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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.

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Table of Contents: Volume 14 Number 32, 12 August, 2015

ARTICLES

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Full Length Research Paper

Assessing the genetic diversity of 48 groundnut (*Arachis hypogaea* L.) genotypes in the Guinea savanna agro-ecology of Ghana, using microsatellite-based markers

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Groundnut (*Arachis hypogaea* L.) is the most important grain legume in Ghana. However, its production is constrained by a myriad of biotic and abiotic stresses which necessitate the development and use of superior varieties for increased yield. Germplasm characterisation both at the phenotypic and molecular level is important in all plant breeding programs. The aim of this study was to characterise selected advanced breeding groundnut lines with different phenotypic attributes at the molecular level using simple sequence repeats (SSR) markers in Ghana. A total of 53 SSR markers were screened and 25 were found to be polymorphic with an average polymorphic information content (PIC) value of 0.57. Of the 48 groundnut genotypes studied, 67% showed very close relationship (~100% similarity) with one or more genotypes among themselves. In fact, there were 14 instances where two to three genotypes within the same sub-cluster exhibited 100% similarity even though they displayed different phenotypic attributes. The remaining 33% of the groundnut genotypes were distant from each other and could therefore serve as effective parental material for future work. In this study, the SSR-based markers were found to be quite discriminatory in discerning variations between and among groundnut lines even where the level of variation was low. Microsatellite-based markers therefore represent a useful tool for dissecting genetic variations in cultivated crops, especially groundnut.

Key words: Phenotypic traits, DNA extraction, PCR amplification, simple sequence repeats (SSR) markers, alleles, polymorphic information content, Jaccard's similarity coefficient.

INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is one of the most important oilseed crops in the world. Currently, China, India and Nigeria account for largest groundnut production in the world, with Ghana ranking 12th in land area under groundnut cultivation (FAO, 2014). Groundnut is therefore the most important grain legume in Ghana (MoFA-SRID, 2011) and is grown in all agro-ecologies of the country with the three northern regions accounting for 70% of production (Tsigbey et al., 2003). The grain, fodder, processed oil and cake serve as a major source

of cash income for smallholder famers. Groundnut grain contains 20 to 30% protein and 40 to 55% oil (Asibuo et al., 2008) and is therefore important for nutritional security. As a nodulated legume, groundnut can contribute substantial amounts of symbiotic N to cropping systems, which ranges from 43 to 171 kg N ha^{-1} in Zambia, Ghana and South Africa (Dakora et al., 1987; Nyemba and Dakora, 2010; Mokgehle et al., 2014). In Ghana, the haulms serve as high-protein fodder for livestock during the dry season (Martey et al., 2015). Despite the importance of groundnut to food and nutritional security and its contribution to soil fertility, increased production is constrained by a variety of factors. The yields obtained on farmers' fields are less than 1.0 t ha^{-1} due largely to biotic and abiotic stress (Naab et al., 2005; 2009). For example, foliar diseases such as early and late leaf spot caused by *Cercospora arachidicola* S. Hori and *Cercosporidium personatum* Berk. and Curt., respectively, are known to reduce groundnut yields on farmers field (Naab et al., 2009). Groundnut rosette virus and insect pest damage can also reduce groundnut yield (Padi, 2008). Abiotic factors such as low phosphorus and calcium in soils, as well as erratic rainfall, can cause poor yields of groundnut in Ghana (Abubakari et al., 2012; Rademacher-Schulz et al., 2014). The use of improved genotypes is a cost-effective and environmentally-safe approach to increasing yields in fields of resource-poor farmers (Holbrook and Stalker, 2003). Therefore, developing groundnut genotypes with tolerance to both biotic and abiotic stress has the potential to achieve higher yields on farmers' fields (Holbrook and Stalker, 2003). Traditionally, morphological characters have been used to describe traits such as seed distinctiveness, uniformity and stability of genotypes (Holbrook and Stalker, 2003). However, this method is sometimes influenced by the environment and is labour intensive. In recent times, molecular markers (especially DNA-based markers) have been employed as an alternative to the use of morphological traits. Even then, using a combination of the two (that is, morphological characters and DNA markers) has been found to offer a more comprehensive characterisation of plant genotypes (Holbrook and Stalker, 2003). Many DNA-based molecular markers have been used to characterise groundnut. These include restriction fragment length polymorphism (RFLP), amplified fragment length polymerphism (AFLP), sequence characterised amplified regions (SCARs), random amplified polymorphic DNA (RAPDs), and simple sequence repeats (SSR) or microsatellites (Kochert et al. 1991; Garcia et al. 1996; He and Prakash 1997; Hopkins et al., 1999; Cuc et al., 2008; Carvalho et al., 2010). More recently, single nucleotide polymorphism (SNP) has also been applied to the characterization of groundnut (Barkley et al., 2011; Nagy et al., 2012). However, SSR markers appear to have wider application because of their presence in genomes of all living organisms, their high level of allelic variation, their co-dominant way of inheritance and their potential for automated analysis (Rakoczy-Trojanowska and Bolibok, 2004). SSR markers have thus remained the common routine tool used in the breeding and genetic analysis of groundnut (Pandey et al., 2012a). In this study, 48 groundnut genotypes, comprising advanced breeding lines and farmer varieties that exhibited varying levels of resistance to drought, foliar diseases and aflatoxin contamination, were screened with 53 SSR markers in order to assess (i) the genetic diversity among the groundnut genotypes, and (ii) their potential as parental material in future groundnut breeding programs.

MATERIALS AND METHODS

Plant materials

In this study, a total of 48 groundnut genotypes exhibiting varying levels of resistance to drought, foliar diseases and aflatoxin contamination were used. These included 45 advanced breeding lines supplied by the International Crop Research Institute for the Semi-Arid Tropics (ICRISAT) and three commonly-grown varieties used by farmers in Northern Ghana (Table 1).

Plant DNA extraction

Total plant genomic DNA was extracted from young newly emerged leaves of 12-day-old plants using a modified CTAB protocol (Mace et al., 2003). The quality and quantity of DNA were estimated by running the extracted DNA on a 0.8% agarose gel stained with ethidium bromide. The DNA samples were diluted to 5 ng/µl prior to use in PCR analysis.

PCR amplification

Polymerase chain reaction was performed with the 53 SSR-based primers as described by Pandey et al. (2012b). The reaction was conducted in a 10 µl reaction volume containing 5 ng of genomic DNA, 0.5 µmoles of each primer, 1.0 µl 10X PCR buffer, 0.25 mM of each dNTPs, 2 mM $MgCl_2$ and 1.0 U Taq DNA Polymerase (Sib enzyme, Russia). Touchdown PCR amplification was performed on an ABI Thermal Cycler (GeneAmp PCR system 9700) with an initial denaturation step (94°C for 3 min), and five cycles of

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Abbreviations: PIC, Polymorphic information content; **RFLP,** restriction fragment length polymorphism; **AFLP,** amplified fragment length polymorphism; **SCARs,** sequence characterised amplified regions; **RAPDs,** random amplified polymorphic DNA; **SSR,** simple sequence repeats **SNP,** single nucleotide polymorphism.

Table 1. Biological traits of groundnut germplasm used in this study and their sources.

Genotype	Pedigree	Phenotypic trait	Source
ICG 6222	Germplasm line (PI 262060, landrace, Brazil)	Aflatoxin tolerance	ICRISAT, Mali
ICGV 03315	(ICGV 91284 x ICGV 87846)	Aflatoxin tolerance	ICRISAT, Mali
ICGV 91177	(ICGV 86012 x ICGV 86407)	Aflatoxin tolerance	ICRISAT, Mali
ICGV 91324	(U4-7-5 x PI 337394F)	Aflatoxin tolerance	ICRISAT, Mali
ICGV 94379	(UF 71513-1 x U1-2-1)	Aflatoxin tolerance	ICRISAT, Mali
ICGV 03323	(J 11 x ICGV 87350)	Aflatoxin tolerance	ICRISAT, Mali
ICGV 92305	(NCAc 343 x ICGV 86309)	Aflatoxin tolerance	ICRISAT, Mali
ICGV 91328	(J 11 x U4-7-5)	Aflatoxin tolerance	ICRISAT, Mali
ICGV 92302	(Ah 7223 x U1-2-1)	Aflatoxin tolerance	ICRISAT, Mali
ICGV 91341	(UF 71513-1 x U1-2-1)	Aflatoxin tolerance	ICRISAT, Mali
ICGV 91317	(U4-7-5 x JL 24)	Aflatoxin tolerance	ICRISAT, Mali
ICGV 86124	[JL 24 x (Dh. 3-20 x Robut 33-1) F_8]	Drought tolerance	ICRISAT, Mali
ICGV 91114	(ICGV 86055 x ICGV 86533)	Drought tolerance	ICRISAT, Mali
ICGV 99247	(ICGV 87354 x SANGDI)	Drought tolerance	ICRISAT, Mali
ICGV 97188	(ICGV 86887 x ICGV 87121)	Drought tolerance	ICRISAT, Mali
ICGV 02313	(ICGV 87290 x ICGV 87846)	Drought tolerance	ICRISAT, Mali
ICGV 02171	[(ICGV 88145 x ICGV 87110)F1 x ICGV 88312]	Drought tolerance	ICRISAT, Mali
ICGV 00369	(ICGV 86300 x TAG 24)	Drought tolerance	ICRISAT, Mali
ICGV 99240	(ICGV 87290 x ICGV 87846)	Drought tolerance	ICRISAT, Mali
ICGV 03056	(ICGV 99160 x ICGV 99240)	Drought tolerance	ICRISAT, Mali
ICGV 99249	(ICGV 87846 x TAG 24)	Drought tolerance	ICRISAT, Mali
ICGV 00350	(ICGV 87290 x ICGV 87846)	Drought tolerance	ICRISAT, Mali
ICGV 00308	(ICGV 95244 x ICGV 96223)	Drought tolerance	ICRISAT, Mali
ICGV 00362	(ICGV 86300 x ICGV 92242)	Drought tolerance	ICRISAT, Mali
ICGV 03196	(ICGV 96342 x ICGV 98266)	Early maturity	ICRISAT, Mali
ICGV 03206	(ICGV 98191 x ICGV 93382)	Early maturity	ICRISAT, Mali
ICGV 03181	(ICGV 95319 x ICGV 92206)	Early maturity	ICRISAT, Mali
ICGV 02022	(ICGV 94361 x ICGV 92267)	Early maturity	ICRISAT, Mali
ICGV 03157	(ICGV 93470 x ICGV 96403)	Early maturity	ICRISAT, Mali
ICGV 03184	(ICGV 95319 x ICGV 92206)	Early maturity	ICRISAT, Mali
FLEU II	Unknown	Early maturity	ICRISAT, Mali
ICGV 03166	(ICGV 87378 x ICGV 96342)	Early maturity	ICRISAT, Mali
ICGV 03169	(ICGV 87378 x ICGV 96342)	Early maturity	ICRISAT, Mali
ICGV 03179	(ICGV 96300 x ICGV 96352)	Early maturity	ICRISAT, Mali
ICGV 02144	(ICGV 97052 x U 4-7-5)	Early maturity	ICRISAT, Mali
ICIAR 19 BT	ICGM/754 x ICGV 87922	Early maturity	ICRISAT, Mali

Table 1. Contd.

Genotype	Pedigree	Phenotypic trait	Source
ICGV 03207	(ICGV 98191 x ICGV 93382)	Early maturity	ICRISAT, Mali
ICGV 03194	(ICGV 98247 x ICGV 98262)	Early maturity	ICRISAT, Mali
ICG 7878	Germplasm line (NC Ac 10811-A)	Foliar disease tolerance	ICRISAT, Mali
ICGV 01276	(ICGV 92069 x ICGV 93184)	Foliar disease tolerance	ICRISAT, Mali
ICGV 99029	(ICGV 94118 x ICGV 93427)	Foliar disease tolerance	ICRISAT, Mali
ICG (FDRS) 4	ICGV 87157 (Argentine x PI 259747)	Foliar disease tolerance	ICRISAT, Mali
ICGV 00064	{[ICGV 88312 x (B4 x ICGV 86885)] x [(JL 24 x ICG(FDRS) 4) x JL 24]}	Foliar disease tolerance	ICRISAT, Mali
ICGV 00068	(ICGV 92069 x ICGV 94088) F2-SSD-SSD-B2-B1-B1(VB)	Foliar disease tolerance	ICRISAT, Mali
ICGV-IS 08837	(Agentine x PI 129747) F_3	Foliar disease tolerance	ICRISAT, Mali
CHINESE	Unknown	Early maturity	SARI, Ghana
NKATIESARI	F-mix X ICG (FDRS) 20	Foliar disease tolerance	SARI, Ghana
SUMNUT 22	Unknown	Foliar disease tolerance	Nigeria

denaturation (94°C for 20 s), annealing (65°C for 20 s with a decrease in 1°C for each cycle), and extension(72°C for 30 s). This was followed by 35 cycles of 94°C for 20 s with a constant annealing temperature of 59°C for 20 s and 72°C for 30 s, followed by a final extension of 72°C for 20 min. PCR products of four different fluorescence dye-labelled primers were mixed with 0.2 µl of Gene scan LIZ Size standard (Applied Biosystems, California, USA) and 9.3 µl of Hi-Di™ formamide (Applied Biosystems, California, USA). The DNA fragments were denatured and size-fractioned using capillary electrophoresis on ABI 3730xl genetic analyzer (Applied Biosystems, California, USA). Allele size estimation was performed using GeneMapper v 4.0 genotyping software (Applied Biosystems, California, USA). The software PowerMarker version 3.2 (Liu and Muse, 2005) was used to analyse major allelic frequencies, polymorphic information content (PIC) of markers, and gene diversity. DARwin version 5.0 (Perrier and Jacquemound-Collet, 2006) was used to assess genetic diversity, and NTSYSpc version 2.1 (Rohlf, 1992) to generate a dendrogram for assessing genotypic relatedness among the test groundnut material.

RESULTS AND DISCUSSION

A total of 53 SSR markers with PIC values ≥ 0.5 were selected and used in this study (Table 2). Twenty five (25) of the 53 SSR markers (47%) successfully amplified polymorphic fragments in all the 48 groundnut genotypes tested (Table 3). These 25 SSR markers amplified a total of 164 alleles (Table 3). However, the number of alleles per marker was highly variable, and ranged from two for marker GM1357 to 21 for GM1577, with an average of 6.56 alleles per marker. Major allele frequency per marker also ranged from 0.13 to 0.91, which indicated the presence of allelic variants. Marker GM1577 (0.13) recorded the lowest allele frequency, with GM2053 (0.91) and S003 (0.91) showing the highest (Table 3). Large variability was observed between and among the markers for gene diversity, which ranged from 0.172 to 0.929. Markers GM2053 and S003, which were found to have the highest major allele frequency, revealed the lowest gene diversity. In contrast, marker GM1577 which showed the lowest major allele frequency, exhibited the highest gene diversity. Although, all the markers selected for use in this study had a PIC value ≥ 0.5 Pandey et al. (2012a), the PIC values obtained here ranged from a low 0.16 for markers GM2053 and S003 to 0.92 for GM1577, yielding a PIC mean value of 0.57. The observed variation in PIC values in this study could be attributed to genotypic differences in the groundnut material used. To assess the diversity among the 48 groundnut genotypes, Jaccard's similarity coefficient was calculated using NTSYSpc v. 2.1 and a dendrogram generated based on the unweighted pair-group method with arithmetic mean (UPGMA) procedure (Figure 1). Overall, two major clusters were formed at 72% coefficient of similarity. Cluster I consisted mainly of genotypes that matured at 85 to 90 days after sowing (early maturity) with the exception of NKATIESARI, which matured at 110 days after planting (medium maturity). Cluster II showed no clear-cut demarcation as it contained both early- and late-maturing genotypes. However, the majority of the genotypes in cluster II exhibited tolerance to foliar diseases and aflatoxin contamination. Two sub-clusters (sub-cluster IA and IB or IIA and IIB) were formed within each cluster at 75% coefficient of similarity. Sub-cluster IA contained two early-maturing genotypes and IB

Table 2. Description of SSR markers used in this study and their sources.

Primer	Forward sequence	Reverse sequence	Repeat motif	Number of repeat units	Allele size (bp)	Reference
GM1937	TTCATCCTCTGCTTCCTTTGA	TGACCAAACCCATCATCATCT	(TC)12	4	107	Guo et al., 2012
GM1043	GAATTCAGCTTGTGGATTGGA	TTGTTGTTGTTAGGCCACCAC	(TGA)6	4	258	Guo et al., 2012
GM1357	ACGCAATGCACATCCTTTAGA	CAGAAACAGGTGAAGGAGCTG	(TTC)6	5	103	Guo et al., 2012
GM1483	GCTGTTACATGGGCATCATTT	TCATCAGAGACCCAAGATCCA	(TCA)13	5	113	Guo et al., 2012
GM2444	CCCTGTTACACACAAGCCATT	TGAGCAAGTGTTAGCCATGAA	(CAA)10	5	268	Guo et al., 2012
GM1477	GTTTGTGTTTGTGCCGAACTT	CAAGCAACCCTTGATGTGTTA	(TTTG)5	5	408	Guo et al., 2012
GM1834	GAAGCAAGAAACCAACCAAGTC	GTGATAAAGCGGCCACAATAG	(CAT)9	5	104	Guo et al., 2012
GM2103	GCAACATGCCCTTAGACATACA	GCTTTCTCTTCTCGCTTCCTC	(TGA/TGA)	5	294	Guo et al., 2012
GM1489	GGAAGATGTGGTTGCAAATTC	CTCCCAGCTATCAACTTCACG	(AT)35	4	408	Guo et al., 2012
S046	ATGGCGAATCGGAGGGTAGGTT	TCCAATCGTGCGTTTCAATCATCT	GAA	4	272	Wang et al., 2007
S108	GCTTACATTACACGTCATCTC	CCGAACTTACAGTTAGGAG	(TC)14(AC)15	4	221	Wang et al., 2007
S003	GCACCAATTTTGTCCCTGAT	AAGGGGTTTGCACGTAAATG	TCT	5	188	Wang et al., 2007
S021	AGTCCTACTTGTGGGGGTTG	TCCCTTTTGCAGTGAAATCC	CT	5	240	Wang et al., 2007
GM2730	GGAGACGAGTTGTTGTTACCG	GGAAAGAGACCCCATCAACTC	(ATG)6	4	100	Guo et al., 2012
S073	AGTCCACTGAACCGAACACCAATC	TCCCTACCACCGAACGAAACAAT	AAG	4	335	Wang et al., 2007
GM2084	CGCAGAAATGAACCGAAATTA	GGATGCATTCTTCTTCCTCCT	(ATG/TGA/AACAAT)	5	418	Guo et al., 2012
S076	ATACTGATAGATAGGGTCGAAGGAGAG	CAACGAAAGAAAAATAAGGACATAGTG	GA	5	305	Wang et al., 2007
S118	TATATGATGCTTGATTGAGACT	CATGTAGAAGGCTTGGAGGGTAT	(AAT)6	5	271	Wang et al., 2007
GM1959	GTGTTCTCAGCCATCTTTTCG	GTGAAGGTGTTGTGAATGCAG	(TC)22	6	158	Guo et al., 2012
GM2053	ACAAGGAAAACCCATCCAATC	ACGTGATGGATTCTTGTGGAG	(TCT)5, (TGATTC)3	4	405	Guo et al., 2012
GM995	GAGGAAGCGGATTAGTTGAGG	GAAAGCTACCGCTGTCAAGTG	(TG)8	5	408	Guo et al., 2012
S084	CAGCCAATATGTCACAACCCTAAT	CTCCCACTACAAATCTCCAATCAAT	GA	4	265	Wang et al., 2007
GM1533	CCATTAGCTGAAGGCATTGAA	CAAGATGGTGACGAGAAGAGC	(TTC)7	5	250	Guo et al., 2012
GM2638	ATGCTCTCAGTTCTTGCCTGA	CAGACATAACAGTCAGTTTCACC	(TC)14	5	107	Guo et al., 2012
S024	GGCAATGCACACGCTACTCT	CGTGAGGCGTGAGAGTTCAT	GA	5	217	Wang et al., 2007
S086	TCCATGAGGGGTTATAGGTGTTT	GGGTGTATTTCTGAAGTTCCATTATC	TTC	5	132	Wang et al., 2007
GM1911	CAGCTTTCTTTCAATTCATCCA	CACTTCGTGTTCTTCCTGCTC	(AG)25	5	136	Guo et al., 2012
S070	CCTTTCCCATTCCATTAGC	GTCCGAGTTGAGGAACAACAA	TTG	5	258	Wang et al., 2007
S080	GGCGTCCCATTGCTTAC	AGAATGCGTTGATGTTATGAA	TCT	5	225-267	Wang et al., 2007
S113	TTGCATGTAGGAAAGAAAGATT	TTGGATGTGGTGGTGATGT	(TC)9	5	263	Wang et al., 2007
GM1577	GCGGTGTTGAAGTTGAAGAAG	TAACGCATTAACCACACACCA	(GA)13	5	278	Guo et al., 2012
GM2602	ACTCGATTGGGAACTGAGGAT	TCTCGTCTCTGCCATTAGTACC	(GAA)7	6	97	Guo et al., 2012
GM2348	ACACAAGAACCACCAAAAGCA	CAGCGCCATTTCTCAACTATC	(CA)8	4	142	Guo et al., 2012
GM1991	GAAAATGATGCCGAGAAATGT	GGGGAGAGATGCAGAAAGAGA	(TC)12	6	122	Guo et al., 2012
S093	TTGGGGAAATACAGAATAACG	CTCCCACATCCCCACCAT	(TG)10(AG)14	5	162	Wang et al., 2007

Table 2. Contd.

Primer	Forward sequence	Reverse sequence	Repeat motif	Number of repeat units	Allele size (bp)	Reference
S052	CCCTGAGAATGAAAGAAAGAAACA	CAACCGCAGCGACGATAGATG	GA	6	142	Wang et al., 2007
GM822	CACGGAACCCAGATAAACTGA	ATCACCATCACCATCGTTGTC	(ACG)5	5	409	Guo et al., 2012
GM1996	CATCCCATCATTTTCCCTCTT	TACAGTGAAGGTGGGATCCTG	(CAA)19	6	150	Guo et al., 2012
GM1864	CAACACACCCAGTCACTCTCTC	TCCTTTCTGATGTTCTGTGTGTG	(CT)15	5	106	Guo et al., 2012
GM1502	TTCCTTTACACACACGCACAC	TGGAGGAAATGTAGGGAAAGG	(TC)19	7	424	Guo et al., 2012
GM2504	ACATCAATCCCTGCCTACCTC	TCGGATTCTGTTACCACCTCA	(AT)18	7	307	Guo et al., 2012
S023	CTGGAAGTGGTCCTGTTGGT	GCTGCTCCTGTCTCTGGAAT	TGT	6	188	Wang et al., 2007
GM2637	ATGCTCTCAGTTCTTGCCTGA	AAGGAGCCAGCTAGCTACATAGT	(GA)12	6	131	Guo et al., 2012
S038	GGCAGCGAAGCACCCATTGTTA	GTAGGGTTGCGTTTCGTTTTCTTATCG	GA	8	212	Wang et al., 2007
GM2009	CAAACGCATACACCCCATAAC	TTTGGTTCTCGTTTGTGTTTT	(AG)16	7	107	Guo et al., 2012
S083	CTTGAACTTATTTTTGGTGGGTGAAC	CAAGGGAGAATGAAGAATGCTAAG	(CA)15TA(CA)11	6	247	Wang et al., 2007
GM1986	GCTGCTGCAAGTCTTAAGGAA	AAAGTGTCAGGTGCAAAGCAT	(TA)14	6	133	Guo et al., 2012
GM2605	ACTGCTGCCATGGTTGAGTTA	TTTCGCACTTTCTCAGTTTCC	(CT)8	7	104	Guo et al., 2012
S001	TGGACTAGACAAGGAACAACCA	GAGCCATGAGCACACAACAC	CT	8	202	Wang et al., 2007
S019	GCTCCACTAGTGCCGAAATC	CAGACACCCGGAGGCTTA	CT	9	103	Wang et al., 2007
GM744	TGATGCCTGAGAGACTTTGGT	GACTCCTTCACCTCCCTAAGC	(GAA)5	8	408	Guo et al., 2012
GM840	GCAGCATACAAGCAATCCACT	TTTGCCATTTGCTGTTCTACC	(GT)9	9	415	Guo et al., 2012
S009	CGCTGTCCTTATCGAACCAT	CTCTCACTCGCGCTTTCTCT	AG	11	125	Wang et al., 2007

Table 3. Summary statistics and polymorphism of markers used in this study, calculated using PowerMarker v.3.25. Map position and distances were obtained from the integrated consensus map of cultivated groundnut (Shirasawa et al., 2013).

Marker	Major allele frequency	Allele number	Gene diversity	PIC	Map position	Distance (cM)
GM1357	0.56	2.00	0.49	0.37		
GM1483	0.56	3.00	0.56	0.47	B03, B10	40.21, 3.239
GM1502	0.82	5.00	0.31	0.29	B03	38.452
GM1577	0.13	21.00	0.93	0.92	A05	53.751
GM1834	0.52	4.00	0.54	0.43		
GM1864	0.31	4.00	0.74	0.69		
GM1911	0.31	8.00	0.79	0.77	A09	83.224
GM1937	0.52	7.00	0.67	0.64	A07	74.214
GM1959	0.21	13.00	0.87	0.86	B04	64.853
GM1986	0.29	13.00	0.84	0.82	A07, B08	84.217, 73.894
GM1991	0.18	12.00	0.90	0.89	B06	92.887

Table 3. Contd.

Marker	Major allele frequency	Allele number	Gene diversity	PIC	Map position	Distance (cM)
GM1996	0.63	5.00	0.55	0.50	B03	63.726
GM2053	0.91	3.00	0.17	0.16	B03	74.424
GM2084	0.23	17.00	0.89	0.88	B05	21.271
GM2348	0.63	5.00	0.53	0.47		
GM2637	0.24	8.00	0.83	0.81	A04	88.62
S3	0.91	3.00	0.17	0.16		
S21	0.33	4.00	0.73	0.68		
S23	0.78	3.00	0.36	0.32		
S38	0.54	4.00	0.61	0.54		
S70	0.52	5.00	0.64	0.59		
S80	0.50	6.00	0.66	0.61		
S83	0.56	3.00	0.57	0.49		
S84	0.54	3.00	0.59	0.52		
S93	0.57	3.00	0.53	0.44		
Mean	0.49	6.56	0.62	0.57		

20 genotypes belonging to minor clusters. Significant among them was a cluster that contained the single genotype ICIAR 19BT, and shared 76% similarity with the others. The remaining genotypes within sub-cluster IB showed similarities ranging from 90 to 100%. Sub-cluster IIA and IIB shared a similarity coefficient of 75%. Sub-cluster IIA had four minor clusters at 80% similarity with one cluster containing a single genotype (ICGV-IS 08837), which was medium-maturing and exhibited high tolerance to foliar diseases. Sub-cluster IIB had two minor clusters with similarity coefficients ranging from 84 to 100%.

Although the groundnut genotypes were grouped based on their phenotypic attributes, the clustering from DNA analysis did not match these phenotypic groupings. Genotypes from different groups were found to cluster together irrespective of their phenotypic characters, and this observation is consistent with the findings of other studies which also found low genetic diversity within cultivated groundnut (Jiang et al., 2007; Janila et al., 2013). Based on these results, there is a strong possibility that the majority of genotypes tested in this study shared a similar pedigree since few parents have historically been used in groundnut breeding programs (Nigam, 2000; Janila et al., 2013). The limited number of parents used in groundnut breeding programs stems from the fact that the hybridization of two possible diploid ancestors (*Arachis duranensis* and *Arachis ipaensis*), followed by chromosome doubling, resulted in the allotetraploid genome of cultivated groundnut (Nagy et al., 2012; Shirasawa et al., 2013; Janila et al., 2013), which introduced a crossing barrier with its wild diploid ancestors and therefore limited the sources of allelic variability needed for groundnut improvement (Nagy et al., 2012; Janila et al., 2013).

A number of reports on the use of SSR markers to characterise groundnut have produced results similar to those obtained in this study. For example, Mace et al. (2006), who used 23 SSR primers to study 22 groundnut genotypes with varying levels of resistance to rust and early leaf spot, recorded 52% polymorphism with a PIC value ≥ 0.5. In a study with 31 groundnut genotypes that exhibited different levels of resistance to bacterial wilt, Jiang et al. (2007) also found that 29 of the 78 SSR primers were polymorphic, and amplified a total of 91 polymorphic loci with an average of 2.25 alleles per primer. Similarly, Tang et al. (2007) employed 34 SSR markers to determine the genetic diversity in four sets of 24 accessions from the four botanical varieties of cultivated groundnut, and found that 16 primers were polymorphic. This led to the conclusion that abundant inter-variety SSR polymorphism exists in groundnut.

A recent study which assessed the diversity of 11 groundnut genotypes using 17 SSR markers, also recorded 24% polymorphism (Shoba et al.,

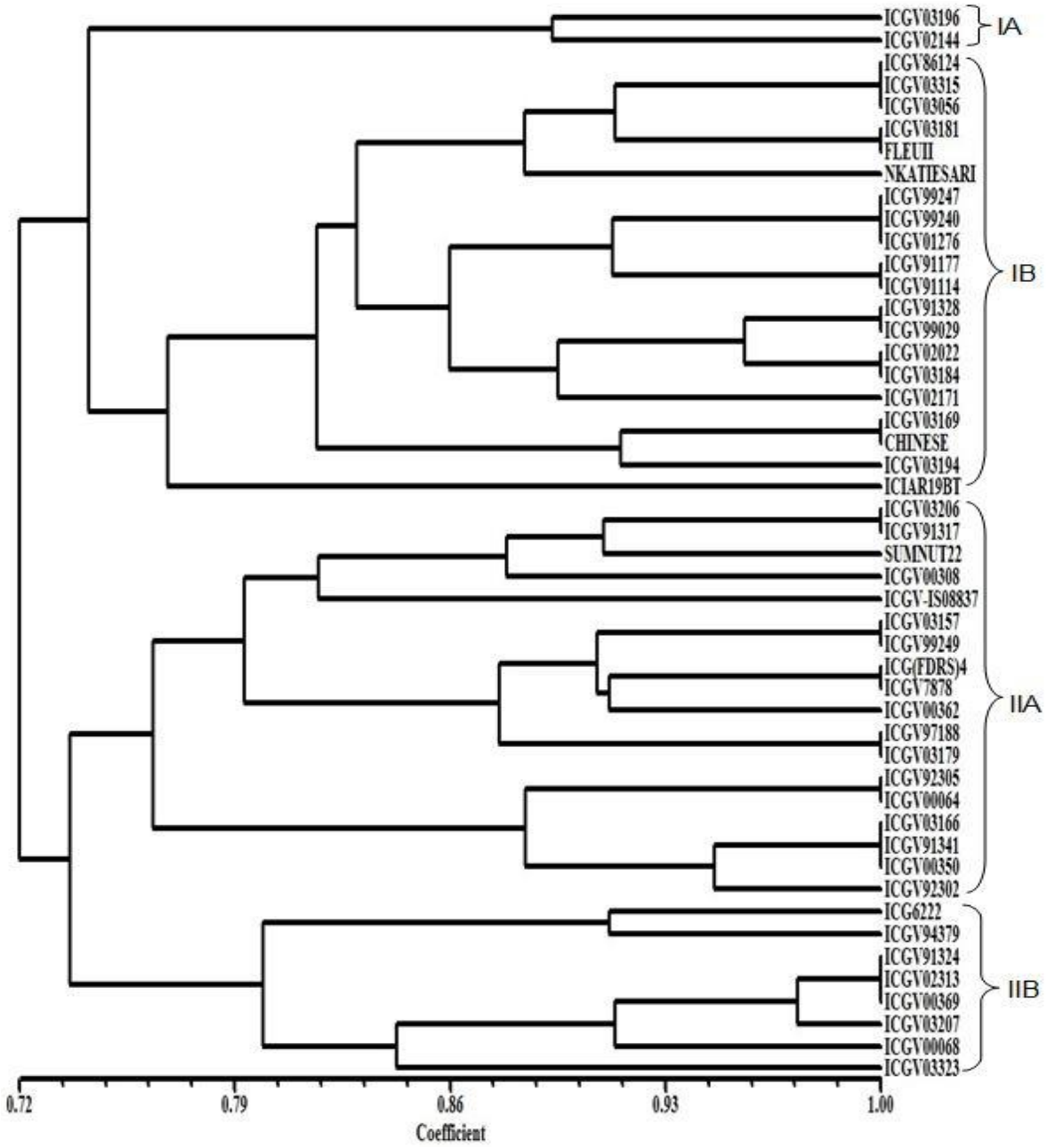

Figure 1. Genetic diversity among 48 groundnut genotypes generated using the unweighted pair group method with arithmetic mean (UPGMA) procedure based on the Jaccard's similarity coefficient created with NTSYSpc v. 2.1.

2010). Mondal and Badigannavar (2010) similarly used 26 SSR primers to amplify 136 bands and showed that 76.5% were polymorphic in 20 cultivated groundnut genotypes that differed in resistance to rust and late leaf spot disease. It is therefore interesting that, in this study, a total of 164 bands were amplified using 54 SSR markers on 48 groundnut genotypes, and 25 were found to be polymorphic. But more importantly, the map position and distance of some of the polymorphic markers used in this study (Table 3) have recently been identified on the consensus genetic map of the *Arachis* genome (Shirasawa et al., 2013). Furthermore, they have also been mapped on different linkage groups from the consensus groundnut maps created to identify QTLs associated with foliar disease resistance and drought tolerance (Table 4). Marker GM1911 in this study is, for example, linked to a drought tolerance QTL (Ravi et al., 2011; Gautami et al., 2012), while markers GM1577 and GM1991 are linked to QTLs associated with tolerance to late leaf spot disease (Sujay et al., 2012). No doubt, the

Table 4. Polymorphic markers associated with traits of interest in cultivated groundnut.

Marker	Linkage group	Position (cM)	Traits of interest	Reference
GM1483	AhIV	293.4	SCMR	Gautami et al., 2012
GM1577	AhV	187.4, 10.6	TE, LLS	Gautami et al., 2012; Sujay et al., 2012
GM1911	AhXIII	11.3, 53.0	SCMR, SLA, LLS, Rust	Ravi et al., 2011; Gautami et al., 2012; Sujay et al., 2012
GM1937	AhXVI	32.1, 6.9	HI, TDW, VW, SCMR	Gautami et al., 2012; Ravi et al., 2011
GM1959	AhXVII	24.6	SCMR, HI	Gautami et al., 2012
GM1991	AhX	21.8	SCMR	Ravi et al., 2011
GM1996	AhXIV	64.1	SLA	Gautami et al., 2012
GM2637	AhVII	55.4	TW	Gautami et al., 2012

SCMR - SPAD chlorophyll meter reading, TE - transpiration efficiency, LLS - late leaf spot resistance, SLA - specific leaf area, rust - Rust tolerance, HI - harvest index, TDW - total dry weight, VW - vegetative weight.

identification of polymorphisms associated with these important agronomic traits has potential for advancing groundnut improvement in Ghana, as they can be used in QTL mapping, and/or marker-assisted breeding activities (for example, marker-assisted backcrossing and marker-assisted recurrent selection).

Taken together, the results of this study demonstrate that SSR markers can be very effective in discerning variations among the 48 different groundnut genotypes despite their close relatedness, a finding consistent with other studies (Hopkins et al., 1999; Cuc et al., 2008; Carvalho et al., 2010). The mean PIC value of 0.57 suggests that the primers were highly polymorphic (Pandey et al., 2012b) and can be applied to different groundnut populations in breeding programs. The clustering of groundnut genotypes in this study was independent of their phenotypic attributes, and thus confirmed the low level of genetic variability in cultivated groundnut (Pandey et al., 2012a; Janila et al., 2013). The relatively low genetic diversity within the genotypes used in this study was likely due to the fact that they were crosses generated from a breeding program. The SSR markers could discern variations and differentiate between the closely related groundnut genotypes, makes this technology a powerful tool for genomic characterisation of groundnut. The relatively diverse genotypes identified in this study are potential candidates for use as parental material in future studies to advance groundnut breeding in Ghana.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Effects of *Moringa oleifera* leaf extract on morphological and physiological growth of cassava and its efficacy in controlling *Zonocerus variegatus*

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Zonocerus variegatus **infestation has been found to cause a lot of devastation in the production of cassava crop plants in tropical and subtropical areas. Understanding the efficacy of *Moringa oleifera* leaf extract on controlling *Z. variegatus* is desired. The present study was conducted in the Department of Crop Science experimental site, Faculty of Agriculture, University of Nigeria, Nsukka to investigate the effects of *M. oleifera* leaf extract (MLE) on the morphological and physiological growth characteristics of cassava (*Manihot utilissima* Pohl.); and its efficacy as organic insecticide for controlling *Z. variegatus* infestation. *M. oleifera* leaf extract diluted in water at the volume to volume (v/v) ratios of 1:10, 1:20, 1:30 and 1:40 were applied weekly while "Uppercut [(R)]" (30 g dimethoate plus 250 g cypermethrin), used as water emulsifiable concentrate, was applied at the rate of 0.2 a.i./ha at three-weekly interval for two months. Result shows that *M. oleifera* leaf extract (MLE) dilution at the ratio of 1:30 MLE in water gave the highest percentage stem height difference (%SHD) at 32 weeks after planting with a value of 20.5% followed by 1:20 (17.8%), 1:10 (10.56%), dimethoate plus cypermethrin (8.98%) and 1:40 (8.05%). The control had the least %SHD and %LND. Dimethoate plus cypermethrin treatment was more efficacious as it eradicated the insects and caused significant ($p < 0.05$) increase in the number of leaves and reduction in the percentage leaf abscission especially from the thirtieth week after planting. However a combined use of Dimethoate plus cypermethrin and *M. oleifera* leaf extract may give a better result and as such recommended than a single treatment application.**

Key words: Moringa extract, dimethoate plus cypermethrin insecticide, cassava hybrid, *Zonocerus variegatus* infestation.

INTRODUCTION

Cassava (*Manihot utilissima* Pohl.) belongs to the *Euphorbiaceae* family, and it is one of the most important root crops in the tropics especially Nigeria. Four African countries including Mozambique, Nigeria, Tanzania and Zaire, were among the 10 largest producers of cassava in the world by 2005 (Anikwe et al., 2005). All the cultivars of cassava belong to the species *Manihot esculenta* Crantz and *M. utilissima* Pohl. These species can be differentiated based on many factors such as shape of their leaves, plant height, petiole color, leaf size, etc.

(Anikwe et al., 2005). Cassava can be used in various ways such as cassava pellets, cassava chips, cassava flakes and fermented cassava. Some of the pests of cassava include termites, mites, mealybugs such as *Phenacoccus manihotis,* and grasshopper such as *Zonocerus variegatus. Z. variegatus* can defoliate the leaves and reduce the photosynthetic capacity of a plant. Some other plants attacked by *Z. variegatus* include cowpea, maize, soybean, sweet potato etc. *Z. variegatus* infestation has been found to cause a lot of devastation in the production of these crop plants.

Some botanicals such as neem (*Azadirachta indica*) seed extract and scent leaf (*Ocimum gratissimum*) extract are used as insect-repellent liquids (Silva et al., 2010). *Moringa oleifera* leaves have been found to possess some antibacterial and antifungal characteristics (Rao et al., 2007; Arya et al., 2010). Moringa is an all-purpose plant. It is a native of India but is widely cultivated in some sub-Saharan African countries like Zimbabwe, Madagascar, Zanzibar, South Africa, Tanzania, Malawi, Benin, Burkina Faso, Cameroon, Chad, Gambia, Ghana, Guinea, Kenya, Liberia, Mali, Mauritania, Nigeria, Niger, Sierra Leone, Sudan, Ethiopia, Somalia, Zaire, Togo, Uganda and Senegal (Amaglo, 2010; Annenber, 2010; Fuglie and Sreeja, 2011). Every part of the plant can be used for one thing or the other. The leaves have very high nutritional value. They are good sources of protein, minerals, vitamins, beta-carotene, amino acids and various phenolic compounds. They provide a rich and rare combination of zeatin, quercetin, beta-sitosterol, caffeoylquinic acid and kaempferol (Moyo et al., 2011). The seed is eaten like a peanut in Malaysia. The thickened root is used as substitute for horseradish. The foliage is eaten as green garnish in salads, in vegetable curries, as pickles, and it can also be used for seasoning.

Moringa is very important for its many impressive ranges of medicinal uses. Various parts of this plant such as the leaves, roots, seeds, fruits, flowers and immature pods act as cardiac and circulatory stimulants. They possess antitumor, antipyretic, antiepileptic, anti-inflammatory, antiulcer, antispasmodic, diuretic, antihypertensive, antidiabetic, hepatoprotective, antibacterial and anti-fungal, cholesterol lowering properties and some antioxidants (Fuglie and Sreeja, 2011; Moyo et al., 2011; Oz, 2014). The leaves are ground and used for scrubbing utensils and for cleaning walls. Its seeds yield about 40% of non-drying oil, known as Ben or Oleic oil, used for cooking, lubricating watches and other delicate machinery, soap and cream making etc. The oil is clear, sweet and odorless, and it is useful in the manufacture of perfumes and weave-on oil in hairdressing. The oil compares favorably with olive oil (Moyo et al., 2011; Oz, 2013). Moringa wood yields a blue dye. The leaves and young branches are eaten by livestock. It is planted as a living fence tree. The bark can serve for tanning; its mature seeds can also be used to purify water. The flowers which are present throughout the year, are good sources of nectar for honey producing bees, thus its presence enhances production in other crops due to increase in pollination activities by bees (Fuglie and Sreeja, 2011). Incorporation of the green leaves of *M. oleifera* into the soil had been successfully used in preventing damping off disease caused by *Pythium debaryanum* in seedlings of okra plant (Rao et al., 2007; Arya et al., 2010). Little or no information had been given on the use of *M. oleifera* leaves or the extract as organic pesticide. The main objectives of this work were to determine the effect of *M. oleifera* leaf extract application at various concentrations on some morphological and physiological characteristics of cassava, and also study the efficacy of *M. oleifera* leaf extract in controlling *Z. variegatus* infestation on cassava.

MATERIALS AND METHODS

The experiment was carried out in the experimental plot (crop garden) of the Department of Crop Science, Faculty of Agriculture, University of Nigeria, Nsukka (UNN). The treatments involved the use of different concentrations of *M. oleifera* leaf extract (as organic insecticide) and dimethoate plus cypermethrin at the rate of 0.2 a.i./ha (as inorganic insecticide) to control *Z. variegatus* in cassava. The dimethoate plus cypermethrin was collected from the Entomology Unit of the Department of Crop Science, UNN. The moringa leaf liquid extraction was done by pounding the freshly harvested leaves in a clean mortar and squeezing out the liquid through a filter paper into a container. Lower mature leaves (6^{th} leaf and below) were used for the experiment because of their high composition of phytochemicals (Ndubuaku et al., 2014). The different concentrations of the moringa leaf extract (MLE) were made by mixing the extract with water at the following v/v ratios: 1:10, 1:20, 1:30 and 1:40. The untreated plot was the control. The *M. oleifera* leaf extract (MLE) was applied to the cassava plants weekly while the dimethoate plus cypermethrin was applied tri-weekly using a hand sprayer to avoid drift. The spraying was done for eight weeks between April and June at the onset of regular rainfall when the *Zonocerus* infestation was heavy relative to other pests. During this period, the cassava plants were between four and six months old (16 weeks before and 24 weeks after spraying). The cassava cultivar used in this study was a hybrid, CR1247 and the cuttings were planted at the spacing of 1 × 1 m. The experiment was laid out in a randomized complete block design (RCBD) with four replications. The four replicates constituted the blocks.

The morphological characteristics studied included the

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Abbreviations: MLE, *Moringa oleifera* leaf extract; **RCBD,** randomized complete block design; **SPSS,** statistical package for social sciences; **SHD,** stem height difference; **WAP,** weeks after planting; **IPM,** integrated pest management.

Table 1. Effect of *Moringa oleifera* leaf extract (MLE) and dimethoate plus cypermethrin on mean population of *Zonocerus variegatus* in a cassava plot between 1 and 7 weeks after spraying (WAS).

Concentration (MLE : Water)	Weeks after spraying (WAS)								Treatment mean
	Before spaying	1 WAS	2 WAS	3 WAS	4 WAS	5 WAS	6 WAS	7 WAS	
1:10	2.12	2.35	2.12	1.87	2.35	2.74	2.92	3.09	2.45
1:20	1.87	2.12	2.35	1.87	2.12	2.55	2.92	2.74	2.32
1:30	2.35	2.35	2.12	2.12	2.12	2.35	2.74	2.92	2.38
1:40	2.12	1.87	2.12	1.87	2.12	2.35	3.24	3.24	2.37
Dimethoate plus cypermethrin	2.35	1.87	1.58	1.22	1.22	1.22	0.71	0.71	1.36
Control	2.35	2.55	2.12	2.35	2.12	2.74	3.39	3.39	2.63
Mean	2.19	2.07	2.07	1.88	2.01	2.33	2.65	2.68	
F-$LSD_{0.05}$	NS	NS	NS	NS	NS	NS	1.39	1.40	

NS means not significant.

percentage stem height difference (% SHD). The stem height was measured with a meter ruler and the percentage of the differences over the different periods of observation was calculated. The number of leaves was determined by the total number of leaves on the plant in each sampling period and the percentage difference (% LND) calculated as the difference in the number of leaves at two consecutive periods. The physiological growth characteristics included the rate of leaf fall (% leaf abscission),

$$\% \text{ Leaf abscission} = \frac{(T_2 - T_1)(L_1 - L_2 - nf)}{L_1 (T_2 - T_1)} \times \frac{100}{1}$$

Where, L_1 = Number of leaves at the periods T_1, L_2 = Number of leaves at the period T_2, nf = the number of new leaves (leaf flushes), and the rate of leaf appearance (leaf flushing),

$$\% \text{ Leaf appearance } \frac{(T_2 - T_1)(L_1 - L_2)}{L_1 (T_2 - T_1)} \times \frac{100}{1}$$

Where, L_1 = Number of leaves at the periods T_1, L_2 = Number of leaves at the period T_2

The insect population was determined by counting the number of insects on the plants at each period of observation and transforming the data collected into indexed values as shown below using linear additive model, Statistical Package for Social Sciences (SPSS) Release 7.22 Edition 3 (Obi, 2002):

$Y^2 = X + 0.5$

Where, Y = insect population (indexed), X = number of insects on the plants.

Data collected were presented in percentages. The transformed data were analyzed using analysis of variance (ANOVA) and significant means separated using Fisher's least significant difference at 5% probability.

RESULTS

The percentage stem height difference (% SHD) in the cassava plants treated with 1:10, 1:20, and 1:30 of *M. oleifera* leaf extract (MLE) in water increased with time as shown in Table 1. The 1:40 MLE in water increased the % SHD from 28 weeks after planting (WAP). There was a decreasing effect of Dimethoate plus Cypermethrin treatment on the percentage stem height difference (% SHD) 26 WAP, after which the value increased steadily from 28 to 32 WAP. The % SHD of the control plants decreased at 30 WAP. The plants treated with 1:30 MLE in water showed the highest % SHD (20.5%) on the 32 WAP followed by those treated with 1:20 MLE in water with the highest value (18.43%) on the 30 week. There were significant differences ($p < 0.05$) in the % SHD of the plants treated with the different ratios of MLE in water from the 26 to 32 weeks after planting (Figure 1). With the exception of the plants treated with dimethoate plus cypermethrin, the percentage difference in the number of leaves (%LND) in the MLE-treated plants, including the control, decreased with time. The 1:10 MLE in water treatment caused a reduction in the % LND at 32 week while in the other treatments, with the exception of dimethoate plus cypermethrin treated plants, the reduction started from 30 weeks (Figure 2). There were significant differences ($p < 0.05$) across the treatments from 26 to 32 weeks after planting.

The percentage leaf abscission (% LA) in all the treatments, except dimethoate plus cypermethrin, increased from 30 weeks with the initial decrease at 26 weeks after planting. The % LA in the dimethoate plus cypermethrin treated plants decreased consistently from the 26 to 32 week after planting. The highest % LA (30%) showed in the control plants at 32 weeks after treatment (Figure 3). There were significant differences ($p < 0.05$) in the % LA of the plants across the treatments from 26 to 32 week after planting. The treatment effect on percentage leaf appearance (leaf flushing) is shown in Figure 4. It increased progressively and consistently in all the treatments, including the control, from the 26 to 32 week after planting. However, 1:40 MLE in water gave

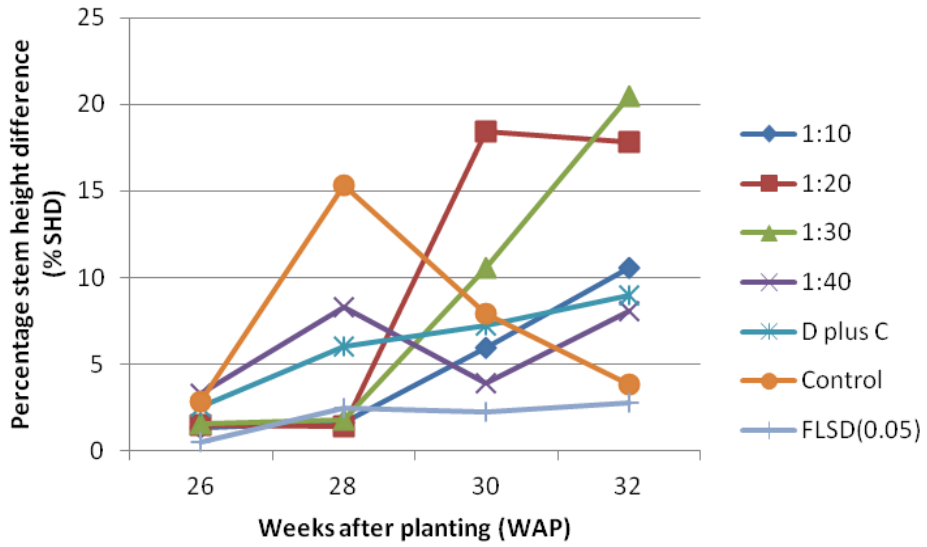

Figure 1. Effect of different concentrations of *Moringa oleifera* leaf extract (MLE) and dimethoate plus cypermethrin on percentage stem height difference (%SHD) of cassava between 26 and 32 weeks after planting (WAP), D plus C = dimethoate plus cypermethrin.

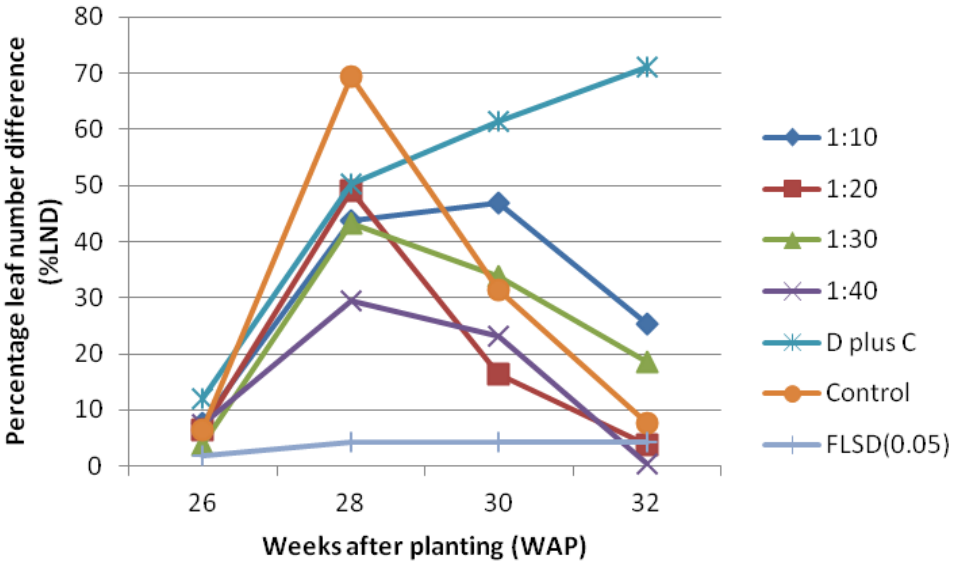

Figure 2. Effect of different concentrations of *Moringa oleifera* leaf extract (MLE) and dimethoate plus cypermethrin on percentage leaf number difference (% LND) of cassava plants between 26 and 32 weeks after planting (WAP), D plus C = dimethoate plus cypermethrin.

the highest percentage leaf appearance at the thirty second week after planting followed by 1:30, 1:20, 1:10, dimethoate plus cypermethrin and control in that order (31, 29, 28, 26, 20 and 17%, respectively). There were no significant differences ($p > 0.05$) across the various treatments in the first two weeks of treatment but there were significant differences ($p < 0.05$) between twenty eighth and thirty second week after planting (Figure 4).

Data comparing the effects of *M. oleifera* leaf extract (MLE) and dimethoate plus cypermethrin on *Z. variegatus* population are shown in Table 1. The population of the insect in the plot treated with dimethoate plus cypermethrin decreased progressively from 1 to 7 weeks after spraying. There was an increase in the insect population in all the *M. oleifera* leaf extract-treated plants, as well as the control, from the fifth week after spraying.

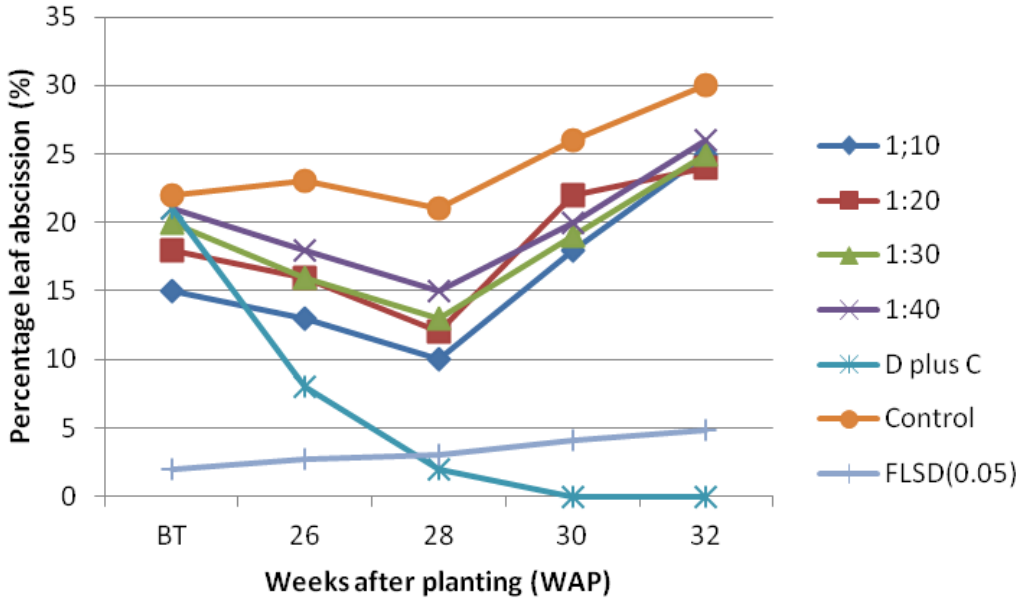

Figure 3. Effect of different concentrations of *Moringa oleifera* leaf extract (MLE) and dimethoate plus cypermethrin on percentage leaf abscission (%LA) in cassava between 26 and 32 weeks after planting (WAP), D plus C = dimethoate plus cypermethrin, BT = before treatment.

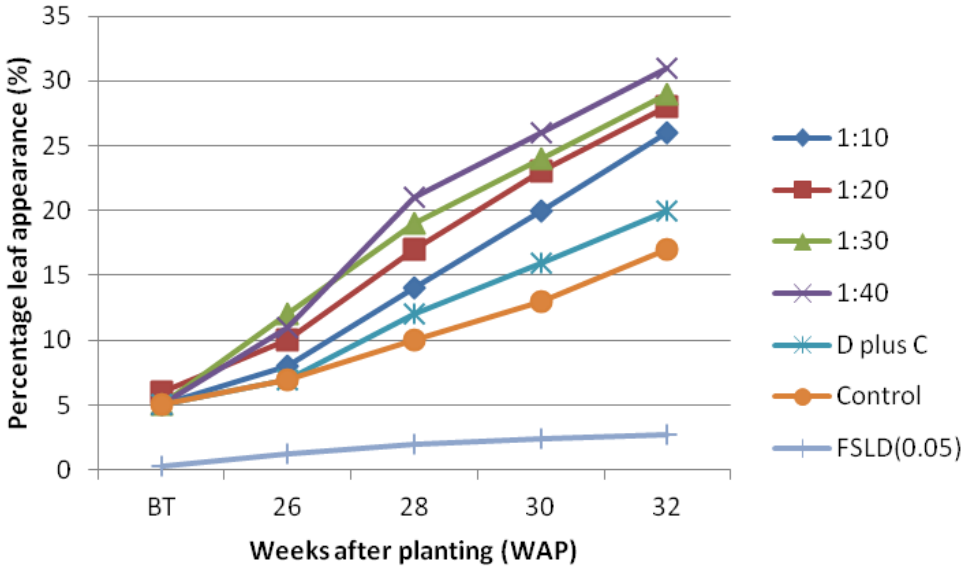

Figure 4. Percentage leaf appearance (flushing) in cassava weeks after treated with different concentrations of *Moringa oleifera* leaf extract (MLE) and dimethoate plus cypermethrin between 26 and 32 weeks after planting (WAP), D plus C = dimethoate plus cypermethrin, BT = before treatment.

The control plants had the highest insect population (2.63) followed by the plants treated with 1:10, 1:30, 1:40 and 1:20 MLE in water in that order (2.44, 2.38, 2.37 and 2.32, respectively). There were significant differences ($p < 0.05$) in the insect population across the treatments from the 6 to 7 week after spraying.

DICUSSION

The high values of percentage stem height difference (% SHD) obtained in 1:10, 1:20 and 1:30 MLE in water especially at the thirty second week after planting could be attributed to the high nutritional values of the extract at

these concentrations. Fuglie and Sreeja (2011) observed that 1:32 MLE in water increased the growth and yield of maize and other crops by 30%. The dimethoate plus cypermethrin treatment gave the highest percentage leaf number difference (70.99) at the 32 weeks after planting and the value increased consistently from 28 to 32 week after planting. This could be ascribed to the total eradication of the insect especially at the 6 and 7 week after spraying, thus, reducing the level of defoliation (leaf abscission) and increasing the total number of leaves mostly with the new flushes emerging over the period. The high nutritional qualities of *M. oleifera* leaf extract further increased the general growth of the plants and enhanced the rate of leaf appearance on the MLE treated plants as earlier reported by Fuglie and Sreeja (2011). Ndubuaku et al. (2006) observed that increase in the number of leaves as a result of leaf flushing enhanced the photosynthetic capacity of cocoa plants, increased the photosynthate accumulation and total yield. The fluctuations in the percentage leaf number especially in the MLE-treated plants could also be due to high percentage leaf abscission obtained in the plants as shown in the result which might not be commensurate with the rate of leaf flushing (percentage leaf appearance). The variations in the %SHD, %LND, %LA and percentage leaf appearance measured fortnightly (biweekly) showed the marginal differences in the growth traits which could also reflect in the weekly measurements. Rain is a major constraint in foliar pesticide/fertilizer application to plants because much of the applied chemical can be washed off after immediate rain following application thus, reducing the amount actually absorbed by the plant. However, in all the applications of the MLE and dimethoate plus cypermethrin, there were no immediate rains before and after applications. Hand sprayer was used for application to reduce wind-drift and ensure direct contact with the plants.

At all the concentrations of *M. oleifera* leaf extract and the control, the insect population increased from the fifth week of treatment application which coincided with the period of heavy rain (around May/June). Fuglie and Sreeja (2011) reported that *M. oleifera* leaves and young shoots could be used for animal feed because of their high nutrient contents. Thus, the leaf extract, instead of acting as organic insecticide, could have been a source of nutrition for the insect to increase its strength and destructive abilities. This reflected in the high rate of defoliation/ leaf abscission recorded in MLE treated plants from 6 week of treatment when the level of insect infestation on the plants increased. Dimethoate plus cypermethrin controlled the insect population better than *M. oleifera* leaf extract. This was evident in the high reduction in the population of the insect in the dimethoate plus cypermethrin -treated plants all through the period of observation. This ultimately enhanced the morphological growth (stem height and number of leaves) of the plants treated with dimethoate plus cypermethrin because they were not prone to much destruction by the insect attack. However, this does not suggest that the organic pesticide cannot be used in a good integrated pest management (IPM) program. Higher concentrations and the undiluted form of the *M. oleifera* leaf extract should be tried in further experiments to ascertain the level that can be toxic to the insect. Efforts to isolate the active ingredients in the leaf extract are important to know if *M. oleifera* leaf extract had any insecticidal quality.

Conclusion

Dimethoate plus cypermethrin controlled the insect population in the plot better than *M. oleifera* leaf extract. This was evident in the high reduction in the population of the insect in the dimethoate plus cypermethrin -treated plants all through the period of observation. This ultimately enhanced the morphological growth (stem height and number of leaves) of the plants treated with dimethoate plus cypermethrin because they were not prone to much destruction by the insect attack. However a combined use of Dimethoate plus cypermethrin and *M. oleifera* leaf extract may give a better result and as such recommended than a single treatment application.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Anti-nutrient, vitamin and other phytochemical compositions of old and succulent moringa (*Moringa oleifera* Lam) leaves as influenced by poultry manure application

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The study was carried out to determine the anti-nutrient, vitamin and other phytochemical compositions of old and succulent leaves of *Moringa oleifera* plants as influenced by poultry manure application. Three levels of poultry manure, that is, 0, 5 and 10 tonnes ha^{-1} were used for treatment. Poultry manure application insignificantly ($p > 0.05$) increased oxalate, phytate and saponin compositions of the leaves. The older leaves had higher values of tannin, oxalate, phytate and saponin than the succulent ones. The poultry manure levels did not show any significant differences ($p > 0.05$) in the proximate/chemical properties and some vitamins (vitamins A, B_1, B_2, B_6, and C). Succulent leaves had higher values of vitamins, proximate and chemical properties. The higher concentrations of the anti-nutrients in the older leaves and higher values of vitamins, proximate and chemical properties in the succulent ones provide a good guide to moringa leaf consumers, to harvest the younger succulent (first to fifth) leaves for consumption.

Key words: Anti-nutrients, moringa leaves, poultry manure, phytochemicals, vitamins.

INTRODUCTION

Anti-nutrients are substances which interfere with the metabolism and utilization of body nutrients; examples are phytates, oxalates, tannins and saponins. Moringa leaves have been found to contain little quantities of the anti-nutrients (Moyo et al., 2011). Phytates decrease the availability of minerals and reduce protein digestion by the formation of phytic acid protein complex (Oz, 2013). Tannins have beneficial effects on health. They curb

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Abbreviations: RCBD, Randomized complete block design; **OM**, organic matter; **MC**, moisture content; **IITA**, International Institute for Tropical Agriculture; **FLSD**, Fisher's least significant difference.

haemorrhage (haemostatic) and bare swelling. They are used for mouth and eye washes, vaginal douches and treatment of rectal disorders (Bamshaiye et al., 2011). At low and moderate quantities, tannins can improve protein digestion in the lumen of ruminants (Oz, 2013). Moringa leaves have been found to contain small quantities of saponins at levels that are relatively harmless to human health. Thus, moringa leaves are consumed with no side effects. Low dietary levels of saponins in soyabeans had been shown to increase growth in tilapia while high quantities tended to retard it (Fuglie and Sreeja, 2011). Oxalic acid combines with divalent metallic cations such as calcium (Ca^{2+}) and iron. Fresh and dried moringa leaves have been found to contain negligible quantities of oxalate which are not harmful to the body if the leaves are consumed in moderate quantities. However, fresh moringa leaves contain more oxalate than the dry ones (Amaglo, 2010; Moyo et al., 2011).

Previous research work by Fuglie and Sreeja (2011) had shown that moringa leaves contained up to 25.1% crude protein, 6.5% lipid, 12% ash, 27.1% protein, 2.3% fat, 38.2% carbohydrate, 19.2% fibre, 20.0% calcium (Ca), 1.37% magnesium (Mg), 0.20% phosphorus (P), 1.32% potassium (K), 0.03% iron (Fe) and 0.87% sulphur (S). Fuglie and Sreeja also obtained high levels of vitamins A, B, C and E in the dried leaves. Moringa is a multipurpose plant with high nutritional, agricultural, medicinal, domestic, industrial and environmental benefits. It is used to combat malnutrition, especially among infants and nursing mothers (Oz, 2014). The leaves are rich in B carotene, amino acids and ascorbic acids. Thus, they can be used to increase milk production in lactating mothers (Fuglie and Sreeja, 2011). The leaves of *Moringa oleifera* plant can be eaten fresh, cooked or stored as dried powder for many months without loss of vitality.

Analysis of poultry manure had shown that it was high in N, K and other nutrient elements (Ogbonna and Umar-shaaba, 2011). Singh (2010) also reported increase in N, P, K Zn, protein and carbohydrate contents of corn (*Zea mays* L.) with poultry manure application. Poultry manure and other organic fertilizers increase the nutrient status of most soils and boost crop productivity (Annenber, 2010; Singh, 2010), but there is little information on the influence of poultry manure application on anti-nutrient, vitamin and other phytochemical compositions of old and succulent leaves of moringa (*Moringa oleifera*) plants.

The objective of the study was to determine the anti-nutrient, vitamin and other phytochemical compositions of old and succulent leaves of moringa plants as influenced by poultry manure application.

MATERIALS AND METHODS

The study was carried out in the Department of Crop Science, Faculty of Agriculture, University of Nigeria, Nsukka. Three levels of poultry manure (0, 5 and 10 tonnes ha^{-1}) were used. The experiment was a 3 × 3 factorial trial in a randomized complete block design (RCBD) with three replications. The poultry manure was incorporated into the soil before planting the seeds. The seeds were planted at stake on the field at planting distance of l m × l m. The moringa seeds used were collected from three locations of Nigeria; Nsukka (Enugu State with rainforest vegetation), Dutse (Jigawa State with semi-arid vegetation) and Jos (Plateau State with savannah vegetation). Prior to seed planting, the field was tractor-ploughed and harrowed. Soil samples were collected from different locations of the field, bulked to form a composite sample and analyzed in the laboratory for physical and chemical properties. The poultry manure was analyzed for N, P, K, Ca, Mg, sodium (Na), pH, organic matter (OM) and organic carbon (C). Tables 1 and 2 represent the physical and chemical properties of the soil of the experimental site and the poultry manure used for the experiment. The poultry manure was incorporated into the soil at the different rates before planting. The moringa seeds were planted two/hole on shallow seed beds of 5 m long × 1 m wide × 15 cm high. Two weeks after emergence, the seedlings were thinned down to

Table 1. Physical and chemical properties of the soil of the experimental site.

Parameter	Value
Clay (%)	22
Silt (%)	13
Fine Sand (%)	24
Coarse Sand (%)	42
pH (H_2O)	5
pH (KCl)	4.7
Carbon (%)	1.65
Organic matter (%)	1.02
Nitrogen (%)	0.06
Sodium (meq 100 g^{-1})	0.34
Calcium (meq 100 g^{-1})	0.6
Magnesium (meq 100 g^{-1})	1.8
CEC (meq 100 g^{-1})	14.9
Base salt (%)	21
H^+ (meq 100 g^{-1})	2.7
Phosphorus (ppm)	14.99

Table 2. Chemical properties of the poultry manure.

Chemical properties	Value
pH (H_2O)	7.3
pH (KCl)	6.7
Organic C (%)	19.89
Organic matter (%)	51
N (%)	1.73
Sodium (%)	0.08
Potassium (%)	0.08
Calcium (%)	4.65
Magnesium (%)	1.35
Phosphorus (%)	1.39

Table 3. Anti-Nutrient contents of succulent and older leaves of *Moringa oleifera* plants.

Anti-nutrient	Succulent leaves	Older leaves	Mean	$t_{(0.05)}$
Tannin (g 100 g^{-1})	6.11	6.48	6.30	Ns
Oxalate (g 100 g^{-1})	2.51	3.24	2.87	Ns
Phytate (g 100 g^{-1})	8	9.75	8.88	Ns
Saponin (g 100 g^{-1})	3.77	4.32	4.04	Ns

ns = Non-significant.

one/hole. Samples of the leaves were taken monthly for laboratory analyses. Sampling was done for three months after planting. First to fifth leaf from the shoot-tip and older leaves from the base were used for laboratory analyses. The leaves were dried and ground into powder for analyses. Analyses of the leaf samples were done at the International Institute for Tropical Agriculture (IITA), Ibadan, Nigeria. The samples were analyzed for vitamins, proximate and chemical properties using the standard methods of the Association of Analytical Chemists (AOAC, 2005). The procedures used for anti-nutrient analyses are stated below:

Determination of tannin content

One gram of the leaf sample (powder) was weighed and macerated with 50 ml of distilled water and filtered. Five milliliter of the filtrate was then pipetted and to it 0.3 ml of 0.1 N ferric chloride in 0.1 N HCl added. Next, 0.3 ml of 0.008 potassium ferricyanide was added to the mixture. Absorbance was measured at 720 nm

Determination of phytate content

A test sample of 0.5 g of Moringa leaves was weighed into a 500 ml flat bottom flask. The flask with the sample was placed in a shaker and extracted with 100 ml of 2.4% HCl for 1 h at room temperature, and then decanted and filtered. Five milliliters of the filtrate was pipetted and diluted to 25 ml with distilled water. To 10 ml of diluted sample, 15 ml of sodium chloride was added and the solution passed through an ampletraisen, then 15 ml of 0.7m sodium chloride was added. The absorbance was taken at 520 nm wavelength. A standard curve and blank were prepared. Absorbance was read at the same wavelength and the concentration of phytate in the test sample was calculated.

Determination of oxalate

Two grams of leaf sample was weighed into a 300 ml flask. Twenty millilitres of 30% HCl was added and allowed to stand for 20 min. Forty grams of ammonium sulphate was added to the solution and allowed to stand for 30 min. The solution was filtered into a 250 ml volumetric flask and made up to the mark with 30% HCl. Ten ml of the filtrate was transferred into a 100 ml centrifuge tube and 30 ml diethyl ether added. The pH was adjusted to x with ammonium hydroxide, then centrifuged at 10 000 g for 15 min. Decantation into a 250 ml conical flask followed. The decant was titrated with 0.1 M potassium tetraoxomanganate (VII) ($KMnO_4$). The volume was recorded and calculations done as follow:

$$\% \text{ Oxalate} = \frac{\text{Titre} \times \text{mol } KMnO_4 \times \text{DF } (12.5)}{\text{Weight of sample}} \times 100$$

Determination of saponin

One gram of the leaf sample was weighed and macerated with 10 ml of petroleum ether. Decantation into a beaker followed. Another 10 ml of petroleum ether was added to the residue left out, macerated and decanted into a beaker. The filtrates were combined and evaporated to dryness. The residue was dissolved with 6 ml of ethanol. Two ml were then transferred into a test tube and 2 ml colour reagent added. This was allowed to stand for 30 min at room temperature. Absorbance was measured at 550 nm.

Statistical analysis

Statistical data collected were analyzed using analysis of variance (ANOVA). The T-test was used to compare two sets of data obtained on two comparable variables, for example, old and succulent leaves. Significant means were compared using Fisher's least significant difference (FLSD) at 5% probability according to Obi (2002). The statistical package used was Genstat 3.0 release 4.23 Discovery Edition (2008).

RESULTS

The older leaves of the moringa plants had higher concentrations of tannin, oxalate, phytate and saponin than the succulent leaves. There were no significant differences ($t > 0.05$) in the anti-nutrient contents of both leaf ages (Table 3). The effect of poultry manure rates on the anti-nutrient contents of moringa leaves is presented in Table 4. The different poultry manure levels showed no significant differences ($p > 0.05$) in the anti-nutrient compositions of the leaves. However, the highest value (6.48 g 100 g^{-1}) of tannin in the leaves was obtained at 0 tonne ha^{-} while the least value (6.19 g 100 g^{-1}) was obtained at 5 tonnes ha^{-1} treatment. The highest value (2.91 g/100 g) of oxalate was obtained at 10 tonnes ha^{-1} followed by 5 tonnes ha^{-1} (2.87 g 100 g^{-}) and 0 tonne ha^{-1} (2.84 g 100 g^{-}) in that order. The highest values of phytate and saponin (9.41 g 100 g^{-1} and 4.63 g 100 g^{-1}) were obtained when the poultry manure was applies at 5 tonnes ha^{-} while the least values (8.47 g 100 and 3.65 g 100 g^{-}) were obtained at the 0 tonne ha^{-1} (control). The different poultry manure levels showed no significant differences ($p > 0.05$) in the anti-nutrient compositions of the leaves (Table 4). The levels of the poultry manure did not have any significant effects ($p > 0.05$) on the

Table 4. Effect of poultry manure on anti-nutrient contents of *Moringa oleifera* leaves.

Parameter	Manure rate				
	0 tonne ha^{-1}	5 tonnes ha^{-1}	10 tonnes ha^{-1}	Mean	FLSD$_{(0.05)}$
Tanin (g 100 g^{-1})	6.48	6.19	6.23	6.30	ns
Oxalate (g 100 g^{-1})	2.84	2.87	2.91	2.87	ns
Phytate (g 100 g^{-1})	8.47	9.41	8.74	8.89	ns
Saponin (g 100 g^{-1})	3.65	4.63	3.86	4.04	ns

ns = Non-significant.

Table 5. Effects of poultry manure on the proximate, vitamin and chemical properties of *Moringa oleifera* leaves.

Proximate values	Manure rates				
	0 tonne ha^{-1}	5 tonnes ha^{-1}	10 tonnes ha^{-1}	Mean	FLSD$_{(0.05)}$
Ash	6.64	6.53	6.47	6.54	ns
% Protein	12.37	12.26	12.57	12.40	ns
% M.C	29.57	27.13	28.58	28.43	ns
% Fat	0.17	0.20	0.20	0.19	ns
% Crude Fibre	16.96	16.29	15.99	16.41	ns
% Carbohydrate	40.93	44.13	42.67	42.58	ns
Vitamins					
Vit A (ppm)	0.57	0.60	0.59	0.58	ns
Vit B1 (ppm)	0.27	0.31	0.27	0.29	ns
Vit B2 (ppm)	0.66	0.66	0.66	0.66	ns
Vit B6 (ppm)	1.06	1.11	1.04	1.07	ns
Vit C (ppm)	0.38	0.33	0.35	0.36	ns
Vit E (ppm)	0.87	1.13	1.00	0.99	0.21
Chemical properties					
% Mg	0.77	0.73	0.75	0.75	ns
% K	0.38	0.35	0.37	0.36	ns
P (ppm)	71.20	71.00	71.20	71.10	ns
Na (ppm)	13.86	15.26	13.68	14.27	ns
Mn (ppm)	22.85	21.95	24.00	22.94	ns
Fe (ppm)	61.30	58.00	62.20	60.50	ns
I (ppm)	1.10	1.01	1.05	1.05	ns
Cu (ppm)	1.43	1.26	1.43	1.38	ns
Zn (ppm)	2.58	2.65	2.86	2.70	ns
% N	1.98	1.96	2.01	1.98	ns

ns = Non-significant.

proximate qualities of the moringa leaves sampled at three months after planting (Table 5). Five tonnes ha^{-1} of poultry manure gave the least values of percentage ash and protein and greatest value of percentage carbohydrate. Ten tonnes ha^{-1} gave the least values of percentage ash and crude fibre, and highest protein value. Zero tonne ha^{-1} gave the least values of percentage carbohydrate and fat contents in the leaves. The poultry manure levels did not show significant differences ($p > 0.05$) in the values of vitamins A, B and C but vitamin E (Table 5). Five tonnes ha^{-1} poultry manure gave the greatest values of vitamins A, $B_{1,}$ B_6, and E and the least value of Vitamin C in the leaves compared to other levels. Zero tonne ha^{-1} gave the highest value of

Table 6. Effects of ages of leaves on the proximate, vitamin and chemical properties of *Moringa oleifera* leaves.

Proximate value	Age		Mean	$t_{0.05}$
	Older	Succulent		
Ash	6.35	6.73	6.54	ns
% protein	11.63	13.17	12.40	ns
% M.C	29.03	27.82	28.43	ns
% fat	0.19	0.19	0.19	ns
% crude fibre	14.87	17.94	16.41	ns
% carbohydrate	44.28	40.87	42.58	ns
Vitamin				
Vit A (ppm)	0.53	0.64	0.58	ns
Vit B1 (ppm)	0.16	0.41	0.29	ns
Vit B2 (ppm)	0.66	0.65	0.66	ns
Vit B6 (ppm)	0.97	1.16	1.07	ns
Vit C (ppm)	0.30	0.41	0.36	ns
Vit E (ppm)	0.88	1.12	0.99	ns
Chemical properties	2.03	2.21	2.12	ns
% Ca				
% Mg	0.74	0.76	0.75	ns
% K	0.34	0.39	0.36	ns
P (ppm)	67.40	74.90	71.10	5.59
Na (ppm)	14.02	14.52	14.27	ns
Mn (ppm)	19.87	26.00	22.94	3.18
Fe (ppm)	54.80	66.20	60.50	6.52
I (ppm)	1.05	1.05	1.05	ns
Cu (ppm)	1.48	1.27	1.38	Ns
Zn (ppm)	2.98	2.60	2.70	Ns
% N	1.86	2.11	1.98	Ns

ns = Non-significant.

vitamin C and least values of vitamins A and E. There were no significant differences ($p > 0.05$) in the effects of the poultry manure levels on N, P, K, Ca, Mg, Mn, Zn, iron (Fe) and copper (Cu) contents of the leaves. However, 10 tonnes ha^{-1} poultry manure gave the highest values of Mn, Fe, Zn and N (Table 5). Data presented in Table 6 showed that the succulent leaves had higher values of ash, crude protein, crude fibre, and vitamins A, B_1, B_6, C and E. The succulent leaves also had higher values of N, P, K, Ca, Mg, Mn and Fe. There were no significant differences ($t > 0.05$) between the two leaf types in their proximate and vitamin compositions. Both, also, did not differ significantly ($t > 0.05$) in their chemical compositions except in P, Mn and Fe contents (Table 6).

The source of seeds did not have any significant ($p > 0.05$) effect on the proximate and vitamin contents of the leaves except vitamin A (Table 7). Similarly, there were no significant differences ($p > 0.05$) in the chemical compositions of the leaves of plants grown from seeds collected from the different locations except Na and Zn contents (Table 7). Nsukka accession had the highest contents of vitamin A and Zn, while Jos accession had the highest leaf Na content. Figures 1 and 2 show the interactions in the effects of poultry manure levels, seed sources and ages of the leaves on proximate and vitamin contents of moringa leaves. Figure 1 shows clearly that crude fibre, protein and ash contents were higher in the young succulent leaves. Young leaves from Dutse (Jigawa State) which received no manure treatment had the highest fibre content. Carbohydrate was higher in the older leaves. Fat was high in the succulent leaves of the plants grown from the seeds collected from Dutse and Jos accessions. Succulent and older leaves from Nsukka, treated with 5 tonnes/ha poultry manure were also high in fats. Older leaves from Nsukka had the highest concentration of moisture. Figure 2 depicts a preponderance of vitamins A, C, B_1 and E in the young succulent leaves mainly from the plants that received some doses

Table 7. Effects of seed source on the proximate, vitamin and chemical properties of *Moringa oleifera* leaves

Proximate value	Accession				
	JOS	NSK	DTS	Mean	$FLSD_{(0.05)}$
Ash	6.43	7.04	6.17	6.54	ns
% Protein	11.79	12.71	12.69	12.40	ns
% M.C	28.83	27.97	28.47	28.43	ns
% Fat	0.18	0.20	0.18	0.19	ns
% Crude Fibre	15.27	16.96	17.00	16.41	ns
% Carbohydrate	43.92	42.16	41.66	42.58	ns
Vitamin					
Vit A (ppm)	0.50	0.66	0.59	0.58	0.12*****
Vit B1 (ppm)	0.28	0.30	0.28	0.29	ns
Vit B2 (ppm)	0.70	0.62	0.66	0.66	ns
Vit B6 (ppm)	1.11	1.14	0.96	1.07	ns
Vit C (ppm)	0.33	0.38	0.36	0.36	ns
Vit E (ppm)	0.93	1.00	1.07	0.99	ns
Chemical properties	2.12	2.22	2.02	2.12	ns
% Ca					
% Mg	0.75	0.79	0.71	0.75	ns
% K	0.37	0.38	0.34	0.36	ns
P (ppm)	72.70	71.80	69.00	71.10	ns
Na (ppm)	17.77	12.86	12.17	14.27	3.20*
Mn (ppm)	22.92	23.01	22.89	22.94	ns
Fe (ppm)	59.00	62.50	60.00	60.50	ns
I (ppm)	1.06	1.05	1.06	1.05	ns
Cu (ppm)	1.46	1.38	1.28	1.38	ns
Zn (ppm)	2.46	3.02	2.62	2.70	0.42*
% N	1.89	2.03	2.03	1.98	ns
NSK = Nsukka (Enugu state)					
DTS = Dutse (Jigawa State)					
JOS = Jos (Plateau State)					

ns = Not significant.

of poultry manure. In contrast, B_3 was highest in older leaves from Dutse grown without manure. Nsukka and Jos accessions treated with 10 tonnes/ha of poultry manure also contained high levels of Vitamin B_2. The highest concentration of Vitamin B_6 was got in older leaves of Jos accession treated with 5 tonnes/ha poultry manure.

DISCUSSION

The higher values of the anti-nutrients (oxalates, phytates and saponins) obtained in the manure-treated moringa leaves suggested positive influence of poultry manure on the composition of the anti-nutrients in the plants. Poultry manure probably had substances which could have contributed to the anti-nutrient compositions of the leaves. Fuglie and Sreeja (2011) reported values of 2.73, 3.1 and 4.1%, respectively for tannins, phytates and oxalates in untreated moringa leaves as against 6.23, 9.41 and 2.91% which were the highest values of these anti-nutrients obtained in the present study for the leaves of poultry manure-treated plants. Oxalates had been found to be negligible or relatively absent in dried leaves of moringa plants according to Fuglie and Sreeja (2011). The higher concentration of the anti-nutrients in the older leaves is an indication of the fact that older leaves are better accumulators of these anti-nutrients. The cells of the older leaves are usually tougher, larger and broader than those of the succulent ones. There was likely to be little or no mobility of the anti-nutrients from the older leaves to the succulent ones as the leaves were aging.

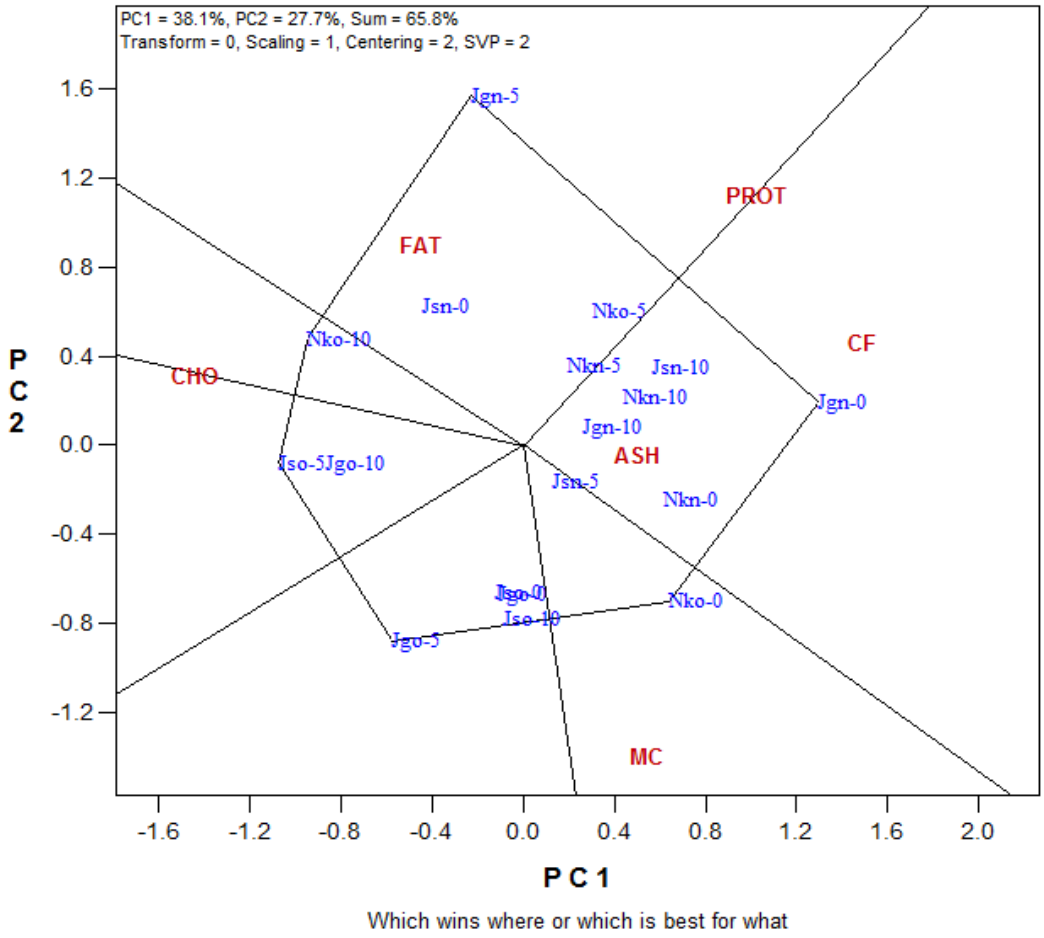

Figure 1. GGE Biplot analysis on effects of poultry manure; accession and age on the proximate compositions of leaves of *M. oleifera*. CHO = carbohydrate; MC = moisture content; CF = crude fibre; PROT = protein; Jso-0; Jso-5 and Jso-10 = Jos older leaves with 0; 5 and 10t/ha PM respectively; Jsn-0; Jsn-5 and Jsn-10 = Jos succulent leaves with 0; 5 and 10t/ha PM respectively; Nko-o; Nko-5 and Nko-10 = Nsukka older leaves with 0; 5 and 10t/ha PM respectively; Nkn-0; Nkn-5 and Nkn-10 = Nsukka succulent leaves with 0; 5 and 10t/ha PM respectively; Jgo-0; Jgo-5 and Jgo-10 = Jigawa older leaves with 0; 5 and 10t/ha PM respectively; Jgn-0; Jgn-5 and Jgn-10 = Jigawa succulent leaves with 0; 5 and 10t/ha PM. PM = poultry manure.

Thus, leaf senescence can be said to be synonymous with and directly proportional to leaf anti-nutrient accumulation. This fact gives a good direction to the consumers to take small quantities of older leaves or concentrate more on the young succulent leaves which contain lesser quantities of anti-nutrients. The insignificant effect of the poultry manure on the proximate qualities, chemical properties and vitamin contents of the moringa leaves within the three months of study could be attributed to the slow nutrient release nature of the manure into the soil coupled with soil factor as well as the physiological conditions of the plants. This agreed with the work of Demir et al. (2010) who also obtained insignificant effects of poultry manure on N, Mg and Mo concentrations of tomato leaves and fruits. It suggests that short-duration crops may not benefit much from poultry manure application. Ndubuaku et al. (2014) observed that poultry manure increased the nutrient status of the soil and boost crop productivity.

According to Ojeniyi et al. (2012), application of liquid agro-industrial by-products increased soil-plant nutrient supply by releasing structurally bound elements such as N, P and Ca in soil solution during decomposition thereby increasing crop growth and yield. However, the values of the proximate properties obtained in this study did not differ significantly from the levels reported by previous workers. Moyo et al. (2011) reported 8.4% ash and 7.9% crude fibre in moringa leaves as against 7.64% ash and 6.54% crude fibre obtained in the current study. Fuglie and Sreeja (2011) in Philippines reported higher values of vitamins A (16.3 ppm against 0.29 ppm obtained in this study). The variations in the levels of the nutrients

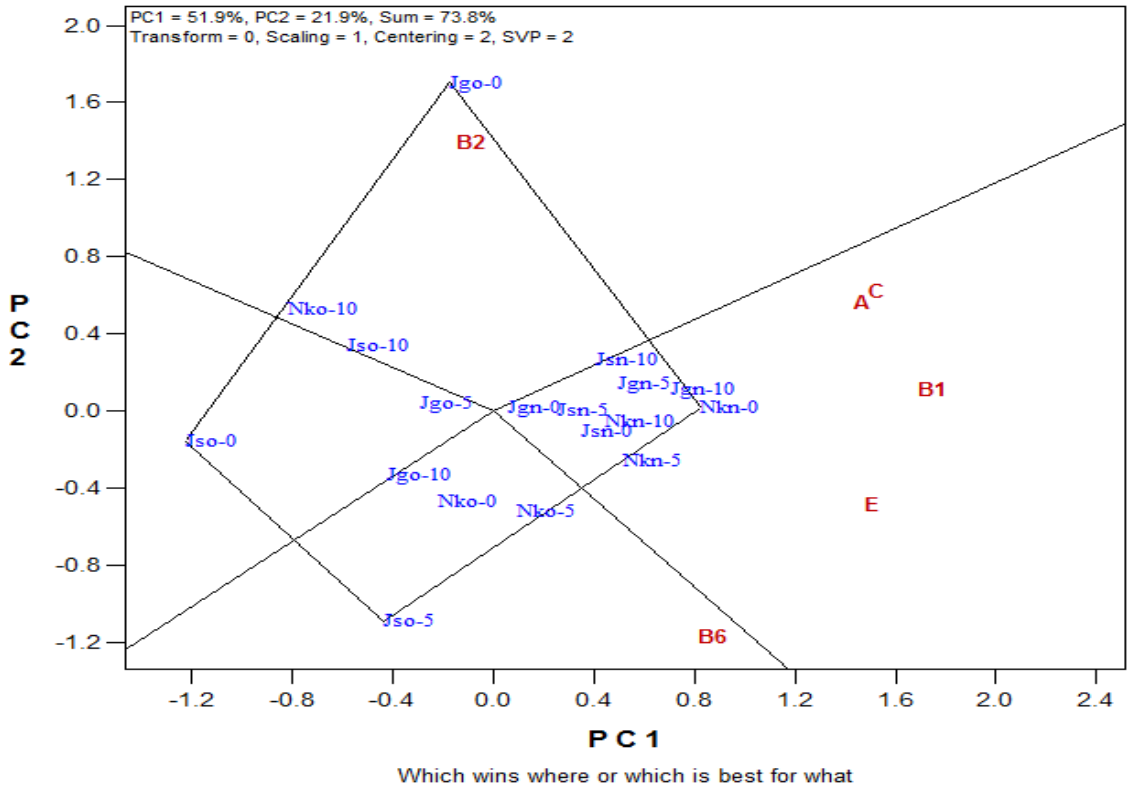

Figure 2. GGE Biplot analysis of the effects of poultry manure; accession and age on the vitamin content of leaves of *Moringa oleifera*. Jso-o; Jso-5 and Jso-10 = older leaves from Jos without PM; with 5 tons/ha and 10 tons/ha PM; respectively; Nko-0; Nko-5 and Nko-10 = older leaves from Nsukka without PM; with 5 tons/ha and 10 tons/ha PM; respectively; Jgo-0; Jgo-5 and Jgo-10 = older leaves from Jigawa without PM; with 5 tons/ha and 10 tons/ha PM; respectively; Jsn-0 = succulent leaves from Jos without PM; with 5 tons/ha and 10 tons/ha PM; respectively; Nkn-0; Nkn-5 and Nkn-10 = succulent leaves from Nsukka without PM; with 5 tons/ha and 10 tons/ha; respectively; while Jgn-0; Jgn-5 and Jgn-10 = succulent leaves from Jigawa without PM; with 5 tons/ha and 10 tons/ha PM; respectively.

obtained by the various workers could be due to the differences in the study locations and the environmental conditions. The higher values of vitamins, proximate and chemical properties in the succulent leaves compared to the older ones could be as a result of higher metabolic activities in the succulent leaves. There is usually translocation of nutrient elements like N from the lower (older) leaves to the younger (succulent) leaves during senescence. This can also account for the higher composition of N in the succulent leaves than the older ones. This is a good direction for moringa leaf consumers, to harvest the younger succulent leaves instead of the older ones for consumption.

Plants of moringa seeds collected from different locations of Nigeria gave no significant differences in their leaf compositions of vitamins, proximate and chemical properties indicating non-phenotypic influences on the genetic constitutions of the seeds. The performances of the plants were determined by the environmental factors prevalent in the study area (Nsukka) which would have also determined the physiological conditions of the plants.

Conclusion

Succulent leaves had higher values of vitamins, proximate and chemical properties. The higher concentrations of the anti-nutrients in the older leaves and higher values of vitamins, proximate and chemical properties in the succulent ones provide a good guide to moringa leaf consumers, to harvest the younger succulent (first to fifth) leaves for consumption.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Effects of ethylene inhibitors, silver nitrate ($AgNO_3$), cobalt chloride ($CoCl_2$) and aminooxyacetic acid (AOA), on *in vitro* shoot induction and rooting of banana (*Musa acuminata L.*)

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Significant increase in shoot regeneration, leaf chlorophyll content and rooting occurred when silver nitrate ($AgNO_3$), cobalt chloride ($CoCl_2$) or aminooxyacetic acid (AOA) were added to banana culture medium. The highest numbers of shoots per explants shoot length and leaf surface area was obtained when media were supplemented with 10 mgl^{-1} $AgNO_3$. Number of shoots per explants increased three fold, shoot length and leaf surface area increased by an average of 4.5 and 2 cm^2, respectively, in comparison to control. $CoCl_2$ and AOA had less promotive effects on shoot generation with maximum shoot number per explant and shoot length achieved at 15 mgl^{-1}. Rooting of banana shoots *in vitro* was enhanced by these compounds. The highest number of roots per shoot (21.7) and the longest roots (12.68 cm) were observed when rooting media was supplemented with 10 mgl^{-1} $AgNO_3$. For $CoCl_2$ and AOA the maximum rooting occurred in media supplemented with 15 mgl^{-1}, although roots number and root length were lower than those achieved by $AgNO_3$. Considerable increase in leaf total chlorophyll content occurred in shoots grown on media containing $AgNO_3$ and AOA. The largest increase in leaf chlorophyll content (120%) was noted when shoots were grown in the presence of 10 mgl^{-1} $AgNO_3$. This was followed by AOA which increased chlorophyll content by 35%. $CoCl_2$ had no significant effect on leaf chlorophyll content. These findings suggest that application of ethylene inhibitors, particularly $AgNO_3$, to culture media may be useful for improving *in vitro* growth performance of banana cultures.

Key words: Ethylene inhibitors, banana, *Musa acuminata* L, *in vitro* culture.

INTRODUCTION

In recent years, several studies have demonstrated that ethylene accumulates in vessels of *in vitro* plant culture systems (Biddington, 1992; Zobayed et al., 1999; Fuentes et al., 2000). Accumulation of ethylene in culture vessels

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Abbreviations: DMRT, Duncan's multiple range test; **AgNO3,** silver nitrate; **CoCl2,** cobalt chloride; **AOA,** aminooxyacetic acid; **AVG,** aminoethoxyvinylglycine; **BAP,** benzylaminopurine; **IAA,** indole-3-acetic acid; **2iP,** 2-isopentenyladenine; **IBA,** indole-3-butyric acid.

may induce growth abnormalities of *in vitro* generated plants including inhibition of growth, leaf epinasty, leaf senescence and diminution of foliar area (Jaksonet al., 1991; Joosten and Woltering, 1994; Kumar et al., 1998; Zobayedet al.,2001; Zhang et al., 2001;Turhan, 2004; Giridhar et al., 2003; Mullins et al., 2006; Hazarika, 2006; Kepczyn´ska et al., 2009; Dang and Wei, 2009; Steinitz et al., 2010). However, the influence of ethylene in plant cells and tissues grown *in vitro* is diverse and often controversial depending on plant species and even the cultivar (Hu et al., 2006; Santana-Buzzy et al., 2006; Jhaet al., 2007). For instance, ethylene was reported to be important for shoot morphogenesis in rice callus (Adkins et al., 1990) and embryogenesis from anther cultures of *Hordeumvulgare* (Cho and Kasha, 1989). In contrast, ethylene accumulation was found to inhibit *in vitro* regeneration of several plant species (Huxteret al., 1981; Purnhauseret al., 1987; Chi et al., 1991; Gong and Pua, 2004). In fact, addition of ethylene inhibitors such as silver nitrate ($AgNO_3$), cobalt chloride ($CoCl_2$), aminooxy-acetic acid (AOA) and aminoethoxyvinylglycine (AVG) to culture media have been demonstrated to improve regeneration and growth performance of both dicot and monocot plant tissue cultures (Beyer, 1976; Duncan et al., 1985; Davies, 1987; Songstad et al.,1988; Chi and Pua, 1989; Veen and Over Beek, 1989; Bais et al., 2000; Giridhar et al., 2003; Kumar et al., 2007; Abdellatef and Khalafalla, 2008; Osman and Kalafalla, 2010; Sandra and Maira, 2013).

Clonal propagation of banana (*Musa acuminata L*.) via *in vitro* culture techniques have been extensively studied using different explants sources, basal media components and phytohormones levels (Al-*amin* et al., 2009; Ngomuo et al., 2014; Devendrakumar et al., 2013). However, to the best of our knowledge, no information on the effect of ethylene or ethylene inhibitors on *in vitro* culture of banana is available despite the recognized positive effects of these inhibitors on plant regeneration and growth *in vitro* (Kumar et al., 1998). In the present investi-gation, we compare the efficacy of the ethylene inhibitors silver nitrate ($AgNO_3$), cobalt chloride ($CoCl_2$) and aminooxyacetic acid (AOA) to assess their effects on shoot and root development of *invitro* cultured banana plants.

MATERIALS AND METHODS

Shoot tip explants of banana (*Musa acuminata L*.) (cultivar Grand Nain) were excised from young suckers grown in pots. Explants were surface sterilized with 75% ethanol for 50 s followed by 30 min with 40% commercial bleach (Clorox 5.75% NaOCl) to which few drops of Tween-20 were added. After complete washing with sterile distilled water, explants were trimmed to final size of 10 to 15 mm in the laminar flow cabinet. For culture initiation, explants were cultured in screw-capped glass vessels containing 30 ml of initiation media composed of MS basal salts (Murashige and Skoog, 1962) supplemented with sucrose (40 g l^{-1}), thiamine (0.1 g l^{-1}), benzylaminopurine (BAP) (12 µM), indole-3-acetic acid (IAA) (3 µM) And cystein HCl (40 mg l^{-1}). Medium was solidified with 2 g l^{-1} gelrite (Sigma Chemical Co., St. Louis) and its pH was adjusted to 5.8 before autoclaving at 121°C for 15 min. All cultures were incubated at 25°C under 16 h photoperiod for 4 weeks. Light intensity was 35 µmol $s^{-1}m^{-2}$.To evaluate the influence of $AgNO_3$, $CoCl_2$ and AOA on shoot multiplication and growth, banana shoot tip explants from *in vitro* initiated cultures were transferred to multiplication media. Multiplication medium contained MS basal salts, sucrose (40 g l^{-1}), thiamine (0.1 g l^{-1}), BAP (20 µM) and cystein HCl (40 mg l^{-1}) supplemented with different concentrations (0 to 25 mg/l) of $AgNO_3$, $CoCl_2$ and AOA individually. Cultures were arranged in a randomized block design with 10 replicates per treatments (3 explants per culture bottle) and incubated at 25°C under16 h photoperiod for 4 weeks. Light intensity was 35 µmol $s^{-1}m^{-2}$. After 4 weeks of culture, the number of shoots formed per explant, shoot length(cm) and leaf surface area (cm^2) were determined.

For the determination of leaf chlorophyll content, 0.25 gfresh leaf material of individual treatments was extracted in 5ml 80% acetone (v/v) and total chlorophyll content was determined according to Lichtenthaler (1987).For evaluating the effect of $AgNO_3$, $CoCl_2$ and AOA on *in vitro* rooting, uniform banana shoots formed on multiplication media were excised and transferred to rooting medium. Rooting medium consisted of MS basal slats, sucrose (40 g l^{-1}), 2-isopentenyladenine (2iP) (5µM) and indole-3-butyric acid (IBA) (0.1 µM) supplemented with different concentrations (0 to 25 mg/l) of $AgNO_3$, $CoCl_2$ and AOA individually. Medium was solidified with 1.8 g l^{-1} gelrite and its pH was adjusted to 5.8. Cultures, consisting of 10 replicates per treatment, were incubated at 25°Cunder16 h photoperiod. After 4 weeks the number of roots formed per shoot and root lengths (cm) were estimated.All data were expressed as means of all replicates ± standard error. Means were separated by Duncan's multiple range test (DMRT) (Duncan, 1955) at 5% significance level.

RESULTS

The effect of the ethylene inhibitors $AgNO_3$, $CoCl_2$ and AOA on *in vitro* shoot regeneration of banana is presented in Table 1. In the control experiment low shoot regeneration (2.37 shoots/explant) with an average shoot length of 2.89 cm and mean leaf surface area of 3.74 cm^2 were observed. Presence of varying concentrations of $AgNO_3$ in the shoot multiplication medium had strong positive effect on shoot multiplicationand a maximum of 6.68 shoots/explant was achieved at10 mgl^{-1} $AgNO_3$ .This concentration was also the most effective in promoting shoot growth increasing shoot length and leaf surface area by 150 and 58%, respectively, compared to control. Addition of $CoCl_2$ or AOA to multiplication media was also beneficial to banana shoot multiplication although less effective than $AgNO_3$. The highest number of shoots/explant was observed in media supplemented with 15mgl^{-1} $CoCl_2$ or AOA giving an average of 4.59 and 5.12 shoots/explants, respectively. Among these treatments only $CoCl_2$ increased shoot length to a maximum of 85% relative to control when added to the medium at15mgl^{-1}. Application of 5 to 15 mgl^{-1} AOA to multiplication media did not, however, influence shoot length and higher concentrations inhibited shoot elongation. Leaf surface area on the other hand, was not significantly affected by all concentrations of $CoCl_2$ tested whereas considerablereduction in leaf surface area was

Table 1. Effects of various concentrations of $AgNO_3$, $CoCl_2$ and AOA on shoot regeneration from shoot tips of bananaafter 4 weeks of culture *in vitro.*

Treatment	Concentration (mg/L)	Mean number of shoots/ explant ± SE	Mean shoot length ± SE (cm)	Mean leaf surface area ± SE (cm^2)
Control	0	2.37 ± 0.18[a]	2.89 ± 0.31[a]	3.74 ± 0.45[a]
Silver nitrate ($AgNO_3$)	5	3.24 ± 0.26[b]	4.62 ± 0.43[b]	4.21 ± 0.32[b]
	10	6.68 ± 0.38[c]	7.42 ± 0.25[c]	5.92 ± 0.58[c]
	15	4.54 ± 0.21[d]	5.87 ± 0.38[d]	4.86 ± 0.44[b]
	20	3.05 ± 0.19[b]	3.95 ± 0.26[e]	4.12 ± 0.36[b]
	25	2.76 ± 0.11[a]	2.85 ± 0.24[a]	3.68 ± 0.14[a])
Cobalt chloride ($CoCl_2$)	5	3.17 ± 0.42[b]	3.45 ± 0.36[e]	3.62 ± 0 .23[a]
	10	3.62 ± 0.38[b]	4.12 ± 0.24[b]	3.78 ± 0.36[a]
	15	4.59 ± 0.43[d]	5.35 ± 0.22[d]	3.96 ± 0.28[a]
	20	3.86 ± 0.35[b]	4.87 ± 0.44[b]	3.72 ± 0.39[a]
	25	3.21 ± 0.21[b]	3.68± 0.38[e])	3.72 ± 0.46[a])
Amino-oxyacetic acid (AOA)	5	2.89 ± 0.32[b]	2.72 ± 0.47[a]	3.07 ± 0.22[a]
	10	3.76 ± 0.38[b]	2.58 ± 0.14[a]	2.83 ± 0.15[a]
	15	5.12 ± 0.35[e]	2.94 ± 0.42[a]	2.62 ± 0.29[a]
	20	3.89 ± 0.46[b]	2.55 ± 0.24[a]	2.46 ± 0.10[b]
	25	2.16 ± 0.12[a]	2.10± 0.22[a]	1.95 ± 0.12[b]

Data are means of 10 replicates with 3 explants per replicate. Means followed by different alphabet denote significant differences within column based on DMRT (p = 0.05).

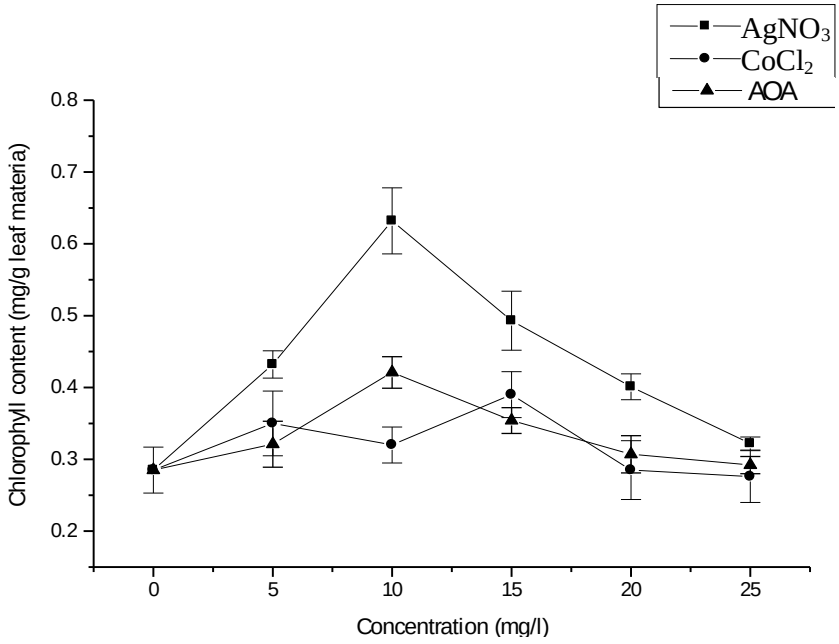

Figure 1. Effects of various concentrations of $AgNO_3$, $CoCl_2$ and AOA on total chlorophyll content in leaves of banana shoots cultivated *in vitro* for 4 weeks. Data are means of 10 replicates ± SE.

noticed in the presence of increasing concentrations of AOA in the medium.

The data presented in Figure 1 shows that application of 5 to 20 mgl^{-1} $AgNO_3$ to banana multiplication media resulted in a significant increase in total leaf chlorophyll content. The highest amount of chlorophyll was observed

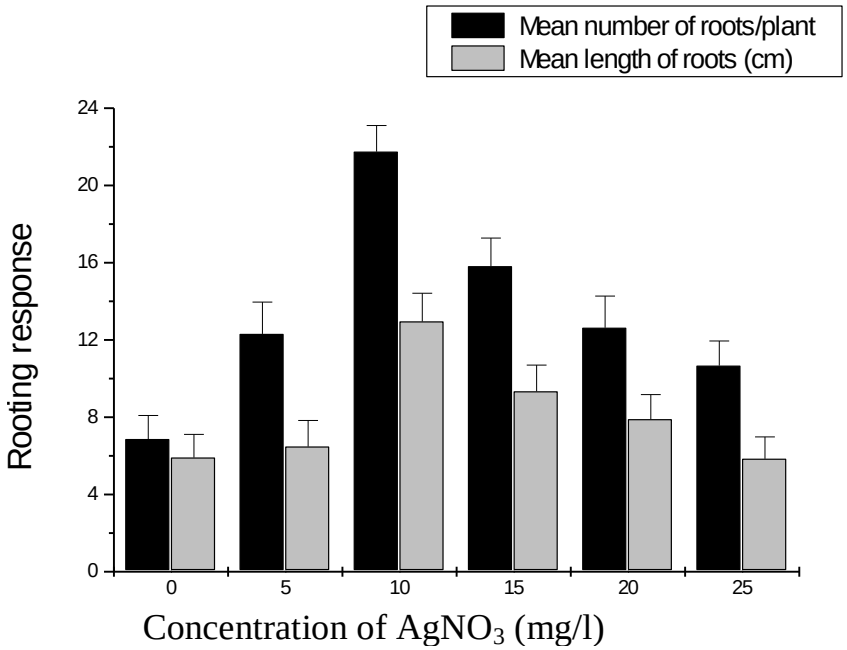

Figure 2. Effects of various concentrations of $AgNO_3$ (mg/L) on rooting of *in vitro* grown banana shoots after 4 weeks of culture. Data are means of 10 replicates± SE.

at 10 mgl^{-1} $AgNO_3$. At this concentration total leaf chlorophyll content increased by 120% compared to control. Treatment with AOA, however, resulted in a lower increase in total leaf chlorophyll content reaching a maximum of 35% over control at a concentration of 10 mgl^{-1}. On the contrary, $CoCl_2$ treatment had no significant effect on leaf chlorophyll content.The presence and concentration of $AgNO_3$ in the rooting medium had significant effect on rooting in banana (Figure 2). The highest number of roots/explant and the highest root growth were achieved on medium containing 10 mgl^{-1} $AgNO_3$ (Plate 1). At this concentration silver nitrate increased root formation and mean root length by 190 and 115%, respectively, relative to control. Incorporation of $CoCl_2$ and AOA in the rooting medium also stimulated rooting but proved to be less effective than $AgNO_3$ (Figures 3 and 4). The best concentrations of $CoCl_2$ and AOA for rooting (15 mgl^{-1}) produced 85 and 115% more roots/ explant, respectively, compared to control. Contrary to $CoCl_2$ treatment which had no significant effect on root length, treatment with 15 mgl^{-1} AOA resulted in 80% increase in root growth relative to control.

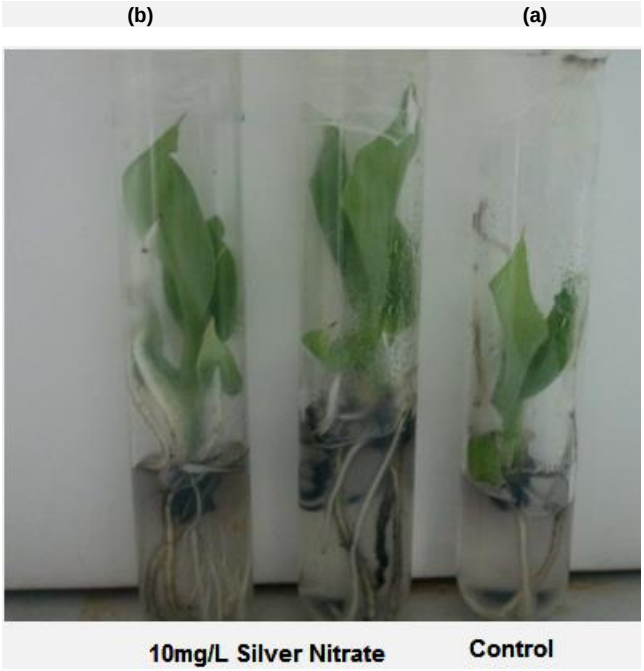

Plate 1. Rooting and shoot growth of *in vitro* grown banana shoots after 4 weeks of culture. **(a)** Control, **(b)** media supplemented with 10 mgl^{-1} $AgNO_3$.

DISCUSSION

In the present study the influence of ethylene inhibitors $AgNO_3$, $CoCl_2$ and AOA on *in vitro* culture of banana (*Musa acuminata L)* was investigated. The results show that the use of ethylene inhibitors in culture media can enhance the ability of banana shoot tip culture to produce higher number of shoots per explant along with shoot

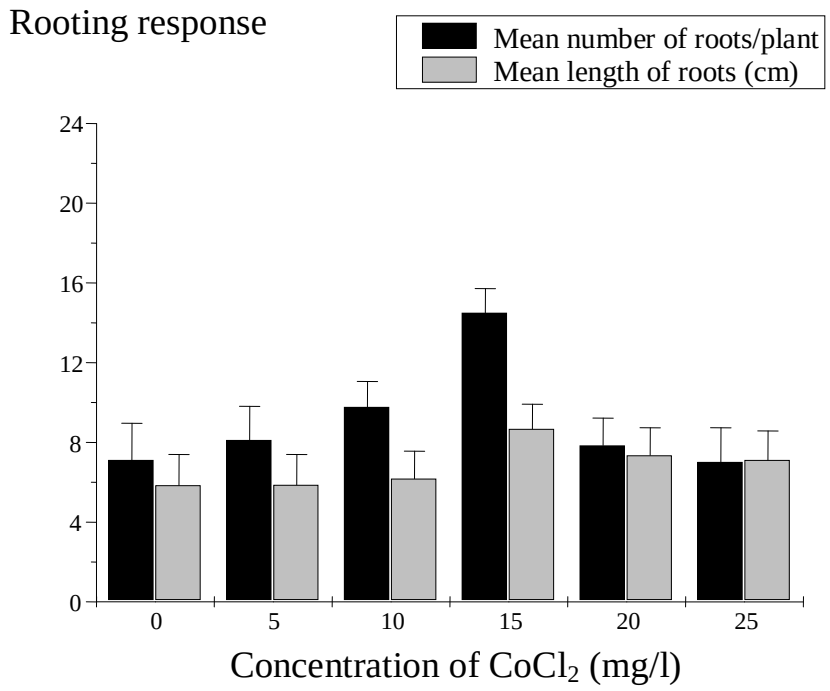

Figure 3. Effects of various concentrations of $CoCl_2$ (mg/L) on rooting of *in vitro* grown banana shoots after 4 weeks of culture. Data are means of 10 replicates± SE.

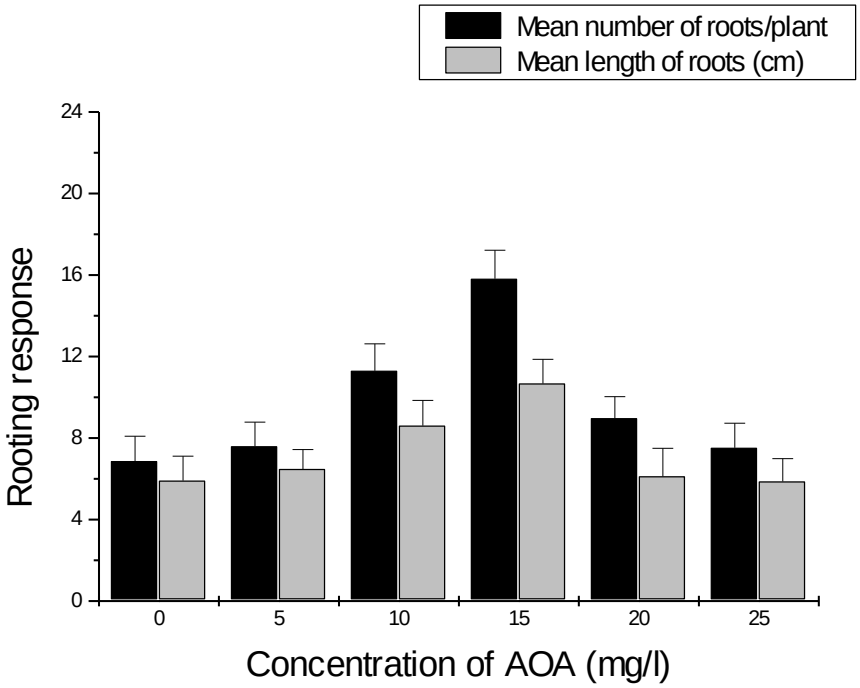

Figure 4. Effects of various concentrations of AOA (mg/L) on rooting of *in vitro* grown banana shoots after 4 weeks of culture. Data are means of 10 replicates± SE.

elongation and leaf expansion. The maximum number of shoots as well as shoot length and leaf surface area was achieved on media supplemented with $10mgl^{-1}$ $AgNO_3$; shoot number/expant were 3 times higher, shoots formed

were 4.5cm longer and leaf surface area were 2 cm^2 greater than those recorded on media without $AgNO_3$. This result agrees with previously reported findings demonstrating the stimulative role of $AgNO_3$ on shoot organogenesis in many plants such as coffee sp., (Giridhar et al., 2003), strawberry (Qin et al., 2005), sweet potato (Gong et al., 2005), sesame (Abdellatef et al., 2010), tomato (Osman and Khalafalla, 2010) and turmeric (Dikashet al.,2012). For $CoCl_2$ and AOA treatments, higher concentration (15 mgl^{-1}) were required to enhanced banana shoot development, although, lower number of shoots/explant were achieved compared to $AgNO_3$ treatment. While, shoot length was improved by $CoCl_2$ treatment, no positive effect on leaf surface area was noted by either of these compounds.

It is well known that $AgNO_3$ is a potent inhibitor of ethylene action (Beyer, 1979; Veen and Overbeek, 1989; Pua and Chi, 1993) whereas $CoCl_2$ and AOA are known to inhibit the enzymes ACC synthase and ACC oxidase involved in ethylene biosynthesis (Satoh and Esashi, 1980; Yang and Hoffman, 1984; Abeles et al., 1992). There is also accumulating evidence suggesting that *in vitro* tissue cultures produce ethylene in sealed containers (Chi et al., 1991). In addition there have been several reports indicating that ethylene produced during *in vitro* culture impairs plant growth and development and could limit *in vitro* propagation of several plants (Biddington, 1992; Pua and Chi, 1993; Chraibi et al., 1991). Accordingly, the findings of this study may suggest that ethylene inhibitors, particularly $AgNO_3$ alleviated the negative effects of ethylene on the growth of banana culture *in vitro.* Support for this suggestion comes from the finding that these compounds are also capable of increasing chlorophyll content in banana leaves (Figure 1). The association of ethylene with senescence of plant parts is well known (Jona et al., 1997) and its negative effect on chlorophyll content of plants was documented. For example, Jakob-Will et al. (1999) reported that ethylene induced expression of chlorophyllase genes (*Chlase*) in citrus fruits. There are also some reports suggesting that inhibition of ethylene action by silver ions increased leaf chlorophyll content (Ehsanpour and Jones, 2001; Perl et al., 1988). Apparently, addition of $AgNO_3$ and to a lesser extent $CoCl_2$ and AOA to banana culture media could improve shoot multiplication and promote the maintenance of green healthy *in vitro* tissue for long time periods.

In addition to their positive effects on *in vitro* shoot growth and development, $AgNO_3$, $CoCl_2$ or AOA incorporated into banana rooting media also improved rooting of *in vitro* produced banana shoots. Among these compounds $AgNO_3$ resulted in the best rooting response. Shoots in rooting media supplemented with 10mgl^{-1} $AgNO_3$ produced 3 times more roots and increased root length by 7 to 8 cm. The optimal concentration of $CoCl_2$ and AOA for rooting was achieved at 15 mgl^{-1}. This concentration resulted in approximately two fold increase in the number of roots formed per shoot, compared to control with limited or no significant influence on root elongation. These observations are in accordance with previous findings demonstrating improvement of *in vitro* rooting of plants by ethylene inhibitors such as *Decalepis hamiltonii* (Bais et al.,2000; Reddy et al.,2001), coffee (Giridhar et al.,2003) and apple (Ma et al.,1998).

In conclusionthe findings of this study demonstrate that ethylene inhibitors particularly $AgNO_3$ and to a lesser extent $CoCl_2$ and AOA enhanced vigour of banana shoots proliferation *in vitro* along with leaf expansion and shoot elongation; possessed the potential to protect developing banana leaves from ethylene induced senescence by maintaining high leaf chlorophyll content and increased the rooting capacity of *in vitro* grown banana shoots. Taken together, this study suggests that $AgNO_3$, $CoCl_2$ and AOA may be used as important tools for improving protocols of banana cultures *in vitro* and for protecting *in vitro* cultured banana tissue from the possible negative effects of ethylene in culture vessels.

Conflict of interests

The author did not declare any conflict of interest.

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Full Length Research Paper

Bacteriocinogenic potential and genotypic characterization of three *Enterococcus faecium* isolates from Algerian raw milk and traditional butter

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The detection of bacteriocinogenic potential of *Enterococcus sp.* isolates from Algerian raw milk coded LO4 and LO12 and from traditional butter coded BRO2 was carried on M17 buffered medium. PCR amplification of *Enterococcus sp.* DNA using specific enterococcal primers gave 733 bp fragments. The phylogenetic analysis using the neighbour joining method further supported the identification of the three strains as *Enterococcus faecium*. These bacteria were bacteriocinogenic against *Pseudomonas sp*, *Proteus mirabilis* and *E. faecium*. Lyophilisate extracts were tested for sensitivity to enzymes, heating and effect of pH. Complete inactivation in bacteriocinogenic activity was observed after treatment with proteolytic enzymes. The antibacterial activity from *E faecium* LO12 was stable (1280 AU/ml) for range pH 2 to 12. Maximal activity from BRO2 strain was at pH 7 (20480 AU/ml) and from LO4 strain was at pH 7 and 6 (2560 AU/ml). Antibacterial activities of *E. faecium* BRO2 (5120 AU/ml) and *E. faecium* LO12 (640 AU/ml) remained stable at 60°C for 30 min. The antibacterial activity of .*E faecium* LO4 was stable at 100°C for 30 min (5120 AU/ml).

Key words: Butter, milk, *Enterococcus faecium,* antibacterial activity, bacteriocinogenic.

INTRODUCTION

Traditional food manufacturing appreciated to this day by some consumers was at the origin of products from the food industry. In old times, an excess of food need to be conserved for a long time to survive in winter or in drought periods. Together with drying and salting, fermentation is one of the oldest methods of food preservation, and embedded in traditional cultures and village life (Marshall and Meijia, 2012). Today, when many secrets of fermentation are known, the nutritional and hygienic quality of food required had been mastered in the food industry. Indeed, some micro-organisms had largely been exploited in the food fermentation including lactic acid bacteria, moulds and yeast (Giraffa, 2004). They are responsible for many proprieties of fermented

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food such as flavor, shelf life, texture and health benefits (Giraffa, 2004). The production of fermented food is based on the use of starter cultures, for instance lactic acid bacteria that initiate rapid acidification of the raw material (Leroy and DeVuyst, 2004). They acidify the food, resulting in a tangy lactic acid taste, frequently exert proteolytic and lipolytic activities, and produce aromatic compounds from, for instance, amino acids upon further bio-conversion (Williams et al., 2001; Yvon and Rijnen, 2001; Van Kranenburg et al., 2002). The group of lactic acid bacteria occupies a central role in these processes, and has a long and safe history of application and consumption in the production of fermented food and beverages (Ray and Daeschel, 1992; Wood and Holzapfel, 1995; Wood, 1997; Caplice and Fitzgerald, 1999). They increase the nutritional quality of food by increasing digestibility as in the fermentation of milk to cheese (Caplice and Fitzgerald, 1999). Their antimicrobial effect through fermentation processes has been appreciated by man and has enabled him to extend the shelf life of many foods (Savadogo et al., 2004). Among lactic acid bacteria genus *Enterococcus* is the object of many studies these last years. The advent of molecular methods has allowed distinguishing an *Enterococcus* genus, which is the largest Lactic Acid Bacteria (LAB) genus after *Lactobacillus* and *Streptococcus* (Franz et al., 2011). On the basis of 16SrRNA gene similarity, the species of *Enterococcus* fall into seven species of groups (Franz et al., 2011). *Enterococcus faecium* is widely distributed in food and their environment. Their ubiquitous nature and resistance to adverse environmental conditions account for their ability to colonize different habitats and underlie their potential to easily spread through the food chain (Fracalanzza et al., 2007).

Generally, enterococcal bacteria were found in milk and dairy product. These bacteria can also be used as starter in the food industry due to their capacity to produce lipase, protease and volatile compounds ensuring desirable organoleptic features in some specific kinds of food (Camargo et al., 2014). This genus endowed similar properties than *Lactococcus* and *Lactobacillus* such as antimicrobial activity. The last two decades had seen an intensive investigation on natural antimicrobial products synthesized by food grade lactic acid bacteria that can be used as food preservatives in place of chemical preservatives (Gautam et al., 2014). According to Šušković et al. (2010) the main antimicrobial effect of starter LAB responsible for biopreservation is the rate of acidification, but in slightly acidified products the bacteriocinogenic activity could play a crucial role to eliminate undesirable microorganisms that display acid tolerance.

Bacteriocins are ribosomally synthesized peptides produced by bacteria that are capable of killing other bacteria by forming pores in target membranes (Abee, 1995; Zacharof and Lovitt, 2012). Most of bacteriocin as is the case for lantibiotic, are initially synthesized with an N-terminal leader peptide (Parada et al., 2007). In general the peptide is modified by the action of other proteins encoded by the bacteriocin gene cluster before export (Deegan et al., 2006). Many bacteriocins from Gram positive bacteria have fairly broad spectra, and have a great potential as antimicrobial agents in food and feed production (Nigutova et al., 2005). They are frequently found as secondary metabolites produced by various microorganisms, such as the Gram-positive bacteria of the genus *Streptomyces*, lactic acid bacteria and genus *Bacillus* (Katz and Demain, 1977; Klaenhammer, 1988). Bacteriocins are widely used in food science to extend food preservation duration (Ghrairi et al., 2012). Bacteriocins inhibit pathogen infection of animal diseases (Van Heel et al., 2011). Pharmaceutical industry and medical society attempt to use bacteriocins to treatment for malignant cancers (Lancaster et al., 2007). Two bacteriocins are used in food technology: Nisin which is produced by *Lactococcus lactis* and the first antibacterial peptide found in lactic acid bacteria (Rogers, 1928), and Pediocin PA-1 marked as Alta ® 2341 which inhibits the growth of *Listeria monocytogenes* in meat product (Settanni and Corsetti, 2008).

Enterococci can produce bacteriocins, called enterocins, with inhibitory activity against strains closely related to the producer microorganism (Poeta et al., 2008). Enterocin produced by *E. faecium* has a broad-spectrum of activity towards food-borne pathogens indicating its application in food processes as a co-culture or as an additive (Leroy et al*.,* 2003). Settanni and Corsetti (2008) reported that enterocin CCM 4231 and EJ97 are used in soy milk and zucchini purée for suppression of contamination, respectively while enterocin AS-48 seems a good candidate for application in biopreservation of fruit juices (Grande Burgos et al., 2014).

In this paper, we were interested to put on evidence the bacteriocinogenic activity of three enterococcal bacteria isolated from traditional butter and raw milk towards unwanted bacteria. The isolates were identified to *E. faecium* by biochemical and molecular methods.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Four *Enterococcus* sp. strains from the collection of the Laboratory of Biology of Microorganisms and Biotechnology (Oran University, Algeria) were used for this study: the strain BRO2 was isolated from a sample of traditional butter, the strains LO4 and LO12 were isolated from a sample of raw milk from a single cow and the strain H3 used as target was isolated from “hammoum” a traditionally fermented barley. They were stored at - 20°C in reconstituted skim milk (10% w/v) and before use they were propagated twice in their M17 broth media (Fluka, Switzerland) at 30°C. Several bacteria used as target strains (Table 1) were incubated at 37°C in nutritive broth (tryptone 10 g/l, meat extract 5 g/l, NaCl 5 g/l pH 7.2) or in nutritive agar.

Table 1. Inhibitory spectrum of enterococcal isolates.

Diameter of inhibitions zones (mm)	BRO2		LO4		LO12	
Target bacteria and origin	M	BM	M	BM	M	BM
Citrobacter freundi EC2	2	ND	2	ND	5	ND
E coli EC3	5	ND	2	ND	5	ND
E coli HB4	ND	ND	ND	ND	2	3
E coli ATCC 25922	ND	ND	ND	ND	ND	ND
Enterococcus faecium H3	14	12	13	15	8	6
Proteus mirabilis HB3	11	13	9	12	8	9
*Pseudomonas sp.*HB2	14	12	11	14	18	9
Pseudomonas aeruginosa HB5	ND	ND	ND	ND	7	9
Pseudomonas aeruginosa ATCC 27853	ND	ND	ND	ND	ND	ND
Staphylococcus aureus HB1	ND	ND	ND	ND	ND	ND
Staphylococcus sp. V3	2	ND	5	ND	8	ND

HB, Isolates from human biological samples; EC, isolates from water; V, isolates from meat; M, unbuffered medium; BM, buffered medium; ATCC, American Type Culture Collection; ND, not detected.

Phenotypic identification of enterococcal strains

Enterococcal strains were grown in bile esculine agar (Institut Pasteur, Algiers). Gram staining and catalase production were tested prior to inoculation of the rapid commercial system API 20 STREP gallery (Biomérieux, France) for the identification of enterococci, according to the manufacturer recommendations.

Molecular identification of enterococcal strains

Amplification of enterococcal DNA

Two colonies of each enterococcal culture on M17-lactose agar were picked up and homogenized with 1 ml of pure water in order to extract DNA, according to the boiling method described by Reischl et al. (1994). The supernatant containing the DNA was directly used for PCR reactions. Primers E1 (5'TCAACCGGGGAGGGT3') and E2 (5'ATTACTAGCGATTCCGG3') designed by Deasy et al. (2000) were used to amplify enterococcal DNA. Universal primers U1 (5'AAYATGATTACIGCIGCICARARATGGA'3) and U2 (5'-AYRTTITCICCIGGCATIACCAT-'3) were used as a control. The master mix composition for 1 reaction was 5 µl of 10X PCR buffer, 0.4 µl of 25 mM dNTP mixture, 2.5 µl of 50 mM $MgCl_2$, 1.25 µl of 10 µM downstream primers, 1.25 µl of 10 µM upstream primers, 12.2 µl pure water and 0.4 µl of *Taq* DNA polymerase (5 U/µl). PCR reaction was carried out by using thermocycler (Techne TC-412) in a final volume of 50 µl. The amplification program used was: initial denaturation step at 95°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, hybridization at 60°C for 1 min, polymerization at 72°C for 1 min, final extension at 72°C for 5 min. 5 µl of each product of PCR reaction were mixed with 2 µl of loading buffer. 5 µl of each sample was electrophoresed (40 min at 135 V) on 1% agarose gel using TAE buffer containing 200 ng/ml ethidium bromide (Sigma-Aldrich). The gels were photographed on a UV transilluminator.

Phylogenetic analysis of enterococcal bacteria

The PCR products were sequenced by 96-Capillary Applied Biosystems 3730*xl* analyzer. The obtained sequences were analyzed by the BLAST software of the NCBI (http://www.ncbi.nlm.nih.gov/). The phylogenetic tree of the 16S rRNA gene was performed using Clustal X2.1 (Thompson et al., 1997) in the Mega 6.06 software (Tamura et al., 2013). The phylogenetic tree was constructed using the neighbor-joining method with bootstrap analysis for 100 replicates.

Bacteriocinogenic activity

Bacteriocinogenic activity tests were performed using a method of Spelhaug and Harlander (1989). In our study, the M17 agar medium was buffered by sodium phosphate buffer (0.1 M; pH 7.2) instead of 1% of ß-glycerophosphate. The isolates (BRO2, LO4, LO12) were grown in M17 broth (30°C, 16 h). 5 µl from each culture were spotted onto M17 agar plate (without buffer) and onto M17 buffered agar plate. The plates were incubated at 30°C for 18 h. The target strains were grown in nutritive broth up to 4 or 5 h (OD_{600nm} 0.3 to 0.5), except for *Enterococcus sp.* H3 which was grown in M17 broth. Each one of these cultures (250 µl) was used to inoculate 7 ml of soft agar in order to overlay the spotted plates. The plates were stored at 5°C for 4 h prior to be further incubated at 30°C for 24 h. Zones of inhibition of growth of target strains revealed inhibitory activity of enterococcal strains.

Sensitivity to enzymes, heat treatment and effect of different pH values on antibacterial activity produced by bacteriocinogenic strains

Culture extract preparation and bacteriocin assay

The overnight bacteriocinogenic cultures were centrifuged (12000 rpm for 15 min). The supernatants were filtered through a Millipore 0.22 µm pore size membrane, than lyophilized in lyophilizer for 6 h. Fifty µl of 200 mg made in 1 ml sodium phosphate buffer (0.1 M; pH 7.2) were tested by the well-diffusion method (Tag and McGiven, 1971).

Enzymes susceptibility

Aliquots (200 µl) of the lyophilisates were incubated at 37°C for 2 h

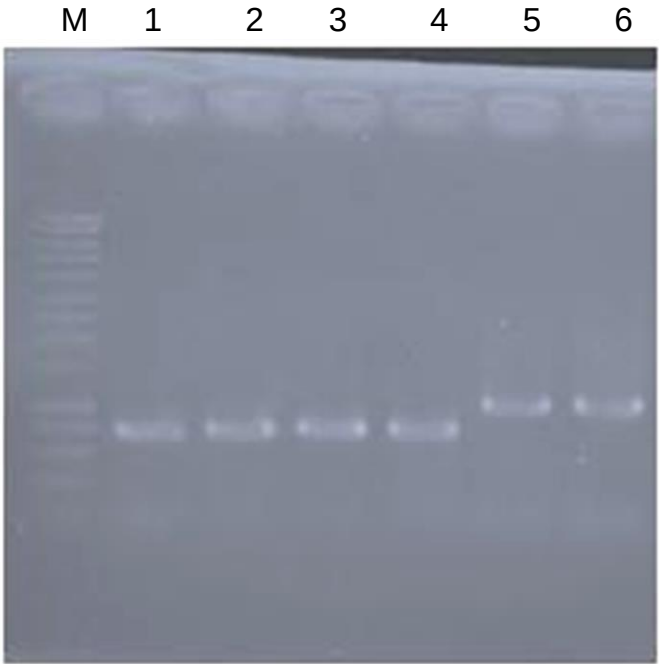

Figure 1. Electrophoresis gel of PCR products. M, Molecular marker 1000 bp; lanes 1 to 4, PCR amplification products with enterococcal primers: lane 1: BRO2; lane 2: LO12; lane 3: LO4; lane 4: H3; lanes 5 to 6, PCR amplification products with universals primers U1/U2: lane 5: LO12; lane 6: BRO2.

(Todorov et al., 2011) in the presence of 1 mg ml^{-1} of catalase, trypsin, pronase E, proteinase K, pepsin or α-chymotrypsin (Aktypis and Kalantzopoulos, 2003). All enzymatic solutions (Sigma-Aldrich) were prepared in buffer sodium phosphate (0.01 M pH 7) except the pepsin that was dissolved in HCl of 0.02 M. The susceptibility of antibacterial activity to enzymes was appreciated by the well-diffusion method (Tag and McGiven, 1971).

Heat treatment

Aliquots (200 µl) of the lyophilisates were heated at 60°C (30 and 60 min), 100°C (30 and 60 min) and at 121°C (20 min). The remaining activity was evaluated according to Graciela et al. (1995) against *E. faecium* H3. The activity was expressed as arbitrary units (AU) per milliliter.

Effect of pH values

The pH of aliquots (200 µl) of the lyophilisate were adjusted to different pH ranging from 2 to 12 with HCl (1 mole l^{-1}) or NaOH (1 mole l^{-1}). After incubation for 4 h at 30°C (Hernandez et al., 2005), and filtration through a Millipore 0.22 µm pore size membrane, the remaining activity was evaluated as described before.

RESULTS

Identification of enterococcal strains

The enterococcal strains used in this study were isolated from raw milk (LO4, LO12), traditional butter (BRO2) and fermented barley (H3) in Oran (Algeria). They are cocci shaped, Gram+, catalase negative and present a degradation of esculine on bile esculine agar medium. Based on API 20 Strep profile strains were identified as *E*. *faecium*. The identification was confirmed by PCR, using specific primers for *Enterococcus* which gave 733 base pairs fragment (Figure 1). According to the designer of primer (Deasy et al., 2000), the products of amplification and their size confirms that the four strains (BRO2, LO4, LO12, H3) belong to *Enterococcus* genus. The isolates (BRO2, LO4 and LO12) were characterized at the genotypic level by sequencing the specific amplified fragment of 16S rRNA gene to determine the species of *Enterococcus*. Nucleotide Blast analysis against the NCBI nucleotide database showed that the sequence of amplified fragment of 16S rRNA gene from three strains BRO2, LO4, LO12 were 99% identical to sequences obtained from different strains of *E. faecium*. The results of alignment were used to construct a phylogenetic tree bootstrapped for 100 times. Analysis of tree (Figure 2) showed that LO4 and LO12 strains are related with 56% of bootstrap analysis. While, BRO2 strain is closely related to cluster of LO4 and LO12 in 100% bootstrap. However, the 3 strains formed a cluster, which is closely related (100% bootstrap) to a cluster including *E. faecium* JCM 5804 and *E. faecium* NRRL B 2354.

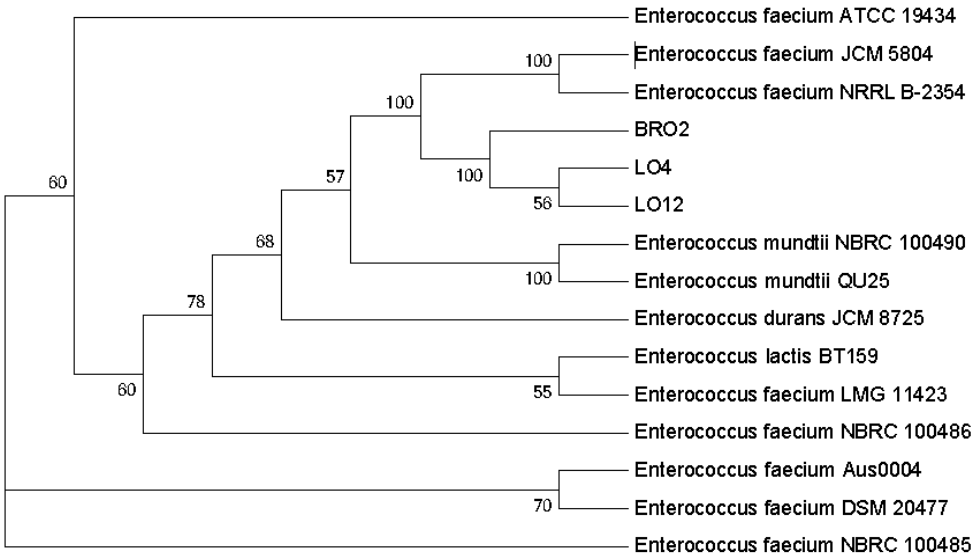

Figure 2. Neighbor-joining tree of 16SrRNA sequences from the isolates (LO4, LO12 and BRO2) and 16SrRNA sequences of 12 known *Enterococcus* species. The phylogenetic tree was bootstrapped for 100 times and clustered by using Mega 6 and Clustal X2.1.

Table 2. Effect of enzymes on inhibitory activity.

Enzymes	Inhibition zone of *E faecium* (mm)		
	BRO2	LO4	LO12
Catalase	10	10	13
α-Chymotrypsin	0	0	0
Trypsin	0	0	0
Pronase E	0	0	0
Proteinase K	0	0	0
Pepsin	10	09	11

Figure 3. Effect of enzymes on culture supernatant of *E. faecium* LO12. 1, α-Chymotrypsin; 2, catalase; 3, native supernatant; 4, trypsin; 5, Pronase E; 6, pepsin; 7, proteinase K.

Bacteriocinogenic activity

The strains *E. faecium* BRO2, LO4 and LO12 inhibited the growth of *Pseudomonas sp.* HB2, *Proteus mirabilis* HB3 and *E. faecium* H3. The strains *E. coli* HB4 and *Pseudomonas aeruginosa* HB5 were inhibited only by LO12 strain. Indeed the strains BRO2, LO4 and LO12 are bacteriocinogenic. All these inhibitions were recorded on solid buffered M17 medium and on unbuffered M17 medium (Table 1). For the target strains *Staphylococcus sp.* V3, *Citrobacter freundi* EC2 and *E. coli EC3,* no activity was recorded on buffered medium.

Effect of enzymes, heating and different pH values

The lyophilized supernatants from the culture of BRO2 LO4 and LO12 were sensitive to all tested proteolytic enzymes except pepsin. The inhibitory activity was maintained after treatment with catalase (Table 2 and Figure 3). These results suggest that inhibitory substances from strain BRO2, LO4 and LO12 are proteinaceous nature. The effect of temperature on the inhibitory activity showed that the antibacterial agent LO4 was more stable to heat treatment than the other agents. Its inhibitory activity against *E. faecium* H3 was maintained stable after heating at 60°C (for 30 and 60 min) and at 100°C for 30 min. While for antibacterial agents BRO2 and LO12, we observed that the inhibitory activity was maintained stable only at 60°C for 30 min but

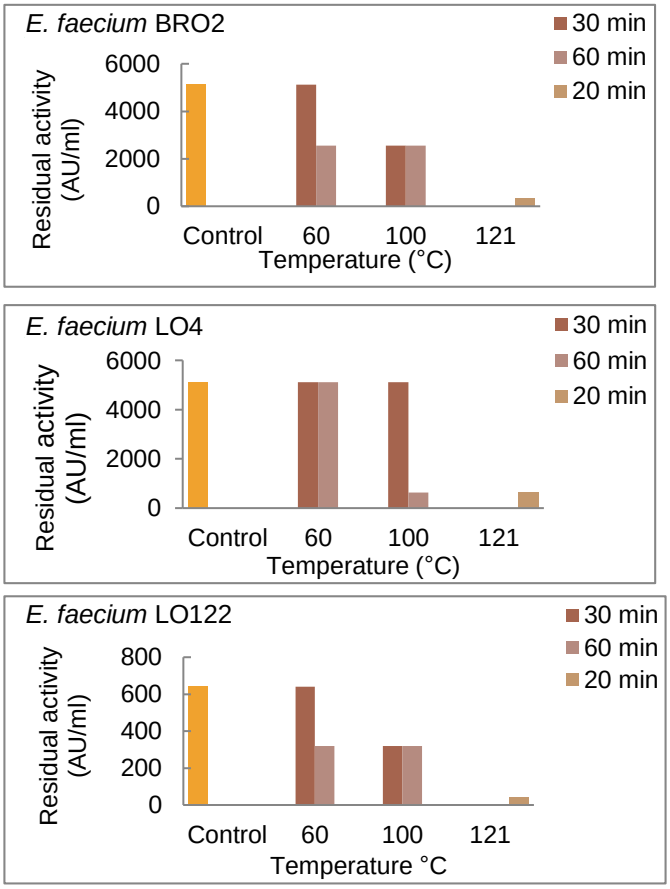

Figure 4. Residual activity of inhibitory substances from isolates BRO2, LO4 and LO12 against *E. faecium* H3.

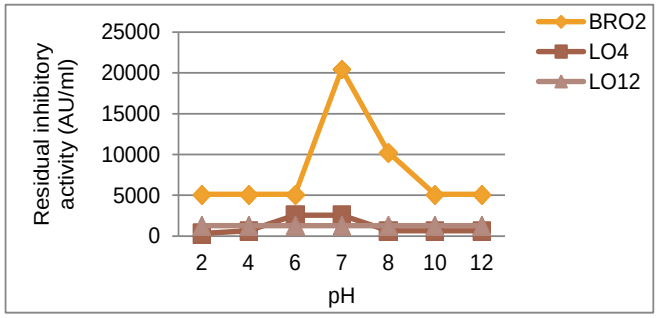

Figure 5. Effect of pH on inhibitory activity of *E. faecium* strains BRO2, LO4 and LO12 against *E. faecium* H3.

decreased to half at 60°C for 60 min and 100°C (30 and 60 min). We also observed that low activity was maintained at 121°C (20 min) for the three antibacterial agents (Figure 4). The inhibitory activity of antibacterial agent BRO2 was maximal at pH 7 (20480 AU/ml). A decrease of activity was observed for acidic and basic pH, but without a total inactivation of this agent. At pH 6 and 7, the antibacterial agent LO4 presented maximal activity (2560 AU/ml), while for acidic pH (4 and 2) and basic pH (8, 10 and 12) the inhibitory activity decrease (Figure 5). The inhibitory activity of LO12 agent was maintained stable for a large range of pH (2 to 12). Pinto et al. (2009) reported that bacteriocin produced by *E. faecium* 130 maintained total activities in the pH range from 2 to 8.

DISCUSSION

Enterococcus species are omnipresent and are alive freely in the soil, on plant and in large numbers in the dairy products where in certain cases, they prevail towards lactobacilli and lactococci (Franz et al., 1999; Giraffa, 2002). Approximately half of the *Enterococcus* species have been relatively recently described (Franz et al., 2011). The interest bearing on *Enterococcus* in recent years and their ubiquitous character encouraged to study their bacteriocinogenic potential for use in food technology. In our study, three *E. faecium* isolates were found to be bacteriocinogenic against *E. faecium* H3 and two human pathogens Gram negative bacteria. *E. faecium* H3 is more sensitive than the other target due to their close phylogenetic relationship to bacteriocinogenic strains. Growth inhibition of Gram negative bacteria was rarely reported. Some previous studies report activity of bacteriocins produced by *E. faecium* against *P. aeruginosa* (De Kwaadsteniet et al., 2005; Line et al., 2008; Gaaloul et al., 2015) and *P. mirabilis* (Line et al., 2008). In contrast, no activity was reported for *E. faecium* R111 towards *P. aeruginosa*, *Proteus vulgaris* or *E. coli* (Khay et al., 2011). The absence of sensitivity recorded for testing *Staphylococcus* strains and *E. coli* can be explained by natural variation in susceptibility and the ability to develop resistance to bacteriocins (Nascimento et al., 2010). At phylogenetic level our strains are related to *E. faecium* strain NRRL B-2354 and *E. faecium* JCM 5804. The last one produces three different types of bacteriocins, enterocin A, enterocin B, and enterocin P-like bacteriocin (Park et al., 2003).

The bacteriocins are known to be resistant to high temperature (Todorov et al., 2011). The antibacterial agent LO4 was more resistant to heat than the other studied antibacterial agents. Similar results were reported by Tulini et al. (2009) for bacteriocins from *E. faecium* 130. In our study inhibitory substances maintained a low activity at 100°C during 60 min and at 121°C during 20 min. The thermal stability of bacteriocins produced by enterococcal bacteria was also reported by Chen et al. (2007). The heat resistance can be due to the formation of small complex structures, stable cross-linkage and the generation of strongly hydrophobic portions (De Vuyst and Vandamme, 1994). It is an important characteristic for the application of these substances as natural food preservatives. The sensitivity of all antibacterial substances to proteolytic enzymes indicates that the inhibitions

are due to bacteriocins. According to Klaenhammer (1988), the bacteriocins were sensitive, at least for one proteolytic enzyme. The sensitivity to pronase E and trypsin suggest that it may be used as a biological preservative in foods and feed, as it will not affect the microbial flora of the gastrointestinal tract (Aktypis and Kalantzopoulos, 2003).

In our study inhibitory substances maintained their activity at any pH. Generally, the bacteriocins are not affected by the pH. However, pH seems to play an important role for the adsorption to target bacteria. Most of the described bacteriocins are active in a range of pH from 2 to 8 and are partially or completely inactivated at pH 10 (Tomé et al., 2009). The resistance to heat and to the large range of pH is an important characteristic for the application of these substances as natural food preservatives.

Conclusion

Three enterococcal strains identified at phylogenetic level as *E. feacium* were found to be bacteriocinogenic. The effectiveness of the bacteriocinogenic strains depends on target bacteria. Their inhibitory activity against *Pseudomonas* sp. and *P. mirabilis* are interesting. These bacteriocinogenic strains produce extracellular substances which are sensitive to proteolytic enzymes supporting strongly the proteinaceous nature. The substances from the three bacteriocinogenic strains *E. faecium* BRO2, LO4 are resisting in a large range of pH and to heat treatment of the antibacterial activity.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

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Multiple protein-domain conservation architecture as a non-deterministic confounder of linear B cell epitopes

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Epitope prediction is a critical step to diagnostic and vaccine discovery. Despite existence of some parameters for epitope discovery, this area remains inconclusive and wanting-for new complementary or stand-alone tools. The phenomenon of multiple protein-domain conservation architecture (MPDCA) as used here refers to homologous motifs unveiled by multiple sequence alignments across strain-variants of the same protein aside of the conserved domains (CD) present within the same super family. Unpublished data suggests that MPDCA might be a confounder of epitope necessitating further investigation as a predictor of the same. The ease of determining MPDCA is appealing when considering protein-analysis; specifically epitope discovery. This study aimed to validate MPDCA as a predictive confounder of epitope. Using two-sets of surface viral glycoproteins of human immunodeficiency virus type I, HIV-1 (gp120) and Ebola virus, EBOV (gp1,2 preprotein) (selected because their CD-architecture has widely been studied, their sequences are available in public databases, and the same are well annotated), the MPDCAs among three different virus-strains in each-set, were compared to epitopes predicted by established tools (Bipred and DiscoTope). 4/6 (66.6%) of the linear epitopes confounded MPDCA, with 3/6 (50%) of these MPDCA's confounding with the predicted linear epitopes (LE) at identities of > 50%, when compared to just 3/6 (50%) of the discontinuous epitopes (DE) that confounded with MPDCA at a < 50% identity. MPDCA is a non-deterministic confounder of Linear B cell epitopy. There is no causal relationship between the two, much as there is an evident co-occurrence. Therefore, MPDCA cannot accurately be used as an additional parameter to predict linear and or non-linear B cell epitopes.

Key words: Epitope, multiple protein-domain conservation architecture (MPDCA), discontinuous epitopes (DE).

INTRODUCTION

Protein-epitopes or antigenic determinants are surface situated protein-motifs that are recognized by either the B or T cell arm of the immune system. Protein-epitopes can either be conformational (non-linear, discontinuous) or linear(Huang and Honda, 2006). Identifying epitopes of particular pathogen-proteins, represents a critical step in the discovery of diagnostics and vaccines for infectious diseases. As a consquence, several groups have previously focused on uncovering the biophysical determinants of epitope (Korber et al., 2006). Despite the

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rigorous inquest to which the subject of epitope prediction has been subjected, the accurate prediction of epitope remains incomplete (Korber et al., 2006; Emini et al., 1985; Chou and Fasman, 1978; Haste Andersen et al., 2006; Karplus and Schulz, 1985; Kolaskar and Tongaonkar, 1990; Larsen et al., 2006; Parker et al. 1986; Zhang et al., 2008). New parameters are sought to complement or even replace the existing ones as a strategy to enhance the process of epitope prediction. Proteins belonging to a particular super family are defined by the presence of conserved domains (CD) therein. CD have previously been grouped together into a conserved domain database (CDD) as a strategy to allow easy annotation of newly sequenced proteins (Sievers et al., 2011; Geer et al., 2002; Marchler-Bauer et al., 2011). On the contrary, multiple sequence alignments of variants of the same protein from say different pathogen-strains within the same species (which are thereby homologous) reveals the occurence of 100% identical sequence-conservation which is not necessarily of CD nature (Sievers et al., 2011). Henceforth, we chose to refer to this phenomenon as 'multiple protein-domain conservation architecture (MPDCA)'. We have co-incidentally previously uncovered a repetitive occurence of B cell epitope within the context of MPDCA (Unpublished data), findings which have prompted us to question if MPDCA may be a confounder useful towards epitope prediction. Such quest is justified by the fact that MPDCA is an easy and fast parameter to investigate which if proven to be predictive of epitopy, will simplify vaccine or diagnostic discovery.

This study aimed to validate MPDCA as a predictive confounder of epitopy. To do so, we used two-sets of surface viral glycoproteins of human immunodeficiency virus type I, HIV-1 (gp120) and Ebola virus, EBOV (gp1,2 preprotein) (selected because their CD-architecture has widely been studied, their sequences are available in public databases, and the same are well annotated). The MPDCAs in these two-sets of viral glycoproteins among three different virus-strains in each-set, were compared to epitopes predicted by established tools (Bipred and DiscoTope). The authors report results to confirm a non-deterministic confounding of MPDCA with linear B cell epitope (LC); but no definitive correlation with discontinuous epitope (DE).

MATERIALS AND METHODS

This study was limited to *in-silico* sequence analyses, which did not necessitate the author to seek ethical approval from his institutional review board(s). All analyses presented below where done using default settings of software and databases described.

Identifying MPDCAs of the case-study HIV-1 and EBOV strain glycoproteins

Affirming super-family evolutionary ancestory across the case-study viral glycoproteins

Design: *In-silico* sequence analysis.

Software, databases and sequences: Conserved domain architecture retrieval tool (cDART) (Geer et al., 2002), reverse position-specific (RPS)- basic local sequence alignment tool (BLAST) (Altschul et al. 1997), the conserved domain database (Marchler-Bauer et al., 2011), accession # of the case-study viral glycoproteins of HIV [HIV-1 Clade B (France) SP "|Q00A19|" corresponding to HIVSeqDB "|DQ863898|" (Labrosse et al., 2006); HIV-1 Clade D (Uganda) SP "|D0EMT9|" corresponding to HIVSeqDB "Z19524" (Bruce et al., 1993); HIV-1 Clade B (USA) SP "|D0EMT9|" corresponding to HIVSeqDB "|GQ859302|" (Liu et al., 2009); and EBOV [EBOV Zaire (Mayinga) SP "|Q05320|" (Sanchez et al., 1993); EBOV Sudan (Uganda-00) SP"|Q7T9D9|" (Sanchez et al., 2004); EBOV Reston (Reston-89) SP "|Q66799|" (Sanchez et al., 1996).

The details of amino acid sequences are listed in supporting file S1.

Intervention: We searched the CDD for conserved domains (CD) by feeding the accession # of the respective case-study viral glycoproteins into the RPS-BLAST linked to the CDD as per user guide.

Measured variables: CD specific to the viral glycoprotein super-family.

Unveiling MPDCAs among the case-study viral glycoproteins

Design: *In-silico* multiple sequence analysis

Software, databases and sequences: Clustal Omega (Sievers et al., 2011), and FASTA format amino acids (Aa) sequences of the case-study viral glycoproteins of HIV-1 and EBOV were used are details are shown in supporting file S1 (Table 1).

Intervention: Multiple sequence alignments of the above HIV-1 and EBOV glycoprotein was done by individually feeding the FASTA formats of the individual virus-group's (either HIV-1 or EBOV) glycoproteins' amino acid sequences into the Clustal omega software at default setting.

Measured variables: MPDCA were defined as peptides of > 6 Aa length and cross-strain homology of 100%. Peptides of > 6 Aa were selected, as this is the recognized minimum peptide length with variable reconstructable immunogenicity *in-vivo* (Emini et al., 1985; Chou and Fasman, 1978; Haste Andersen et al., 2006; Karplus and Schulz, 1985; Kolaskar and Tongaonkar, 1990; Larsen et al., 2006; Parker et al. 1986; Zhang et al., 2008).

Exposing B cell epitopes within the case-study viral glycoproteins

Linear B cell epitope prediction by bipred

Design: Immuno-informatics.

***Software,* databases and sequences:** Bepipred linear B cell prediction software (Larsen et al., 2006) and FASTA format amino acids (Aa) sequences of the case-study viral glycoproteins of HIV-1 and EBOV are detailed in supporting file S1 (Table 1).

Intervention: Linear B cell epitopes were derived by feeding the FASTA formats of the amino acids (Aa) sequences of the case-study viral glycoproteins of HIV-1 and EBOV into the Bepipred user interface at default.

Measured variables: Linear B cell epitopes.

Table 1. Description of additional files.

Additional file	File type	Description
S1	MS.doc	This file offers details of HIV Sequence Database/Swiss-prot accession numbers, FASTA formats and Amino acid loci of all HIV-1 and EBOV glycoproteins used in this study
S2	MS.xcel	This file details superfamily conserved domains of the HIV-1 clade B (France) glycoprotein used in this study
S3	MS.xcel	This file details superfamily conserved domains of the HIV-1 clade D (Uganda) glycoprotein used in this study
S4	MS.xcel	This file details superfamily conserved domains of the HIV-1 clade B (USA) glycoprotein used in this study
S5	MS.xcel	This file details superfamily conserved domains of the EBOV Zaire (Mayinga) glycoprotein used in this study
S6	MS.xcel	This file details superfamily conserved domains of the EBOV Sudan (Uganda-00) glycoprotein used in this study
S7	MS.xcel	This file details superfamily conserved domains of the EBOV Reston (Reston-89) glycoprotein used in this study
S8	Pdf	This file shows RST-BLAST results obtained by searching one of the HIV-1 clade (B, France) glycoproteins across the conserved domain database, CDD
S9	Pdf	This file shows RST-BLAST results obtained by searching one of the EBOV (Zaire, Mayinga) glycoproteins across the conserved domain database, CDD
S10	MS.doc	This file details multiple protein domain conservation architecture revealed by multiple sequence alignment of all three HIV-1 strains' glycoproteins
S11	MS.doc	This file details multiple protein domain conservation architecture revealed by multiple sequence alignment of all three EBOV strains' glycoproteins
S12	MS.doc	This file shows correlation of MPDCA with linear epitopes predicted by Bepipred across sequences of HIV-1 Clade B (France)'s glycoprotein
S13	MS.doc	This file shows correlation of MPDCA with linear epitopes predicted by Bepipred across sequences of HIV-1 Clade D (Uganda)'s glycoprotein
S14	MS.doc	This file shows correlation of MPDCA with linear epitopes predicted by Bepipred across sequences of HIV-1 Clade B (USA)'s glycoprotein
S15	MS.doc	This file shows correlation of MPDCA with linear epitopes predicted by Bepipred across sequences of EBOV Zaire (Mayinga)'s glycoprotein
S16	MS.doc	This file shows correlation of MPDCA with linear epitopes predicted by Bepipred across sequences of EBOV Sudan (Uganda-00)'s glycoprotein
S17	MS.doc	This file shows correlation of MPDCA with linear epitopes predicted by Bepipred across sequences of EBOV Reston (Reston-89)'s glycoprotein
S18	MS.doc	This file shows correlation of MPDCA with non-linear (discontinuous) epitopes predicted by Bepipred across sequences of HIV-1 Clade B (France)'s glycoprotein
S19	MS.doc	This file shows correlation of MPDCA with non-linear (discontinuous) epitopes predicted by Bepipred across sequences of EBOV Zaire (Mayinga)'s glycoprotein

Non-linear B cell prediction by discotope

Design: Immuno-informatics.

Software, databases and sequences: DiscoTope conformational B cell prediction software (Haste Andersen et al., 2006) and 3-D crystal structure accession # for the case-study viral glycoproteins of HIV-1 and EBOV (PDB entry "3dnl" and "3CSY" respectively).

Intervention: Conformational B cell epitopes were derived by individually feeding the PDB entry accessions of the case-study viral glycoproteins of HIV-1 and EBOV into the DiscoTope user interface at default.

Measured variables: Conformational B cell epitopes.

Correlating multiple domain conservation architectures with predicted epitope among the case-study vira; glycoproteins

This was more of a mathematical or statistical analysis of the data above; with a focus on ascertaining the correlation between epitopes and MPDCA. The few cases used for this validation stage could not permit derivation of variation coefficients with statistical significance. Instead, a cross-tabulation of MPDCA with either linear epitope (LE) or discontinuous epitope (DE) was done.

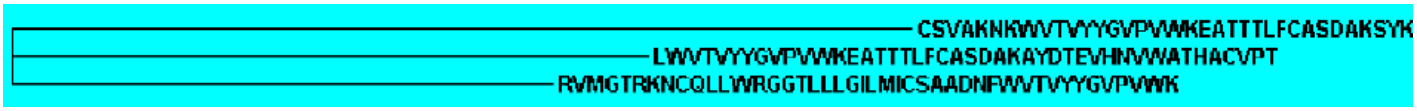

Figure 1. Evolutionary relationship between the strain-specific variants of the HIV-1 glycoprotein gp120 as revealed by conserved domain architecture. Description: phylogenetic tree revealing the evolutionary relationship between the strain-specific variants of the HIV-1 glycoprotein gp120 as revealed by conserved domain architecture.

Availability of supporting data

"The data set(s) supporting the results of this article is (are) included within the article (and its additional file(s)" (Table 1).

RESULTS AND DISCUSSION

The results confirm a non-deterministic confounding of MPDCA with linear B cell epitope (LC); and no definitive correlation with discontinuous epitope (DE).

MPDCA among EBOV and HIV-1 strain glycoproteins

First, the presence of conserved-domains consistent with the viral glycoprotein super-families studied was affirmed and subsequently unveiled multiple protein domain conservation architectures across the same case-study viral glycoproteins.

Super-family conserved domains (CD) across the case-study viral glycoproteins

All case-study viral glycoproteins were affirmed to lie within the HIV-1 and EBOV super-family of glycoproteins (for details, see additional files S1, S2, S3, S4, S5, S6, and S7) (Table 1). A phylogenetic illustration of this evolutionary ancestry across the HIV-1 case study glycoproteins, gp120 is shown in Figure 1. Schematics of the conserved domain architecture within the case-study viral glycoproteins are shown in the RST-BLAST results detailed in additional supporting files S8 and S9 (Table 1).

These data served to justify our choice of strain-type variants of the study viral glycoproteins within the same species of virus. CD may be viewed as functional protein motifs, which by virtue of their inter-network molecules within a network assume evolutionary patterns of hub-proteins. Therefore as pathogens evolve (presumably across strains in the same species), the CD are maintained to sustain their functionality. Further, because CDs are functional motifs which must interact with ligands in the network, the same are often located on the surface, thereby explaining the confounding between CD and epitope (Geer et al., 2002; Marchler-Bauer et al., 2011). The multiple protein-domain conservation architecture (MPDCA) revealed that, on the other hand, may or may not be functional motifs as is elucidated by prior studies and further evidence provided. Nonetheless, it appears that the same MPDCA are under pressure from other interaction within the network; be they functional or structural (proxy).

MPDCA among the case-study viral glycoproteins

Six (6) MPDAs with more than six amino acids (Aa) length and 100% identity were unveiled in each of the case-study glycoprotein groups, HIV-1 gp120 and EBOV gp1,2. The respective details of these findings are shown by different color shades in supporting files S10 and S11 (Table 1). Fraser et al. (2002) previously found that the connectivity of well-conserved proteins in the network is negatively correlated with their rate of evolution. Overall, this group showed that proteins with more interactions evolve more slowly not because they are more important to the organism, but because a greater proportion of the protein is directly involved in its function. In contrast to this claim that proteins with more interaction partners (sometimes called hubs) are- owing to an assumed high density of binding sites, both physiologically more important and slow evolving; Batada et al. (2006) found that hub proteins are indeed more important for cellular growth rate and are under tight regulation but are not slow evolving. These studies suggest that at sites important for interaction between proteins (such as the MPDCA which were studied), evolutionary changes occur largely by coevolution, in which substitutions in one protein result in selection pressure for reciprocal changes in interacting partners. He argued that, in the same manner as evolutionary changes in the B cell paratrope could potentially influence epitope architecture, MPDCA may be under pressure from their indeterminate interactions. Such analogy to hub proteins with regards to the conservation patterns of protein domains is, however, debatable. Specifically, the primary reason for conservation of sequence across a domain is preservation of the domain fold and secondary structure elements (internal interactions). Conservation of active site residues and structure, and conservation of binding regions contribute to local sequence conservation (that is, within the same functional sub-family). Also, the assumption of a co-evolutionary model may not be universal, since this will only occur when the domain

Table 2. Correlation of MPDCA with linear and discontinuous epitope among the case-study HIV-1 glycoproteins.

(Position)_Multiple protein-domain conservation architecture (MPDCA)	Length (Aa)	Occupied by linear epitope (LE/MPDCA)	Occupied by discontinuous epitopy (DE/MPDCA)
74_DIISLWDQSLKPCVKLTPLCVTLNCT	27	0/27	0/27
177_ITQACPKVTFEPIPI	15	7/15	2/15
192_HYCAPAGFAILKC	13	0/13	0/13
229_PVVSTQLLLNGSLAE	15	1/15	0/15
401_VGKAMYAPPI	10	8/10	1/10
442_FRPGGGDMRDNWRSELYKYK	21	12/21	6/21

Note that this was only a validation step, and the small sample size used could not allow for derivation of variation coefficients.

Table 3. Correlation of MPDCA with linear and discontinuous epitope among the case-study EBOV glycoproteins.

(Position)_Multiple protein-domain conservation architecture (MPDCA)	Length (Aa)	Occupancy by linear epitope (LE/MPDCA)	Occupancy by discontinuous epitopy (DE/MPDCA)
99_YEAGEWAENCYNLEIKK	17	10/17	0/17
130_RGFPRCRYVHK	11	1/11	0/11
157_GAFFLYDRLASTVIYRG	17	0/17	0/17
559_RQLANETTQALQLFLRATTELR	22	3/22	0/22
586_LNRKAIDFLLQRWGGTC	17	0/17	0/17
645_WTGWRQWIPAGIG	13	2/13	0/13

interacts with another protein (or DNA/RNA segment). However, most domain function (enzymes, signalling) involve interactions with small molecules which do not "evolve". Also, protein domains occur in proteins throughout the cell, and are not predominantly associated with cell walls or membranes (Huang and Honda, 2006; Korber et al., 2006; Emini et al., 1985; Chou and Fasman, 1978; Haste Andersen et al., 2006; Karplus and Schulz, 1985; Kolaskar and Tongaonkar, 1990; Larsen et al., 2006; Parker et al. 1986; Zhang et al., 2008; Sievers et al., 2011; Geer et al., 2002; Marchler-Bauer et al., 2011; Fraser et al., 2002; Batada et al., 2006; Altschul et al., 1997; Labrosse et al., 2006; Bruce et al., 1993; Liu et al., 2009; Sanchez et al., 1993; Sanchez et al., 2004; Sanchez et al., 1996).

B cell epitopes within the case-study viral glycoproteins

We uncovered both linear and non-linear B cell epitopes in all case-study viral glycoproteins as is further detailed below.

Linear B cell epitopes prediction by bepipred

Several linear B cell epitopes were unveiled in all case-study viral glycoproteins that are shown further in supporting files S12, S13, S14, S15, S16 and S17 (Table 1).

Non-linear B cell epitopes prediction by discotope

The conformational B cell epitopes unveiled across the case- study viral glycoproteins are shown further in supporting files S18 and S19 (Table 1), respectively for HIV-1 and EBOV.

Correlation of MPDCA and predicted epitope among the case-study viral glycoproteins

The author observed an arbitrarily non-deterministic confounding of MPDCA with linear B cell epitope (LC); but no definitive correlation with discontinuous epitope (DE). Specifically, 4/6 (66.6%) of the linear epitopes confounded MPDCA, with 3/6 (50%) of these MPDCA's confounding with the predicted linear epitopes (LE) at identities of > 50% (Tables 1 and 2) when compared to just 3/6 (50%) of the discontinuous epitopes (DE) that confounded with MPDCA at a < 50% identity (Tables 2 and 3). There are several weaknesses in our approach and findings. Since this was only a first-step in proof of concept, and the small sample size used (there are over

100,000 sequences for HIV GP120) could not allow for derivation of variation coefficients, he argued that further expanded work is sought in this direction to better inform the performance of MPDCA. Further, the methods to which he compare the performance of MPDCA as a predictor of B cell epitope, have variable precision and are not necessary the best (Emini et al., 1985; Chou and Fasman, 1978; Haste Andersen et al., 2006; Karplus and Schulz, 1985; Kolaskar and Tongaonkar, 1990; Larsen et al., 2006; Parker et al. 1986; Zhang et al., 2008). Certainly in the case of the linear predictor the false positive prediction rate is very high making it unusable as a benchmark (no false negative rate given, because not much could be determined as this stage).

Overall, these data show that MPDCA is a non-deterministic confounder of linear B cell epitope. Moreover, there appears to be no causal relationship between the two, much as there is an evident co-occurrence. Therefore, MPDCA cannot accurately be used as an additional parameter to predict linear and or non-linear B cell epitopes. The only possible applicability of MPDCA in epitope discovery is that of rapidly scanning across proteins to see areas that may be potentially epitopic. More important to note outside of our findings is that MPDCA cannot predict antigenicity or immunogenicity. Further, another major shortcoming with using MPDCA to predict linear epitopy, is that MPDCA have the potential to interact with other players in the network, a behavior that might mask or even conceal their architecture *in-vivo*, making them inappropriate vaccine or diagnostic targets.

Conflict interests

The author declare that there is no conflict of interest.

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Abbreviations: MPDCA, Multiple protein-domain conservation architecture; **CD,** conserved domains; **DE,** discontinuous epitopes; **LE,** linear epitopes; **CDD,** conserved domain database; **cDART,** conserved domain architecture retrieval tool; **RPS,** reverse position-specific; **BLAST,** basic local sequence alignment tool.

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