Sons, Inc. Hoboken, New Jersey.

Sugiyama, M. and K. Imamura, 2006. Dose-, time-, and tissue-dependent effects of 5-bromo-2_-deoxyuridine on the *in-vitro* organogenesis of *Arabidopsis thaliana*. Plant Cell Tiss. Org. Cult., 87: 17-25.

Sujatha, M. and N. Mukta, 1996. Morphogenesis and plant regeneration from tissue cultures of *Jatropha curcas.* Plant Cell Tissue Organ Culture, 44: 135-141.

Sujatha, M., T.P. Reddy and M.J. Mahasi, 2008. Role of biotechnological interventions in the improvement of castor (*Ricinus communis* L.) and *Jatropha curcas* L. Biotechnology Advances, 26: 424-435.

Tyagi, R.K., A. Agrawal, C. Mahalakshmi, Z. Hussain and H. Tyagi, 2007. Low cost media for *in vitro* conservation of turmeric (*Curcuma longa* L.) and genetic stability assessment using RAPD markers. *In vitro* Cell. Dev. Biol.—Plant, 43: 51-58.

Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey, 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res., 18: 6531-6535.

Wong, H.L., H.H. Yeoh, S.H. Lim and K.C.L. Looi, 1997. Design of primers for RAPD analyses of cassava (*Manihot esculenta*). Phytochemistry, 46 (5): pp X05 Xii).

Yang, Z.-S., G.-D. Chen, Y.X. Li and J. Chen, 2009. Characterization of callus formation in leaf of *Euphorbia helioscopia*. African Journal of Plant Science, 3(6): 122-126.