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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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Full Length Research Paper

Genetic variability in coat protein gene of sugarcane mosaic virus in Pakistan and its relationship to other strains

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Sugarcane mosaic virus (SCMV) is one of the three causative viruses of mosaic in sugarcane, a sugar crop widely grown under tropical and subtropical conditions worldwide. Although molecular characterization of SCMV strains was reported from many countries, strain occurring in Pakistan, a major sugarcane producer has not been reported so far. One hundred and two (102) sugarcane samples from foremost sugarcane growing districts in Pakistan were transcribed by reverse-transcription polymerase chain reaction (RT-PCR) by means of a pair of primers. All of them were found infected with SCMV. The sequences of SCMV coat protein gene amplified in this study varied between 406 and 457 nt. The sequence assessment of 12 Pakistani SCMV isolates revealed extensive range of sequence resemblances, 82-100% nucleotide (nt) and 5.0-95% amino acid (aa) respectively. A deletion of aa residues was observed in the amplified segments of the Pakistani amplicons. 50 SCMV CP sequences (12 from Pakistan and 38 from other sugarcane growing countries) were subjected to phylogenetic and *in-silico* restriction analyses grouped the isolates mostly in order of their geographical origin. The 12 Pakistani SCMV isolates were included in one group. Nearly 97.0% of isolates from Pakistan have no signs for close association with earlier categorized sugarcane mosaic virus strains SCMV-A, SCMV-B, SCMV-D, SCMV-E, and SCMV-SC described from various countries. Our studies discovered that the sugarcane mosaic in Pakistan is instigated by a new group/strain SCMV-PAK. It is the first representation on the diversity and existence of novel SCMV population in Pakistan.

Key words: Sugarcane, sugarcane mosaic virus, geographical, Pakistan, reverse transcriptase polymerase chain reaction (RT-PCR).

INTRODUCTION

Mosaic of sugarcane is a significant disease in sugarcane which is nearly a couple of centuries ancient and disseminated worldwide (Koike and Gillespie, 1989). In Pakistan, the occurrence of mosaic disease in sugarcane

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was validated by Jensen and Hall (1993), and since then it broadly prolonged its acclimatization in all the sugarcane growing areas of Pakistan (Yasmin et al., 2011). Sugarcane mosaic is considered as least problematic in some regions but it affects considerable crop damages in many countries by its extreme epidemics. Epidemics of mosaic of sugarcane is about 100% in the world (Mandahar, 1987) and is a major threat to gigantic area under sugarcane farming, which causes damages up to 46% (Singh, 1971; Mandahar, 1987).

The sugarcane mosaic virus can also infect other economic crops such as corn and sorghum (Teakle et al., 1989). Four distinct potyviruses, sugarcane mosaic virus (SCMV), Johnson grass mosaic virus (JGMV), sorghum mosaic virus (SrMV) and maize dwarf mosaic virus (MDMV) are accepted as SCMV subgroups (Teakle et al., 1989; Shukla et al., 1992). Previously, mosaic in sugarcane was recognized to only a Potyvirus named as sugarcane mosaic virus (SCMV) possessing several strains (Shukla et al., 1989; Koike et al., 1989). Presently, sugarcane mosaic virus subgroup from the genus Potyvirus comprises of seven various species SCMV, MDMV, SrMV, JGMV (Shukla et al., 1989; McKern et al., 1991), Zea mosaic virus (ZeMV) (Seifers et al., 2000), Cocksfoot streak virus (CSV) (Gotz and Maiss, 2002), and Pennisetum mosaic virus (PenMV) (Fan et al., 2003a, b). Amongst these viruses, only SCMV and SrMV are considered to be infectious in sugarcane naturally, hence it is a major causative organism of mosaic in sugarcane (Grisham et al., 2000). Five strains of SCMV, SCMV-A, SCMV-B, SCMV-D, SCMV-E and SCMV-SC, and three strains of SrMV, SrMV-SCH, SrMV-SCI and SrMV-SCM have been categorized on the basis of sequencing of coat protein gene from United States and Australia (Shukla et al., 1992, 1994; Frenkel et al., 1991; Yang and Mirkov, 1997). On the basis of differences in mosaic pattern on sugarcane, 12 SCMV strains: SCMV-A, SCMV-B, SCMV-C, SCMV-D, SCMV-E, SCMV-F, SCMV-H, SCMV-I, SCMV-J, SCMV-K, SCMV-M and SCMV-N were described from India (Farrag and Kandaswamy, 1979; Kondaiah and Nayudu, 1984a, b, 1985; Rishi and Rishi, 1985; Gopal and Reddy, 1988; Gopal et al., 1991). SCMV is transmitted by several aphid species, particularly *Rhopalosiphum maidis*. A very important secondary dissemination method is vegetative propagation of sugarcane through infected setts (Chen and Adams, 2002; Li et al., 2013).

Nucleotide and amino acid sequences of coat protein gene have been used as molecular marker to differentiate eight strains of SCMV but they symbolize only United States of America, Australia and India amongst the countries growing sugarcane. Based on serological and differential host interactions, dissemination of different SCMV strains in Pakistan has been described and minute exertions have been tried up to now to detect the prevailing strains of SCMV in Pakistan using molecular biology techniques. The present study of SCMV was carried out against different sugarcane cultivars grown in Pakistan.

MATERIALS AND METHODS

RNA isolation from infected varieties/cultivars of sugarcane

Isolation of RNA was carried out from 102 samples of the nine different cultivars/varieties of sugarcane (SPF-234, NSG-555, NSG-646, HSF-240, BF-162, HSF-245, HSF-242, SP-98-133 and SPF-213) from 18 districts of Punjab Province, Pakistan. All samples were collected on the basis of symptoms of mosaic disease and were selected based on the following conditions: (i) SCMV dissemination pattern in *Saccharum* spp. (ii) detection of the widespread variants of SCMV strains; and (iii) assessment of degree of discrimination among the variants of the strains and certain isolates prevailing globally.

Primer designing

A pair of primers SCMV-F454 (5'-GAGCAACCAGAGAGGAGTTTG-3') and SCMV-R454 (5'-CCAGACCGAACAATCGTGTG-3') was synthesized for the amplification of ~456 bp of coat protein from core region based on reported sequences of viral strains SCMV-A, SCMV-B, SCMV-D, SCMV-E (Yang and Mirkov, 1997), and SCMV-SC (Frenkel et al., 1991), and isolates conveyed earlier from School of Biological Sciences, University of the Punjab (GenBank Acc. AM040436; DQ648195) (Haider et al., 2011).

RT-PCR

RNAs were subjected to reverse transcription by the enzyme M-*MuLV* H-minus reverse transcriptase, using Oligo(dT) primer. cDNA were subjected to PCR using *Taq* DNA polymerase (1.0 U) (Enzymology Lab. National Centre of Excellence in Molecular Biology, University of the Punjab Lahore, Pakistan), 1.0x reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), primers (10 pmol each), and 0.2 mM of each dNTP in a 20 µl reaction. Polymerase chain reaction was carried out by heating for 4 min at 94°C, following 35 cycles (94°C for 1 min, 55°C for 1 min and 72°C for 1 min), and finally a single cycle of final extension at 72°C for 10 min. DNA fragments were separated on 1.5% agarose gel, stained with 100 ng/ml ethidium bromide and visualized under UV light.

Cloning and sequencing of CP genes

The amplified segments of SCMV CP gene from all positive were eluted from the high melting agarose gel using QIAquick Gel Extraction Kit (QIAGEN) and following manufacturer's instructions. The purified PCR fragments were cloned into the pCR®2.1 vector using the TA Cloning® Kit (invitrogen™) and transformation was carried out into the *Escherichia coli* DH5α competent cells. Using GeneJET Plasmid Miniprep Kit (Ferments, USA) the plasmids having inserts of estimated size were purified. Two cloned inserts, for each virus isolate, were subjected to complete sequencing on both strands by DNA Sequencing Lab. National Centre of Excellence in Molecular Biology, Lahore, Pakistan. The amplicons with 1.0% sequence differences was assumed as distinct isolate.

Phylogenetic analysis and sequence comparison

Sequences of nucleotide and amino acid of the coat protein from amplified region of 50 SCMV isolates, 12 from this study and 38

from worldwide locations including Pakistan (two sequences) were aligned separately using ClustalW (Thompson et al., 1994) and matched themselves. The trimming of 5'-terminal and 3'-terminal regions was carried out in accordance with the isolate SCMV-CEMB-5 evenly by BioEdit version 7.2.0.0 with built in Treeview software (Hall, 1999). Phylogenetic tree was produced and envisaged, and grouping patterns were compared. Similarities were calculated by creating similarity index and converting it into percent similarities using the same alignment (Table 2).

Genetic diversity and *in-silico* restriction analysis

Strain differentiation among potyviruses was reported in sugarcane mosaic virus and sorghum mosaic virus based on restriction profile (Yang and Mirkov, 1997) using a group of restriction enzymes. Using the enzymes *Alu*I, *Bst*MWI, *Cvi*JI, *Eco*RI, *Hinf*I, *Hpy*CH4V and *Taq*I (Table 3), the restriction analysis of nucleotide sequences were executed.

RESULTS AND DISCUSSION

All the 102 samples of the nine different cultivars/varieties of sugarcane were observed with different intensity of mosaic symptoms. All the samples under investigation were found infected with SCMV using RT-PCR (Singh et al., 2009). On the other hand, Hema et al. (1999) stated that SCMV subgroup viruses are not the cause of mosaic in sugarcane, it is due to SCSMV.

In the same way, in many Asian countries which grow sugarcane, at least two strains of SCSMV are the major cause of mosaic in sugarcane (Chatenet et al., 2005). Conversely, Xu et al. (2008) collected 164 field samples from Guangdong, Guangxi, Hainan and Yunnan provinces of China and reported that all of them were infected either with sugarcane mosaic virus or sorghum mosaic virus alone or with both viruses and SCSMV was not the cause of sugarcane mosaic except a few germplasm from India.

In Louisiana, Grisham and Pan (2007) failed to categorize SrMV or sugarcane mosaic virus in sugarcane presenting symptoms of mosaic. In the same way, the primers used to detect SrMV and SCMV could not amplify the virus genes in the mosaic diseased sugarcane in Argentina (Perara et al., 2007). Hence, it can be concluded that sugarcane mosaic virus is the main cause of mosaic in sugarcane worldwide. Either sugarcane mosaic virus or sugarcane streak mosaic virus alone or their combinations are the cause of sugarcane mosaic in Pakistan (Yasmin et al., 2011; Li et al., 2013).

Using a pair of primers (SCMV-F and SCMV-R), we observed a noteworthy difference among the lengths of the amplified sequences (406-457 nt) used in this study. The amplified sequences varied from 406 (SCMV-CEMB-2) to 457 (SCMV-CEMB-5) nt for different isolates of SCMV (Table 1). The variation in sequence segments of SCMV amplified from sugarcane varieties from Pakistan predicted the genetic variation among different SCMV strains going through different levels of mutations. The diversity in the CP sequence is inevitable in the hyper variable region (Oertel et al., 1997) in order to meet the selection pressure, possibly when it is exposed to the insects and the environment.

The overall nt similarity in the core region (CR) of the CP gene of sugarcane mosaic virus isolates occurring worldwide varied between 26 and 100% (mean 84.6%) while aa identity ranged between 1.0 and 100% (average 53.5%) (Table 2). The lowest range of resemblances was observed with SCMV-VN/SC1 and SCMV-VN/SC2; isolates from Veitnam (Ha et al., 2008) present the greater inconsistency.

Though, the isolate SCMV-CEMB-9 (GenBank Acc. KC249914) of this study expressed the lowest nt (59-99%) and aa (4.0-100%), sequence identities in the CR of the CP were compared with the strains from all over the world, and it exhibited extreme nucleotide identity of 100% with Indian isolate SCMV-CB89003-4 (GenBank Acc. EF 655890) and amino acid similarity of 92% with SCMV-CEMB-6 (GenBank Acc. KC249911).

The overall genetic similarities among our 12 SCMV isolates, and the isolate cssg-668 (GenBank Acc. DQ648195) reported from Pakistan (Haider et al., 2011), ranged between 82 and 100% at nucleotide and between 6.0 and 90% at amino acid levels respectively in the sequenced region (Table 2). The corresponding resemblances among the isolates prevailing worldwide (except Pakistani isolates) fluctuated between 26 to 100% and from 1.0 to 100%. The mean of the sequence identity level of isolates from all over the world was 84.6 and 53.5% at nt and aa. The isolates SCMV-CEMB-4 (GenBank Acc. KC249909), SCMV-CEMB-7 (GenBank Acc. KC249912) and SCMV-CEMB-8 (GenBank Acc. KC249913) of this study presented the least nt (27%) and aa (4.0%) identities with the strains from USA and Veitnam.

Recurrence of short peptide motifs was observed in the isolates from Pakistan. They are recurring in a similar fashion as perceived by Frenkel et al. (1991) and Xiao et al. (1993) in SCMV-SC and USA strains (Yang and Mirkov, 1997). The sequence form ‘‘EQPERSLIGGMKP’’ (Figure 1) has accurately recurred in 4 of 12 (33.3%) Pakistani isolates. Similarly, the sequence patterns “MDGDEQRVF-PLKPVI” and “ENASPTFRQIMHHFSDAAEAYIEYRNSTERYMPRYGL QR” have exactly been repeated in 6 of 12 (50%). Among the isolates/type strains from worldwide included in this study, the conserved motif (EQPERSLIGGMKP) is repeated exactly in 18 of 38 (47%) and the motif “HDMDFSEISPTIA” has been repeated in 21 of 38 (55.25%) isolates. Several other amino acid sequence motives have repeats with residual variation of 1 or 2 aa, which range between 40 to 60%.

Phylogenetic tree was built from the nucleotide sequences of our 12 and 38 selected isolates from all over the world (Figure 2). In the phylogenetic tree, most of the virus isolates were grouped with respect to their

Table 1. Details of SCMV-CEMB isolates and other SCMV types strains/isolates used in the comparison study.

SCMV isolates/strains	Size (bp)	Source/variety	State/Country	GenBank Acc. No.	Reference
SCMV-CEMB-1	455	Saccharum hybrid cultivar	Punjab, Pakistan	KC200152	This study
SCMV-CEMB-2	406	Saccharum hybrid cultivar	Punjab, Pakistan	KC249907	This study
SCMV-CEMB-3	421	Saccharum hybrid cultivar	Punjab, Pakistan	KC249908	This study
SCMV-CEMB-4	455	Saccharum hybrid cultivar	Punjab, Pakistan	KC249909	This study
SCMV-CEMB-5	457	Saccharum hybrid cultivar	Punjab, Pakistan	KC249910	This study
SCMV-CEMB-6	456	Saccharum hybrid cultivar	Punjab, Pakistan	KC249911	This study
SCMV-CEMB-7	456	Saccharum hybrid cultivar	Punjab, Pakistan	KC249912	This study
SCMV-CEMB-8	455	Saccharum hybrid cultivar	Punjab, Pakistan	KC249913	This study
SCMV-CEMB-9	432	Saccharum hybrid cultivar	Punjab, Pakistan	KC249914	This study
SCMV-CEMB-10	431	Saccharum hybrid cultivar	Punjab, Pakistan	KC249915	This study
SCMV-CEMB-11	422	Saccharum hybrid cultivar	Punjab, Pakistan	KC249916	This study
SCMV-CEMB-12	423	Saccharum hybrid cultivar	Punjab, Pakistan	KC249917	This study
mosIAC	360	Sugarcane	Brazil	JF699509	Sawazaki et al., 2013
AP1	852	Sugarcane	India	GQ386846	Reddy et al., 2011
SCMV-Mfc	286	Maize	France	HM014060	Marie-Jeanne et al., 2011
Q86	700	NA	Iran	AY648298	Ghasemi et al., 2005
SCMV-VN/SC1	939	Sugarcane(*Saccharum officinarum*)	Yenbai, Vietnam	DQ925431	Ha et al., 2008
SCMV-VN/SC2	939	Sugarcane (*Saccharum officinarum*)	Hoabinh, Vietnam	DQ925427	Ha et al., 2008
CB44-101	866	*Saccharum officinarum* cv. CP-44-101	India	EF655894	Viswanathan et al., 2008
KhzL66	939	Sugarcane (*Saccharum officinarum*)	Iran	DQ369960	Ghasemi et al., 2005.
GX-1	888	Saccharum sp.	China	DQ227694	Zhou et al., 2007
KhzQ86	939	Sugarcane	Iran	DQ438949	Masumi et al., 2006
PIR-2	886	NA	Brazil	AY819718	Goncalves et al., 2012
CSSG 676	888	Sugarcane	Punjab, Pakistan	AM040436	Haider et al., 2011
cssg-668	888	Sugarcane	Pakistan	DQ648195	Haider et al., 2011
SC	889	NA	Sichun, China	AJ421468	Jiang and Zhou 2002
ZAF 53-1	852	Saccharum hybrid cultivar	South Africa	AJ491973	Alegria et al., 2003
USA Flo 36-1	852	Saccharum hybrid cultivar	Florida, USA	AJ491971	Alegria et al., 2003
USA Lou 40-1	828	Saccharum hybrid cultivar	Louisiana, USA	AJ491965	Alegria et al., 2003
EGY7-1	852	Saccharum hybrid cultivar	Egypt	AJ491963	Alegria et al., 2003
CON98-1	852	Saccharum hybrid cultivar	Republic of Congo	AJ491961	Alegria et al., 2003
CAM94-1	852	Saccharum hybrid cultivar	Cameroon	AJ491939	Alegria et al., 2003
ZJ1	802	NA	Zhejiang, China	AJ421465	Jiang and Zhou 2002
NJ1	802	NA	Jiangsu, China	AJ421463	Jiang and Zhou 2002
SCMV-Seehausen/S26	942	NA	Germany	X98165	Oertel, et al., 1997
SCMV-CB72-1	898	Saccharum hybrid cultivar BO 72	Bihar, India	DQ842502	Viswanathan et al., 2008

Table 1. Contd.

SCMV-CB617	818	Saccharum hybrid Co 617	Tamil Nadu, India	EU089686	Viswanathan et al., 2008.
SCMV-CB89003-4	898	*Saccharum officinarum* cv. Co 89003	Haryana, India	EF655890	Viswanathan et al., 2008
SCMV-CBA7701	871	*Saccharum officinarum* cv. CoA7701	Andhra Pradesh, India	EF655899	Viswanathan et al., 2008
SCMV-CB84213-3	898	*Saccharum officinarum* cv. Co Pant 84213	Uttaranchal, India	EF655889	Viswanathan et al., 2008
PB-CoJ85	899	NA	Punjab, India	DQ866746	Singh et al., 2009
KL-Co86032	899	NA	Kerala, India	DQ866744	Singh et al., 2009
IND	898	NA	India	AY241923	Gaur et al., 2003
TUC-1C	900	Sugarcane	Argentina	EU196423	Perera et al., 2007
E	939	NA	USA	U57357	Yang and Mirkov 1997
D	927	NA	USA	U57356	Yang and Mirkov 1997
A	915	NA	USA	U57354	Yang and Mirkov 1997
B	927	NA	USA	U57355	Yang and Mirkov 1997
SC	939	NA	Australia	D00948	Frenkel et al., 1991
BRIS	942	NA	Australia	AF006734	Handley et al., 1998

Table 2. Percent nucleotide (above diagonal), amino acid (below diagonal) sequence similarity of Pakistani SCMV isolates, strains, and other isolates from worldwide at core region of the coat protein spanning 457 nt, corresponding to SCMV-CEMB-5 (GenBank Acc. KC249910).

Acc. No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
AJ421465	100	100	94	59	92	89	89	89	69	89	89	89	70	91	91	91	89	89	90	89	90	89	88	83	88
AJ421463	99	100	93	59	92	89	89	89	69	89	89	89	71	91	91	91	89	89	90	89	90	89	88	83	88
X98165	82	81	100	58	91	88	89	88	69	89	89	89	69	90	89	89	89	89	90	90	89	88	88	83	88
HM014060	5	5	5	100	6	57	57	57	53	56	56	57	70	56	56	57	56	56	57	57	57	57	56	59	56
AJ421468	76	76	73	6	100	89	90	89	69	88	89	89	70	89	89	89	88	88	89	89	89	89	88	83	88
DQ866746	69	69	67	5	69	100	100	98	75	95	95	95	74	95	95	96	95	95	96	96	95	95	95	90	95
DQ866744	68	69	67	5	70	99	100	98	75	95	96	95	74	95	95	96	95	95	96	96	95	95	95	90	95
GQ386846	68	69	67	4	69	94	94	100	75	95	95	96	74	94	95	96	94	94	95	95	95	95	95	90	95
JF699509	51	52	52	4	52	68	69	68	100	75	75	75	53	75	75	75	74	74	75	76	76	75	75	79	75
AJ491971	68	69	69	6	68	87	87	86	71	100	99	96	74	95	95	96	95	95	96	97	96	96	95	91	95
U57357	69	70	69	6	69	87	88	87	71	99	100	97	74	96	96	97	96	96	97	97	96	97	96	91	96
AJ491973	66	66	67	6	67	85	85	86	70	91	91	100	75	87	96	97	96	96	97	97	97	96	97	91	97
AY648298	6	6	6	4	7	6	7	7	5	7	7	7	100	77	76	76	74	74	75	76	75	75	75	70	75
DQ369960	73	73	71	5	69	83	84	81	69	89	90	87	6	100	97	97	96	96	97	97	96	96	96	91	96
DQ438949	74	75	69	6	69	84	85	85	69	89	90	87	6	90	100	99	95	95	96	96	96	96	96	91	96
AJ491963	73	73	69	6	69	87	88	87	70	92	93	90	7	90	96	100	96	96	97	98	97	97	97	92	97
EF655890	69	70	69	6	64	84	85	82	67	89	90	87	7	89	87	89	100	100	98	98	97	96	96	91	96

Table 2. Contd.

EF655889	67	68	69	6	66	84	85	84	69	91	91	89	9	90	87	90	93	100	98	98	97	96	96	91	96
DQ842502	70	70	71	6	67	87	88	85	70	92	93	90	7	91	89	91	96	96	100	99	98	97	97	92	97
EU089686	69	70	70	6	67	87	88	87	71	94	94	91	7	92	90	93	94	96	97	100	99	98	97	92	97
EF655899	70	71	70	5	69	87	88	87	71	93	94	91	7	91	91	94	94	95	96	98	100	97	96	91	96
EU196423	68	68	67	6	69	85	86	85	70	93	94	89	7	89	87	90	90	91	93	94	64	100	96	91	96
KC249911	65	66	66	6	66	85	86	85	69	91	91	90	7	88	89	91	89	91	91	92	91	89	100	94	100
KC249914	59	60	59	6	60	79	80	79	73	86	86	84	7	82	81	84	82	86	85	86	86	84	92	100	94
KC200152	12	12	12	44	13	15	15	15	14	16	16	14	5	14	16	16	16	16	17	16	16	16	16	16	100
KC249910	14	14	16	3	16	18	19	18	18	20	20	20	79	18	18	20	19	22	21	20	20	20	21	20	17
KC249907	8	8	8	4	8	8	8	8	7	9	9	9	87	8	8	8	8	10	9	9	9	9	9	8	6
KC249908	7	7	7	5	8	7	7	7	6	8	8	7	77	7	7	7	7	9	8	8	8	8	8	7	19
KC249916	6	6	5	48	7	5	5	5	4	7	7	5	6	5	6	6	6	6	7	6	6	6	5	5	88
KC249917	8	8	6	47	8	6	6	6	4	7	8	5	5	6	8	8	8	8	8	8	8	8	7	5	86
KC249912	8	8	9	3	9	9	10	10	9	11	10	11	71	9	9	10	9	11	10	10	10	10	10	11	8
KC249915	7	7	7	4	9	7	8	8	7	9	9	9	80	7	7	8	8	9	9	9	9	9	9	8	7
KC249913	58	59	56	6	59	77	78	77	69	83	83	81	7	79	79	82	79	83	82	84	83	82	89	90	16
KC249909	11	11	12	3	12	13	14	14	13	15	15	16	71	13	13	14	14	16	15	15	15	15	15	14	12
AM040436	67	68	67	6	67	87	88	87	71	92	93	91	7	90	90	93	90	93	93	94	94	91	98	91	16
DQ648195	68	68	66	6	68	85	86	86	69	90	91	91	7	88	88	91	88	91	91	92	93	89	96	89	15
AY819718	68	67	67	5	66	85	86	86	71	91	92	90	8	88	89	91	89	91	92	94	94	90	90	84	17
AF006734	67	68	66	6	66	87	88	87	71	94	94	91	7	91	90	93	91	94	94	96	95	90	94	88	16
AJ491965	68	69	70	6	66	85	86	85	70	93	94	89	7	89	88	91	91	94	94	94	93	92	91	86	16
U57354	69	70	71	6	66	84	85	84	69	92	93	89	7	89	87	90	90	93	93	93	92	91	91	85	16
AY241923	67	68	65	6	64	87	86	82	67	89	90	87	7	89	86	89	89	90	91	91	91	70	89	84	16
D00948	68	69	66	6	65	88	87	84	69	91	91	89	7	90	87	90	90	91	93	93	92	91	91	85	16
U57356	68	68	68	6	67	89	89	87	71	95	96	91	7	91	90	93	91	94	94	96	95	94	94	88	16
U57355	68	68	68	6	63	85	86	83	70	91	92	88	7	91	88	91	89	91	92	92	91	91	91	86	16
AJ491961	67	68	68	6	66	87	88	87	70	93	94	89	8	87	89	91	89	90	92	93	92	89	89	84	16
AJ491939	69	69	69	5	65	85	86	85	69	91	91	87	8	87	85	88	87	89	89	91	90	91	90	84	16
DQ227694	67	68	65	5	65	84	84	84	67	87	88	85	9	84	84	87	84	87	87	88	89	87	87	82	16
EF655894	69	70	69	6	64	84	85	82	67	89	90	87	7	89	87	89	100	93	96	94	94	91	89	82	16
DQ925431	4	4	5	5	1	4	4	4	5	3	3	4	2	3	3	3	4	4	4	4	4	4	4	4	3
DQ925427	1	1	3	5	1	4	4	4	5	3	3	4	5	1	2	3	4	4	4	4	4	4	4	4	4

place of isolation, except the isolates IND (GenBank Acc. AY241923) and AP1 (GenBank Acc. GQ386846), isolated from India and the strain type GX-1 (GenBank Acc. DQ227694) from China. The hyper variable region (HVR) of the CP gene undergoes much variations due to

Table 2. Contd.

Acc. No.	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
AJ421465	87	78	80	81	81	87	83	87	86	89	89	89	89	89	89	88	89	89	89	89	89	88	89	29	28
AJ421463	87	79	80	81	81	87	83	88	86	89	89	88	89	89	89	88	89	89	89	89	89	88	89	29	28
X98165	87	78	80	81	80	87	82	87	86	89	88	89	89	90	90	88	88	89	89	89	89	87	89	29	27
HM014060	56	62	60	61	60	56	59	56	56	56	56	57	57	57	57	55	56	57	56	56	56	56	56	34	32
AJ421468	87	78	80	81	80	87	82	87	86	89	89	88	88	89	88	87	88	89	88	88	88	87	88	28	27
DQ866746	94	84	86	87	86	94	89	94	93	96	95	95	96	95	95	95	96	96	95	95	95	94	95	28	26
DQ866744	95	84	86	87	87	94	89	95	93	96	95	95	96	95	95	95	96	96	95	96	95	94	95	29	27
GQ386846	94	84	86	87	86	93	88	94	93	95	95	95	96	95	95	94	94	95	94	95	95	94	95	28	26
JF699509	74	64	66	67	67	75	70	75	75	75	75	76	76	75	75	74	75	76	75	75	75	74	75	23	22
AJ491971	95	84	86	87	86	94	89	94	93	96	95	96	96	96	96	95	95	97	96	97	97	95	96	28	27
U57357	95	85	87	88	87	95	89	95	94	97	96	97	97	97	97	96	96	98	96	97	96	95	96	29	27
AJ491973	96	86	88	89	88	95	90	96	95	97	97	97	98	97	97	96	96	97	96	96	96	95	96	29	27
AY648298	75	84	81	81	81	74	78	74	73	75	74	75	76	75	75	74	75	75	75	74	74	73	75	37	35
DQ369960	95	85	88	88	88	95	90	95	94	97	96	96	97	97	96	96	96	97	97	96	95	94	96	30	28
DQ438949	95	85	87	88	87	95	89	95	94	96	96	96	97	96	96	95	95	96	96	96	95	94	95	29	28
AJ491963	96	86	88	89	88	96	90	96	95	98	97	97	98	97	97	96	96	98	97	97	96	95	96	29	28
EF655890	95	84	87	88	87	94	89	95	94	96	95	96	97	97	96	96	96	97	96	96	95	94	97	29	27
EF655889	95	84	87	88	87	94	89	95	94	96	95	96	97	97	96	96	96	97	96	96	95	94	97	29	27
DQ842502	96	85	88	89	88	95	90	96	95	97	96	97	98	98	98	97	97	98	97	97	96	95	98	30	28
EU089686	96	86	88	89	88	96	91	96	95	98	97	98	98	98	98	97	97	98	97	98	97	95	98	30	28
EF655899	96	85	88	88	88	95	90	96	95	97	97	97	98	97	97	96	96	98	96	97	96	96	98	30	28
EU196423	96	85	87	88	88	95	90	95	94	97	96	97	98	97	97	96	96	98	97	97	96	95	97	30	27
KC249911	99	88	90	91	91	98	93	99	97	99	98	97	98	97	97	96	96	98	97	96	96	95	96	30	28
KC249914	93	82	85	86	86	93	89	93	93	94	93	91	93	92	92	91	91	92	92	91	91	90	92	30	28
KC200152	99	88	90	91	91	98	93	99	97	99	98	97	98	97	97	96	96	98	97	96	96	95	96	30	28
KC249910	100	87	90	90	90	97	92	98	97	99	98	96	97	96	96	95	96	97	96	95	96	95	96	30	28
KC249907	84	100	95	95	94	87	91	87	86	88	87	85	86	86	86	85	85	86	86	85	85	84	85	33	31
KC249908	75	79	100	97	96	89	92	90	88	90	89	88	89	88	88	87	88	89	88	87	87	86	88	32	30
KC249916	7	7	21	100	98	90	93	91	89	91	90	89	90	89	89	88	88	89	89	88	88	87	88	32	30
KC249917	6	6	20	95	100	90	94	90	89	91	90	88	89	88	88	87	88	89	88	88	88	86	88	32	30
KC249912	80	80	67	6	6	100	92	98	97	98	97	95	96	96	96	95	95	96	96	95	95	94	95	29	27
KC249915	81	89	76	8	8	82	100	93	91	93	92	90	91	91	91	90	90	91	90	90	90	89	90	31	29
KC249913	20	9	8	6	6	11	9	100	97	98	98	96	97	96	96	95	96	97	96	95	95	94	96	29	27
KC249909	84	79	66	6	6	88	82	14	100	97	96	95	96	95	95	94	95	96	95	94	94	93	95	29	27
AM040436	20	9	8	5	7	10	9	89	15	100	99	97	98	98	98	97	97	98	97	97	96	95	97	30	28
DQ648195	20	9	8	5	7	10	9	87	15	98	100	96	98	97	97	96	96	97	96	96	96	95	96	29	27

Table 2. Contd.

AY819718	20	9	8	7	8	10	8	82	14	92	90	100	98	97	97	96	97	98	97	97	96	95	97	29	27
AF006734	20	8	8	6	8	10	8	85	14	96	94	95	100	98	98	97	98	99	98	97	97	96	98	29	27
AJ491965	20	8	7	6	8	10	8	83	15	94	91	91	95	100	100	97	97	99	98	97	97	96	98	29	27
U57354	19	8	7	6	8	10	8	82	14	93	91	91	94	99	100	96	97	98	97	97	97	96	97	29	27
AY241923	19	8	7	6	8	10	8	81	14	91	89	89	93	91	90	100	100	98	96	96	95	94	96	29	27
D00948	19	8	7	6	8	10	8	82	14	93	91	91	94	92	91	99	100	98	97	96	96	95	96	29	27
U57356	20	9	8	6	8	10	9	85	15	96	94	94	97	96	96	94	95	100	98	98	97	96	98	29	27
U57355	20	8	7	6	8	10	8	84	14	92	90	90	94	93	92	91	92	95	100	96	97	96	96	29	27
AJ491961	20	10	8	6	7	11	9	81	16	92	89	92	93	92	92	89	90	94	91	100	98	96	96	29	27
AJ491939	21	10	8	6	7	11	9	82	16	91	89	90	92	91	91	89	90	94	91	94	100	96	96	29	27
DQ227694	22	10	9	6	7	12	10	79	16	88	88	87	89	89	88	86	87	91	89	88	91	100	95	29	27
EF655894	19	8	7	6	8	9	8	79	14	90	88	89	91	91	90	89	90	91	89	89	87	84	100	29	27
DQ925431	3	4	3	3	3	3	3	4	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	100	81
DQ925427	5	5	3	4	5	5	5	4	5	4	4	4	4	4	4	4	4	4	4	4	4	4	4	51	100

Table 3. *In-silico* restriction mapping and discrimination of SCMV strains/variants (12 CEMB isolates and 38 from all over world).

Isolates/	Restriction sites						
Acc. No.	AluI	BstMWI	CviJI	EcoRI	HinfI	HpyCH4V	TaqI
KC200152	248, 380, 386	226, 251, 289, 341, 392	36, 248, 380, 386, 395, 413	364	171	229, 245, 288, 403	261
KC249907	199, 331, 337	177, 202, 240, 292, 343	199, 331, 337, 346, 364	315	122	180, 196, 354	212
KC249908	214, 346, 352	192, 217, 255, 307, 358	5, 214, 346, 352, 361, 379	330	137	195, 211, 369	227
KC249909	250, 382, 388	228, 253, 291, 343, 394	37, 250, 382, 388, 397, 415	366	173	231, 247, 405	263
KC249910	250, 382, 388	228, 253, 291, 343, 394	36, 250, 382, 388, 397, 415	366	173	231, 247, 405	263
KC249911	249, 381, 387	227, 252, 290, 342, 393	36, 249, 381, 387, 396, 414	365	172	230, 246, 289, 404	262
KC249912	250, 382, 388	228, 253, 291, 343, 394	37, 250, 382, 388, 397, 415	366	173	231, 247, 405	263
KC249913	249, 381, 387	227, 252, 290, 342, 393	36, 249, 381, 387, 396, 414	365	172	230, 246, 289, 404	262
KC249914	249, 381, 387	227, 252, 290, 342, 393	36, 249, 381, 387, 396, 414	365	172	230, 246, 289, 404	262
KC249915	226, 358, 364	204, 229, 267, 319, 370	16, 226, 358, 364, 373, 391	342	149	207, 223, 381	239
KC249916	215, 347, 353	193, 218, 256, 308, 359	5, 215, 347, 353, 362, 380	331	138	196, 212, 370	228
KC249917	215, 347, 353	193, 218, 256, 308, 359	5, 215, 347, 353, 362, 380	331	138	196, 212, 370	228
JF699509	249, 331	227, 252, 290, 342	36, 249, 331			230, 246	262
GQ386846	249, 331, 381, 387	252, 290, 342, 393	36, 249, 331, 381, 387, 396, 414, 451			230, 246, 404, 417	196, 262
HM014060	131, 213, 263, 269	134, 172, 224, 275	131, 213, 263, 269, 278			86, 112, 128	

Table 3. Contd.

AY648298	154, 236, 286, 292	132, 157, 195, 247, 298	154, 236, 286, 292, 301, 319	270	77	11, 135, 151, 309	14, 167, 254
DQ925431		209	61, 171, 198		217	19, 117, 124, 186	167
DQ925427		206	60, 168, 195		214	49, 114	164
EF655894	249, 331, 381, 387	227, 252, 290, 342, 393	36, 249, 331, 381, 387, 396, 414	365	172	230, 246, 404	262
DQ369960	249, 331, 381, 387	227, 252, 290, 342, 393	36, 249, 331, 381, 387, 396, 414	365	172	230, 246, 404	109, 262, 349
DQ227694	249, 331, 381, 387	227, 252, 290, 342, 393	36, 249, 331, 381, 387, 396, 414	365	172	230, 246, 404	262
DQ438949	249, 331, 381, 387	252, 290, 342, 393	36, 188, 249, 331, 381, 387, 396, 414	365	172, 314	106, 230, 246, 404	262, 349
AY819718	249, 331, 381, 387	227, 252, 290, 342, 393	36, 91, 249, 331, 381, 387, 396, 414		172	230, 246, 365, 404	262
AM040436	249, 381, 387	227, 252, 290, 342, 393	36, 249, 381, 387, 396, 414	365	172	230, 246, 404	262
DQ648195	249, 381, 387	227, 252, 290, 342, 393	36, 249, 381, 387, 396, 414	365	172	230, 246, 404	262
AJ421468	249, 331, 381, 387	252, 290, 342, 393	249, 331, 381, 387, 396, 414			106, 204, 230, 246, 404	109, 196
AJ491973	249, 381, 387	227, 252, 290, 342, 393	36, 249, 381, 387, 396, 414	365	172	230, 246, 404	262
AJ491971	174, 249, 331, 381, 387	227, 252, 290, 342, 393	36, 174, 249, 331, 381, 387, 396, 414			230, 246, 345, 404	262
AJ491965	249, 331, 381, 387	227, 252, 290, 342, 393	36, 249, 331, 381, 387, 396, 414	365	172	230, 246, 345, 404	262
AJ491963	249, 331, 381, 387	252, 290, 342, 393	36, 188, 249, 331, 381, 387, 396, 414	365	172	230, 246, 404	262, 349
AJ491961	249, 331, 381, 387	227, 252, 290, 342, 393	36, 249, 331, 381, 387, 396, 414	365	314	33, 230, 246, 345, 404	262, 349
AJ491939	249, 331, 381, 387	227, 252, 290, 342, 393	36, 249, 331, 381, 387, 396, 414	365		230, 246, 345, 404	262
AJ421465	249, 331, 381, 387	252, 290, 342, 393	188, 249, 331, 381, 387, 396, 414			106, 230, 246, 404	109, 349
AJ421463	249, 331, 381, 387	252, 290, 342, 393	188, 249, 331, 381, 387, 396, 414			106, 230, 246, 404	109, 349
X98165	249, 331, 387	252, 290, 342, 393	249, 331, 387, 396, 414			106, 230, 246, 345, 404	109
DQ842502	249, 331, 381, 387	227, 252, 290, 342, 393	36, 249, 331, 381, 387, 396, 414	365	172	230, 246, 404	262
EU089686	249, 331, 381, 387	227, 252, 290, 342, 393	36, 249, 331, 381, 387, 396, 414	365	172	230, 246, 404	262

Table 3. Contd.

EF655890	249, 331, 381, 387	227, 252, 290, 342, 393	36, 249, 331, 381, 387, 396, 414	365	172	230, 246, 404	262
EF655899	249, 331, 381, 387	227, 252, 290, 342, 393	36, 249, 331, 381, 387, 396, 414	365	172	230, 246, 404	262
EF655889	249, 331, 381, 387	227, 252, 290, 342, 393	36, 249, 331, 381, 387, 396, 414	365	172	230, 246, 404	262
DQ866746	249, 331, 381, 387	252, 290, 342, 393	36, 249, 331, 381, 387, 396, 414, 451			230, 246, 404, 417	196, 262
DQ866744	249, 331, 381, 387	252, 290, 342, 393	36, 249, 331, 381, 387, 396, 414, 451			230, 246, 404, 417	196, 262
AY241923	249, 331, 381, 387	227, 252, 290, 342, 393	36, 249, 331, 381, 387, 396, 414	365		230, 246, 404	262, 383
EU196423	249, 331, 381, 387	227, 252, 290, 342, 393	36, 249, 331, 381, 387, 396, 414	365		230, 246, 345, 404	262
U57357	174, 249, 331, 381, 387	227, 252, 290, 342, 393	36, 174, 249, 331, 381, 387, 396, 414			230, 246, 345, 404	262
U57356	249, 331, 381, 387	227, 252, 290, 342, 393	36, 249, 331, 381, 387, 396, 414	365	172	230, 246, 345, 404	262
U57354	249, 331, 381, 387	227, 252, 290, 342, 393	36, 249, 331, 381, 387, 396, 414	365	172	230, 246, 345, 404	262
U57355	249, 331, 381, 387	227, 252, 290, 342, 393	36, 249, 331, 381, 387, 396, 414	365	172	230, 246, 345, 404	262, 349
D00948	249, 331, 381, 388	227, 252, 290, 342, 394	36, 249, 331, 381, 387, 396, 415	366		230, 246, 404	262, 383
AF006734	249, 331, 381, 387	227, 252, 290, 342, 393	36, 91, 249, 331, 381, 387, 396, 414	365	172	230, 246, 404	262

addition or deletion mutations, and evolutionary process frequently as it has surface exposure to the environment. Hence, phylogenetic analysis, performed with conserved region (CR) is consistent. Shukla et al. (1994) recommended that conserved region CR is the most reliable marker to discriminate the viral strains in the genus potyvirus. Thus, only the conserved region of coat protein gene of SCMV isolates was amplified and sequenced for phylogenetic analysis. The phylogenetic and restriction analyses resembled in grouping the isolates.

Considering the restriction profile of all 12 isolates in this study, they are identical for *Alu*I, *Bst*MWI, *Eco*RI, *Hin*fI and *Taq*I, but differed for *Cvi*JI and *Hpy*CH4I. Though the sequences from the amplified region resembled highly (nt 82-100% and aa 92%), the level of similarity was not enough to classify them as separate variants, but the phylogenetic analyses and CP sequence assessments did not resemble those of the type strains SCMV-A, SCMV-B, SCMV-D, SCMV-E and most of the isolates reported from all over the world; possibly it characterizes a novel strain SCMV-PAK. Previously, isolates from India, China and Congo were described as cause of mosaic in the sugarcane under Pakistani environments, based on serology and differential host interactions (Haider et al., 2011). Moreover, the cutting pattern by restriction enzymes varied for other isolates from the world, however they match sequence identities in the reported region of CP gene at high level, perhaps representing a new

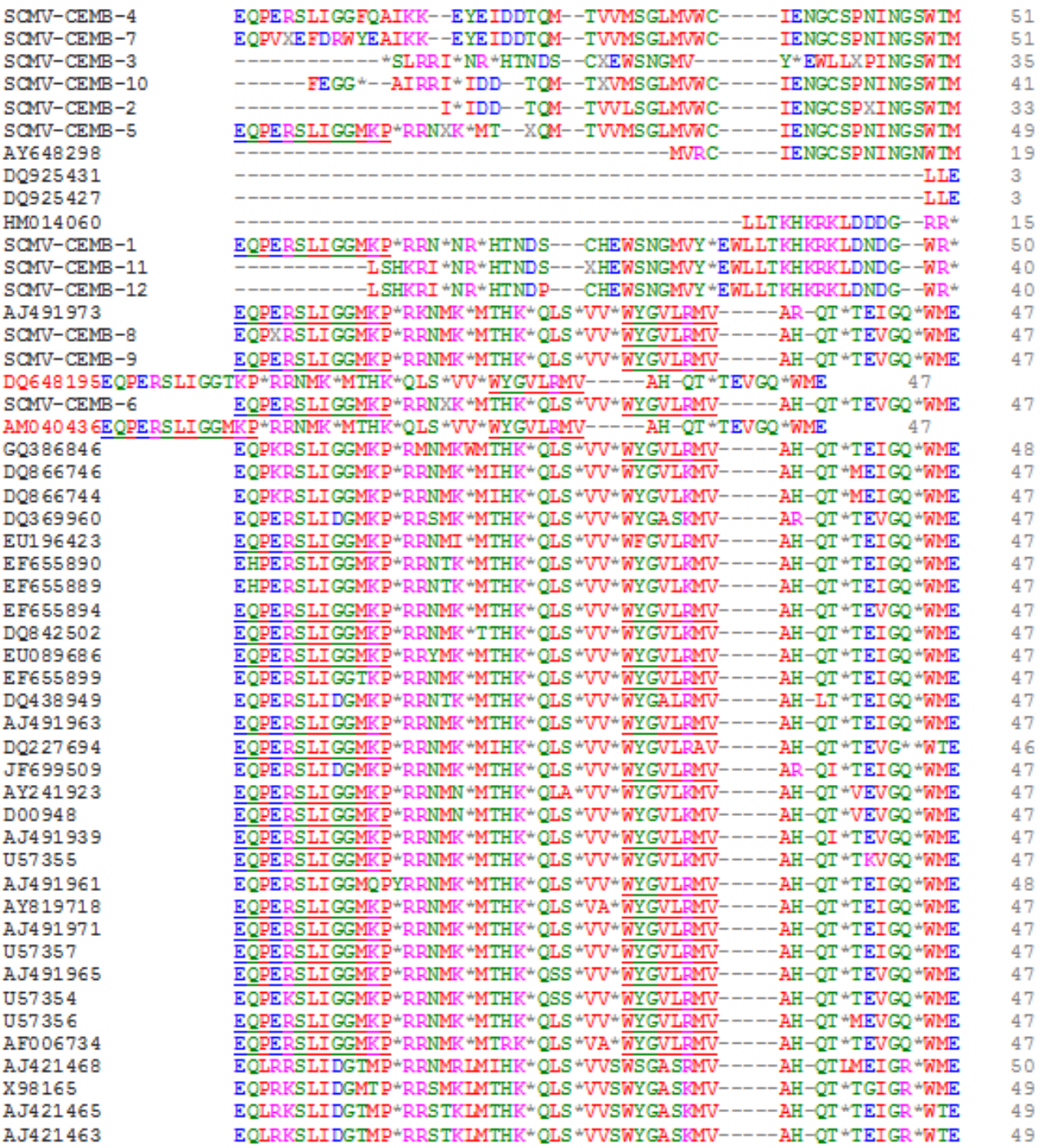

Figure 1. Comparison of the aa sequences of the CPs of different SCMV type strains/strains/isolates in the N-terminal region, with reference to the coordinates 1-151 aa of SCMV-CEMB-5 (GenBank Acc. No. KC249910). Sequences were aligned using CLUSTAL W program (Thompson et al., 1994). '-' in the alignment indicates gap introduced for better alignment/deletion mutation undergone by corresponding sequences; C, conserved aa residues; the underlined aa residues are conserved and are repeated twice in 40-60% of the isolates/strains; for accession numbers (see Table 1).

variant, SCMV-PAK.

The cutting pattern of the SCMV-VN/SC1 and SCMV-VN/SC2, isolates from Veitnam (Ha et al., 2008) was entirely different for all the enzymes. They have no restriction sites for *Alu*I and *Eco*RI. The restriction patterns of *Alu*I, *Bst*MWI, *Cvi*JI, *Hpy*CH4I and *Taq*I, were in a similar fashion as the isolates from Argentina, USA, Cameroon, Republic of Congo and Australia but *Hinf*I and

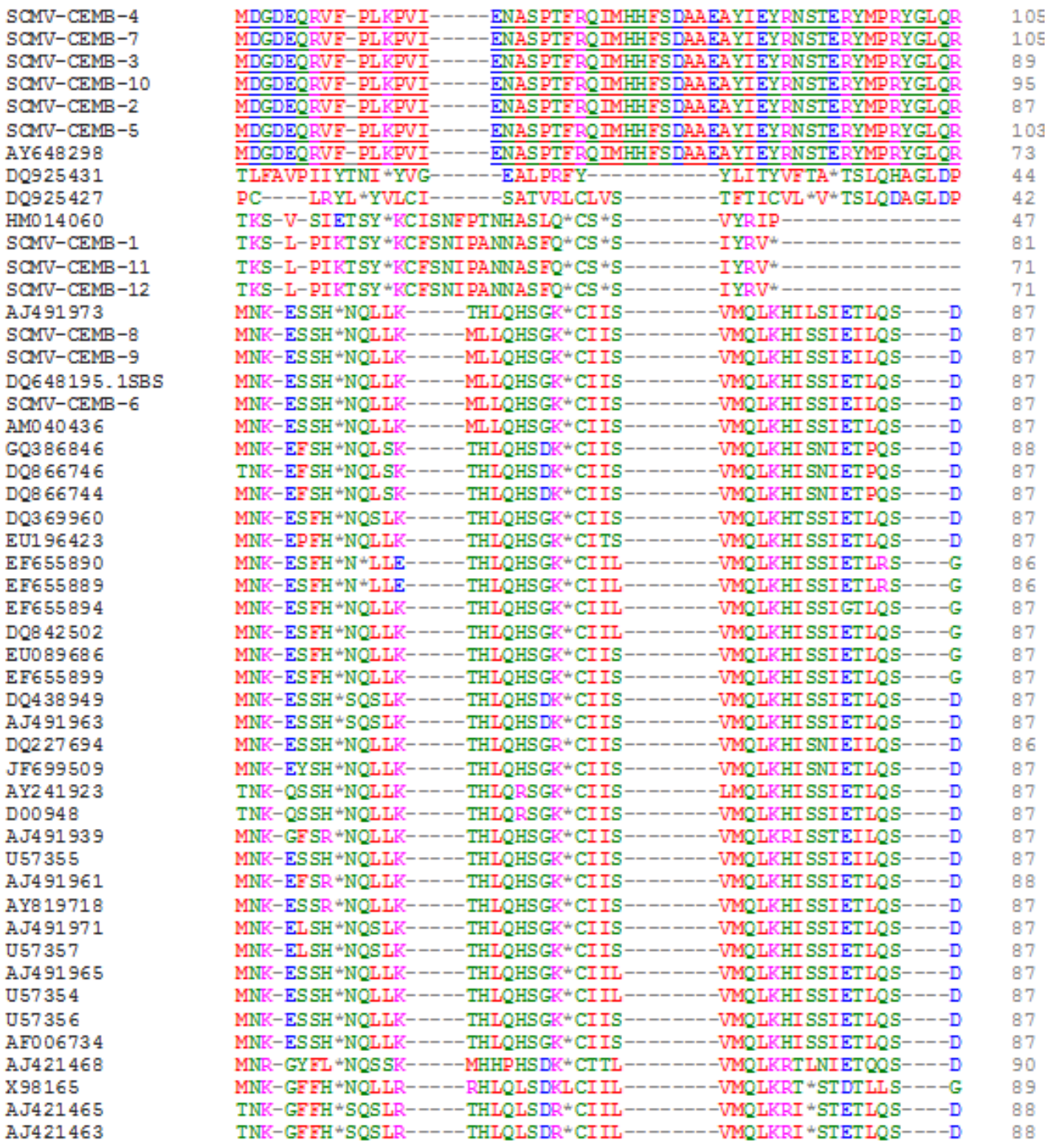

Figure 1. Contd.

*Eco*RI restriction profile was unique to distinguish them (Table 3).This massive difference in the CR of CP gene concludes its intensity of evolution, subsequently evolving as a compelling virus, extensively widespread in varieties/cultivars of sugarcane in Pakistan.

We have shown the molecular basis of genetic variation of the CP gene which are usually identified for genetic understanding of potyviruses (Shukla et al., 1994; Garcia-Arenal et al., 2003; Frenkel et al., 1989, 1992; Gemechu et al., 2006; Alegria et al., 2003). The 12 Pakistani SCMV isolates expressed a deviation of 0-18 and 0.00-95% at nucleotide and amino acids level

Figure 1. Contd.

respectively in the reported sequences as compared with the deviation of 0-73 and 0-96% at nucleotide and amino acids level respectively in the compared region (data based on Table 2), noticed amongst the isolates of SCMV from sugarcane cultivated all over the world. This huge divergence may be based on the addition anddeletion mutations in the CP gene of SCMV isolates/variants. Though, more than 12 strains of SCMV causing mosaic in sugarcane have been described worldwide, but only five strains SCMV-A, SCMV-B, SCMV-D, SCMV-E from USA (Yang and Mirkov, 1997) and SCMV-SC from Australia (Frenkel et al., 1991) have been characterized on molecular level. Serological and host interaction based approaches (Haider et al., 2011;

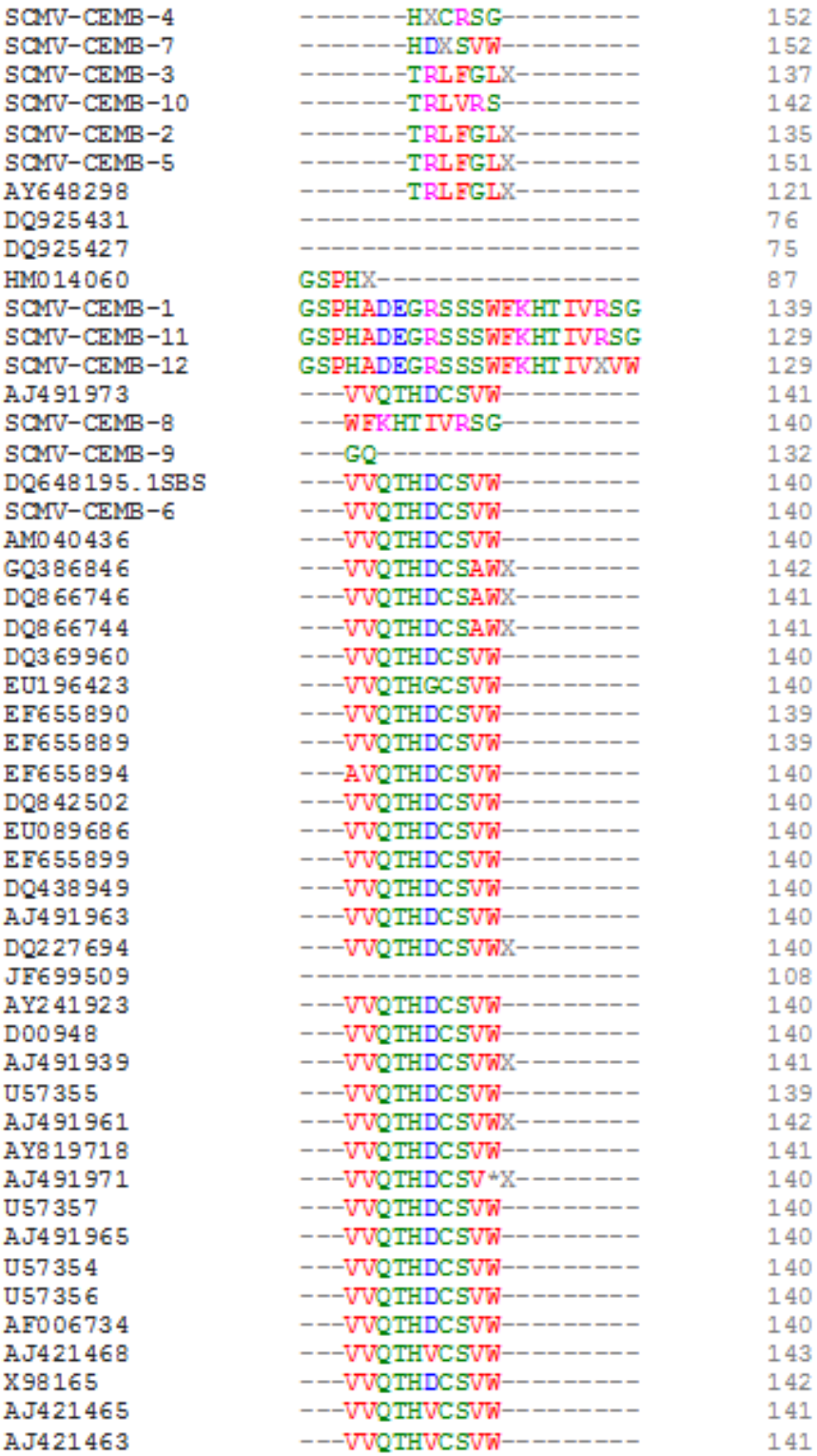

Figure 1. Contd.

Mansoor et al., 2003) have been used to identify the strains SCMV-A, SCMV-B, SCMV-D and SCMV-E in Pakistan earlier; the SCMV isolates characterized in this study are genetically different. This effort presents the detection of population of SCMV in Pakistan by molecular methodologies and it proved thought-provoking results on

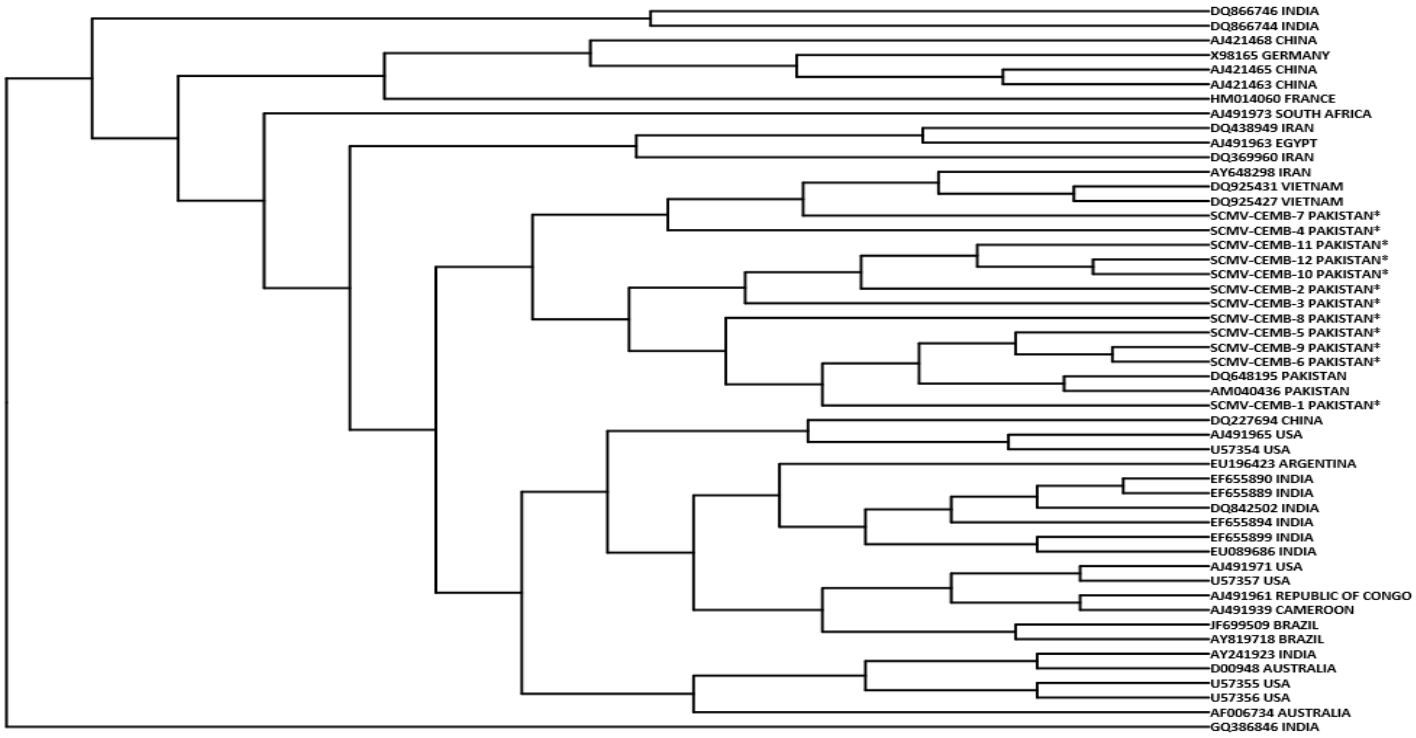

Figure 2. Phylogenetic relatedness of Pakistani SCMV isolates with the SCMV type strain/strains and other SCMV isolates from sugarcane reported from worldwide. Phylogram presented is derived from DNA Maximum Likelihood program with molecular clock, based on a nucleotide sequence alignment of core region in the coat protein coding sequence corresponding to nt 1-457 of SCMV-CEMB-5 (GenBank Acc. No. KC249910) in the CP coding sequence. A 97.0% sequence identity limit and position in the phylogenetic tree was used to assign the virus isolates to different phylogenetic groups. *The isolates from this study. The details of the sequences and their accession numbers are given in Table 1.

the incidence of new sugarcane mosaic virus population. Further investigations regarding the sequencing of complete genome of certain variants are under way which might be helpful to understand and characterize strainal variations more precisely.

Conflict of Interests

The author(s) have not declared any conflict of interests.

REFERENCES

Alegria O M, Royer M, Bousalem M, Chatenet M, Peterschmitt M, Girard J C, Rott P (2003). Genetic diversity in the coat protein coding region of eighty-six sugarcane mosaic virus isolates from eight countries, particularly from Cameroon and Congo. Arch. Virol. 148:357-372.

Chatenet M, Mazarin C, Girard JC, Fernandez E, Gargani D, Rao GP, Royer M, Lockhart B, Rott P (2005). Detection of sugarcane streak mosaic virus in sugarcane from several Asian countries. Sugar Cane Int. 23:12-15.

Chen J, Adams MJ (2002). Characterization of potyviruses from sugarcane and maize in China. Arch. Virol. 147(6):1237-1246.

Fan Z, Chen H, Cai S, Deng C, Wang W, Liang X, Li H (2003a). Molecular characterization of a distinct potyvirus from white grass in China. Arch. Virol. 148(6):1219-1224.

Fan ZF, Chen HY, Liang XM, Li HF (2003b). Complete sequence of the genomic RNA of the prevalent strain of a potyvirus infecting maize in China. Arch. Virol. 148(4):773-782.

Farrag SH, Kandaswamy TK (1979). Soybean and Greengram as Local Lesion Assay Hosts for Sugarcane Mosaic Virus. Curr. Sci. 48(19):865.

Frenkel MJ, Jilka JM, McKern NM, Strike PM, Clark JMJr, Shukla DD, Ward CW (1991). Unexpected sequence diversity in the amino-terminal ends of the coat proteins of strains of sugarcane mosaic virus. J. Gen. Virol. 72(PT2):237-242.

Frenkel MJ, Jilka JM, Shukla DD, Ward CW (1992). Differentiation of potyviruses and their strains by hybridization with the 3' non-coding region of the viral genome. J. Virol. Methods 36:51-62.

Frenkel MJ, Ward CW, Shukla DD (1989). The use of 3'-noncoding sequences in the taxonomy of potyviruses: application to watermelon mosaic virus 2 and soybean mosaic virus-N. J. Gen. Virol. 70:2775-2783.

Garcia-Arenal F, Fraile A, Malpica JM (2003). Variation and evolution of plant virus populations. Int. Microbiol. 6:225-232.

Gemechu AL, Chiemsombat P, Attathom S, Reanwarakorn K, Lersrutaiyotin R (2006). Cloning and sequence analysis of coat protein gene for characterization of Sugarcane mosaic virus isolated from sugarcane and maize in Thailand. Arch. Virol. 151:167-172.

Gopal K, Reddy KS (1988). Studies on mosaic disease of sugarcane (*Saccharum officinarum* L.). Plant Pathol. Newsl. 6:1-2.

Gopal K, Reddy KS, Upadhyaya HD, Reddy DR (1991). Host range of sugarcane mosaic virus (SCMV-B) from Rayalaseema. Ind. J. Virol. 7:117-119.

Gotz R, Maiss E (2002). The complete sequence of the genome of Cocksfoot streak virus (CSV), a grass infecting Potyvirus. Arch. Virol. 147(8):1573-1583.

Grisham MP (2000). In: A Guide to Sugarcane Diseases, ed. by Rott, P., Bailey, R.A., Comstock, J.C., Croft, B.J., Saumtally, A.S. (CIRADISSCT, CIRAD Publication Services, Montepellier, France), pp. 249-254.

Grisham MP, Pan YB (2007). A genetic shift in the virus strains that cause mosaic in Louisiana sugarcane. Plant Dis. 91:453-458.

Ha C, Revill P, Harding RM, Vu M, Dale JL (2008). Identification and sequence analysis of potyviruses infecting crops in Vietnam. Arch. Virol. 153:45-60.

Haider MS, Afghan S, Riaz H, Tahir M, Javed MA, Rashid N, Iqbal J (2011). Identification of two sugarcane mosaic virus (SCMV) variants from naturally infected sugarcane crop in Pakistan. Pak. J. Bot. 43(2):1157-1162.

Hall TA (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41:95-98.

Hema M, Venkataramana M, Savithri HS, Sreenivasulu P (1999). Biological, antigenic and genomic relationships among the virus isolates causing mosaic disease of sugarcane in South India. Curr. Sci. 77(5):698-702.

Jensen SG, Hall JS (1993). Characterization of a Krish-sorghum infecting potyvirus. Sorghum Newsl. 34:17.

Koike H, Gillespie AG (1989). Mosaic. In: Disease of Sugarcane-Major Diseases, ed. by C. Ricaud, B.T. Egan, A.G. Gillespie, C.G. Hughes (Elsevier, Amsterdam, 1989). pp. 301-322.

Kondaiah E, Nayudu MV (1984a). A key to the identification of sugar cane mosaic virus (SCMV) strains. Sugar Cane 6:3-8.

Kondaiah E, Nayudu MV (1985). Strain N, a new strain of sugarcane mosaic virus. Sugar Cane 4:11-14.

Kondaiah E, Nayudu MV (1984b). Sugarcane mosaic virus strain H, a new record from India. Curr. Sci. 53:273-275.

Li Y, Liu R, Zhou T, Fan Z (2013). Genetic diversity and population structure of Sugarcane mosaic virus. Virus Res. 171:242-246.

Mandahar CL (1978). Introduction to Plant Viruses. S. Chand and Co. New Delhi. Second Edition. pp. 568.

Mansoor H, Sahi GM, Wakil W, Imanat Y (2003). Aphid Transmission of Sugarcane Mosaic Virus (SCMV). Pak. J. Agric. Sci. 40(1-2):74-76.

McKern NM, Shukla DD, Toler RW, Jensen SG, Tosic M, Ford RE, Leon D, Ward CW (1991). Confirmation that the sugarcane mosaic virus subgroup consists of four distinct potyviruses by using peptide profiles of coat proteins. Phytopathology 81:1025-1029.

Oertel U, Schubert J, Fuchs E (1997). Sequence comparison of the 30-terminal parts of the RNA of four German isolates of sugarcane mosaic potyvirus (SCMV). Arch. Virol. 142:675-687.

Perera MF, Filippone MP, Ramallo J, Cuenya MI, Garcia ML, Castagnaro AP (2007). Zgenetic diversity of Sugarcane mosaic virus complex in Tucuman, Argentina. Proc. Int. Soc. Sugar Cane Technol. 26:988-992.

Rishi N, Rishi S (1985). Purification, electron microscopy and serology of strain A to F of Sugarcane Mosaic Virus. Ind. J. Virol. 1:79-86.

Seifers DL, Salomon R, Marie-Jeanne V, Alliot B, Signoret P, Haber S, Loboda A, Ens W, She YM, Standing KG (2000). Characterization of a novel potyvirus isolated from maize in Israel. Phytopathology 90:505-513.

Shukla DD, Frenkel MJ, McKern NM, Ward CW, Jilka J, Tosic M, Ford RE (1992). Present status of sugarcane mosaic subgroup of potyviruses. Arch Virol. 5(Suppl):363-373.

Shukla DD, Tosic M, Jilka J, Ford RE, Toler RW, Langham MAC (1989). Taxonomy of potyviruses infecting maize, sorghum and sugarcane in Australia and the United States as determined by reactivities of polyclonal antibodies directed towards virus-specific N-termini of coat proteins. Phytopathology 79:223-229.

Shukla DD, Ward CW, Brunt AA (1994). Sugarcane mosaic virus subgroup in the Potyviridae. CAB, Inter. Wallingford, UK. pp. 360-370.

Singh D, Tewari AK, Rao GP, Karuppaiah R, Viswanathan R, Arya M, Baranwal VK (2009). RT-PCR/PCR analysis detected mixed infection of DNA and RNA viruses infecting sugarcane crops in different states of India. Sugar Tech. 11:373-380.

Singh K (1971).Virus diseases of sugarcane and the seed programme. Adv. Agric. 1:9-87.

Teakle DS, Shukla DD, Ford RE (1989). Sugarcane mosaic virus. AAB Descriptions of Plant viruses. No. 342.

Thompson JD, Higgins DG, Gibson TJ (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22(22):4673-4680.

Viswanathan R, Balamuralikrishnan M, Karuppaiah R (2008). Identification of three genotypes of sugarcane yellow leaf virus causing yellow leaf disease from India and their molecular characterization. Virus Genes 37:368-379.

Xiao XW, Frenkel MJ, Teakle DS, Ward CW, Shukla DD (1993). Sequence diversity in the surface exposed amino-terminal region of coat proteins of seven strains of sugarcane mosaic virus correlates with their host range. Arch. Virol. 132:399-408.

Xu DL, Park JW, Mirkov TE, Zhou GH (2008). Viruses causing mosaic disease in sugarcane and their genetic diversity in southern China. Arch. Virol. 153:1031-1039.

Yang ZN, Mirkov TE (1997). Sequence and relationships of sugarcane mosaic and sorghum mosaic virus strains and development of RT-PCR-based RFLPs for strain discrimination. Phytopathology 87:932-939.

Yasmin T, Iqbal S, Farooq A, Zubair M, Riaz A (2011). Prevalence, Distribution and Incidence of Major Sugarcane Infecting Viruses in NWFP and Punjab. Pak. J. Phytopathol. 23(1):24-30.

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Full Length Research Paper

Effect of exogenous phytohormones and sucrose on micropropagation and microtuberisation of *Manihot esculenta* Crantz var. TMS 96/0023

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The effect of exogenous phytohormones and sucrose on micropropagation and microtuberization from nodal cuttings of *Manihot esculenta* was studied. Direct and indirect organogeneses were established from these explants. When nodal cuttings were cultured in the presence of 0.01 to 0.1 mg.L^{-1} of BAP or NAA there was induction of the budding of axillary buds, with the highest percentages of nodal cuttings with axillaries buds budded of 98 and 73% with 0.07 mg.L^{-1} BAP and 0.05 mg.L^{-1} NAA, respectively. When sub cultured in the presence of 0.05 to 0.1 mg.L^{-1} NAA, the nodal cuttings with axillaries buds budded produced yellowish and friable callus with the highest percentage of callogenesis of 59.8% with 0.08 mg.L^{-1} NAA. The highest fresh weight of callus of 1334.7 mg was obtained with 0.07 mg.L^{-1} of NAA. When sub cultured in the presence of 0.05 to 0.1 mg.L^{-1} BAP, the nodal cuttings and callus induced shoots with the highest percentages of 72.3% and 92.3% for nodal cutting and callus respectively with 0.08 mg.L^{-1}. Also, the highest numbers of shoots per nodal cutting 17.9 and per callus 28.1 were obtained with 0.08 mg.L^{-1}. Multiple shoots, when isolated from nodal cuttings or callus and sub cultured in the presence of different ratio of BAP/NAA, differentiated into plantlets. With 0.07/0.05 mg.L^{-1} and 0.08/0.05 mg l^{-1} ratio, all shoots (100%) gave rise to plantlets with the highest growth parameters (14.8 ± 3.1 to 15.3 ± 1.7 cm of high, 12.3 to 12.7 leaves and 7.8 to 8.3 roots). These plantlets were used to induce microtubers on the basal medium supplemented with 0.1 to 0.6 mg.L^{-1} of NAA or Kin or NAA/Kin ratio and 10 to 60 g.L^{-1} sucrose. The ratio NAA/Kin of 0.4/0.4 mg.L^{-1} combined with 20 to 30 g.L^{-1} sucrose was more effective on the microtuberization than NAA and Kin used separately. In fact, it gave rise to 57.8 to 61.3% of plantlets which produce microtubers with the highest number of microtuber per plantlet (4 to 5), the highest diameter (130.4 to 131.8 mm) and the highest fresh weight of microtubers (403.3 to 408.1 mg). When the plantlets were acclimatized in different substrates, 100 % survived in the mixture red soil/black soil at equal volume (V/V) These results of this work show that BAP, NAA, and Kin used separately or in ratio can be used for organogenesis and micropropagation in *Manihot esculenta* var TMS 96/0023 *in vitro*.

Key word: NAA, Kin, BAP, sucrose, organogenesis, microtuberization, cassava.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a major staple crop for millions of people in East and Central Africa, mostly in the rural areas and it is the second most important staple crop in Africa after maize. Because of it importance in food security and poverty alleviation, cassava has been prioritized by the New Partnership for Africa's Development (NEPAD) as a 'poverty fighter' which will spur industrial development in Africa (Whingwiri, 2004). Acedo and Labana (2008) reported that the demand for cassava has been increase in the recent past years because of its potential use in biofuel industry. It is grown for its starchy tuberous roots which provide food for over 500 million people, mostly in a small-scale plantation by the farmers in the developing countries (Roca et al., 1992). Thus, anything that affects the production of the cassava crop may have serious consequences in food availability and the economy of the people in such growing areas. It is therefore not surprising that every year millions of dollars are allocated by the government in these growing areas to combat pests and diseases which affect the yields of the crop. Some challenges of cultivation include low nutritive value of cassava (1.2 to 1.8 % crude protein, 0.1 to 0.8 % crude lipid, 1.5 to 3.5 % crude fiber and 1.3 to 2.8 % ash) (Albert et al., 2005) and occasionally accumulations of toxic cyanogenic glucosides (White et al., 1998). This therefore, necessitates the adoption of breeding programmes to introduce new varieties of higher nutritional quality.

Tissue culture is one of the most successful, comercially exploited components of biotechnology and has been used for rapid clonal multiplication (micropropagation) of selected genotypes of diverse groups of plant species (Rani and Raina, 2000). The first study on tissue culture of cassava was done by Kartha et al. (1974) who reported regeneration of shoots from meristems of five cassava varieties cultured on MS medium supplemented with 0.1 $mg.L^{-1}$ Benzylaminopurine (BAP), 0.04 $mg.L^{-1}$ Gibberellic acid (GA3) and 0.2 $mg.L^{-1}$ Naphylacetic acid (NAA). Bhagwat et al. (1996) reported regeneration of multiple shoots from nodal explants of cassava using 0.11 to 0.22 $\mu M.L^{-1}$ thidiazuron (TDZ), 2.2 $\mu M.L^{-1}$ BAP and 1.6 $\mu M.L^{-1}$ GA3. A frequent *in vitro* culture manipulation for cassava involves standard media such as the Murashige and Skoog (1962) (MS medium), but with altered macro- and/or micronutrient concentrations. This manipulation was initially restricted to embryo culture and nodal micro propagation, but was later extended to somatic embryogenesis (Taylor et al., 1996).

The aim of this study was to test the effect of exogenous phytohormones concentration and sucrose on micropropagation and microtuberisation of cassava. Many factors such as the presence or the absence of growth regulators, the concentration and of sugars, the mineral composition of the medium and the photoperiod were known to influence the organogenesis *in vitro* (Koda and Kikuta, 1991; Mantell and Hugo, 1989; Santos and Salema, 2000; Ovono Ondo et al., 2007). Therefore, the effect of growth regulators (BAP and NAA) and sucrose, together or in combination with the micropropagation of *M. esculenta* was studied.

MATERIALS AND METHODS

Plant material and disinfection

TMR 96/0023 cassava variety was obtained from the Internal Institute of Tropical Agriculture (IITA) PMB 2008, Messa Yaounde, Cameroon. Nodal cuttings (explants) for 1.5 cm long were isolated from stems of this variety and then, cleaned under running tap water for 2 h. These explants were disinfected in 70% ethanol for 5 min followed by 3.5% sodium hypochlorite for 10 min and then rinsed four times (10 min each) in sterilized distilled water. All stages of disinfection were done in the laminar flow hood.

Budding of axillaries buds from nodal cuttings

For all experiments the basal medium (BM) consists of Murashige and Skoog (MS) salt (1962) supplemented with Morel and Wetmore (1951) vitamins, 20 $g.L^{-1}$ sucrose and 7 $g.L^{-1}$ Difco agar. For the budding of axillaries buds, disinfected nodal cuttings were cultured in closed test tubes containing 10 ml of BM supplemented with 0.01 to 0.1 $mg.L^{-1}$ of 6- benzylaminopurine (BAP) or 0.01 to 0.1 $mg.L^{-1}$ of naphthalene acetic acid (NAA). The pH of all media was adjusted to 5.8 with NaOH solution (1N) or HCl solution (0.1N) before autoclaving at 115°C for 30 min under a pressure of 1.6 ± 0.1 $kg.cm^{-2}$. All cultures were incubated under 80 $\mu mol.m^{-2}.s^{-1}$ light provided by cool white fluorescent tube lamps (Mazda) at a photoperiod of 16 h at 26 ± 1°C. 100 nodal cuttings were cultured for each medium and the experiment was repeated twice. After 11 days, axillaries buds develop and the percentage of budding was calculated per medium. The nodal cutting with budded axillaries buds constituted the explants for the following experiments.

Induction of callus from nodal cuttings

For the induction of callus, the nodal cuttings with budded buds were sub cultured in the closed test tubes (150 × 80 mm) containing10 ml of BM supplemented with 0.05 to 0.1 $mg.L^{-1}$ NAA. The pH of all media was adjusted to 5.8 before autoclaving. All sub cultures were incubated under the same conditions as during budding of axillaries buds. 50 explants were sub cultured per

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Abbreviations: BAP: Benzylaminopurine**, NAA:** Naphthalene Acetic Acid**, Kin:** Kinetin**, etc**

medium and the experiment was repeated thrice. The percentages of explants inducing callus and the fresh weight of callus were evaluated per medium after 28 days. For all experiments, the control was the BM without phytohormones.

Induction and proliferation of multiple shoots from nodal cuttings or from callus

The effect of BAP on the induction and proliferation of multiple shoots was tested directly by using nodal cuttings with budding auxiliary buds or indirectly by using callus as explants. For direct method, nodal cuttings were sub cultured in BM supplemented with 0.05 to 0.1 mg.L^{-1} BAP. The pH of all media was adjusted to 5.8 before autoclaving. All sub cultures were incubated under the same conditions as during budding of axillaries buds. 50 explants were sub cultured per medium and the experiment was repeated thrice. The percentage of explants inducing multiple shoots and the average number of shoots per explant were evaluated per medium after 45 days.

For indirect method, callus induced in the presence of NAA were first sub cultured in basal medium without phytohormones for 7 days. This sub culture was followed by a second sub culture in BM supplemented with 0.05 to 0.1 mg.L^{-1} BAP. Also, the pH of all media was adjusted to 5.8 before autoclaving. All second sub cultures were incubated under the same conditions as during induction of callus. 50 callus were sub cultured per medium and the experiment was repeated thrice. The percentage of callus inducing multiple shoots and the average number of shoots per callus were evaluated per medium after 45 days. For all experiments, the control was the BM without phytohormones.

Regeneration of plantlets from multiple shoots

For the regeneration of plantlets, multiple shoots were isolated from different explants (nodal cuttings and callus) and cultured firstly for 7 days in closed test tubes containing each 10 ml of basal medium and secondly on the basal medium supplemented with 0.05 to 0.1 mg.L^{-1} BAP and 0.05 mg.L^{-1} NAA (BAP / NAA ratio) (Table 3). The pH of all media was adjusted to 5.8 before autoclaving. All cultures were incubated under the same conditions as during budding of axillaries buds. 30 isolated multiple shoots were cultured per medium and the experiments was repeated thrice. The growth of plantlets was measured after 26 days by counting the number of leaves and roots differentiated and by measuring the height of plantlets in the presence of each BAP/NAA ratio. The control medium was the BM without phytohormones.

Induction and growth of microtubers

Regenerated plantlets from multiple shoots were subcultured in closed test tubes containing each 10 ml of semi liquid (3.5 g.L^{-1} Difco agar) basal medium supplemented with 2 µM Jasmonic acid, 0.1 to 0.6 mg l^{-1} NAA or Kinetin (Kin) or 0.1 to 0.6 mg.L^{-1} NAA and 0.4 mg.L^{-1} Kin (NAA/Kin ratio) (Table 4) The pH of all media was adjusted to 5.8 before autoclaving. All cultures were incubated under the same conditions as during plantlets regeneration. 20 plantlets were sub cultured for each concentration of phytohormones or each ratio and all experiments were repeated thrice. After 65 days, the percentage of plantlets inducing microtubers, the average number of microtubers produced, the average diameter and the average fresh weight of microtubers were evaluated for each treatment. For all experiments, control was the semi liquid basal medium without phytohormones. Simultaneously the effect of sucrose on the inducing and growth of microtubers was also studied. The semi liquid basal medium was supplemented with 10, 20, 30, 40, 50 and 60 g.L^{-1} sucrose together with 0.4 mg.L^{-1} NAA or Kin or 0.4/0.4 mg.L^{-1} NAA/Kin. The control was the semi liquid basal medium without phytohormones.

Acclimatization of plantlets

Some regenerated plantlets from multiple shoots were acclimatized in polyethylene bags containing the following sterilized substrates; red soil, black soil, vermiculite, mixture red soil/ black soil at equal volume (V/V), red soil/vermiculite (V/V), and black soil/vermiculite (V/V). However, the effects of these substrates were tested for acclimatization, 20 plantlets were cultured per substrate and the experiment was repeated thrice. All cultures were incubated under a temperature of 26 ± 1°C, 70 to 72% of relative humidity and a photoperiod of 16 under a light period of 80 µmol.m^{-2}.s^{-1} during 36 days. During this period, the relative humidity was progressively reduced as plantlets were watered firstly with sterilized tap water during 10 days and then with tap water during 26 days. The percentage of survival was evaluated per substrate.

Data analysis

All experiments were set up in a completely randomized design. Differences between means were scored with Duncan's Multiplication Range Test. The analysis of samples from each treatment was statically evaluated by analysis of variance (ANOVA, $P < 0.05$) and the interactive effect of two phytohormones was assessed by two-way ANOVA. The program used was SPSS (version 12 for windows).

RESULTS

Effect of BAP and NAA on budding of axillaries buds of nodal cuttings

Nodal cuttings were cultured (Figure 1a) in the presence of 0.01 to 0.1 mg.L^{-1} of BAP or NAA for inducing the budding of axillaries buds. After 11 days of culture, the highest percentages of nodal cuttings with axillaries buds budded were 98 and 73% with 0.07 and 0.03 mg.L^{-1} BAP and NAA respectively (Figure 2). The lowest percentages of 59 and 17% were obtained with 0.1 mg.L^{-1} of both phytohormones (Figure 2). Comparatively, BAP is more effective on the budding than NAA. In fact, the percentages of budding obtained with BAP at all concentrations were higher than that of NAA (Figure 2). The budding is mainly characterized by increasing the volume and elongation of axillaries buds (Figure 1b).

Effect of NAA on the production and growth of callus from budded nodal cuttings

When cultured in the basal medium supplemented with 0.05 to 0.1 mg.L^{-1} NAA, the budded nodal cuttings produced callus after 28 days. These callus were yellowish and friable (Figure 1c). The highest percentage of callogenesis (59.8%) was obtained with 0.08 mg.L^{-1}

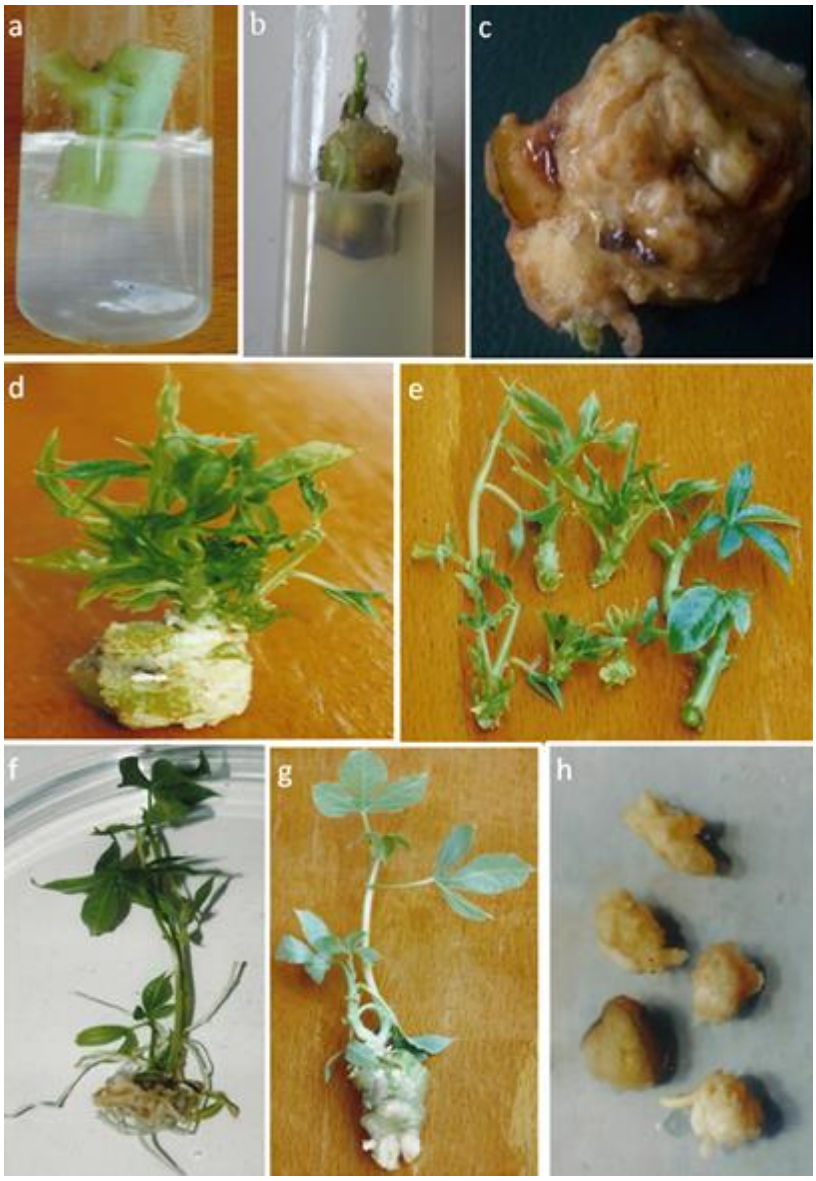

Figure 1. Micropropagation and microtuberization of *Manihot esculenta.* (a) Nodal cutting cultured on BM supplemented with 0.01 to 0.1 mg.L^{-1} BAP or NAA; (b) budded axillary buds after 11 days of culture on the same media; (c) callus produced from nodal cutting after 28 days of culture on BM supplemented with 0.08 mg.L^{-1} NAA; (d) multiple shoots differentiated on callus after 45 days of culture on BM supplemented with 0.08 mg.L^{-1} BAP; (e) isolated shoots from nodal cuttings or from callus ; (f) Plantlets regenerated from shoots after 26 days of culture on BM supplemented with 0.07/0.05 or 0.08/0.05 g.L^{-1} BAP/NAA; (g) microtubers (mc) induced on plantlets sub cultured on BM supplemented with 0.4 / 0.4 NAA/Kin and 30 g.L^{-1} sucrose after 35 days of culture; (h) isolated microtubers from plantlets after 65 days of culture.

NAA (Table 1). The fresh weight of callus varied with the concentration of NAA. The highest fresh weights of 1344.7 mg and 1337.4 mg were obtained with 0.07 and 0.08 mg.L^{-1} of NAA respectively (Table 1). With the other concentrations, the value of fresh weight was between 529.3 mg and 1023.2 mg (Table 1).

Effect of BAP on the induction of shoots from nodal cuttings and callus

When cultured in the presence of 0.05 to 0.1 mg.L^{-1} BAP, the nodal cuttings induce shoots after 30 days and the callus after 45 days (Figure 1d). The percentages of

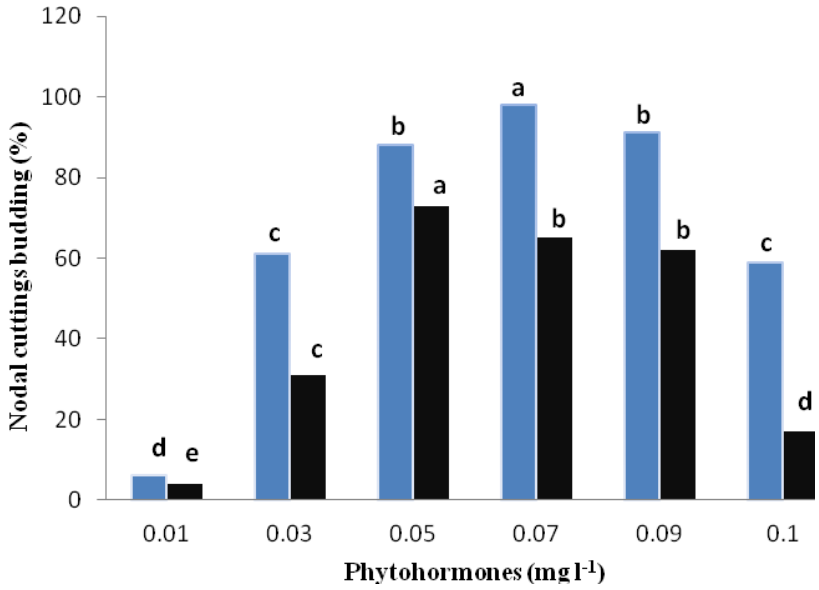

Figure 2. Effect of BAP( ) and NAA ( ) on budding of axillaries buds of nodal cuttings of *Manihot esculenta* after 11 days of culture. DMRT was used to differentiate the percentage of axillaries buds budding. For each treatment the share carrying the same letter were not significantly different at 5% level.

Table 1. Effect of NAA on the callogenesis and fresh weight of callus from nodal cuttings with budded axillary buds of *Manihot esculenta* after 28 days of culture.

NAA ($mg.L^{-1}$)	% of callogenesis	Average fresh weight of callus (mg)
0	0	0
0.05	3.6^{f}	801.5^{c}
0.06	15.4^{e}	1023.2^{b}
0.07	28.3^{c}	1344.7^{a}
0.08	59.8^{a}	1337.4^{a}
0.09	31.4^{b}	648.8^{d}
0.1	17.2^{d}	529.3^{e}

DMRT was used to differentiate the percentages of callogenesis and the fresh weight of callus. Data carrying the same letter in the same column were not significantly different at 5% level.

explants inducing shoots and the average number of shoots per explants varied according to the concentration of BAP and the type of explants. The highest percentages of 72.3 % and 92.3 % were obtained respectively with nodal cutting and callus with 0.08 $mg.L^{-1}$ BAP and the lowest percentages of 12.4 % for nodal cuttings and 13.3 % for callus were obtained with 0.06 $mg.L^{-1}$ and 0.05 $mg.L^{-1}$ BAP respectively (Table 2). Also the highest numbers of shoots per nodal cutting (17.9) and per callus (26.8 to 28.1) were obtained with 0.08 $mg.L^{-1}$ and 0.07 $mg.L^{-1}$, respectively (Table 2). When compare the performance of explants to induce shoots, callus was more effective than nodal cuttings. In fact the percentages of callus inducing shoot and the average numbers of shoots per callus were generally higher than that of Nodal cutting almost for all concentrations of BAP tested (Table 2).

Effect of BAP / NAA ratio on the regeneration and growth of plantlets from multiple shoots

When isolated from nodal cuttings or callus (Figure 1e) and sub cultured in the presence of different ratio of BAP/NAA, the multiple shoots gave rise to plantlets (Figure 1f) after 26 days. With 0.07/0.05 and 0.08/0.05 $mg.L^{-1}$ ratio, all shoots (100 %) gave rise to plantlets (Table 3). Also with these ratio, plantlets had the highest height (14.7 ± 3.6 to 15.3 ± 1.7 cm), the highest number of leaves (11.9 to 12.7) and the highest number of roots (7.8 to 8.3) (Table 2). The lowest parameters of regeneration (percentage of differentiation, 21 % to 31 %; height of plantlets, 9.6 cm to 11.2 cm; number of leaves, 4.1 to 7.1 and number of roots, 3.3 to 4.5) were obtained with 0.05/0.05 and 0.1/0.05 $mg.L^{-1}$ BAP/NAA (Table 3).

Effect of NAA, Kin and NAA/Kin ratio on the production and growth of microtubers from plantlets

When sub cultured in the basal medium supplemented with NAA or Kin or NAA/Kin ratio, the plantlets differentiate microtubers at their base in 35 days (Figure 1 g). These microtubers grew and after 65 days, they

Table 2. Effect of BAP on the inducing of shoots from nodal cuttings and callus of *Manihot esculenta* after 45 days of culture.

BAP (mg.L^{-1})	% of explants inducing shoots		Average number of shoots per explants	
	Nodal cuttings	Callus	Nodal cuttings	Callus
0	0	0	0	0
0.05	0	13.3^{f}	0	4.0^{c}
0.06	12.4^{e}	22.2^{e}	3.2^{c}	1.6^{d}
0.07	61.8^{b}	71.7^{b}	8.4^{b}	26.8^{a}
0.08	72.3^{a}	92.3^{a}	17.9^{a}	28.1^{a}
0.09	44.1^{c}	51.9^{c}	7.8^{b}	5.8^{b}
0.1	23.3^{d}	36.7^{d}	4.2^{c}	6.1^{b}

DMRT was used to differentiate the percentage of explants inducing shoots and the average number of shoots per explants. Data carrying the same letter in the same column were not significantly different at 5% level.

Table 3. Effect of BAP/NAA ratio on the regeneration and growth of plantlets from isolated shoots of *Manihot esculenta* after 26 days of culture.

BAP/NAA (mg.L^{-1})	% of shoots growth to plantlets	Height of plantlets (cm)	Average number of leaves per plantlets	Average number of roots per plantlets
0	0	0	0	0
0.05/0.05	21	9.6 ± 3.1^{d}	4.1^{d}	3.3^{d}
0.06/0.05	68	12.2 ± 2.8^{b}	6.0^{c}	5.6^{b}
0.07/0.05	100	14.7 ± 3.6^{a}	11.9^{a}	8.3^{a}
0.08/0.05	100	15.3 ± 1.7^{a}	12.7^{a}	7.8^{a}
0.09/0.05	48	14.8 ± 3.1^{a}	12.3^{a}	4.1^{c}
0.1/0.05	31	11.2 ± 3.3^{c}	7.1^{b}	4.5^{c}

χ^2 and DMRT were used to differentiate the height of plantlets, the average number of leaves and average number of roots per plantlets. Data carrying the same letter in the same column were not significantly different at 5% level.

were isolated from plantlets (Figure 1h) and weighed. The percentages of plantlets producing microtubers, the number of microtubers per plantlet, their diameter and fresh weight varied with the type and the concentrations of phytohormones. The highest percentage of plantlets producing microtubers (57.6%) was obtained with 0.4/0.4 mg.L^{-1} NAA/Kin; follow by 0.4 mg.L^{-1} NAA (34.6%) and by 0.4 mg.L^{-1} Kin (33.2%) (Table 4). The number of microtubers per plantlet varied from 1 to 4 for all treatments (Table 4). The microtubers with the highest diameter (108.4 mm) were obtained with 0.4/0.4 NAA/Kin ratio; follow by 0.4 mg.L^{-1} Kin (61.3 mm) and by 0.3 and 0.4 mg.L^{-1} NAA (55.8 mm) (Table 4). With all other treatments, the diameter of microtubers was below these higher values (Table 4). The highest fresh weights of microtubers (326.9 mg and 320.4 mg) were obtained with 0.4/0.4 mg.L^{-1} and 0.3/0.4 mg.L^{-1} NAA/Kin, follow by 0.4 and 0.3 mg.L^{-1} Kin (284.1 and 248.6 mg, respectively) and by 0.2 mg l^{-1} NAA (248.3 mg) (Table 4). The lowest fresh weights of microtubers (151.2 mg, 195.6 mg and 154.1mg) were obtained with 0.1 mg.L^{-1} NAA; 0.6 mg.L^{-1} Kin and 0.1/0.4 mg.L^{-1} NAA/Kin respectively (Table 4).

Effect of sucrose on the production and growth of microtubers from plantlets

When plantlets were sub cultured in the presence of 10 to 60 g.L^{-1} sucrose supplemented with 0.4 mg.L^{-1} NAA or 0.4 mg.L^{-1} Kin or 0.4/0.4 mg.L^{-1} NAA/Kin, they differentiated microtubers in 35 days. They were then harvested after 65 days and Weighed. The highest percentages of plantlets producing microtubers (61.3 and 41.2%) were obtained with 30 g.L^{-1} sucrose in the presence of 0.4/0.4 mg.L^{-1} NAA/Kin and 0.4 mg.L^{-1} NAA respectively, while in the presence of 0.4 mg.L^{-1} Kin, a maximum of 48.5% plantlets produce microtubers with 40 g.L^{-1} sucrose (Table 5). With all concentrations of sucrose tested in the presence of NAA and Kin used sparely, the number of microtubers per plantlets varied from 1 to 4, while in the presence of NAA/Kin, a maximum of 5 microtubers per plantlet was obtained with 30 and 40 g.L^{-1} sucrose (Table 5). The largest microtubers (130.4 to 131.8 mm of diameter) were obtained with 40 and 30 mg.L^{-1} sucrose respectively in the presence of NAA/Kin compared to other treatments (Table 5). Also with 40 and

Table 4. Effect of NAA, Kin and NAA/Kin ratio on the production and growth of microtubers from plantlets of *Manihot esculenta* after 65 days of culture.

Phytohormones ($mg.L^{-1}$)	% of plantlets producing microtuber	Growth of microtubers		
		Number per plantlets	Average diameter (mm)	Average fresh weight (mg)
NAA				
0	0	0	0	0
0.1	22.4^{b}	2^{b}	30.2^{c}	151.2^{f}
0.2	21.8^{c}	2^{b}	56.9^{a}	248.3^{a}
0.3	21.7^{c}	3^{a}	41.7^{b}	219.7^{c}
0.4	34.6^{a}	3^{a}	55.8^{a}	233.4^{b}
0.5	21.4^{c}	2^{b}	55.8^{a}	201.2^{e}
0.6	8.1^{d}	2^{b}	42.6^{b}	213.6^{d}
Kin				
0	0	0	0	0
0.1	0	0	0	0
0.2	12.3^{d}	1^{d}	38.3^{d}	198.2^{d}
0.3	24.7^{b}	3^{b}	47.8^{b}	248.6^{b}
0.4	33.6^{a}	4^{a}	61.3^{a}	284.1^{a}
0.5	14.1^{c}	2^{c}	46.1^{c}	218.8^{c}
0.6	12.4^{d}	2^{c}	38.1^{d}	195.6^{e}
NAA/Kin				
0	0	0	0	0
0.1/0.4	6.2^{e}	2^{c}	30.3^{f}	154.1^{f}
0.2/0.4	24.8^{d}	3^{b}	56.7^{d}	242.8^{d}
0.3/0.4	26.9^{c}	4^{a}	101.2^{b}	320.4^{b}
0.4/0.4	57.6^{a}	4^{a}	108.4^{a}	326.9^{a}
0.5/0.4	39.3^{b}	2^{c}	58.3^{c}	277.3^{c}
0.6/0.4	3.2^{f}	3^{b}	48.9^{e}	201.2^{e}

DMRT was used to differentiate the percentage of plantlets production microtubers, the number of microtubers per plantlet, the average diameter of microtubers and average fresh weight of microtubers. Data carrying the same letter in the same column were not significantly different at 5% level.

Table 5. Effect of sucrose on the production and growth of microtubers from plantlets of *M. esculenta* after 65 days of culture.

Phytohormones	Sucrose ($g.L^{-1}$)	% of plantlets producing microtubers	Average number of microtubers per plantlets	Average diameter of microtubers (mm)	Average of fresh weight of microtuber (mg)
NAA ($0.4\ mg.L^{-1}$)	0	0	0	0	0
	10	0	0	0	0
	20	39.4^{b}	3^{a}	55.6^{a}	234.6^{a}
	30	41.2^{a}	3^{a}	52.8^{b}	229.8^{b}
	40	28.4^{c}	2^{b}	40.3^{d}	216.4^{c}
	50	11.3^{d}	1^{c}	39.9^{d}	212.3^{d}
	60	2.6^{e}	1^{c}	41.0^{c}	202.9^{e}
Kin ($0.4\ mg.L^{-1}$)	0	0	0	0	0
	10	3.2^{f}	2^{c}	49.1^{c}	217.6^{d}
	20	33.7^{b}	4^{a}	62.1^{a}	288.3^{a}
	30	32.8^{c}	3^{b}	58.6^{b}	275.9^{b}
	40	48.5^{a}	3^{b}	49.7^{c}	221.8^{c}

Table 5. Contd

	50	21.4^{d}	2^{c}	30.9^{e}	161.4^{f}
	60	9.5^{e}	2^{c}	43.4^{d}	198.7^{e}
NAA/Kin (0.4/0.4 mg.L^{-1})	0	0	0	0	0
	10	12.2^{e}	3^{c}	98.3^{d}	297.8^{d}
	20	57.8^{b}	4^{b}	109.2^{c}	372.4^{c}
	30	61.3^{a}	5^{a}	131.8^{a}	408.1^{a}
	40	40.4^{c}	5^{a}	130.4^{b}	403.3^{b}
	50	26.2^{d}	1^{d}	91.8^{e}	286.9^{e}
	60	11.7^{e}	1^{d}	1.3^{f}	249.2^{f}

DMRT was used to differentiate the percentage of plantlets production microtubers, the average number of microtubers per plantlet, the average diameter of microtubers and the average fresh weight of microtubers per plantlets. Data carrying the same letter in the same column were not significantly different at 5% level.

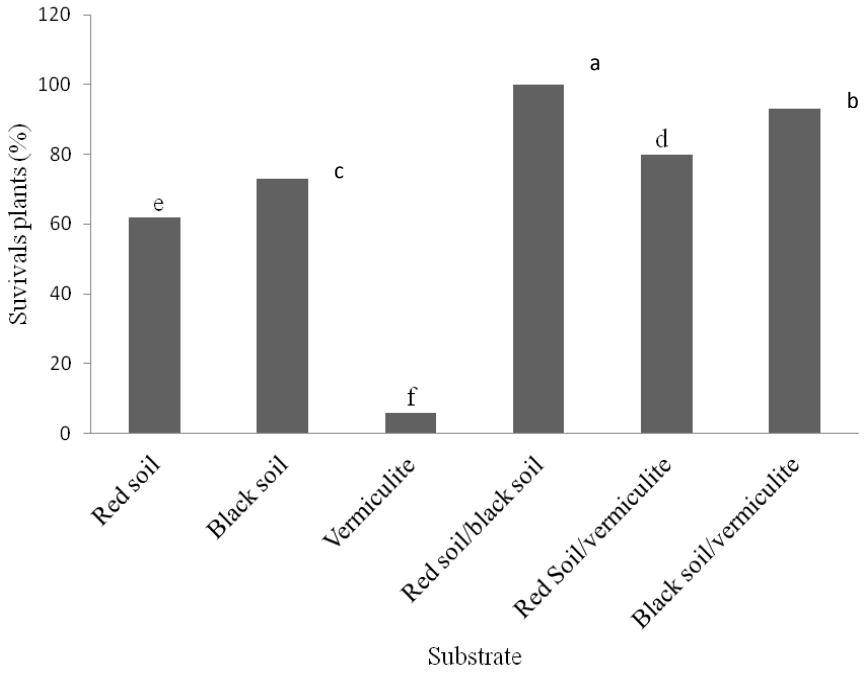

Figure 3. Effect of substrate on the acclimatization of plantlets regenerated from multiples shoot of *M. esculenta* after 36 days. DMRT was used to differentiate the percentage of survival plants on different substrates share carrying the same letter were not significantly different at 5% level.

30 mg L^{-1}sucrose and in the presence of 0.4/0.4 mg.L^{-1} NAA/Kin, microtubers presented the highest fresh weights of 403.3 mg and 408.1 mg, respectively compared to all other treatments (Table 5).

Effect of substrate on the acclimatization of plantlets

When transferred on different substrates, the plantlets regenerated from shoots gave rise to vigorous plants after 36 days. During this period, each surviving plant had an average height of 19.8 ± 1.7 m and average number of existing leaves of 14.6. The percentages of surviving plants varied with the type of substrate. In fact, a significant percentage of 100% of survival plants was exposed to mixture red soil/black soil (V/V), followed by a mixture of black soil/vermiculite (v/v) (93%), a mixture of red soil/vermiculite (V/V) (80%). The red soil and black soil gave average percentages of surviving plants (62 to 73%) and vermiculite gave the lowest percentage of 6% (Figure 3).

DISCUSSION

Manihot esculenta as many tropical crops can be successfully propagated *in vitro*. Different explants such as nodal cuttings (Konan et al., 1997; Smith et al., 1986), meristems (Kartha et al., 1974), auxiliary buds (Konan et al., 1994), shoot-tips (Roca et al., 1992) can be used for this propagation. The propagation of *Manihot esculenta* can be direct by inducing shoots directly from the explants or indirectly by inducing shoot from the callus. This has been obtained in the same species by Acedo and Labana (2008); Ma and Xu (2002) or in other species such as *Saussurea obvaiiata* (Dhar and Joshi, 2005), *Dioscorea zingiberensis* (Yongqin et al., 2003; Yuan et al., 2005), *Dioscorea alata* (Fotso et al., 2013) and *Colocassia esculenta* (Yam et al. 1990; 1991). The nodal cuttings when cultured on MS supplemented with BAP or NAA budded after 11 days with higher percentages of 98% (0.07 mg l^{-1} BAP) and 73% (0.05 mg l^{-1} NAA). Comparable results have been showed in many herbaceous and ligneous species (Blagwat et al., 1996; Gulati and Jaiwal, 1996; Fotso, 2005). But in general, the rate of budding varies not only according to the species but also according to the nature and the concentration of phytohormone used. The results of this work show that BAP is more effective on budding (98%) than NAA (73%). Similar results were obtained on tree varieties of the same species by Deden and Herni (2011) but with the combination of thidiazuron. In this work, the callus of *Manihot esculenta* was induced from budded nodal cuttings in the presence of NAA. Many works have been carried out on the establishment of callus culture from cassava using different explants (Peter et al., 2011; Fietosa et al., 2007; Atehnkeng et al., 2005; Schopke et al., 1996), but this is the first time that callus is established from budded nodal cutting of this species. The highest percentage of callogenesis (59.8%) was obtained with 0.08 $mg.L^{-1}$ NAA.

Similar results were obtained by Peter et al. (2011) on two cultivars of the same species of cassava (cv Afisiati and cv Afebankye) but with higher concentration of NAA (8 to 15 $mg.L^{-1}$). The shoots of cassava were induced directly from budded nodal cuttings or indirectly from callus in the presence of BAP. This confirms the highest potential of BAP to induce shoots in different species as reported in several works (Anh Hong et al., 2007; Konan et al., 1997; Fotso et al., 2013; Davies, 2004). The percentages of explants inducing shoots were ranged from 12.4% (0.06 $mg.L^{-1}$ BAP) to 72.3% (0.08 $mg.L^{-1}$ BAP) for nodal cuttings and from 13.3% (0.05 $mg.L^{-1}$ BAP) to 92.3% (0.08 $mg.L^{-1}$ BAP) for callus. Also with the same concentrations of BAP, the average numbers of shoots per explants were ranged from 3.2 to 17.9 for nodal cuttings and from 4.0 to 28.1 for callus. Then in the presence of BAP, callus of *Manihot esculenta* seem to be more effective to induce shoots than nodal cuttings. These results are comparable to those obtained by Deden and Herni (2011) on three cassava varieties but BAP was used in combination with 0.1 to 1 $mg.L^{-1}$ thidiazuron and 80 $mg.L^{-1}$ adenine sulfate. In this work, plantlets were regenerated when shoots were subcultured in the presence of different ratio BAP/NAA. With the ratio 0.07/0.05 and 0.08/0.05 $mg.L^{-1}$, all shoots (100 %) gave rise to plantlets with the highest growth parameters The regeneration of plantlets in the presence of BAP/NAA ratio was already reported in the same specie (Ma and Xu, 2002) or in the other species (Mantell and Hugo, 1989; Fotso et al., 2013; Dhar and Joshi, 2005).This result confirms the complementary and synergize action of auxins and cytokinins in the process of growth and development that has been reported by some authors (Dhar and Joshi, 2005; Vines, 2001; Guo and Zhang, 2005).When acclimatized, plantlets regenerated from *Manihot esculenta* survived more (100 %) in the mixture red soil/black soil (V/V).This result is in contrasts with acclimatization of many species where plantlets survived in vermiculite or in mixture of vermiculite and soil (Yongqin et al., 2003; Yassen et al., 1995).

In this study, the microtubers were produced when the basal medium was supplemented with 2 µM jasmonic acid different concentration of NAA, Kin, NAA/Kin ratio and sucrose. Till date very few works has been reported on microtuberization in cassava species while several has been reported on other crop tuber species such as *Solanum tuberosum* (Gami et al., 2013; El-Sawy et al., 2007), *Xanthosoma sagittifolium* (Tsafack et al., 2009), *Dioscorea sp* (Ovono Ondo et al., 2007; Fotso et al., 2013). These different works show that, the percentage of plantlets producing microtubers, the number of microtubers per plantlet and their size varies not only according to species but also according to the culture conditions and this is confirmed in this study. In fact, jasmonic acid has been shown to be effective on microtuberization (Santos and Salema, 2000), but this effectiveness also depend on the other culture conditions mainly the type and the concentration of phytohormones used and the concentration of sucrose in the culture media (Hoque, 2010; Yu et al., 2000; Zakaria et al., 2008; Bazabakana et al., 2003). The results of this study shows that, microtubers in *Manihot esculenta* can be produced in the presence of 2 µM jasmonic acid and 0.1 to 0.6 $mg.L^{-1}$ NAA or Kin used separately or in ratio (NAA/Kin).

The highest percentages of plantlets producing microtuber were 34.6 % (0.4 $mg.L^{-1}$ NAA), 33.6 % (0.4 $mg.L^{-1}$ Kin) and 57.6 % (0.4/0.4 NAA/Kin $mg.L^{-1}$). With these concentrations the highest number of 3 and 4 microtubers was obtained with the respective diameter of 55.8 cm, 61.3 cm and 108 cm and the respective fresh weight of 233.4 mg, 248.1 mg and 326.9 mg for NAA, Kin and NAA/Kin. Then, NAA and Kin were more effective on the microtuberization in *Manihot esculenta* when they were used in ratio or combination. These results were comparable to those obtained by Tsafack et al. (2009) in *Xanthosoma sagittifolium* during which the effect of

photoperiod and thermoperiod were also studied on the microtuberilization, but are in contrast with those obtained by Gami et al. (2013) which showed that NAA or Kin used separately were more effective on the microtuberilization in *Solanum tuberosum.* When the basal medium is supplemented with 10 to 60 g l^{-1} sucrose and 0.4 mg l^{-1} NAA or Kin or 0.4/0.4 mg l^{-1} NAA/Kin, the highest percentage of plantlets producing microtubers (61.3 %) the highest numbers of microtubers per plantlet (5) having bigger sizes (131.8 cm of diameter and 408.1 mg of fresh weight) were obtained with NAA/Kin ratio and 30 $g.L^{-1}$sucrose. This result contrasts those obtained by Debeljak et al. (2002) in *Pterostylis sanguinea* which showed that in the presence of 1.5 and 2 mM jasmonic acid and NAA or Kin the microtuberization is induced at least with 60 $g.L^{-1}$ sucrose, and those of Alizadeh et al. (1998) which show that in *Dioscorea composita,* microtuberilization is induced with 80 to 100 $mg.L^{-1}$ sucrose.

Conclusion

Direct and indirect organogenesis of *M. esculenta* was achieved in this study from micro cuttings and induced callus cultured in the presence of BAP and NAA. Plantlets were fully regenerated when these phytohormones were used in combination or ratio with 100% of success with ratio BAP/NAA of 0.07/0.05 and 0.08 / 0.05 $mg.L^{-1}$. Microtuberization was induced on regenerated plantlets in the presence of NAA, Kin and 10 to 60 $g.L^{-1}$ sucrose. 100 % of regenerated plantlets survived when acclimatized in a mixture of red soil/black soil. Hence, the micropropagation of *M. esculenta* by *in vitro* culture method is more effective and more significant than the conventional method where apex culture produced generally a single plant. Further studies are needed to evaluate the performance of plantlets transferred to the fields.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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REFERENCES

Acedo VZ, Labana C (2008). Rapid propagation of released Philippine cassava varieties through tissue culture. J. Root Crops 34(2):108-14.

Albert CL, Klanarong S, Tzou-chi H (2005). Proximate composition, mineral contents, hydrogen cyanide and phytic acid of 5 cassava genotypes. Food Chem. 92:615-620.

Alizadeh S, Mantell SH, Viana AM (1998). *In vitro* shoot culture and microtuber induction in the steroid yam *Dioscorea composita* Hemsl. Plant Cell Tissue Organ Cult. 53:107-112.

Anh Hong LT, Le BV, Anh BL, Soytong K, Danh ND (2007). Plant regeneration of cassava (*Manihot esculenta* CRANTZ) plants. J. Agric. Technol. 3(1):121-127.

Atehnkeng J, Adetimirin VO, Ng SYC (2005). Exploring the African cassava (*Manihot esculenta* Crantz) germplasm for somatic embryogenic competence. Afr. J. Biotechnol. 5:1324-1329.

Bazabakana R, Baucher M, Diallo B, Jaziri M (2003). Effect of jasmonic acid on developmental morphology during *in vitro* tuberisation of *Dioscorea alata.* Plant Growth Regul. 40:229-237.

Bhagwat B, Luiz, GF, Vieiral and Larry R, Erickson (1996). Stimulation of *in vitro* shoot proliferation from nodal explants of cassava by thidiazuron, benzyladenine and gibberellic acid. Plant Cell Tissue Organ Cult. 46:1-7.

Davies PJ (2004). The plant hormones: Their Nature, Occurrence and Function In: Davies PJ (Editor) Plant Hormones Biosynthesis, Signal Transduction, Action. Kluwer Academic Press. 1-15

Debeljak N, Regvar M, Dixon KW, Sivasithamparam K (2002). Induction of tuberization *in vitro* with jasmonic acid and sucrose in an Australian terrestrial orchid, *Pterostylis sanguinea*. Plant Growth Regul. 36:253-260.

Deden S, Herni W (2011). Effects of plant growth regulators on shoot multiplication and root induction of cassava varieties *in vitro* culture. Biotropia 18 (1): 50 - 60.

Dhar U, Joshi M (2005). Efficient plant regeneration protocol through callus for *Saussurea obvallata* (DC.) Edgew. (Asteraceae): effect of explant type, age and plant growth regulators. Plant Cell Rep. 24: 195-200.

El-sawy A, Bekhetet S, Aly UI (2007). Morphological and molecular characterization of potato microtubers production on coumarin inducing medium. Int. J. Agric. Biol. 9 (5):675-680.

Fietosa T, Bastos JLP, Ponte LFA, Jucá TL, Campos F (2007). Somatic Embryogenesis in Cassava Genotypes from the Northeast of Braz. Arch. Biol. Technol. 50:201-206.

Fotso (2005). *In vitro* organogenesis of some wild plants at multiple uses. Ph D Thesis, UYI Cameroon. 160 p.

Fotso, Ngo Ngwe MFS, Mbouobda HD, Djocgoue PF, Omokolo ND (2013). Micropropagation of *Dioscorea alata* L. from microtubers induced *in vitro.* Afr. J. Biotechnol. 12 (10): 1057-1067.

Gami RA, Parmar SK, Patel PT, Tank CJ, Chauhan RM, Bhadauria HS, Solanki SD (2013). Microtuberization, minitubers formation and *in vitro* shoot regeneration from bud sprout of potato *(Solanum tuberosum* L.) cultivar *K. badshah.* Afr. J. Biotechnol. 12(38):5640-5647.

Gulati A, Jaiwal PK (1996). Micropropagation of *Dalbergia sissoo* from nodal explants of mature trees. Biol. Plantarum. 3(2):169-175.

Guo Y, Zhang Z (2005). Establishment and plant regeneration of somatic embryogenic cell suspension cultures of the *Zingiber officinale* Rosc. Sci. Hortic. 107: 90-96.

Hoque ME (2010). *In vitro* tuberization in potato (*Solanum tuberosum* L.). Plant Omics J. 3(1):7-11.

Kartha KK, Gamborg OL, Constabel F, Shyluk JP (1974). Regeneration of cassava plants from apical meristems. Plant Sci. Lett. 2:107-113.

Koda Y, Kikuta Y (1991). Possible involvement of jasmonic acid in tuberization of yam plants. Plant Physiol. 32:629-633.

Konan NK, Chopke C, Carcamo R, Beachy RN, Fauket C (1997). An efficient mass propagation system for cassava (*Manihot esculenta* Crantz) based on nodal explants and axilarry bud-deived meristem. Plant Cell Rep. 16:444-449.

Konan NK, Sangwan RS, Sangwan-Norreel BS (1994). Efficient *in vitro* shoot-regeneration systems in cassava (*Manihot esculenta* Crantz). Plant Breed. 113:227-236.

Ma GH, Xu QS (2002). Induction of somatic embryogenesis and adventitious shoots from immature leaves of cassava. Plant Cell Tissue Organ Cult. 70:281-282.

Mantell SH, Hugo SA (1989). Effects of photoperiod, mineral medium strength, inorganic ammonium, sucrose and cytokinin on root, shoot and microtuber development in shoot culture of *Dioscorea alata* L. and *D. bulbifera* L. yams. Plant Cell Tissue Organ Cult. 16:23-37.

Morel G, Wetmore RH (1951). Fern callus tissue culture. Ann. J. Bot.

38:141-143.
Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol. 15:473-479.
Ovono Ondo P, Kevers C, Dommes J (2007). Axillary proliferation and tuberisation of *Dioscorea cayenensis –D. rotundata* complex. Plant Cell Tissue Organ Cult. 91:107-114.
Peter T, Eugene KAF, Amoako TNE (2011). Effect of 2.4-D, explants type and cultivar on the callogenesis expression of cassava (*Manihot esculenta* Crantz) in Ghana. Afr. J. Biotechnol. 10 (46): 9396-9401.
Rani V, Raina SN. (2000). Genetic Fidelity of Organized Meristem-Derived Micropropagated Plants: A Critical Reappraisal. *In Vitro* Cell Dev. Biol. Plant 36(5):319-330.
Roca WM, Henry G, Angel F, Sarria R (1992). Biotechnology research applied to cassava improvement at the International Center of Tropical Agriculture (CIAT). 8-15.
Santos I, Salema R (2000). Promotion by jasmonic acid of buld formation in shoot culture of *Narcissus triandus* L. Plant Growth Regul. 30:133-138.
Schopke C, Taylor N, Carcamo R, Konan NDK, Marmey P, Henshaw GG, Beachy RN, Fauquet C (1996). Regeneration of transgenic cassava plants (*Manihot esculenta* Crantz) from microbombarded embryogenic suspension cultures. Nat. Biotechnol. 14:731-735.
Smith MK, Biggs BJ, Scott KJ (1986). *In vitro* propagation of cassava (*Manihot esculenta* Crantz). Plant Cell Tissue Organ Cult. 6:221-228.
Taylor NJ, Edwards M, Kiernan RJ, Davey CDM, Blakesley D. Henshaw GG (1996). Development of friable embryogenic callus and embryogenic suspension culture systems in cassava (*Manihot esculenta* Crantz). Nat. Biotechnol. 14:726-730.
Tsafack TJJ, Charles GP, Hourmant A, Omokolo ND, Branchard M (2009). Effect of photoperiod and thermoperiod on microtuberization and carbohydrate levels in cocoyam (*Xanthosoma sagittifolium* (L.) Schott). Plant Cell Tissue Organ Cult. 96:151-159.
Vines R (2001). Plant Biotechnology. Biotechnology Information, Virginia Tech. p. 443.
Whingwiri E (2004). NEPAD Pan Africa Cassava Initiative. Paper read at ISTRC-AB Symposium, 31st October-5th November 2004, Whitesands Hotel, Mombasa, Kenya.
White WLB, Arias-Garzon DI, McMahon JM, Sayre RT (1998). Cyanogenesis in Cassava - The Role of Hydroxynitrile Lyase in Root Cyanide Production. Plant Physiol. 116:1219-1225.
Yam TW, Ishihashi S, Arditti J (1991). Callus growth and plantlet regeneration in taro, *Colocassia esculenta var esculenta* (L.). Ann. Bot. 67:317-323.
Yam TW, Webb EL, Arditti J (1990). Callus formation and plantlet development from axillary buds of taro. Planta 180: 458-460.
Yassen RN, Shell RJ, Splittstoesser WE (1995). *In vitro* shoot proliferation and micropropagation of guava (*Psidium guajava* L.) from germinated seedlings. Plant Cell Rep. 14:525-528.
Yongqin C, Jinyu F, Fei Y, Zhongxun L, Yunsheng F (2003). Rapid clonal propagation of *Dioscorea zingiberensis.* Plant Cell Tissue Organ Cult. 73:75-80.
Yu WC, Joyce PJ, Cameron DC, McCown BH (2000). Sucrose utilization during potato microtuber growth in bioreactors. Plant Cell Rep. 19:407-413.
Yuan S, Yan Y-C, Lin H-H (2005). Plant regeneration through somatic embryogenesis from callus cultures of *Dioscorea zingiberensis*. Plant Cell Tissue Organ Cult. 80:157-161.
Zakaria M, Hossain M M, Khalequemain M A, Hossain T, Uddin M Z (2008). *In vitro* tuberization of potato influenced by Benzyl Amino Purine (BAP) and Chloro chloline chloride (CCC). Bangladesh J. Agric. Res. 33(3):419-425.

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Microbial and heavy metal contamination of pineapple products processed by small and medium scale processing enterprises in Rwanda

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Fruit products are increasingly consumed but highly prone to microbial deterioration if not adequately processed and stored. The present study was conducted to evaluate the microbial and heavy metal concentrations of packed pineapple nectars, syrups and jams processed by 10 Small and Medium scale pineapple processing Enterprises (SMEs) over a storage duration of 12 months. Collected samples were analysed to determine whether the levels of microbial and heavy metal concentrations were in line with maximum permissible limits set by Codex Alimentarius Commission (CAC), East African Standards (EAS) and Rwanda Bureau of Standards (RBS). The samples were tested for yeasts and moulds, total plate counts, Faecal coliforms, total coliforms, *Escherichia coli*, *Salmonella*, *Shigella* and *Staphylococcus aureus* using tested International Organization for Standardization (ISO) microbial determination methods. Quantitative determination of heavy metals: zinc, iron, lead, copper, cadmium and aluminium was carried out by Atomic Absorption Spectrophotometer (AAS). Pineapple products were free from most of the microorganisms but only nectars from 30% of SMEs were highly contaminated above the permissible Codex and RBS limits with total plate counts >300 CFU/ml and yeasts and mould counts >300 CFU/ml. The mean levels of zinc, iron, copper and aluminium were within the acceptable recommended Codex and RBS standard values but the levels of lead and cadmium were above those permissible standard values. These results indicated that some fruit processors in Rwanda may not be observing good manufacturing and hygienic practices, leading to a need for improved post-harvest and processing guidelines, better monitoring and enforcement, and additional research into heavy-metal ingress in the manufacturing process.

Key words: Pineapple, juices, jams, microbial contamination, heavy metal, Rwanda.

INTRODUCTION

Pineapple [*Ananas comosus* (L.) Merrill.] is an important tropical fruit. The Philippines, Brazil, Costa Rica, Thailand and China are the main pineapple producing countries in the world, whereas France, Belgium, USA, Netherlands and Japan are the global lead consumers of pineapple products (United Nations Conference on Trade and Development /UNCTAD, 2012). In the last 10 years, the world trade of fresh pineapple, juices and canned fruit

has doubled and the pineapple export industry has developed into a complex export supply chain (UNCTAD, 2012). In Rwanda, pineapple production has increased and it was expected that the production would increase from 30,000 tonnes in 2006 to 120,000 tonnes in 2012 (RHODA, 2008). In order to improve on their income, small and medium scale pineapple processing enterprises have started value addition processes to the pineapple fruit by transforming the fruit into varied processed products namely juices, jams, wines and dried slices (Austin et al., 2009). These products are highly prone to microbial deterioration if not adequately processed and stored (Osuntogum and Aboaba, 2004). A large number of lactic acid bacteria, coliforms, yeasts and moulds cause spoilage of fruit products by fermenting carbohydrates to produce undesirable changes such as production of acids, alcohols and diacetyls affecting the organoleptic properties of the food products (Tribst et al., 2009). Such spoiled products cannot get to the export market since their quality cannot meet the international quality requirements including microbiological quality. Food spoilage can result in health problem as well as economic losses (Loureiro and Querol, 1999).

Codex Alimentarius Commission has limits for certain microorganisms including yeasts and moulds, total plate counts, faecal coliforms, total coliforms, *Escherschia coli*, *Salmonella*, *Staphylococcus aureus* and *Streptococcus faecalis* (Rwanda Standard, 2005). For example the maximum limits which should not be exceeded in concentrated pineapple juice are: total viable counts (10^3 CFU/ml), yeasts and moulds (<1 CFU/ml), coliforms (<1CFU/ml), *E. coli* (<1CFU/ml) *S. aureus* (<1 CFU/ml) and *Salmonella* (<1 CFU/ml). For the jam, the maximum limits are the same as juice but the values are expressed as CFU/g with the exception of total plate counts that should not exceed 10^2 CFU/g (Rwanda Standard, 2008).

Fruit products including pineapple products may provide significant exposure routes to heavy metal contamination and the effect of some metals such as lead and cadmium at low levels have been well proven and their levels above the permissible limits are shown to contribute to serious health problems such as cardiovascular, nervous, kidney as well as bone diseases (Jarup, 2003). Other elements such as aluminium, zinc, iron and copper are very important to human health at very low levels but can lead to toxicity once ingested in high doses (Plum et al., 2010).

Heavy metals are found in the environment either naturally or anthropogenically and their concentration are elevated through waste disposal, smelter stacks atmospheric deposition, fertiliser and pesticides and the application of sludge in arable land (Sobukola et al., 2010; Zheng et al., 2007; Damirözü and Saldamli, 2002). They are non-biodegradable, thermo-stable and can accumulate to toxic levels (Ramesh and Murphy, 2012). Under certain conditions, either essential micronutrients such as copper, zinc and iron or toxic elements such as mercury (Hg), lead (Pb) and cadmium (Cd) in the environment can accumulate to a toxic concentration level, which can result in health damages (European Environment and Health Information System, 2009; Jefferies et al., 1984). Food consumption is one of the major pathways of human exposure to heavy metals and it counts more than 90% of the rest of the other ways of exposure namely inhalation and dermal contact (Loutfy et al., 2006).

Since the increase in numbers of small scale pineapple processing enterprises in Rwanda, there has not been adequate information regarding microbiological and toxicological quality of the processed products. The objective of the current study was therefore to evaluate the effect of storage conditions on microbiological quality and heavy metal contamination of the processed pineapple products by small and medium scale pineapple processing enterprises in Rwanda. The results were measured against their compliance to Rwanda Bureau and Codex Standards requirements.

MATERIALS AND METHODS

Microbiological analysis of juice and jam samples

Processed pineapple juices (nectars and syrups) and jams were collected from 10 pineapple processing enterprises across the country in October 2012. All samples were bottled in plastic bottles for juices and plastic jars for jams at about 40°C. Bottles were manually capped. From each enterprises, 18 bottles (500 ml each bottle) of nectars, 18 bottles (500 ml each bottle) of syrups and 18 jars (250 g each jar) of jams were randomly collected. Samples were immediately transported to the Laboratory of Analysis of Foodstuff, Drugs, Water and Toxics (LADAMET) of the faculty of Medicine at the University of Rwanda (UR). Samples which were not analyzed within 24 h were kept in a refrigerator at 4°C for further analysis of microbial contamination of pineapple products at the initial storage time. Another batch of samples was kept at room temperature (21 to 25°C) for further microbial analysis over storage times of 12 months for syrups and jams and two months for nectars. Microbial analysis was performed every three months for syrups and jams and every one month for nectars. All samples were analyzed for microbial contamination using International Organization for Standardization (ISO) methods (ISO, 1999; ISO, 2001; ISO, 2002; ISO, 2006). All culture media used were manufactured by Biokar, France.

Enumeration of microorganisms

One gram and or one milliltre sample was taken from each pineapple jam jar and or juice bottle under asceptic conditions. It was then placed in sterile test tube containing 9 ml of 0.2%

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Table 1. Microbiological quality of pineapple nectars over storage period of two months (CFU/ml).

Sample	YM	TPC	TC	FC	*E. coli*	SA	SF	S
N_1	<1*	<1	<1	<1	nd	<1	<1	nd
N_2	<1	<1	<1	<1	nd	<1	<1	nd
N_3	>300	>300	<1	<1	nd	<1	<1	nd
N_4	<1	<1	<1	<1	nd	<1	<1	nd
N_5	<1	<1	<1	<1	nd	<1	<1	nd
N_6	>300	>300	<1	<1	nd	<1	<1	nd
N_7	<1	<1	<1	<1	nd	<1	<1	nd
N_8	>300	>300	<1	<1	nd	<1	<1	nd
N_9	<1	<1	<1	<1	nd	<1	<1	nd
N_{10}	<1	<1	<1	<1	nd	<1	<1	nd

N1-N10 represent nectar samples from different pineapple enterprises; *: The values represent the mean of microbial population counts detected at the initial storage time, the end of first and second month of storage. nd, not detected; YM, yeasts and moulds; TPC, total plate count; TC, total coliforms; FC, faecal coliforms; E. Coli, *Escherichia coli*; SA, *Staphyloccocus Aureus*; SF, *Streptococcus faecalis*; S, *Salmonella* spp.

peptone water and shaken at a speed of 2200 revolution per minute (rpm) for 2 min. This gives a 10^{-1} dilution. Further serial dilutions were prepared up to 10^{-5}. Each serial dilution for nectars, syrups and jams was spread-plated in terms of 0.1 ml in triplicate on Sorbitol McConkey Agar (SMAC) for determination of *E. coli* (ISO 16649-2:2001), xylose lysine, deoxycholate agar for determination of *Salmonella* spp. after pre-enrichment in peptone water and enrichment on Rappaport Vassiliadis medium with soya as indicated by ISO (ISO 6579:2002), horizontal method for detection of *Salmonella* spp). Total plate count agar was used for total plate counts (ISO 4833:2003 colony counting technique at 30°C). The counting of microorganisms was done after 72 h of incubation of Petri dishes at 30°C. Yeasts and moulds were counted on Potato Dextrose Chloramphenicol agar after 72 h of incubation of Petri dishes at 25°C (ISO 7954:1987, colony counting technique at 25°C). Baird Parker Agar medium with addition of egg yolk tellurite emulsion was used for determination of *S. aureus* (commercial preparation was available) (ISO6888-1). The counting of all Enterobacteriaceae was done after incubation of Petri dishes for 48 h at 37°C (ISO 4832:1991 colony counting method). The enumeration of total coliforms was done after 48 h of incubation at 37°C of Petri dishes containing the violet red bile lysine agar medium (ISO 4832-2006 (E)). All culture media were prepared according to the indicated guidelines by the manufacturer (Biokar, France) and the results were expressed as CFU/ml for juices or CFU/g for jams.

Determination of heavy metals

Acid digestion was performed by placing 20 g of each jam sample and or 20 ml of juice/water in a 100 ml volumetric flask. Then, 10 ml of a concentrated hydrochloric acid (1%) were added to the sample and thoroughly mixed. After 30 min, the mixture was made up to volume of 100 ml with distilled deionised water. The solution was again mixed thoroughly and filtered using filter paper (no. 389) and a funnel. The solution was then stored in a refrigerator at 4°C for analysis. Minimal dilution of the sample was chosen to provide a more concentrated digestate solution so that a lower concentration can be measured in the sample. In this case, dilution factor of five was used (Perkin-Elmer Corporation,1996).

Standard stock solutions for Cu, Zn, Fe, Pb, Cd and Al were used. Series of working standards were prepared by addition of the blank solution of HCl (1%), distilled- deonised water and HNO_3 (1%) as cleaning solutions. The blanks were used for zeroing the instrument before each analysis to avoid matrix interference. Appropriate quality assurance procedures and precautions (including calibration of AAS repeated periodically) were carried out to ensure reliability of the results and samples were carefully handled to avoid cross-contamination. All chemicals used were of reagent grade (high purity HNO_3 (65%), HCl (37%); Merck, Germany). Before starting any analysis, all equipment and containers were soaked in 10% HNO_3 for 24 h and rinsed touroughly with distilled water before use. Distilled-deonised water was used throughout the study. Reagent blank determinations were used to correct the instrument readings. The analyses were performed using Atomic Absorption Spectrophometer (AAS) (Perkin-Elmer, model 200). For validation of the analytical procedure, repeated analyses of the samples were done three times and the appropriated lamps such as multilamp for copper and iron (Fe) and unilamp for zinc (Zn), Cadmium (Cd), lead (Pb) and Aluminium (Al) were used.

Statistical analysis

Microbiological and heavy metal data was captured into Microsoft Excel Software, version 2010 which was also used to calculate means and standard deviations.

RESULTS AND DISCUSSION

Microbial counts

Results show that at the initial stage, after the second and the third month of storage at room temperature, pineapple nectars from 70% of both enterprise categories complied with the Codex and Rwanda Standard requirements. The results for two months of storage are given in (Table 1). According to Codex Alimentarius Commission (2003), yeasts and moulds, total coliforms, faecal coliforms, *E. coli*, *Salmonella* spp. and *S. aureus* should be absent in pineapple fruit juices and jams.

The remaining 30% of the visited enterprises had their

Table 2. Microbiological quality of pineapple syrups over storage period of 12 months (CFU/ml).

Sample	YM	TPC	TC	FC	*E. coli*	SA	SF	S
S_1	<1*	<1	<1	<1	nd	<1	<1	nd
S_2	<1	<1	<1	<1	nd	<1	<1	nd
S_3	<1	<1	<1	<1	nd	<1	<1	nd
S_4	<1	<1	<1	<1	nd	<1	<1	nd
S_5	<1	<1	<1	<1	nd	<1	<1	nd
S_6	<1	<1	<1	<1	nd	<1	<1	nd
S_7	<1	<1	<1	<1	nd	<1	<1	nd
S_8	<1	<1	<1	<1	nd	<1	<1	nd
S_9	<1	<1	<1	<1	nd	<1	<1	nd
S_{10}	<1	<1	<1	<1	nd	<1	<1	nd

S_1-S_{10} represent syrups samples different pineapple enterprises. The values represent the mean of microbial population counts detected at 0, 3,6,9 and 12 month of storage. nd, not detected; YM,Yeasts and Moulds; TPC, Total Plate Count; TC, Total Coliforms; Faecal Coliforms; E. Coli: *Escherichia coli*; SA, *Staphyloccocus Aureus*; SF, *Streptococcus Faecalis*; S, *Salmonella* spp

Table 3. Microbiological quality of pineapple jam samples over storage period of 12 months (CFU/ml).

Sample	YM	TPC	TC	FC	*E. coli*	SA	SF	S
J_1	<1*	<1	<1	<1	nd	<1	<1	nd
J_2	<1	<1	<1	<1	nd	<1	<1	nd
J_3	<1	<1	<1	<1	nd	<1	<1	nd
J_4	<1	<1	<1	<1	nd	<1	<1	nd
J_5	<1	<1	<1	<1	nd	<1	<1	nd
J_6	<1	<1	<1	<1	nd	<1	<1	nd

J_1-J_6 represent jam samples from different pineapple enterprises. The values represent the mean of microbial population counts detected at 0,3,6,9 and 12 month of storage. nd: not detected ; . nd, not detected; YM, yeasts and moulds; TPC, total plate count; TC, total coliforms; FC, Faecal coliforms; E. Coli, *Escherichia coli*; SA, *Staphyloccocus Aureus*; SF, *Streptococcus faecalis*; S, *Salmonella* spp

ready to drink pineapple nectars contaminated above the acceptable limits of yeasts and moulds. Total plate counts were estimated at > 2.47 log CFU/ml. These results corroborate with the results reported by Tournas et al. (2006) where 22% of 65 pasteurised juices from the retail markets were contaminated with yeasts and moulds with counts ranging from 1 to 6.83 log CFU/ml. Tribst et al. (2009) argued that such contamination may be due to post-pasteurisation contamination or highly contaminated raw material used. This was obvious in this case since at the second month of storage, the juices nectars from those three enterprises had already shown abundant gas formation, which deformed and sometimes broke up the plastic packages. Besides that, there was formation of films and off flavour with a fermentation smell (alcohol and esters) and undesirable taste. These are typical indication of yeast and mould contamination in fruit juices during their storage time (Tournas et al., 2006; Loureiro and Queroly, 1999).

The presence of high load of yeasts and moulds in these ready to drink juices nectars may be due to poor hygiene conditions at these small scale enterprises. These enterprises do not have a proper food processing environment as set by Codex Alimentarius Commission in the good hygienic and good manufacturing practices regulations at food processing enterprise levels (Austin et al., 2009). In addition, the sterilization of the processed products was not fully completed because since it has been proven that the shelf-life of fruit juice can be prolonged by conventional heat treatment to inactivate enzymes and microorganisms (Bates et al., 2001). The product has therefore to be subjected to a heat treatment of 70°C for 30 min, followed by filling and a rapid heating at 90°C for a holding time of 30 s with rapid cooling as suggested by Aguilar-Rosas et al. (2007) and Bates et al. (2001). This is a challenge to the studied enterprises that do not have in place a standard pasteurizing system and which sterilise the product employing boiling pots and package in plastic containers at 40°C (Mukantwali et al., 2013). Pineapple syrups and jams evaluated quarterly for 12 months did not show any microbial growth during the storage period (Tables 2 and 3).

The inhibition of microbial growth in these products may be due to the presence of sulphur dioxide, high

Table 4. Heavy metal concentrations in pineapple nectars (mean±SD in mg/l).

Nectars	Cu	Fe	Pb	Zn	Cd	Al
N_1	0.74±0.01	1.35±0.01	0.86±0.00	1.74±0.01	0.43±0.22	3.00±0.00
N_2	1.01±0.00	1.53±0.11	0.59±0.00	0.83±0.00	0.32±0.16	2.31±0.02
N_3	3.13±0.11	1.76±0.01	0.74±0.02	0.9±0.11	0.37±0.15	2.36±0.02
N_4	2.66±0.15	1.2±0.12	0.55±0.02	2.7±0.15	0.30±0.11	2.56±0.02
N_5	2.86±0.05	0.96±0.01	0.78±0.00	2.34±0.057	0.41±0.14	3.28±0.02
N_6	1.15±0.01	1.05±0.01	0.80±0.00	1.85±0.011	0.21±0.10	2.34±0.02
N_7	1.01±0.00	1.39±0.01	0.25±0.00	1.82±0.006	0.23±0.12	2.36±0.02
N_8	1.13±0.05	1.76±0.01	0.98±0.00	0.64±0.057	0.21±0.10	3.15±0.05
N_9	1.75±0.01	1.48±0.02	0.75±0.00	0.95±0.015	0.05±0.03	3.73±0.02

N_1-N_{10} represent sample nectars from different enterprises. The results of the heavy metals given in this table are the average of three independent experiments. However, the levels of Pb and Cd, which ranged from 0.25 to 0.98 mg/l and 0.05 to 0.93 mg/l were above the permissible limits 0.3 mg/l and 0.1mg/l, respectively for these minerals. Higher levels of Pb and Cd than the recommended levels in the nectars, make these products unacceptable at local, regional and international markets.

Table 5. Heavy metal concentrations in pineapple syrups (mean±SD in mg/l).

Syrup	Cu	Fe	Pb	Zn	Cd	Al
S_1	3.26±0.34	0.95±0.00	0.08±0.00	2.17±0.05	0.75±0.15	4.10±0.05
S_2	3.50±0.00	1.15±0.00	0.42±0.00	2.49±0.00	1.23±0.22	3.18±0.07
S_3	3.19±0.00	1.01±0.01	0.03±0.00	1.22±0.00	0.26±0.26	2.80±0.00
S_4	0.24±0.15	0.95±0.64	1.74±0.53	1.01±0.31	0.13±0.19	3.76±0.02
S_5	2.66±0.00	1.92±0.02	0.87±0.00	1.32±0.00	0.77±0.44	3.31±0.02
S_6	3.17±0.00	1.23±0.02	0.98±0.00	2.21±0.00	0.75±0.63	3.38±0.05
S_7	2.55±0.00	1.65±0.00	0.05±0.00	0.63±0.00	0.00±0.00	2.65±0.00
S_8	2.68±0.00	1.13±0.05	0.25±0.00	2.67±0.00	0.02±0.03	3.31±0.02
S_9	2.94±0.00	1.35±0.00	0.52±0.00	1.80±0.00	0.02±0.03	3.93±0.05
S_{10}	2.77±0.00	1.05±0.00	0.81±0.00	1.45±0.00	0.65±0.24	2.93±0.02

S_1-S_{10} represents syrup samples from different enterprises. The results of the heavy metals given in this table are an average of three independent experiments

acidity, high sugar content and the effect of the heat treatment during processing as all these four factors are known to be inhibitors of microbial growth (Graham, 2000; Ewaidah, 1988). It has been reported that few bacteria grow below a pH value of 3.5 and this may be the reason why no single bacteria had grown in these products because the pH value of a pineapple juice was expected to be between 3 and 4 (United States Food and Drug Administration, 2007). In addition, inhibition of microbial growth could have been due to high levels of sugars (14.54 to 24.47%) resulting from overheating in syrup and jam products and use of preservatives. A preservative like sulphur dioxide (SO_2) is very detrimental to respiratory system of individuals (Fowie et al., 2006). Similar findings were reported by Basal and Rahman (2007) in Bangladesh where and the absence of total viable count growth in fruit juices was attributed may have been due to the use of higher preservatives than the recommended amount.

Heavy metals

Levels in nectars

Levels in ready to drink nectars from 10 processing enterprises (Table 4) show that, the concentrations of copper, iron, zinc and aluminium were lower than the maximum permissible levels set by the Codex Alimentarius Commission. Their values (with maximum permissible limits in brackets) ranged from 0.74 to 3.1 (5) mg/L for Cu; 0.96 to 1.76 (15) mg/L for Fe; 0.64 to 2.77 (5) mg/L for Zn and 2.28 to 3.73 (8) mg/L for Al.

Levels in syrups

Lower levels than the permissible limits of Cu, Fe, Zn and Al in juice syrups were observed (Table 5). Their concentrations ranged from 0.511 to 0.701 (5) mg/L for

Table 6. Heavy metal concentrations in pineapple jams (mean±SD in mg/kg).

Jams	Cu	Fe	Pb	Zn	Cd	Al
J_1	1.38±0.00	1.73±0.05	0.77±0.00	3.55±0.00	0.01±0. 01	5.00±0.25
J_2	2.04±0.00	2.83±0.05	0.55±0.00	2.16±0.00	0.31±0.32	7.91±0.28
J_3	2.47±0.00	2.53±0.05	0.46±0.00	2.97±0.00	0.58±0.23	7.67±0.06
J_4	3.29±0.00	2.13±0.05	0.33±0.00	0.82±0.00	0.80±0.13	4.50±0.00
J_5	.633±0.05	2.95±0.00	0.35±0.00	2.16±0.00	0.80±0.29	6.50±0.00
J_6	2.63±0.00	2.73±0.05	0.38±0.00	1.53±0.05	1.46±0.38	7.00±0.00

J_1-J_{10} represents jam samples from different enterprises.The results of the heavy metals given in this table are the average of three independent experiments. Knowing the side effects of high ingestions of some of these minerals, there is a need of improving jam processing techniques so as to lower the concencetartions of the heavy metals before being taken to any market.

Cu; 1 to 2.7 (15) mg/L for Fe ; 0.63 to 2.675 (5) mg/L for Zn and 2.65 to 4.10 (8)mg/L for Al. The levels of Pb and Cd were above the permissible limits for 60 and 70% of syrups, respectively and ranged from 0.42 to 1.74 mg/L and 0.13 to 1.23 mg/L, respectively. Only 40 and 30% of the syrups had lower levels of Pb and Cd , respectively, ranging from 0.03 to 0.25 mg/L and 0.00 to 0.02 mg/L, respectively than the maximum limits of 0.3 and 0.1mg/L for Pb and Cd, respectively. It is important to note that the concentrations of Pb in the juices syrups were 11 to 18 times higher than the permissible limit of 0.05 mg/kg set by the Commission of the European Communities (CEC, 2006). Therefore, though the concentrations of the majority of measured heavy metals in both syrups and nectars were within the permissible concentration levels, their commercialisation is not still allowed either in Rwanda or elsewhere. High concentration of Pb causes detrimental side effects such as colic, constipation, anaemia, inducing high blood pressure and cardiovascular diseases in adults and foetal neuro-development and reduced learning capacity in children. High Cd ingestion is known to cause hemorrhaging digestive tract, damage of liver, kidneys and heart and many others (European Environment and Health Information System, 2009).

Levels in jams

Lower levels than the maximum permissible limits of Fe, Al and Pb in 67% of the jams were observed (Table 6). Their concentrations ranged from 1.73 to 2.95 mg/kg (15) mg/l for Fe and 4.50 to 7.91 (8) mg/kg for Al and 0.33 to 0.77 (1) mg/kg for Pb . The levels of Cu, Zn and Cd were above the permissible levels and ranged from 0.63 to 2.47 (0.03) mg/kg for Cu; 0.82 to 3.55 (0.05) mg/kg for Zn and 0.01 to 1.46 (0.01) mg/kg for Cd. Though copper and zinc are recognised to be essential trace elements for several biological functions in human body and though their toxicity is rare (Plum et al., 2010); it is of paramount that their levels be of acceptable standards in processed food products. From the current findings, it is possible that post-harvest, processing and preservation techniques were the source of the high levels of Zn and Cu since fruits, and consequently fruit products, are known to have very low concentrations in these trace elements (Wilson, 2014).

The damaging side effects of high concentrations of specific heavy metals indicates a need of improving jam processing techniques so as to lower the concentrations of the heavy metals prior to further marketing.

Levels in water from enterprises processing sites

The concentrations of some heavy metals in the samples of water collected from the enterprises are shown in Table 7. The results showed low concentrations of heavy metals in water that is used for processing pineapple products. The concentrations ranged from 0.02 to 0.30 mg/L for Cu; not detected to 0.041 for Fe; 0.03 to 2.54 for Zn; 0.03 to 0.56 for Mn and not detected to 0.08 for Cd. Lead was not detected in water samples. The present study revealed therefore that for commercial purposes, processed pineapple products by studied SMEs may pose risks to human health due to the high concentrations of heavy metals, such as Pb and Cd in juices and Cu and Zn in jams. This leads to suggestion that these products are not acceptable at local and even export market levels, knowing their side effects to human health once they are in high concentration. In addition, accessing EU market will not be possible since most of the EU countries had for example reported lead concentration in fruit juices ranging between 0.005 to 0.024 mg/kg (CEC, 2006). Since currently there is no knowledge about possible sources of contamination, it is necessary to establish levels of these metals by monitoring water quality, soil, plant and processing equipment for concen-tartion of these metals that could be a source of contamination and pose potential health hazards (Ramesh and Murphy, 2012).

Conclusions and recommendations

Results showed that pineapple products produced by SMEs in Rwanda were free from microorganisms except

Table 7. Heavy metal concentrations in water from processing enterprises (mean±SD in mg/l).

Water	Cu	Fe	Pb	Zn	Mn	Cd
W_1	0.17±0.05	nd*	nd	0.23±0.09	0.17±0.02	nd
W_2	0.24±0.03	0.412	nd	0.92±0.11	0.14±0.00	nd
W_3	0.30±0.07	0.027	nd	0.03±0.03	0.02±0.03	0.08±0.00
W_4	0.11±0.01	nd	nd	1.58±0.47	0.08±0.00	0.04±0.00
W_5	0.09±0.05	0.102	nd	0.85±0.06	0.56±0.03	nd
W_6	0.02±0.02	nd	nd	0.30±0.14	0.25±0.04	nd
W_7	0.22±0.00	nd	nd	2.54±0.13	0.06±0.02	0.05±0.00
W_8	0.14±0.04	nd	nd	0.21±0.07	0.04±0.01	0.05±0.01
W_9	0.04±0.00	0.214	nd	0.93±0.07	0.03±0.00	nd
W_{10}	0.16±0.02	nd	nd	0.36±0.13	0.04±0.02	0.08±0.00

W_1-W_{10} represents water samples from different enterprises. *nd=not detected. The results of the heavy metals given in this table are the average of three independent. The current findings showing very low levels of evaluated heavy metals in water used for processing lead to suggestion that there could be other sources of heavy metals found in the processed pineapple products, such as pineapple fruits, soil where the pineapple fruits were grown and/or processing equipment.

for 30%; the nectar samples that had higher levels of yeasts and moulds than the permissible levels set by Rwanda Bureau of Standards and Codex Alimentarius Commission. The levels of heavy metal contamination for copper, iron and zinc in juices and lead in jams were within the permissible limits set by the Rwanda Bureau of Standards. However, the lead and cadmium levels were above the permissible limits in nectars and syrups while the levels of copper, zinc and cadmium were above the permissible levels in jams. Therefore, additional research is needed to establish the actual source of heavy metal contamination in pineapple processed products displayed in the open markets and different supermarkets in the country. While doing more research, there is also a need of considering the time of sample collection as the concentrations may depend on the time and season the samples were collected. The research will facilitate intervention to lower the levels in the products due to their health concerns. In addition, processors need to be informed on the requirements by local, regional and international markets so that they strive to comply with the set limits.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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REFERENCES

Aguillar-Rosas SF, Ballinos-Garrubias ML, Nevarez-Morrillon GV, Martin-Belloso O, Ortega Rivas E (2007). Thermal and pulsed electric fields pasteurisation of apple juice. Effects on phytochemical properties and flavour compounds. J. Food Eng. 83(1):41-46.

Austin JE, Jones C, Cavanaugh G (2009). Study on Marketing, Post Harvest, and Trade Opportunities for Fruit and Vegetables in Rwanda. World Bank Group, Final Report. p. 57

Basal MA, Rahman SR (2007). Assessment of Microbiological Quality of ProcessedFruit Juice. Short Communication. Bangladesh J. Microbiol. 24(2):166-168.

Bates RP, Morris JR, Grandall PG (2001). Principles and practices of small and medium scale fruit juice processing. Food and Agriculture Organisation of the United Nations, Rome, Italy. p. 226

Codex Alimentarius Commission (2003). Recommended International Code of Practice General Principles of Food Hygiene CAC/RCP 1-1969, rev. 4-2003. FAO, Rome.

Commission of the European Communities (2006). Commission Regulation (EC) No 1881/2006 of 19 December 2006. Setting maximum levels for certain contaminants in foodstuffs (Text with EEA relevance). Official Journal L. 364:1-5.

Damirözü B, Saldamli I (2002). Metallic contamination problem in a pasta production plant. Turkish J. Eng. Environ. Sci. 26:316-365.

European Environment and Health Information System (2009). Expose of children to chemical Hazards in Food. Fact sheet 4.4. WHO, Europe. 9pp.

Ewaidah EH (1988). Studies on Commercially Canned Juices Produced Locally in Saudi Arabia: Part 2--Physicochemical, Organoleptic and Microbiological Assessment. Food Chem. 29:88-96.

Fowie J, Gasso P, Benford DJ (2006). The short term effects of carcinogens and sulphur dioxide on the nuclear size of rat nasal epithelial cell. J. Appl. Toxicol. 10(1):29-38.

Graham WG (2000). Preservation: past, present and future. British Medical Bulletin 56 (1):84-94.

International Organization for Standardisation (1999). Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) - Part 1:Technique using Baird-Parker

agar medium. Reference number ISO 6888-1:1999(E). First edition. ISO, Geneva. 11p.
International Organization for Standardisation (2001). Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of glucuronidase positive *Escherichia coli*. Reference number ISO 16649-1. First edition. ISO,Geneva. 14pp.
International Organization for Standardisation (2002). Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp. Reference number ISO 6579:2002(E). Fourth edition. ISO, Geneva. 34pp.
International Organization for Standardisation (2006). Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of coliforms - Colony-count technique. Reference number ISO 4832:2006(E). Third edition. ISO, Geneva.12pp.
Jarup L (2003). Hazards of heavy metals contamination. Br. Med. Bull. 68:167-182.
Jefferies DJ, Freestone P (1984). Chemical analysis of some coarse fish from a Suffolk River carried out as part of the preparation for the firstrelease of captive-bred otters. J Otter Trust 1:17-22.
Loureiro V, Querol A (1999). The prevalence and control of spoilage yeasts in foods and beverages. Trends in Food Science & Technology 10:356-365.
Loutfy N, Fuerhacker M, Tundo P, Raccanelli S, El Dien AG, Ahmed MT (2006). Dietary intake of dioxins and dioxin-like PCBs, due to the consumption of dairy products, fish/seafood and meat from Ismailia city, Egypt. Sci Total Environ 370:1-8.
Mukantwali C, Laswai H, Tiisekwa B, Wiehler S, Brat P (2013). Could good hygienic practices reduce the microbial population on pineapple fruits? Acad. J. Agric. Res. 1(7):114-121.
Osuntogum B, Aboaba OO (2004). Microbial and physicochemical evaluation of non alcoholic Beverages . Pak. J. Nutr. 3(3):188-192.
Plum LM, Rink L, Haase H (2010). The essential Toxin: Impact of zinc on human health. Int. J. Environ. Res. Public Health 7(4):1342-1365.
Ramesh HL, Murthy VNY (2012). Assessment of Heavy Metal Contamination in Green Leafy Vegetables Grown in Bangalore Urban District of Karnataka. Advances in Life Science and Technology 6:40-51.
RHODA (2008). A Survey report on the Status of Horticulture in Rwanda. Rwanda Horticultural Development Authority, Kigali, Rwanda. 85pp.
Rwanda Standard (2005). Concentrated Pineapple Juice Preserved Exclusively by Physical means Specification. Reference number . RS CODEXSTAN 85-2005. First edition. RS, Kigali, Rwanda. 7pp.
Rwanda Standard (2008). Jam, Jellies, Marmalades specifications. First edition. RS 136:2008. Rwanda Bureau of Standards, Kigali, Rwanda. 22pp.
Sobukola OP, Adeniran OM, Odedairo AA, Kajihausa OE (2010). Heavy metal of some fruits and leafy vegatables from selected markets in Lagos, Nigeria. Afr. J. Food Sci. 4(2):389-393.
Tournas VH, Heeres J, Burgess L (2006). Moulds and yeasts in fruit salads and fruit juices. Food Microbiol. 23:684-688.
Tribst AAL, de Souza Sant Ana A, de Massaguer PR (2009). Review: Microbiological quality and safety of fruit juices past, present and future perspectives. Crit. Rev. Microbiol. 35(4):310-339.
United Nations Conference on Trade And Development (2012). Agricultural Products.[http://www.unctad.info/en/Infocomm/Agricultural_Products/] site visited 6/6/2014.
United States Food and Drug Administration (2007). Approximate pH of foods and food products. [http://www.cfsan.fda.gov/~comm/lacf-phs.html] site visited 5/6/2014.
Wilson, L (2014). Copper toxicity Syndrome.[http://drlwilson.com/articles/copper_toxicity_syndrome.htm] site visited 7 /6/2014.
Zheng N, Wang Q, Zhang X, Zheng D, Zhang, Zhang S (2007). Population health risk due to dietary intake of heavy metals in the industrial area of Huludao city, China. Science of the Total Environment 387:96-104.

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Evaluation of genotoxicity of profenofos to freshwater fish *Channa punctatus* (Bloch) using the micronucleus assay

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The present study investigates the genotoxicity of profenofos (PFF), an organophosphate pesticide in erythrocyte cells of freshwater fish, *Channa punctata* (Bloch) using micronucleus assay. The 96 h LC_{50} value of PFF (50% EC) was estimated for the fish species in a semistatic system and then 50% of LC_{50} was determined as 1.15 ppb. The fish specimens were exposed to 1.15 ppb sublethal concentration of the pesticide and samplings were done on 24, 48, 72 and 96 h post exposure for assessment of DNA damage by micronucleus assay. The study confirms that PFF is toxic for aquatic organism and the micronucleus assay is a useful tool in determining the potential genotoxicity and mutagenicity of xenobiotic compounds and can be considered as sensitive parameter for toxicity monitoring program.

Key words: Genotoxicity, profenofos, *Channa punctatus,* DNA damage, micronucleus assay.

INTRODUCTION

The pesticides and related chemicals originating from human activities or agricultural farming are discharged directly or indirectly into waterbodies. The presence of these chemicals in the environment has become a global problem. The studies have shown that the reproduction, growth and development of organisms, including invertebrates, amphibians, reptiles, fish, birds and mammals may have interacted with these chemicals and interfere with the endocrine system and other hormonal processes (Khan and Law, 2005; Kayhan et al., 2007).

Profenofos (O-4-bromo-2-chlorophenyl-O-ethyl S-propyl phosphorothioate), a broad-spectrum organophosphate pesticide is widely used for agricultural and household purposes in India and also in developing and developed countries. Profenofos (PFF) had been investigated to be highly toxic to different organisms including mammals, insects and fish. It has also been classified as moderately hazardous (toxicity class II) pesticide by WHO and it has a moderate order of acute toxicity following oral and dermal administration (Pandey

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Table 1. Characteristics/ properties/ specification of the test chemical.

Parameter	Characteristics/ properties/ specification of pesticide
Common Name	Profenofos
Chemical/ Product Name	(*RS*)-*O*-4-bromo-2-chlorophenyl *O*-ethyl S-propyl phosphorothioate
Chemical/ Product Name	CELCRON
Grade	EXCEL crop care Ltd., Mumbai
WHO and EPA toxicity class	II, Moderately toxic
CAS No.	41198-08-7
Alkyl groups	*S*. propyl-*O*. ethyl

et al., 2011a).

Fishes are considered as sensitive organism in toxicity studies and can play a significant role in assessing potential risks associated with contamination in aquatic environment by environmental contaminants. Fishes can respond to mutagens at low concentrations of toxicants in a manner similar to higher vertebrates (Pandey et al., 2011b).

The micronucleus (MN) test is an *in vivo* and *in vitro* short-time screening test, developed by Schmid (1975), and is widely used to detect genotoxic assessment (Saleh and Alshehri, 2011). It is the most frequently used bio-markers for genotocixity testing in aquatic environments. The MN test has gained high relevance in bio-monitoring of aquatic environments due to nucleated nature of fish erythrocytes. The MN is a chromatin mass in the form of small nuclei which appear within the cytoplasm and close to the main nucleus in interphase cells. They are originated spontaneously or as consequence of clastogenic and/or aneugenic effects, which ultimately generate acentric chromosomal fragments and/or lagging chromosomes during the mitotic anaphase (Betancur et al., 2009). Several researcher showed that the micronucleus (MN) test is one of the simple, sensitive, reliable, least expensive and rapid screening system for both clastogenic (chromosome breakage, formation of a centric fragments), eugenic (chromosome lagging and effects on spindle) and genotoxic effects of xenobiotic chemicals under field and laboratory conditions (Pandey et al., 2009; Chaudhary et al., 2006; Ali et al., 2008).

The information regarding the mutagenic and genotoxic nature of profenofos in aquatic organism is rare, especially the data pertaining to its effects on fishes. The present study investigates the genotoxic effects of profenofos using MN assay in erythrocytes cells of *C. punctatus* exposed *in vivo*.

MATERIALS AND METHODS

Experimental fish specimens

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The fish *C. punctatus* (Bloch) belongs to family: Channidae and order: Perciformes. It was obtained from the local market and acclimatized in the laboratory condition for 10 days before experimentation. They were kept in a large holding tank of 1000 L in capacity during acclimatization. Length and weight of the fish ranged from 12.0 ± 3.0 cm and 23 ± 2.0 g, respectively. A set of 10 acclimatized fish specimens was randomly selected for experiment. Fishes were fed on boiled chicken, eggs or poultry waste material daily at the rate of approximately 4% of fish body weight.

Test chemical

The pesticide used for this study, technical-grade profenofos (EC50) with product name CELCRON (manufactured by EXCEL Crop Care Ltd., Mumbai) was purchased from local market. The test chemical specifications are summarized in Table 1.

In vivo exposure experiment

The fishes were exposed to profenofos 1.15 ppb (50% of LC 50); aforementioned test concentrations of PFF in a semi-static system for 96 h, Keeping 10 fish in each test concentration in 20 L of water in a 50-L plastic tub as an aquarium without change of water. No crowding stress was observed during experimentation. The exposure was continued up to 96 h and tissue sampling was done at intervals of 24, 48, 72 and 96 h on each sampling day. The erythrocytes were collected and immediately processed for MN. Feeding was stopped 24 h before exposure, and fish were not fed during the experimentation period (Pandey et al., 2011a). The physico-chemical properties of test water, namely temperature, pH, dissolved oxygen, electrical conductivity and total hardness were analysed by using a digital analyzer.

Micronucleus test

Peripheral blood samples obtained from the caudal vein were smeared on clean microscopic slide. The slides were then fixed by dipping in methanol for 10 min and left to air-dry at room temperature and finally stained with 6% Giemsa in 0.04 M phosphate buffer (pH = 6.8) for 30 min. After dehydration through graded alcohol and clearing in xylene, slides were mounted in DPX which is a mixture of distyrene (Polystyrene), plasticizer (Tricresyl phosphate) and xylene. The slides were observed under a light microscope (Leitz Wetzlar Germany; Type 307-083.103; oil immersion lens, 100/1.25) and 1000 cells from each specimen were examined for the presence of MN. The MN frequency was calculated as:

$$\text{MN \%} = \frac{\text{Number of cells containing micronucleus}}{\text{Total number of cells counted}} \times 100$$

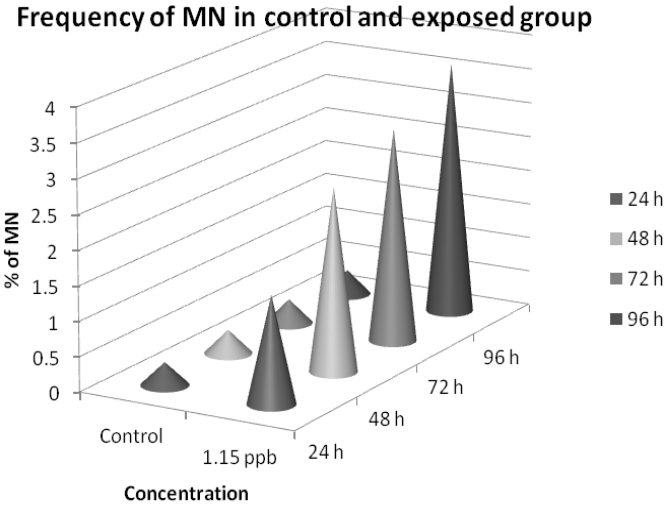

Figure 1. Profenofos concentration response relationship of MN frequency in the erythrocytes of *C. punctatus* for multiple sampling times.

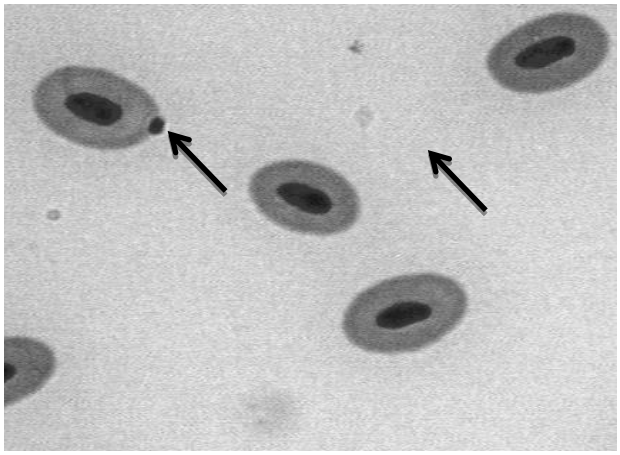

Figure 2. Micronuclei formation in the erythrocytes after exposure to profenofos.

Statistical analysis

The percentage of MN frequency among different exposure intervals and concentrations were compared using the Mann-Whitney test.

RESULTS AND DISCUSSION

The temperature of test water varied from 18.2 to 24.6°C and pH values ranged from 7.1 to 8.3. The dissolved oxygen (DO) ranged from 6.0 to 8.4 mgl^{-1}. The electrical conductivity of the water ranged from 250 to 304 µM cm^{-1} while total hardness ranged from 166 to 185 mg l^{-1} during experiment period.

The fish specimens were exposed *in vivo* to the aforementioned test concentrations of PFF than the control groups at different time periods and the result of MN analysis in erythrocytes of *C. punctatus* for the control and exposed group concentrations of PPF was significant in the fish specimens. Higher induction of MN with the highest MN frequency was recorded at 50% of LC_{50} at 96 h (3.653%), whereas the lowest induced MN frequency was recorded at 50% of LC_{50} at 24 h (1.538%) in blood erythrocyte with 96 h exposure. The observed MN varied from cell to cell. In some cells MN were found attached to the cell wall or boundary while others were located near the main nucleus. The results are summarized in Figures 1 and 2.

Several researchers reported a dose-dependent increase in the induction of MN in peripheral blood of Fish (Chaudhary et al., 2006; Ali et al., 2008; Normann, et al., 2008; Ali et al., 2009; Betancur et al., 2009; Nwani et al., 2010; Saleh and Alshehri, 2011) in response to pesticides, heavy metals and other toxicants. In fishes, the damages are very significant, making it possible to monitor the environmental health state through this parameter (Normann et al., 2008). This study emphasizes the importance of the peripheral blood MN assay and suggests its broader application as an early biological marker of exposure of fish to clastogenic pollutants in the aquatic environment.

Erythrocyte MN test in fish was also widely applied for genotoxicity assessment of aquatic organism *in situ* using native or caged animals following different periods of exposure. Genotoxicity biomarkers must be an integral part of the suite of biomarkers considered as exposure to genotoxic agents which may exert damage beyond that of the individual and may be active through several generations (Magni et al. 2006).

The fish respond to toxic agents similar to higher vertebrates and can allow the assessment of substances that are potentially hazardous to humans. However, the low amount of DNA per cell, the large numbers of small chromosomes and the low mitotic activity in many fish species impaired the metaphase analysis of chromosomal damage and sister chromatid exchanges (Bolognesi and Hayashi, 2011). The MN test is one of the suitable methods for assessing DNA damage at the chromosome level. It permits to measure chromosome loss and chromosome breakage (Fenech, 2000; Hovhannisyan, 2010). The MN assay was developed as a simpler short-term screening test and now accepted as valid alternative to the chromosome aberration assay. In this method, chromosome aberrations are detected indirectly *via* chromatin loss from the nucleus leading to MN in the cytoplasm of the cell (Kirsch-Volders et al., 2003; Hovhannisyan, 2010). It is crucial to assess genotoxicity and cytotoxicity of the environmental pollutants on aquatic organisms. In fish, there are several

types of nuclear lesions whose origin is not still understood (Ayllon and Garcia-Vazquez, 2000; Guner and Muranli, 2011). Toxic chemicals produced nuclear abnormalities thus could help to know the potential risks of water quality as well as the health of fish species.

The results demonstrate that the technical-grade PFF was found to be genotoxic to fishes, which indicates that there is serious apprehension about the potential danger of this pesticide to aquatic organisms. Thus, this is encouraging for judicious and careful use of pesticide in agricultural and non-agricultural practices and also ensures that adverse effects on aquatic organism, human health and the environment are prevented.

Conflict of Interests

The author(s) have not declared any conflict of interests.

REFERENCES

Ali D, Nagpure NS, Kumar S, Kumar R, Kushwaha B (2008). Genotoxicity assessment of acute exposure of chlorpyrifos to freshwater fish *Channa punctatus* (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. Chemosphere 71:1823-1831.

Ali D, Nagpure NS, Kumar S, Kumar R, Kushwaha B, Lakra WS (2009). Assessment of genotoxic and mutagenic effects of chlorpyrifos in freshwater fish *Channa punctatus* (Bloch) using micronucleus assay and alkaline single cell gel electrophoresis. Food Chem. Toxicol. 47:650-656.

Ayllon F, Garcia-Vazquez E (2000). Induction of micronuclei and other nuclear abnormalities in European minnow Phoxinus and mollie Poecilia latipinna: an assessment of the fish micronucleus test. Mutat. Res. 467(2):177-186.

Betancur PI, Palacio-Baena JA, Camargo-Guerrero M (2009). Micronuclei test application to wild tropical ichthyic species common in two lentic environments of the low zones in Colombia. Actual. Biol. 31(90):67-77.

Bolognesi C, Hayashi M (2011). Micronucleus assay in aquatic animals. Mutagenesis 26 (1):205-213.

Chaudhary R, Pandey S, Kushwaha B, Gaur KK, Nagpure NS (2006). Fish micronucleus assay: a sensitive tool for ecogenotoxicity studies. J. Ecophysiol. Occup. Health 6:143-147.

Fenech M (2000). The *in vitro* micronucleus technique. Mutat. Res. 455:81-95.

Guner U, Muranli FDG (2011). Micronucleus test, nuclear abnormalities and accumulation of Cu and Cd on *Gambusia affinis* (Baird and Girard, 1853). Turk. J. Fish. Aquat. Sci. 11:615-622.

Hovhannisyan GG (2010). Fluorescence *in situ* hybridization in combination with the comet assay and micronucleus test in genetic toxicology. Mol. Cytogenet. 3:17.

Kayhan FE, Golak S, Kog ND, Golak A (2007). Investigation of organochlorine pesticide residues in liver and muscle tissues of Atlantic Bluefin tuna (*Thunnus thynnus* L., 1758) from gulf of antalya, mediterranean, Turkey. Rapp. Comm. Int. Mer Médit. 38: 276.

Khan MZ, Law FCP (2005). Adverse effects of pesticides and related chemicals on enzyme and hormone systems of fish, amphibians and reptiles: a review. Proc. Pak. Acad. Sci. 2:315-323.

Kirsch-Volders M, Sofuni T, Aardema M, Albertini S, Eastmond D, Fenech M, Ishidate MJ, Kirchner S, Lorge E, Morita T, Norppa H, Surrallés J, Vanhauwaert A, Wakata A (2003). Report from the *in vitro* micronucleus assay working group. Mutat. Res. 540: 153-163.

Magni P, De Falco G, Falugi C, Franzoni M, Monteverde M, Perrone E, Sgro M, Bolognesi C (2006). Genotoxicity biomarkers and acetylcholinesterase activity in natural populations of Mytilus galloprovincialis along a pollution gradient in the Gulf of Oristano (Sardinia, western Mediterranean). Environ. Pollut. 142 (1): 65-72.

Normann CABM, Moreira JCF, Cardoso VV (2008). Micronuclei in red blood cells of armored catfish *Hypostomus plecotomus* exposed to potassium Dichromate. Afr. J. Biotechnol. 7(7):893-896.

Nwani CD, Lakra WS, Nagpure NS, Kumar R, Kushwaha B, Srivastava SK (2010). Mutagenic and genotoxic effects of carbosulfan in freshwater fish *Channa punctatus* (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. Food Chem. Toxicol. 48(1):202-208.

Pandey AK, Gurbani D, Bajpayee M, Parmar D, Ajmani S, Dhawan A (2009). *In silico* studies with human DNA topoisomerase-II alpha to unravel the mechanism of *in vitro* genotoxicity of benzene and its metabolites. Mutat. Res. 661: 57-70.

Pandey AK, Nagpure NS, Trivedi SP, Kumar R, Kushwaha B (2011b). Kushwaha B. Profenofos induced DNA damage in freshwater fish, *Channa punctatus* (Bloch) using alkaline single cell gel electrophoresis. Mutat. Res. 726:209-214.

Pandey AK, Nagpure NS, Trivedi SP, Kumar R, Kushwaha B, Lakra WS (2011a). Investigation on acute toxicity and behavioral changes in *Channa punctatus* (Bloch) due to organophosphate pesticide profenofos. Drug Chem. Toxicol. 34:424-428.

Saleh KA, Alshehri MA (2011). The intensity of pollutant genotoxicity in lake uluabat: investigation of the peripheral erythrocytes of *Cyprinus carpio*. Afr. J. Biotechnol. 10 (71): 16045-16050.

Schmid W (1975). The micronucleus test. Mutat. Res. 31:9-15.

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Nigerian mistletoe *(Loranthus micranthus* Linn) aqueous leaves extract modulates some cardiovascular disease risk factors in monosodium glutamate induced metabolic dysfunction

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The safety of monosodium glutamates (MSG's) usage has generated much controversy locally and globally. This study investigates the efficacy of *Loranthus micranthus* in modulating the metabolic disorder associated with MSG intake. Thirty five (35) female rats randomized to three groups labeled 1 (7 rats), 2 (21 rats) and 3 (7 rats), respectively were used. They were administered with normal saline, MSG (200 mg/Kg) and MSG (200 mg/kg) + *L. micranthus* (600 mg/Kg), respectively for 28 days. Seven rats were then sacrificed from each group. The remaining rats in group 2 were then redistributed to groups labeled 4 and 5 consisting of 7 rats each. MSG was then withdrawn from all the rats. Group 4 was treated with *L. Micranthus* (600 mg/Kg) while normal saline was administered to group 5. All administrations were carried out for 30 days. The rats were then sacrificed and the serum was used for analysis. Analysis showed increase total cholesterol, triglyceride, low-density lipoprotein (LDL) - cholesterol, glucose and total protein concentrations and a reduced high-density lipoprotein (HDL) - cholesterol (when MSG was administered). These parameters were restored to normal with *L. micranthus* treatment. Although no histological change was observed in the uterus, there was a significant increase in organs relative weight when rats were placed on MSG but this was reversed with *L. micranthus*. The study suggests the efficacy of *L. micranthus* in reversing cardiovascular disorder and its ability to prevent MSG induced fibroid in rat.

Key words: Cardiovascular diseases, cholesterol, Lipid profile, *Loranthus micranthus*, of monosodium glutamates (MSG).

INTRODUCTION

A report by World Health Organization (WHO) indicates that over 80% of the world population still relies on herbs for treatment of diseases and in the past few decades, there has been an increasing demand for medicinal plants

and plant products as alternative to orthodox medicines especially in developing countries (Bright, 2013; WHO, 2008).

Mistletoes are a polyphyletic group of flowering plants comprising over 1300 species from a broad range of habitats across all continents except Antarctica (Nickrent, 2001). They are semiparasitic plants and grow on various host trees and shrubs where they survive by depending on their respective host for mineral nutrition and water, although they produce their own carbohydrates through photosynthesis (Griggs, 1991). Mistletoe was described as “an all-purpose herb” due to its rich traditional uses and it has been widely used in ethnomedicine for various purposes, including antihypertensive, anticancer, anti-spasmodic, antidiabetic and for treatment of epilepsy, headache, infertility, menopausal syndrome and rheumatism (Moghadamtousi et al., 2013).

Nigeria has wide distribution of mistletoes with different local names that depend on the area where they occur. *L. micranthus*, a member of the Loranthaceae family is a Nigeria species of the African mistletoe. It grows on various host trees including *Persia americana*, *Baphia nitida*, *Kola acuminata*, *Pentaclethra macrophylla*, and *Azadirachta indica* (Osadebey et al., 2012, Moghadamtousi et al., 2013). The plant is widely reputed in folk medicine for its use as antimicrobial, antihypertensive, anticancer, and antidiabetic agent and also for the treatment of headache, infertility, epilepsy, cardiovascular diseases, menopausal syndrome, agglutination, and rheumatism. Some of these ethnomedicinal uses have been supported with scientific data in the literatures (Nkanu et al., 2002; Osadebe and Ukweze; 2004, Grossarth et al., 2007; Orji et al., 2013). Of particular interest to this study is the reported use of the plant among some local people in Nigeria for the management of fibroid [an overgrowth of tissue in the endometrium (inner lining of the uterus)] in women.

Monosodium glutamate (MSG), (2-amino pentane dioic or 2-amino glutaric acid) is the sodium salt of the non-essential amino acid glutamic acid commonly used as a flavor enhancer especially in foods. It is a popular condiment in Nigeria and marketed as Ajinomoto*, v*edan, vetsin, Accent and Tasting powder (Palamisamy et al., 2012; Ogunlabi et al., 2014). Despite its wide uses, the safety of MSG's usage has generated much controversy. In Nigeria, many people often use MSG as a bleaching agent for the removal of stains from clothes (Obochi et al., 2009; Eweka, 2007). As a result, there is a growing apprehension that its excellent bleaching properties could be harmful to the stomach mucosa or worse still inducing terminal diseases in consumers when ingested as a flavor enhancer in food (Huthman et al., 2009; Eweka, 2007). To support this, some authors have reported brain damaging potentials, stunted skeletal development, behavioral aberration, neuro-endocrine disorder, possible learning deficits, seizures (epileptic fits), learned taste aversion and hyperglycemia as possible adverse effects of abuse of the use of MSG (Farombi and Onyema, 2006; Onyema et al., 2006; Ortiz et al., 2006).

Some studies have shown that MSG induces oxidative stress and hepatotoxicity in rats (Onyema et al., 2012; Onyema et al., 2006; Diniz et al., 2004) as well as impaired glucose-induced insulin secretion by pancreatic islets of obese mice (Andreazzi et al., 2009). Another report by Park et al. (2010) proposed that MSG produces altered lipid profile with elevation in reactive oxygen species (ROS) formation and reduction of antioxidant activities. This observation agrees with earlier studies that reported hyperleptinemia (Hollopeter et al., 1998), adiposity and increase of plasma fatty acids and triacylglycerols (Dawson et al., 1997) with oral administration of MSG into rats. Obochi et al. (2009) also reported that MSG administration leads to increased cholesterol, protein and oestrogen which lead to induction of fibroid in rats.

Therefore, the present study was carried out to investigate the efficacy of *L. micranthus* in preventing or reversing alter plasma lipid profile and cardiovascular disease risk factors associated with MSG administration in rats.

MATERIALS AND METHODS

Monosodium glutamate

MSG was obtained from Sigma Chemical Company Japan. A solution was prepared by dissolving 10 g of MSG in 500 ml distilled water.

Plant authentication and extraction

Fresh leaves of mistletoe were obtained from a farm garden at Odogbolu, Ogun State, Nigeria in August 2013. The plant was identified as *L. micranthus* at the Botany Department of Olabisi Onabanjo University, Ago-Iwoye, Ogun State. A voucher number EH135IO was thereafter assigned after which specimen was deposited at the herbarium. The leaves were then washed and air dried at room temperature for two weeks. The dried leaves were pulverized into coarse form. The coarse powdered form was weighed and 362 grams was soaked in 1000 ml distilled water. The mixture was left to stand for 48 h with occasional stirring. The extracted product was then sieved into a clean container and further concentrated using a rotary evaporator at 40°C. The concentrated product was then lyophilized. The yield of the extract was 8.7%.

Experimental design

Thirty five (35) mature virgin female Wistar rats weighing 155 to

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164 g were used in the study. The rats were obtained from the animal house, Department of Physiology, Olabisi Onabanjo University, Ago-Iwoye, Nigeria and were acclimatized for seven days at the Animal Facility Centre, Department of Biochemistry, Olabisi Onabanjo University, Nigeria. All the animals were housed in metallic cages and maintained in well ventilated room provided with 12:12 h light and dark cycle for each 24 h period at a temperature of approximately 25°C. They were all maintained on standard rat pellets and tap water *ad libitum* throughout the period of the study. All the rats used in the study showed regular estrous cycle length (4 to 5 days). The phases of estrous cycle were determined by observing the vaginal smear in the morning (08: 00h to 10:00 h) according to procedure described previously (Solomon et al., 2010). The animals were initially randomly assigned to three groups labeled as normal control/group 1 (7 rats), test control /group 2 (21 rats) and test group / group 3 (7rats). Rats in each group were treated as follow: Group 1 (Normal control): administered with normal saline; Group 2 (Test control): administered with MSG (200 mg/kg body weight); Group 3 (Test group): administered with MSG (200 mg/kg b. w) and *L. micranthus* 600 mg/kg body weight).

All administrations were carried out as a single dose daily for 28 days by oral galvage. After 28 days of administration, seven rats were sacrificed from each group after 12 h fasting; the rats were then evaluated on the basis of preventive effects of *L. micranthus*. The remaining rats in group 2 were redistributed to two groups of seven rats each, labeled, group 4 (extract treated) and 5 (self-recovery). Administration of MSG was discontinued in the two groups while rats in group 4 were administered with *L. micranthus* (600 mg/kg) extract; rats in group 5 were treated with normal saline. All administrations were carried out for the next 30 days after which the rats were sacrificed.

All the rats were sacrificed after anethesia with diethyl ether in a closed jar. Blood was collected by cardiac puncture into plain bottles and the rats were evaluated based on the curative potential of the extract. The uterus, kidney and liver were harvested cleaned of blood and weighed. The uterus was thereafter used for histopathology study.

Biochemical assay

Initial and the final body weight of all the rats were measured using a Mettler weighing balance (Mettler Toledo Type BD6000, Greifensee, Switzerland). Serum was prepared from the collected blood samples by centrifugation and used for analysis. Fasting blood glucose was measured according to method adopted previously by Miwa et al. (1972) using a glucose kit (enzymatic method) (Sigma). Serum protein determination was by the method of Lowry et al. (1951). Triglyceride was determined using enzymatic colorimetric kits (Wahlefeld, 1974). Both total cholesterol and HDL-C were determined in the serum by the methods previously described (Stein, 1986). From the results, LDL cholesterol was calculated based on the method of Friedewald et al. (1972). According to the method, LDL can be calculated as follows:

LDL = Total cholesterol - HDL-TG/5

Atherogenic index was calculated from serum HDL and cholesterol levels using the equation previously reported by Gillies et al. (1986).

$$\text{Atherogenic index} = \frac{\text{Serum cholesterol level- serum HDL level}}{\text{Serum HDL level}}$$

Coronary risk index was obtained by the method of Alladi and Khada (1989).

$$\text{Coronary risk index (CRI)} = \frac{\text{Total cholesterol}}{\text{HDL- cholesterol}}$$

Histopathology study

The dissected uterus was immersion-fixed in bouins fluid over night at room temperature after which the tissues were transferred to ascending grades of alcohol for dehydration. The tissues were cleared with two changes of xylene for one and half hours each, transferred into two changes of molten paraffin wax I and II for one and half hour each and wax- III for overnight in an oven at 65°C for infiltration.

The tissue was then processed according to the method previously described (Solomon et al., 2010). The tissue block was serially sectioned at 6 µm thickness using microtome. Strips of sections were gently lowered into the surface of a warm water bath at 40°C. The floated sections were mounted on egg albumin coated microscopic slides, and put in an oven maintained at 60°C for 30 min to fix the tissue firmly on the slide. The slides were dewaxed with two changes of xylene and hydrated with decreasing alcohol concentration and then immersed in water for 5 min. The sectioned tissues were then stained regressively with Ehrlich's hematoxylin and counter stained with Eosin.

After staining with eosin, tissues were washed in tap water and dehydrated by rinsing in increasing concentration of alcohol and then xylene-I. They were then placed in xylene-II until mounting. Finally, a drop of mountant DPX (A mixture of Distyrene, a Plasticizer, and Xylene) was placed on top of the sections and the cover slip was applied.

Animal care

The care of the animals was in accordance with the U.S. Public Health Service Guidelines (NRC, 1999) and was approved by the Olabisi Onabanjo University, College of Health Sciences Animal Ethics Com.

Statistics

All data were expressed as mean ±SEM. One-way analysis of variance (ANOVA) was used to analyze the data. Comparisons between the groups were made at a two-sided alpha level of 0.05. $p < 0.05$ was considered statistically significant.

RESULT

Table 1 depicts the variation in serum lipid profile, atherogenic index (AI) and coronary risk index (CRI) with MSG administration and during treatment with *L. micranthus*. There was a significant increase ($p<0.05$) in plasma triglyceride, total cholesterol and LDL-cholesterol and a reduction in HDL-cholesterol when MSG was administered. Combined administration of MSG with *L. micranthus* significantly brought these parameters to the pre-treatment values. Table 1 also indicates that when the rats were treated with *L. micranthus* for the 30 days after the 28 days of continuous administration of MSG, the observed values of serum triglyceride, total cholesterol, LDL-cholesterol and HDL-cholesterol (96.41±5.93, 120.16±5.93, 41.27±5.15, 26.90±4.02 mg/dl

Table 1. The results of the effect of *L. micranthus* treatment on plasma lipid profile, atherogenic and coronary risk indexes.

Group	Treatment	Plasma lipid profile				Atherogenic index (AI)	Coronary risk index (CRI)
		Triglyceride (mg/dl)	Total cholesterol (mg/dl)	HDL (mg/dl)	LDL (mg/dl)		
1	Normal control (normal saline)	87.03±5.90[a]	102.42±2.11[a]	45.91±3.63[a]	20.64±4.79 [a]	0.45±9.01[a]	2.21±0.11[a]
2	Test control (MSG)	114.41±9.95[b]	174.18±16.20[b]	24.59±5.57[b]	54.98±11.39[b]	2.24±0.01[b]	7.08±0.30[b]
3	Test group (co-administration of MSG and *L. micranthus)*	92.71±3.82[a]	118.11±5.75[a]	42.09±5.19[a]	26.99±4.02[a]	0.64±0.03[c]	2.82±0.01[a]
4	Treatment with *L. micranthus* after MSG withdrawal from group 2	96.41±4.27[a]	120.16±5.93[a]	41.27±5.15[a]	30.01±5.11 [a]	0.73±0.11[c]	2.91±0.10[a]
5	Normal saline after withdrawal of MSG from group 2	113.88±8.70[b]	171.63±15.31[b]	25.45±5.59[b]	54.09±11.00[b]	2.43±0.02[b]	6.74±0.10[b]

Values are mean± SEM of 7 determinations, All mean in the same column with similar superscripts are not significantly different from each other, Significant between the mean were established at p<0.05.

Table 2. Effect of *L. micranthus* treatment on fasting blood glucose and total protein.

Group	Treatment	Fasting blood glucose (mg/dl)	Serum protein (mg/dl)
1	Normal control (normal saline)	82.00±0.93[a]	9.89±0.48[a]
2	Test control (MSG)	112.00±3.86[b]	24.55±2.80[b]
3	Test group (co-administration of MSG and *L. micranthus)*	89.83±2.57[a]	11.13±0.88[a]
4	Treatment with *L. micranthus* after MSG withdrawal from group 2	91.67±2.11[ab]	19.82±0.84[c]
5	Normal saline after withdrawal of MSG from group 2	97.67±1.65[b]	25.46±3.00[b]

Values are mean± SEM of 7 determinations. All mean in the same column with similar superscripts are not significantly different from each other. Significance between the mean were established at p<0.05.

respectively) were not significantly different from the values obtained when MSG and *L. micranthus* were co-administered for 28 days (92.71±3.82, 118.11±5.75, 42.09±5.19, 26.99±4.02 mg/dl respectively). When compared however, the serum lipid of rats that were allowed to recover by itself (without treatment after 28 days administration of MSG) were not different from the observed values prior to withdrawal of MSG. Table 1 also show a significant increase (p<0.05) in atherogenic and coronary risk indexes during MSG administration and when the rats were allowed to recover from MSG administration without treatment (when compared with the normal control values). However, combined administration of MSG and *L. micranthus* and treatment with *L. micranthus* after the initial administration of MSG brought the coronary risk index to the pre-treatment values. Although the atherogenic index values were raised above the normal control level both during combined administration of MSG and *L. micranthus* and when *L. micranthus* was administered after withdrawal of MSG, the observed AI were significantly lowered than the values observed when MSG was administered alone and was also higher than the value that was obtained during the self-recovery process.

Administration of MSG significantly increased (p<0.05) the fasting blood glucose above the normal control value (Table 2). Thirty (30) days after withdrawal of MSG without subsequent treatment, the fasting blood glucose level was still observed to be significantly higher (p<0.05) than the normal control value. When MSG was however co-administered with *L. micranthus,* the

Table 3. Effect of treatment on body weight changes and relative organ weight ratio.

Group	Treatment	Body weight (g)	Organ weight (g)			Organ/body weight ratio (%)		
			Kidney	Uterus	Liver	Kidney	Uterus	Liver
1	Normal control (normal saline)	175.83±2.71[a]	1.22±0.05	4.81±0.35	0.33±0.02	0.73±0.02[a]	0.20±0.02[a]	2.90±0.62[a]
2	Test control (MSG)	175.00±4.28[a]	1.32±0.02	4.90±0.26	0.43±0.04	0.88±0.06[b]	0.36±0.04[b]	3.51±0.30[b]
3	Test group (co-administration of MSG and *L. m-icranthus)*	175.67±4.00[a]	1.27±0.10	4.84±0.55	0.35±0.02	0.76±0.05[a]	0.23±0.05[a]	2.93±0.19[a]
4	Treatment with *L. micranthus* after MSG withdrawal from group 2	179.72±5.83[a]	1.29±0.11	4.82±0.41	0.40±0.02	0.76±0.02[a]	0.23±0.01[a]	2.98±0.34[a]
5	Normal saline after withdrawal of MSG from group 2	174.17±6.38[a]	1.41±0.03	4.88±0.26	0.44±0.03	0.73±0.06[a]	0.52±0.02[b]	3.61±0.19[b]

Values are mean± SEM of 7 determinations. All mean in the same column with similar superscripts are not significantly different from each other. Significance between the mean were established at $p<0.05$.

observed fasting blood glucose level of 89.83±2.57 mg/dl was neither different from the normal control value nor was it different from the fasting blood glucose level of 91.67±2.11 mg/dl observed when *L. micranthus* was administered continuously for 30 days after MSG withdrawal. Similarly, administration of MSG was observed in the study to increase the serum total protein significantly ($p<0.05$) above the normal control value. The observed serum protein concentration of 24.55±2.80 mg/dl after 28 days of MSG administration was not different from the value of 25.46±3.00 mg/dl observed 30 days after withdrawal of MSG without treatment. Combined administration of MSG and *L. micranthus* significantly brought the serum protein concentration to the pre-treatment level. When *L. micranthus* was administered for 30 days after withdrawal of MSG, the serum protein concentration was observed to be lower than the value obtained in the group of rats that were not treated though the observed value was still higher than the value in the normal control group.

Table 3 is the result of the effect of treatment on percentage relative change in kidney, liver and uterus weights. Administration of MSG significantly increased ($p<0.05$) the percentage relative weight of the uterus, kidney and liver above the normal control value. The same trend was maintained when the weights were monitored 30 days after MSG withdrawal without treatment. The result however showed that combined administration of MSG and *L. micranthus* and treatment of the rats with *L. micranthus* for 30 days after MSG withdrawal restored the relative organ weights to the pre-treatment level.

Figures 1 to 5 is the result of the effect of treatment on the histology of endometrium of the uterus. The uterine histology of the control rats showed normal features: single layered columnar epithelial cell with elongated nuclei at the base of the cells, highly folded epithelial lining, numerous and tortuous endometrial glands (Figure 1). No significant alterations were also observed in the histology of all other treatment groups.

DISCUSSION

Iwase et al. (1998) and Suga et al. (1999) has reported that post-natal administration of MSG in rats induces insulin resistance, body weight gain, hyperleptinemia and serum glucose level alteration even though the exact mechanisms for these alterations were not clearly defined. In an effort to find out the exact mechanisms by which MSG predispose to tissue injuries, a study by Onyema et al. (2006) reported that MSG is capable of inducing oxidative stress which leads to hepatotoxicity in rats. Another study with obese mice also reported that MSG may impair insulin secretion by pancreatic islets (Andreazzi et al., 2009).

The report from our study confirmed that MSG is capable of causing metabolic alterations in rats. In line with previous study, our study has demonstrated that oral administration of MSG in rats could cause metabolic abnormalities in lipid metabolism which is indicated by marked elevation serum total cholesterol, triglyceride and LDL-cholesterol (hyperlipidemia) with concomitant decrease in plasma HDL-cholesterol level. These observations are in agreement with previous reports by some other authors (Obochi et al., 2009; Pal, 2009; Ogunlabi et al., 2014). This alteration in plasma lipid profile was also reflected in increased atherogenic and coronary risk indices. This is in agreement with report by

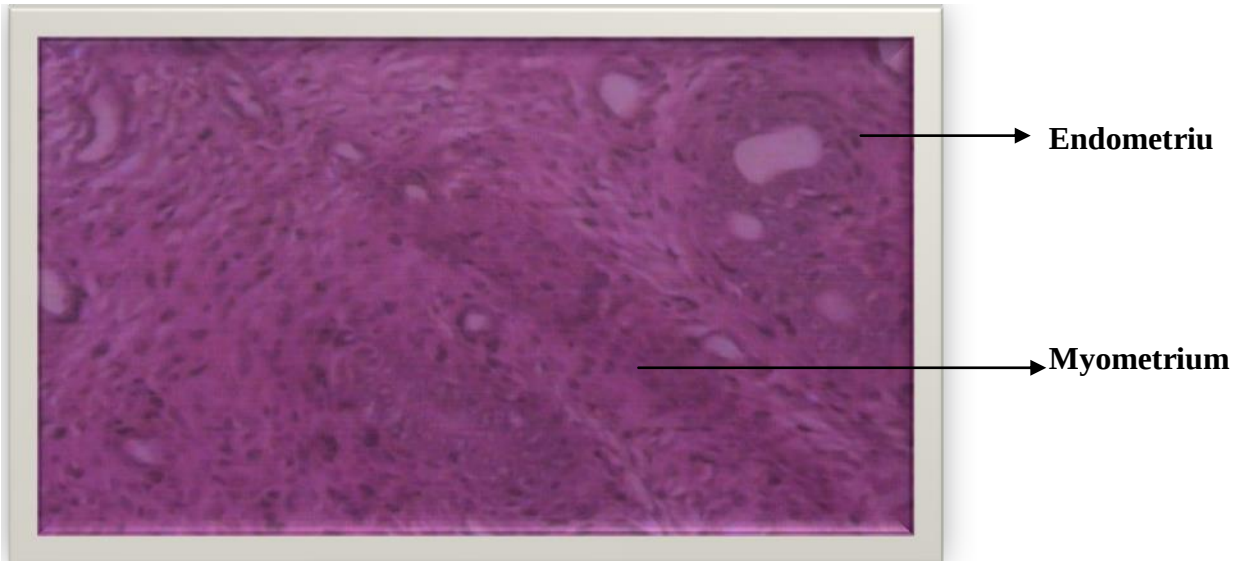

Figure 1. Photomicrographs of hematoxylin and eosin stained tissue cross-section of the control rat showing a normal histological picture of a uterine tissue. Photographed at a magnification x 400

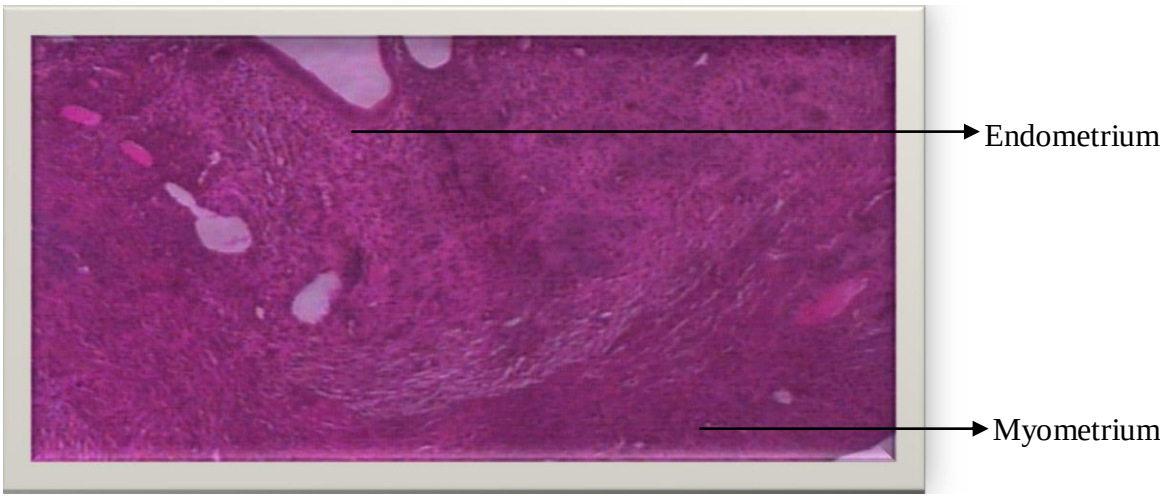

Figure 2. Photomicrographs of hematoxylin and eosin stained tissue cross-section of the MSG administered rat showing a normal histological picture of a uterine tissue. Photographed at a magnification x 400.

Nagata et al. (2006). Our study also shows that both combined administration of MSG and *L. micranthus* and treatment with *L. micranthus* after prolonged exposure to MSG is capable of ameliorating these serum lipid alterations. It has been proposed that MSG elevates reactive oxygen species (ROS) formation and reduction of antioxidant activities (Park et al., 2010) which we opined may be the biochemical basis for the altered lipid profile. Some authors have reported that MSG induces oxidative stress and Reactive Oxygen Species (ROS) production which have been noted play specific roles in the modulation of cellular events (Diniz et al., 2005; Nagata et al., 2006). The ROS react with protein thiol moieties to produce a variety of sulfur oxidations, thus diminishing the insulin receptor signal and inhibiting cellular uptake of triacylglycerol from the blood (Mohamed et al., 2008; Chen et al., 2003). Some studies have linked hypertriglyceridemia to higher serum small dense LDL particles, atherothrombosis and impaired endothelial function, the hallmarks of several prevalent cardiovascular diseases as well as their complications (Lupattelli et al., 2000; Lundman et al., 2001; Ginsberg,

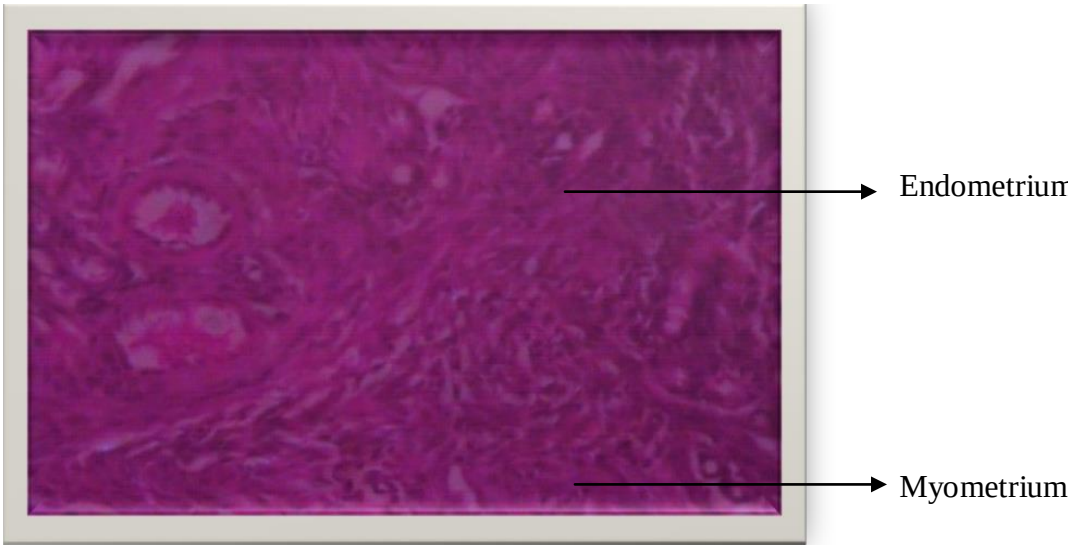

Figure 3. Photomicrographs of hematoxylin and eosin stained tissue cross-section of the MSG + *L. micranthus* administered rat showing a normal histological picture of a uterine tissue. Photographed at a magnification x 400.

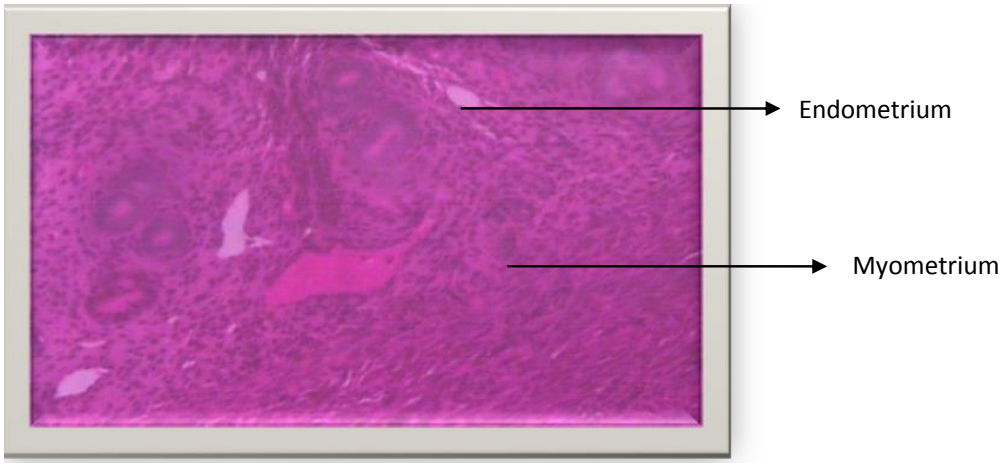

Figure 4. Photomicrographs of hematoxylin and eosin stained tissue cross-section of the MSG + *L. micranthus* treated rat (post administration) showing a normal histological picture of a uterine tissue. Photographed at a magnification x 400.

2002).

The elevated serum concentration of total cholesterol noted in the rats administered with MSG suggests that MSG predispose to hypercholesterolemia. The increase in the levels of LDL-cholesterol with a simultaneous decreased concentration of HDL-cholesterol reflected that MSG will induce abnormalities in lipoprotein metabolism. Because of the changes in the cholesterol profile and other lipid molecules noted in the present study in the MSG-treated rats, we opined that they may be due to the ability of MSG toxicity to inhibit the activity of hydroxy 3-methylglutaryl-coenzyme. A reductase (HMG-CoA) which plays an important regulatory role in cholesterol biosynthesis, and inhibition of its activity is known to alter the metabolism of all lipids, including cholesterol (Ness and Chambers, 2000). Hypercholesterolemia and abnormalities in lipoprotein metabolism are considered as serious risk factors and important early events in the pathogenesis of atherosclerosis in both peripheral and coronary circulation (Maxfield and Tabas, 2000; Mallick, 2007; Grover-Paez and Omez, 2009). Lipid compounds and products of their oxidation especially LDL accumulate during formation of atherosclerotic lesions (Mallick, 2007).

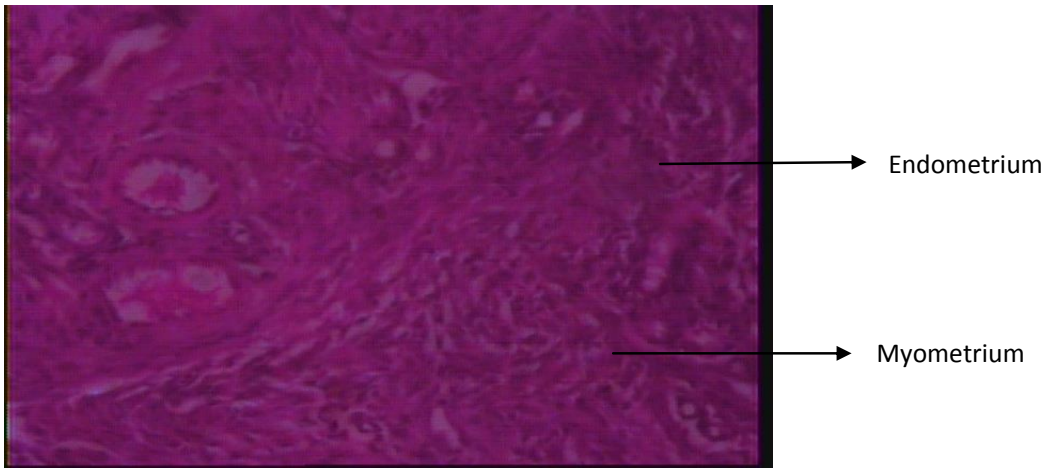

Figure 5. Photomicrographs of hematoxylin and eosin stained tissue cross-section of the MSG administered rat treated with normal saline showing a normal histological picture of a uterine tissue. Photographed at a magnification x 400.

The LDL functions in the atheroma formation whereas the HDL plays an important role in inhibiting the formation of atheroma (Maxfield and Tabas, 2000; Mallick, 2007). The antiatherosclerotic action of HDL resides in its ability to remove cholesterol from vascular wall, stimulate prostacyclin formation and inhibit the synthesis of adhesive molecules (Pal, 2009). So, lowering the plasma lipid levels through dietary or drug therapy may be beneficial in decreasing the risk of vascular disease.

As regards the ability of *L. micranthus* to reverse these alterations as reported in this study, we opined, that it is an indication of the efficacy of *L. micranthus* to prevent predisposition to cardiovascular disease and atherogenesis during MSG intake. Specie of mistletoe, *Viscum album* was reported in a previous study carried out by Yusuf et al. (2013) to show a dose-dependent relationship for the reducing ability, free radical scavenging and the Fe^{2+} chelating tendency. The study observed that *V. album* extracts from cola tree compares favorably with the standards agents (ascorbic acid, ethylenediamine tetraacetic acid and buthylated hydroxytoluene used. A phytochemical screening report by Orji et al. (2013) indicated the presence of tannins, flavonoids, saponins, phenols, alkaloids and anthocyanins in the leaves of ethanolic extracts of *L. micranthus* leaves. A correlation between the total phenol content of plant food and their antioxidant properties has been well reported. This may be the biochemical explanation for the efficacy of *L. micranthus* leaves extract as reported in this study. Another hypothesis may be that *L. micranthus* is efficacious in decreasing HMG-CoA reductase activity (an enzyme required for cholesterol synthesis) thereby decreasing the predisposition to increase atherogenic and coronary risk index of MSG (Arai et al., 2000; Lapointe et al., 2006).

The results of our study also show that, administration of MSG to rats induced hyperglycemia which is indicated by elevated level of fasting blood glucose when compared with the normal control rats. Our result corroborates previous report by some other authors who attributed the increase in serum glucose in response to MSG administration to hypertrophy of pancreatic islets with associated hyperinsulinemia, an early marker of insulin resistance, together with impaired glucose uptake by tissues due to the decrease in the number of glucose transporter-4 (GLUT 4) (Seraphim et al., 2001; Diniz et al., 2005; Nagata et al., 2006). Insulin resistance is often associated with clustering of coronary risk factors, which leads to an increased risk of cardiovascular disease, presumably due to promotion of atherosclerosis (Zavaroni et al., 1989; Roberts and Sindhu, 2009). The observed hypertriglyceridemia induced with MSG administration reported in our study may thus be a response to the increased blood glucose level. A positive correlation between hypertriglyceridemia and hyperglycemia induced by MSG has been previously reported (Nayira et al., 2009). The regulation of energy balance is essential to maintain the control of metabolism and body composition (Scharrer, 1999). Importantly, MSG has been widely used to induce obesity through hypothalamic lesions in neonatal period (Nakayama et al., 2003). Though a study by Grassiolli et al. (2007) reported glucose disturbances only in neonatal MSG-treated animals compared to adult MSG treatment our study observed elevated fasting blood glucose in adult female rats placed on MSG. For one of the main causes of hyperglycemia in these rats, we opined, it may be related to a decreased amount of GLUT 4 protein found in adipocytes (Macho et al., 2000). It seems true that insulin sensitivity is influenced by the redox state of the organism, whereby oxidative processes may trigger the development of insulin resistance (Evans et al., 2002). Data has been reported to prove that ROS react with protein thiol moieties to produce a variety of sulfur oxida-

tions which attenuates insulin receptor signal and inhibits cellular uptake of triglyceride from blood stream (Chen et al., 2003). This may be the basis for the observed increased plasma triglyceride levels in MSG animals reported in this study.

MSG was also observed in this study to increase the level of total protein. Elevated total protein and cholesterol among some other alterations has been implicated in the induction of fibroid in rats (Obochi et al., 2009). The effect of MSG on protein alteration has been attributed to the activation of transcriptional promoter and enhancer elements used for the control of gene expression which promoted the ability of RNA polymerase to recognize the nucleotide at the initiation stage thereby increasing protein synthesis (Obochi et al., 2009). Our report also shows the efficacy of *L. micranthus* extract to reverse these alterations.

Organ weights are widely accepted in the evaluation of test article-associated toxicities (Black, 2002; Bucci, 2002; Woolley, 2004). In toxicological experiments, comparison of organ weights between treated and untreated groups of animals have conventionally been used to evaluate the toxic effect of the test article and its changes often precede morphological changes. Data from our study indicates that oral administration of MSG predispose to increased relative organ weight of the tissues investigated. This result corroborates previous report by Manal and Nawal (2012) where treatment with MSG was reported to lead to increased liver and kidney relative weight. *L. micranthus* was also observed in this study to be able to restore the relative organ weight in the treated rats to the normal level. When the histopathology of the uterus was however compared, these variations in relative organ weight were not seen to be accompanied with gross morphological changes.

Conclusion

In conclusion, MSG was seen to have predisposed to serious biochemical alterations and that both pretreatment with *L. micranthus* and administration of *L. micranthus* subsequent to MSG withdrawal successfully improves these parameters and ameliorates the metabolic alterations caused by MSG administration. Furthermore, *L. micranthus* administration is reported in this study to normalize the MSG induced weight changes in the liver, kidney and uterus of rats.

Conflict of Interests

The author(s) have not declared any conflict of interest.

REFERENCES

Alladi S, Khada SM (1989). Induction of hypercholesterolemia by supplementing soy protein with acetate generating amino acids. Nutr. Rep. Int. 40: 893-899.

Andreazzi AE, Scomparin DX, Mesquita FP, Balbo SL, Gravena C, De Oliveira JC (2009). Swimming exercise at weaning improves glycemic control and inhibits the onset of monosodium L-glutamate-obesity in mice. J. Endocrinol. 201:351-359

Arai Y, Watanabe S, Kimira M, Shimoi K, Mochizuki R, Kinae N (2000). Dietary intakes of flavonols, flavones and isoflavones by Japanese women and the inverse correlation between quercetin intake and plasma LDL cholesterol concentration. J. Nutr. 30:2243-2250

Black H (2002). Preparation of the report for a toxicology/pathology study. In: Haschek WM, Rousseaux CG., Wallig MA, eds. Handbook of Toxicologic Pathology. Academic Press, San Diego, CA. pp. 419-433.

Bright A (2013). Utilization of traditional herbal mediocine and its role in health care delivery in Ghana: The case of Wassa Amenfi West District. A Thesis submitted to the Department of Geography and Rural Development. Kwame Nkrumah University of Science and Technology.

Bucci TJ (2002). The practice of toxicologic pathology: basic techniques. In: Haschek WM, Rousseaux CG, Wallig MA, eds. Handbook of Toxicologic Pathology. Academic Press, San Diego, CA. pp. 681-784.

Chen K, Thomas SR, Keaney JF (2003). Beyond LDL oxidation: ROS in vascular signal transduction. Free Radic. Biol. Med. 35: 117-132.

Dawson R, Pelleymounter MA, Millard WJ, Liu S (1997). Attenuation of leptin-mediated effects by monosodium glutamate-induced arcuate nucleus damage. Am. J. Physiol. 22:E202-E206

Diniz YS, Faine LA, Galhardi CM, Rodrigues HG (2005). Monosodium glutamate in standard and high-fiber diets: Metabolic syndrome and oxidative stress in rats. Nutrition 21:749-755.

Diniz YS, Fernandes AA, Campo KE, Mani F, Ribas BO, Novelli EL. (2004). Toxicity of hypercaloric diet and monosodium glutamate: oxidative stress and metabolic shifting in hepatic tissue. Food Chem. Toxicol. 42(2):319-325.

Evans JL, Goldfine ID, Maddux BA, Grodsky GM (2002). Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. Endocr. Rev. 23: 599-622

Eweka AO (2007). Histological studies of the effects of Monosodium-glutamate on the medial geniculate body of adult wistar rats. Internet J. Health 6(2):121-128.

Farombi EO, Onyema OO (2006). Monosodium glutamate - induced oxidative damage and genotoxicity in the rat: modulatory role of vitamin C, vitamin E and quercetin. Hum. Exp. Toxicol. 25: 251-259.

Friedewald WT, Levy RI, Fredrickson DS (1972). Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin. Chem. 18: 499-502

Gillies PJ, Rathgeb KA, Perri MA, Robinson JL (1986). Regulation of acyl-oA: Cholesterol acyltransferase activity in normal and atherosclerotic rabbit aortas: Role of a cholesterol substrate pool. Exp. Mol. Pathol. 44: 329-339.

Ginsberg HN (2002). New perspectives on atherogenesis: Role of abnormal triglyceride-rich lipoprotein metabolism. Circulation 106: 2137-2142

Grassiolli S, Gravena C, de Freitas M, Mathias PC, Muscarinic M (2007). H receptor is active on pancreatic islets from hypothalamic obese rat. Eur. J. Pharmacol. 556: 223-228.

Griggs P (1991). Mistletoe, myth, magic and medicine. Biochemist 13: 3-4.

Grossarth MR, Ziegler R (2007). Prospective cohort studies on long term therapy of cervical cancer patients with mistletoes preparations Forsch Komplementmed. 14: 140-147.

Grover-Paez F, Omez ABZ (2009). Endothelial dysfunction and cardiovascular risk factors. Diabetes Res. Clin. Practice. 84: 1-10.

Hollopeter G, Erickson JC, Palmiter RD (1998). Role of neuropeptide Y in diet-, chemical-and genetic-induced obesity of mice. Int. J. Obes. Relat. Metab. Disord. 22:506-512.

Huthman I, Shoyebo O, Akinbowale O, Ajayi R, Huthman A, Adenowo T, Adefule A. (2009). *Monosodium Glutamate:* A Good Replacement For Hydrogen Peroxide In Bone Preparations. Internet J. Biol. Anthropol. 4 (1): 45-51.

Iwase M, Yamamoto M, Lino K, Ichikawa K, Shinohara N, Yoshinari M

(1998). Obesity induced by neonatal monosodium glutamate treatment in spontaneously hypertensive rats: an animal model of multiple risk factors. Hypertens. Res. 21:1-6.
Lapointe A, Couillard C, Lemieux S (2006). Effects of dietary factors on oxidation of low-density lipoprotein particles. J. Nutr. Biochem. 17:645-658.
Lowry DH, Rosebrough NJ, Farr AL (1951). Protein measurement with the folin-phenol reagent. J. Biol. Chem.193:265-275.
Lundman P, Eriksson MJ, Stuhlinger M, Cooke JP, Hamsten A, Tornvall P (2001). Mild-to-moderate hypertriglyceridemia in young men is associated with endothelial dysfunction and increased plasma concentrations of asymmetric dimethylarginine. J. Am. Coll. Cardiol. 38:111-116.
Lupattelli G, Lombardini R, Schillaci G, Ciuffetti G, Marchesi S, Siepi D, Mannarino E (2000). Flow-mediated vasoactivity and circulating adhesion molecules in hypertriglyceridemia: Association with small, dense LDL cholesterol particles. Am. Heart J. 140:521-526.
Macho L, Ficková M, Jezová C, Zórad S (2000). Late effects of postnatal administration of monosodium glutamate on insulin action in adult rats. Physiol. Res. 49: S79-S85.
Mallick HN (2007). Understanding safety of glutamate in food and brain. Ind. J. Physiol. Pharmacol. 51:216-234.
Manal ST, Nawal AB (2012). Adverse Effects of Monosodium Glutamate on Liver and Kidney Functions in Adult Rats and Potential Protective Effect of Vitamins C and E. Food Nut. Sci. 3:651-659.
Maxfield FR, Tabas I (2005). Role of cholesterol and lipid organization in disease. Nature 438:612-621
Miwa I, Okuda J, Maeda K, Okuda G (1972). Mutarotase effect on colorimetric determination of blood glucose with D-glucose oxidase. Clin. Chem. Acta. 37: 538-540.
Moghadamtousi SZ, Hajrezaei M, Abdul Kadir H, Zandi K. (2013). *Loranthus micranthus* Linn.: Biological Activities and Phytochemistry. Evid. Based Complement. Alternat. Med. 9 pages. Article ID 273712.
Mohamed ME, Nagawa MNE, Inas ED (2008). The possible cytoprotective effect of vitamin C and E and L-carnithine on monosogium glutamate induced oxidative stress in the rat. Bull. Alex. Fac. Med. 44(1):205-211
Nagata MW, Suzuki S, Iizuka M, Tabuchi C, Maruyama H (2006). Type 2 diabetes mellitus in obese mouse model induced by monosodium glutamate. Exp. Anim. 55: 109-115.
Nakayama D, Magami Y, Azuma T, Inokuchi H, Furukawa M, Ohyashiki J (2003). Turnover of acinar and islet cells in the pancreas of monosodium glutamate-treated obese mice. Obes. Res. 11:87-94.
National Research Council (NRC) (1999). Guide for the Care and Use of Laboratory Animals. National Academy Press, Washington, DC.
Nayira A, Abdel BA, Mohamed M, Faddah LM (2009). Protective Effect of N-acetyl Cysteine and/or Pro Vitamin A against Monosodium Glutamate-Induced Cardiopathy in Rats. J. Pharmacol. Toxicol. 4:178-193.
Ness GC, Chambers CM (2000).Feedback and hormonal regulation of hepatic 3-hydroxy-3-methylglutaryl coenzyme a reductase: The concept of cholesterol buffering capacity. Proc. Soc. Exp. Biol. Med. 224: 8-19.
Nickrent DL (2001). Mistletoe Phylogenetics: Current relationships gained from analysis of DNA sequences. In Geils B and Mathiasen R eds. Proc. West. Int. For. Dis. Work Conf., USDA for. Serv., Kona JI.
Nkanu RE, Umoh FB, Ofem DT, Eno AE (2002). The effect of ageous extract from the leaves of European Mistletoe on blood glucose level and lipid profile in male wister rats. NG J. Physiol. Sci. 7: 108-112.
Obochi GO, Malu SP, Obi-Abang M, Alozie Y, Iyam MA (2009). Effect of garlic extracts on monosodium glutamate (MSG) induced fibroid in wistar rats. Pak. J. Nut. 8(7):970-976.
Ogunlabi OO, Adegbesan BO, Sumaoya OB, Ajani EO (2014). Modulating effects of *Allium sativum* (garlic) extract in monosodium glutamate induced injuries in rats. Med. Plants Int. J. Phytomed. Rel. Ind. 6(1):28-35.
Onyema OO, Alisi CS, Ihetuge AP (2012). Monosodium Glutamate Induces Oxidative Stress and Affects Glucose Metabolism in the Kidney of Rats. Int. J. Biochem. Res. Rev. 2(1):1-11.
Onyema OO, Farombi EO, Emerole GO, Ukoha AI, Onyeze GO (2006). Effect of vitamin E on monosodium glutamate induced hepatotoxicity and oxidative stress in rats. Indian J. Biochem. Biophys. 43:20-24.
Orji FA, Nwachukwu NC, Onyia AU, Nkwocha JO (2013). Phytochemical and antimicrobial properties of leaves of African mistletoe (Loranthus micranthus) on some selected microbial pathogens in Abia State, Nigeria. Glob. Adv. Res. J. Microbiol. 2(1):011-016.
Ortiz GG, Bitzer-Quintero OK, Zarate CB, Rodriguez-Reynoso S, Larios-Arceo F, Velázquez-Brizuela IE, Pacheco-Moisés F (2006). Rosales-Corral SA, Monosodium glutamate- induced damage in liver and kidney: a morphological and biochemical approach. Biomed. Pharmacol. 60: 86-89.
Osadebe PO, Abba CC, Agbo MO (2012). Antimotility effects of extracts and fractions of Eastern Nigeria mistletoe (*Loranthus micranthus* Linn). Asian Pac. J. Trop. Med. 5(7):556-60.
Osadebe PO, Ukwueze SE (2004). A comparative study of the phytochemical and antimicrobial properties of the Eastern Nigerian Species of African Mistletoe (*Loranthus micranthus*) sourced out from different host trees. J. Biol. Biotechnol. 2 (1):18-23.
Pal M (2009). HDL Therapeutics for the treatment of atherosclerosis: A brief overview of the synthetic approaches. Tetrahedron 65:433-447.
Palanisamy P, Selvaraju S, Krishnamoorthy SS, Natarajan M, Ragunathan Y, Perumal P (2012). Monosodium glutamate modulates the circadian rhythms of biochemical variables and behavioral activity in rats under constant light. Int. J. Nutr. Pharmacol. Neurol. Dis. 2: 251-257.
Park CH, Kim MY, Sok DE, Kim JH, Lee JH, Kim MR (2010). Butterbur (Petasites japonicas Max.) extract improves lipid profiles and antioxidant activities in monosodiumL-glutamate-challenged mice. J. Med. Food. 13: 1216-1223.
Roberts CK, Sindhu KK (2009). Oxidative stress and metabolic syndrome. Life Sci. 84: 705-712.
Scharrer E (1999). Control of food intake by fatty acid oxidation and ketogenesis. Nutrition 15:704-714.
Seraphim PM, Nunes MT, Machado UF (2001). GLUT 4 protein expression in obese and lean 12-month-old rats. Braz. J. Med. Biol. Res. 34:1353-1362.
Solomon TL, Mekbeb Z, Eyasu AM, Asfaw D (2010). Effect of *Rumex steudelii* methanolic root extract on ovarian folliculogenesis and uterine histology in female albino rats. Afr. Health Sci. 10: 353-361.
Stein EA (1986). Textbook of Clinical Chemistry. W.B. Saunders, Philadelphia. pp. 583-584.
Suga A, Hirano T, Kageyama H, Kashiba M, Oka J, Osaka T (1999). Rapid increase in circulating leptin in ventromedial hypothalamus-lesioned rats: role of hyperinsulinemia and implication for upregulation mechanism. Diabetes 48: 2034-2038.
Wahlefeld AW (1974). Method of Enzymatic Analysis. Vol. 5, Academic Press, New York. pp. 1831-1835.
WHO (2008). Traditional Medicine. World Health Organization Fact Sheet N 134, December 2008. (Accessed from http://www.who.int/mediacentre/f actsheets/fs134/en/ on 11 August 2010.
Woolley A (2004). Determination—General and reproductive toxicology. In: Taylor and Francis, ed. A Guide to Practical Toxicology Evaluation, Prediction and Risk. , New York. pp. 80-106.
Yusuf L, Oladunmoye MK, Ogundare AO, Akinyosoye FA, Daudu OA, Hassan GA (2013). Antimicrobial and antioxidant properties of mistletoe (viscum album) growing on cola (cola nitida) tree in Akure North, Nigeria. J. Microbiol. Res. Rev. 1(3):35-41.
Zavaroni IE, Bonora M, Pagliara E, Dall'Aglio C, Luchetti L (1989). Risk factors for coronary artery disease in healthy persons with hyperinsulinemia and normal glucose tolerance. N. Engl. J. Med. 320:702-706.

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